Univerzita Karlova v Prahe

Prírodovedecká fakulta

Dizertačná práca

2014 **Mgr. Veronika Vlková**

Univerzita Karlova v Prahe

Prírodovedecká fakulta

Doktorské študijné programy v biomedicíne

Odbor: Molekulárna a bunková biológia, genetika a virológia

Mgr. Veronika Vlková

Epigenetické mechanizmy v regulácii prezentácie antigénu a protinádorová imunita.

Epigenetic mechanisms in the regulation of antigen presentation and anti-tumour immunity.

Dizertačná práca

Školiteľ: RNDr. Milan Reiniš, CSc.

Praha, 2014

Čestne prehlasujem, že som predloženú dizertačnú prácu spracovala samostatne s použitím uvedenej literatúry a ďalších informačných zdrojov.

V Prahe, 20.04.2014 ……..

Poďakovanie

Chcela by som poďakovať všetkým, ktorí mi pomohli pri spracovaní mojej dizertačnej práce. Moje poďakovanie patrí vedúcemu mojej dizertačnej práce RNDr. Milanovi Reinišovi, CSc. za vedenie, cenné rady a podporu pri záverečnom spracovaní práce. Ďalej by som veľmi rada poďakovala kolegom v našom laboratóriu za dobrú spoluprácu.

Osobité poďakovanie patrí mojej rodine, najmä mojim rodičom a manželovi, že ma v celom štúdiu podporovali a pomáhali mi.

Zoznam skratiek

DNA	Deoxyribonukleová kyselina (Deoxyribonucleic acid)
MHC	Hlavný histokompatibilný komplex (Major histocompatibility complex)
IFN	Interferon
DAC	5-aza-2'-deoxycytidin (5-aza-2'-deoxycytidine)
5-azaC (5AC)	5-azacytidin (5-azacytidine)
TAP-1	Transporter associated with Antigen Processing 1
TAP-2	Transporter associated with Antigen Processing 2
LMP-2	Low molecular mass polypeptide 2
PSMB9	Proteasome subunit beta type-9
LMP-7	Low molecular mass polypeptide 7
PSMB8	Proteasome subunit beta type-8
β2m	Beta-2-microglobulin
CBP	CREB-binding protein
HPV16	Ľudský papillomavírus typ 16 (Human papillomavirus Type 16)
MDSC	Myeloidné supresorové bunky (Myeloid-derived supressor cells)
CD11b	Cluster of differentiation molecule 11B
Gr-1	Granulocyte receptor 1
CD marker	Cluster of differentiation marker
TGF-β	Transformujúci rastový faktor (Transforming growth factor-β)
ROS	Reaktívne formy kyslíka (Reactive oxygen species)
Arg-1	Arginása 1 (Arginase 1)
NO	Oxid dusnatý (Nitric oxide)
IRFs	Interferon regulatory factors
STAT	Signal Transducers and Activators of Transcription
CpG ODN	CpG oligodeoxynukleotidy (CpG oligodeoxynucleotides)
CY	Cyklofosfamid (Cyclophosphamide)
VEGF	Vaskulárny endoteliálny rastový faktor (Vascular endothelial growth factor)
CY-MDSC	Myeloidné supresorové bunky akumulované po liečbe s cyklofosfamidom
TU-MDSC	Myeloidné supresorové bunky akumulované počas rastu nádoru
CYTU-MDSC	Myeloidné supresorové bunky akumulované počas rastu nádoru a liečby cyklofosfamidom
Ly6g	Lymphocyte antigen 6 complex, locus G
Ly6c	Lymphocyte antigen 6 complex, locus C
ATRA	All-trans-retinová kyselina (All Trans-Retinoic Acid)

IL	Interleukin
TC-1	Názov pre myšaciu nádorovú pľúcnu líniu, MHC I pozitívnu
TC-1/A9	Názov pre myšaciu nádorovú pľúcnu líniu, MHC I deficientnú
TRAMP-C2	Názov pre myšaciu nádorovú prostatickú líniu, MHC I deficientnú
PC61	Protilátka proti regulačným T-lymfocytom (Anti-CD25 Ab)
Ab	Protilátka (Antibody)
TH bunky	Pomocné T-bunky (T helper cells)
CTL	Cytotoxické T lymfocyty (Cytotoxic T lymphocyte)
NK T cells	Subpopulácia T-lymfocytov (Natural killer T cells)
NK cells	Prirodzení zabíjači (Natural Killer Cell)
C. Elegans	Caenorhabditis elegans
S. Cerevisiae	Saccharomyces cerevisiae
A-N6	Metylácia adenínu v polohe N6
C-N4	Metylácia cytozínu v polohe N4
C5	Metylácia cytozínu C5
DNMT	DNA metyltransferáza (DNA methyltransferase)
t-RNA	Transferová ribonukleová kyselina (Transfer ribonucleic acid)
LINEs	Dlhé včlenené retrotranspozibilné elementy (Long interspersed elements)
SINEs	Krátke včlenené retrotranspozibilné elementy (Short iterspersed elements)
A	Adenín
G	Guanín
C	Cytozín
T	Tymín
SP1	Specificity protein 1
LTR	Dlhé koncové opakujúce sa poradia nukleotidov DNA (Long terminal repeats)
Alu elementy	Krátke úseky DNA, ktoré sú charakterizované činnosťou restrikčnej endonukleázy Alu (Arthrobacter luteus)
GAG	Názov proteínu kódovaného v rámci genómu retrovírusov ,,Group antigens‘‘
POL	Názov proteínu kódovaného v rámci genómu retrovírusov s funkciou reverznej transkriptázy
Xist	Transkript inaktivujúci chromozóm X (X-inactive specific transcript)
MBDs	Metyl CpG väzobné proteíny (Methyl-CpG binding proteins)
MED1	Methyl-CpG-binding endonuclease 1
HDAC	Histón-deacetyláza (Histone deacetylase)

MECP2	Metyl CpG väzobný proteín 2 (Methyl CpG binding protein 2)
PCNA	Proliferating Cell Nuclear Antigen
Rb	Retinoblastoma protein
E2F1	E2 promoter binding factor 1
PHD doména	Plant Homeo Domain
SUV39H1	Suppressor Of Variegation 3-9 Homolog 1
NuRD	Nucleosome Remodeling Deacetylase
HpaII	Deoxyribonukleáza z Haemophilus parainfluenzae
HPLC	Vysokoúčinná kvapalinová chromatografia (High performance liquid chromatography)
SAM	S-adenozyl-L-metionín
TSA	Trichostatin A
HAT	Histón-acetyltransferáza (Histone acetyltransferase)
AcetylCoA	Acetylkoenzým A
ncRNA	Nekódujúca RNA (Non-coding RNA)
siRNA	Malá interferujúca RNA
miRNA	Micro RNA
RISC	RNA-induced silencing complex
5mC	5-metylcytozín
5hmC	5-hydroxymetylcytozín
5fC	5-formylcytozín
5caC	5-carboxylcytozín
5 hmU	5 hydroxymetyluracyl
TET	Ten-eleven translocation 5mC-hydroxylase
BER	Bázová excízna oprava (Base excision repair)
ICAM1	Intercellular adhesion molecule 1
LFA-1	Lymphocyte function-associated antigen 1
TEM	Transendoteliálna migrácia leukocytov (Leukocyte transendothelial migration)
ICOS-L	Inducibilný kostimulačný ligand (Inducible costimulator ligand)
PD-L1	Ligand 1 programovanej smrti (programmed death-1 ligand)
PD-L2	Ligand 2 programovanej smrti (programmed death-2 ligand)
B7RP1	B7-related protein-1
B7-H1	B7 Homolog 1
B7-H3	B7 Homolog 3

B7-H4	B7 Homolog 4
B7.DC	B7-dendritic cells
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
APC	Antigén prezentujúca bunka (Antigen-presenting cell)
TCR	T-bunkový receptor (T cell receptor)
IDO	Indolamín 2,3- dioxygenáza (Indoleamine 2, 3-dioxygenase)
DC	Dendritická bunka (Dendritic cell)
MØ	Makrofágy (Macrophage)
TNF	Faktor nekrotizujúci nádory (Tumor necrosis factor)
TRAIL	TNF-Related Apoptosis-Inducing Ligand
FoxP3	Forkhead box P3
iNOS	Inducible nitric oxide synthase
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
NFκB	Jadrový faktor κB (Nuclear factor-kappaB)
JAK	Janus kinase
IFN-γR	Interferon gamma receptor
GAS	Oblasti promótora génov aktivované interferonom gamma (Interferon gamma activated sites)
TCP45	T-cell protein tyrosine phosphatase
SOCS	Suppressor of cytokine signaling
TDG	Thymine DNA glycosylase
AID	Activation-induced (DNA-cytosine) deaminase
APOBEC1	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1
T-bet	T-cell-specific T-box transcription factor
GATA-3	GATA binding protein 3
α-GalCer	α-Galactosylceramide
PCR	Polymerázová reťazová reakcia (Polymerase chain reaction)
UTR	Untranslated region

Abstrakt

Veronika Vlková: **Epigenetické mechanizmy v regulácii prezentácie antigénu a protinádorová imunita.**
Univerzita Karlova v Prahe, Prírodovedecká fakulta, Doktorské študijné programy v biomedicíne
Odbor: Molekulárna a bunková biológia, genetika a virológia
Dizertačná práca, 2014

Reverzibilné zníženie expresie MHC I na povrchu nádorových buniek je bežný spôsob, ktorým nádorové bunky unikajú imunitnému dohľadu a je často spojené s koordinovaným znížením génov antigén-prezentujúcej mašinérie. Expresia týchto génov môže byť obnovená pomocou IFN-γ. V predkladanej práci poukazujeme na spojenie demetylácie DNA regulačných oblastí vybraných génov antigén-prezentujúcej mašinérie so zvýšenou expresiou MHC I na povrchu nádorových buniek po ovplyvnení IFN-γ, čo znamená, že IFN-γ by mohol plniť úlohu epigenetického agensu. Naše výsledky objasňujú úlohu metylácie DNA pri úniku nádorových buniek imunitnému dohľadu. Použitie epigenetických modifikátorov môže obnoviť expresiu MHC I a tak môžu zviditeľniť nádory pre imunitný systém. Naše dáta poskytujú tiež informácie o chemoterapii pomocou diferenciačných liečiv, prednostne pre použitie v kombinácii s ďalšími liečivami pre dosiahnutie nízkeho imunosupresívneho rozsahu mikroprostredia nádoru. Navyše, naše dáta poskytujú dôkazy, že mimo známych cieľov epigenetických agensov alebo imunoregulačných protilátok, ďalšie nešpecifické alebo nepriame účinky musia byť zvážené počas terapie. Práca detailne popisuje reverzibilné mechanizmy, ktorými nádorové bunky unikajú špecifickej imunite (s dôrazom kladeným na úlohu metylácie DNA v regulácii génovej expresie).

Abstract

Veronika Vlková: Epigenetic mechanisms in the regulation of antigen presentation and anti-tumour immunity.
Charles University in Prague, Faculty of Science, Molecular and Cellular Biology, Genetics and Virology
Dissertation, 2014

Reversible downregulation of MHC class I expression on tumour cells, a common mechanism by which tumour cells can escape from specific immune responses, is frequently associated with coordinated silencing of antigen-presenting machinery genes. The expression of these genes can be restored by IFN-γ. Here we describe association of DNA demethylation of selected antigen-presenting machinery gene regulatory regions upon IFN-γ treatment with MHC class I upregulation on tumour cells thus demonstrating that IFN-γ acts as an epigenetic modifier. Our results cast more light on the role of DNA methylation in tumour cell escape from specific immunity. Treatment of MHC class I deficient tumour by epigenetic modifiers sensitized neoplasia to the immunotherapy. Our data also provide knowledge about differentiation cancer chemotherapies, especially for use in combination with other drugs to achieve lower immunosuppressive function of tumour microenvironment. In addition, our data provide evidence that besides the known targets of epigenetic agents or immunoregulatory antibodies other unspecific or indirect activities should be considered during the therapy. The aim of whole work was to describe in detail reversible mechanisms in the tumour cell escape from specific immunity.

Obsah

1. Úvod

Epigenetické zmeny, napríklad chybná metylácia DNA, plnia dôležitú úlohu v karcinogenéze (Jones and Baylin, 2007; Esteller, 2008) a tiež pri úniku nádorových buniek imunitnému systému (Sigalotti et al., 2005; Tomasi et al., 2006). Zníženie expresie MHC I na povrchu nádorových buniek je bežný spôsob, ktorým nádorové bunky unikajú imunitnému dohľadu (Garrido et al., 1997; Bubeník, 2003; Reiniš, 2010; Seliger, 2012). Molekulárne defekty, ktoré sú zodpovedné za narušenú expresiu na povrchu nádorových buniek môžu byť ireverzibilné a reverzibilné (Garrido et al., 2010). Reverzibilné zníženie expresie MHC I na povrchu nádorových buniek je často spojené s koordinovaným znížením expresie génov antigén-prezentujúcej mašinérie (Seliger et al., 2000; Garcia-Lora et al., 2003). Expresia týchto génov môže byť obnovená pomocou IFN-γ (Gabathuler et al., 1994; Seliger et al., 2000) alebo pomocou inhibítorov DNA metyltransferáz, 5-aza-2'-deoxycytidinom (DAC) alebo 5-azacytidinom (5-azaC, 5AC), čo sú cytostatické agensy s epigenetickými účinkami (Manning et al., 2008). Preto je možné, že zvýšená expresia umlčaných génov antigén-prezentujúcej mašinérie je spojená s demetyláciou DNA v regulačných oblastiach daných génov, ktorá zvýši citlivosť nádorových buniek k špecifickej imunitnej odpovedi. Poznatky, že epigenetické mechanizmy sú dôležité pri znížení expresie MHC I molekúl na povrchu buniek deficientných nádorov v génoch antigén-prezentujúcej mašinérie a jej obnovu po ovplyvnení IFN-γ priniesla Setiadi spolu s kolektívom (Setiadi et al., 2007). Zníženie expresie génu *TAP-1* bolo spojené s nízkou hladinou histón-acetyltransferázy CBP (z angl. CREB-binding protein) v oblasti promótora génu *TAP-1*. Po ovplyvnení IFN-γ dochádza k zvýšeniu expresie génu *TAP-1* cez zvýšenú acetyláciu histónu 3 v oblasti promótora génu *TAP-1*. Je známe, že metylácia DNA a acetylácia histónov má významnú úlohu v reverzibilnej strate MHC I na povrchu bunky, kde táto môže byť znovu obnovená ošetrením buniek inhibítormi DNA metyltransferáz a histón-deacetyláz (Manning et al., 2008; Setiadi et al., 2008; Khan et al., 2008). Toto zvýšenie je spojené so zvýšenou expresiou génov antigén-prezentujúcej mašinérie, menovite *TAP-1, TAP-2, LMP-2 (PSMB9), LMP-7 (PSMB8*) a tiež s demetyláciou DNA v oblasti regulačných sekvencií daných génov. Zmeny DNA metylácie po aktivácii dráhy IFN-γ neboli dosiaľ hlbšie študované. Preto cieľom práce bolo zistiť a nájsť spojenie medzi demetyláciou DNA a zvýšenou expresiou génov antigén-prezentujúcej mašinérie sprostredkovanou IFN-γ v MHC I deficientných myšacích nádorových líniách, inými slovami, či by mohol IFN-γ plniť úlohu epigenetického agensu. Tiež nás zaujímal dopad

terapie pomocou epigenetických agensov na expresiu molekúl MHC I na povrchu MHC I deficientných nádorových buniek. Pre zlepšenie účinku imunoterapie boli vyvinuté viaceré postupy, ktoré zvyšujú expresiu MHC I na povrchu nádorových buniek. Epigenetické agensy tiež podporujú nádorové bunky v dráhe programovanej bunkovej smrti a pomáhajú pri ich ničení cez cytotoxické T-lymfocyty (Fulda and Debatin, 2006). Preto kombinácia účinnej chemoterapie s príslušným epigenetickým agensom a imunoterapie by mohla byť vhodným spôsobom liečby nádorových ochorení.

Ďalším mechanizmom, ktorým nádorové bunky unikajú imunitnému dohľadu, je nádorom-indukovaná imunosupresia. Myeloidné supresorové bunky (z angl. myeloid-derived supressor cells; MDSC) patria k hlavným zložkám, ktoré sprostredkujú nádorom-indukovanú imunosupresiu (Gabrilovich et al., 2007). Ide o heterogénnu populáciu nediferencovaných buniek, ktoré u myší charakterizuje marker monocytov (CD11b) a neutrofilov (Gr-1). Hlavným znakom MDSC je ich schopnosť rušiť protinádorovú imunitu a umožňujú rast nádorov, pretože blokujú aktiváciu $CD4^+$, $CD8^+$ T buniek a teda ich cytotoxicitu voči nádorovým bunkám (Bronte et al., 2001; Huang et al., 2006). MDSC prispievajú k poruchám indukcie T buniek cez produkciu TGF-β (z angl. transforming growth factor-β), ROS (z angl. reactive oxygen species), arginázy 1 (Arg-1) a oxidu dusnatého (NO) (Kusmartsev and Gabrilovich, 2006). V posledných rokoch sa objavuje čoraz viac štúdií, ktoré zaznamenali zvýšenú hladinu regulačných T-lymfocytov pri nádorových ochoreniach (Facciabene et al., 2012; Whiteside et al., 2012). Táto skutočnosť je často zodpovedná za slabú protinádorovú efektorovú odpoveď a tak je ohrozená a znížená protinádorová imunita (Elkord et al., 2010; Nishikawa and Sakaguchi, 2010).

Práca detailne popisuje reverzibilné mechanizmy, ktorými nádorové bunky unikajú špecifickej imunite (s dôrazom kladeným na úlohu metylácie DNA v regulácii génovej expresie) a tiež sa zaoberá sledovaním expresie génov s imunosupresívnym účinkom v nádorových a imunitných bunkách počas rastu a liečby nádoru.

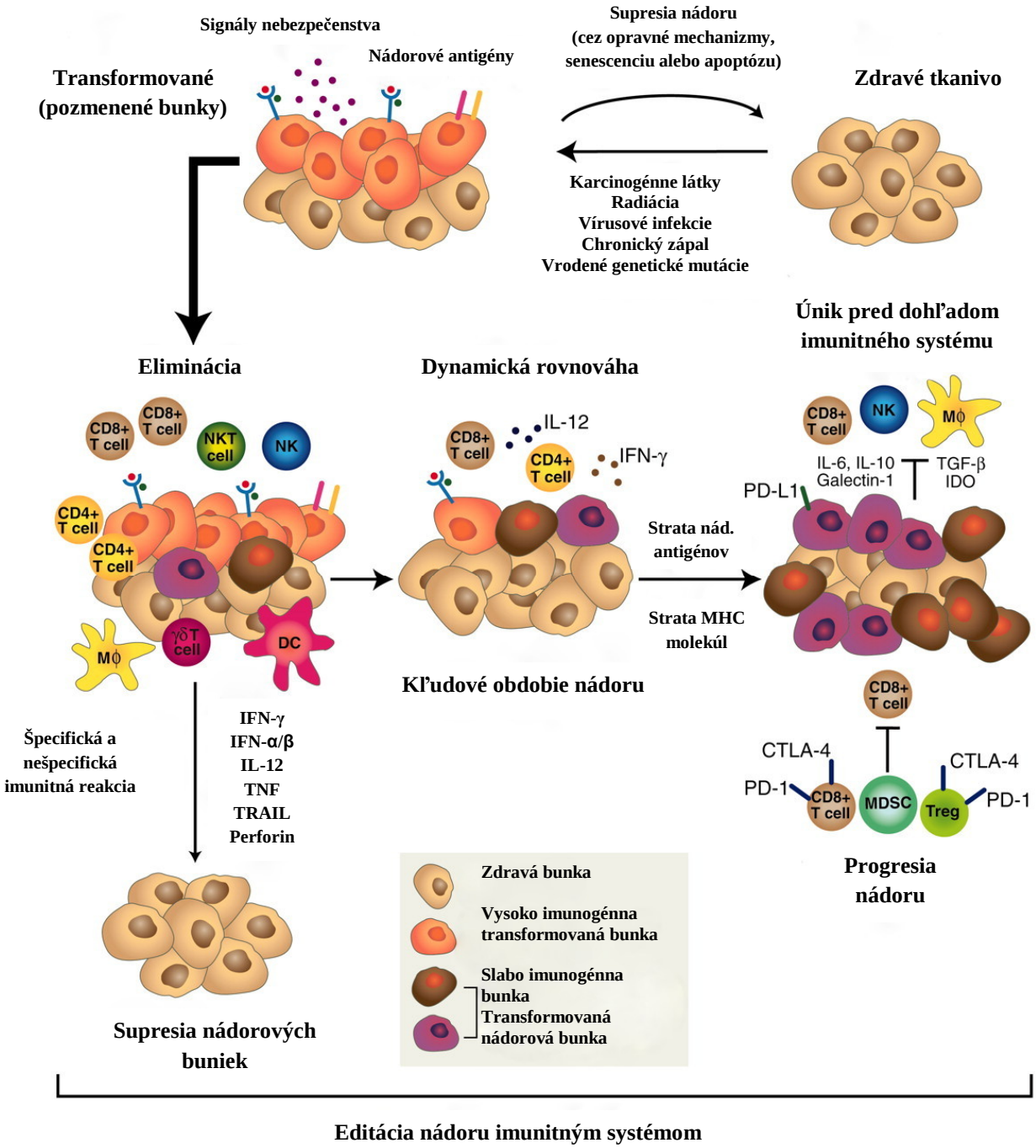
2. Úloha imunitného systému v obrane proti nádorovým bunkám-teória imunoeditácie

Imunitný systém je schopný rozpoznať a eliminovať nádorové bunky. Na druhej strane, imunitný systém vyvíja na nádorové bunky nepretržitý selekčný tlak. Tento tlak vedie k vzniku takých foriem transformovaných buniek, ktoré dokážu uniknúť efektorovým bunkám imunitného systému. Môžeme teda povedať, že imunitný systém má dvojakú úlohu v boji proti nádorom. Potláča rast nádoru tým, že ničí nádorové bunky alebo inhibuje ich vznik a rast, ale tiež môže napomáhať progresii nádoru selekciou takých foriem nádorových buniek, ktoré sú schopné prežívať v imunokompetentnom hostiteľovi alebo vytvorením takých podmienok v rámci mikroprostredia nádoru, ktoré uľahčujú vznik a rast nádoru (Schreiber et al., 2011).

Nádorová imunoeditácia, inými slovami editácia nádoru imunitným systémom, je dynamický proces, ktorý sa skladá z troch fáz (Dunn et al., 2002; Vesely et al., 2011; Schreiber et al., 2011).

1) **Eliminácia transformovanej bunky (z angl. elimination) -** Vrodená (aktivované makrofágy, neutrofilné granulocyty, NK bunky) aj adaptívna (T a B lymfocyty) zložka imunitného systému sa zúčastňuje chránenia organizmu elimináciou vznikajúcich transformovaných buniek, ničia vznikajúce nádorové bunky ešte predtým, ako sú klinicky pozorovateľné. Ak je štádium eliminácie úspešne dokončené, organizmus ostáva bez nádorových buniek a proces editácie nádoru je týmto ukončený a neprechádza do ďalšieho štádia.
2) **Rovnováha medzi transformovanou bunkou a organizmom (z angl. equilibrium) -** Nádorové bunky, ktoré však prežijú a nie sú zničené v procese eliminácie, vstupujú ďalej do fázy equilibria, kde adaptívna imunita bráni ich rastu. T bunky, IL-12 a IFN-γ sú potrebné pre udržanie nádorových buniek v štádiu funkčného pokoja. Equilibrium je fázou kontroly nádorovej imunogenicity. Predstavuje tiež štádium pokoja nádoru, v ktorom je rast skrytých nádorových buniek zreteľne kontrolovaný imunitným systémom. Toto štádium predstavuje aj najdlhšiu fázu editácie nádoru imunitným systémom. Equlibrium môže tiež predstavovať konečnú fázu nádorovej imunoeditácie a môže v ňom dôjsť k potlačeniu rastu skrytých nádorových buniek organizmu.
3) **Únik transformovanej bunky pred dohľadom imunitného systému (z angl. escape) -** Prechod zo štádia equilibria do štádia úniku sa deje v dôsledku toho, že

populácia nádorových buniek sa zmení pod tlakom funkcie imunitného systému alebo hostiteľský imunitný systém sa zmení v dôsledku narastajúceho imunosupresívneho prostredia nádoru alebo v dôsledku celkového oslabenia imunitného systému. Môžeme teda povedať, že ako dôsledok stáleho tlaku imunitného systému na geneticky nestabilné nádorové bunky vo fáze equilibria, môžu vznikať nové nádorové varianty, ktoré sa vyznačujú zníženou imunogenicitou, nie sú pozorované zložkami adaptívnej imunity kvôli strate antigénu alebo v dôsledku porúch spracovania a prezentácie antigénu. Môže dôjsť k tvorbe imunosupresívneho stavu v mikroprostredí nádoru. Takéto bunky môžu vstúpiť do fázy úniku, kde ich nárast nie je už blokovaný imunitným systémom. Rovnováha medzi nádorovými a imunitnými bunkami je narušená tvorbou takých variantov, ktoré sa úplne vymknú z kontroly imunitného systému. Takéto nádorové bunky spôsobujú klinické nádorové ochorenie.



Obr. č. 1 **Teória protinádorového dohľadu, editácia nádoru imunitným systémom a klinické porovnanie jednotlivých štádií.**

Upravené podľa Schreiber et al, Science 2011

Popis obrázku:

Nádorová bunka je vo veľkej väčšine rozpoznaná v skorých štádiách nádorovej transformácie a je zničená imunitným systémom. Proces editácie nádoru imunitným systémom môže takto skončiť alebo pokračovať do ďalšej fázy. Ak niektoré nádorové bunky ďalej prežívajú, môže dôjsť k nastoleniu dynamickej rovnováhy medzi hostiteľským imunitným systémom a prežívajúcimi nádorovými bunkami. Tieto nádorové bunky majú

čoraz väčšiu prispôsobivosť, ktorá vyplýva z rastúcej genetickej instability a pod týmto tlakom vznikajú nové genotypy s menšou imunogenicitou až nakoniec môže dôjsť k úniku takejto nádorovej bunky pred dohľadom imunitného systému. Nastolenie rovnováhy je najdlhším procesom a v tomto štádiu ostávajú prežívajúce nádorové bunky najčastejšie nediagnostikované. Takto môžeme rozdeliť proces editácie nádoru imunitným systémom do troch fáz: Úplná eliminácia nádorových buniek; Dlhodobé udržiavanie dynamickej rovnováhy medzi imunitným systémom a prežívajúcimi nádorovými bunkami; Únik pred imunitným dohľadom a rozvoj klinicky diagnostikovateľného ochorenia (Dunn et al., 2002).

3. Únik nádorových buniek imunitnému systému

Únik nádorových buniek imunitnému systému predstavuje zlyhanie imunitného systému eliminovať alebo kontrolovať nádorové bunky, čo im dovoľuje rásť v imunologicky nekontrolovaných podmienkach. Nádorové bunky podstupujú genetické a epigenetické zmeny, čo množí modifikácie potrebné k obídeniu vrodenej a adaptívnej imunitnej obrany. Navyše, imunitný systém prispieva k progresii nádoru selekciou viac agresívnych foriem nádorových buniek, čo naopak utlmuje protinádorovú imunitnú odpoveď a podporuje proliferáciu nádorových buniek. Nádorové bunky majú vyvinuté viaceré mechanizmy ako unikať imunitnému dohľadu. Môžeme ich nasledovne rozčleniť na autonómne modifikácie buniek na úrovni tých nádorových buniek, s tým, že nádorová bunka dokáže priamo uniknúť detekcii imunitným systémom a deštrukcii a na modifikácie imunitných buniek, ktoré sú postihnuté imunosupresívnym prostredím tvoreným nádorovými bunkami (Vesely et al., 2011). Medzi hlavné mechanizmy úniku nádorových buniek pred rozpoznaním bunkami imunitného systému patria zníženie alebo strata povrchovej expresie MHC molekúl I triedy, adhezívnej molekuly CD54 (z angl. intercellular adhesion molecule 1; ICAM1) alebo kostimulačných molekúl CD80 a CD86 z rodiny B7. CD54 je prezentovaná v nízkej koncentrácii na povrchu endoteliálnych buniek a leukocytov. Po stimulácii cytokínmi koncentrácia stúpa. CD54 je ligand pre LFA-1 (z angl. lymphocyte function-associated antigen 1) (Rothlein, 1986). LFA1 patrí do rodiny leukocytárnych integrínov, transmembránových receptorov. Po aktivácii dôjde k väzbe medzi leukocytmi a endoteliálnymi bunkami prostredníctvom CD54/LFA-1 a migrujú cez tkanivo, cez tzv. transendoteliálnu migráciu leukocytov (z angl. leukocyte transendothelial migration; TEM). Toto je kľúčovým krokom, aby sa leukocyty dostali do miesta zápalu (Yang et al., 2005). Významnú úlohu v boji proti nádorom plní aj cytokín IFN-γ. IFN-γ podporuje produkciu antigén-špecifických $CD4^+$ TH1 buniek a cytotoxických T lymfocytov (CTL), umožňuje aktiváciu dendritických buniek a makrofágov a tiež utlmuje nádorom-

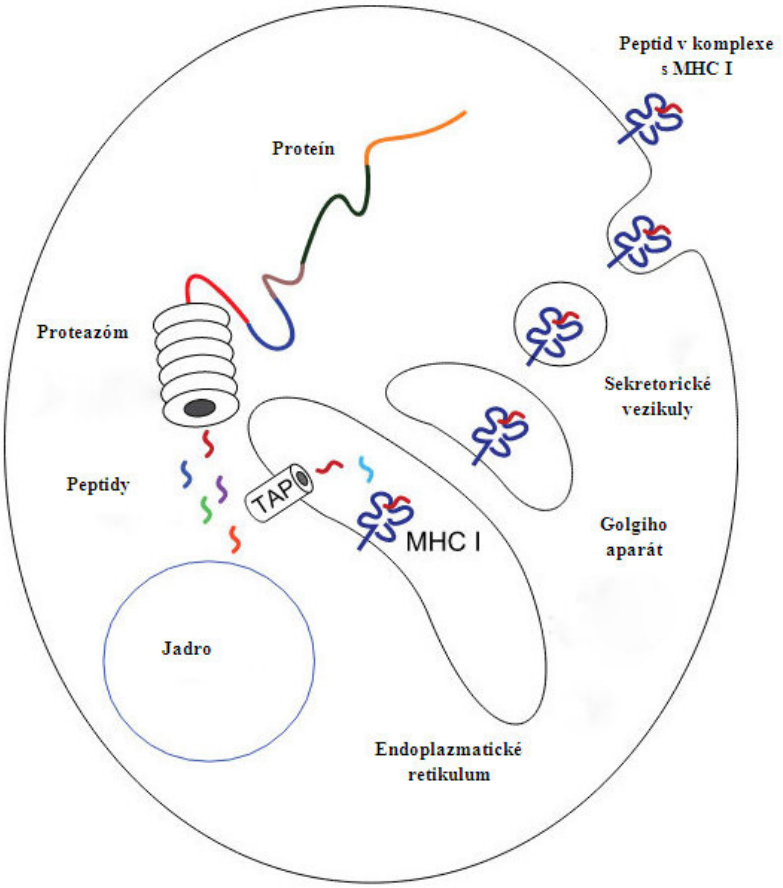
indukovanú angiogenézu (Hayakawa et al., 2002). Môžeme tiež povedať, že nádorové bunky sú cieľom účinku IFN-γ, pretože tento cytokín navodzuje zvýšenie expresie zložiek spracovania a prezentácie antigénu.

Medzi ďalšie mechanizmy úniku nádorových buniek pred rozpoznaním bunkami imunitného systému patria indukcia expresie inhibičných molekúl, produkcia inhibičných cytokínov, zvýšená tvorba indolamín 2,3- dioxygenázy a nereagovanie na apoptotické signály a tiež schopnosť ovplyvniť mikroprostredie nádoru smerom k zvýšeniu hladiny komponentov s imunosupresívnym účinkom, ako sú regulačné T-lymfocyty, myeloidné supresorové bunky alebo nezrelé dendritické bunky.

3.1. Poruchy spracovania a prezentácie antigénu nádorovými bunkami- znížená povrchová expresia molekúl MHC I ako spôsob úniku nádorových buniek imunitnému systému

Efektívne spracovanie a prezentácia nádorových antigénov je dôležitým faktorom v imunitnom dohľade (Setiadi et al., 2005). Rozpoznanie nádorových buniek bunkami efektorovými sa deje pomocou spracovania a prezentácie endogénnych nádorových antigénov v komplexe s molekulami MHC I. Bunkové proteíny sú degradované v proteazóme, pomocou podjednotiek LMP-2 a LMP-7, v cytoplazme nádorových buniek. Peptidy sú následne transportované do lumenu endoplazmatického retikula pomocou transportérov TAP-1 a TAP-2, kde každý peptid tvorí komplex s β-2-mikroglobulinom a ťažkým reťazcom MHC I. Tento proces je podporený proteínmi z triedy chaperónov (calnexin, calreticulin, tapasin). Takýto komplex je následne transportovaný na bunkový povrch a rozpoznaný cytotoxickými T-lymfocytmi, ktoré zabijú takú bunku, ktorá prezentuje nie vlastný antigén (Setiadi et al., 2005). Štúdie ukazujú, že nádorové bunky majú narušené komponenty dráhy spracovania a prezentácie antigénu (Alimonti et al., 2000; Seliger et al., 2000) a takto unikajú detekcii imunitným systémom (Vesely et al., 2011). Ide najmä o stratu transportérov TAP-1, TAP-2, molekúl MHC I, beta-2-microglobulinu (β2m), podjednotiek protezómu LMP-2, LMP-7 a rozvoj necitlivosti k IFN-γ alebo IFN-α/β, čím sa bránia nádorové bunky eliminácii prostredníctvom T buniek, čo v konečnom dôsledku umožňuje rast nádoru (Jäger et al., 1996; Restifo et al., 1996; Khong et al., 2004; Dunn et al., 2006). Môže dôjsť až k tomu, že nádorové bunky stratia schopnosť odpovedať na IFN-γ cez mutácie alebo epigenetické umlčanie génov kódujúcich komponenty signalizačnej dráhy IFN-γ (IFN-γR1, IFN-γR2, JAK1, JAK2 a STAT1) (Dunn et al., 2005). V tomto prípade, postihnuté nádorové

bunky zlyhávajú v regulácii molekúl MHC I na povrchu bunky, ale aj v produkcii a regulácii komponentov mašinérie spracovania a prezentácie antigénu (TAP-1, TAP-2, LMP-2, LMP-7) (Vesely et al., 2011). Nízka expresia alebo chýbanie molekúl TAP-1 alebo TAP-2 je bežným javom pri mnohých nádorových ochoreniach (Gabathuler et al., 1994; Seliger et al., 1997; Ritz and Seliger, 2001). Táto skutočnosť narúša formáciu komplexu v lumene endoplazmatického retikula a výsledkom je chýbanie MHC I na povrchu bunky, tým pádom špecifické cytotoxické T-lymfocyty nie sú schopné rozpoznať a zničiť mnoho nádorových buniek (Seliger et al., 1997).



Obr. č. 2 **Dráha MHC I. triedy.**

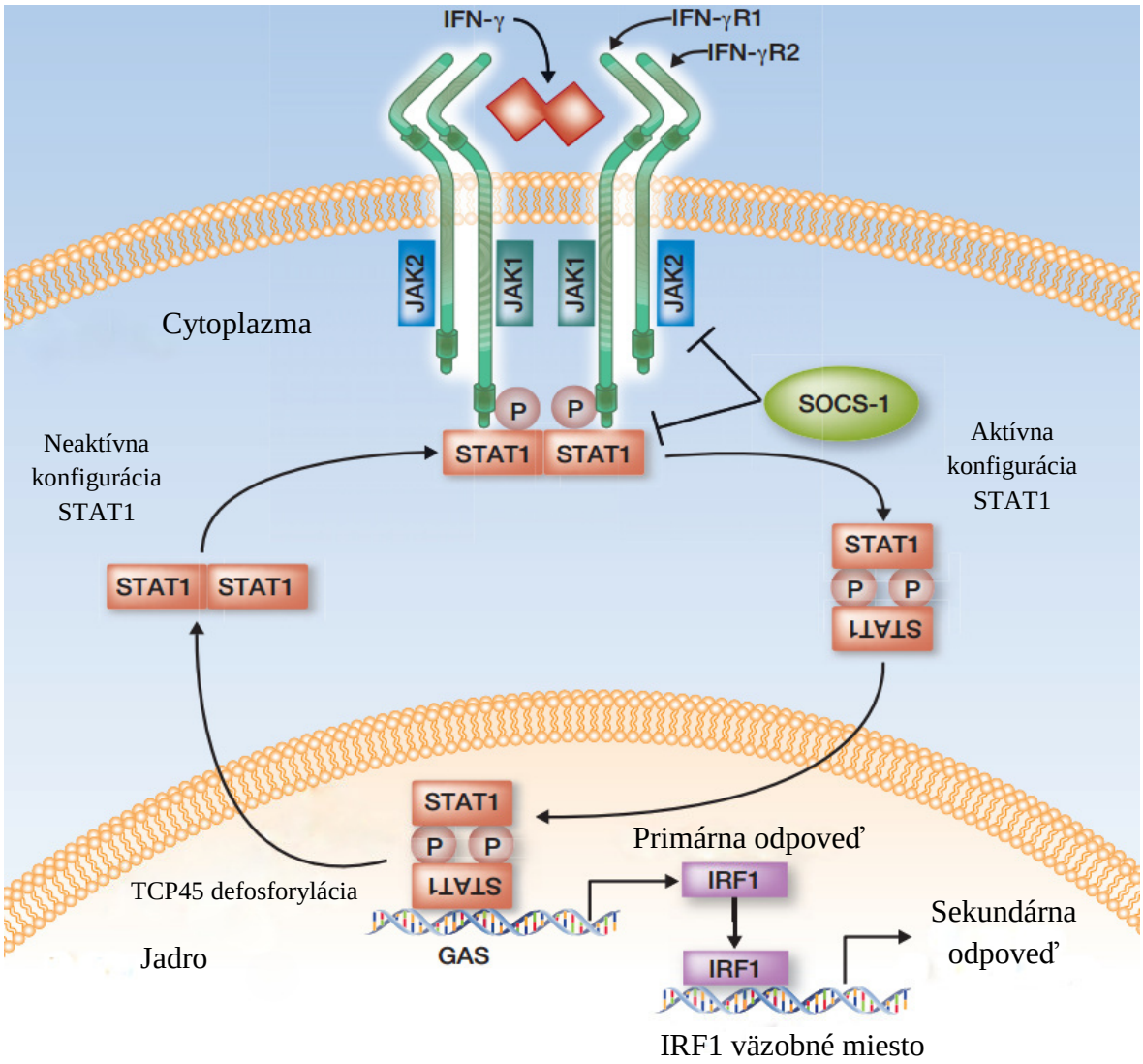
Upravené podľa: http://en.wikipedia.org/wiki/File:MHC_Class_I_processing.svg

Popis obrázku:

Zjednodušený diagram degradácie cytosolických proteínov v proteazómoch, transportu peptidov do endoplazmatického retikula pomocou molekúl TAP, tvorby komplexu s MHC I a jeho transportu na povrch bunky za účelom prezentácie antigénu.

3.1.1. Signalizačná dráha IFN-γ a jeho účinok na povrchovú expresiu MHC I

Interferóny sú pleiotropné cytokíny, ktoré majú významnú úlohu v intracelulárnej komunikácii počas vrodenej a adaptívnej imunitnej odpovede a obrany proti vírusovým a bakteriálnym infekciám a tiež aj v protinádorovom dohľade. Pri cicavcoch rozdeľujeme tieto cytokíny do troch hlavných kategórií, typ I, typ II, typ III. Jednotlivé kategórie sa podstatne líšia v imunomodulačných vlastnostiach. Do skupiny I patria IFN-α a IFN-β. Ich signalizácia prebieha cez receptor typu I a sú stálo exprimované. Jediným členom interferónov typu II je IFN-γ. Do tretej kategórie interferónov patria interferóny λ (Bartlett et al., 2005). IFN-γ a jeho expresia je viac obmedzená v porovnaní s IFN typu I. Je štruktúrne a aj funkčne odlišný od interferónov typu I a má vlastný receptor, ktorý pozostáva z podjednotiek IFN-γR1 a IFN-γR2. Biologicky aktívna forma IFN-γ je v stave diméru, ktorý interaguje s extracelulárnou doménou podjednotky IFN-γR1 receptora. Väzba ligandu aktivuje podjednotku IFN-γR2 receptora, ktorá je zodpovedná za intracelulárny prenos signálu. Kinázy JAK1 a JAK2 fosforylujú receptor po väzbe ligandu. Táto fosforylácia tvorí väzobné miesto pre proteíny STAT, v prvom rade STAT1. Po jeho fosforylácii sa aktívny homodimér STAT1 presúva do jadra, kde sa viaže na miesta aktivované IFN-γ (z angl. interferon gamma activated sites; GAS) v oblasti promótora génov, ktoré sú regulované IFN-γ. Jeden z hlavných faktorov, ktoré odpovedajú na signalizáciu IFN-γ je faktor IRF1. IRF1 ďalej aktivuje ďalšie gény v rámci sekundárnej odpovede. Homodimér STAT1 sa vyskytuje v cytoplazme v neaktívnej konfigurácii. Fosforylácia vedie k zmene konfigurácie, čo odhalí jadrový lokalizačný signál, čím je umožnený presun do jadra. Intracelulárna defosforylácia fosfatázou TCP45 inaktivuje STAT1 homodimér a spôsobí návrat neaktívneho STAT1 homodiméru do cytoplazmy. Dráhu IFN-γ/JAK/STAT negatívne reguluje supresor cytokínovej signalizácie (SOCS), ktorý blokuje aktivitu Janusových kináz (Zaidi and Merlino, 2011).



Obr. č. 3 **Dráha IFN-γ.**

Upravené podľa Zaidi and Merlino, Clinical Cancer Research 2011

Popis obrázku:

Biologicky aktívna forma IFN-γ je v stave diméru, ktorý interaguje s extracelulárnou doménou podjednotky IFN-γR1 receptora. Väzba diméru IFN-γ aktivuje podjednotku IFN-γR2 receptora, ktorá je zodpovedná za intracelulárny prenos signálu. Kinázy JAK1 a JAK2 fosforylujú receptor po väzbe ligandu. Táto fosforylácia tvorí väzobné miesto pre STAT1. Po jeho fosforylácii neaktívny homodimér STAT1 zmení konfiguráciu, stáva sa aktívnym a presúva sa do jadra, kde sa viaže na miesta aktivované IFN-γ (z angl. interferon gamma activated sites; GAS) v oblasti promótora génov, ktoré sú regulované IFN-γ. Jeden z hlavných faktorov, ktoré odpovedajú na signalizáciu IFN-γ je faktor IRF1. IRF1 ďalej aktivuje ďalšie gény v rámci sekundárnej odpovede. Intracelulárna defosforylácia fosfatázou TCP45 inaktivuje STAT1 homodimér a spôsobí návrat neaktívneho STAT1 homodiméru do cytoplazmy. Dráhu IFN-γ/JAK/STAT negatívne reguluje supresor cytokínovej signalizácie (SOCS), ktorý blokuje aktivitu a fosforyláciu Janusových kináz a STAT1 (Zaidi and Merlino, 2011).

Ako už bolo spomenuté, IFN-γ patrí medzi rodinu IFN typu II, pre jeho vzdialenú sekvenčnú homológiu s rodinou IFN typu I a jeho produkciou pomocou NK buniek a aktivovaných T buniek (Borden et al., 2007). Reverzibilné zníženie expresie MHC I na povrchu nádorových buniek je často spojené s koordinovaným znížením génov antigén-prezentujúcej mašinérie (Seliger et al., 2000; Garcia-Lora et al., 2003) cez epigenetické umlčanie týchto génov. Expresia génov antigén-prezentujúcej mašinérie môže byť obnovená pomocou IFN-γ (Gabathuler et al., 1994; Seliger et al., 2000) a tiež pomocou inhibítorov DNA metyltransferáz, 5-aza-2'-deoxycytidinom (DAC) alebo 5-azacytidinom (5-azaC, 5AC), čo sú cytostatické agensy s epigenetickými účinkami (Manning et al., 2008). Zvyšujú spracovanie a prezentáciu antigénu, zvyšujú expresiu génov antigén-prezentujúcej mašinérie *TAP1/2*, *LMP2/7*. Zvýšenie expresie MHC I molekúl na povrchu buniek je jedna z najlepšie charakterizovaných funkcií IFN-γ. Zvyšujú tým nádorovú imunogenicitu. Zvýšenie expresie MHC I molekúl na povrchu buniek umožňuje rozpoznanie a elimináciu nádorových buniek cytotoxickými T-lymfocytmi, ktoré sa dostanú k nádorom cez chemokínovú signalizáciu sprostredkovanú IFN-γ (Zaidi and Merlino, 2011). Ošetrenie TAP-1 deficientných buniek pomocou IFN-γ zvyšuje acetyláciu histónu 3 a aktívnu transkripciu génu *TAP-1*. Takto Setiadi a kol. (Setiadi et al., 2007) popísali nový mechanizmus, ktorým IFN-γ zvyšuje expresiu génu TAP-1. Christova a kol. (Christova et al., 2007) popísali zmeny v štruktúre chromatínu celého lokusu MHC I, ktorá bola indukovaná IFN-γ. V tejto oblasti sa nachádzajú gény rodiny *TAP* a aj *LMP*. Je veľmi málo známe o DNA demetylácii v rámci regulácie génov sprostredkovanej IFN-γ. Doposiaľ bolo zistené, že indukcia expresie indolamín 2,3-dioxygenázy 1 (IDO1) je spojená s DNA demetyláciou oblasti promótora génu pre *IDO1* (Xue et al., 2012). Zaujímavé zistenie tiež je, že aj ďalšie cytokíny dokážu navodiť demetyláciu DNA, konkrétne TGF-β spôsobil aktívnu demetyláciu DNA a expresiu tumor-supresorového génu $p15^{ink4b}$ (Thillainadesan et al., 2012). Avšak, podobne ako aj TGF-β alebo TNF-α, aj IFN-γ môže mať duálne kontrastné účinky a môže pôsobiť pronádorovo. Bolo popísané, že IFN-γ umožňuje vývoj regulačných T-lymfocytov a tiež môže utlmovať cytotoxické T-lymfocyty indukciou indolamín 2,3-dioxygenázy v melanómových bunkách. Monocytárne (Mo-MDSC) a granulocytárne (G-MDSC) myeloidné supresorové bunky sú tiež závislé na IFN-γ a ich prítomnosť v mikroprostredí nádoru utlmuje odpoveď T buniek. Závisí od súvislostí nádorovej špecificity, faktorov v mikroprostredí nádoru a intenzity signalizácie, ktoré účinky IFN-γ prevládnu, či tie protinádorové alebo pronádorové (Zaidi and Merlino, 2011).

3.2. Supresia imunitného systému sprostredkovaná mikroprostredím nádoru

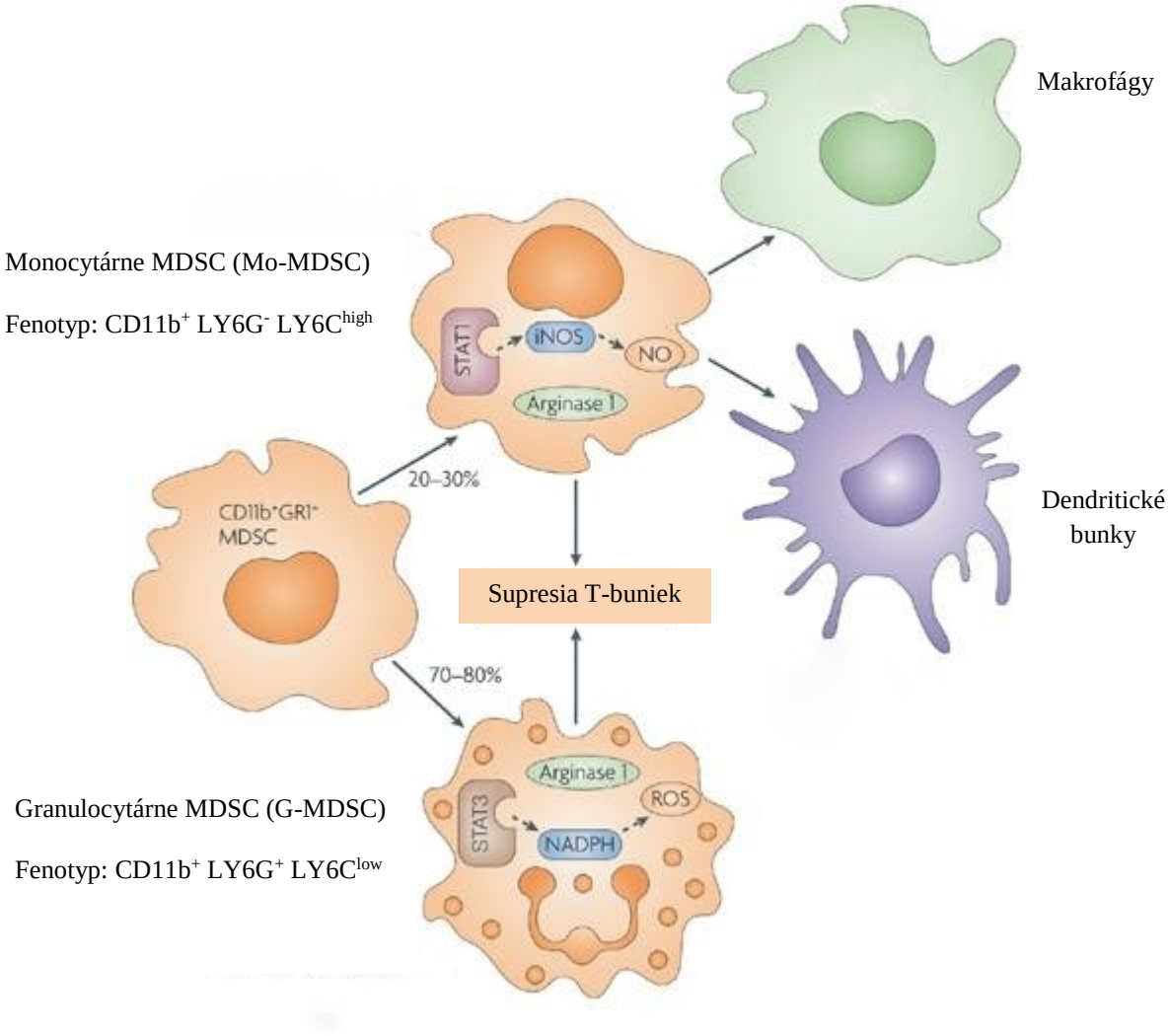
Únik nádorových buniek môže byť sprostredkovaný tvorbou imunosupresívneho stavu v rámci mikroprostredia nádoru (Radoja et al., 2000; Radoja and Frey, 2000). Nádorové bunky sú schopné produkovať imunosupresívne faktory a tieto majú ďalší vplyv na funkciu imunitného systému (Chambers et al., 2003). Sú to napríklad imunosupresívne faktory ako vaskulárny endoteliálny rastový faktor (VEGF), transformujúci rastový faktor (TGF-β), galectin alebo indolamín 2,3-dioxygenáza (IDO) (Vesely et al., 2011). Regulačné T-lymfocyty a myeloidné supresorové bunky sú dôležité imunosupresívne populácie buniek, ktoré majú významnú úlohu v inhibícii ochrannej protinádorovej odpovede (Schreiber et al., 2011). Nádorom indukovaná imunosupresia patrí medzi kritické mechanizmy, akými nádory unikajú imunitnému dohľadu.

3.2.1. Produkcia inhibičných cytokínov a rastových faktorov

Funkcie jednotlivých bunkových populácií v mikroprostredí nádoru sú výrazne potlačené. VEGF (z angl. vascular endothelial growth factor), ktorý môže byť produkovaný nádorovými bunkami, inhibuje diferenciáciu a maturáciu dendritických buniek. Ďalšími inhibične pôsobiacimi faktormi, ktoré produkujú nádorové bunky, sú IL-10 a TGF-β. Utlmujú napríklad diferenciáciu T-buniek, inhibujú produkciu cytokínov, znižujú schopnosť prezentácie antigénu, znižujú expresiu kostimulačných molekúl, a narúšajú cytolýzu efektorovými bunkami (Chambers et al, 2003). Nádorové bunky tvoria tiež prostaglandíny. Tie utlmujú proliferáciu T a B buniek a inhibujú NK bunky. Indukujú expresiu IL-10 (Huang et al, 1996). Prostaglandíny ďalej zvyšujú expresiu anti-apoptotických faktorov, napríklad Bcl-2 (Sheng et al., 1998).

3.2.2. MDSC-myeloidné supresorové bunky

Myeloidné supresorové bunky sú heterogénna populácia nediferencovaných buniek akumulovaná počas rastu nádorov, ktoré sú schopné inhibovať odpoveď T-buniek (Gabrilovich and Nagaraj, 2009). Inhibujú funkciu lymfocytov indukciou regulačných T-lymfocytov, produkciou TGF-β, depléciou alebo vychytávaním aminokyselín arginínu, tryptofánu alebo cysteínu potrebných pre správnu funkciu T buniek a nakoniec nitráciou receptora T buniek alebo receptorov chemokínov na nádor-špecifických T bunkách (Schreiber et al., 2011). U myší ich charakterizuje marker monocytov (CD11b) a neutrofilov (Gr-1).



Obr. č. 4 **Rozdelenie MDSC.**

Upravené podľa Gabrilovich and Nagaraj, Nature Reviews Immunology 2009

Popis obrázku:

Populáciu MDSC rozdeľujeme na monocytárnu (Mo-MDSC) a granulocytárnu (G-MDSC). G-MDSC zvyšujú hladinu STAT3 (z angl. signal transducer and activator of transcription 3), čo spôsobuje zvýšenú tvorbu ROS, ktoré posttranslačne modifikujú receptor T-buniek. Táto skutočnosť je zodpovedná za neodpovedanie na antigén. Minoritné Mo-MDSC spôsobujú zvýšenú expresiu STAT1, iNOS (z angl. inducible nitric oxide synthase) a zvýšenú hladinu NO. NO je produkovaný cez metabolizáciu L-arginínu prostredníctvom iNOS, čo utlmuje funkciu a proliferáciu T-buniek, cez viaceré mechanizmy: inhibíciou JAK3 (z angl. Janus kinase 3) a STAT5, inhibíciou expresie MHC II, indukciou apoptózy T-buniek. Iba táto populácia môže v konečnom dôsledku diferencovať na makrofágy alebo dendritické bunky. Obe populácie produkujú vo zvýšenej miere arginázu-1, čo vedie k supresii T-buniek kvôli deplécii L-arginínu (Gabrilovich and Nagaraj, 2009).

3.2.3. Regulačné T-lymfocyty

Regulačné T-lymfocyty sú definované ako $CD4^+$, $CD25^+$ T bunky, ktoré konštitutívne exprimujú transkripčný faktor FoxP3 (Hori et al., 2003). Patria do skupiny populácie leukocytov s imunosupresívnymi vlastnosťami. Po stimulácii inhibujú funkciu cytotoxických T-lymfocytov pomocou expresie koinhibičných molekúl ako, CTLA-4, PD-1 a PD-L1 a produkciou imunosupresívnych cytokínov ako, IL-10 a TGF-β a odstránením cytokínu IL-12, ktorý je kritický pre udržovanie funkcie cytotoxických T-lymfocytov (Schreiber et al., 2011).

3.2.4. Mikroprostredie nádoru a jeho ovplyvnenie chemoterapiou

V súčasnosti sa vyvíja čoraz väčšia snaha ustanoviť vhodnú chemoterapiu pomocou diferenciačných liečiv, prednostne pri použití v kombinácii s ďalšími liečivami pre dosiahnutie nízkeho imunosupresívneho rozsahu mikroprostredia nádoru. Cyklofosfamid (CY) je používaný ako alkylačné cytostatikum, chemoterapeutické činidlo. Okrem priameho cytotoxického účinku CY slúži na podporovanie adaptívnej a aj vrodenej imunity (Bass and Mastrangelo, 1998; Lake and Robinson, 2005). CY v nižších dávkach ako sa zvyčajne používa, môže podporiť protinádorovú imunitu zničením regulačných T-lymfocytov (Treg buniek) (Lake and Robinson, 2005). Naopak, vyššie dávky CY podporujú akumuláciu MDSC (Angulo et al., 2000; Peláez et al., 2001; Diaz-Montero et al., 2009). Snahou imunoterapie MDSC, je navodiť ich diferenciáciu do maturovaných myeloidných buniek, ktoré už nebudú vykazovať supresívne účinky. Tkanivá obsahujú časť buniek, ktoré sú schopné sa deliť a obnovovať. Časť buniek tkanív môže diferencovať. Proces diferenciácie vedie k terminálne diferencovanej bunke, ktorá sa nemôže ďalej deliť. Preto indukcia diferenciácie nediferencovaných buniek s imunosupresívnym účinkom môže byť dobrým terapeutickým kľúčom. V súčasnosti sa spomína all-trans-retinová kyselina (ATRA), metabolit vitamínu A, ktorá dokáže eliminovať MDSC u pacientov s nádorovým ochorením, dokáže navodiť diferenciáciu MDSC (Almand et al., 2001; Gabrilovich et al., 2001). Táto diferenciácia je sprostredkovaná cez zníženú produkciu ROS. ATRA by teda mohla byť používaná ako diferenciačné činidlo pre MDSC a eliminácia MDSC pomocou kyseliny ATRA by mohla zlepšiť efekt liečby pacientov s nádorovým ochorením. 5-azacytidin znižuje expresiu Arg-1, čo je marker imunosupresívneho prostredia a hlavným producentom Arg-1 sú MDSC (Zea et al., 2005). Aj cytokín IL-12 indukuje diferenciáciu MDSC na zrelé antigén-prezentujúce bunky (dendritické bunky), čo môže tiež ovplyvniť akumuláciu MDSC a ich funkciu. Štúdie naznačujú, že MDSC potlačujú produkciu IL-12, čo by mohlo znamenať,

že IL-12 má funkciu v regulácii aktivity MDSC. Ošetrenie s IL-12 mení supresívnu funkciu MDSC a znižuje percento MDSC v mikroprostredí nádoru (Steding et al., 2011).

3.3. Eliminácia imunitných efektorových buniek

Eliminácia imunitných efektorových buniek v mikroprostredí nádoru môže prebiehať cez aktiváciu indukovanej programovanej smrti (z angl. activation-induced cell death; AICD). Ide o indukciu apoptózy aktivovaných T-buniek. Stimulácia T-buniek spôsobí zvýšenú hladinu povrchového ligandu smrti, ktorý tvorí väzbu s receptorom na tej istej alebo susediacej bunke. AICD je sprostredkovaná vo veľkej miere cez spoluprácu medzi receptorom Fas (CD95) a ligandom Fas ligand (FasL; CD95L), ale často je vyžadovaná signalizácia cez TNF alebo ligandy TRAIL (z angl. TNF-related apoptosis-inducing ligand) (Zheng et al., 1995; Martinez-Lorenzo et al., 1998). AICD je zodpovedná za zvýšenú mieru apoptózy v rámci nádor infiltrujúcich lymfocytov (Radoja et al., 2001). Ak nádorové bunky exprimujú FasL a dôjde k interakcii s lymfocytom cez receptor Fas, indukuje sa apoptóza daného nádor infiltrujúceho lymfocytu (O'Connell et al., 1996; O'Connell et al, 1999). Nádorové bunky teda používajú ten istý mechanizmus, ktorý bežne používajú T bunky v boji proti nádorovým bunkám.

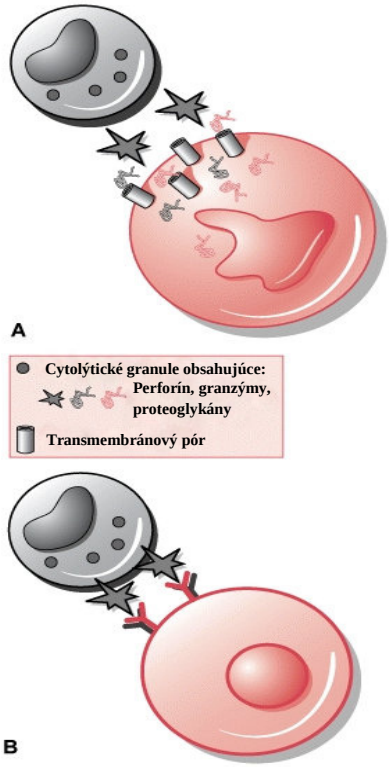
3.4. Poruchy v signalizačných dráhach imunitných buniek

Imunitné bunky pacientov s nádorovými ochoreniami často vykazujú porušenú signalizáciu jadrového faktora κB (NFκB). Dráha NFκB je veľmi dôležitá pre expresiu inhibítorov bunkovej smrti, ako Bcl2 alebo BclXL (Tamatani et al., 1999), inými slovami sprostredkuje signály pre prežitie bunky. Ak je táto dráha narušená, dochádza vo zvýšenej miere k bunkovej smrti imunitných buniek.

3.5. Mechanizmus rezistencie nádorových buniek na cytotoxicitu T buniek

Cytotoxické T-lymfocyty poskytujú veľmi účinnú obranu proti infekciám a intracelulárnym patogénom. Ich lýtická mašinéria však môže byť namierená aj proti bunkám hostiteľského organizmu pri autoimunitných ochoreniach (Barry and Bleackley, 2002) alebo pri nádorových ochoreniach. Efektorové bunky sprostredkujú lýzu cieľových nádorových buniek cez viaceré mechanizmy: 1. Exocytózou obsahu cytoplazmatických granúl, napríklad cytolyzínu alebo granzýmov z rodiny serínových proteáz, ktoré sú schopné indukovať nekrotickú bunkovú smrť alebo indukovať apoptózu. 2. Indukciou apoptózy cez väzbu ligandu Fas na bunkách imunitného systému s receptorom Fas na bunkách nádorových. 3. Uvoľňovaním rozpustných toxínov, ktoré sa viažu na príslušný

receptor na nádorových bunkách a následnou indukciou apoptózy (Chambers et al, 2003). Rezistenciu nádorových buniek k apoptóze si nádorové bunky môžu vyvinúť cez zmeny komponentov apoptotickej dráhy. Takéto zmeny v konečnom dôsledku vyselektujú nádorové bunky, ktoré sú odolné k lýze cez cytotoxické T-lymfocyty a dokážu prežiť pod tlakom imunitného systému.



Obr. č. 5 **Expresia molekúl, ktoré bránia tvorbe transmembránového póru na nádorových bunkách cez perforín efektorových buniek a porucha vstupu granzýmu do nádorových buniek a tým pádom porucha indukcie apoptózy nádorových buniek.**

Upravené podľa Chambers et al, in: Cancer Medicine. 6th edition 2003

Popis obrázku:

A. Imunitné efektorové bunky vypustia obsah cytolytických granúl, ktoré formujú transmembránové póry na plazmatickej membráne nádorových buniek, čo napomáha vstupu molekúl, ktoré následne indukujú apoptózu.

B. Niektoré nádorové bunky exprimujú vysokú hladinu molekúl, ktoré naopak blokujú aktivitu komponentov cytolytických granúl vytvárajúcich transmembránový pór (Chambers et al, 2003).

3.6. B7 rodina-kostimulácia vs. koinhibícia

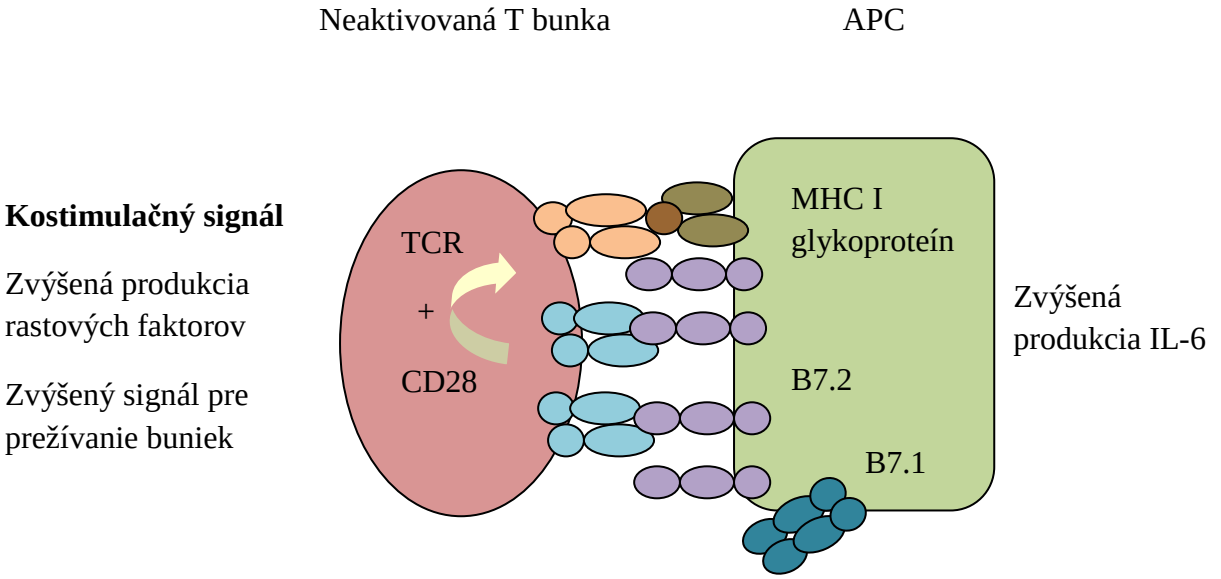
Ligandy z rodiny B7 sa viažu na receptory na lymfocytoch a regulujú imunitnú odpoveď. Aktivácia T a B lymfocytov prebieha cez spojenie antigénu s receptorom, antigén-

špecifickým T alebo B bunkovým receptorom. K tejto aktivácii je súčasne vyžadovaný aj druhý signál, ktorý prichádza zo strany ligandov rodiny B7. Tento kostimulačný signál je sprostredkovaný ligandami B7 cez rodinu receptorov CD28 na povrchu lymfocytov. Spomínaná interakcia členov rodiny B7 zvyšuje imunitnú odpoveď. Poznáme 7 členov rodiny B7: B7.1 (CD80), B7.2 (CD86), inducibilný kostimulačný ligand (z angl. inducible costimulator ligand; ICOS-L alebo aj B7RP1- z angl. B7-related protein-1), ligand 1 programovanej smrti (z angl. programmed death-1 ligand; PD-L1 alebo aj B7-H1 či CD274), ligand 2 programovanej smrti (z angl. programmed death-2 ligand; PD-L2), B7-H3 a B7-H4. Pri ich mutáciách sa vyskytujú imunodeficientné a autoimunitné ochorenia (Collins et al., 2005). Všetky ligandy B7 sú exprimované antigén-prezentujúcimi bunkami, ako sú dendritické bunky, makrofágy a B bunky. B7.1, B7.2 a PD-L2 sú exprimované lymfoidnými bunkami. PD-L1, B7-H3, B7-H4 a ICOS-L aj nelymfoidnými bunkami. PD-L2 je exprimovaný aj na dendritických bunkách a makrofágoch a PD-L1 je exprimovaný aj na B a T bunkách a tiež aj na nelymfoidných bunkách (Collins et al., 2005).

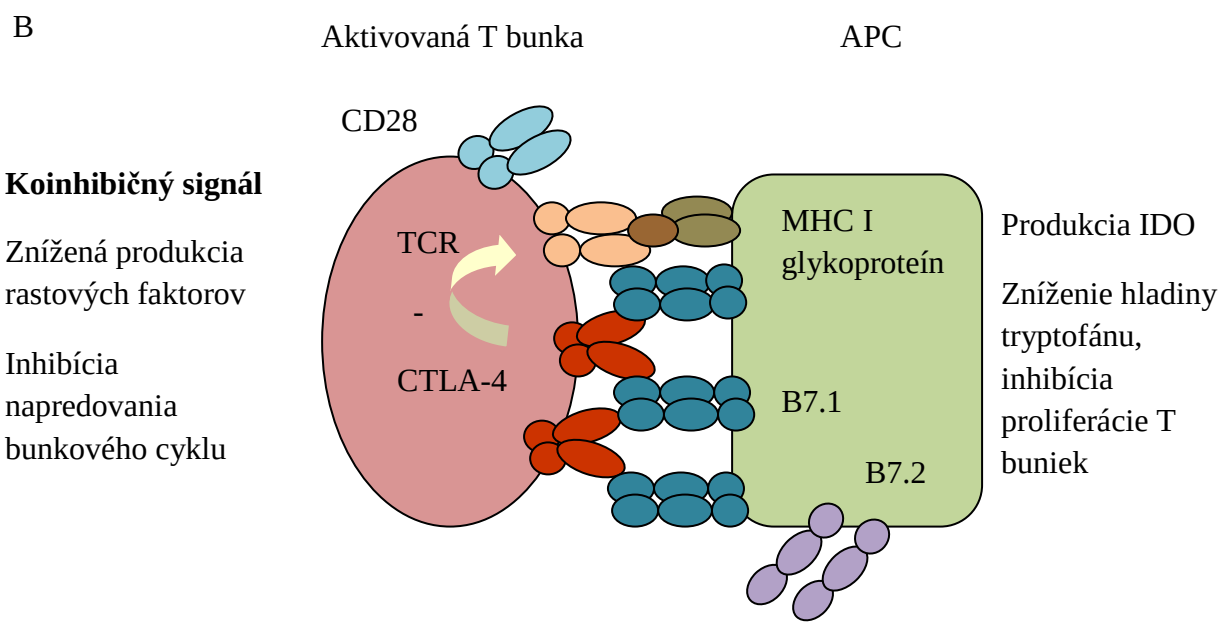
3.6.1. Nerovnováha medzi expresiou stimulačných a inhibičných členov z rodiny B7

Ligandy B7 poskytujú kostimulačný a koinhibičný signál po naviazaní antigénu na receptor na povrchu buniek. Koinhibičný signál utlmuje signalizáciu, zmierňuje odpoveď na väzbu antigén/receptor, znižuje aktiváciu buniek, inhibuje indukcie rastových faktorov, inhibuje prechod bunkovým cyklom a môže indukovať apoptózu. Kostimulačný signál podporuje aktiváciu, expanziu a diferenciáciu T buniek, čo umožňuje efektívnu imunitnú odpoveď (Collins et al., 2005). Interakcia medzi B7.1 a B7.2 cez receptor CD28 poskytuje kostimulačný signál, naopak interakcia cez receptor CD 152, alebo tiež CTLA-4 (z angl. cytotoxic T-Lymphocyte Antigen 4) poskytuje koinhibičný signál.

A



B



Obr. č. 6 **Kostimulácia a koinhibícia.**

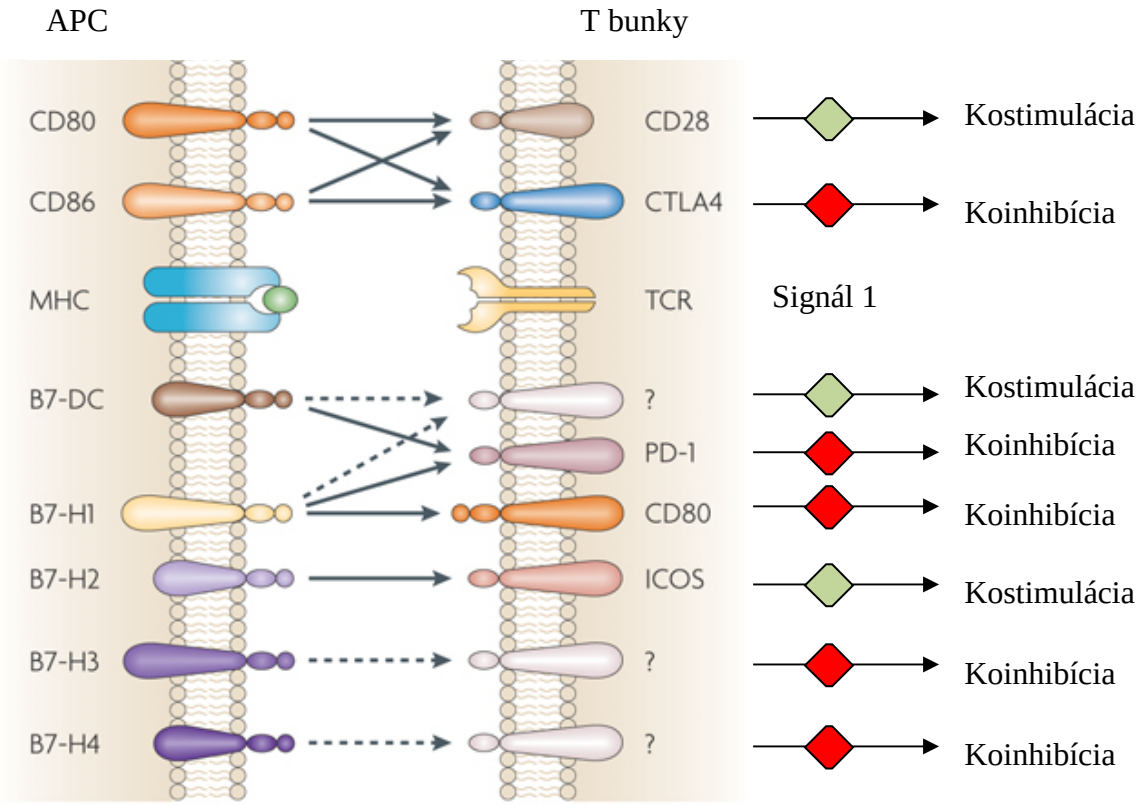
Upravené podľa Collins et al., Genome Biology 2005

Popis obrázku:

Väzba receptora CD28 alebo CTLA-4 na T bunkách s ligandami B7.1 a B7.2 na antigén-prezentujúcich bunkách (z angl. antigen-presenting cells; APC) môže viesť ku kostimulácii alebo ku koinhibícii, v závisloti od presného vzoru expresie génov pre receptory a ligandy a od stupňa aktivácie T buniek. **A.** CD28 je exprimovaný na neaktivovaných T bunkách a môže sa viazať aj s ligandom B7.1 alebo aj s ligandom B7.2 na APC. Na obrázku vidíme väzbu s B7.2, čo vedie k aktivácii neaktivovanej T bunky. Táto kostimulácia vedie

T bunky k zvýšenej tvorbe rastových faktorov a k zvýšenej tvorbe faktorov dôležitých pre prežívanie bunky. Bola popísaná aj tzv. reverzná signalizácia, kedy ligand na povrchu bunky môže plniť úlohu receptora na spúšťanie vnútrobunkových signálov v bunke, ktorá má na svojom povrchu daný ligand. Takáto reverzná signalizácia pri väzbe CD28 s ligandami B7.1 alebo B7.2 vedie antigén-prezentujúce bunky k produkcii IL-6. **B.** CD28 a CTLA-4 sú exprimované na aktivovaných T bunkách a oba receptory na T bunkách (z angl. T cell receptor; TCR) sa môžu viazať s B7.1 a B7.2 na APC. Na obrázku vidíme väzbu CTLA-4 s B7.1, ktorá vedie k utlmeniu aktivácie T buniek. Reverzná signalizácia CTLA-4 s ligandom B7.1 alebo B7.2 vedie antigén-prezentujúce bunky k produkcii indolamín 2,3- dioxygenázy (z angl. indoleamine 2, 3-dioxygenase; IDO) a k redukcii hladiny tryptofánu (Collins et al., 2005). Zvýšenú hladinu inhibičného receptora zvyčajne pozorujeme po aktivácii T buniek (Pardoll, 2012).

Receptor PD-1 viaže ligand B7-H1 (PD-L1, CD274) a B7-DC (PD-L2). Receptor je exprimovaný na B bunkách, $CD4^+$T bunkách, $CD8^+$T bunkách a na NKT bunkách. Ligand B7-H1 je konštitutívne exprimovaný na T, B bunkách, na makrofágoch a na dendritických bunkách ako aj na bunkách pankreasu, srdca a pľúc. Ligand B7-DC je limitovaný na dendritické bunky a makrofágy. Signál PD-1/ B7-H1, B7-DC je koinhibičný, ochraňuje pred autoimunitnými ochoreniami. Signalizácia je založená na tyrozínových doménach receptora PD-1. Po väzbe ligandu dôjde k fosforylácii tyrozínových domén, čo priláka špecifickú tyrozín-fosfatázu, ktorá poskytne inaktivačnú defosforylačnú signalizáciu, čo redukuje aktiváciu lymfocytov, zastavuje bunkový cyklus (Shi et al., 2013)**.** Bolo popísané, že ligand B7-H1 je exprimovaný myeloidnými bunkami v mikroprostredí nádoru (Curiel et al., 2003; Kuang et al., 2009; Liu et al., 2008).



Obr. č. 7 **Rodina ligandov B7 a prezentácia antigénu T bunkám.**

Upravené podľa Zou and Chen, Nature Reviews Immunology 2008

Popis obrázku:

Antigén-prezentujúce bunky (dendritické bunky, makrofágy a B bunky) prezentujú antigén v komplexe s molekulami MHC T bunkovému receptoru na T bunkách (z angl. T cell receptor; TCR). Prezentácia cez molekuly MHC II vedie k aktivácii $CD4^{+}$T lymfocytov a prezentácia cez molekuly MHC I umožňuje aktiváciu $CD8^{+}$T lymfocytov. Nutný je aj kostimulačný signál. Ligandy z rodiny B7 a ďalšie kostimulačné/koinhibičné molekuly kontrolujú alebo vylepšujú odpoveď T buniek. Napríklad molekuly B7-H1 alebo B7-H4 poskytujú negatívny signál, ktorý kontroluje a utlmuje odpoveď T buniek. Ľudské nádorové bunky a s nádorom asociované APC (z angl. antigen-presenting cells; APC) exprimujú limitované množstvo stimulačných ligandov z rodiny B7 CD80 (B7.1) a CD86 (B7.2) a vysokú hladinu inhibičných členov z rodiny B7 B7-H1 a B7-H4. Nerovnováha medzi expresiou stimulačných a inhibičných členov z rodiny B7 môže viesť k úniku nádorových buniek imunite v nádorovom mikroprostredí. CTLA4; cytotoxic T-lymphocyte antigen 4; ICOS, inducible T-cell co-stimulator; PD-1, programmed cell death 1. (Zou and Chen, 2008).

Tolerancia T buniek je jedným zo základných programov, ktorý zabraňuje imunitnému systému odpovedať na vlastný antigén, kde inhibičný signál zabezpečený cez receptor PD-1 vo väzbe s ligandom B7-H1 je veľmi dôležitý. Najnovšie štúdie dokazujú, že ligand B7-H1 sa viaže aj s molekulou CD80 a táto interakcia B7-H1/CD80 tiež T bunkám poskytuje inhibičný signál a reguluje indukciu a udržovanie T bunkovej tolerancie (Park et al., 2010).

Zdá sa, že ligand B7-H1 a jeho zvýšená expresia je jedným z mechanizmov úniku nádorových buniek imunitnému systému a terapeutické možnosti blokovania interakcií dráhy B7-H1/PD-1 sú intenzívne študované (McDermott and Atkins, 2013).

4. DNA metylácia-hlavný regulátor epigenómu

Epigenetika sa zaoberá dedičnými zmenami na bunkovej úrovni, napríklad profilom génovej expresie bunky, ktoré ale nie sú zapríčinené zmenami v nukleotidovej sekvencii DNA (Eccleston et al., 2007). Epigenetické zmeny plnia dôležitú úlohu pri normálnom vývine ako aj pri rôznych ochoreniach. Epigenomika sa zaoberá štúdiom kompletného súboru epigenetických zmien v rámci genetického materiálu bunky, zvaného epigenóm.

4.1. DNA metylácia a cicavčí genóm

Pri cicavcoch, metylácia cytozínov v CpG pozíciách DNA sekvencie je jedným zo znakov epigenetického umlčania génov. Počas evolúcie, oblasti bohaté na CpG, CpG ostrovy, boli vyčlenené ako prominentné oblasti promótora génu. Kým ostatné regióny genómu sú konštantne metylované, tieto elementy v promótore sú demetylované, umožňujúc tak tvorbu otvoreného chromatínu a iniciáciu transkripcie. Písmeno "P" v spojení CpG značí fosfodiesterovú väzbu (z angl. phosphodiester bond) medzi cytozínom a guanínom, čo naznačuje, že C a G sú vedľa seba v sekvencii bez ohľadu na to, či je DNA jedno- alebo dvoj-vláknová. C a G v rámci CpG miest sa nachádzajú na to istom reťazci DNA alebo RNA a sú spojené cez fosfodiesterovú väzbu. DNA metylácia sa tiež uplatňuje v umlčaní repetitívnych sekvencií, pohyblivých elementov, znižuje možnosť nežiadúcich rekombinácií a udržuje stabilitu genómu. DNA metylácia je epigenetický signál kovalentnou väzbou viazaný k DNA. Z evolučného hľadiska je táto epigenetická modifikácia jedna z najstarších, pretože sa s ňou stretávame pri baktériofágoch, baktériách, hubách, rastlinách, rovnako aj pri živočíchoch a cicavcoch (Paulsen et al., 2008). Počet organizmov, ktoré nevyužívajú metyláciu svojej DNA ako epigenetický mechanizmus počas evolúcie je malý, ale môžeme do tejto skupiny zaradiť významné modelové organizmy ako C. Elegans alebo S. Cerevisiae. Týmto eukaryotickým organizmom chýba detekovateľná metylácia cytozínov v ich genóme a nebola u nich identifikovaná žiadna kódujúca sekvencia pre cytozín-metyltransferázu (Goll and Bestor, 2005). Prokaryoty oplývajú mnohými typmi DNA metylácie (A-N6, C-N4 a C5), kým DNA metylácia rastlín a vyšších živočíchov je obmedzená na metyláciu cytozínu C5. Pri cicavcoch dochádza k metylácii DNA v konkrétnej sekvencii 5'-CpG- 3'. Zaujímavosť CpG ostrovov ako

epigenetického signálu je v tom, že nie každý CpG ostrov je metylovaný v rámci genómu. Regulačné sekvencie, ako promótory prevádzkových génov sú udržované bez metylácie pri väčšine bunkových typov. Naopak, tkanivovo špecifické gény sa vyskytujú bez metylácie DNA v tých bunkách, kde sú dané gény transkribované (Paulsen et al., 2008). Tkanivovo špecifické gény môžu časom podľahnúť metylácii DNA a tak môže časom dôjsť k strate ich CpG ostrovov kvôli náchylnosti k hypermutáciám, bez straty schopnosti expresie. Naopak prevádzkové gény sú stále asociované s CpG ostrovmi (Antequera and Bird, 1993). Komplexnosť regulácie vzoru DNA metylácie je tiež odzrkadľovaná množstvom a rôznorodou funkciou DNA metyltransferáz a proteínov reagujúcich s 5mC. Pri cicavcoch rozlišujeme päť proteínov podobných DNA metyltransferázam, DNMT1, 2, 3A, 3B a 3L (Goll and Bestor, 2005). DNMT1, 3A a 3B majú funkciu DNA metyltransferáz, enzýmy DNMT3A a 3B sú zodpovedné za ustanovenie vzoru DNA metylácie (*de novo* metyltransferázy), enzýmy DNMT1 sa zúčastňujú na udržovaní ustáleného vzoru metylácie. DNMT3L má zrejme funkciu regulačnú. DNMT2 vykazuje *in vitro* nízku DNA metylačnú aktivitu, ale súčasné poznatky dokazujú, že ide o t-RNA špecifickú metyltransferázu (Goll et all., 2006; Jeltsch et al., 2006). Pochopenie vzájomných súvislostí medzi metyláciou DNA, štruktúrou chromatínu a CpG ostrovmi nám pomôže lepšie porozumieť patogénnym procesom, ktoré sú spájané s nesprávnou funkciou génov. Ustanovenie a udržovanie vzoru metylácie v genóme nezávisí od rozpoznania špecifickej sekvencie, ale namiesto toho je kontrolovaný citlivosťou k repetitívnym sekvenciám, interakciou s RNA a DNA a tiež metyláciou histónov (Goll and Bestor, 2005). U ľudí sa stretávame s dvoma triedami genómových elementov, ktoré sú spojené s metyláciou DNA: CpG ostrovy, ktoré sú znakom pre nemetylovaný región promótora a retrotranspozibilné/repetitívne elementy, ako LINE alebo SINE, ktoré sú znakom metylovanej heterochromatínovej štruktúry. Repetitívne elementy sú teda vo veľkej väčšine metylované a v heterochromatínovom stave, tým sú udržované v transkripčne utlmenej hladine a zostávajú neprístupné rekombinácii. Je možné, že DNA metylácia vznikla pôvodne ako obranný mechanizmus pred pohyblivými elementmi a až počas evolúcie nadobudla aj transkripčne regulačný potenciál, napríklad genómový imprinting, alebo inaktivácia chromozómu X (Paulsen et al., 2008).

4.2. CpG dinukleotidy

Cieľom pre metyláciu DNA sú CpG dinukleotidy, v rámci genómu sa vyskytujú menej ako je celkový obsah báz G+C. Vysvetlenie pre depléciu CpG počas evolúcie je také, že 5-

metyl-cytozín má tendenciu byť náchylný k spontánnej deaminácii na tymín, ktorý nie je odstránený pomocou enzýmu DNA-uracil glykosidázy (Coulondre et al., 1978; Bird, 1980), pretože sa aj prirodzene vyskytuje v DNA. Výsledkom je zvýšená mutabilita CpG dinukleotidov, ktorá sbôsobuje, že metylovaná oblasť stráca veľmi rýchlo CpG dinukleotidy (Cohen et al., 2011). Táto deplécia však nie je uniformná v rámci cicavčieho genómu. Sú oblasti s bohatým výskytom GpG dinukleotidov. Toto je spôsobené chýbaním metylácie DNA (Bird et al., 1985). Takéto nemetylované oblasti bohaté na CpG dinukleotidy nie sú náchylné k hypermutáciám (Cohen et al., 2011). Tieto nemetylované CpG dinukleotidy sa zhromažďujú v oblasti promótorov génov. Časť CpG dinukleotidov sa nachádza v repetitívnych elementoch, tieto oblasti sú v metylovanom stave a sú náchylné k C-T tranzícii (Rollins et al., 2006).

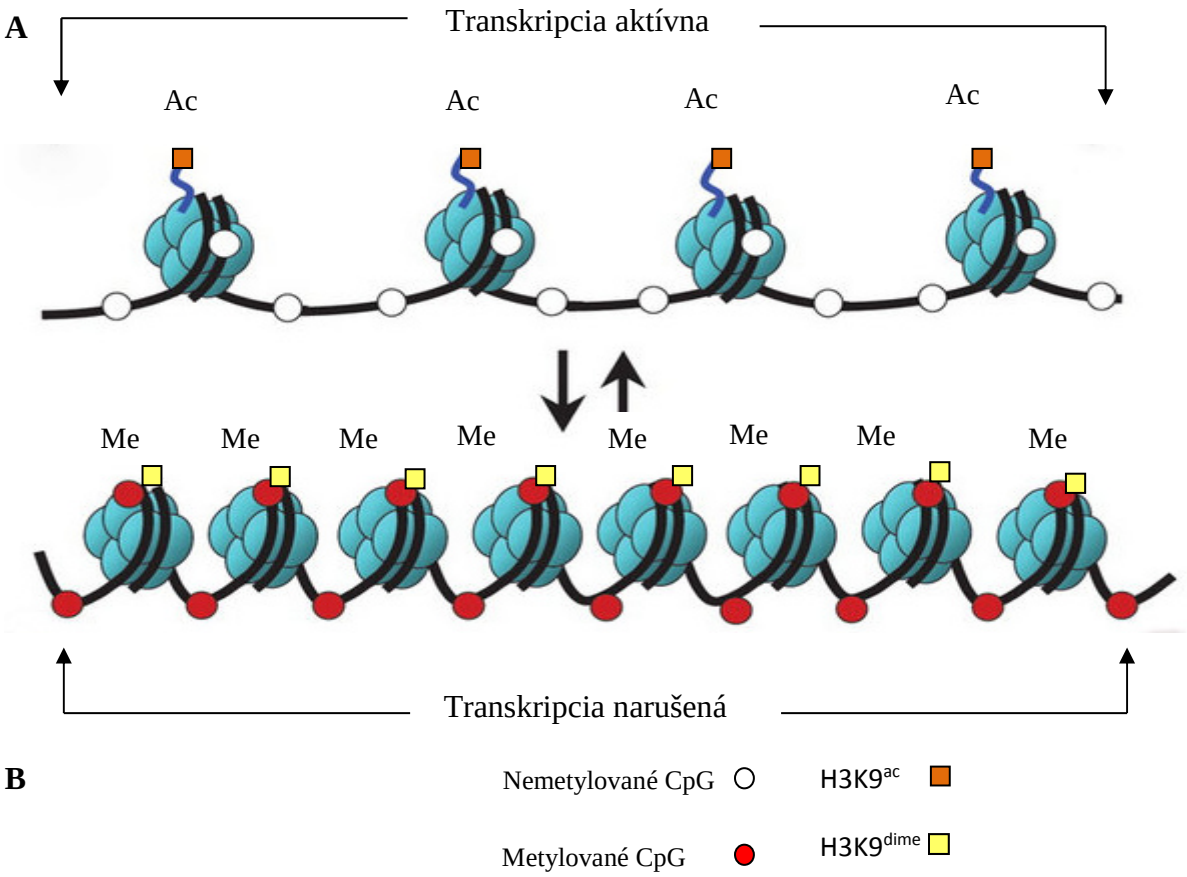
4.2.1. Definícia CpG ostrovov

Prvá snaha definovať vlastnosti CpG ostrovov a umožniť ich detekciu vyšla od Gardiner-Gardena a Frommera (Gardiner-Garden and Frommer, 1987), táto bola ďalej rozvinutá Takaiom a Jonesom (Takai and Jones, 2002), ktorí sprísnili pravidlá pre detekciu funkčných CpG ostrovov. Sú spájané s vysokým obsahom G+C párov. Bola ustálená nasledovná definícia: CpG ostrov by mal obsahovať viac ako 55% C+G párov, pričom pomer pozorovaných CpG vs. očakávaných CpG je viac ako 0,65 pri minimálnej dĺžke 500 bázových párov. Táto definícia zahrňuje všetky regulačné CpG ostrovy v 5' oblasti génu , ale vyčleňuje tie v repetitívnych elementoch, tie nazývame oblasti bohaté na CpG dinukleotidy (Takai and Jones, 2002). Keďže väčšina promótorov obsahuje CpG ostrovy, sú tieto považované za regulátory génovej expresie. Väčšina CpG dinukleotidov v rámci CpG ostrovov promótorov je v stave bez metylácie DNA v zdravom tkanive ľudského genómu. Naopak, metylované oblasti bohaté na CpG dinukleotidy obsahujú často repetitívny sekvenčný motív (Bock et al., 2006).

4.2.2. CpG ostrovy a štruktúra chromatínu

Aktivácia transkripcie je spojená s otvorenou štruktúrou chromatínu, čo umožňuje väzbu transkripčných faktorov a RNA polymerázy. Popri metylácii DNA, stav kondenzácie chromatínu tiež súvisí aj s modifikáciami histónov. Ide o rôzne typy modifikácií histónov: metylácia, acetylácia, fosforylácia, ubiquitinácia. Metylácia histónu H3 v štvrtej polohe lyzínu (H3-Lys4) a acetylácia histónu 3 v polohe lyzín 9 (H3-Lys9) vedie k aktivácii transkripcie, dekondenzácii štruktúry chromatínu a je spájaná s nemetylovanými CpG ostrovmi (Santos-Rosa et al., 2002; Bernstein et al., 2005). Naopak, metylácia histónu H3-

Lys9 je charakteristická pre kondenzáciu a následne heterochromatínovú uzavretú štruktúru príslušného regiónu v genóme, neprístupnú pre transkripčné faktory a RNA polymerázu (Nakayama et al., 2001). Vyplýva z toho, že modifikácie histónov majú významnú regulačnú funkciu v kontrole transkripcie.



Obr. č. 8 **Spojitosť modifikácie histónov a DNA v súvislosti s otvorenou a uzevretou štruktúrou chromatínu.**

Upravené podľa Paulsen et al, in: Epigenetics. 2008

Popis obrázku:

Obrázok znázorňuje štruktúru nukleozómov (s DNA, ktorú znázorňuje samostatná čierna čiara), okolo štartovacieho miesta transkripcie génov (čierna šipka). Metyláciu DNA vykresľujú otvorené biele (nemetylované) a plné červené (metylované) kruhy v rámci DNA. Hlavné modifikácie lyzínu v pozícii 9 N-koncového histónového chvosta sú znázornené štvorčekmi. Oranžové: acetylácia (Ac) histónu 3 v polohe lyzín 9; žlté: dimetylácia (Me) histónu 3 v polohe lyzín 9

A. Otvorený aktívny chromatín, nemetylované cytozíny (biele kruhy), acetylované históny (oranžové štvorčeky).

B. Kondenzovaný chromatín, metylované cytozíny (červené kruhy), deacetylované históny, dimetylácia histónu 3 v polohe lyzín 9 (žlté štvorčeky).

4.2.3. CpG ostrovy a promótory-čo zamedzuje metylácii DNA v oblasti promótora

Hlavným znakom CpG ostrovov je ich prítomnosť okolo oblasti promótora a ich udržovanie v otvorenom, hypometylovanom a tým pádom v transkripčne aktívnom stave. Avšak, pozorovania, ktoré odhalili, že CpG ostrovy sú náchylné na zmeny v stave metylácie súvisiace s vekom a ochoreniami, podnietili zvýšený záujem o analýzu epigenómu (Antequera, 2003; Laird, 2005). Kým DNA metyltransferázy sprostredkujú metyláciu cytozínov, menej je známe ako sú chránené nemetylované oblasti v genóme cicavcov pred *de novo* metyláciou a či sa tu uplatňuje aktívna demetylácia (Cortellino et al., 2011). Tymidín-DNA glykozyláza je potrebná pre príchod acetyltransferázy p300 do oblasti promótorov regulovaných kyselinou retinovou, pre ochranu CpG ostrovov pred hypermetyláciou a pre aktívnu demetyláciu tkanivovo špecifických promótorov a enhancerov, ktoré sú regulované hormonálne a počas vývoja. Toto naznačuje duálnu úlohu tymidín-DNA glykozylázy v udržovaní správnej epigenetickej hladiny počas vývoja a predznamenáva dvojkrokový mechanizmus DNA demetylácie cicavcov, a to že 5-metylcytozín a 5-hydroxymetylcytozín je najprv deaminovaný pomocou deaminázy AID (z angl. activation-induced (DNA-cytosine) deaminase) z 5-metylcytozínu na tymín a z 5-hydroxymetylcytozínu na 5-hydroxymetyluracyl a následne sú odstránené pomocou tymidín-DNA glykozylázy (Cortellino et al., 2011). Najnovšie poznatky podporujú myšlienku, že ide o spoločnú úlohu transkripčnej mašinérie a miestnych modifikácií histónov, ktoré sa zapájajú v udržovaní CpG ostrovov v hypometylovanom stave pri prevádzkových génoch. Odhaduje sa, že približne 88% aktívnych promótorov je spätých s CpG bohatými sekvenciami, čo naznačuje, že CpG ostrovy plnia podstatnejšiu úlohu v regulácii expresie génov, ako sa predpokladalo (Kim et al., 2005). Kým promótory, v ktorých nie sú obsiahnuté CpG bohaté sekvencie zvyčajne obsahujú konkrétne štartovacie miesto transkripcie a TATA boxy, promótory asociované s CpG ostrovmi sa vyznačujú alternatívnymi miestami štartu transkripcie a nemusia obsahovať vždy TATA box (Carninci et al., 2006). CpG ostrovy sú asociované s 5′ koncom všetkých prevádzkových génov a mnohých tkanivovo-špecifických génov (Gardiner-Garden and Frommer, 1987). Promótory asociované s CpG ostrovmi reprezentujú znak, že ide o gény, ktoré musia byť stabilne exprimované. Niektoré tkanivovo-špecifické gény majú promótor bez znaku stabilnej expresie a je tu lepšia možnosť ich zablokovania, keď nie sú potrebné v danej chvíli.

4.3. DNA metylácia ako obranný mechanizmus

Repetitívne sekvencie obsahujú okolo 50% všetkých CpG nukleotidov (Rollins et al., 2006), sú však konštantne metylované, čo je kontrast k CpG ostrovom. Metylácia repetitívnych sekvencií je bežná pre živočíchy, rastliny aj huby a je možné, že tento mechanizmus bol vyvinutý ako obranný voči včleneniu cudzorodej DNA do hostiteľského genómu (Yoder et al., 1997). Promótory transpozibilných elementov sú inaktivované metyláciou DNA a mnoho transpozónov bolo v priebehu času zničených kvôli C-T tranzícii v mieste metylácie (Yoder et al., 1997). Jednoduchý avšak účinný mechanizmus bol popísaný pri bakteriálnych organizmoch. Tento mechanizmus je založený na metylácii DNA. Nazýva sa aj restrikčno-modifikačný systém. Všetky špecifické miesta pre restrikčné endonukleázy sú označené pomocou DNA metyltransferáz a sú chránené, keď vstúpi cudzorodá nemetylovaná DNA, okamžite je štiepená restrikčnými enzýmami, kým hostiteľská DNA, metyláciou označená, je ňou zároveň chránená. U eukaryotov je úloha metylácie DNA odlišná. V týchto organizmoch podporuje a udržuje metylácia DNA heterochromatínovú štruktúru a transkripčné umlčanie integrovanej cudzorodej DNA (Paulsen et al., 2008). Repetitívne elementy sú udržované v metylovanom stave, lebo by inak mohli brániť transkripcii génov a mohli by narúšať integritu genómu. Medzi metylované repetitívne elementy môžeme zaradiť pseudogény, DNA transpozóny, retrotranspozóny, endogénne retrovírusy a priame repetície, ktoré môžeme nájsť v oblasti centromér alebo mikrosatelitov. Najmä endogénne retrovírusy, retrotranspozibilné elementy a transpozóny, sekvencie v genóme, ktoré pochádzajú z infekcií zárodočných buniek u cicavcov a ostatných stavovcov a vo forme provírusu sa prenášajú do ďalšej generácie, majú potenciál byť nebezpečné pre organizmus, pretože môžu byť reaktivované a môžu sa tým pádom včleniť do funkčného regiónu genómu, ako napríklad do kódujúcej oblasti alebo do regulačného elementu. Metylácia týchto elementov je klúčovým mechanizmom pre ochranu genómu pred transkripciou a transpozíciou týchto elementov (Hedges and Batzer, 2005). Nežiadúca hypometylácia môže znovu aktivovať retrotranspozóny a endogénne retrovírusy. Po ošetrení inhibítormi DNA metyltransferáz (napr. 5AC) je možné znovu aktivovať retrotranspozóny, prechádzajú do demetylovaného stavu, čiže je zrejmé, že metylácia sa uplatňuje pri ich deaktivácii (Liu et al., 1994). Globálna, celogenómová hypometylácia je bežným javom a znakom rôznych typov nádorových ochorení (Shulz, 2006) a súvisí práve s hypometyláciou repetitívnych elementov.

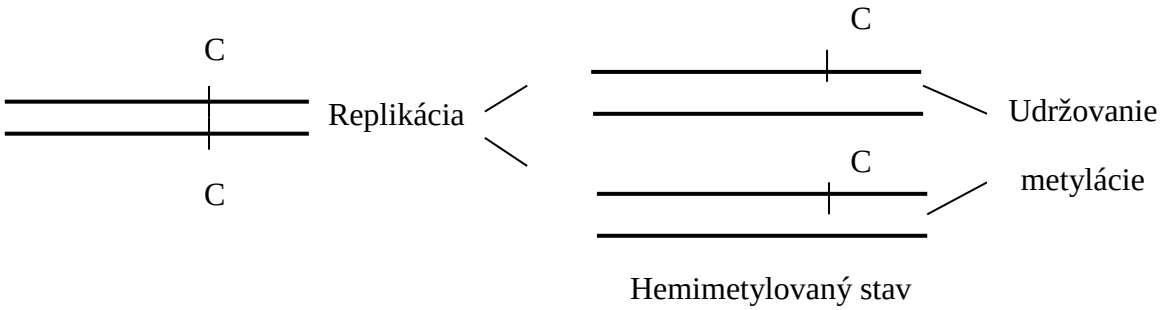
4.4. DNA metyltransferázy a metyl-CpG väzobné proteíny ako multifunkčné regulátory štruktúry chromatínu

Epigenetická modifikácia DNA 5-metylcytozínom je významná v rámci regulácie štruktúry chromatínu, udržovania genómovej stability, genómového imprintingu, inaktivácie chromozómu X, regulácie transkripcie a v umlčovaní retrotranspozónov a v embryonálnom vývoji (Robertson, 2001; Bird, 2002; Li, 2002). Tento proces je umožnený vďaka DNA metyltransferázam a je interpretovaný metyl-CpG väzobnými proteínmi. DNA metyltransferázy nemajú funkciu iba v katalýze metylácie DNA, ale zúčastňujú sa tiež v regulácii expresie génov cez spoluprácu s ostatnými proteínmi, ktoré utlmujú transkripciu a modifikujú štruktúru chromatínu. Deje sa to na cytozíne v rámci CpG dinukleotidov a skoro 70% CpG miest v cicavčom genóme obsahuje 5-metylcytozín, s výnimkou tých, ktoré sa nachádzajú v CpG ostrovoch promótorov génov (Bird, 2002), tie sú zvyčajne nemetylované (Jones and Laird, 1999). Analýza DNA metylácie počas embryonálneho vývoja myší odhalila, že po fertilizácii genóm podstupuje aktívnu a pasívnu demetyláciu (Hajkova et al., 2002; Kelly and Trasler, 2004). Vzor metylácie je následne znovu ustanovený cez vlnu *de novo* metylácie (Hajkova et al., 2002). Niektoré repetitívne elementy sú však čiastočne odolné voči vlne demetylácie a vykazujú neúplné odstránenie metylačného vzoru, čo je dôležité pre zachovanie chromozómovej stability a pre zabránenie aktivácii transpozónov a tým pádom pre zníženie rizika zárodočných mutácií (Hajkova et al., 2002). Metylácia DNA v cicavčích bunkách pozostáva z dvoch procesov, ktoré sú závislé na: DNA metyltransferázach (DNMT), ktoré katalyzujú pridanie metylovej skupiny na DNA, a metyl-CpG väzobných proteínov (MBD), ktoré čítajú alebo sprostredkujú tento znak metylácie (Van Emburgh and Robertson, 2008). DNA metyltransferázy sú enzýmy, ktoré vytvárajú a udržujú genómový vzor metylácie DNA. U cicavcov rozlišujeme tri aktívne DNA metyltransferázy, ktoré rozdeľujeme nasledovne: udržovacia DNA metyltransferáza DNMT1, *de novo* metyltransferáza DNMT3A a DNMT3B a nakoniec DNMT2. DNMT1, DNMT3A a DNMT3B metyltransferázy spolupracujú s histón-deacetylázami (HDAC), ktoré sú schopné modifikovať štruktúru chromatínu deacetyláciou histónov (Fuks et al., 2000, 2001; Geiman et al., 2004; Ling et al., 2004). Efekt metylácie DNA je tiež sprostredkovaný cez metyl-CpG väzobné proteíny (MBD). Medzi tieto môžeme zaradiť: MeCP2, MBD1, MBD2, MBD3 a MBD4. Úloha proteínov MBD je spolupracovať s ďalšími komponentmi, ktoré udržujú utlmený stav transkripcie. MeCP2, MBD1, MBD2, MBD3 sú priamo spojené s utlmením transkripcie, kým MBD4 plní funkciu v oprave DNA (Van Emburgh and Robertson, 2008). Všeobecná

enzymatická reakcia C5-metyltransferáz vyžaduje prenos metylovej skupiny z kofaktora a univerzálneho donora metylu S-adenosyl-L-metionínu (AdoMet) na cytozín v pozícii 5 (Van Emburgh and Robertson, 2008).

4.4.1. DNMT1

Počas replikácie DNA sú produkované hemimetylované oblasti v dcérskom reťazci, ak boli rodičovské CpG oblasti pôvodne metylované. Aby bol zachovaný metylačný vzor v dcérskych bunkách, nemetylovaná CpG oblasť oproti tej metylovanej musí byť tiež metylovaná. Ľudská aj myšacia DNMT1 prednostne metyluje DNA, ktorá je v hemimetylovanom stave (Hitt et al., 1988; Pradhan et al., 1999; Fatemi et al., 2001; Yokochi and Robertson, 2002) a preto ju môžeme nazvať aj udržovacou metyltransferázou. Hladina DNMT1 sa mení počas bunkového cyklu, s akumuláciou na začiatku fázy S a so zníženou hladinou po delení bunky, čo je podobné aj pri ostatných proteínoch, ktoré sú spojené s replikáciou DNA (Vogel et al., 1988; Robertson et al., 2000b).



Obr. č. 9 **Udržovanie metylácie.**

Popis obrázku:

Počas replikácie DNA sú produkované hemimetylované oblasti v dcérskom reťazci, ak boli rodičovské oblasti CpG pôvodne metylované. Aby bol zachovaný metylačný vzor v dcérskych bunkách, nemetylovaná CpG oblasť oproti tej metylovanej musí byť tiež metylovaná.

Metylácia DNA je koordinovaná s replikáciou DNA (Araujo et al., 1998), čo je dôležité pre dedičnosť ustáleného vzoru metylácie. DNMT1 sa vyznačuje rôznymi funkciami sprostredkovanými cez proteín-proteín interakcie. Je schopná znížiť transkripciu nezávisle od jej metylačnej funkcie. DNMT1 obsahuje transkripčne represívnu doménu, ktorá interaguje s histón-deacetylázou HDAC1 a táto doména je spojená s histón-deacetylázovou aktivitou (Fuks et al., 2000). DNMT1 obsahuje väzobnú doménu pre jadrový antigén PCNA (Chuang et al., 1997). PCNA je potrebný pri replikácii DNA ako aj pri oprave DNA. DNMT1 je takto dovedená do miesta replikačného ohniska. Ďalej DNMT1 sa uplatňuje v regulácii komplexu DNMT1, HDAC1, Rb, E2F1 a tento komplex utlmuje

transkripciu génov s promótormi s väzobným miestom pre E2F1 a utlmuje prechod bunkového cyklu z fázy G do fázy S (Robertson et al., 2000a) a udržuje E2F v metylovanom stave a vyznačuje sa inaktívnym znakom transkripcie a to metyláciou lyzínu v polohe 9 histónu 3. Po DNA poškodení DNMT1 metyluje a utlmuje gény, ktoré inhibujú funkciu proteínu p53. V cicavčích bunkách, funkčný tumor-supresorový proteín p53 odpovedá na bunkový stres, ako napríklad na poškodenie DNA a je schopný indukovať zastavenie bunkového cyklu ako odpoveď na poškodenie DNA a indukovať apoptózu (Levine, 1997; Vogelstein, 2000). V bežných podmienkach v bunke je proteín p53 udržovaný v nízkej koncentrácii. Poškodenie DNA spôsobí aktiváciu dráhy p53, toto následne spôsobí zvýšenie hladiny p53 v bunke a aktiváciu p53 ako transkripčného faktora (Levine, 1997). Pretože dráha proteínu p53 je aktivovaná pred apoptózou, je potrebné vyradiť z funkcie antiapoptotické gény. Aktivácia p53 vedie k utlmeniu *survivinu*, čo je člen rodiny inhibítorov apoptózy. Promótorová oblasť *survivinu* obsahuje CpG ostrov v rámci väzobného miesta pre p53 a dôjde k metylácii inhibítora génu apoptózy, *survivinu* (Estève et al., 2005).

4.4.2. DNMT2

Myši, ktorým chýbala DNMT2 vykazovali normálnu *de novo* a udržovaciu metyláciu DNA (Okano et al., 1998). DNMT2 by mohla plniť úlohu ako RNA metyltransferáza, prednostne metylujúca $tRNA^{Asp}$ (Van Emburgh and Robertson, 2008).

4.4.3. DNMT3

Ide o rodinu DNMT3 metyltransferáz, kde rozlišujeme DNMT3A, DNMT3B a DNMT3L (DNMT3-like) metyltransferázy. DNMT3A je dôležitá pri ustálení maternálneho i paternálneho imprintingu. Nezúčastňuje sa pri metylácii retrotranspozibilných elementov. Vykazuje vyššiu afinitu k nemetylovaným substrátom v porovnaní s hemimetylovanými substrátmi (Yokochi and Robertson, 2002), čiže môžeme povedať, že nejde o udržovaciu metyltransferázu. Dôležitá pred *de novo* metyláciou je modifikácia chromatínu. Chromatín musí podstúpiť štrukturálne zmeny pred *de novo* metyláciou prostredníctvom DNMT3A (Takeshima et al., 2006), čo priláka *de novo* metyltransferázy. Štruktúra chromatínu je jedným z dôležitých mechanizmov regulácie *de novo* metylácie, ktorá zabraňuje nesprávnej metylácii prostredníctvom DNMT3A. DNMT3B sa uplatňuje pri metylácii pohyblivých elementov a oblasti centromér. Spolupracuje s DNMT1 pri utvorení a udržovaní vzoru metylácie. Rovnako ako DNMT3A a DNMT1, aj DNMT3B formuje represívne proteínové komplexy (Van Emburgh and Robertson, 2008). DNMT3L sa

uplatňuje v udržovaní metylácie DNA retrotranspozónov (Bourc'his and Bestor, 2004). Ako aj ostatné metyltransferázy, aj DNMT3L formuje represívne proteínové komplexy s histón-deacetylázami cez PHD doménu (Plant Homeo Domain). Touto doménov sa proteíny môžu viazať na modifikované históny ako histón H3 tri-metylovaný v polohe lyzín 9 (H3K9me3) (Iwase et al., 2007; Maksakova et al., 2011) alebo H3K4me3 (Peña et al., 2006). Domény, ktoré viažu modifikované históny môžeme nazvať čitateľmi epigenetického znaku, pretože špecificky rozpoznávajú modifikované verzie histónových koncov a viažu ich. Modifikácia H3K4me3 je spojená s miestom štartu transkripcie a s aktívnymi génmi a modifikácia H3K9me3 je spojená s inaktívnymi génmi (Santos-Rosa et al., 2002; Sims III et al., 2003). DNMT3L tiež spolupracuje s enzymaticky aktívnymi *de novo* metyltransferázami DNMT3A a DNMT3B (Hata et al., 2002). Nesprávna regulácia funkcie DNA metyltransferáz je závažným krokom v progresii vzniku nádorov. Jedným zo znakov nádorových ochorení je epigenetická zmena, ktorá zahŕňa globálnu hypometyláciu a promótorovú hypermetyláciu (Van Emburgh and Robertson, 2008).

4.5. Metyl-CpG väzobné proteíny

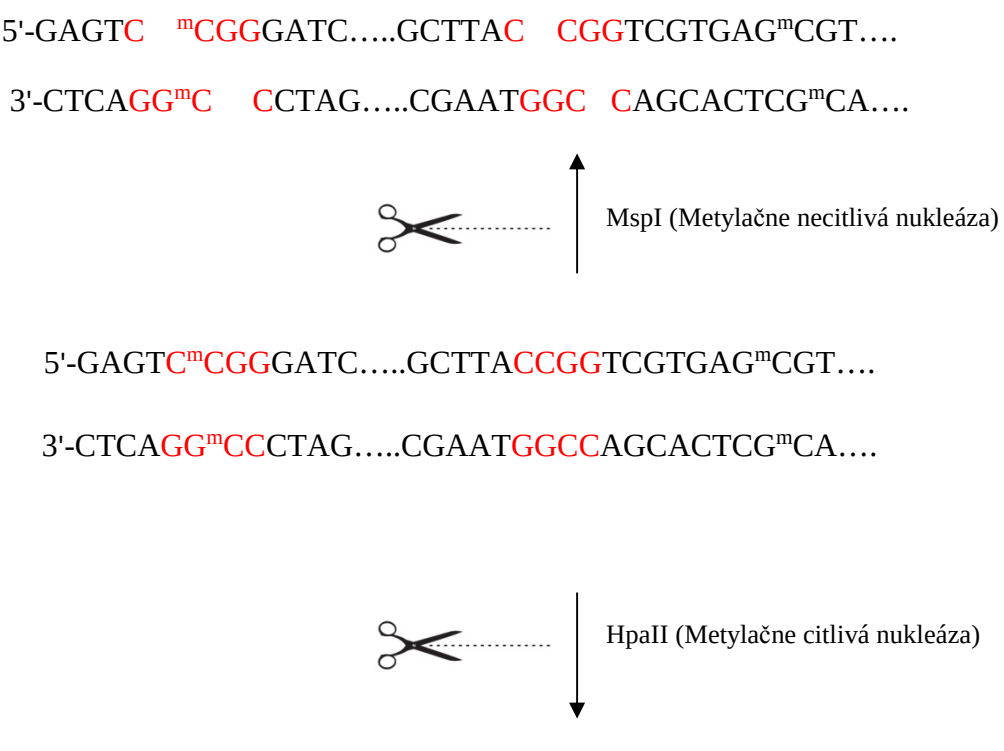
Metylácia v promótorovom regióne v CpG miestach utlmuje transkripciu. Metylová skupina môže brániť väzbe príslušných transkripčných faktorov, ktoré obsahujú CpG miesta v rámci svojej rozpoznávajúcej sekvencie, čo následne inhibuje transkripciu. Metyl-CpG väzobné proteíny sa viažu do tejto oblasti a zabraňujú tak väzbe transkripčných faktorov (Van Emburgh and Robertson, 2008). MeCP2 vykazuje represívnu aktivitu cez spoluprácu a tvorbu komplexu s histón-deacetylázami HDAC1 a HDAC2. MeCP2 je spojený s histón H3K9 metyltransferázovou aktivitou (Fuks et al., 2003). MBD1 interaguje napríklad s histón-metyltransferázou SUV39H1 a zvyšuje jej represívnu schopnosť (Fujita et al., 2003). MBD2 (MeCP1 komplex) bol identifikovaný cez prítomnosť metyl-CpG väzobnej domény (Hendrich and Bird, 1998) a táto doména bola identifikovaná ako súčasť komplexu MeCP1 (Ng et al., 1999). MBD2 tiež utlmuje transkripciu cez spoluprácu s ďalšími represívnymi proteínmi. Je členom represívneho komplexu s histón-deacetylázami HDAC1 a HDAC2 (Ng et al., 1999). MBD3 podobne ako ostatné metyl-CpG väzobné proteíny interaguje s proteínmi, ktoré sú vyžadované v prestavbe chromatínu. Je súčasťou komplexu NuRD, ktorý obsahuje aj histón-deacetylázy HDAC1 a HDAC2 (Zhang et al., 1999). MBD4 bol identifikovaný ako opravný proteín DNA pod názvom metyl-CpG väzobná endonukleáza 1 (z angl. methyl-CpG-binding endonuclease 1; MED1). Táto aktivita nie je závislá od DNA metylácie, ale MBD4 sa uplatňuje pri

opravách C → T tranzícií pri metylovaných cytozínoch, kvôli spontánnej deaminácii 5-metylcytozínu (Petronzelli et al., 2000). MBD4 vykazuje tiež represívnu funkciu cez interakciu s histón-deacetylázou HDAC1 (Kondo et al., 2005).

4.6. Metódy pre celogenómovú a génovo špecifickú analýzu hladiny a vzoru metylácie DNA

Všetky bunky mnohobunkového organizmu nesú tú istú genetickú informáciu kódovanú ich sekvenciou DNA, ale bunky zjavne majú širokú morfologickú a funkčnú rôznorodosť. Táto heterogenita je spôsobená odlišnou expresiou génov, ktorá je riadená epigenetickými modifikáciami. Metylácia cytozínu je dôležitá pre správnu embryogenézu a vývoj cicavcov a pre imprinting. Zapája sa do inaktivácie chromozómu X a plní významnú úlohu v udržovaní integrity genómu cez umlčanie transkripcie repetitívnych sekvencií DNA a endogénnych transpozónov. Metylácia v oblasti CpG ostrovov, čo sú CpG bohaté oblasti, ktoré sú zvyčajne v nemetylovanom stave a nachádzajú sa v oblasti promótora a prvého exónu množstva génov, je dôležitá pre ustanovenie a udržovanie expresie génov závislej od bunkového typu a metylácia DNA je často spojená s umlčaním expresie génov. Nahromadené genetické a epigenetické zmeny môžu viesť k nekontrolovanému rastu bunky a až k vzniku nádorového ochorenia. Metylácia DNA môže byť ideálnym biomarkerom pre diagnózu, klasifikáciu a prognózu nádorových ochorení u ľudí. Metylácia DNA je veľmi úzko spätá s inými epigenetickými modifikáciami, ktoré spolu určujú fenotyp špecifickej bunky. Medzi tieto modifikácie patria modifikácie koncov histónov, napríklad acetylácia a metylácia zbytkov lyzínu ako aj fosforylácia a ubiquitinácia ďalších aminokyselinových zbytkov. Profil modifikácií histónu príslušného regiónu je zvyčajne určovaný pomocou imunoprecipitácie chromatínu s použitím protilátky proti modifikácii histónu, ktorá nás zaujíma a následnej analýzy precipitátu (Tost, 2008). V súčasnosti poznáme množstvo princípov, na ktorých sú založené reakcie na vyšetrenie metylácie DNA. Napríklad Maxam-Gilbert sekvenovanie, ktoré je založené na princípe chemického štiepenia rádioaktívne značenej DNA (Maxam and Gilbert, 1977). Cytozíny sú štiepené po ošetrení hydrazínom, kým metylované cytozíny nie sú (Tost, 2008). K ďalším metódam patrí napríklad štiepenie pomocou metylačne-citlivých restrikčných endonukleáz. Z metylačne-citlivých restrikčných endonukleáz najviac používaným párom enzýmov je pár HpaII/MspI, ktoré rozpoznávajú a štiepia palindrómovú sekvenciu CCGG v dvojvláknovej DNA, ale kým MspI štiepi nezávisle od stavu metylácie, HpaII nie je schopný štiepiť sekvenciu, ak je druhý cytozín metylovaný

(C | ^{me}CGG). Čiže štiepenie je blokované, keď je v špecifickom mieste rozpoznaný metylovaný cytozín. Pri cicavcoch môže dôjsť k metylácii DNA iba v kontexte CG, teda iba také restrikčné enzýmy s príslušným štiepnym miestom môžu byť použité (Tost, 2008).



Obr. č. 10 **Restrikčné štiepenie pomocou metylačne citlivej nukleázy HpaII a pomocou metylačne necitlivej nukleázy MspI.**

Upravené podľa Tost, in: Epigenetics. 2008

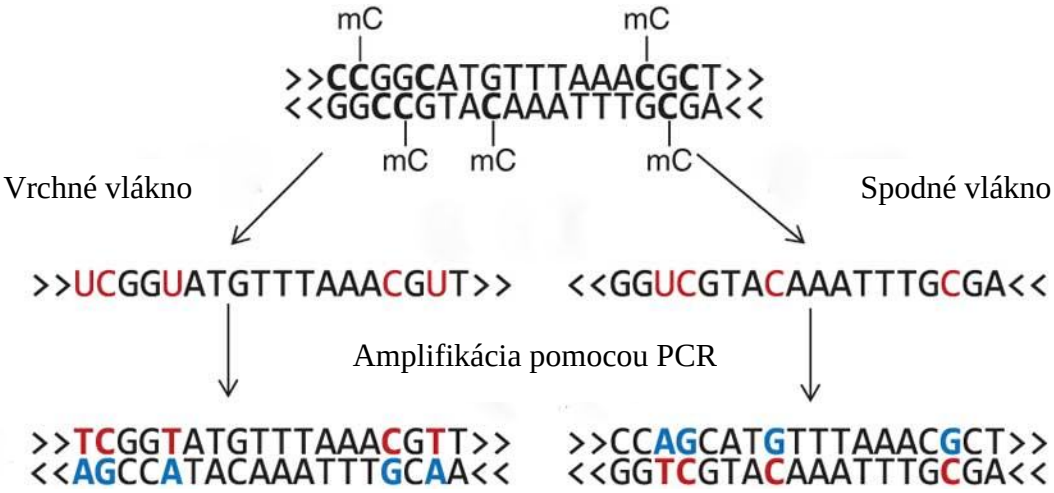
Popis obrázku:

Enzýmy sú špecifické pre štiepenie v palindrómovej sekvencii CCGG, ale HpaII nie je schopná štiepiť, ak druhý cytozín je metylovaný. Čiže štiepenie je blokované, keď je v špecifickom mieste rozpoznaný metylovaný cytozín. MspI štiepi nezávisle od metylácie. Štiepenie nie je blokované.

Restrikčné štiepenie a ošetrenie hydrogénsiričitanom sodným sú dva najbežnejšie používané prístupy pre analýzu metylácie DNA. Princíp konverzie hydrogénsiričitanom sodným je v tom, že nemetylované cytozíny sú deaminované na uracily prostredníctvom hydrolytickej deaminácie, ktoré sú následne nahradené tymínmi počas amplifikácie pomocou PCR. Metylované cytozíny sú rezistentné k ošetreniu a ostávajú po amplifikácii PCR cytozínmi. Takto každý cytozín, ktorý je stále prítomný v hydrogénsiričitanom

sodným ošetrenej DNA odzrkadľuje predchádzajúci metylovaný cytozín (Krueger et al., 2012).

Konverzia hydrogénsiričitanom sodným



Obr. č. 11 **Princíp konverzie genómovej DNA s hydrogénsiričitanom sodným.**

Upravené podľa Krueger et al, Nature Methods 2012

Popis obrázku:

Nemetylované cytozíny sú konvertované na uracily, ktoré sú následne nahradené tymínmi počas amplifikácie pomocou PCR. Metylované cytozíny sú rezistentné k ošetreniu a ostávajú po amplifikácii PCR cytozínmi (Krueger et al., 2012).

Je nutné pamätať na to, že ošetrenie DNA s hydrogénsiričitanom sodným indukuje degradáciu DNA kvôli oxidačnému poškodeniu a depurinácii DNA, čo môže spôsobiť stratu veľkej časti vzorky, preto musia byť podmienky reakcie prísne kontrolované (Raizis et al., 1995). Existujú aj ďalšie obmedzenia daného ošetrenia. Patria sem napríklad, neúplná konverzia, čo by mohlo poskytnúť falošne pozitívne výsledky na metyláciu a už spomínaná degradácia DNA po ošetrení hydrogénsiričitanom sodným alebo neúplná desulfonácia pyrimidínov, čo môže inhibovať DNA polymerázu.

Celkovo princípy pre štúdium metylácie DNA môžeme rozdeliť na metódy:

1. Globálna analýza DNA metylácie

Metóda analýzy globálnej metylácie DNA určuje celkový obsah 5-metylcytozínu alebo zmeny postihujúce celý epigenóm, ako napríklad celková hypometylácia pri nádorových ochoreniach. Nedáva však informáciu o konkrétnej pozícii metylácie DNA. Epigenóm

obsahuje všetky epigenetické zmeny a modifikácie v rámci celého genómu. Využíva sa napríklad metóda vysokoúčinnej kvapalinovej chromatografie (z angl. High performance liquid chromatography; HPLC). DNA je pri tejto metóde poštiepená na jednotlivé deoxyribonukleozidy a nukleozidy sú separované na základe rôznej rozpustnosti v polárnom rozpúšťadle a identifikované cez porovnanie ku kalibračnému štandardu. Táto metóda je používaná pri globálnej analýze metylačných zmien po ovplyvnení demetylačnými liečivami pri rôznych ochoreniach (Tost, 2008). Ďalšou metódou je napríklad metyltransferázová analýza. Táto metóda využíva fakt, že metyltransferáza prenáša metylovú skupinu z univerzálneho donora S-adenozyl-L-metionínu (AdoMet) na nemetylovanú pozíciu CpG (Miller et al., 1974; Bestor and Ingram, 1983). Metóda teda využíva tieto enzýmy a analyzuje množstvo inkorporovanej rádioaktívne značenej metylovej skupiny z donora vo vzorke a porovnávame so stupňom metylácie pred značením. Ďalšou metódou je *in situ* hybridizácia s protilátkami priamo proti 5-metylcytozínu, čo umožňuje zmeranie obsahu metylácie a jej distribúciu. Pred samotnou analýzou je nutná denaturácia, pretože protilátky rozpoznávajú 5-metylcytozín iba na jednovláknovej DNA. Po inkubácii s primárnou protilátkou je pridaná fluorescenčne značená sekundárna protilátka na detekciu a obrázok je následne analyzovaný fluorescenčným mikroskopom (Tost, 2008).

2. Metóda pre celogenómovú analýzu vzoru metylácie

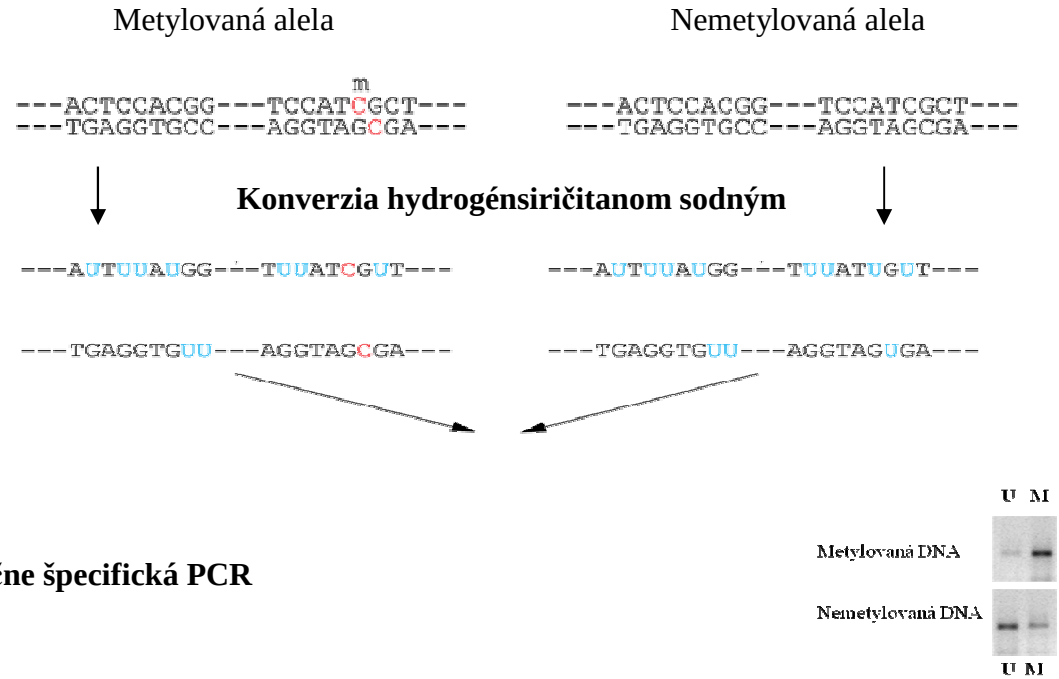
Metóda tzv. odstránenia metylácie DNA indentifikuje vzor metylácie, nie však analýzou samotných zmien vzoru DNA metylácie, ale cez monitorovanie expresie mRNA po ošetrení buniek s inhibítormi DNA metylácie (Suzuki et al., 2002). Farmakologické liečivá ako napríklad analóg nukleozidu azacytidin (5-azacytidin) inhibujú metyláciu DNA vznikom kovalentného komplexu medzi metyltransferázou a DNA počas udržovania metylácie pri hemimetylovaných templátoch, ktorá nasleduje po replikácii DNA. Následne dôjde ku globálnej demetylácii (Yoo and Jones, 2006). Bunky môžu byť kultivované s azacytidinom samostatne alebo v kombinácii s inhibítorom histón-deacetyláz, ako trichostatin A (TSA), čo spôsobuje aktiváciu epigeneticky umlčaných génov (Suzuki et al., 2002). Väčšinou ide o porovnávanie dvoch rôznych vzoriek, napríklad zdravé vs. nádorové tkanivo.

3. Techniky, ktoré umožňujú analýzu DNA metylácie v oblasti, ktorá nás zaujíma, napríklad promótorová oblasť

Nasledujúce metódy dovoľujú analýzu DNA metylácie v amplifikovanom úseku pomocou špecifických primerov po ošetrení hydrogénsiričitanom sodným (Tost, 2008). Po ošetrení hydrogénsiričitanom sodným nasleduje amplifikácia pomocou špecifických primerov, aby sme získali úsek, ktorý nás zaujíma. Ďalej nasleduje purifikácia amplifikovanej DNA, klonovanie a po izolácii plazmidovej DNA nasleduje sekvenovanie. Klonovanie a sekvenovanie jednotlivých klonov sú bežne využívané pri štúdiu metylácií DNA na zisťovanie metylačného statusu CpG nukleotidov. Môžeme sa dozvedieť, ktoré cytozíny boli metylované a teda, ktoré boli odolné voči ošetreniu hydrogénsiričitanom sodným. Metódu môžeme nazvať aj nemetylačne špecifickú, pretože nepotrebujeme dva sety primerov (jeden pár metylačne špecifických a druhý pár nemetylačne špecifických primerov), ako je tomu pri metylačne špecifickej PCR. Primery sú pri nemetylačne špecifickej metóde špeciálne navrhnuté tak, aby sme získali úsek, ktorý nás zaujíma, ale neobsahujú CpG nukleotidy, tým pádom nám stačí jeden pár primerov, aby sme získali oblasť záujmu. Týmito primermi získame úsek, ktorý môže obsahovať aj nemetylované ale aj metylované cytozíny v CpG dinukleotidoch. Všetky nemetylované cytozíny sú amplifikované pomocou PCR ako tymíny v zmyslovom reťazci (z angl. sense strand) a ako adeníny v protizmyslovom reťazci (z angl. antisense strand). Všetky metylované cytozíny sú amplifikované pomocou PCR ako cytozíny v zmyslovom reťazci (z angl. sense strand) a ako guaníny v protizmyslovom reťazci (z angl. antisense strand).

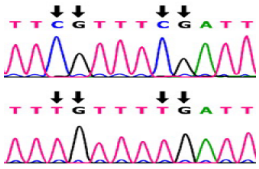
4. Detailná analýza jednotlivých CpG pozícií

V súčasnosti sa prikladá DNA metylácii významná pozornosť pre jej potenciál ako biomarker skorej diagnózy, prognózy a odpovede na liečbu pri mnohých nádorových ochoreniach (Laird, 2003). Metylačne špecifická PCR analýza je vhodná metóda na vyšetrenie metylácie DNA v jednotlivých CpG miestach. DNA je modifikovaná hydrogénsiričitanom sodným a na PCR reakciu potrebujeme dva sety primerov, metylačne špecifický pár primerov a nemetylačne špecifický pár primerov. Metylačne špecifický pár primerov komplementuje iba nekonvertovaný 5-metylcytozín. Nemetylačne špecifický pár primerov komplementuje tymíny (uracily), konvertované z nemetylovaných cytozínov. Metylácia je následne určená podľa schopnosti amplifikácie špecifických primerov. Metódu teda nazývame ako metylačne špecifickú. Primery obsahujú CpG dinukleotidy a CpG dinukleotidy v 3' pozícii primeru zvyšujú citlivosť. Takto získame informáciu o metylačnom stave konkrétneho/konkrétnych CpG dinukleotidov v oblasti záujmu (Herman et al., 1996).



Metylačne špecifická PCR

Nemetylačne špecifická PCR



Obr. č. 12 **Konverzia genómovej DNA hydrogénsiričitanom sodným a následná analýza.**

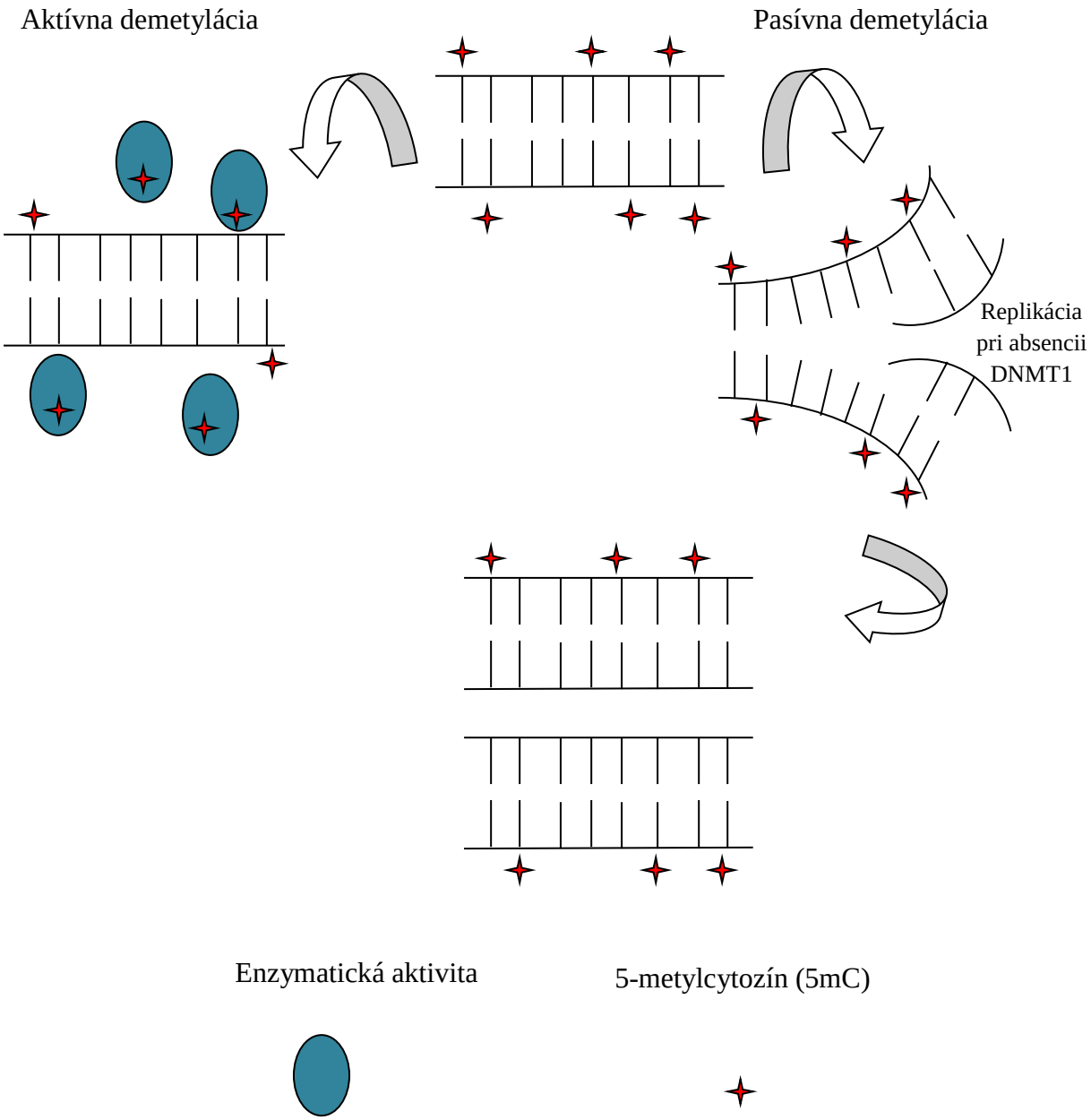
Popis obrázku:

Modré nukleotidy prezentujú nemetylované cytozíny, ktoré boli konvertované na uracily, kým červené nukleotidy prezentujú 5-metylcytozíny odolné voči konverzii. Analýza jednotlivých CpG dinukleotidov môže prebehnúť pomocou metylačne špecifickej PCR s párom primerov špecifickým na metylovanú DNA a s párom primerov špecifickým na nemetylovanú DNA. Primery, ktoré sú špecifické na metylovanú DNA rozoznávajú iba sekvenciu, ktorá je metylovaná a teda obsahuje 5-metylcytozín, ktorý je odolný voči konverzii. Primery, ktoré sú špecifické na nemetylovanú DNA rozoznávajú iba sekvenciu, ktorá nie je metylovaná a teda obsahuje uracil, ktorý vznikol konverziou nemetylovaného cytozínu. Detekcia prebieha napríklad pomocou gélovej elektroforézy. U znamená nemetylovaná DNA (z angl. unmethylated) a M znamená metylovaná DNA (z angl. methylated). Analýza celej oblasti, ktorá nás zaujíma, prebieha pomocou nemetylačne špecifickej PCR, kedy pomocou špecifických primerov získame požadovaný úsek konvertovanej DNA, ktorý nás zaujíma. Na rozdiel od metylačne špecifickej PCR nepotrebujeme dva páry primerov, pretože v sekvencii primerov pri nemetylačne špecifickej PCR nie sú CpG dinukleotidy a teda tieto primery nerobia rozdiely medzi metylovanou a nemetylovanou sekvenciou DNA. Nasleduje klonovanie a sekvenovanie a detekcia metylovaných cytozínov ako cytozínov a nemetylovaných cytozínov ako tymínov.

4.7. Pasívna a aktívna demetylácia DNA

K demetylácii môže dochádzať pasívne alebo aktívne (Teperek-Tkacz et al., 2011). K pasívnej demetylácii dochádza počas replikácie DNA pri absencii udržovacej DNA metyltransferázovej aktivity, takže nemetylované cytozíny sú inkorporované do nového vlákna DNA. Počas aktívnej demetylácie, metylované cytozíny sú nahradené nemetylovanými cytozínmi cez enzymatický proces nezávislý od replikácie DNA (Teperek-Tkacz et al., 2011). Demetylácia DNA môže byť teda pasívna, závislá od replikácie DNA, kedy dcérske vlákno DNA nie je metylované kvôli defektom v DNA metyltransferázach alebo aktívna, ktorá je rýchla a nie je závislá od replikácie DNA (Niehrs, 2009).

Súčasný objav novej modifikovanej bázy 5-hydroxymetylcytozínu (5hmC) v cicavčích bunkách má veľký význam pre objasnenie mechanizmu aktívnej demetylácie (Chen and Riggs, 2011). 5-hydroxymetylcytozín je kľúčovým faktorom v demetylácii, ktorý môže byť pasívne odstránený počas replikácie DNA alebo môže dôjsť k obnoveniu cytozínu aktívne, kedy je 5-hydroxymetylcytozín odstránený cez oxidáciu a bázovú excíznu opravu založenú na glykozyláze TDG (z angl. thymine DNA glycosylase) (Kohli and Zhang, 2013). Dráha aktívnej demetylácie prebieha cez oxidáciu 5mC pomocou TET 5mC hydroxylázy. Tento enzým katalyzuje konverziu z 5mC na 5hmC, 5fC a nakoniec na 5caC, ktorý je odstránený tymidín-DNA glykozylázou (Plass et al., 2013). Takto vznikne abázické miesto, ktoré je opravené pomocou bázovej excíznej opravy (z angl. base excision repair; BER). Ďalším procesom demetylácie cytozínov zahŕňa proces založený na deaminácii a následnej bázovej excíznej oprave alebo nukleotidovej excíznej oprave (Plass et al., 2013). Medzi deaminázy patria napríklad deamináza AID (z angl. activation-induced (DNA-cytosine) deaminase) a APOBEC1 (z angl. apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1), ktoré konvertujú 5mC na tymín. Takéto miesto je opravené pomocou BER a táto oprava je iniciovaná tymidín-DNA glykozylázou (Chen and Riggs, 2011). Zdá sa, že 5hmC by mohol byť tiež deaminovaný na 5 hydroxymetyluracyl (5hmU). 5hmU môže byť následne rozpoznaný tymidín-DNA glykozylázou, ktorá ho odstráni z DNA vlákna a vznikne abázické miesto, ktoré je opravené pomocou bázovej excíznej opravy (Piccolo and Fisher, 2014). Tieto dráhy demetylácie sú nezávislé od replikácie a ponúkajú veľmi rýchlu konverziu z metylovaného do nemetylovaného stavu.



Obr. č. 13 **Dva hlavné modely demetylácie DNA. Porovnanie aktívnej a pasívnej demetylácie DNA.**

Upravené podľa Hajkova, in: Epigenetic reprogramming in mouse germ cells 2004

Popis obrázku:

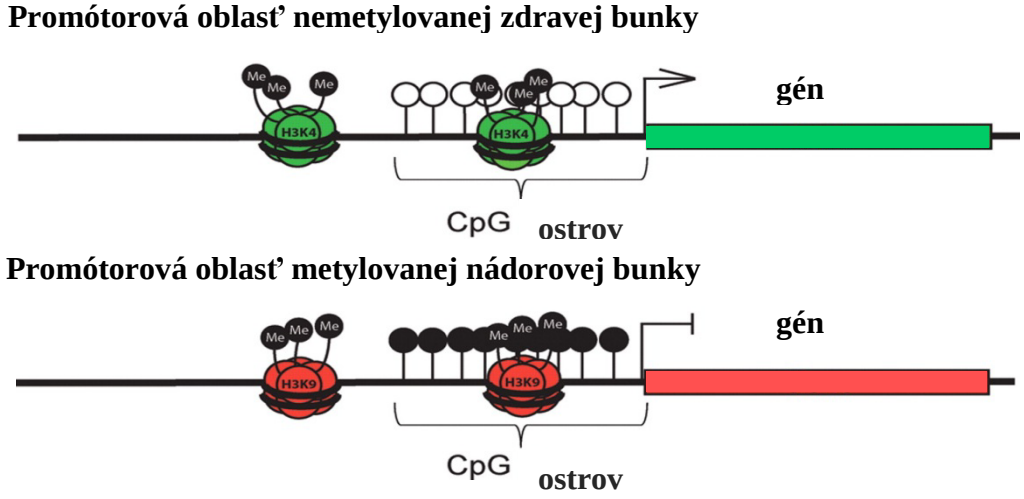
K pasívnej demetylácii DNA dochádza v dôsledku replikačného procesu pri absencii udržovacej metylácie sprostredkovanej prostredníctvom metyltransferázy DNMT1. V kontraste s pasívnou demetyláciou DNA je aktívna demetylácia DNA, ktorá nie je závislá od replikácie DNA a vyžaduje enzymatickú aktivitu.

4.8. Globálne epigenetické zmeny pri nádorových ochoreniach

Epigenetické zmeny, napríklad metylácia DNA regulujú expresiu génov počas vývoja cicavcov. Avšak, hypermetylácia v oblasti promótorov génov môže viesť k vzniku nádorového ochorenia práve kvôli transkripčnému umlčaniu dôležitých tumor-supresorových génov. Ďalšie modifikácie chromatínu, napríklad deacetylácia histónov, ovplyvňujú štruktúru chromatínu a spolu s metyláciou DNA regulujú transkripciu génov (Baylin, 2005). Epigenóm je vlastne súbor všetkých epigenetických modifikácií DNA a histónov v genóme. Zmeny v epigenetickej regulácii aktivity genómu sú veľmi závažné pre vznik nádorov, rovnako ako aj zmeny samotnej kódujúcej genetickej informácie genómu. Každá epigenetická drahá regulácie vyžaduje enzýmy, ktoré sprostredkujú danú modifikáciu ("writers"), prostredníctvom nich daná modifikácia vzniká. Ďalej sú potrebné enzýmy, ktoré modifikujú alebo zvrátia danú modifikáciu ("editors") a enzýmy, ktoré sprostredkujú vytvorenie proteínových komplexov s danou modifikáciou ("readers") a sprostredkujú efekt na transkripciu. V súčasnosti sa objavuje veľa štúdií, ktoré poukazujú, že nádorové genómy vykazujú časté zmeny v epigenóme (Plass et al., 2013). To zahrňuje epigenetické umlčanie génov s tumor-supresorovým efektom, ktoré sa zúčastňujú signalizačných dráh ako apoptóza, proliferácia buniek, migrácia buniek alebo oprava DNA (Esteller, 2002).

4.8.1. Modifikácie histónov

Zmeny v metylácii DNA pri nádorových ochoreniach sú často spájané so zmeneným znakom modifikácie histónov. Spolu modifikácie DNA a histónov determinujú stav chromatínu, ktorý odzrkadľuje, či je gén transkripčne aktívny alebo neaktívny (Plass et al., 2013). Zlyhanie správnej metylácie v somatických bunkách môže byť tiež znakom nádorových ochorení, kde vzor metylácie DNA je porušený, čo sa prejavuje globálnou genómovou hypometyláciou sprevádzanou hypermetyláciou v promótorových oblastiach génov, čo je opak bežného stavu pri zdravých bunkách (Jones and Laird, 1999; Baylin and Herman, 2000; Laird, 2005).



Obr. č. 14 **Hypermetylácia a nádorové ochorenia.**

Upravené podľa Lahtz and Pfeifer, Journal of Molecular Cell Biology 2011

Popis obrázku:

Promótorové oblasti génov sú zvyčajne bohaté na CpG dinukleotidy. Tieto oblasti bývajú v zdravých bunkách v nemetylovanom stave a tento stav sa spája s aktívnym znakom modifikácie histónu, 3-metyláciou histónu 3 v polohe lyzín 4 (H3K4me3). Táto modifikácia histónu zabraňuje metylácii DNA. Počas rozvoja nádorového ochorenia, CpG dinukleotidy v oblasti promótora génu prechádzajú do metylovaného stavu, čo sa spája s inaktívnym znakom modifikácie histónu, napríklad 3-metyláciou histónu 3 v polohe lyzín 9 (H3K9me3) a gén je transkripčne umlčaný (Lahtz and Pfeifer, 2011).

Regulácia štruktúry chromatínu má významnú úlohu v kontrole génovej expresie. Chromatín je komplex DNA a histónových proteínov. Konce histónov poskytujú miesta pre posttranslačné modifikácie, napríklad acetylácia lyzínu, fosforylácia serínu, metylácia arginínu a lyzínu (Grant, 2001; Esteller, 2011). Konkrétna kombinácia modifikácií koncov histónov formuje histónový kód, ktorý reguluje aktivitu génov (Esteller, 2011). Acetylácia patrí medzi najviac študované modifikácie histónov. Acetylované históny sú zvyčajne spájané s transkripčne aktívnym chromatínom a neacetylované históny sa zvyčajne spájajú s transkripčne neaktívnym chromatínom (Grant, 2001). Acetylácia znižuje pozitívny náboj, teda afinitu N-koncových domén histónov k DNA, tým pádom majú transkripčné faktory lepší prístup k regulačným oblastiam DNA. Porucha regulácie génov, ktoré sa zapájajú v úprave štruktúry chromatínu sa spája s nekontrolovaným rastom buniek a rozvojom nádorov (Klenova et al., 2002; Lund and van Lohuizen, 2004). Aktívna transkripcia je spojená s vysokou hladinou acetylácie histónov chromatínu (Struhl, 1998; Berger, 2002; Eberharter and Becker, 2002; Legube and Trouche, 2003; Eberharter et al., 2005).

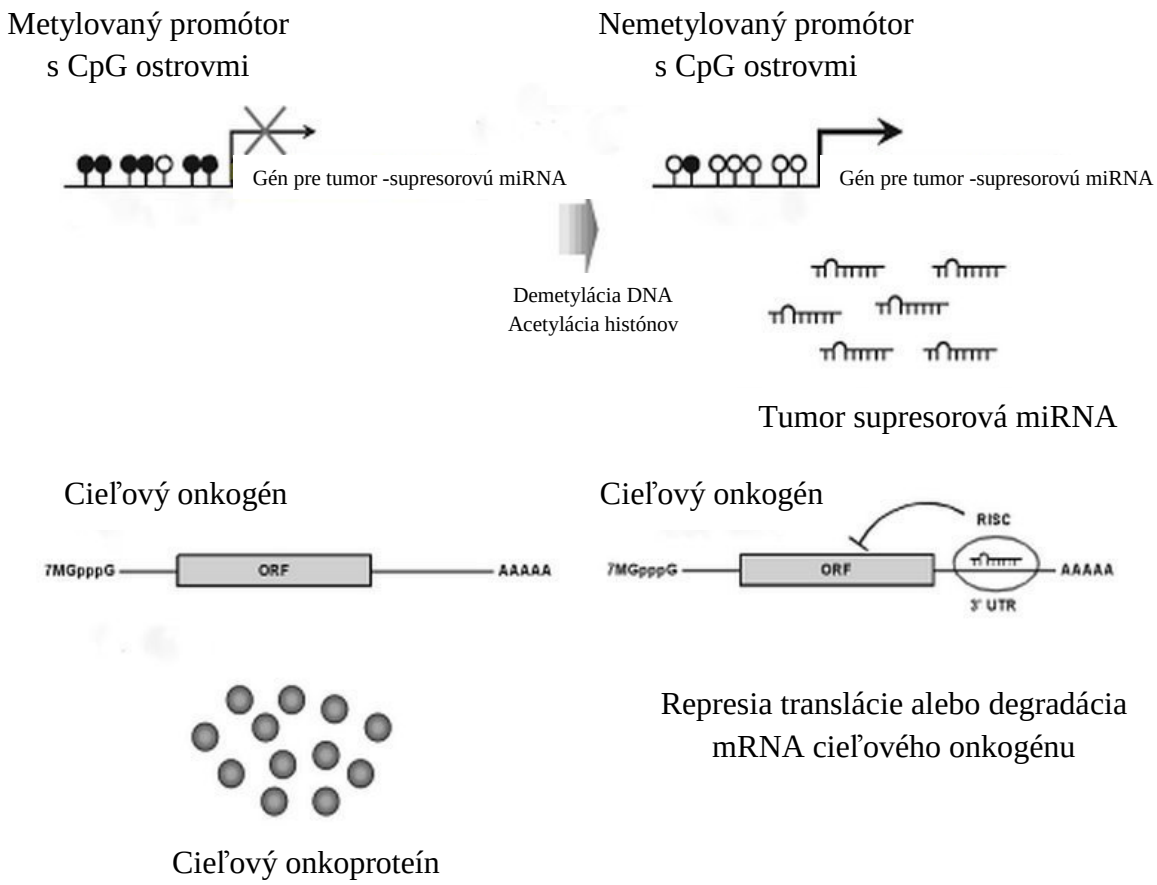
Histón-acetyltransferázy (z angl. histone acetyltransferase; HAT) acetylujú lyzínové N-terminálne zvyšky histónov, inými slovami prenášajú acetylovú skupinu z acetylkoenzýmu A (acetylCoA) za tvorby N-acetylovaného lyzínu. V súvislosti s nádorovými ochoreniami bola popísaná vysoká hladina histón-deacetyláz a zníženie acetylácie histónu 3 (Hellebrekers et al., 2006; Glozak and Seto, 2007; Setiadi et al., 2007). CBP (z angl. CREB-binding protein) je známa histón-acetyltransferáza, ktorá acetyluje históny v oblasti promótora, čo spôsobuje vyššiu prístupnosť promótora pre transkripčné faktory (Kalkhoven, 2004). Acetylácia histónov je spojená s aktívnou transkripciou, acetylované históny sú rozpoznávané transkripčnými faktormi a ich bromodoménami. Teda dochádza k zvýšenej väzbe transkripčných faktorov, ktoré sú zodpovedné za iniciáciu transkripcie. Histónové konce sú pozitívne nabité kvôli lyzínom a arginínom. Tento pozitívny náboj ich drží spolu a sprostredkuje väzbu na záporne nabitú fosfodiesterovú kostru DNA. Acetylácia ruší tento pozitívny náboj, uvoľňuje priestor pre transkripčné faktory a umožňuje transkripciu. Často ide o acetyláciu histónu 3 v polohe lyzín 9, 14, 18 a 23 a acetyláciu histónu 4 v polohe lyzín 5, 8, 12 a 16 (Grant, 2001). Naopak deacetylácia obnovuje pozitívny náboj a zabraňuje v prístupe transkripčných faktorov a zabraňuje transkripcii. Trichostatin A (TSA) je špecifický inhibítor histón-deacetylázovej aktivity (HDAC). Štúdie potvrdili sľubné využitie protinádorovej aktivity TSA proti rakovine prsníka *in vitro* a *in vivo*, čo výrazne podporuje fakt, že histón-deacetylázová aktivita je dobrým molekulárnym cieľom pre protinádorovú terapiu rakoviny prsníka (Vigushin et al., 2001) pomocou inhibítorov histón-deacetylázovej aktivity. Ošetrenie TAP-1 deficientných buniek pomocou IFN-γ zvýši väzbu histón-acetyltransferázy CBP k promótoru génu antigén-prezentujúcej mašinérie *TAP-1*. Táto skutočnosť bola spojená so zvýšenou acetyláciou histónu 3 a aktívnou transkripciou génu *TAP-1* (Setiadi et al., 2007). Prvé štúdie o modifikáciách histónov ukázali, že histón 3 v polohe lyzín 4, 9 a 27 a histón 4 v polohe lyzín 20 sú prednostne metylované. Môže ísť o mono-, di- alebo tri-metyláciu daného histónu. 3-metylácia histónu 3 v polohe lyzín 4 (H3K4me3) je aktívnym znakom modifikácie histónu. Táto modifikácia histónu zabraňuje metylácii DNA. Počas rozvoja nádorového ochorenia, CpG dinukleotidy v oblasti promótora génu prechádzajú do metylovaného stavu, čo sa spája s inaktívnym znakom modifikácie histónu, napríklad 3-metyláciou histónu 3 v polohe lyzín 9 (H3K9me3) a gén je transkripčne umlčaný (Lahtz and Pfeifer, 2011). S umlčaním transkripcie sa ďalej spája napríklad 3-metylácia histónu 3 v polohe lyzín 27 (H3K27me3) a mono-metylácia histónu 4 v polohe lyzín 20 (H4K20me1) (Sims et al., 2008).

4.8.2. Modifikácie DNA

Epigenóm je oveľa variabilnejší ako genóm, aj v rámci jedného organizmu sa mení v priebehu života. Epigenetické zmeny pri nádorových ochoreniach sú študované už mnoho rokov. Mnoho štúdií sa orientuje na 5-metylcytozín (5mC), ale je tiež známe, že cytozíny môžu byť ďalej modifikované na 5-hydroxymetylcytozíny (5hmC), 5-formylcytozíny (5fC) a 5-carboxylcytozíny (5caC). Hypermetylácia CpG ostrovov v oblasti promótorov génov je spájaná s umlčaním transkripcie, čo bolo dokázané pre mnohé tumor-supresorové gény. Metylácia DNA je relatívne stabilným epigenetickým znakom, ktorá udržuje gény v utlmenom štádiu, ale v súčasnosti sa objasňujú dráhy, ktoré sa zapájajú do odstránenia metylácie DNA (Eccleston et al., 2013). Nádorové genómy sú charakterizované globálnou stratou 5mC (hypometyláciou), čo najčastejšie postihuje repetitívne sekvencie. Model dráhy demetylácie DNA prezentovali Kohli a Zhang v roku 2013, ktorý zdôraznili úlohu enzýmov TET v mnohých biologických procesoch (Eccleston et al., 2013).

4.8.3. Epigenetika a nekódujúce RNA

Súčasné štúdie odhalili, že v eukaryotickej bunke vzniká mnoho RNA. Veľká časť z nich neplní funkciu mRNA, tRNA alebo rRNA a preto ich nazývame ako nekódujúce RNA (z angl. non-coding RNA; ncRNA). ncRNA sa však zúčastňujú mnohých biologických procesov, napríklad slúžia ako regulátory génovej expresie na úrovni modifikácie chromatínovej štruktúry, transkripcie, kontroly stability a translácie RNA, regulácie zostrihu RNA. Najlepšie preštudované sú malé interferujúce RNA (z angl. small interfering RNA; siRNA) a micro RNA (z angl. micro RNA; miRNA), ktoré regulujú expresiu génov cez dráhu RNA interferencie (Yu, 2008). miRNA môžu plniť úlohu ako tumor-supresorové gény alebo ako onkogény (Esquela-Kerscher and Slack, 2006). Často dochádza k zníženej regulácii miRNA pri mnohých nádorových ochoreniach, čo naznačuje ich funkciu nádorových supresorov. V niektorých prípadoch naopak dochádza k ich zvýšenej regulácii, čo naznačuje ich funkciu ako onkogénov a zvyšujú rozvoj nádorového ochorenia (Saito et al., 2008).



Obr. č. 15 **Model aktivácie tumor supresorovej miRNA pomocou epigenetických agensov pri nádorovom ochorení.**

Upravené podľa Saito et al, Epigenetics 2008

Popis obrázku:

Gén pre tumor supresorovú miRNA je umlčaný cez hypermetyláciu DNA a cez kondenzovanú štruktúru chromatínu v promótorovej oblasti pri mnohých nádorových ochoreniach. Epigenetickými agensami, napríklad inhibítormi metylácie DNA alebo deacetylácie histónov, môžeme znovu navodiť transkripciu tumor supresorovej miRNA. Maturovaná miRNA je inkorporovaná do komplexu RISC (z angl. RNA-induced silencing complex) a takýto komplex miRNA/RISC sa asociuje so sekvenčne špecifickou mRNA onkogénu na základe párovania báz a tak tumor supresorová miRNA sprostredkuje umlčanie alebo degradáciu cieľového onkogénu (Saito et al., 2008).

Metylácia DNA plní úlohu aj v inaktivácii chromozómu X a v genómovom imprintingu (Sado et al., 2000). Proces inaktivácie chromozómu X je regulovaný zo špecifického regiónu na chromozóme X, ktorý sa nazýva stred inaktivácie X (Wutz and Jaenisch, 2000). V tejto oblasti je kódovaných množstvo neprekladajúcich sa transkriptov, ktoré sú

vyžadované pri inaktivácii celého chromozómu X. Najvýznamnejším z nich je dlhá nekódujúca RNA Xist, ktorá je transkribovaná z budúceho inaktívneho chromozómu X (Xi), ale nie z toho aktívneho (Xa) (Norris et al., 1994). Xist pokrýva inaktívny chromozóm X a umožňuje začiatok procesu inaktivácie cez nevratnú heterochromatizáciu (Brown and Willard, 1994; Csankovszki et al., 1999; Wutz and Jaenisch, 2000). Udržovanie v inaktívnom stave závisí od metylácie DNA (Sado et al., 2000). Gén Xist je v nemetylovanom stave na inaktívnom chromozóme X, ale oblasti promótorov množstva ďalších génov, ktoré sa tu nachádzajú sú v metylovanom stave, kým na aktívnom chromozóme X sú v nemetylovanom stave. Udržovanie inaktívneho stavu chromozómu Xi môžeme vysvetliť prítomnosťou mnohonásobných CpG miest. Inaktívny stav je udržovaný cez tieto miesta aj napriek tomu, že sa môže stať, že niektoré náhodne uniknú metylácii. Ďalej, úlohu v inaktivácii chromozómu X majú retrotranspozibilné elementy LINE1, slúžia ako pomocný motor šírenia inaktivácie chromozómu X (Lyon, 1998; Bailey et al., 2000) a môžu byť rozpoznávané ako elementy, ktoré je nutné umlčať. Metylácia DNA plní tiež dôležitú úlohu v genómovom imprintingu. Imprintované gény sú inaktivované metyláciou DNA na jednej alele podľa rodičovského pôvodu. Inými slovami, imprintované gény sú transkribované iba z jednej alely, a to alely určitého rodičovského pôvodu. Druhá alela génu je neaktívna – imprintovaná. Väčšinou sa v genóme nenachádzajú tieto gény izolované, ale naopak sa zhlukujú a je tak možné identifikovať celé chromozomálne oblasti s imprintovanými génmi. Tu sa aj nachádza zásadná štruktúra pre samotnú realizáciu imprintingu, tzv. oblasť riadiaca imprinting (z angl. imprinting control region; ICR) (Weaver and Bartolomei, 2014). Akceptované sú dva modely mechanizmu regulácie génového imprintingu: model inzulátorov a enhancerov a model nekódujúcich RNA. Princíp modelu inzulátorov a enhancerov môžeme vysvetliť nasledovne. Intergénová oblasť riadiaca imprinting je na paternálnej alele metylovaná, čo bráni väzbe inzulátora. Metylácia je rozšírená aj na gén, ktorý sa nachádza medzi inzulátorom a enhancerom a jeho aktivácia je utlmená. Enhancer má teda dosah na vzdialenejší gén za väzobným miestom pre inzulátor a dochádza k jeho aktivácii. Na maternálnej alele je intergénová oblasť riadiaca imprinting nemetylovaná, čo umožňuje väzbu inzulátora. V tomto prípade iba gén, ktorý sa nachádza medzi inzulátorom a enhancerom môže byť aktivovaný. Na vzdialenejší gén za inzulátorom enhancer nemá dosah, pretože mu v tom bráni naviazaný inzulátor. Model nekódujúcich RNA môžeme vysvetliť nasledovne. Oblasť riadiaca imprinting plní funkciu promótora pre ncRNA. Na paternálnej alele je táto oblasť nemetylovaná, čo umožňuje expresiu ncRNA. ncRNA následne na paternálnej alele

v danej oblasti utlmuje gény, ktoré majú byť umlčané. Na maternálnej alele je oblasť riadiaca imprinting metylovaná a nedochádza k vzniku ncRNA a gény v danej oblasti na maternálnej alele sú exprimované (Weaver and Bartolomei, 2014).

4.9. Chemoterapia pomocou epigenetických agensov a jej dopad na protinádorovú imunitu

Inhibítory metylácie DNA 5-azacytidin (Vidaza) a 5-aza-2'deoxycitidin (Dacogen) indukujú obnovenie expresie chybne umlčaných génov pri nádorových ochoreniach, čo vyústi do zastavenia bunkového cyklu a spustenia apoptózy nádorových buniek (Baylin, 2005). Doposiaľ štyri epigeneticky aktívne liečivá dostali povolenie od zastupiteľstva USA FDA (z angl. US Food and Drug Administration) na základe ich preukázaných klinických účinkov. Sú to, inhibítory DNA metyltransferáz 5-azacytidin (Vidaza) a 5-aza-2'deoxycitidin (Dacogen) a ďalej sú to inhibítory histón-deacetyláz kyselina suberoylanilid hydroxamová (Vorinostat) a romidepsin (Istodax). Oboje triedy liečiv obnovujú expresiu chybne umlčaných génov, ktorá je spôsobená činnosťou DNA metyltransferáz a histón-deacetyláz. Štúdie naznačujú obnovenú transkripčnú aktivitu sprevádzanú epigenetickou prestavbou a protinádorovou aktivitou *in vitro* aj *in vivo*. Protinádorový efekt môžeme vysvetliť už spomínanou indukciou zastavenia bunkového cyklu, indukciou diferenciácie alebo apoptózy nádorových buniek, či zvýšenou citlivosťou nádorových buniek voči chemoterapii alebo rádioterapii (Plass et al., 2013). Inhibítory DNA metyltransferáz, napríklad 5-aza-2'deoxycitidin, dokážu indukovať aj regulačné T-lymfocyty prostredníctvom aktivácie expresie *FoxP3* (Lal et al., 2009). Bol tiež popísaný negatívny efekt inhibítorov histón-deacetylázovej aktivity na maturáciu dendritických buniek a na ich schopnosť prezentácie antigénu (Nencioni et al., 2007; Kim and Lee, 2010). Na druhej strane, 5-aza-2'deoxycitidin dokáže indukovať diferenciáciu myeloidných supresorových buniek na imunogénne antigén-prezentujúce bunky (Daurkin et al., 2010). Inhibítory DNA metyltransferáz rozdeľujeme na nukleozidové a nenukleozidové. Nukleozidové sú včlenené do DNA a dokážu interagovať s DNA metyltransferázami, dôjde k zachyteniu enzýmu kovalentnou väzbou, jeho inaktivácii a teda k demetylácii po dokončení replikácie DNA. Patria sem 5-azacytidin alebo 5-aza-2'deoxycytidin. Majú aj cytotoxické vlastnosti, takže ich vplyv na expresiu génov a ich protinádorový účinok nemusí byť vždy z ich demetylačných vlastností, ale aj z ich cytotoxických vlastností. 5-azacytidin je analóg cytidínu, ktorý sa inkorporuje do RNA aj do DNA. Pred včlenením do DNA musí byť chemicky upravený na deoxyribonukleozid trifosfát, aby mohlo dôjsť k inkorporácii do

DNA. Avšak, kým je 5-azacytidin chemicky upravený na deoxyribonukleozid trifosfát, časť z neho sa včlení do RNA, čo ovplyvňuje mnohé funkcie RNA, napríklad biogenézu ribozómov. Tieto účinky ošetrenia 5-azacytidinom sú nezávislé od demetylácie DNA. 5-aza-2'deoxycytidin je tiež analóg cytidínu, ale nie je potrebná chemická úprava na deoxy formu a včleňuje sa priamo a iba do DNA (Lyko and Brown, 2005). Nádorové bunky majú často zrýchlený bunkový cyklus a 5-azacytidin inhibuje regulátory prechodu bunkovým cyklom a tak spomaľuje rast a množenie sa nádorových buniek. Medzi nenukleozidové inhibítory DNA metyltransferáz patrí napríklad RG108. Blokuje priamo aktívne miesto DNA metyltransferáz (Lyko and Brown, 2005). Trichostatin A (TSA) je špecifický inhibítor histón-deacetylázovej aktivity (HDAC). Štúdie potvrdili sľubné využitie protinádorovej aktivity TSA proti rakovine prsníka *in vitro* a *in vivo*, čo výrazne podporuje fakt, že histón-deacetylázová aktivita je vhodným molekulárnym cieľom pre protinádorovú terapiu rakoviny prsníka (Vigushin et al., 2001) pomocou inhibítorov histón-deacetylázovej aktivity. Inhibítory histón-deacetyláz tiež zvyšujú expresiu adhezívnej molekuly CD54 (z angl. intercellular adhesion molecule 1; ICAM1) na povrchu endoteliálnych buniek derivovaných z nádoru. Tým sa zvýši schopnosť lymfocytov priľnúť k endoteliálnym bunkám, čo im umožňuje migrovať cez tkanivo do miesta nádoru. Promótorová oblasť génu *ICAM1* endoteliálnych buniek derivovaných z nádoru obsahuje hypoacetylovaný histón H3 a hypometylovaný histón H3 v polohe lyzín 4. Ošetrenie s inhibítormi histón-deacetyláz a inhibítormi DNA metyltransferáz reaktivuje adhezívnu molekulu ICAM1 prostredníctvom zvýšenej acetylácie histónu H3 a metylácie histónu H3 v polohe lyzín 4 v promótorovej oblasti génu *ICAM1* (Hellebrekers et al., 2006; Glozak and Seto, 2007).

5. Ciele práce

Záujmom práce je imunoterapia MHC I negatívnych a pozitívnych nádorov myší a regulácia protinádorovej imunity. Špeciálnu pozornosť venuje epigenetickým mechanizmom v regulácii prezentácie antigénov nádorovými bunkami, efektom epigenetických agensov na nádorové a supresorové bunky a problematike potlačenia imunosupresie v protinádorovej terapii. Hlavnými cieľmi sú:

1) Sledovať spôsob, akým IFN-γ zvyšuje expresiu MHC I na povrchu buniek a faktorov regulovaných IFN-γ (z angl. interferon regulatory factors; IRF) v nádorových bunkách a či sa tu uplatňujú epigenetické mechanizmy.
    a) Zistiť, či komponenty dráhy IFN-γ môžu byť ovplyvnené 5-azacytidinom (IRF alebo STAT1).
    b) Určiť a porovnať hladinu transkripcie vybraných imunoaktívnych génov a určiť epigenetické zmeny v rámci genómu nádorových buniek po ovplyvnení epigenetickými agensami a s IFN-γ.
    c) Preskumáť, či IFN-γ môže plniť úlohu epigenetického agensu, ktorý zvyšuje expresiu génov potrebných pre prezentáciu antigénu a kostimuláciu cez DNA demetyláciu.
    d) Podrobne opísať reverzibilné mechanizmy pri úniku nádorových buniek špecifickej imunite.
2) Sledovať ako epigenetický agens 5-azacytidin ovplyvňuje interakcie nádorových buniek a imunitného systému a ich citlivosť k imunoterapii.
    a) Sledovať inhibičný efekt na nádorové bunky v kombinovanej terapii 5-azacytidinom a krátkymi nemetylovanými oligodeoxynukleotidmi (CpG ODN) alebo podaním IL-12.
    b) Efekt epigenetického agensu 5-azacytidinu na expresiu MHC I na povrchu nádorových buniek a na expresiu génov antigén-prezentujúcej mašinérie u MHC I deficientných nádorov.
    c) Zistiť metylačný status regulačných sekvencií génov antigén-prezentujúcej mašinérie počas rastu a terapie nádorov.
3) Rozšíriť predmet záujmu o monitorovanie génov s imunosupresívnym účinkom mikroprostredia nádoru počas rastu a liečby nádoru.

a) Podrobne preskúmať mechanizmus akumulácie MDSC po chemoterapii s CY (úloha prozápalových cytokínov) a následne identifikovať možnú imunoterapiu s cieľom zoslabiť indukovanú imunosupresiu.
b) Porovnať fenotyp a funkciu akumulovaných MDSC v slezine po terapii s CY (CY-MDSC) s tými, kde sú MDSC akumulované počas rastu nádoru TC-1 (TU-MDSC) a s tými MDSC, ktoré sú akumulované počas rastu nádoru TC-1 pri ošetrení s CY, čo podporuje ich ďalšiu akumuláciu v slezine (CYTU-MDSC).
c) Zistiť účinok terapie s induktorom diferenciácie kyselinou ATRA alebo s IL-12 na MDSC akumulované po liečbe s CY (CY-MDSC).

4) Zistiť efekt epigenetických agensov, konkrétne 5-azacytidinu na MDSC, ktoré sú akumulované v mikroprostredí nádoru a v slezine počas jeho rastu a počas terapie nádorov TC-1/A9 a TRAMP-C2 s CY.
   a) Zistiť, či 5-azacytidin dokáže indukovať diferenciáciu MDSC na imunogénne antigén-prezentujúce bunky.
5) V neposlednom rade zistiť efekt protilátky PC61 (anti-CD25 Ab) na regulačné T-lymfocyty a aktivované NKT bunky (z angl. natural killer T cells) a zistiť použitie danej protilátky v kombinovanej imunoterapii experimentálnych nádorov myší. Zistiť účinok protilátky v kombinácii s aktiváciou NKT buniek na rast nádoru TC-1.

6. Výsledky

Výsledky tejto práce boli zhrnuté do piatich publikácií. V nasledujúcej kapitole sú tieto publikácie uvedené vo forme, v ktorej boli zverejnené v zahraničných časopisoch (okrem publikácie č.1, ktorá bola zaslaná do časopisu *International Journal of Cancer* a je v menovanom časopise posudzovaná). Komentár pred každou publikáciou zhŕňa výsledky práce a hodnotí jej samotný význam.

6.1. Publikácia I

Epigenetické regulácie v signalizačnej dráhe IFN-γ: Zvýšená expresia molekúl MHC I na povrchu nádorových buniek sprostredkovaná IFN-γ je spojená s DNA demetyláciou génov antigén-prezentujúcej mašinérie.

Epigenetic regulations in the IFN-γ signalling pathway: IFN-γ-mediated MHC class I upregulation on tumour cells is associated with DNA demethylation of antigen-presenting machinery genes.

Veronika Vlková, Ivan Štěpánek, Veronika Hrušková, Filip Šenigl, Veronika Mayerová, Martin Šrámek, Jana Šímová, Jana Bieblová, Marie Indrová, Tomáš Hejhal, Nicolas Dérian, David Klatzmann, Adrien Six a Milan Reiniš

Reverzibilné zníženie MHC I na povrchu nádorových buniek je bežný mechanizmus, ktorým nádorové bunky unikajú imunitnej odpovedi a je spájané s koordinovaným umlčaním génov antigén-prezentujúcej mašinérie. Táto expresia môže byť znovu obnovená po ovplyvnení IFN-γ. IFN-γ je cytokín s pleiotropným účinkom na nádorové bunky, ktorý je tiež považovaný za hlavného sprostredkovateľa efektívnej protinádorovej imunity s priamym efektom na nádorové bunky (Dunn et al., 2006). Hlavným cieľom práce bolo určiť, či IFN-γ môže plniť úlohu epigenetického agensu, či má efekt napríklad na DNA demetyláciu príslušných regulačných oblastí génov, ktoré aktivuje a či je DNA demetylácia súčasťou mechanizmu, ktorým IFN-γ zvyšuje expresiu vybraných génov v myšacích nádorových líniách deficientných na MHC I. V tejto práci sme dokázali spojitosť demetylácie DNA v promótorových oblastiach vybraných génov antigén-prezentujúcej mašinérie (*TAP-1*, *TAP-2*, *LMP-2*, *LMP-7*) po ovplyvnení IFN-γ so zvýšenou expresiou MHC I na povrchu nádorových buniek v myšacích MHC I deficientných nádorových líniách. Naše výsledky tiež hovoria o vysokej hladine metylácie DNA pri MHC I deficientnej línii TC-1/A9 v porovnaní s jej pôvodnou MHC I pozitívnou líniou TC-1, čo naznačuje, že metylácia génov antigén-prezentujúcej mašinérie, ktorá spôsobuje zníženú expresiu MHC I na povrchu nádorových buniek, sa uplatňuje pri úniku nádorových buniek pred špecifickou imunitnou odpoveďou. Zatiaľ je veľmi málo známe o DNA demetylácii v rámci regulácie génov sprostredkovanej IFN-γ. Našou prácou sme prispeli zistením, že DNA demetylácia sprostredkovaná IFN-γ je závislá na signalizácii cez JAK/STAT dráhu, pretože inhibítor Janusových kináz blokoval demetyláciu DNA a indukciu expresie MHC I na povrchu buniek. Ďalej DNA demetylácia sprostredkovaná

IFN-γ je dynamickejšia v porovnaní s demetyláciou DNA indukovanou pomocou inhibítora DNA metyltransferáz 5-azacytidinom, čo naznačuje, že proces demetylácie je aktívny a nezávislý od replikácie DNA na rozdiel od DNA demetylácie indukovanej 5-azacytidinom, ktorá je od replikácie DNA závislá a vyžaduje inkorporáciu liečiva do DNA a blokuje metyláciu nascentného reťazca DNA kvôli inhibícii metyltransferáz (Creusot et al., 1982). Nakoniec DNA demetylácia sprostredkovaná IFN-γ je asociovaná s acetyláciou histónu H3 v oblasti promótorov génov antigén-prezentujúcej mašinérie. V publikácii sme priniesli dáta, ktoré naznačujú, že IFN-γ môže plniť úlohu epigenetického agensu a môže navodiť demetyláciu DNA mnohých génov, s dôrazom kladeným najmä na gény antigén-prezentujúcej mašinérie.

Prínos autora dizertačnej práce k danej publikácii:

Ako prvá autorka danej publikácie som spravila väčšinu experimentálnej práce, čo zahrňuje napríklad monitorovanie povrchovej expresie MHC I molekúl na vybraných nádorových modeloch a monitorovanie relatívnej expresie vybraných génov antigén-prezentujúcej mašinérie. Mojou úlohou bolo ďalej sledovať efekt IFN-γ na demetyláciu DNA oblastí promótorov génov antigén-prezentujúcej mašinérie pomocou metylačne špecifickej PCR a sekvenovaním príslušnej oblasti promótora vybraných génov. Tiež som prispela do kinetickej štúdie efektu IFN-γ a 5-azacytidinu na demetyláciu DNA oblastí promótorov génov antigén-prezentujúcej mašinérie. A v neposlednom rade som sa zúčastnila pokusov s inhibítorom Janusových kináz, ktorý blokoval demetyláciu DNA a indukciu expresie MHC I molekúl na povrchu buniek. Vyhodnocovala som výsledky do danej publikácie a výrazne som sa podieľala pri písaní publikácie.

Epigenetic regulations in the IFNγ signalling pathway: IFNγ-mediated MHC class I upregulation on tumour cells is associated with DNA demethylation of antigen-presenting machinery genes.

Veronika Vlková[1], Ivan Štěpánek[1], Veronika Hrušková[1], Filip Šenigl[2], Veronika Mayerová[1], Martin Šrámek[1], Jana Šímová[1], Jana Bieblová[1], Marie Indrová[1], Tomáš Hejhal[1], Nicolas Dérian[3,4,5,6], David Klatzmann[3,4,5,6], Adrien Six[3,4,5,6] and Milan Reiniš[1]*

[1]Department of Tumour Immunology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, v. v. i., Prague
[2]Department of Viral and Cellular Genetics, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, v. v. i., Prague
[3] UPMC Univ Paris 06, UMR 7211, Immunology-Immunopathology-Immunotherapy (I3), F-75013, Paris, France
[4]CNRS, UMR 7211, Immunology-Immunopathology-Immunotherapy (I3), F-75013, Paris, France
[5]INSERM, UMR_S 959, Immunology-Immunopathology-Immunotherapy (I3), F-75013, Paris, France
[6]AP-HP, Hôpital Pitié-Salpêtrière, CIC-BTi Biotherapy & Département Hospitalo-Universitaire (DHU) Inflammation-Immunopathology-Biotherapy (i2B), F-75013, Paris, France

* **Corresponding author:**
Laboratory of Tumour Immunology, Institute of Molecular Genetics, v. v. i.
Academy of Sciences of the Czech Republic
Vídeňská 1083, 142 20 Prague 4
Czech Republic
Phone: (+420) 296 443 461
FAX: (+420) 224 310 955
e-mail: reinis@img.cas.cz

Short title: IFNγ-induced demethylation of antigen-presenting machinery gene promoters

Keywords: IFNγ signalling, DNA demethylation, 5-azacytidine, MHC class I downregulation, tumour immunology

Brief Description of Novelty: MHC class I deficiency on tumour cells is commonly caused by coordinated silencing of antigen-presenting machinery genes and restorable by IFNγ. Here we describe association of DNA demethylation of selected antigen-presenting machinery gene regulatory regions upon IFNγ treatment with MHC class I upregulation on tumour cells thus demonstrating that IFNγ acts as an epigenetic modifier. Our results cast more light on the role of DNA methylation in tumour cell escape from specific immunity.

Abstract

Reversible downregulation of MHC class I expression on tumour cells, a common mechanism by which tumour cells can escape from specific immune responses, is frequently associated with coordinated silencing of antigen-presenting machinery genes. The expression of these genes can be restored by IFNγ. In this study we documented association of DNA demethylation of selected antigen-presenting machinery genes located in the MHC genomic locus (*TAP-1, TAP-2, LMP-2, LMP-7*) upon IFNγ treatment with MHC class I upregulation on tumour cells in two MHC class I-deficient murine tumour cell lines, TC-1/A9 and TRAMP-C2. Our data also documented higher methylation levels in these genes in TC-1/A9 cells, as compared to their parental MHC class I-positive TC-1 cells. IFNγ-mediated DNA demethylation was dependent on the JAK/STAT signalling, relatively fast in comparison with demethylation induced by DNA methyltransferase inhibitor 5-azacytidine, and associated with increased histone H3 acetylation in the promoter regions of APM genes. Comparative transcriptome analysis in distinct MHC class I-deficient cell lines upon their treatment with either IFNγ or epigenetic agents revealed that, although there was a clear difference in the responsiveness of the cell lines to the same treatment and in the responsiveness of the same cells to IFNγ or epigenetic drug treatments, a set of genes, significantly enriched for the antigen presentation pathway, was regulated in the same manner. Collectively, our data demonstrate that IFNγ acts as an epigenetic modifier when upregulating the expression of antigen-presenting machinery genes.

Introduction

Epigenetic changes, such as aberrant DNA methylation, play important roles in carcinogenesis[1,2] and namely in the tumour cell escape from anti-tumour immune responses.[3,4]. MHC class I downregulation on tumour cells represents a frequent mechanism by which tumour cells can escape from anti-tumour specific immunity.[5-8] The molecular defects responsible for impaired expression on the tumour cell surface can be either irreversible ("hard") or reversible ("soft").[9] The latter can be associated with coordinated silencing of antigen-presenting machinery (APM) genes in tumour cells[10,11] and the expression of these genes can be restored by IFNγ.[12,10]

An important task is whether epigenetic events, such as changes in DNA methylation, take place in concerted APM gene silencing and IFNγ-induced restoration of their expression. Evidence that epigenetic mechanisms are important in MHC class I downregulation in APM-deficient tumours and its IFNγ-mediated induction was brought by Setiadi et al.[13]. The lack of *TAP-1* transcription in TAP-deficient cells was associated with low levels of recruitment of histone acetyltransferase CBP (CREB-binding protein) to the *TAP-1* promoter. IFNγ-mediated MHC class I expression corresponded to upregulation of the *TAP-1* expression by increasing histone H3 acetylation at the *TAP-1* promoter locus. Another study documented higher-order chromatin remodelling and subsequent histone hyperacetylation of the human MHC locus upon IFNγ-mediated activation of the JAK/STAT signalling pathway.[14]

We, as well as other laboratories, have previously documented that DNA methylation and histone acetylation might play a role in reversible MHC class I deficiency on the tumour cell surface, since it could be partially restored by the treatment with DNA methyltransferase or histone deacetylase inhibitors.[15-17] This increase was associated with elevated expression of antigen-presenting machinery genes, such as *TAP-1*, *TAP-2*, *LMP-2 (PSMB9), LMP-7 (PSMB8*), as well as with DNA demethylation of their corresponding regulatory sequences. We have also shown that chemotherapy of MHC class I-deficient tumours with 5-azacytidine (5AC) in mice increased the expression of the APM genes and associated MHC class I molecule cell surface expression and we have demonstrated 5AC additive effects against MHC class I-deficient tumours when combined with immunotherapy. Notably, the efficacy of this chemoimmunotherapy was partially dependent on the $CD8^+$-mediated immune responses.[18]

Unlike chromatin remodelling and histone acetylation dynamics, the changes in DNA methylation upon activation of the IFNγ signalling pathway have not been studied so far. Based on the fact that a set of the APM genes is upregulated by both IFNγ and DNA methyltransferase inhibitors, we have hypothesized that IFNγ-mediated re-activation of silenced APM and some other genes is also associated with their DNA demethylation. The objective of this study was to uncover the association of DNA methylation with IFNγ-mediated upregulation of genes encoding the components of APM in MHC class I-deficient murine tumour cell lines.

Materials and Methods

Cell culture

MHC class I-positive cell line TC-1 was obtained by *in vitro* co-transfection of murine lung C57BL/6 cells with HPV16 *E6/E7* and activated human *Ha-ras* (G12V) oncogenes.[19] The TC-1/A9 (MHC class I-deficient) cell line[20] was obtained from TC-1 tumours developed in immunized mice. The TRAMP-C2 tumour cell line (ATCC collection) was established from a prostate of a PB-Tag C57BL/6 (TRAMP) mouse.[21] TC-1/A9 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and antibiotics; the TRAMP-C2 cells in D-MEM medium supplemented with 5% FCS, Nu-Serum IV (5%; BD Biosciences, Bedford, MA, USA), 0.005 mg/ml bovine insulin (Sigma, St Louis, MO), dehydroisoandrosterone (DHEA, 10 nM; Sigma) and antibiotics. Both cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2. The RVP3 cell line[22] was maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and antibiotics.

Cells were cultured in fresh medium for 24 h, after which the medium was removed and the cells were grown in medium containing either rIFNγ (50 U/ml, R&D Systems, Minneapolis, USA) or 5 μM 5AC (Sigma). Except for the kinetic studies, cells were cultured for 48 h and harvested for analysis.

Flow cytometry

Cell suspensions were prepared from the cell cultures. Cell surface MHC class I expression on tumour cells was determined using PE anti- H-2D^b (clone KH95) and PE anti- H-2K^b (AF6-88.5) antibodies. Flow cytometry was performed using an LSR II flow cytometer (BD

Biosciences, San Jose, CA), and 10,000 cells were counted. Antibodies used, including the relevant isotypic control, were obtained from Pharmingen, San Diego, CA.

Real-time quantitative RT-PCR

Total RNA was extracted with RNeasy Mini Kit (Qiagen). The amount of 1 μg of RNA was reverse transcribed to cDNA using random hexamer primers from GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA) in a 20 μL reaction volume at 42ºC for 30 min. Quantification of PCR products was performed in 10 μL of Lightcycler 480 SYBR Green I Master mix (Roche) using a real-time PCR Lightcycler (Roche). DNA was denatured at 95ºC for 2 min; then followed 45 cycles of denaturation at 95 ºC for 25 s, annealing at 60 ºC for 45 s, elongation at 72 ºC for 1 min and incubation at 80 ºC for 5 s. cDNAs were amplified with specific primers for β-actin, *TAP-1, LMP-2, TAP-2,* and *LMP-7*. The list of the *TAP-1, TAP-2, LMP-2, LMP-7* and reference genes and their primer sequences have been described elsewhere.[15,18] Fold changes in the transcript levels were calculated using C_T values standardized to *β-actin*, used as the endogenous reference gene control. All samples were run in biological triplicates. For statistical analysis of qPCR the Student's t-test was used. Differences between experimental and control samples with $P< 0.05$ were considered to be statistically significant. The levels of relative gene expression were presented as fold changes compared to the levels found in control samples.

Bisulphite modification, methylation-specific PCR (MSP) and bisulphite sequencing

Total DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen). Treatment of DNA from TC-1/A9, TC-1, TRAMP-C2 and RVP3 cells with sodium bisulphite and methylation-specific PCR (MSP) analysis of the *TAP-1, TAP-2, LMP-2, LMP-7* promoter regions were performed with Bisulphite Epitect kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In order to identify CpG islands within the promoter region of the antigen-processing genes, MSP analysis was performed with primers designed with the program METHPRIMER. The list of the *TAP-1/LMP-2* and *TAP-2* primer sequences has been described elsewhere.[15,18] Two CpG island containing regions within the *LMP-7* upstream sequences were investigated, the primer sequences spanning the more distant to the transcription start have been published previously[15] and the sequences of the primers spanning CpG islands more proximal to the transcription start were as follows: *LMP-7* MSP Un, 5' TAGGAGGGATATATGAAAAGGTTTG (forward) and

AAAATATTAAACAAATCCACCTAAACATA (reverse); *LMP-7* MSP Me, 5' TAGGAGGGATATATGAAAAGGTTC (forward) and TATTAAACAAATCCACCTAAACGTA (reverse). Within *TAP-2*, upstream sequences were investigated for CpG islands at positions -207 and -214 from the transcription start with forward primer and CpG islands at positions -26 and -40 from the transcription start with reverse primer. Within the *TAP-1/LMP-2* upstream sequences a CpG island was investigated at position +119 from the transcription start with forward primer and CpG islands at positions +278 and +281 from the transcription start with reverse primer. With the *LMP-7* distant primers, CpG islands were investigated at positions -1219, -1233 and -1238 from the transcription start with forward primer and a CpG island at position -1087 from the transcription start with reverse primer. With the *LMP-7* proximal primers, a CpG island was investigated at position -335 from the transcription start with forward primer and CpG islands at positions -186 and -190 from the transcription start with reverse primer.

The program for PCR was as follows: 95°C for 2 min, then 35 cycles of 95°C for 2 min, 55°C for 2 min, 73°C for 1:30 min. At the end a final extension period of 73°C for 10 min was added. The PCR products were analysed with gel electrophoresis.

For bisulphite sequencing, another set of primers that amplified both methylated and unmethylated sequences were designed to directly determine the nucleotides resistant to bisulphite conversion. Their sequences were as follows: *TAP-2* BSP, 5' TTTTGGGTTTAGGTAAGTTTTTTT (forward) and TCTTCTCAAACTAAATCTCCTAAA (reverse); *TAP-1/LMP-2* BSP, 5' AGTTTTAGGGGTTTTTGATTATTTTAT (forward) and AACTAATAAAACTAACTAAAAATACTA (reverse); *LMP-7* BSP, 5' GTAGTTTTTGGGTAGATAATGTTT (forward) and AAAACCACAATACCAAAAAAAAA (reverse). Twenty-five CpG islands within the *TAP-2* upstream sequences were investigated, the primer sequences spanning the region from -264 to +76 from the transcription start. Thirty CpG islands within the *TAP-1/LMP-2* upstream sequences were investigated, the primer sequences spanning the region from -335 to +168 from the transcription start. Twenty-five CpG islands within the *LMP-7* upstream sequences were investigated, the primer sequences spanning the region from -502 to +130 from the transcription start. The program for PCR was as follows: 95°C for 5 min, then 25 cycles of 95°C for 50 s, 58°C for 2 min, 72°C for 1:30 min and then 15 cycles of 95°C for 45 s, 54°C for 2 min, 72°C of 1:30 min (+2 s every cycle). At the end, a final extension period of 72°C for 10 min was added. The PCR products were cloned using pGEM®-T Easy Vector System I

(Promega). Five clones for each of the different DNA sources were sequenced (Applied Biosystem, USA) after thermo-cycle sequencing reaction using the 3.1 version kit.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously[23] with minor modifications. Briefly, for ChIP experiments, TC-1 or TC-1/A9 cells were grown in 150 cm^2 culture flasks (TPP) and stimulated with IFNγ (50 U/mL) or left untreated. Two days later the cells were fixed directly in the flasks by addition of 1/10 volume of CRS buffer (11% (v/v) formaldehyde, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES pH 8; Sigma) for 10 min at 4 °C. Cross-linking was stopped by the addition of glycine (final concentration 0.125 M) at 4°C for 5 min. Then, the cells were harvested and resuspended in RIPA buffer (50 mM Hepes, pH 8.0, 150 mM NaCl, 1.0% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 0.5 mM EGTA; Sigma) containing protease inhibitor cocktail-Complete (Roche), sonicated (Branson 450) and subjected to ChIP analysis. Cell lysate was pre-cleared with 0.05 mg/ml yeast tRNA (Sigma)/protein A/G-agarose beads (Santa Cruz) and then incubated with the rabbit antibody for acetylated histone H3 (Lys18) (Cell Signaling, dilution 1/25). After several washes, DNA bound to the immunocomplexes was obtained and decross-linked by overnight incubation at 65°C. DNA was recovered via phenol/chloroform extraction. The amount of precipitated DNA was analysed using the LightCycler 480 Real-Time PCR System (Roche). Purified (by phenol extraction) input chromatin (chromatin that was not subjected to ChIP) was analysed in control PCR reactions. For the promoter analysis, we designed the following PCR primers which span the *TAP-1/LMP-2* bidirectional promoter: forward GGCAAATCTGCCCAGAGA and reverse CCTAGCCTGGGACTCTCGAC; *TAP-2* promoter: forward CACGGCAGTGAAGTGAAAGC and reverse CAAAAGAACTCACCTGCGGC; *LMP-7* promoter: forward GGACCTAAAGACCCCTGTGC and reverse AGCGGAGGACTGAATAGGGT. The negative control experiments were performed with primers amplifying the gene desert region of DNA: forward CCATGCACATGCTAGCGCTCGA and reverse TCCGAAAGCTGGGAGAAGGGGT.

Transcriptome analysis

Cell culture protocol published by Suzuki et al. in 2002[24] was applied with minor modifications. Briefly, for comparative transcriptome analysis, TC-1/A9 and RVP-3 cells

were treated either with IFNγ (100 U/ml) for 24 h or with a combination of 3 μM 5-2´-deoxyazacytidine (DAC) for the 48 h (medium was replaced with fresh one with inhibitor after 24 h) and 30 ng/ml Trichostatin A (TSA) for the additional 24 h. RNA was extracted from biological triplicates with RNeasy Mini Kit (Qiagen). The amount of 1 μg of RNA was subjected to the transcriptomic analysis, using Illumina Mouse WG6 bead chips in the Genomic Core Facility at the Institute of Molecular Genetics in Prague. Raw data extraction was performed using Illumina BeadStudio version 2. Raw data analyses were performed using R 3.0.0 software with the packages lumi and limma for data normalization (quantile), transformation (log2) and probe filtering (probes with detection p-values > 0.01 in all samples removed). Statistical analyses were performed using eBayes algorithm with Benjamini-Hochberg p-value correction. Functional annotations were performed using the IPA module from Ingenuity® software. The transcriptome analysis data were deposited at the GEO public functional genomics data repository under the reference Series No. GSE53469.

Results

MHC class I molecule upregulation on tumour cells upon the IFNγ treatment

First, we assessed the level of the MHC class I and APM molecule expression and its modulation by IFNγ on the selected tumour cells (**Fig. 1a**). MHC class I expression on tumour cells after IFNγ treatment was upregulated as compared to the tumour cells without treatment. As expected and as has been published previously,[20,25] the MHC class I upregulation induced by IFNγ was associated with increased expression of APM genes (**Fig. 1b**). As a negative control, we used the MHC class I-deficient RVP-3 cell line that did not respond to the IFNγ treatment. TC-1 cells, an MHC class I-positive parental cell line to the TC-1/A9 cells, which also displayed higher APM expression levels, as compared to TC-1/A9 cells, served as an MHC class I-positive control.

The IFNγ-mediated increase of MHC class I expression on tumour cells and APM gene machinery induction are associated with DNA demethylation of the corresponding APM gene regulatory sequences

Enhanced APM gene expression in the MHC class I-deficient tumour cells was associated with DNA demethylation of the corresponding gene promoter regions determined by MSP (**Fig. 2**). We demonstrated DNA demethylation of the promoter sequences of selected

antigen-presenting machinery genes (*TAP-1/LMP-2*, *TAP-2*) upon IFNγ treatment both in the MHC class I-deficient tumour cell line TC-1/A9 (**Fig. 2a**) and in the prostate cancer cell line TRAMP-C2 (**Fig. 2b**). For *LMP-7*, we did not see any dramatic changes in the MSP analysis targeting cytosines located at positions -186, -190 and -335 upstream from the LMP-7 transcription start (proximal primers). We therefore analysed CpGs in a more distant region covering CpGs at the positions -1219, -1233 and -1238 and -1087. In this region, we indeed noticed massive demethylation upon IFNγ treatment (distant primers). As a positive control, we used DNA from the MHC class I-positive TC-1 cell line (**Fig. 2a**), and as a negative control, we used MHC class I-deficient RVP-3 cell line, which did not respond to the IFNγ treatment (**Fig. 2c**). Comparative analysis of the TC-1 and TC-1/A9 cell lines demonstrated association of the cell surface MHC class I expression levels with DNA demethylation of the APM genes. No demethylation of the APM genes upon IFNγ treatment was seen in the RVP-3 cells.

Results from the MSP were confirmed by bisulphite sequencing using the TC-1/A9 cell line (**Fig. 3**). Again, strong DNA demethylation of both the *TAP-2* and *TAP-1/LMP-2* gene promoter regions was observed after the treatment with IFNγ. For LMP-7, we did not see any dramatic changes in a bisulphite sequencing analysis targeting cytosines located at the positions -502 upstream to +130 downstream from the LMP-7 transcription start site. This corresponds with the result from MSP analysis with LMP-7 proximal primers. Based on these results, we can suggest that the methylation status of the distant rather than proximal regulatory sites in the *LMP-7* region is crucial for their expression.

DNA demethylation corresponds to the histone H3 acetylation levels

ChIP assay was performed to determine whether the dose of IFNγ that was sufficient to reverse the methylation of the *TAP-1/LMP-2* bidirectional promoter region, as well as *LMP-7* and *TAP-2* promoter regions, was able to modify the histones associated with this promoter (**Fig. 4**). The assay demonstrated that histone H3 on lysine 18 was re-acetylated after IFNγ treatment in all three tested regions. Acetylated histone H3 was detected in untreated TC-1/A9 cells at a low level. The TC-1 cell line served as a positive control with high levels of acetylated histone H3 and, as expected, the acetylation levels were higher in untreated TC-1 cells than in untreated TC-1/A9 cells.

Kinetics of the DNA demethylation

To examine the kinetics by which the APM promoter regions undergo IFNγ-mediated changes in DNA methylation, as compared to the effects of a DNA methyltransferase inhibitor, TC-1/A9 cells were treated with either IFNγ or 5AC for various time periods and then by sodium bisulphite conversion and MSP. In untreated cells, the core CpG island was highly methylated, and demethylation was noticed within 2 hours after IFNγ treatment, while nearly maximal demethylation was evident by 6 h (**Fig. 5**). After 5AC treatment, strong demethylation was evident by 24 h. The kinetics of the 5AC-induced demethylation is in agreement with the fact that 5AC-induced demethylation required DNA replication. On the other hand, the kinetics of the IFNγ-mediated DNA demethylation suggests that DNA replication was not crucial.

JAK inhibition studies

The changes in gene expression by IFNγ involve transient increases in the activities of cellular protein tyrosine kinases, including the Janus kinases Jak1 and Jak2, leading to tyrosine phosphorylation of the transcription factor Stat1.[26] To assess whether the JAK/STAT pathway was crucial for demethylation of the APM gene promoter regions in TC-1/A9 cells after IFNγ treatment, the impact of an inhibitor of Janus kinases on IFNγ-induced demethylation was investigated (**Fig. 6**). Indeed, the inhibitor of Janus kinases caused impaired demethylation of the corresponding gene promoter regions, accompanied by decreased relative gene expression of selected APM genes, along with reduction of the MHC class I cell surface expression.

Comparison of the impacts of IFNγ and epigenetic agent treatments on the transcriptome of the tumour cell lines

In these experiments, the aim was to analyse (i) the global impact of IFNγ compared to epigenetic treatment (namely DAC/TSA) on the gene expression in the TC-1/A9 cell line and (ii) whether IFNγ-induced APM genes can be upregulated upon epigenetic treatment. We compared transcriptome changes upon IFNγ *vs.* DAC/TSA treatments (compared to untreated control cells) of the TC-1/A9 cell line as compared to those of the IFNγ non-responding RVP3 cells. IFNγ-treated TC-1/A9 cells presented 105 significantly upregulated genes (FDR<0.01) and only two downregulated genes (*RIN2* and *LBH*). Treatment with DAC/TSA

provided 2732 significantly upregulated and 2815 downregulated genes. This result can be explained by the fact that IFNγ targets specific genes, while DAC acts on the overall genome.
Out of the 105 upregulated genes in IFNγ-treated TC-1/A9 cells, we defined two gene sets: GS-IFN comprises the 73 genes that were specific to IFNγ treatment, when GS-COM comprises the 32 genes that are upregulated upon both treatments (GS-COM and GS-IFN gene lists are can be seen as Supplementary material **Tab. 1** and **Tab. 2**, respectively). Gene sets were annotated using IPA (Ingenuity®) for pathway enrichment (Benjamini-Hochberg controlled p-values). Both gene sets are significantly enriched for the "Cell Death Of Tumour Cell Lines" pathway (p-value = 1.1e-4 for GS-IFN and p-value = 5.64e-3 for GS-COM): 19 genes are specifically upregulated upon IFNγ treatment (*CASP4, CASP7, CLEC2D, DDX58, ENC1, FST, GDNF, IL15RA, IL7, IRAK2, IRF1, LGALS3BP, MLKL, SOCS3, STAT2, STAT3, TRIM21, UACA, UBA7*). Eight genes are upregulated in both treatments (*BID, CREM, EIF2AK2, FAS, HAP1, IDO1, STAT1, USP18*). As expected, some GS-IFN genes are related to the "Interferon Signaling" pathway (*IFIT3, IFI35, STAT2, IRF9, TAP1, IRF1*; p-value = 2.38e-7). Strikingly, GS-COM annotation identifies the "Antigen Presentation" pathway (*HLA-G, LMP-2 (PSMB9), HLA-B, LMP-7 (PSMB8), TAP2, TAPBP, HLA-E*; p-value = 1.23e-11).
Consistent results were obtained when comparing the impact of IFNγ on TC-1/A9 cells (IFNγ and DAC/TSA sensitive) and DAC/TSA effects on the RVP3 cell line (IFNγ resistant though DAC/TSA sensitive). The 40 upregulated genes in both conditions show enrichment for the "Antigen Presentation" pathway (*HLA-B, HLA-E, HLA-G, NLRC5, TAP2, TAPBP*; p-value=1.6e-8) and the 65 IFNγ-specific gene set is also enriched for the "Interferon Signaling" pathway (*IFIT3, IRF1, IRF9, LMP-7 (PSMB8), STAT2, TAP1*; p-value = 1.16e-7).

Discussion

IFNγ is a cytokine with pleiotropic effects on tumour cells, which is also considered as a crucial mediator of effective antitumour immunity displaying direct impacts on tumour cells.[27] The principal aims of our study were to determine whether IFNγ acts as an epigenetic modifier inducing DNA demethylation and whether the mechanisms by which IFNγ upregulates the expression of selected genes in MHC class I-deficient tumour cells and thus modifies their interactions with the immune system are associated with DNA demethylation of the corresponding regulatory genes. Our data demonstrate that IFNγ-mediated activation of the APM genes and MHC class I expression in two tumour cell lines with reversible MHC

class I expression defects was strongly associated with DNA demethylation of multiple APM genes located in the MHC locus. The promoter sequences of the studied APM genes in TC-1/A9 cells were also more methylated as compared to parental TC-1 cells, suggesting that APM gene methylation is involved in MHC class I downregulation on tumour cells that escape from the specific immunity. The finding that the IFNγ-mediated cell signalling can change the methylation status of the promoter regions of multiple genes contributes to our knowledge of the mechanisms underlying regulation of antigen processing and presentation in the context of MHC class I at the transcriptional level.

So far, very little is known about the involvement of DNA demethylation in the regulation of multiple genes mediated by the IFNγ signalling pathway. To our knowledge, there is only one study showing that IFNγ-mediated induction of the indole 2,3-dioxygenase (IDO)-1 expression was associated with DNA demethylation of the *IDO-1* gene.[28] Setiadi et al. in 2007 observed that changes in histone acetylation and chromatin remodelling underlie induction of the *TAP-1* expression by IFNγ in TAP-deficient tumour cells[13] and, moreover, massive IFNγ-induced chromatin remodelling of the entire MHC locus, in which both *TAP* and *LMP* genes are located, has been shown by Christova et al.[14] The MHC genomic region became decondensed, and this was associated with STAT1 phosphorylation followed by binding of the chromatin remodelling enzyme BRG1 at specific sites and subsequent RNA-polymerase II recruitment and histone hyperacetylation, which appeared 2-4 h after the treatment. Our data add to the story and suggest that IFNγ-induced MHC locus chromatin remodelling and histone modifications are associated with DNA demethylation of multiple regions within this genomic locus, resulting in multiple gene expression and increased MHC class I molecule number on the cell surface. Interestingly, the *LMP-7* gene expression was regulated in our experiments in the same manner as other tested APM genes, although the methylation status in the region close upstream to the initiation codon rather remained demethylated. However, demethylation was observed in the more distant region. It is therefore plausible that this gene is controlled by elements located in the wider region of the MHC locus.

In addition, our data indicate that JAK/STAT signalling takes place, since an inhibitor of the JAK kinases significantly blocked both DNA demethylation and induction of the MHC class I expression on the cell surface. Important conclusions can be drawn from the kinetics of these phenomena. DNA demethylation can be either passive, which means dependent on DNA replication when the nascent DNA strains are not methylated due to the DNA

methyltransferase deficiency, or active, fast and replication-independent.[29] The data from our kinetic study demonstrated that the DNA demethylation of the *TAP/LMP* gene promoter regions was relatively fast, as massive DNA demethylation was seen 6 h after the IFNγ treatments, which roughly corresponded with the kinetics of histone acetylation reported elsewhere and discussed above[14], suggesting an interplay between histone acetylation and DNA demethylation. This is in agreement with the current view on the epigenetic transcription regulation and gene silencing in tumour cells.[30] For comparison, maximum levels of DNA demethylation in the cells treated with DNMTi inhibitor 5-AC were observed 24 after the treatment. This fast kinetics suggests that the demethylation process was active and not dependent on DNA replication, unlike 5AC-mediated demethylation, which requires drug incorporation into DNA and blocks methylation of nascent DNA chains due to methyltransferases inhibition.[31] Interestingly, cytokine-induced DNA demethylation was demonstrated in a study in which TGFβ signalling resulted in active DNA demethylation and $p15^{ink4b}$ tumour suppressor gene expression.[32] We also demonstrated strong association of the DNA demethylation with increased histone acetylation. It is of note that we demonstrate induction of acetylation at histone H3K18, since hypoacetylation at this position has been linked to poor prognosis in several cancers.[33]

The data from this and our previous studies[15,18] show that the expression of the studied APM genes in MHC class I-deficient tumours can be increased both by IFNγ and DNA methyltransferase inhibitors. Thus, IFNγ can in some instances serve as a DNA demethylating agent and, on the other hand, DNA methyltransferase inhibitors can upregulate part of the genes that are under the IFNγ signalling pathway control. This means that DNA methyltransferase inhibitors can partially mimic the IFNγ effects on selected immunomodulatory genes, which can be very important for explanation of the immunomodulatory antitumour effects of these compounds.

The data were complemented by a set of transcriptome analyses based on the comparison of cells treated either with a DNMTi DAC, in combination with a histone deacetylase inhibitor TSA or with IFNγ. Two cell lines were included into this experiment: TC-1/A9 is sensitive to both DAC/TSA and IFNγ treatments, when RVP3 cell line is sensitive to DAC/TSA treatment but resistant to IFNγ. Comparative analysis of TC-1/A9 cells treated with either molecules showed a set of 32 genes commonly upregulated (gene set GS-COM) and a set of 73 genes upregulated in IFNγ treatment only (GS-IFN). Both gene sets present significant enrichment for “Cell Death Of Tumour Cell lines”. Interestingly, genes implicated in this term in GS-

COM gene set comprise IDO-1 which has already been demonstrated as demethylated by IFNγ in previous studies.[28]

GS-COM presents a clear and significant enrichment for antigen-presenting machinery and immunomodulatory genes. It is of importance that the upregulation of APM genes by the epigenetic agents was also seen in an IFNγ non-responding cell line. This represents further evidence demonstrating epigenetic regulation of selected immunomodulatory genes controlled by IFNγ-mediated signalling. Consistent with this finding, the RVP3 cell line (resistant to IFNγ) treated with DAC/TSA, shows a similar list of APM pathway-enriched upregulated genes in common with those found upregulated in IFNγ-treated TC-1/A9 cells. This further confirms the implication of a downstream machinery implicating DNA demethylation for regulating the expression of antigen-presentation molecules triggered by, but not specific to, the IFNγ-transduction cascade.

Collectively, this study documents that IFNγ can act as an epigenetic modifier and induce DNA demethylation of a number of genes, especially those involved in antigen processing and presentation.

Acknowledgements

This work was supported by grant No. 301/10/2174 from the Grant Agency of the Czech Republic, by RVO 68378050, and in part by the Clinigene Network of Excellence for the Advancement of Gene Transfer and Therapy, EU-FP6 Project No. 018933, the European Union FP6 project CompuVac (LSHB-CT-04-005246), by French state funds within the Investissements d'Avenir program (ANR-11-IDEX-0004-02; LabEx Transimmunom) and by IGA NT14461 from the Grant Agency of the Ministry of Health of the Czech Republic. The authors are grateful to Renata Turečková for excellent technical help and to Šárka Takáčová for editorial help. V. V. and I. S. are Ph.D. students supported in part by the Faculty of Science, Charles University, Prague.

References

1. Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007;**128**:683-92.
2. Esteller M. Epigenetics in cancer. *N Engl J Med* 2008; **358**:1148-59.
3. Sigalotti L, Coral S, Fratta E, Lamaj E, Danielli R, Di Giacomo AM, Altomonte M, Maio M. Epigenetic modulation of solid tumors as a novel approach for cancer immunotherapy. *Semin Oncol* 2005;**32**:473-8.
4. Tomasi TB, Magner WJ, Khan AN. Epigenetic regulation of immune escape genes in cancer. *Cancer Immunol Immunother* 2006;**55**:1159-84.
5. Garrido F, Ruiz-Cabello F, Cabrera T, Perez-Villar JJ, Lopez-Botet M, Duggan-Keen M, Stern PL. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today* 1997;**18**:89-95.
6. Bubeník J. Tumour MHC class I downregulation and immunotherapy (Review). *Oncol Rep.* 2003;**10**:2005-8.
7. Reiniš M. Immunotherapy of MHC class I-deficient tumors. *Future Oncol* 2010; **6:**1577-89.
8. Seliger B. Novel insights into the molecular mechanisms of HLA class I abnormalities. *Cancer Immunol Immunother* 2012;**61**:249-54.
9. Garrido F, Cabrera T, Aptsiauri N. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy (Review). *Int J Cancer* 2010; **127**:249-56.
10. Seliger B, Wollscheid U, Momburg F, Blankenstein T, Huber C. Coordinate downregulation of multiple MHC class I antigen processing genes in chemical-induced murine tumor cell lines of distinct origin. *Tissue Antigens*. 2000;**56**:327-36.
11. Garcia-Lora A, Martinez M, Algarra I, Gaforio JJ, Garrido F. MHC class I-deficient metastatic tumor variants immunoselected by T lymphocytes originate from the coordinated downregulation of APM components. *Int J Cancer*. 2003;**106**:521-7.
12. Gabathuler R, Reid G, Kolaitis G, Driscoll J, Jefferies WA. Comparison of cell lines deficient in antigen presentation reveals a functional role for TAP-1 alone in antigen processing. *J Exp Med* 1994;**180**:1415-25.
13. Setiadi AF, David MD, Seipp RP, Hartikainen JA, Gopaul R, Jefferies WA. Epigenetic control of the immune escape mechanisms in malignant carcinomas. *Mol Cell Biol* 2007;**27:** 7886-94.

14. Christova R, Jones T, Wu PJ, Bolzer A, Costa-Pereira AP, Watling D, Kerr IM, Sheer D. P-STAT1 mediates higher-order chromatin remodelling of the human MHC in response to IFNgamma. *J Cell Sci* 2007;**120**:3262-70.
15. Manning J, Indrova M, Lubyova B, Pribylova H, Bieblova J, Hejnar J, Simova J, Jandlova T, Bubenik J, Reinis M. Induction of MHC class I molecule cell surface expression and epigenetic activation of antigen-processing machinery components in a murine model for human papilloma virus 16-associated tumours. *Immunology* 2008;**123:**218-27.
16. Setiadi AF, Omilusik K, David MD, Seipp RP, Hartikainen J, Gopaul R, Choi KB, Jefferies WA. Epigenetic enhancement of antigen processing and presentation promotes immune recognition of tumors. *Cancer Res* 2008;**68:**9601-7.
17. Khan AN, Gregorie CJ, Tomasi TB. Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. *Cancer Immunol Immunother* 2008;**57:**647-54.
18. Simova J, Pollakova V, Indrova M, Mikyskova R, Bieblova J, Stepanek I, Bubenik J, Reinis M. Immunotherapy augments the effect of 5-azacytidine on HPV16-associated tumours with different MHC class I-expression status. *British Journal of Cancer* 2011;**105**:1533-41.
19. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, Wu TC. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996;**56**:21-6.
20. Smahel M, Síma P, Ludvíková V, Marinov I, Pokorná D, Vonka V. Immunisation with modified HPV16 E7 genes against mouse oncogenic TC-1 cell sublines with downregulated expression of MHC class I molecules. *Vaccine* 2003;**21:**1125-36.
21. Foster, B. A., Gingrich, J. R., Kwon, E. D., Madias, C., Greenberg, N. M. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. *Cancer Res* 1997;**57:**3325-30.
22. Bubeník J, Koldovský P, Svoboda J, Klement V, Dvorák R. Induction of tumours in mice with three variants of rous sarcoma virus and studies on the immunobiology of these tumours. *Folia Biol (Praha)* 1967;**13**:29-39.

23. Lukas J, Mazna P, Valenta T, Doubravska L, Pospichalova V, Vojtechova M, Fafilek B, Ivanek R, Plachy J, Novak J, Korinek V. Dazap2 modulates transcription driven by the Wnt effector TCF-4. *Nucleic Acids Res* 2009;**37**:3007-20.
24. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG, Baylin SB. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet.* 2002:**31**: 141-9.
25. Martini M, Testi MG, Pasetto M, Picchio MC, Innamorati G, Mazzocco M, Ugel S, Cingarlini S, Bronte V, Zanovello P, Krampera M, Mosna F, Cestari T, Riviera AP, Brutti N, Barbieri O, Matera L, Tridente G, Colombatti M, Sartoris S. IFN-gamma-mediated upmodulation of MHC class I expression activates tumor-specific immune response in a mouse model of prostate cancer. *Vaccine* 2010;**28**:3548-57.
26. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 1994;**264**:1415-21.
27. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immuno.* 2006;**6**:836-48.
28. Xue ZT, Sjögren HO, Salford LG, Widegren B. An epigenetic mechanism for high, synergistic expression of indoleamine 2,3-dioxygenase 1 (IDO1) by combined treatment with zebularine and IFN-γ: Potential therapeutic use in autoimmune diseases. *Molecular Immunology* 2012; **51:**101-11.
29. Niehrs C. Active DNA demethylation and DNA repair. *Differentiation* 2009;77:1-11.
30. Vaissière T, Sawan C, Herceg Z. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. *Mutat Res* 2008;**659**:40-8.
31. Creusot F, Acs G, Christman JK. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. *J Biol Chem* 1982;**257**:2041-8.
32. Thillainadesan G, Chitilian JM, Isovic M, Ablack JN, Mymryk JS, Tini M, Torchia J. TGF-β-dependent active demethylation and expression of the p15ink4b tumor suppressor are impaired by the ZNF217/CoREST complex. *Mol Cell* 2012; **46**:636-49.
33. Seligson DB, Horvath S, McBrian MA, Mah V, Yu H, Tze S, Wang Q, Chia D, Goodglick L, Kurdistani SK. Global levels of histone modifications predict prognosis in different cancers. *Am J Pathol*. 2009;**174**:1619-28.

Figure Legends

Figure 1

IFNγ upregulation of the cell-surface MHC class I expression cells is associated with APM gene expression in experimental tumour cells.

MHC class I expression (H-2D^b and H-2K^b together) was determined by the FACS analysis of control tumour cells and after the treatment with IFNγ. Representative results are presented. (A) Upregulation of APM genes in TC-1/A9 and TRAMP-C2 tumours after treatment with IFNγ. (B) Expression levels of selected APM genes in TC-1/A9 and TRAMP-C2 control tumour cells and after the treatment with IFNγ. As a negative control, MHC class I-deficient RVP-3 cell line that did not respond to the IFNγ treatment was used. TC-1 cells, a MHC class I-positive parental cell line to the TC-1/A9 cells, that also displayed higher APM expression levels compared to TC-1/A9 cells, served as a MHC class I-positive control. *denote significant changes ($P<0.05$ determined in Student's t-test) as compared to the values for untreated cells. Biological triplicates were used for the analysis. In all experiments, error bars show standard deviations. Relative expression numbers represent the percentage of the β-actin expression levels. The levels of relative gene expression were presented as fold changes compared to the levels found in control samples. Experiments were repeated three times with similar results.

Figure 2

IFNγ stimulates DNA demethylation of the APM gene promoter regions.

DNA from tumour cell lines cultured in the absence or presence of IFNγ were bisulphite treated and subjected to MSP analysis of the *TAP-1/LMP-2*, *TAP-2* and *LMP-7* promoter sequences. Higher proportion of DNA methylation, as compared to TC-1 cells and DNA demethylation induced by IFNγ, is documented in TC-1/A9 cells (A). Similar results were obtained in TRAMP-C2 cells (B), while no effects were noticed in IFNγ-insensitive RVP-3 tumour cells (C). U = unmethylated primer, M = methylated. Experiments were repeated three times with similar results.

Figure 3

IFNγ induced DNA demethylation of the *TAP-2* promoter in TC-1/A9 cells analysed by bisulphite sequencing.

DNA isolated from treated and control untreated TC-1/A9 cells was subjected to bisulphite conversion and cloned. Sequences from 11 clones from each sample are presented. After treatment with IFNγ, strong DNA demethylation of both the *TAP-2* and *TAP-1/LMP-2* gene promoter regions was observed. For LMP-7, we did not see any dramatic changes in a bisulphite sequencing analysis targeting cytosines located at the positions -502 upstream to +130 downstream from the LMP-7 transcription start site. Based on these results, we can suggest that the methylation status of the distant rather than proximal regulatory sites in the *LMP-7* region is crucial for their expression. White and black circles indicate unmethylated and methylated CpGs, respectively. Rhombuses indicate the CpG islands that were investigated with bisulphite sequencing. White colour marks the CpG islands investigated with MSP. TS: transcription start.

Figure 4

Histone H3 acetylation levels in the APM regulatory gene sequences in TC-1/A9 cells are lower than those in TC-1 cells, but can be increased by IFNγ.

ChiP analysis of chromatin from the *TAP-1/LMP-2, TAP-2*, and *LMP-7* promoter sequences isolated from control and treated TC-1/A9 cells demonstrates an increase in acetylated histone H3 (H3K18) after IFNγ treatment. Results were normalized to the levels of the relative input in TC-1 cells. Experiments were repeated five times with similar results. * denotes significant changes ($P<0.05$ determined in Student's t-test) as compared to the values from TC-1 cells. ** denotes significant changes ($P<0.05$ determined in Student's t-test) as compared to the values from TC-1/A9 cells.

Figure 5

Comparative analysis of the kinetics of DNA demethylation of the APM genes induced by IFNγ or 5AC.

TC-1/A9 cells were cultured in the presence of either IFNγ or 5AC. For the indicated time periods, DNA samples were isolated, bisulphite treated and subjected to MSP analysis of the *TAP-1, TAP-2, LMP-2 & LMP-7* promoter sequences. U = unmethylated primer, M = methylated. In untreated cells, the core CpG island was highly methylated, and demethylation

was detected within 2 hours after the IFNγ treatment, while nearly complete demethylation was evident by 6 hours (A). After 5AC treatment, strong demethylation was evident by 24 hours (A). The amount of 1 µg of RNA was reverse transcribed to cDNA and the PCR products were quantified. Upregulation of APM genes in TC-1/A9 tumours after the treatment with IFNγ after 2 hours (A) and with 5AC after 48 hour (B). * denote significant changes ($P<0.05$ determined in Student's t-test) as compared to the values from untreated cells. Biological triplicates were used for the analysis. In all experiments, error bars show standard deviations. Relative expression numbers represent the percentage of the β-actin expression levels. The levels of relative gene expression were presented as fold changes compared to the levels found in control samples. MHC class I expression ($H\text{-}2D^b$ and $H\text{-}2K^b$ together) was determined by FACS analysis of the control tumour cells and after the treatment with IFNγ and 5AC. Representative results are presented (C). Experiments were repeated three times with similar results.

Figure 6

Inhibitor of Janus kinases abrogated IFNγ-induced DNA demethylation of the APM gene promoters in TC-1/A9 cells.

Inhibitor of Janus kinases blocked the IFNγ induction of the MHC class I cell-surface expression (A), as well as APM gene activation **(**B), and caused impaired demethylation of the corresponding gene promoter regions (C). All experiments were performed in triplets and repeated three times. * denotes significant changes ($P<0.05$ determined in Student's t-test) as compared to the values from untreated control cells.

* denotes significant changes ($P<0.05$ determined in Student's t-test) as compared to the values from TC-1/A9 cells after IFNγ treatment. Biological triplicates were used for the analysis. In all experiments, error bars show standard deviations. Relative expression numbers represent the percentage of the β-actin expression levels. The levels of relative gene expression were presented as fold changes compared to the levels found in control samples.

Supplementary material Table 1

GS-COM, a gene set composed of genes commonly upregulated by IFNγ and DAC/TSA.

Transcriptomic analysis showed 32 genes significantly upregulated in TC-1/A9 cultured cells treated by either IFNγ or DAC/TSA (FDR<0.01). IPA provides 30 matches with Gene names and Entrez Gene IDs.

Supplementary material Table 2

GS-IFN, a gene set composed of genes specifically upregulated by IFNγ.

Transcriptomic analysis showed 73 genes significantly upregulated in TC-1/A9 cultured cells treated by IFNγ (FDR<0.01). IPA provides 73 matches with Gene names and Entrez Gene IDs.

Figure 1

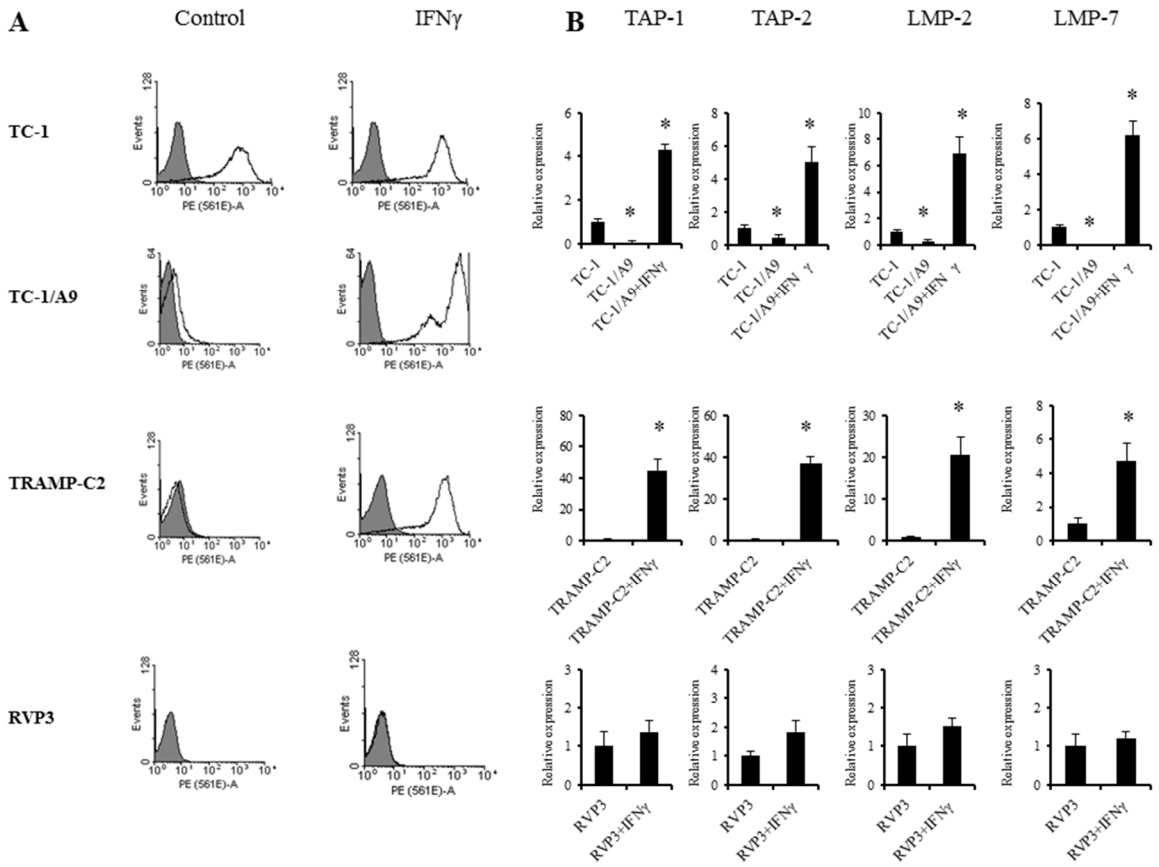


Figure 2

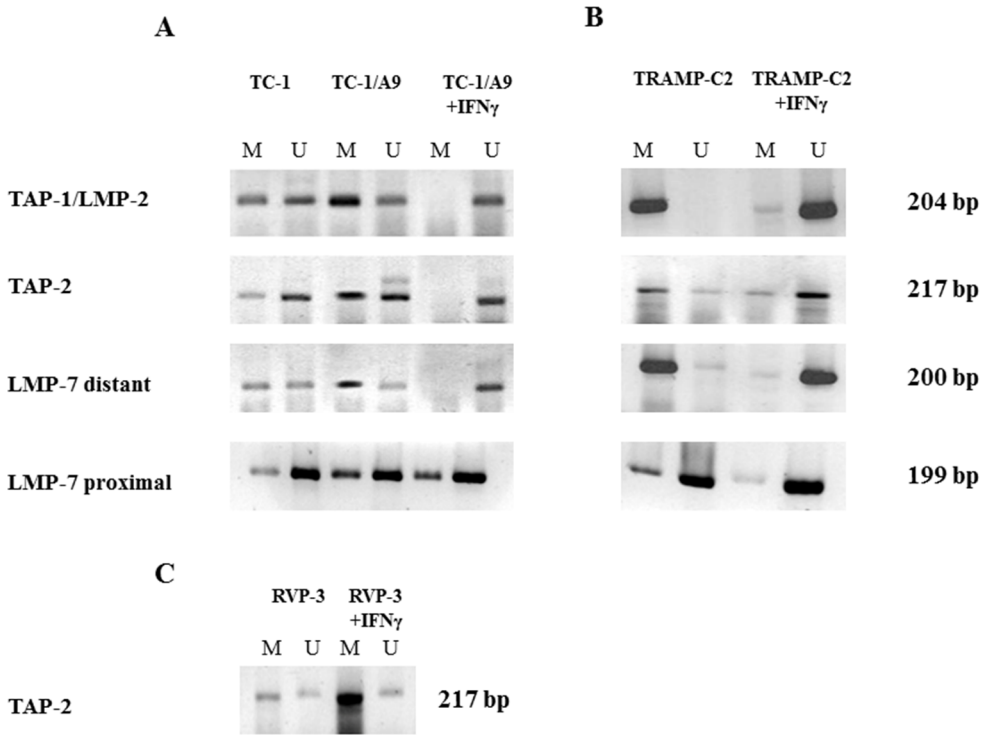


Figure 3

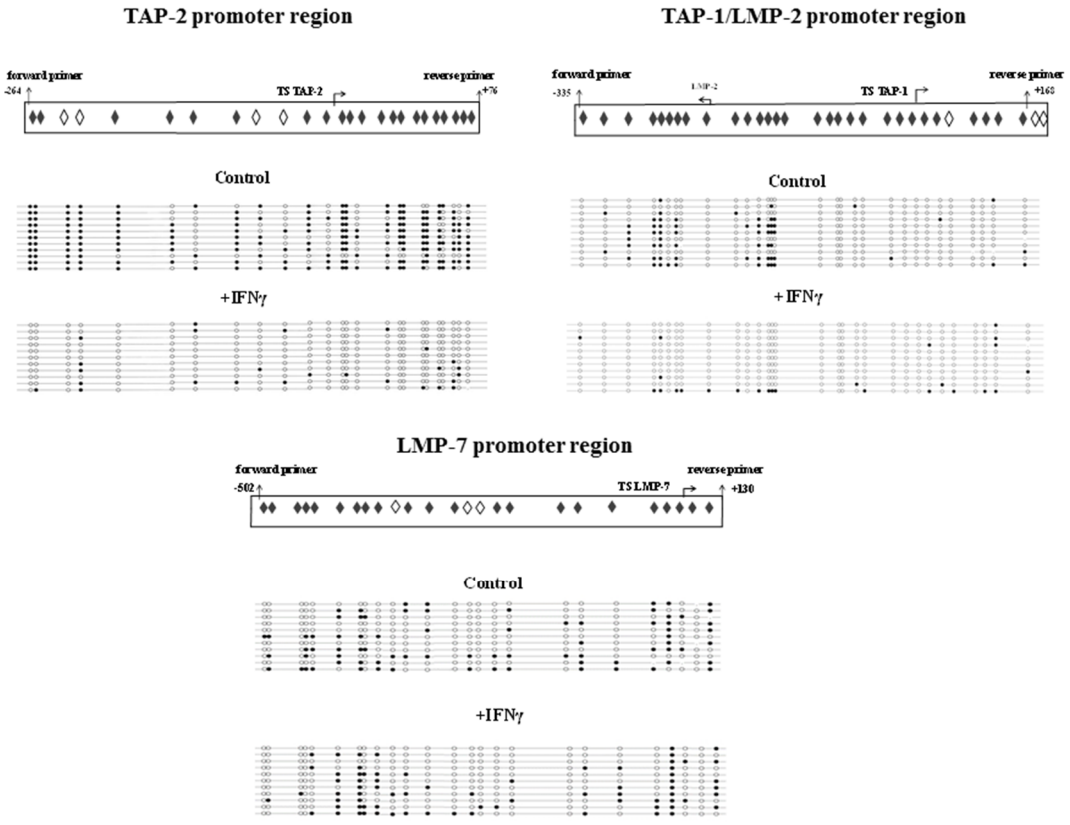


Figure 4

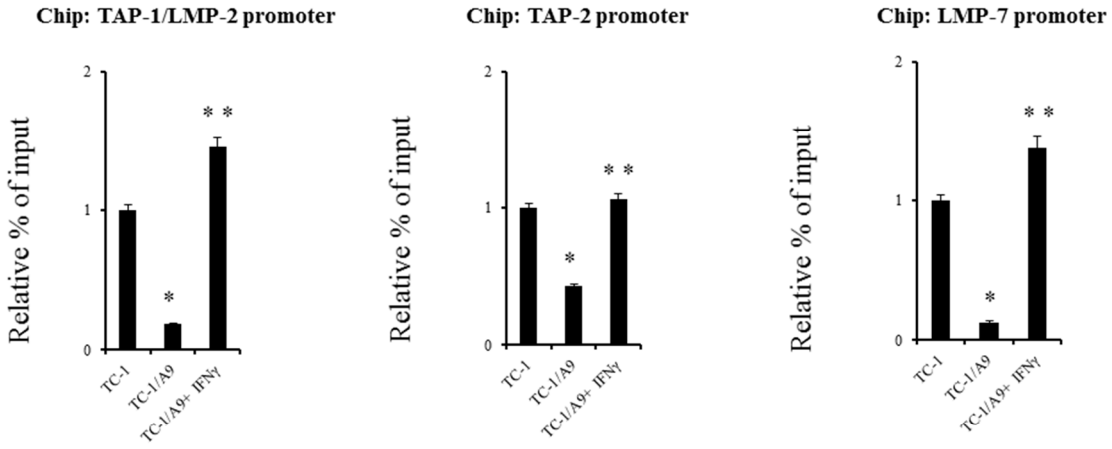


Figure 5

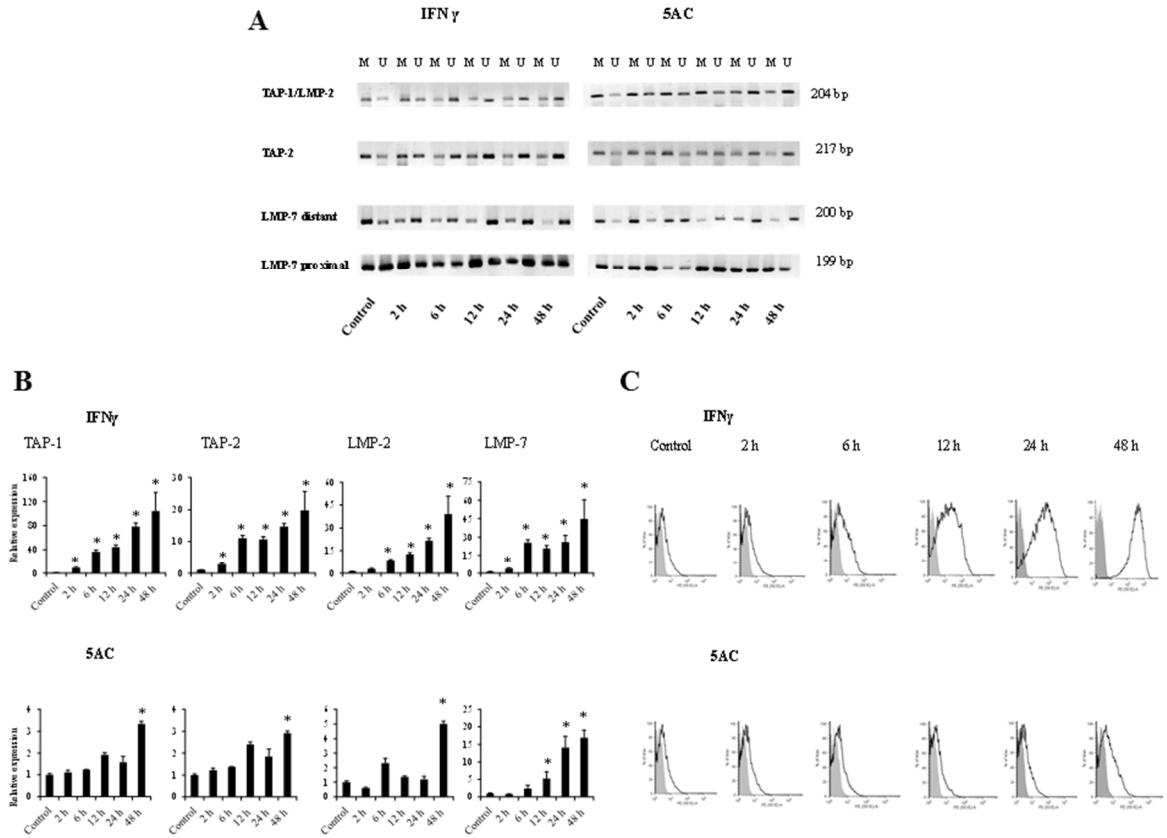
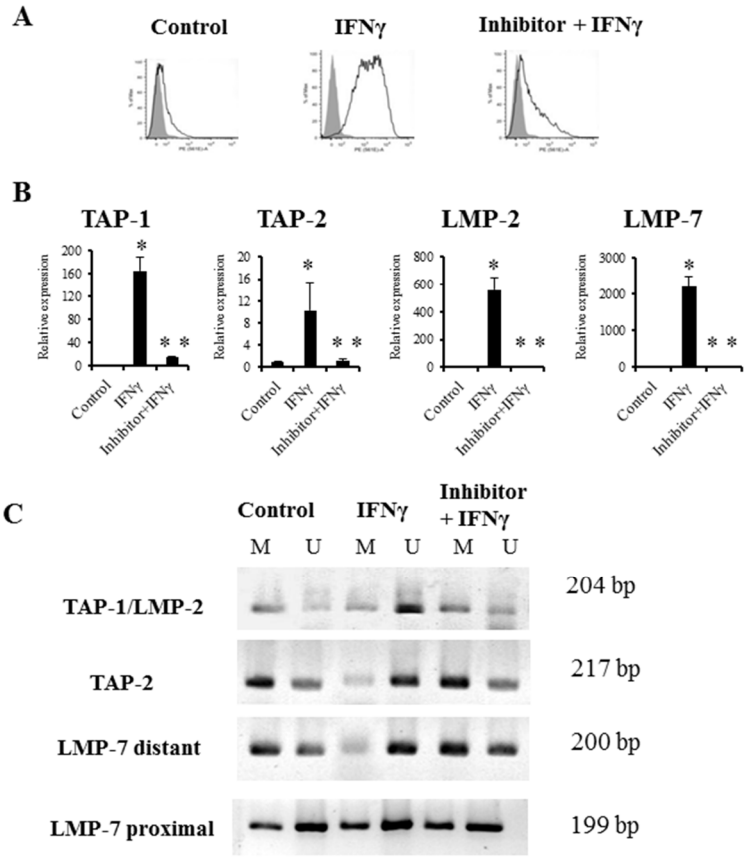


Figure 6



Supplementary material Table 1: GS-COM, a gene set composed of genes commonly upregulated by IFNγ and DAC/TSA.

Symbol	Entrez Gene Name	Entrez Gene ID for Mouse
Apol9a/Apol9b	apolipoprotein L 9b	71898|223672
BID	BH3 interacting domain death agonist	12122
CREM	cAMP responsive element modulator	12916
CX3CL1	chemokine (C-X3-C motif) ligand 1	20312
CXCL9	chemokine (C-X-C motif) ligand 9	17329
CXCL10	chemokine (C-X-C motif) ligand 10	15945
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	19106
FAS	Fas cell surface death receptor	14102
H2-Q5	histocompatibility 2, Q region locus 5	15016
HAP1	huntingtin-associated protein 1	15114
HLA-B	major histocompatibility complex, class I, B	667977|110557|15013|15006|14972|69717|15015|15018|14964|14963
HLA-E	major histocompatibility complex, class I, E	15040
HLA-G	major histocompatibility complex, class I, G	14991
IDNK	idnK, gluconokinase homolog (E. coli)	75731
IDO1	indoleamine 2,3-dioxygenase 1	15930
IFI27	interferon, alpha-inducible protein 27	52668
IL13RA1	interleukin 13 receptor, alpha 1	16164
IL17RC	interleukin 17 receptor C	171095
INSL6	insulin-like 6	27356

IRGM	immunity-related GTPase family, M	15944
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8	16913
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9	16912
RNF19B	ring finger protein 19B	75234
SAMD9L	sterile alpha motif domain containing 9-like	209086
SNX10	sorting nexin 10	71982
STAT1	signal transducer and activator of transcription 1, 91kDa	20846
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	21355
TAPBP	TAP binding protein (tapasin)	21356
USP18	ubiquitin specific peptidase 18	24110
WARS	tryptophanyl-tRNA synthetase	22375

Transcriptomic analysis showed 32 genes significantly upregulated in TC-1/A9 cultured cells treated by either IFNγ or DAC/TSA (FDR<0.01). IPA provides 30 matches with Gene names and Entrez Gene IDs.

Supplementary material Table 2: GS-IFN, a gene set composed of genes specifically upregulated by IFNγ.

Symbol	Entrez Gene Name	Entrez Gene ID for Mouse
4933412E12Rik	RIKEN cDNA 4933412E12 gene	71086
ADAR	adenosine deaminase, RNA-specific	56417
AMIGO2	adhesion molecule with Ig-like domain 2	105827
APOL6	apolipoprotein L, 6	71939
BATF2	basic leucine zipper transcription factor, ATF-like 2	74481
C15orf48	chromosome 15 open reading frame 48	433470
CASP4	caspase 4, apoptosis-related cysteine peptidase	12363
CASP7	caspase 7, apoptosis-related cysteine peptidase	12369
CD274	CD274 molecule	60533
CLEC2A	C-type lectin domain family 2, member A	
CLEC2D	C-type lectin domain family 2, member D	93694|93675|70809
CYTIP	cytohesin 1 interacting protein	227929
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	230073
Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	
DTX3L	deltex 3-like (Drosophila)	209200
ENC1	ectodermal-neural cortex 1 (with BTB domain)	13803
FAM214A	family with sequence similarity 214, member A	235493
FGF10	fibroblast growth factor 10	14165

FST	follistatin	14313
GBP2	guanylate binding protein 2, interferon-inducible	14468
GBP4	guanylate binding protein 4	55932
GBP6	guanylate binding protein family, member 6	
GDNF	glial cell derived neurotrophic factor	14573
GSDMD	gasdermin D	69146
Gvin1 (includes others)	GTPase, very large interferon inducible 1	434223|74558|100042856
IFI35	interferon-induced protein 35	70110
Ifi47	interferon gamma inducible protein 47	15953
Ifi204 (includes others)	interferon activated gene 204	100504287|545384|15951|226691|236312|15950|381308|100040462|226695
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	667370|15959
Igtp	interferon gamma induced GTPase	16145
IL7	interleukin 7	16196
IL15RA	interleukin 15 receptor, alpha	16169
IRAK2	interleukin-1 receptor-associated kinase 2	108960
IRF1	interferon regulatory factor 1	16362
IRF9	interferon regulatory factor 9	16391
Irgm2	immunity-related GTPase family M member 2	54396
KLHL25	kelch-like family member 25	207952
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	19039
Ly6a	lymphocyte antigen 6 complex, locus A	
MLKL	mixed lineage kinase domain-like	74568

MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)	17454
NLRC5	NLR family, CARD domain containing 5	434341
NUB1	negative regulator of ubiquitin-like proteins 1	53312
Oasl2	2'-5' oligoadenylate synthetase-like 2	23962
OGFR	opioid growth factor receptor	72075
PARP3	poly (ADP-ribose) polymerase family, member 3	235587
PARP12	poly (ADP-ribose) polymerase family, member 12	243771
PARP14	poly (ADP-ribose) polymerase family, member 14	547253
PDZRN3	PDZ domain containing ring finger 3	55983
PRKD2	protein kinase D2	101540
PSMB10	proteasome (prosome, macropain) subunit, beta type, 10	19171
PSME1	proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	19186
RMDN3	regulator of microtubule dynamics 3	67809
RNF213	ring finger protein 213	
SAMHD1	SAM domain and HD domain 1	56045
SOCS3	suppressor of cytokine signaling 3	12702
ST5	suppression of tumorigenicity 5	76954
STAT2	signal transducer and activator of transcription 2, 113kDa	20847
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	20848
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	21354

TAPBPL	TAP binding protein-like	213233
Tgtp1/Tgtp2	T cell specific GTPase 1	21822|100039796
TMCO4	transmembrane and coiled-coil domains 4	77056
TRAFD1	TRAF-type zinc finger domain containing 1	231712
TRIM21	tripartite motif containing 21	20821
TRIM25	tripartite motif containing 25	217069
UACA	uveal autoantigen with coiled-coil domains and ankyrin repeats	72565
Uaca	uveal autoantigen with coiled-coil domains and ankyrin repeats	
UBA7	ubiquitin-like modifier activating enzyme 7	74153
UBE2L6	ubiquitin-conjugating enzyme E2L 6	56791
VWA5A	von Willebrand factor A domain containing 5A	67776
XDH	xanthine dehydrogenase	22436
ZNFX1	zinc finger, NFX1-type containing 1	98999

Transcriptomic analysis showed 73 genes significantly upregulated in TC-1/A9 cultured cells treated by IFNγ (FDR<0.01). IPA provides 73 matches with Gene names and Entrez Gene IDs.

6.2. Publikácia II

Imunoterapia zvyšuje efekt 5-azacytidinu pri nádoroch asociovaných s vírusom HPV-16, ktoré majú rozdielny status povrchovej expresie MHC I.

Immunotherapy augments the effect of 5-azacytidine on HPV16-associated tumours with different MHC class I-expression status.

Jana Šímová*, Veronika Polláková (Vlková)*, Marie Indrová, Romana Mikyšková, Jana Bieblová, Ivan Štěpánek, Jan Bubeník a Milan Reiniš

(*Jana Šímová a Veronika Polláková (Vlková) prispeli rovnakým dielom k práci.)

Epigenetické mechanizmy plnia významnú úlohu v úniku nádorových buniek pred imunitným systémom, ide napríklad o zníženú povrchovú expresiu MHC I na povrchu nádorových buniek alebo o zmenenú expresiu komponentov antigén-prezentujúcej mašinérie. Preto chemoterapia inhibítormi DNA metyltransferáz môže ovplyvniť interakciu nádorových buniek s imunitným systémom a jej citlivosť k imunoterapii. Inhibítory DNA metyltransferáz, ako 5-azacytidin, majú dobrý potenciál využitia ako chemoterapeutiká. Zvyšujú imunogenicitu nádorových buniek a tiež aj ich citlivosť ku cytotoxickým T-lymfocytom, tým pádom môžu byť použité pre kombinovanú chemoimunoterapiu. Zhodnotili sme efekt inhibítora DNA metyltransferáz 5-azacytidinu na MHC I deficientné a pozitívne nádory. Cieľom publikácie bolo optimalizovať terapeutický protokol založený na kombinácii imunoterapie s inhibítorom DNA metyltransferáz. Dokázali sme, že 5-azacytidin má prídavný efekt na MHC I deficientné a pozitívne nádory v kombinácii s imunoterapiou pomocou nemetylovaných CpG oligodeoxynukleotidov alebo s bunkovou vakcínou produkujúcou IL-12. Výsledky naznačujú, že pre maximálny terapeutický výsledok by mohla byť zvýšená citlivosť nádorových buniek k imunitnému systému po chemoterapii pomocou epigenetického agensu kombinovaná s aktiváciou imunitnej odpovede pomocou imunoterapie. Pozorovali sme tiež pri nádoroch explantovaných zo zvierat ošetrených 5-azacytidinom zvýšenú povrchovú expresiu MHC I na povrchu nádorových buniek, ktorá bola asociovaná so zvýšenou expresiou génov antigén-prezentujúcej mašinérie a génov dráhy IFN-γ. Toto zvýšenie pri nádoroch explantovaných zo zvierat ošetrených 5-azacytidinom korešpondovalo s DNA demetyláciou promótorových oblastí génov antigén-prezentujúcej mašinérie. Naše dáta naznačujú, že chemoterapia MHC I deficientných nádorov

s inhibítormi DNA metyltransferáz kombinovaná s nešpecifickou imunoterapiou je sľubný terapeutický protokol v boji proti MHC I deficientným nádorom.

Prínos autora dizertačnej práce k danej publikácii:

Ako prvá autorka danej publikácie, spolu s Janou Šímovou, som spravila veľkú časť *in vitro* a *ex vivo* experimentálnej práce, čo zahrňuje napríklad monitorovanie povrchovej expresie MHC I molekúl na vybraných nádorových modeloch a monitorovanie relatívnej expresie vybraných génov antigén-prezentujúcej mašinérie a génov dráhy IFN-γ. Mojou úlohou bolo ďalej sledovať efekt chemoterapie pomocou 5-azacytidinu na DNA demetyláciu oblastí promótorov génov antigén-prezentujúcej mašinérie pomocou metylačne špecifickej PCR.

British Journal of Cancer (2011) 105, 1533 – 1541

www.bjcancer.com

Immunotherapy augments the effect of 5-azacytidine on HPV16-associated tumours with different MHC class I-expression status

J Šímová[1,2], V Polláková[1,2], M Indrová[1], R Mikyšková[1], J Bieblová[1], I Štěpánek[1], J Bubeník[1] and M Reiniš*[,1]

[1]*Department of Tumour Immunology, Institute of Molecular Genetics, v.v.i., Academy of Sciences of the Czech Republic, Videnska 1083, Prague 4 14220, Czech Republic*

BACKGROUND: Epigenetic mechanisms have important roles in the tumour escape from immune responses, such as in MHC class I downregulation or altered expression of other components involved in antigen presentation. Chemotherapy with DNA methyltransferase inhibitors (DNMTi) can thus influence the tumour cell interactions with the immune system and their sensitivity to immunotherapy.

METHODS: We evaluated the therapeutic effects of the DNMTi 5-azacytidine (5AC) against experimental MHC class I-deficient and -positive tumours. The 5AC therapy was combined with immunotherapy, using a murine model for HPV16-associated tumours.

RESULTS: We have demonstrated 5AC additive effects against MHC class I-positive and -deficient tumours when combined with unmethylated CpG oligodeoxynucleotides or with IL-12-producing cellular vaccine. The efficacy of the combined chemoimmunotherapy against originally MHC class I-deficient tumours was partially dependent on the $CD8^+$-mediated immune responses. Increased cell surface expression of MHC class I cell molecules, associated with upregulation of the antigen-presenting machinery-related genes, as well as of genes encoding selected components of the IFNγ-signalling pathway in tumours explanted from 5AC-treated animals, were observed.

CONCLUSION: Our data suggest that chemotherapy of MHC class I-deficient tumours with 5AC combined with immunotherapy is an attractive setting in the treatment of MHC class I-deficient tumours.

British Journal of Cancer (2011) **105,** 1533 – 1541. doi:10.1038/bjc.2011.428 www.bjcancer.com
Published online 20 October 2011

Keywords: 5-azacytidine; MHC class I downregulation; tumour chemoimmunotherapy; epigenetics; IL-12; CpG oligodeoxynucleotides

Epigenetic modifications of the mammalian genome, including aberrant DNA methylation, represent tumourigenic events that are functionally equivalent to genetic changes (Jones and Baylin, 2007). Cellular pathways can be affected by 'epigenetic' agents, such as histone deacetylase and DNA methyltransferase inhibitors (DNMTi), which can reverse aberrant DNA methylation and/or histone acetylation in tumour cells. Their therapeutic benefit has been successfully tested in clinical trials and several compounds, including DNMTi 5-azacytidine (5AC) and 5-aza-2′-deoxycytidine (DAC), have been approved for clinical use (Mai and Altucci, 2009).

MHC class I downregulation on tumour cells in the course of their growth represents a frequent mechanism by which tumour cells can escape from the specific immune responses (reviewed by Garrido *et al*, 2010). Effective anti-tumour immunotherapy should thus be optimised to cope with MHC class I-deficient tumours (Bubenik, 2003; Reinis, 2010). Notably, procedures that lead to MHC class I upregulation on tumour cells have been examined to augment the efficacy of the immunotherapy. Various mechanisms, both reversible and irreversible, underlie the MHC class I downregulation. DNA methylation was found to be responsible for the MHC class I heavy chain gene inhibition (Nie *et al*, 2001; Serrano *et al*, 2001), while both the DNA methylation and histone acetylation changes were associated with inhibition of the antigen-presenting machinery (APM) gene expression (Seliger *et al*, 2006; Setiadi *et al*, 2007, 2008; Campoli and Ferrone, 2008; Khan *et al*, 2008; Manning *et al*, 2008; Adair and Hogan, 2009). Epigenetic agents can also induce (re)expression of a number of silenced genes encoding other immunoactive molecules, such as co-stimulatory molecules, adhesive ICAM-1 (CD54), NKG2D receptor and tumour-associated antigens (Tomasi *et al*, 2006; Fonsatti *et al*, 2007; Sers *et al*, 2009). The epigenetic agents can also sensitise tumour cells to apoptosis and facilitate their killing by cytotoxic T lymphocytes (Fulda and Debatin, 2006). Thus, the combination of chemotherapy with DNA methytransferase inhibitors and immunotherapy can be a promising therapeutic setting.

In vivo administration of epigenetic agents can influence tumour cell interactions with the immune system not only by affecting the tumour cells, but also by exerting their effects on immunocytes. The effects on immunocytes may be immunosuppressive. It has been shown that 5AC induced regulatory T cells by *FoxP3* expression activation (Lal *et al*, 2009). Negative effects of histone deacetylase inhibitors on dendritic cell maturation and antigen-presenting capacity have also been described (Nencioni *et al*, 2007, Kim and Lee, 2010). Moreover, histone deacetylase inhibitors decreased toll-like receptor-mediated activation of proinflammatory gene expression (Bode *et al*, 2007). The histone deacetylase inhibitors exert their anti-inflammatory effects by blocking the secretion of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-12 (Leoni *et al*, 2002). On the other hand, 5AC has

*Correspondence: Dr M Reiniš; E-mail: reinis@img.cas.cz
[2] These authors contributed equally to this work.
Revised 12 August 2011; accepted 21 September 2011; published online 20 October 2011

been shown to induce differentiation of the myeloid-derived suppressor cells towards immunogenic antigen-presenting cells (Daurkin *et al*, 2010).

The aim of this study was to determine the *in vivo* effects of 5AC on expression of the MHC class I molecules and co-stimulatory molecules on tumour cells, using an animal model for MHC class I-deficient, HPV16-associated tumours (Bubenik, 2008). The question was how 5AC as the epigenetic agent can influence the anti-tumour immune responses *in vivo* and whether the treatment with epigenetic agents can be successfully combined with some immunotherapeutic protocols. We determined the additive/synergistic effects of 5AC with immunotherapy performed by the treatment with unmethylated CpG oligodeoxynucleotides (CpG ODN) or IL-12-producing cellular vaccines. Special attention was paid to the role of CTLs in 5AC-treated MHC class I-deficient tumour-bearing animals, as well as to the impacts of the 5AC treatment on the CpG ODN-induced activation of the immune system.

MATERIALS AND METHODS

Mice

C57BL/6 males, 2–4 months old, were obtained from AnLab Co., Prague, Czech Republic. The mice were housed in the animal facility of the Institute of Molecular Genetics AS CR. Experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics AS CR, Prague.

Cell culture

MHC class I-positive cell line TC-1 was obtained by *in vitro* co-transfection of murine lung C57BL/6 cells with HPV16 *E6/E7* and activated human *Ha-ras* (G12V) oncogenes (Lin *et al*, 1996). TC-1/A9 (MHC class I-deficient) cell line (Smahel *et al*, 2003) was obtained from the TC-1 tumours developed in immunised mice. IL-12-gene-modified TC-1/IL-12 (231/clone 15) cells used for vaccination produced *in vitro* 40 ng IL-12/1×10^5 cells ml^{-1} medium/48 h and were irradiated (150 Gy) before use (Indrova *et al*, 2006). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine and antibiotics.

In vivo experiments

TC-1 or TC-1/A9 tumour cells were transplanted subcutaneously (s.c.) in a dose of 1×10^4 into syngeneic mice. Mice were injected s.c. with 100 μg of 5AC (Sigma, Saint Louis, MO, USA) in the vicinity of tumour transplantation on days 3, 7, 10, 14, 17, 21, 24 and 28. CpG ODN 1826 was injected s.c. on days 3 and 10. On day 4, mice were treated s.c. with 1×10^7 150 Gy irradiated TC-1/IL-12 cells. The mice (eight per group) were observed twice a week, and the number of mice with tumours, as well as the size of the tumours was recorded. All *in vivo* experiments were repeated at least twice with similar results. CpG ODN 1826 (5′-TCCATGACG TTCCTGACGTT-3′, phosphorothioate) (Gramzinski *et al*, 2001) was purchased from Genosys, Hradec Kralove, Czech Republic.

In vivo depletion studies

In vivo depletion of NK1.1$^+$, CD4$^+$ and CD8$^+$ cells was performed using monoclonal antibodies PK 136, GK 1.5 and 2.43, as described previously (Reinis *et al*, 2006). To deplete the effector cells, 0.1 mg of the antibody was i.p. injected into mice, during the first week, injections were given three times and in the following 2 weeks, mice received injections once a week. Depletion was verified by the staining of spleen cells with labelled antibodies and FACS analysis.

Flow cytometry

Cell suspensions were prepared from tumours explanted from killed animals. Cells were further cultured for 7 days *in vitro* and subjected, together with original cell lines, to the FACS analysis as described previously (Mikyskova *et al*, 2005). In selected experiments, rIFNγ (50 U ml^{-1}) was added into the culture medium 48 h before analysis. Cell surface MHC class I expression on tumour cells was determined using PE anti-H-2D^b (clone KH95) and PE anti-H-2K^b (AF6-88.5) antibodies. The following antibodies were used: PE anti-CD80 (16-10A1), PE anti-CD86 (B7-2) (GL1), PE anti-CD54 (ICAM-1) (3E2), PE anti-B7-H1 (CD274) (MIH5) and FITC anti-MHC class II I-A^b molecules (AF6-120.1). All cells were initially pre-incubated with anti-CD16/CD32 to determinate non-specific binding. Flow cytometry was performed using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA), 10 000 cells were counted. All antibodies used, including the relevant isotypic control, were obtained from BD Pharmingen (San Diego, CA, USA). For the phenotypic analysis of different populations of spleen cells, mice were killed (15 days after the TC-1/A9 transplantation and treatment with 5AC and CpG ODN 1826) and the suspension of spleen cells was prepared. After lysis of the erythrocytes with Tris–NH_4Cl buffer, the expression of selected markers on spleen cells was analysed by flow cytometry. The following labelled antibodies were used: APC anti-CD45 (LCA, LY5), APC anti-CD11c (Integrin alpha$_x$ chain) (HL3), APC anti-Gr-1 Ly-6G and Ly-6C (Rb6-8C5), FITC anti-CD11b (M1/70), FITC anti-CD4 (L3T4) GK1.5, PE anti-CD25 (IL-2-Receptor-Chain p55) (PC61), FITC anti-CD69 (H1.2F3), PE anti-NK1.1 (NKR-P1B and NKR-P1C) (PK136), FITC anti-CD19 (1D3) and PE anti-F4/80 (CIA3-1). As isotype controls, FITC-, APC- and PE-labelled antibodies of irrelevant specificity were utilised. All antibodies but anti-F4/80 (Biolegend, San Diego, CA, USA) were purchased from BD Pharmingen. For the tetramer assay, 100 000 spleen cells were counted. Cells were stained with PE tetramers containing mouse E7 (49–57) CTL epitope (Sanquin, Amsterdam, The Netherlands), followed by staining APC with anti-CD3e (145-2C11) and FITC anti-CD8a (53–6.7). In all experiments, samples from at least three mice per group were analysed.

Real-time quantitative RT–PCR

Total RNA was extracted with High Pure RNA isolation kit (Roche, Basel, Switzerland). The amount of 1 μg of RNA was reverse transcribed to cDNA using random hexamer primers from GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA, USA) in a 20-μl reaction volume at 42 °C for 30 min. Quantitation of PCR products was performed in 10 μl of Lightcycler 480 SYBR Green I Master mix (Roche) using a real-time PCR lightcycler (Roche). DNA was denatured at 95 °C for 5 min; 45 cycles of denaturation at 95 °C for 25 s, annealing at 60 °C for 45 s, elongation at 72 °C for 1 min and incubation at 80 °C for 5 s. cDNAs were amplified with specific primers for β-actin, TAP-1, LMP-2, TAP-2, LMP-7, tapasin, IRF-1, IRF-8 and STAT-1. The list of the TAP-1/2, LMP-2/7 and reference genes and their primer sequences have been described elsewhere (Manning *et al*, 2008). The remaining PCR primer sequences are as follows: tapasin, 5′-GCTATACTTC AAGGTGGATGACC (forward) and TGCAAGACAGAGCAGTTCT GGG (reverse); IRF-1, 5′-GCCCGGACACTTTCTCTGATG (forward) and AGACTGCTGCTGACGACACACG (reverse); IRF-8, 5′-CGGGG CTGATCTGGGAAAAT (forward) and CACAGCGTAACCTCGTCT TC (reverse); STAT-1, 5′-TCACAGTGGTTCGAGCTTCAG (forward) and GCAAACGAGACATCATAGGCA (reverse); HPV16E6, 5′-GCA AGCAACAGTTACTGCGA (forward) and GTTGTCTCTGGTTGCA AATC (reverse); HPV16E7, 5′-ATGCATGGAGATACACCTAC (forward) and CGCACACAATTCCTAGTG (reverse). Fold changes in transcript levels were calculated using C_T values standardised to β-actin, used as the endogenous reference gene control. All samples were run in biological triplicates.

Bisulphite modification and methylation-specific PCR

Treatment of DNA from TC-1/A9 cells with sodium bisulphite and methylation-specific PCR (MSP) analysis of the TAP-2, TAP-1 and LMP-7 promoter regions were performed by Bisulphite Epitect kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In order to identify CpG islands within the promoter region of the antigen-processing genes, MSP analysis, performed with primers designed with the program METHPRIMER, was described elsewhere (Manning *et al*, 2008).

Proliferation and ELISPOT assays

To determine the portion of the IFNγ-secreting spleen cells, an ELISPOT kit for detection of murine IFNγ (BD Pharmingen) was used. Spleen cells were cultured for 48 h and then placed to the wells of ELISPOT plates (concentration 5×10^5 cells per well) for 24 h. The plates were then processed according to the manufacturer's instructions. Coloured spots were counted with CTL Analyser LLC (CTL, Cleveland, OH, USA) and analysed using the ImmunoSpot Image Analyser software.

For proliferation assay, splenocytes were resuspended at the concentration of 10^7 cells ml^{-1} in PBS supplemented with 5% FCS and labelled with 5,6-carboxy-fluorescein diacetate succinimidyl ester (CFSE; Sigma) by incubation for 5 min in 37 °C and 5% CO_2 at the final concentration of 2.5 μM. Labelling was quenched with RPMI 1640 supplemented with 10% FCS and the cells were washed twice before culturing in flat-bottom 24-well plates (1.5×10^6 ml^{-1}). After CFSE staining, splenocytes were cultured alone or in the presence of immobilised anti-CD3 antibody (145-2C11; 1 μl ml^{-1}) and anti-CD28 (37.51; 1 μl ml^{-1}). For FACS analysis, anti-CD3e (145-2C11), CD4 (GK1.5), CD8a (53–6.7) antibodies were used to determine proliferation of CD3, CD8 and CD4-positive cells. Flow cytometry was performed using an LSR II flow cytometer (BD Biosciences), 10 000 cells were counted. All antibodies used, including the relevant isotope control, were obtained from BD Pharmingen.

Statistical analysis

For statistical analyses of differences between the growth curves of tumours, the analysis of variance (Newman–Keuls and Tukey–Kramer tests) from NCSS, Number Cruncher Statistical System (Kaysville, UT, USA), statistical package was used. For statistical analysis of qPCR and ELISPOT assays, the Student's *t*-test was used. Differences between experimental and control samples with $P < 0.05$ were considered to be statistically significant.

RESULTS

Anti-tumour effects of 5AC on TC-1 and TC-1/A9 tumours are augmented by immunotherapy

The therapeutic effect of DNMTi 5AC against both TC-1/A9 and TC-1 tumours was demonstrated. Mice were transplanted with TC-1/A9 or TC-1 tumour cells and repeatedly treated with the DNA methytransferase inhibitor 5AC, administered intratumourally or into the vicinity of the site of tumour cell injection when the tumours were not yet palpable. As expected, significant inhibition of the tumour growth was observed in mice bearing both TC-1 and TC-1/A9 tumours (Figure 1). Further, the CpG ODN 1826 treatment significantly augmented the 5AC therapeutic effect. The effects of intratumoural administration of 5AC and CpG ODN 1826 and their combinations on the growth of palpable TC-1/A9 tumours were also investigated. In this setting, only the combination but not the 5AC or CpG ODN monotherapies significantly inhibited the tumour growth (Figure 1C). On the other hand, additive or synergistic effects were not observed in the therapy of the TC-1 tumours in which CpG ODN monotherapy significantly

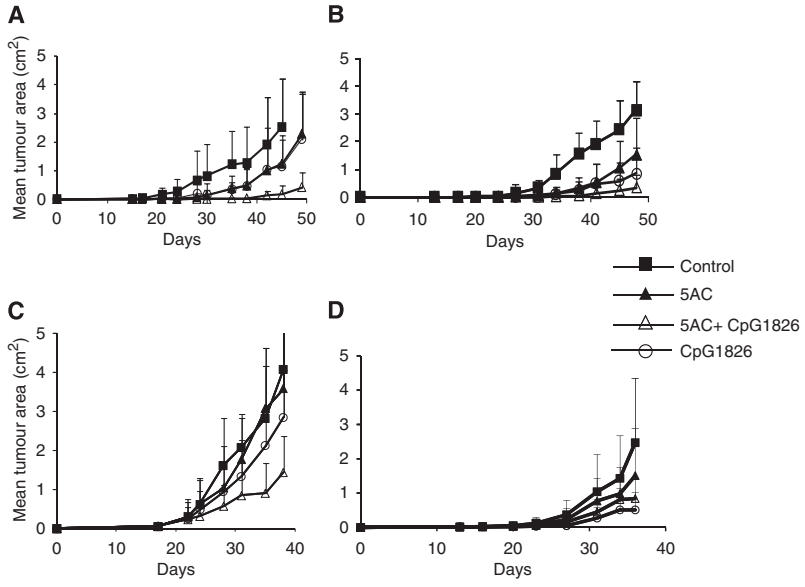


Figure 1 Tumour inhibitory effects of combined 5AC and CpG ODN 1826. Chemoimmunotherapy of the TC-1/A9 and TC-1 tumours. TC-1/A9 (**A**) and TC-1 (**B**) tumour cells were transplanted on day 0. In experimental groups, 5AC was repeatedly administered on days 3, 7, 10, 14, 17, 21, 24 and 28. CpG ODN 1826 was administered on days 3 and 10. Significant inhibition ($P < 0.05$ determined by Newman–Keuls and Tukey–Kramer tests, eight mice per group were used for the experiments) was observed in all treated mice, as compared with the untreated controls. Combined therapy was significantly more effective as compared with monotherapies only. (**C**) CpG ODN and 5AC treatment started when the TC-1/A9 tumours became palpable with ~1 mm in diameter. Significant inhibition was observed only after the combined therapy. (**D**) Treatment of TC-1 palpable tumours. The level of tumour growth inhibition by monotherapies was not different from the effects of the combined therapy.

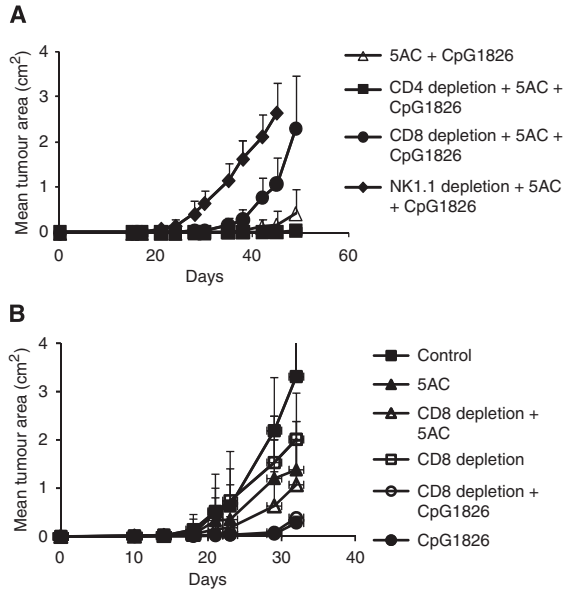


Figure 2 The impact of NK1.1$^+$, CD4$^+$ and CD8$^+$ *in vivo* depletion on the 5AC and CpG ODN 1826 therapeutic effects against the TC-1/A9 tumours. (**A**) The efficacy of the combined 5AC and CpG ODN treatment of the TC-1/A9 tumours was significantly abrogated in mice with depleted CD8$^+$ and NK1.1$^+$ but not the CD4$^+$ cells. (**B**) Tumour growth in mice subjected to the CpG ODN or 5AC monotherapies was not significantly changed upon CD8$^+$ cell depletion, as compared with the treated-only controls. In all experiments, error bars show standard deviations. All experiments were repeated twice with similar results.

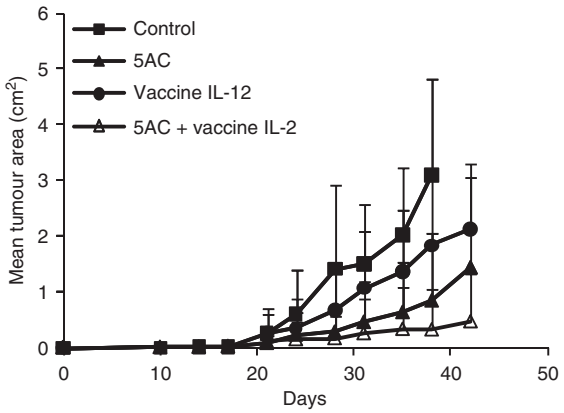


Figure 3 Tumour inhibitory effects of combined 5AC and TC-1/IL-12-producing cellular vaccine therapy of the TC-1/A9 tumours. TC-1/A9 (cells were transplanted on day 0). In experimental groups, 5AC was repeatedly administered on days 3, 7, 10, 14, 17, 21, 24 and 28; IL-12-producing cells were transplanted on day 4. Significant inhibition ($P<0.05$ determined by Newman–Keuls and Tukey–Kramer tests) was observed in all treated mice, as compared with the untreated control. The combined therapy was significantly more effective as compared with the 5AC treatment only.

inhibited the tumour growth (Figure 1D). Since the MHC class I upregulation was observed on cells of growing TC-1/A9 tumours from 5AC-treated animals, we raised the question whether the role of the MHC class I-restricted mechanisms was increased in the immune response leading to the inhibition of the tumour growth. The role of CD4$^+$, CD8$^+$ and NK cells was assessed in the *in vivo* depletion experiments (Figure 2). Although the NK1.1$^+$ cells remained critical for the tumour growth inhibition, the depletion of CD8$^+$ cells also resulted in acceleration of the tumour growth in 5AC- and CpG ODN-treated animals, while the growth of the TC-1/A9 tumours in CpG ODN 1826 only-treated animals was not affected by the CD8$^+$ cell depletion. These results indicate that 5AC therapy increased tumour cells' sensitivity to CTL-mediated cytotoxicity. Further, the efficacy of the 5AC monotherapy, which led to MHC class I upregulation on tumour cells, was not dependent on the CD8$^+$ cell population. This result suggests that besides the 5AC treatment, which increased the MHC class I expression on tumour cells, induction of immune response by immunotherapy was also crucial for the development or efficiency of the CD8$^+$-mediated immunity. In the next series of *in vivo* experiments, the efficacy of combined therapy of MHC class I-deficient TC-1/A9 tumours with IL-12-producing vaccine (irradiated TC-1/IL-12 cell line) and 5AC was tested. As for the CpG ODN treatment, the combined therapy (i.e., treatments with 5AC on days 3, 7, 10, 14, 17, 21, 24, 28 and with TC-1/IL-12 on day 4) resulted in significantly more efficient inhibition of the tumour growth, as compared with monotherapy (Figure 3).

In vivo and *in vitro* cell surface molecule modulation on tumour cells after the 5AC treatment

We have assessed the level of the MHC class I and selected immunoactive molecules expression on the TC-1/A9 tumour cells excised from the tumour-bearing animals and cultured *ex vivo* and compared these levels with the expression level on the cells treated *in vitro* with the epigenetic agents or IFNγ (Figure 4 and Table 1). Indeed, the MHC class I expression on tumour cells from 5AC-treated animals was upregulated as compared with the tumour cells from untreated animals. Explanted tumour cells remained fully sensitive to the IFNγ treatment. We have also investigated the expression of other selected co-stimulatory molecules from the B7 family, as well as of CD54 (ICAM-1). The cell surface expression of CD80 was decreased in the tumour cells from the 5AC-treated animals, as compared with those from the untreated controls, while the B7-H1 molecules were moderately upregulated (a significant change was observed only in the CpG ODN/5AC-treated group). No significant changes were observed in CD54 expression (Table 1). Tumour cells remained CD86-, MHC class II- and B7-H2-negative after all treatments (data not shown). The results indicate that cell treatment with 5AC *in vivo* and *in vitro* results in a similar pattern of the monitored cell surface molecules. However, the MHC class I expression from explanted tumours was higher than that could be achieved upon the treatments of the tumour cell lines *in vitro*. As expected, the MHC class I upregulation induced by 5AC was associated with increased expression of APM genes (Figure 5). DNMTi effects on the APM and co-stimulatory/inhibitory gene expression *in vivo* in some tumour cells resemble the impacts of IFNγ on the expression of these genes. We have hypothesised that DNMTi can also act through the activation of the IFNγ-signalling pathway components and we therefore focused on the expression levels of the selected genes from this pathway, namely interferon responsible factors 1 (*IRF-1*) and 8 (*IRF-8*) and *STAT-1* in tumour cells. Indeed, the expression levels of these genes were increased in the cells from the 5AC-treated animals, as compared with the samples from the untreated animals or from mice after immunotherapy only. Increased sensitivity of tumour cells to the specific lysis could also be attributed to increased expression of tumour rejection antigens. Our analysis demonstrates the increased expression of both HPV16 E6 and E7 oncogenes. It is noteworthy that the TC-1 cell line has been engineered by transfection of plasmids carrying both these oncogenes. Therefore, analysis of their expression regulation is not relevant for understanding of the biology of cervical carcinoma cells. We have previously shown that upregulation of

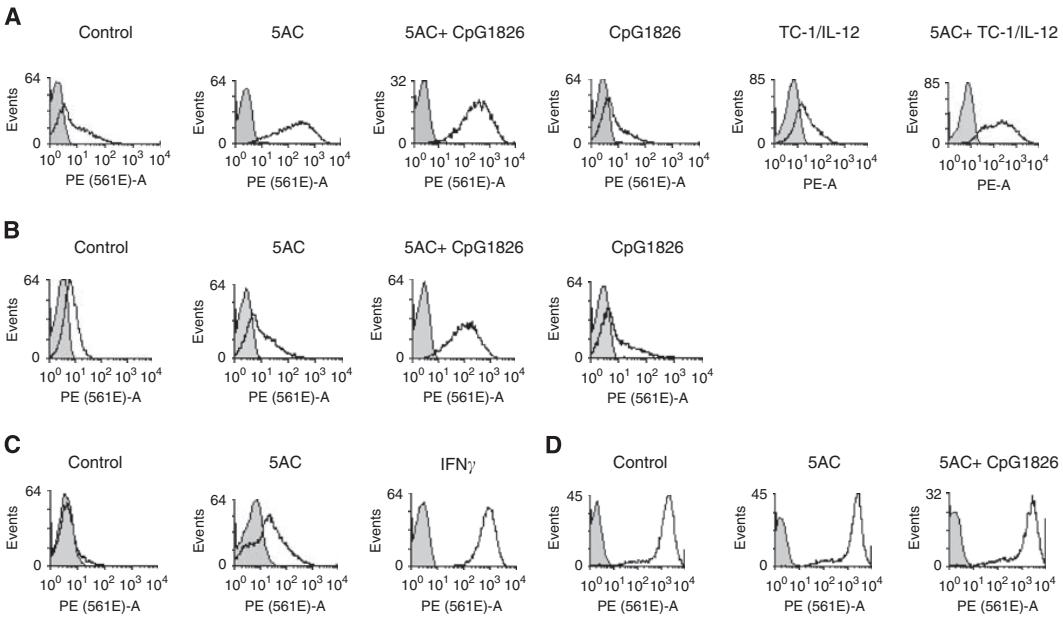


Figure 4 MHC class I expression on TC-1/A9 tumour cells. MHC class I expression (determined by using a mix of anti-H-2D^b and anti-H-2K^b) was determined by the FACS analysis of tumour cells explanted from experimental and control animals, which underwent therapy either immediately after tumour cell transplantation (**A**) or when tumours became palpable (**B**). Control data of the TC-1/A9 cultured cells and treated *in vitro* with either 5AC or IFNγ (**C**) and of the explanted tumour cells from control and treated animals subsequently treated *in vitro* with IFNγ (**D**) are also shown. Representative results are presented; the statistical analysis of at least triplicate analyses is shown in Table 1.

Table 1 Expression of MHC class I and co-stimulatory molecules on explanted TC-1/A9 tumour cells

	Fluorescence intensity (Gmean)			
Group of mice	**MHC class I**	**CD80**	**B7-H1**	**CD54**
Control	14.1 ± 6.9	619.2 ± 76.4	5.6 ± 1.1	7.3 ± 2.4
5AC	189.9 ± 71.1*	413.2 ± 244.4	12.0 ± 7.7	8.6 ± 6.0
5AC + CpG1826	196.0 ± 50.0*	279.0 ± 151.1*	10.9 ± 3.4*	6.5 ± 3.1
CpG1826	10.9 ± 6.2	739.3 ± 163.3	6.1 ± 1.0	5.9 ± 0.5

Abbreviations: MHC = major histocompatibility complex; 5AC = 5-azacytidine.
*$P < 0.05$ as compared with control group.

the APM gene expression upon *in vitro* treatment of the TC-1/A9 cells with the epigenetic agents is associated with DNA demethylation of the corresponding regulatory gene sequences (Manning *et al*, 2008). Here, we document a similar association after 5AC administration *in vivo* by the MSP analysis of the regulatory sequences of the selected APM genes (Figure 6). Explanted tumour cell retained their sensitivity to the IFNγ treatment. The expression of monitored genes was further increased upon 48 h *in vitro* treatment with 50 U ml^{-1} IFNγ (Supplementary Figure S1).

Analysis of immunocyte populations and immune responses in 5AC-treated animals

We have monitored the immune responses after the treatment with 5AC combined with CpG ODN therapy or cell therapy with the IL-12-producing cells and, importantly, the impact of the 5AC administration on immune cells. The results are summarised in Figure 7 and Table 2. The percentage of IFNγ-producing spleen cells, as determined by ELISPOT assay, was significantly higher upon the 5AC and/or CpG ODN treatments, as compared with the healthy controls (Figure 7A). However, these levels were lower, as compared with the untreated tumour-bearing mice. Administration of 5AC also inhibited the activation effect of the IL-12-producing cellular vaccine.

The total numbers of the spleen cells were significantly lower in the 5AC-only treated animals ($54 \times 10^6 \pm 14 \times 10^6$), as compared with the tumour-bearing or healthy mouse controls ($106 \times 10^6 \pm 34 \times 10^6$ and $92 \times 10^6 \pm 19 \times 10^6$, respectively). The total numbers of the spleen cells in all other experimental groups were not significantly different from the control groups. Although the percentage of proliferating cells in spleens were lower in all treated mice, as compared with tumour/bearing untreated mice, the capacity of the CD8$^+$ spleen cells to proliferate upon CD3/CD28 activation was not significantly impaired (Figure 7B). Similar results as for the CD8$^+$ cells were obtained for the CD4$^+$ spleen cells (data not shown).

Detailed analysis of the spleen cell populations in 5AC- and/or CpG ODN-treated and untreated tumour-bearing and control mice is presented in Table 2. This analysis documents that 5AC treatment did not influence the increased proportion of activated (CD69$^+$) T and B lymphocytes, as well as of NK cells induced by the CpG ODN treatment. As expected, CpG ODN increased the numbers of matured dendritic cells (CD11c$^+$/CD86$^+$/MHC class II$^+$) and also the expression of B7-H1. 5AC had no effect on these numbers. In agreement with the *in vivo* therapeutic data showing that the CD8$^+$ population had a role in the inhibition of the tumour growth only when 5AC and CpG were used in combination, the increased specific CD8$^+$ spleen cell population recognising E7 antigen was documented by the tetrameric assay only in the spleens from 5AC and CpG ODN-treated animals. Collectively, this analysis reveals that the 5AC treatment does not dramatically influence the proportion of particular cell populations or their activation status, as well as the changes induced by CpG ODN.

Translational Therapeutics

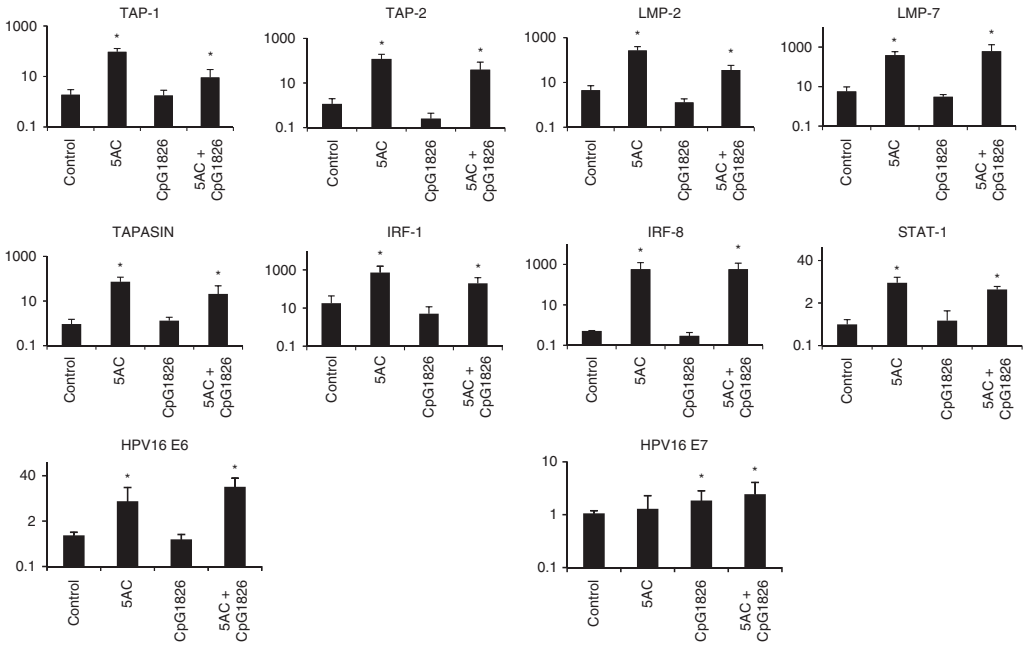


Figure 5 Upregulation of APM genes, IFNγ pathway components and E6/E7 oncogenes in TC-1/A9 tumours explanted from the 5AC- and/or CpG ODN-treated animals. Expression levels of selected APM genes, as well as *IRF-1*, *IRF-8* and *STAT-1* in TC-1/A9 tumour cells explanted from experimental and control animals. *Denotes significant changes ($P<0.05$ determined in Student's *t*-test) as compared with the values from untreated animals. Biological triplicates were used for the analysis. In all experiments, error bars show standard deviations. Relative expression numbers represent the percentage of the β-actin expression levels.

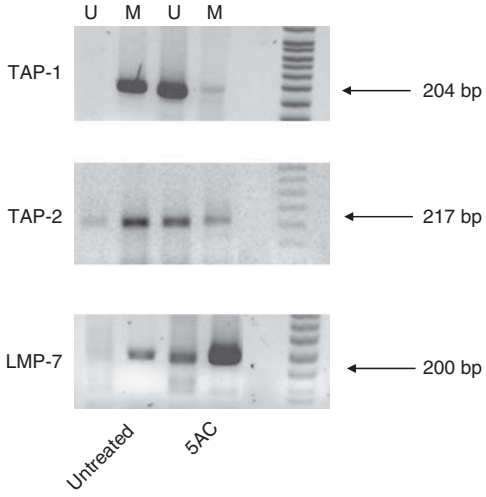


Figure 6 Methylation status of the APM gene regulatory sequences in the TC-1/A9 tumour cells from 5AC-treated and -untreated animals DNA from the TC-1/A9 explanted tumour cells from control 5AC-treated animals was bisulphite treated and subjected to the MSP analysis. The methylation status of the *TAP-1*, *TAP-2* and *LMP-7* promoter sequences was analysed. Bands in the lanes designated U represent the PCR products amplified from unmethylated DNA, bands from the M lanes represent the PCR products from methylated DNA.

DISCUSSION

DNMTi, such as 5AC, display a strong potential to be used as anti-tumour chemoterapeutics. Since they have been described to increase immunogenicity of tumour cells, as well as their sensitivity to the cytotoxic cells, they are attractive candidates for combination chemoimmunotherapy. Two studies, including ours (Manning *et al*, 2008; Setiadi *et al*, 2008), have recently documented MHC class I molecule upregulation on MHC class I-deficient TC-1/A9 tumour cells after DNMTi or HDACi treatments *in vitro* and, subsequently, after the treatment, these cell became sensitive for specific lysis by CTLs. Our aim in this study was to optimise the therapeutic protocols based on immunotherapy combined with DNMTi treatment, using the same model for MHC class I-deficient tumours. Previously, we have demonstrated that CpG ODN can inhibit the tumour growth of tumours with a different MHC class I cell surface expression status (Reinis *et al*, 2006). Similarly, the therapeutic effect of the IL-12-producing cellular vaccine was demonstrated in the treatment of the minimal residual tumour disease after chemotherapy (Indrova *et al*, 2003, 2008; Bubenik 2008).

In this study, we have shown the synergistic/additive effects of DNMTi treatment with non-specific immunotherapy using CpG ODN or cellular vaccine producing IL-12. Our data indicate that the *in vivo* treatment modulates immunogenicity of the TC-1/A9 tumour cells, since the *in vivo* cell depletion study revealed induction of $CD8^+$ cell-dependent mechanisms in protective immune responses against these tumours. It is noteworthy that the $CD8^+$ cell dependence of the therapeutic effect was not observed after 5AC monotherapy but only after combined treatment with CpG ODN. This result suggests that for maximal therapeutic effects, tumour cell sensitisation to immune responses by convenient chemotherapy with epigenetic agents should be combined with activation of the immune responses by immunotherapy. The *in vivo* depletion experiments revealed that the tumour growth in both 5AC-treated and untreated animals was strongly controlled by the $NK1.1^+$ cells. This result documents the role of innate immunity against tumours regardless of their MHC class I expression status. Indeed, we have previously shown, using the TC-1 and TC-1/A9

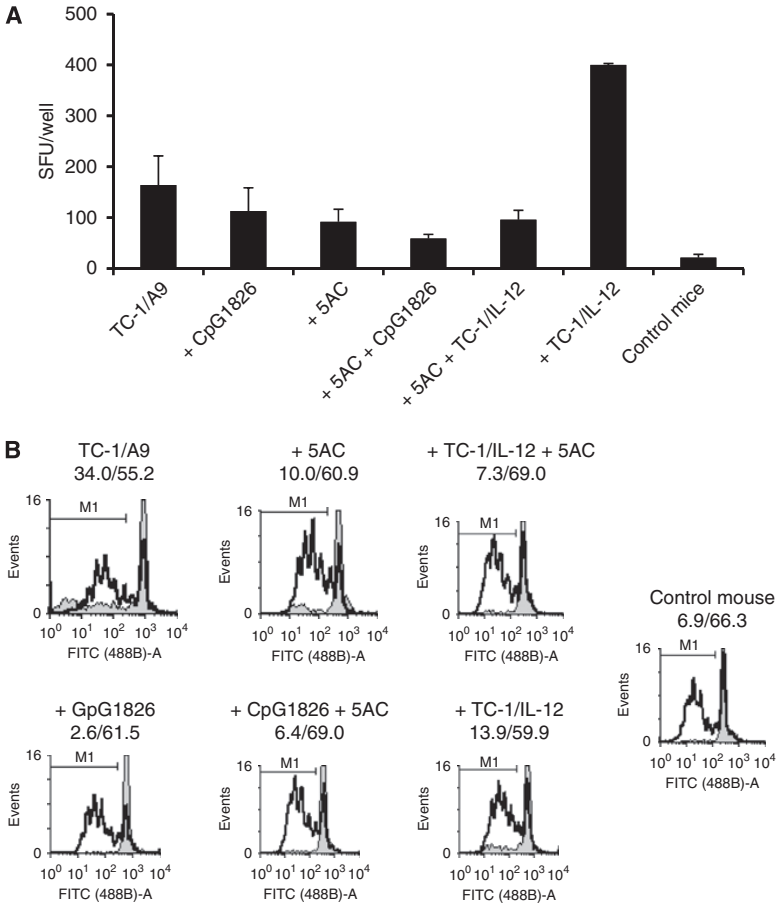


Figure 7 Analysis of spleen cell activation and immune responses in treated animals. Spleen cells were isolated from experimental and control groups 15 days after TC-1/A9 tumour cell transplantation and treatment commencement. (**A**) Spleen cells were subjected to the analysis of IFNγ production by ELISPOT assay. Biological triplicates were used for the analysis. In all experiments, error bars show standard deviations. In all experimental groups, the percentage of IFNγ-producing cells were significantly higher, as compared with healthy controls and lower, as compared with untreated tumour-bearing mice; 5AC treatment significantly decreased the percentage of IFNγ-producing cells from CpG- and TC-1/IL-12- treated, as well as untreated mice, respectively ($P<0.05$ determined in Students' *t*-test). (**B**) $CD8^+$ spleen cell proliferation was determined by CFSE analysis. Representative data show $CD8^+$ proliferation in unstimulated samples (grey histograms) and after CD3/CD28 mAb stimulation (white histograms). Numbers indicate the percentage of proliferating cells from unstimulated/stimulated samples.

Table 2 Phenotypic characterisation of spleen cells from mice 15 days after TC-1/A9 transplantation and treatment with 5AC and/or CpG ODN 1826

	% Positive $CD45^+$ spleen cells from mice treated with				
Marker expression	**Control TC-1/A9**	**5AC**	**5AC + CpG 1826**	**CpG 1826**	**Control mice**
CD8	13.3 ± 2.4	15.7 ± 0.5	13.1 ± 2.4	11.8 ± 0.6	14.8 ± 1.4
CD8/CD69	0.9 ± 0.2	0.9 ± 0.0	2.5 ± 0.1*	2.5 ± 0.5*	0.8 ± 0.0
CD4	17.5 ± 1.6	20.4 ± 1.3	17.1 ± 2.3	17.3 ± 1.2	21.7 ± 2.8
CD4/CD69	3.1 ± 0.2	3.9 ± 0.5	6.0 ± 1.9*	5.6 ± 0.5*	2.8 ± 0.1
NK1.1	7.4 ± 0.4	6.5 ± 0.5	18.1 ± 2.4*	13.7 ± 1.5*	4.9 ± 0.2
NK1.1/CD69	3.8 ± 0.7	3.7 ± 0.3	10.3 ± 1.6*	7.2 ± 0.3*	2.2 ± 0.2
CD19	58.7 ± 4.2*	69.0 ± 2.4†	65.6 ± 5.3†	72.1 ± 3.8†	76.1 ± 4.0†
CD19/CD69	3.3 ± 0.6	4.8 ± 0.2	8.0 ± 0.8*	12.5 ± 1.6*	4.4 ± 0.6
Gr-1	7.9 ± 1.9	5.6 ± 0.6	12.5 ± 0.3*	11.6 ± 1.6*	9.3 ± 0.3
Gr-1/CD11b	3.0 ± 0.8	2.8 ± 0.5	4.0 ± 0.9	4.9 ± 1.1*	3.0 ± 0.3
F4/80	10.3 ± 1.5§	10.3 ± 0.8§	14.0 ± 2.8§	10.3 ± 1.8§	6.3 ± 0.8
CD11c	11.2 ± 0.2**	9.5 ± 0.8**	6.5 ± 0.5	6.7 ± 1.1	7.0 ± 0.8
CD45/B7-H1	3.2 ± 0.6	4.8 ± 0.2	8.0 ± 0.8*	12.5 ± 1.6*	4.4 ± 0.6
CD86/MHC II	7.1 ± 0.6	7.1 ± 1.0	14.3 ± 2.4*	16.1 ± 1.8*	6.5 ± 1.1
CD86/MHC II (in $CD11c^+$ population)	50.6 ± 4.7	53.1 ± 1.8	76.4 ± 4.7*	77.2 ± 2.1*	51.6 ± 5.8
Tetramer H2-D^b/E7 (in $CD8^+$ population)	0.17 ± 0.03	0.2 ± 0.02	0.36 ± 0.06***	0.17 ± 0.06	0.18 ± 0.04

Abbreviations: MHC = major histocompatibility complex; ODN = oligodeoxynucleotides; 5AC = 5-azacytidine. Data from at least three mice were used for analysis. *$P<0.05$ as compared with: control TC-1/A9, treated with 5AC, control mice. †$P<0.05$ as compared with: control TC-1/A9. §$P<0.05$ as compared with: control mice. **$P<0.05$ as compared with: treated with 5AC+CpG 1826, CpG 1826, control mice. ***$P<0.05$ as compared with: 5AC, CpG1826, control TC-1/A9, control mice.

models, that NK1.1$^+$ cell population is an important player controlling the early phases of the parental, MHC class I-positive, TC-1 tumour growth (Simova *et al*, 2004; Reinis *et al*, 2006).

The additive effect of 5AC and CpG ODN administration was surprisingly not observed on palpable TC-1 tumours. The possible explanation might be that CpG ODN monotherapy was more effective against more immunogenic TC-1 tumours than against TC-1/A9 tumours so that it was difficult to see the increased efficacy of combined therapy in our experimental setting.

The phenotypic analyses showed significant MHC class I upregulation on the explanted TC-1/A9 tumour cells upon *in vivo* 5AC treatment. 5AC administration increased the expression of a number of APM genes (*TAP-1*, *TAP-2*, *LMP-2*, *LMP-7*, *Tapasin*). Interestingly, the MHC class I cell surface expression levels after *in vivo* administration of 5AC were higher, as compared with the expression levels achieved upon the *in vitro* treatment of the TC-1/A9 cells. This could be attributed to repeated treatments with 5AC or to the additive effects of endogenous cytokines in the tumour microenvironment, as the MHC class I expression tends to increase even in the tumours from mice that were not subjected to any therapy (Mikyskova *et al*, 2005).

Both *in vitro* and *in vivo* treatment with 5AC induced the expression of the APM and other genes, which are inducible by IFNγ. 5AC treatment combined with CpG ODN (in 5AC only-treated mice, the upregulation was not significant) moderately (much less than can be seen upon *in vitro* IFNγ treatment) increased the expression of the B7-H1-negative regulator on tumour cells, which is known to be regulated through the IFNγ-inducible IRF-1 factor (Lee *et al*, 2006). Further, it is also known that the *IRF-8* gene is frequently epigenetically silenced in a number of tumours and that DNMTi can increase tumour cell sensitivity to apoptosis through upregulation of IRF-8 (Fulda and Debatin, 2006). Therefore, we have decided to select for monitoring, besides *STAT-1*, the *IRF-1* and *IRF-8* gene expression upon the treatment with 5AC. Our data indicate that expression of these crucial players in the IFNγ-signalling pathway is increased in tumour cells from the 5AC-treated animals. Although more studies have to be done, this observation suggests that both, direct demethylation of the corresponding regulatory sequences of the upregulated genes, as well as upregulation of the critical components of the IFNγ-signalling pathway can take place in the modulation of the MHC class I and, or co-stimulatory or regulatory molecules.

Immunomodulatory effects of the hypomethylating agents can also be mediated by their effects on immune cells. Thus, it was important to assess how the *in vivo* administration of these agents influenced subsequent immunotherapy and anti-tumour immune responses upon non-specific immunotherapy. The results demonstrate that the 5AC treatment in our experimental settings can display adverse effects on the immune system, since the number of spleen cells was lower as compared with the 5AC-untreated controls. The 5AC treatment also decreased the percentage of the IFNγ-producing spleen cells in the tumour-bearing animals. However, the proliferative capacity and the proportion of particular spleen cell populations of the spleen cells remained unaffected. Also, 5AC also did not alter the numbers of activated T, B and NK cells induced by CpG ODN. Importantly, the synergistic effect of combined immunochemotherapy was observed on the induction of specific anti-E7 immunity. We have concluded that the potential 5AC adverse effects on the immune system were not an obstacle for an effective combination treatment with immunotherapy. Since chemo- or immunotherapy can also induce negative regulators of the immune responses, the therapeutic efficacy could be increased by combining the treatment with anti-immunosuppressive treatments.

Taken together, our data document that chemotherapy of MHC class I-deficient tumours with DNMTi combined with non-specific immunotherapy is a promising therapeutic setting against MHC class I-deficient tumours, although both positive and detrimental effects of DNMTis have to be considered and the immunotherapeutic settings have to be optimised.

Translational Therapeutics

ACKNOWLEDGEMENTS

This work was supported by Grants Nos. 301/07/1410, 301/10/2174 and 301/09/1024, Grant Agency of the Czech Republic, and in part by the Clinigene Network of Excellence for the Advancement of Gene Transfer and Therapy, EU-FP6 Project No. 018933. We are grateful to M Maleckova and R Tureckova for excellent technical help and to Sarka Takacova for editorial help. VP and IS are PhD students supported in part by the Faculty of Science, Charles University, Prague. This work was presented in part at the CITIM 2011 conference held in Budapest, 2–5 May 2011 and CIMT 2011 meeting held in Hannover, 25–27 May 2011.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

REFERENCES

Adair SJ, Hogan KT (2009) Treatment of ovarian cancer cell lines with 5-aza-2′-deoxycytidine upregulates the expression of cancer-testis antiaagens and class I major histocompatibility complex-encoded molecules. *Cancer Immunol Immunother* **58:** 589–601

Bode KA, Schroder K, Hume DA, Ravasi T, Heeg K, Sweet MJ, Dalpke AH (2007) Histone deacetylase inhibitors decrease Toll-like receptor-mediated activation of proinflammatory gene expression by impairing transcription factor recruitment. *Immunology* **122:** 596–606

Bubenik J (2003) Tumour MHC class I downregulation and immunotherapy (Review). *Oncol Rep* **10:** 2005–2008

Bubenik J (2008) Genetically modified cellular vaccines for therapy of human papilloma virus type 16 (HPV 16)-associated tumours (Review). *Curr Cancer Drug Targets* **8:** 180–186

Campoli M, Ferrone S (2008) HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene* **27:** 5869–5885

Daurkin I, Eruslanov E, Vieweg J, Kusmartsev S (2010) Generation of antigen-presenting cells from tumor-infiltrated CD11b myeloid cells with DNA demethylating agent 5-aza-2′-deoxycytidine. *Cancer Immunol Immunother* **59:** 697–706

Fonsatti E, Nicolay HJ, Sigalotti L, Calabrò L, Pezzani L, Colizzi F, Altomonte M, Guidoboni M, Marincola FM, Maio M (2007) Functional up-regulation of human leukocyte antigen class I antigens expression by 5-aza-2′-deoxycytidine in cutaneous melanoma: immunotherapeutic implications. *Clin Cancer Res* **13:** 3333–3338

Fulda S, Debatin KM (2006) 5-Aza-2′-deoxycytidine and IFN-gamma cooperate to sensitize for TRAIL-induced apoptosis by upregulating caspase-8. *Oncogene* **25:** 5125–5133

Garrido F, Cabrera T, Aptsiauri N (2010) Hard and soft lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy (Review). *Int J Cancer* **127:** 249–256

Gramzinski RA, Doolan DL, Sedegah M, Davis HL, Krieg AM, Hoffman SL (2001) Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice. *Infect Immun* **69:** 1643–1649

Indrova M, Bieblova J, Bubenik J, Reinis M (2008) IL-12 immunotherapy of minimal residual disease in murine models of HPV16-associated tumours: induction of immune responses, cytokine production and kinetics of immune cell subsets. *Int J Oncol* **32:** 499–507

Indrova M, Bieblova J, Jandlova T, Vonka V, Pajtasz-Piasecka E, Reinis M (2006) Chemotherapy, IL-12 gene therapy and combined adjuvant therapy of HPV 16-associated MHC class I-proficient and -deficient tumours. *Int J Oncol* **28:** 253–259

Indrova M, Bubenik J, Mikyskova R, Mendoza L, Simova J, Bieblova J, Jandlova T, Jinoch P, Smahel M, Vonka V, Pajtasz-Piasecka E (2003) Chemoimmunotherapy in mice carrying HPV16-associated, MHC class I+ and class I- tumours: effects of CBM-4A potentiated with IL-2, IL-12, GM-CSF and genetically modified tumour vaccines. *Int J Oncol* **22:** 691 – 695

Jones PA, Baylin SB (2007) The epigenomics of cancer. *Cell* **128:** 683 – 692

Khan AN, Gregorie CJ, Tomasi TB (2008) Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. *Cancer Immunol Immunother* **57:** 647 – 654

Kim ES, Lee JK (2010) Histone deacetylase inhibitors decrease the antigen presenting activity of murine bone marrow derived dendritic cells. *Cell Immunol* **262:** 52 – 57

Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE, Bromberg JS (2009) Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J Immunol* **182:** 259 – 273

Lee SJ, Jang BC, Lee SW, Yang YI, Suh SI, Park YM, Oh S, Shin JG, Yao S, Chen L, Choi IH (2006) Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-gamma-induced upregulation of B7-H1 (CD274). *FEBS Lett* **580:** 755 – 762

Leoni F, Zaliani A, Bertolini G, Porro G, Pagani P, Pozzi P, Donà G, Fossati G, Sozzani S, Azam T, Bufler P, Fantuzzi G, Goncharov I, Kim SH, Pomerantz BJ, Reznikov LL, Siegmund B, Dinarello CA, Mascagni P (2002) The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. *Proc Natl Acad Sci USA* **99:** 2995 – 3000

Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, Wu TC (1996) Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* **56:** 21 – 26

Mai A, Altucci L (2009) Epi-drugs to fight cancer: from chemistry to cancer treatment, the road ahead. *Int J Biochem Cell Biol* **41:** 199 – 213

Manning J, Indrova M, Lubyova B, Pribylova H, Bieblova J, Hejnar J, Simova J, Jandlova T, Bubenik J, Reinis M (2008) Induction of MHC class I molecule cell surface expression and epigenetic activation of antigen-processing machinery components in a murine model for human papilloma virus 16-associated tumours. *Immunology* **123:** 218 – 227

Mikyskova R, Bubenik J, Vonka V, Smahel M, Indrova M, Bieblová J, Símová J, Jandlová T (2005) Immune escape phenotype of HPV16-associated tumours: MHC class I expression changes during progression and therapy. *Int J Oncol* **26:** 521 – 527

Nencioni A, Beck J, Werth D, Grünebach F, Patrone F, Ballestrero A, Brossart P (2007) Histone deacetylase inhibitors affect dendritic cell differentiation and immunogenicity. *Clin Cancer Res* **13:** 3933 – 3941

Nie Y, Yang G, Song Y, Zhao X, So C, Liao J, Wang LD, Yang CS (2001) DNA hypermethylation is a mechanism for loss of expression of the HLA class I genes in human esophageal squamous cell carcinomas. *Carcinogenesis* **22:** 1615 – 1623

Reinis M (2010) Immunotherapy of MHC class I-deficient tumors. *Future Oncol* **6:** 1577 – 1589

Reinis M, Simova J, Bubenik J (2006) Inhibitory effects of unmethylated CpG oligodeoxynucleotides on MHC class I-deficient and -proficient HPV16-associated tumours. *Int J Cancer* **118:** 1836 – 1842

Seliger B, Ritz U, Ferrone S (2006) Molecular mechanisms of HLA class I antigen abnormalities following viral infection and transformation. *Int J Cancer* **118:** 129 – 138

Serrano A, Tanzarella S, Lionello I, Mendez R, Traversari C, Ruiz-Cabello F, Garrido F (2001) Rexpression of HLA class I antigens and restoration of antigen-specific CTL response in melanoma cells following 5-aza-2′-deoxycytidine treatment. *Int J Cancer* **94:** 243 – 251

Sers C, Kuner R, Falk CS, Sueltmann H, Braun M, Buness A, Ruschhaupt M, Conrad J, Mang-Fatehi S, Stelniec I, Krapfenbauer U, Poustka A, Schäfer R (2009) Down-regulation of HLA Class I and NKG2D ligands through a concerted action of MAPK and DNA methyltransferases in colorectal cancer cells. *Int J Cancer* **125:** 1626 – 1639

Setiadi AF, David MD, Seipp RP, Hartikainen JA, Gopaul R, Jefferies WA (2007) Epigenetic control of the immune escape mechanisms in malignant carcinomas. *Mol Cell Biol* **27:** 7886 – 7894

Setiadi AF, Omilusik K, David MD, Seipp RP, Hartikainen J, Gopaul R, Choi KB, Jefferies WA (2008) Epigenetic enhancement of antigen processing and presentation promotes immune recognition of tumors. *Cancer Res* **68:** 9601 – 9607

Simova J, Bubenik J, Bieblova J, Jandlova T (2004) The role of NK1.1+ cells in the protection against MHC class I+ HPV16-associated tumours. *Folia Biol (Praha)* **50:** 200 – 202

Smahel M, Síma P, Ludvíková V, Marinov I, Pokorná D, Vonka V (2003) Immunisation with modified HPV16 E7 genes against mouse oncogenic TC-1 cell sublines with downregulated expression of MHC class I molecules. *Vaccine* **21:** 1125 – 1136

Tomasi TB, Magner WJ, Khan AN (2006) Epigenetic regulation of immune escape genes in cancer. *Cancer Immunol Immunother* **55:** 1159 – 1184

6.3. Publikácia III

Populácia cyklofosfamidom akumulovaných myeloidných supresorových buniek je imunosupresívna, ale nie identická s tými myeloidnými supresorovými bunkami, ktoré sú akumulované rastúcimi nádormi TC-1.

Cyclophosphamide-induced myeloid-derived suppressor cell population is immunosuppressive but not identical to myeloid-derived suppressor cells induced by growing TC-1 tumors.

Romana Mikyšková, Marie Indrová, Veronika Polláková (Vlková), Jana Bieblová, Jana Šímová a Milan Reiniš

Myeloidné supresorové bunky majú významnú úlohu v úniku nádorových buniek imunitnému systému a veľmi prispievajú k nádorom indukovanej imunosupresii. Ide o heterogénnu populáciu nediferencovaných buniek, ktoré u myší charakterizuje marker monocytov (CD11b) a neutrofilov (Gr-1). Akumulujú sa v lymfoidných orgánoch a krvi počas rastu nádorov a ich akumulácia bola popísaná aj po podaní cyklofosfamidu. V predkladanej publikácii sme sa snažili preskúmať podrobne mechanizmus akumulácie MDSC po chemoterapii s CY (úloha prozápalových cytokínov) a následne identifikovať možnú imunoterapiu s cieľom zoslabiť indukovanú imunosupresiu. Úlohou bolo porovnať fenotyp a funkciu akumulovaných MDSC v slezine po terapii s CY (CY-MDSC) s tými, kde sú MDSC akumulované počas rastu nádoru TC-1 (TU-MDSC) a s tými MDSC, ktoré sú akumulované počas rastu nádoru TC-1 pri ošetrení s CY, čo podporuje ich ďalšiu akumuláciu v slezine (CYTU-MDSC). Aj napriek tomu, že CY-MDSC a aj TU-MDSC podporujú rast nádorov TC-1 *in vivo*, ich fenotyp sa odlišoval. CY-MDSC populácia obsahovala vyššie percento monocytárnej populácie a táto skutočnosť bola asociovaná s nižšou expresiou imunosupresívnych génov a nižšou supresiou proliferácie T-buniek. Môžeme teda tvrdiť, že MDSC akumulované po podaní CY vykazujú viac monocytárny fenotyp ako MDSC akumulované rastúcim nádorom TC-1 a keď porovnáme ich fenotyp s ich nižšiou expresiou imunosupresívnych génov, vykazujú CY-MDSC menšie supresívne vlastnosti. Fenotyp a funkcia CYTU-MDSC populácie bola medzi populáciami CY-MDSC a TU-MDSC. Ďalšou úlohou bolo zistiť účinok terapie s induktorom diferenciácie kyselinou ATRA alebo s cytokínom IL-12 na MDSC akumulované po liečbe s CY (CY-MDSC). ATRA mala účinok na diferenciáciu MDSC a inhibovala MDSC akumulované po terapii s CY. Zistili sme teda rozdiely medzi CY-MDSC a TU-MDSC a podporili sme

využitie kyseliny ATRA alebo cytokinu IL-12 pre upravenie akumulácie MDSC po chemoterapii s CY. Takáto modulácia MDSC kyselinou ATRA alebo cytokínom IL-12 počas chemoterapie nádorov môže zvýšiť protinádorový efekt daného chemoterapeutického agensu.

Prínos autora dizertačnej práce k danej publikácii:

Mojou úlohou pri vypracovaní predkladanej publikácie bolo monitorovanie relatívnej expresie imunosupresívnych génov MDSC akumulovaných po terapii s CY (CY-MDSC) a MDSC akumulovaných počas rastu nádoru TC-1 (TU-MDSC) a MDSC akumulovaných počas rastu nádoru TC-1 pri ošetrení s CY (CYTU-MDSC). Ďalšou úlohou bolo zistiť účinok terapie s induktorom diferenciácie kyselinou ATRA alebo s cytokínom IL-12 na MDSC akumulované po liečbe s CY (CY-MDSC), konkrétne zistiť účinok terapie na relatívnu expresiu imunosupresívnych génov.

Cyclophosphamide-induced Myeloid-derived Suppressor Cell Population Is Immunosuppressive But Not Identical to Myeloid-derived Suppressor Cells Induced By Growing TC-1 Tumors

Romana Mikyšková, Marie Indrová, Veronika Polláková, Jana Bieblová, Jana Šímová, and Milan Reiniš

Summary: Myeloid-derived suppressor cells (MDSC) play an important role in tumor escape from antitumor immunity. MDSC accumulate in the lymphoid organs and blood during tumor growth and their mobilization was also reported after cyclophosphamide (CY) administration. In this communication, spleen MDSC accumulating after CY therapy (CY-MDSC) were compared with those expanded in mice bearing human papilloma viruses 16-associated TC-1 carcinoma (TU-MDSC). Although both CY-MDSC and TU-MDSC accelerated growth of TC-1 tumors in vivo, their phenotype and immunosuppressive function differed. CY-MDSC consisted of higher percentage of monocyte-like subpopulation and this was accompanied by lower relative expression of immunosuppressive genes and lower suppression of T-cell proliferation. After interferon-γ stimulation, the expression of immunosuppressive genes increased, but the suppressive ability of CY-MDSC did not reach that of TU-MDSC. The phenotype and function of MDSC obtained from mice bearing TC-1 tumors treated with CY was, in general, found to lie between CY-MDSC and TU-MDSC. After in vitro cultivation of MDSC in the presence of interleukin 12 (IL-12), the percentage of $CD11b^+/Gr\text{-}1^+$ cells decreased and was accompanied by an increase in the percentage of $CD86^+/MHCII^+$ cells. The strongest modulatory effect was noticed in the group of CY-MDSC. The susceptibility of CY-MDSC to all-trans-retinoic acid (ATRA) was also evaluated. In vitro cultivation with ATRA resulted in MDSC differentiation, and ATRA inhibited MDSC accumulation induced by CY administration. Our findings identified differences between CY-MDSC and TU-MDSC and supported the rationale for utilization of ATRA or IL-12 to alter MDSC accumulation after CY chemotherapy with the aim to improve its antitumor effect.

Key Words: myeloid-derived suppressor cells, cyclophosphamide, all-trans-retinoic acid, IL-12, HPV16

(*J Immunother* 2012;35:374–384)

Tumor-induced immunosuppression belongs to the critical mechanisms of the tumor escape from immune response. Myeloid-derived suppressor cells (MDSC) are major contributors to the mechanisms that are thought to mediate tumor-induced immunosuppression.[1] MDSC represents a heterogeneous population of undifferentiated cells that are characterized in mice by markers of monocytes (CD11b) and neutrophils (Gr-1). In healthy mice, $CD11b^+/Gr\text{-}1^+$ cells can be detected in sizeable numbers in the bone marrow; a small number of these cells (< 4%) can be found in the blood and spleen.[2,3] Disturbances in cytokine homeostasis induced by tumor growth, infection, inflammation, or immune stress can alter the equilibrium of this population leading to its accumulation in lymphoid organs and blood.[4] The main feature of MDSC is their ability to interfere with antitumor immunity and promote tumor growth by inhibiting tumor cell cytotoxicity mediated by blocking the activation of tumor-reactive $CD4^+$, $CD8^+$ T cells,[5,6] and natural killer cells.[7] MDSC have been linked to the induction of T-cell dysfunction through the production of transforming growth factor-β (TGFβ), reactive oxygen species (ROS), arginase 1 (Arg-1), and nitric oxide (NO).[8]

Cyclophosphamide (CY) is a widely used antineoplastic drug, DNA alkylating agent, employed alone or in combination with other products.[9] CY appears as an exceptional chemotherapeutic agent. Besides its direct cytotoxic effect CY was reported to modulate both adaptive and innate immunity.[10,11] When CY is administered in relatively lower doses than those routinely used in the clinical regimen, it can augment antitumor responses as it causes depletion of Treg cells. Low-dose CY was also associated with increased expression of dendritic cell maturation markers.[12] CY administration induces transient lymphopenia that is associated with augmented in vivo proliferation and expansion of antigen-specific T cells [13] and reduction in the number of Tregs.[14] In contrast, MDSC accumulation was reported by several authors.[15–17] CY increased levels of interferon (IFN)γ and other proinflammatory cytokines in the serum[13] and elevated the number and activation status of $CD11b^+$ myeloid cells in vivo.[13,18,19] Our previous experiments showed that MDSC accumulate in the spleens of mice after intraperitoneal (IP) administration of a therapeutic dose of Racemic chlorobromofosfamide-4A (150 mg/kg), an ifosfamide derivative.[20]

Recent studies have shown that all-trans-retinoic acid (ATRA) exhibited a potent activity in eliminating MDSC in cancer patients and in tumor-bearing mice. It has been shown that ATRA was able to differentiate MDSC in vitro.[21,22] Differentiation of MDSC by ATRA was induced primarily by neutralization of high ROS production in these cells.[23] Treatment of cancer patients and tumor-bearing mice with ATRA resulted in a substantial reduction of these cells and improvement of immune responses.[24,25] ATRA has also been shown to improve differentiation of dendritic cells in cancer patients.[26] These data suggest that

Received for publication September 27, 2011; accepted March 3, 2012.
From the Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, v. v. i., Prague, Czech Republic.
Reprints: Milan Reiniš, Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, v. v. i., Vídeňská 1083, 142 20 Prague 4, Czech Republic (e-mail: milan.reinis@img.cas.cz).

ATRA could be used as an effective differentiation agent for MDSC and that the elimination of MDSC with ATRA may improve the effect of cancer vaccines.

Interleukin 12 (IL-12) is a promising antitumor and immunostimulatory cytokine shown to be effective against tumors in various experimental models and human clinical trials.[22,27–30]

Moreover, IL-12 could induce differentiation of MDSC into antigen-presenting cells (dendritic cells), which can also affect MDSC accumulation and function. Studies indicated that MDSC decreased IL-12 production from macrophages, suggesting a possible role for IL-12 in modulating MDSC activity. Treatment with IL-12 altered the suppressive function of MDSC and decreased the percentage of MDSC in the tumor microenvironment.[31] In our previous work, we showed the efficacy of recombinant IL-12 (rIL-12) or cellular vaccines engineered to produce IL-12 in the treatment of early tumor transplants of human papilloma viruses (HPV)16-associated tumors, as well as in the treatment of tumor recurrences after surgery or cytoreductive chemotherapy.[32–35] Further, immunotherapy with IL-12-producing, genetically modified TC-1 cells increased tumor infiltration with $CD8^+$ and $CD4^+$ cells and decreased the percentage of $CD11b^+/Gr\text{-}1^+$ cells,[20] which suggests that IL-12 can be used as an immunotherapeutic cytokine for inhibition of immunosuppression caused by MDSC accumulation.

Because the mechanism of CY-induced MDSC accumulation (transient suppression of myelopoiesis together with release of proinflammatory and protumorigenic cytokines caused by inflammatory organ reaction) seems to be different from the mechanism leading to MDSC accumulation during tumor growth (release of proinflammatory and protumorigenic cytokines), in this study we have compared the phenotype and function of spleen $CD11b^+/Gr\text{-}1^+$ MDSC accumulating after CY therapy (CY-MDSC) with those of cells expanding in mice bearing HPV16-associated murine TC-1 tumors (TU-MDSC). To mimic the clinically relevant setting, a group of mice bearing TC-1 tumors treated with CY (CYTU-MDSC) was also included to this comparison. Further, the susceptibility of CY-MDSC to the ATRA or rIL-12 treatment in vitro and in vivo was evaluated.

MATERIALS AND METHODS

Mice

C57BL/6 male mice, 6–8 weeks old, were obtained from AnLab Co., Prague, Czech Republic. Experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics, Prague.

Tumor Cell Lines

For the experiments, the nonmetastasizing TC-1 tumor cell line established by transformation of primary C57BL/6 mouse lung cells with HPV16 E6/E7 and activated Ha-ras encoding DNA[36] was used. TC-1 cells were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, and antibiotics and cultured at 37°C in a humidified atmosphere with 5% CO_2.

Accumulation of MDSC in Spleens of Mice

Mice bearing large TC-1 tumors (approximate size of 1.5 cm in diameter), mice 7 days after IP administration of 200 mg/kg of CY (Baxter, Deerfiel, IL), and mice bearing large TC-1 tumors treated with CY (7 d after IP administration of 200 mg/kg CY) were killed and the percentage of $CD11b^+/Gr\text{-}1^+$ cells in their spleens was analyzed by flow cytometry. As a control, healthy naive mice were used. The dose of CY was established after performing the experiment in which mice bearing small TC-1 tumors (size of 2–3 mm in diameter) were treated with different doses of CY. The dose of 200 mg/kg was selected as optimal for both combined chemoimmunotherapy and study of MDSC and their possible modulation (data not shown).

Preparation and Characterization of CY-MDSC, TU-MDSC, and CYTU-MDSC

$CD11b^+$ cells from spleens of CY-treated (CY-MDSC), TC-1 tumor-bearing (TU-MDSC), and CY-treated TC-1 tumor-bearing mice (CYTU-MDSC) mice were isolated by magnetic-activated cell sorting (MACS) using anti-mouse CD11b (Mac-1α) antibodies (Miltenyi Biotech, Auburn, CA) conjugated to magnetic beads in accordance with the manufacturer's instructions. Cell separation was performed with autoMACS (Miltenyi Biotec). The purity of cells was controlled by flow cytometry and the percentage of $CD11b^+/Gr\text{-}1^+$ cells achieved 86%–91%. All cultivations of MDSC were performed in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, antibiotics, and 2-mercaptoethanol in a humidified atmosphere with 5% CO_2 at 37°C.

Immediately after isolation or after 24 hours of incubation (1×10^6 cells/mL) with 100 U/mL IFNγ (RD Systems, Minneapolis, MN), CY-MDSC, TU-MDSC, and CYTU-MDSC were analyzed by flow cytometry (FACS) for the percentage of $CD11b^+/Gr\text{-}1^+$ cells and the expression of Ly6G, Ly6C, F4/80, CD11c, CD80, and CD86 markers. Relative expression of genes responsible for immunosuppressive function, Arg-1, inducible nitric oxide synthase (iNOS), TGFβ, ROS, and vascular endothelial growth factor (VEGF) was analyzed immediately after cell isolation or after 6 hours of incubation in the RPMI medium supplemented with 100 U/mL IFNγ (1×10^6 cells/mL) by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). VEGF production was analyzed by enzyme-linked immunosorbent assay (ELISA) after 24 hours incubation in RPMI medium (5×10^6 cells/mL) supplemented with 100 U/mL IFNγ.

Flow Cytometry

To determine the percentage of $CD11b^+/Gr\text{-}1^+$ cells, fluorescein isothiocyanate (FITC) rat anti-mouse CD11b (M1/70) and allophycocyanin (APC) rat anti-mouse Ly6G and Ly6C (Gr-1) (RB6-8C5) were utilized. To determine whether MDSC possess the receptor for IL-12, phycoerythrin (PE) anti-CD212 (IL-12–receptor β chain) was used. Expression of Ly6G, Ly6C, F4/80, CD11c, MHC class II, CD80, and CD86 was analyzed using following antibodies: PE anti-Ly6G (1A8), FITC anti-Ly6C (AL-21), PE-CyTM7 rat anti-mouse CD11b (M1/70), PE anti-F4/80 (BM8), APC anti-CD11c (HL3), FITC anti-MHCII (AF6-120.1), PE anti-CD80 (16-10A1), and PE anti-CD86 (GL1). Relevant isotype controls, FITC, APC, PE-CyTM7, and PE-labeled antibody of irrelevant specificity were used. PE anti-F4/80 was purchased from BioLegend (San Diego, CA); other products were purchased from Pharmingen (San Diego, CA). Flow cytometry was performed using an LSR II flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo v. 9.4.11.

qRT-PCR

Total RNA was extracted with High Pure RNA isolation kit (Roche, Basel, Switzerland). The amount of 1 μg of RNA was reverse transcribed to cDNA using random hexamer primers from GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA) in a 20-μl reaction volume at 42°C for 30 minutes. Quantification of PCR products was performed in 10 μl of Lightcycler 480 SYBR Green I Master mix (Roche) using a real-time PCR lightcycler (Roche). DNA was denatured at 95°C for 2 minutes; 45 cycles of denaturation at 95°C for 25 seconds, annealing at 60°C for 45 seconds, elongation at 72°C for 1 minutes, and incubation at 80°C for 5 seconds followed. cDNAs were amplified with specific primers for *β-actin*, *Arg-1*, *iNOS*, *TGF-β1* (designated as *TGFβ*), *ROS*, and *VEGF-164* (designated as *VEGF*). The list of the reference genes and their primer sequences has been described elsewhere.[37] The remaining PCR primer sequences are as follows: *Arg-1*, 5′ CTCCAAGCCAAAGTCCTTAGAG (forward) and AGGAGCTGTCATTAGGGACATC (reverse); *iNOS*, 5′ GTTCTCAGCCCAACAATACAAGA (forward) and GTG GACGGGTCGATGTCAC (reverse); *TGFβ-1*, 5′CCGCA ACAACGCCATCTATG (forward) and CTCTGCACGG GACAGCAAT (reverse); *VEGF-164*, 5′ CTTGTTCA GAGCGGAGAAAGC (forward) and ACATCTGCAAGT ACGTTCGTT (reverse); *ROS*, 5′ CCAACTGGGATAAC GAGTTCA (forward) and GAGAGTTTCAGCCAAGGC TTC (reverse).

Fold changes in transcript levels were calculated using cycle threshold values standardized to β-actin, used as the endogenous reference gene control. All samples were run in biological triplicates.

ELISA Assay

For VEGF production, supernatants from cultivated cells were analyzed by ELISA kits (BD Biosciences) according to the manufacturer's instructions.

Proliferation Assay

For proliferation assay, splenocytes were resuspended at the concentration of 10^7 cells/mL in phosphate buffered saline supplemented with 5% FCS and labeled with 2.5 μM 5,6-carboxy-fluorescein diacetate succinimidyl ester (CFSE, Sigma-Aldrich, St. Louis, MO) by incubation for 5 minutes in 37°C in 5% CO_2. Labeling was quenched with cold RPMI 1640 medium supplemented with 10% FCS and the cells were washed. After CFSE staining, splenocytes were stimulated with 1.0 μg/mL anti-CD3 (clone 145-2C11) and 1.0 μg/mL anti-CD28 (clone 37.51) antibodies (Pharmingen) and cultured alone, or admixed with CY-MDSC, TU-MDSC, and CYTU-MDSC (1:1 ratio) alone or in the presence of IFNγ 100 U/mL for 4 days. Cells were gated on $CD4^+$ and $CD8^+$ cells using PE anti-CD4 (GK1.5) and PE anti-CD8 (53-6.7) antibodies (Pharmingen) and analyzed for dilution of CFSE by flow cytometry.

Immunosuppressive Function of CY-MDSC and TU-MDSC In Vivo

Mice were subcutaneously (SC) inoculated with 5×10^4 TC-1 cells alone or in a mixture (ratio, 1:5) with spleen CY-MDSC or TU-MDSC. The growing tumors were checked twice a week and the size of the tumors was recorded.

Modulation of MDSC With ATRA In Vitro

$CD11b^+$ CY-MDSC obtained by magnetic separation from spleens of mice 7 days after CY treatment were cultivated (2×10^6 cells/3 mL) in vitro for 5 days in the presence of 1.5 μM ATRA (Sigma-Aldrich) in the RPMI medium supplemented with 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) (RD Systems) in the ultralow cluster 6-well plate (Corning Incorporated, Costar, NY). Control samples were cultivated without presence of ATRA. After cultivation, cells were analyzed by flow cytometry for the percentage of $CD11b^+/Gr\text{-}1^+$ cells and the expression of Ly6G, Ly6C, F4/80, and CD11c markers. Relative expression of genes for Arg-1, iNOS, TGFβ, ROS, and VEGF was analyzed by qRT-PCR. Supernatants from cultivated cells were analyzed by ELISA for VEGF production.

Modulation of CY-MDSC With ATRA In Vivo

Mice were administered IP with CY (200 mg/kg). Five days later, mice were treated for 2 weeks with ATRA (10 mg/kg/d SC once a day). The percentage of $CD11b^+/Gr\text{-}1^+$ cells in the spleens of treated mice was subsequently analyzed by flow cytometry. Relative expression of genes responsible for immunosuppressive function (Arg-1, iNOS, TGFβ, ROS, and VEGF) was followed by qRT-PCR. The ex vivo obtained spleen cells were cultivated in vitro for 48 hours in the RPMI medium and the supernatants were analyzed for VEGF production by ELISA.

Treatment of TC-1 Tumors With CY and ATRA

Mice were inoculated SC with 5×10^4 TC-1 tumor cells. After approximately 12 days, when the tumors reached a size of 2–3 mm in diameter, the mice were randomly divided into 4 experimental groups. One experimental group of mice was left as a control group (untreated TC-1 tumor-bearing mice). Two experimental groups of mice were injected IP with CY (200 mg/kg). A week later, 1 group of mice previously injected with CY was treated with ATRA (10 mg/kg/d SC once a day) for 2 weeks (CY + ATRA-treated mice) and the second experimental group of mice was left without further treatment as CY-only–treated mice. The last experimental group without previous CY treatment was injected with ATRA (10 mg/kg/d SC once a day) for 2 weeks (ATRA-treated mice). The growing tumors were checked twice a week and the size of the tumors was recorded.

Modulation of MDSC With rIL-12 In Vitro

$CD11b^+$ CY-MDSC, TU-MDSC, and CYTU-MDSC (IL-12–receptor negative, FACS analysis, data not shown) obtained after magnetic separation from spleens of relevant mice were cultivated in vitro for 4 days in the presence of 0.2 μg/mL rIL-12 (RD Systems) in the RPMI medium supplemented with 10 ng/mL GM-CSF in 12-well Transwell plates with polycarbonate membrane (Corning Incorporated). $CD11b^+$ MDSC were seeded into Transwell inserts (1×10^6 cells/mL), and the $CD11b^-$ cells were seeded into lower compartments of the wells (2×10^6 cells/mL). Control samples were cultivated without the presence of rIL-12. After cultivation, cells were analyzed by flow cytometry for the percentage of $CD11b^+/Gr\text{-}1^+$ cells and the expression of CD86/MHCII markers. Relative expression of genes for Arg-1, iNOS, TGFβ, ROS, and VEGF was analyzed by qRT-PCR. Supernatants from cultivated cells were analyzed by ELISA for VEGF production.

Statistical Analyses

For statistical analyses of in vitro experiments, Student's *t* test for paired and unpaired data was used. For the evaluation of in vivo experiments, analysis of variance and log-rank test from the Number Cruncher Statistical System (Kaysville, UT) statistical package were utilized. SDs are indicated in the figures.

RESULTS

Characterization of MDSC Derived From CY-treated, TC-1 Tumor-bearing Mice and CY-treated TC-1 Tumor-bearing Mice

A significant accumulation of $CD11b^+/Gr\text{-}1^+$ cells in the spleens of CY-treated mice, mice bearing established growing HPV16-associated TC-1 tumor transplants, and CY-treated TC-1 tumor-bearing mice was found when compared with control naive healthy mice (FACS analysis, $P < 0.05$, Fig. 1A). Further, when $CD11b^+$ cells from CY-treated mice (CY-MDSC), $CD11b^+$ cells from TC-1 tumor-bearing mice (TU-MDSC), and $CD11b^+$ cells from CY-treated mice TC-1 tumor-bearing mice (CYTU-MDSC) were isolated by MACS separation and analyzed by flow cytometry, significant differences between CY-MDSC and TU-MDSC were found. The percentage of $Ly6G^-Ly6C^{high}$ [monocytes (MO)-like] population was higher in CY-MDSC when compared with TU-MDSC ($P < 0.05$), whereas the percentage of $Ly6G^+Ly6C^{low}$ [polymorphonuclear (PMN)-like] population was higher in TU-MDSC ($P < 0.05$) (Fig. 1B). Expression of F4/80 (corresponding to the MO-MDSC phenotype) and CD11c was significantly higher in CY-MDSC ($P < 0.05$). There was no significant difference in the expression of CD80 and CD86 (Fig. 1C). In the next step, relative expression of genes for Arg-1, iNOS, TGFβ, ROS, and VEGF in CY-MDSC, TU-MDSC, and CYTU-MDSC was compared by qRT-PCR. Relative expression of all immunosuppressive genes analyzed was significantly higher in TU-MDSC, compared with CY-MDSC ($P < 0.05$) (Fig. 1D). The group of mice bearing TC-1 tumors treated with CY (CYTU-MDSC) was also included into this comparison. In general, the CYTU-MDSC phenotype ($Ly6G^-Ly6C^{high}$ vs. $Ly6G^+Ly6C^{low}$ ratio, proportion of $F4/80^+$ or $CD11c^+$ cells) tended to lie between those of CY-MDSC or TU-MDSC. The relative expression of Arg-1 and TGFβ was similar to the relative expression of these genes in CY-MDSC, whereas the relative expression of iNOS, ROS, and VEGF ranged between CY-MDSC and TU-MDSC.

Comparison of CY-MDSC, TU-MDSC, and CYTU-MDSC After Stimulation With IFNγ

As it is known that IFNγ activates MDSC and thereby increases T-cell suppression, the effect of IFNγ on CY-MDSC, TU-MDSC, and CYTU-MDSC phenotype and function was determined. Isolated MDSC were cultivated in vitro for 24 hours in the medium supplemented with 100 U/mL IFNγ. As can be seen in Figure 2A, the percentage of $CD11b^+/Gr\text{-}1^+$ was higher after cultivation of CY-MDSC and CYTU-MDSC with IFNγ when compared with TU-MDSC. However, no significant difference in the cell-surface expression of F4/80, CD11c, CD80, and CD86 was found (data not shown) in CY-MDSC and TU-MDSC after stimulation with IFNγ. As expected, relative expression of genes for iNOS, ROS, and VEGF was significantly increased in CY-MDSC, TU-MDSC, and CYTU-MDSC after stimulation with IFNγ for 6 hours. It is noteworthy that after stimulation the relative expression of the iNOS gene was significantly higher in CY-MDSC than in TU-MDSC ($P < 0.05$). Relative expression of genes for Arg-1 and TGFβ was not significantly changed (Fig. 2B). As can be seen in Figure 2C, CY-MDSC, TU-MDSC, and CYTU-MDSC produced significantly higher amounts of VEGF after stimulation with IFNγ when compared with unstimulated samples ($P < 0.05$). In addition, CY-MDSC and CYTU-MDSC produced significantly higher amounts of VEGF (both without stimulation and after stimulation with IFNγ) when compared with TU-MDSC ($P < 0.05$).

Immunosuppressive Function of CY-MDSC, TU-MDSC, and CYTU-MDSC In Vitro

The immunosuppressive function of CY-MDSC, TU-MDSC, and CYTU-MDSC was compared in vitro (CFSE proliferation assay). For proliferation assay, stimulated cells labeled with CFSE were admixed with CY-MDSC and TU-MDSC (1:1 ratio) for 4 days (without or with the addition of 100 U/mL IFNγ) and the dilution of CFSE in $CD4^+$ and $CD8^+$ cells was analyzed by flow cytometry. As can be seen in Figure 3A, TU-MDSC suppressed T-cell proliferation more strongly than CY-MDSC ($P < 0.05$). When stimulated with IFNγ, the suppressive capacity of CY-MDSC increased but did not reach the suppressive capacity of TU-MDSC (Fig. 3B). The suppressive capacity of CYTU-MDSC lay between CY-MDSC and TU-MDSC (Fig. 3A).

Immunosuppressive Function of CY-MDSC and TU-MDSC In Vivo

The immunosuppressive function of CY-MDSC and TU-MDSC was also compared in vivo (adoptive transfer experiments). Despite the difference in the immunosuppressive capacity detected in vitro, both CY-MDSC and TU-MDSC accelerated significantly the growth of TC-1 tumors in vivo ($P < 0.05$) (Fig. 3C).

Modulation of CY-MDSC With ATRA In Vitro

As ATRA is an agent that is known to stimulate differentiation of immature myeloid cells, CY-MDSC were cultivated in vitro in the RPMI medium supplemented with ATRA and GM-CSF. After in vitro treatment of CY-MDSC with ATRA, the percentage of $CD11b^+/Gr\text{-}1^+$ cells decreased; the decrease was caused by the loss of Gr-1 marker. It was accompanied by the increase in F4/80 and CD11c expression (Fig. 4A). CY-MDSC cultivated in vitro in the presence of ATRA also exhibited significantly reduced relative expression of genes for Arg-1, iNOS, TGFβ, ROS, and VEGF ($P < 0.05$) (Fig. 4B). Significantly decreased VEGF production ($P < 0.05$) was also found in the supernatant obtained after in vitro cultivation of CY-MDSC with ATRA (Fig. 4C).

Modulation of CY-MDSC With ATRA In Vivo

To confirm the in vitro effect of ATRA on MDSC accumulating after CY treatment, mice were IP administered with CY and subsequently SC treated for 2 weeks with ATRA. When compared with CY-only–treated mice, a significant decrease ($P < 0.05$) in the percentage of $CD11b^+/Gr\text{-}1^+$ cells was found (Fig. 5A). It was accompanied by a significant reduction ($P < 0.05$) of relative expression of genes for Arg-1 and reduction of relative

expression of genes for iNOS, TGFβ, ROS, and VEGF (Fig. 5B). Further, decreased VEGF production by spleen cells cultivated in the RPMI medium for 48 hours was found in the group treated with ATRA after CY administration ($P < 0.05$) (Fig. 5C). To analyze whether ATRA can improve the antitumor effect of the CY treatment, mice bearing established TC-1 tumor transplants were treated with CY in combination with ATRA. The cytoreductive chemotherapy with CY resulted in a strong tumor-inhibitory effect ($P < 0.05$) and the subsequent therapy with ATRA significantly enhanced the CY antitumor efficacy ($P < 0.05$) (Fig. 5D).

Modulation of CY-MDSC, TU-MDSC, and CYTU-MDSC With IL-12 In Vitro

As we have shown previously,[34] the accumulation of CD11b$^+$/Gr-1$^+$ cells was significantly decreased after subsequent IL-12 immunotherapy of TC-1 tumors. This

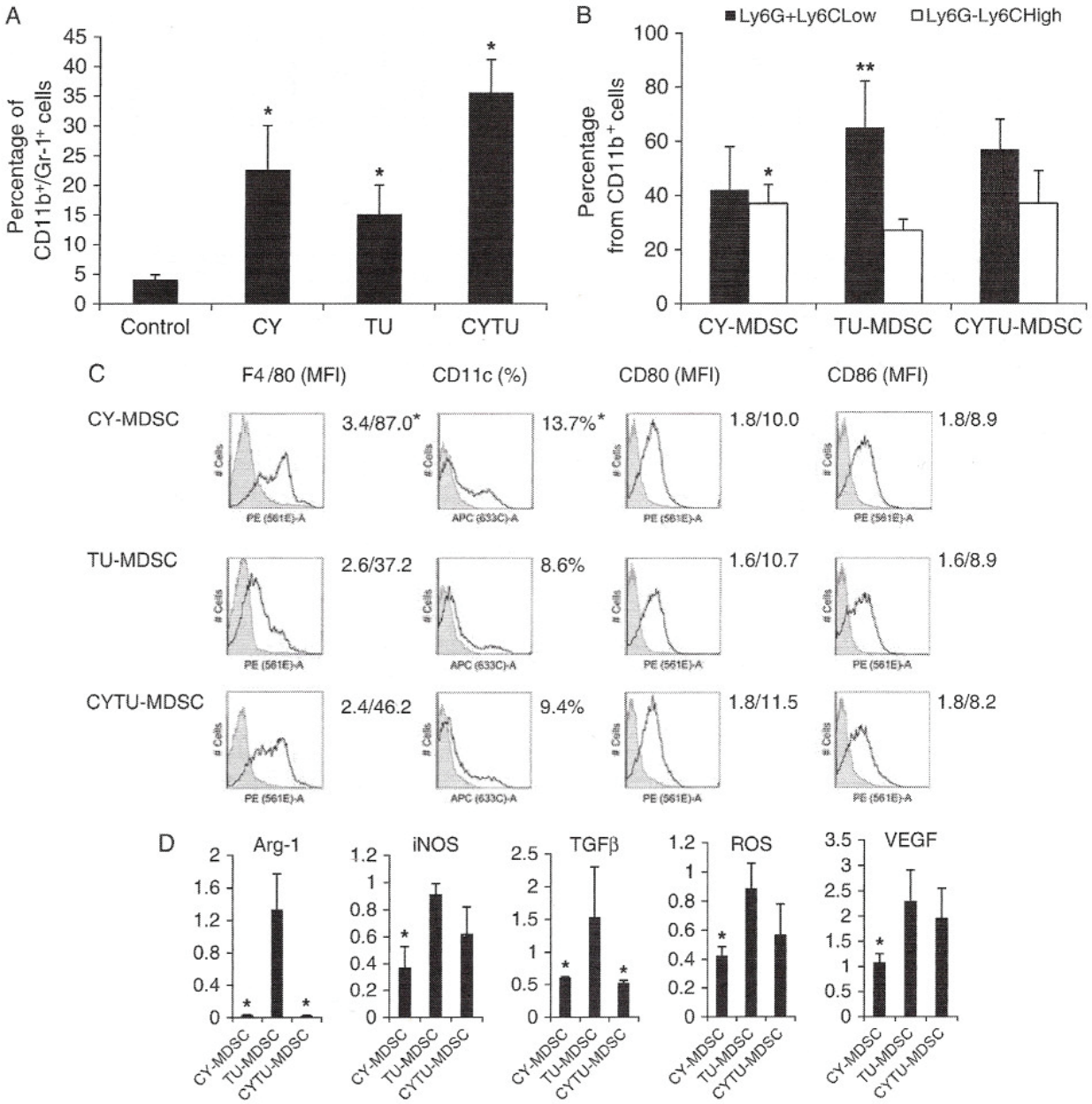


FIGURE 1. Comparison of CY-MDSC, TU-MDSC, and CYTU-MDSC phenotypes and expression of immunosuppressive genes. A, Percentage of CD11b$^+$/Gr-1$^+$ cells in the spleens of mice after cyclophosphamide (CY) treatment, in the spleens of mice with TC-1 tumors, and in the spleens of mice with CY-treated TC-1 tumors in comparison with control mice (FACS; $^*P<0.05$ as compared with control, n = 3–12, *t* test). B, Percentage of monocytes-like population (Ly6G$^-$Ly6C^{high}) (FACS; $^*P<0.05$ as compared with TU-MDSC; n = 12, *t* test) and percentage of polymorphonuclear -like population (Ly6G$^+$Ly6C^{low}) (FACS; $^{**}P<0.05$ as compared with CY-MDSC, n = 12, *t* test). C, Expression of F4/80 and CD11c (FACS; $^*P<0.05$ when compared CY-MDSC vs. TU-MDSC, n = 12, *t* test); expression of CD80, CD86 (FACS; $P>0.05$). D, Relative expression of genes for arginase 1(Arg-1), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGFβ), reactive oxygen species (ROS), and vascular endothelial growth factor (VEGF) (real-time quantitative polymerase chain reaction; $^*P<0.05$ as compared with TU-MDSC, n = 3–12, *t* test). MFI indicates mean flurescence intensity.

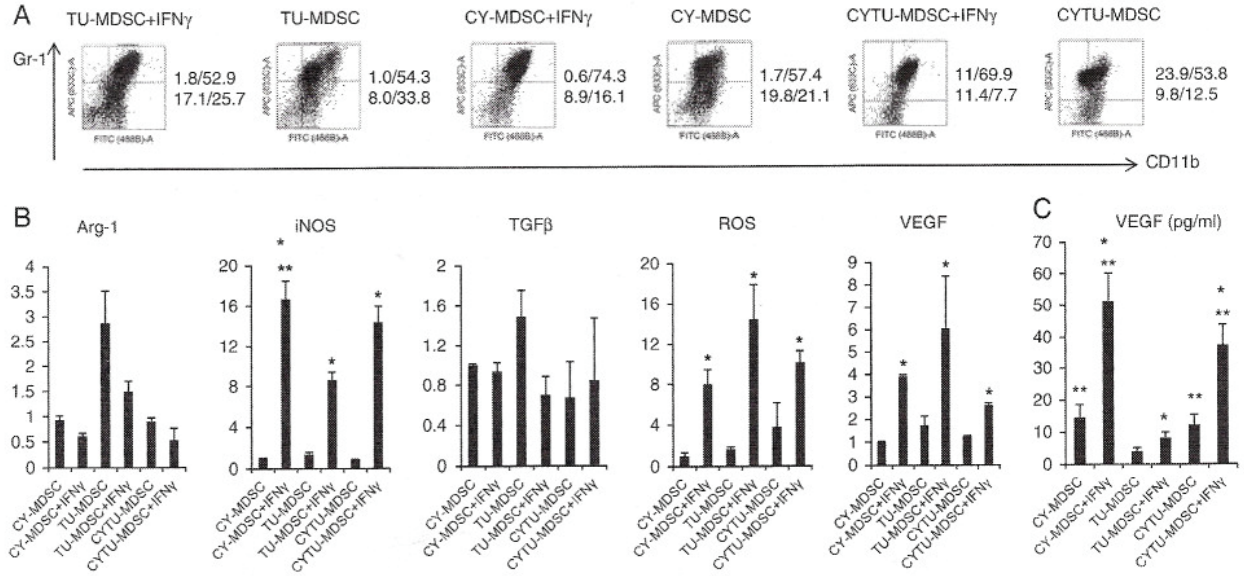


FIGURE 2. Comparison of CY-MDSC and TU-MDSC phenotype and expression of immunosuppressive genes after stimulation with interferon (IFN)γ. A, Percentage of $CD11b^+/Gr\text{-}1^+$ cells after stimulation with 100 U/mL IFNγ for 24 hours (FACS); representative experiment of 3 experiments is given. B, Relative expression of genes for arginase 1 (Arg-1), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGFβ), reactive oxygen species (ROS), and vascular endothelial growth factor (VEGF) in CY-MDSC, TU-MDSC, and CYTU-MDSC after stimulation with 100 U/mL IFNγ for 6 hours (real-time quantitative polymerase chain reaction; $*P<0.05$ as compared with samples without IFNγ, *t* test; $**P<0.05$ as compared with TU-MDSC, n = 9, paired *t* test). C, VEGF production (enzyme-linked immunosorbent assay; $*P<0.05$ as compared with samples without IFNγ, n = 3-9, *t* test; $**P<0.05$ as compared with TU-MDSC).

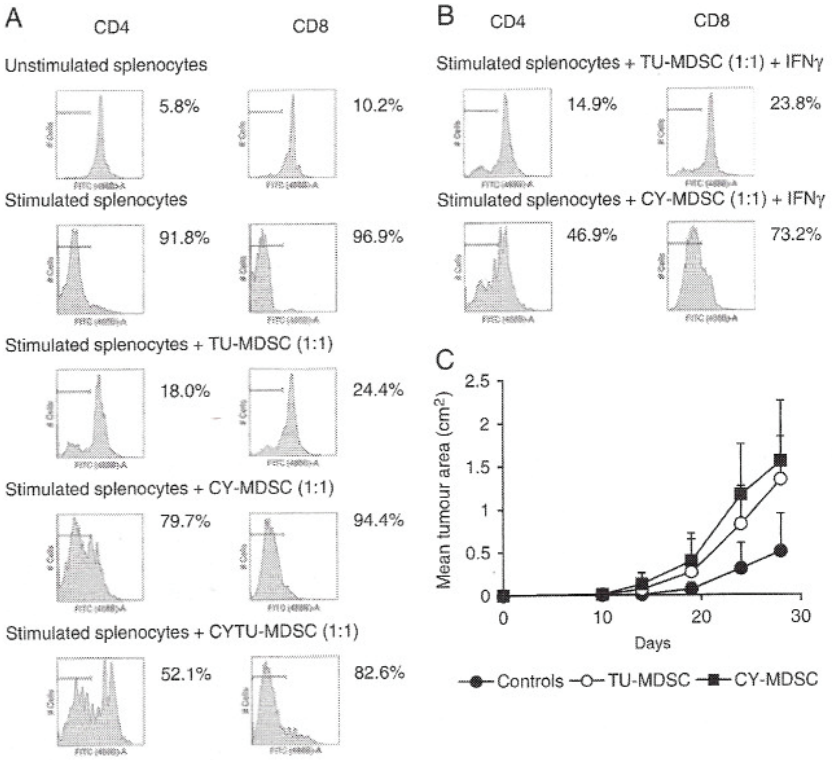


FIGURE 3. Immunosuppressive effect of CY-MDSC, TU-MDSC, and CYTU-MDSC. A, In vitro suppression of 5,6-carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled spleen-cell proliferation (percentages indicate percent of activation). Representative experiment of 3 experiments is given. Each experiment included 3 mice per group. Significant inhibition of $CD4^+$ and $CD8^+$ cells proliferation was found only in case of TU-MDSC (TU-MDSC vs. stimulated splenocytes, CY-MDSC $P<0.05$, *t* test). B, In vitro suppression of CFSE-labeled spleen-cell proliferation by CY-MDSC and TU-MDSC stimulated by 100 U/mL interferon (IFN)γ. C, In vivo acceleration of TC-1 tumor growth after CY-MDSC or TU-MDSC administration (TU-MDSC, CY-MDSC vs. controls $P<0.05$, analysis of variance).

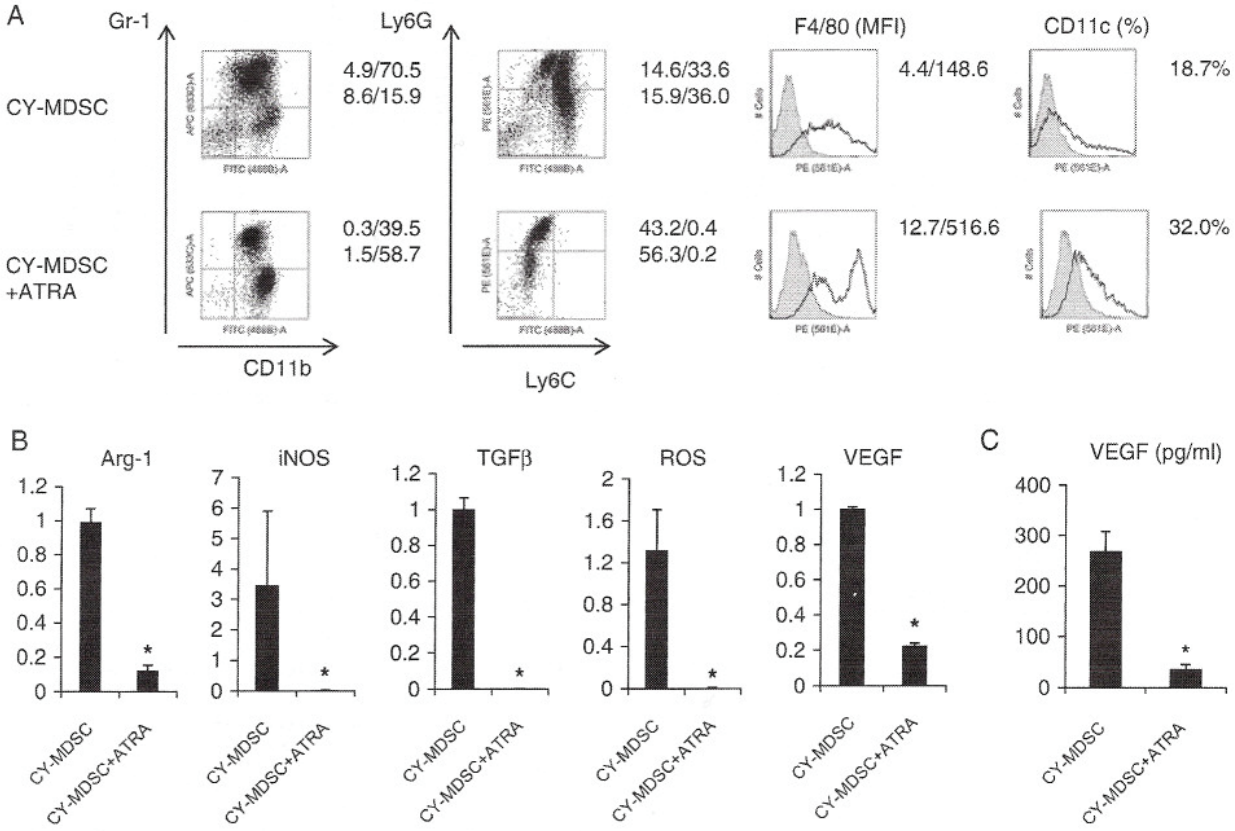


FIGURE 4. In vitro susceptibility of CY-MDSC to all-trans-retinoic acid (ATRA). A, Percentage of CD11b$^+$/Gr-1$^+$ cells and expression of Ly6G, Ly-6C, F4/80, and CD11c after in vitro cultivation of CY-MDSC for 5 days with ATRA (FACS). Representative experiment of 3 experiments is given. B, Relative expression of genes for arginase 1 (Arg-1), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGFβ), reactive oxygen species (ROS), and vascular endothelial growth factor (VEGF) after ATRA treatment (real-time quantitative polymerase chain reaction; $*P<0.05$, n=9, paired *t* test). C, VEGF production (enzyme-linked immunosorbent assay ; $*P<0.05$, n=9, *t* test). MFI indicates mean flurescence intensity.

modulatory effect is indirect, as MDSC in C57BL/6 mice do not have receptor for IL-12 (data not shown). To investigate whether rather soluble factors than cell-to-cell contact of MDSC with the CD11b$^-$ cell population take place, CD11b$^+$ and CD11b$^-$ spleen-cell fractions from CY-treated mice, TC-1 tumor-bearing mice, and CY-treated TC-1 tumor-bearing mice were separately cultivated in vitro in 2 compartments of wells divided with polycarbonate membrane (Transwell plates) in the RPMI medium supplemented with rIL-12 and GM-CSF. The membrane was permeable for culture medium but did not allow cell-to-cell contact. After in vitro cultivation of spleen cells from CY-treated mice with rIL-12, a significant decrease ($P < 0.05$) in the percentage of CD11b$^+$/Gr-1$^+$ cells accompanied by a significant increase ($P < 0.05$) in the percentage of CD86$^+$/MHCII$^+$ (APC-like) cells was observed (Figs. 6A and B). After in vitro cultivation of spleen cells from TC-1 tumor-bearing mice with rIL-12, the percentage of CD11b$^+$/Gr-1$^+$ cells also decreased and was accompanied by a significant increase ($P < 0.05$) in the percentage of CD86$^+$/MHCII$^+$, similarly as in CY-treated mice (Figs. 6A and B). A similar reduction was also observed in the group of CY-treated TC-1 tumor-bearing mice. As can be seen in Figure 6C, spleen cells from CY-treated mice cultivated in vitro in the presence of rIL-12 exhibited significantly reduced relative expression of genes for Arg-1, TGFβ, and ROS. The relative expression of genes for iNOS and VEGF was also reduced. Reduced relative expression of genes for Arg-1, iNOS TGFβ, ROS, and VEGF was also found in the group of TC-1 tumor-bearing mice and CY-treated TC-1 tumor-bearing mice. Significant decrease in VEGF production ($P < 0.05$) was found in the supernatant obtained after in vitro cultivation of spleen cells from CY-treated mice and TC-1 tumor-bearing mice with rIL-12. A similar reduction was also observed in the group of CY-treated TC-1 tumor-bearing mice (Fig. 6D).

DISCUSSION

During the past decade, multiple reports have shown that MDSC accumulate during tumor growth.[1–8] Recently, 2 main different subsets of MDSC, MO-like (Ly6G$^-$ Ly6C^{high}) population, and PMN-like (Ly6G$^+$ Ly6C^{low}) population have been described.[38–41] Accumulation of MDSC was also reported after administration of high-dose CY,[15–17] but controversial results concerning the proper function of CY-MDSC were published. Myeloid cells from spleens of mice that underwent CY administration were found to have tumor-inhibitory effect in mouse mammary carcinoma model.[16] However, it was reported that CY-induced immature myeloid cells were able to suppress T-cell proliferation.[15] So far, the phenotype and function of CY-MDSC and their comparison with the phenotype and function of TU-MDSC have not been characterized in

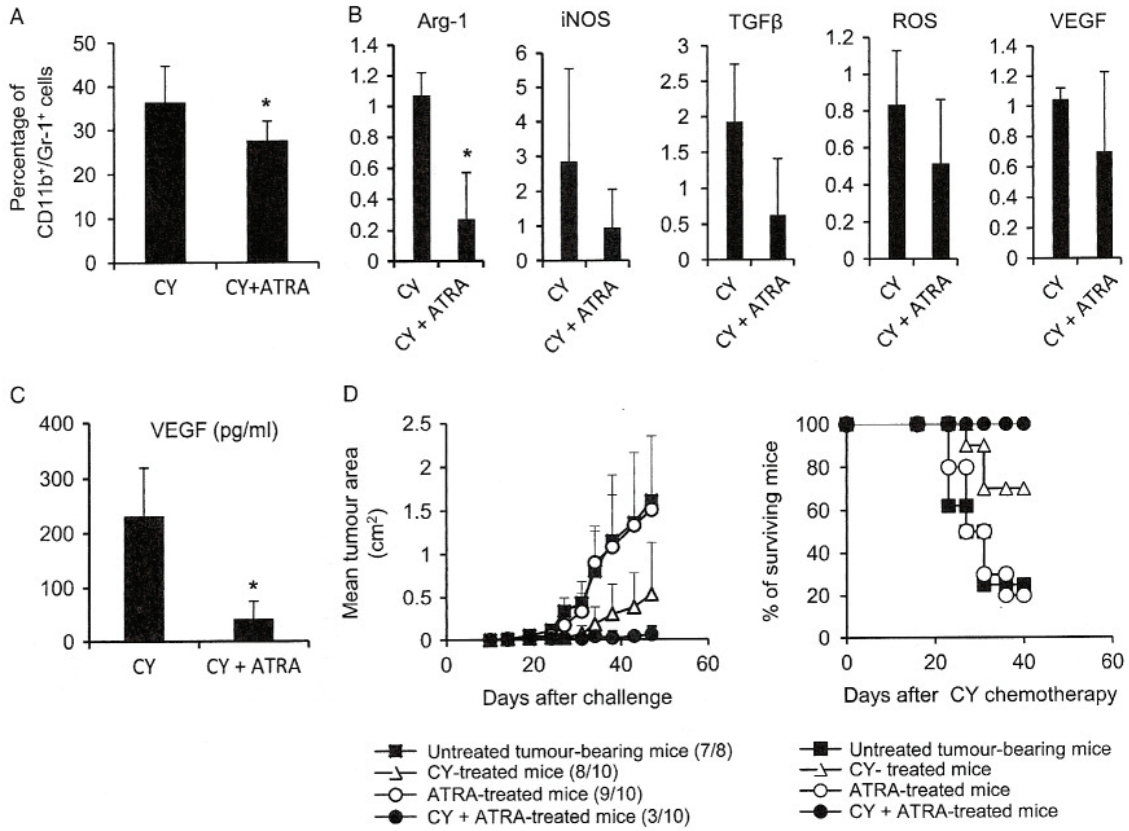


FIGURE 5. The effect of all-trans-retinoic acid (ATRA) on myeloid-derived suppressor cells accumulation after cyclophosphamide (CY) treatment and the effect on CY antitumour efficacy. A, Percentage of $CD11b^+/Gr\text{-}1^+$ cells in the spleens of mice after CY and subsequent ATRA treatment (FACS; $^*P<0.05$; n=12, *t* test). B, Relative expression of genes for arginase 1 (Arg-1), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGFβ), reactive oxygen species (ROS), and vascular endothelial growth factor (VEGF) (real-time quantitative polymerase chain reaction; $^*P<0.05$; n=9, *t* test). C, VEGF production (enzyme-linked immunosorbent assay; n=12, $^*P<0.05$, *t* test). D, Growth inhibition of TC-1 tumor transplants after CY and subsequent ATRA treatment: growth curves (untreated vs. CY+ATRA, untreated vs. CY, ATRA vs. CY+ATRA, ATRA vs. CY $P<0.05$, analysis of variance) and Kaplan-Mayer survival plot (untreated vs. CY+ATRA, ATRA vs. CY+ATRA, untreated vs. CY, ATRA vs. CY, CY vs. CY+ATRA; $P<0.05$, log-rank test). Mice were euthanized when the tumor area reached 2.0 cm^2 and data are presented as percent of survival. Representative experiment of 3 experiments is given.

detail. In this study, we have demonstrated that MDSC that accumulate after CY administration are composed of a higher percentage of MO-like population in comparison with MO-like population in MDSC that are expanded in mice bearing TC-1 tumors, whereas the percentage of PMN-like population was notably higher in tumor-induced MDSC and notably outnumbered the MO-like population. The difference in the MO-like population was not very dramatic, however, in the light of the experiments that compared expression of immunosuppressive genes and immunosuppressive function we suppose that it could have the biological meaning. This latter observation is in agreement with results of Movahedi et al,[41] who reported that mainly PMN cells with suppressive activity were expanded in tumor-bearing mice. The expression of F4/80 and CD11c was significantly higher in CY-MDSC. This finding is consistent with the statement of Movahedi and colleagues that MO-like MDSC are typified with F4/80 marker. It supports our finding that CY-induced MDSC population consists of a higher percentage of MO-like and differentiated cells than tumor-induced MDSC. Not to overestimate the biological meaning of subpopulation differences between the CY-MDSC and TU-MDSC phenotypes, in the next step we compared the relative expression of immunosuppressive genes for Arg-1, iNOS, TGFβ, ROS, and VEGF in CY-MDSC and TU-MDSC and found that the relative expression of all these immunosuppressive genes was lower in CY-induced MDSC. We can conclude that CY-induced MDSC display more monocytic phenotype than tumor-induced MDSC and, moreover, that their phenotype together with the expression of immunosuppressive gene expression display less suppressive features. As it has been described that IFNγ enhanced the suppressive function of tumor-induced MDSC,[39] we compared the sensitivity of CY-MDSC and TU-MDSC to IFNγ in vitro. Our results showed that the percentage of $CD11b^+/Gr\text{-}1^+$ cells was higher in CY-MDSC after cultivation with IFNγ when compared with TU-MDSC. However, no significant difference in the expression of F4/80, CD11c, CD80, and CD86 was found. As expected, the relative expression of immunosuppressive genes for iNOS, ROS, and VEGF was significantly increased in both CY-MDSC and TU-MDSC. It is interesting to note that, the relative expression of the iNOS gene was higher in CY-MDSC after stimulation with IFNγ than in TU-MDSC. This functional analysis revealed that CY-MDSC behavior reflected the higher percentage of MO-like MDSC as iNOS induction is typical for MO-like MDSC. This is in

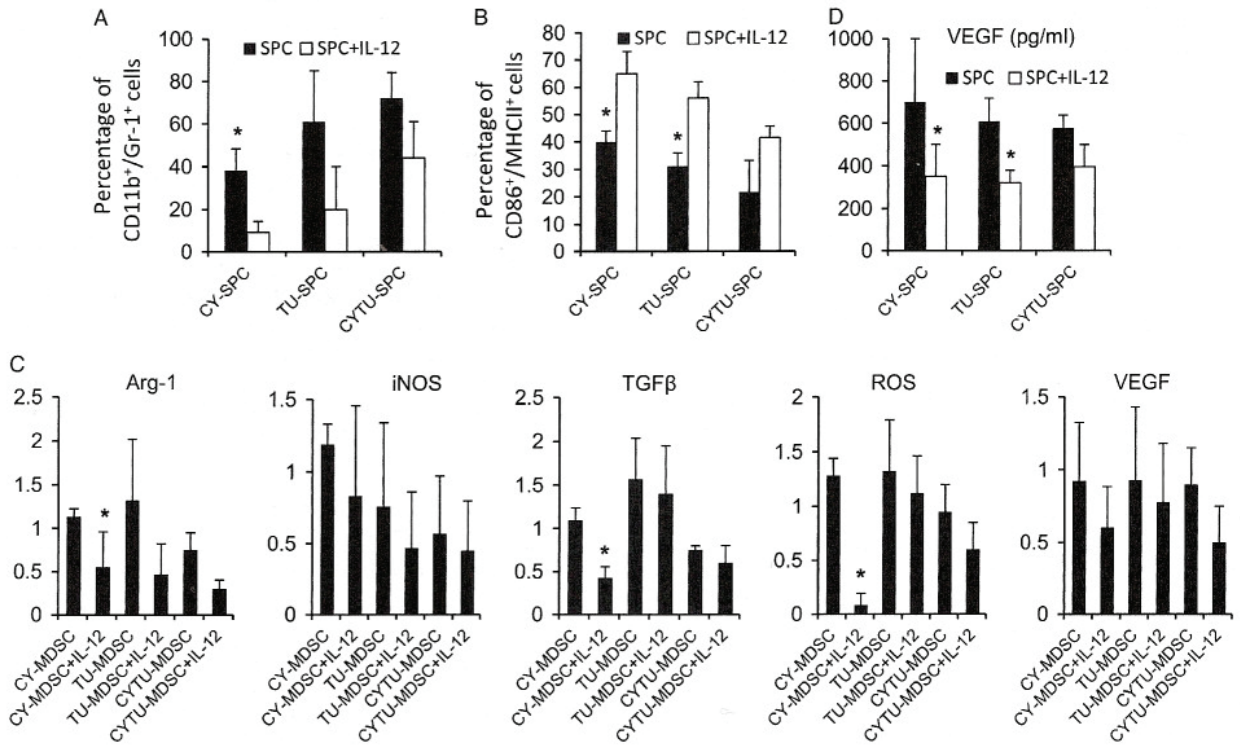


FIGURE 6. Spleen cells from mice after cyclophosphamide (CY) treatment, from mice with TC-1 tumors, and from mice with CY-treated TC-1 tumors, and their cultivation with recombinant interluekin (rIL)-12. A, Percentage of $CD11b^+/Gr\text{-}1^+$ cells after spleen-cell (SPC) cultivation with (white bars) or without rIL-12 (black bars) (FACS; $^*P<0.05$, n=6-12, *t* test). B, Percentage of $CD86^+/MHCII^+$ cells after spleen-cell cultivation with (white bars) or without rIL-12 (black bars) (FACS; n=6-12, $^*P<0.05$, *t* test). C, Relative expression of genes for arginase 1 (Arg-1), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGFβ), reactive oxygen species (ROS), and vascular endothelial growth factor (VEGF) (quantitative reverse transcription-polymerase chain reaction; $^*P<0.05$, n=9, *t* test). D, VEGF production (enzyme-linked immunosorbent assay; $^*P<0.05$, n=3–12, *t* test).

agreement the finding of Angulo et al,[15] that the suppressive activity of MDSC induced by CY is mainly dependent on NO production. As we have found that CY-induced MDSC display more monocytic phenotype and that their phenotype including gene expression indicates their less suppressive function, we decided to compare the degree of the suppressive function of these 2 types of MDSC in vitro and in vivo. The ability of MDSC from tumor-bearing mice to suppress T-cell proliferation was described previously[5,6] and the suppressor activity of CY-induced MDSC was reported by Angulo and colleagues, but the degree of their suppressive function has not been compared yet. Our in vitro experiments indicated that tumor-induced MDSC suppressed T-cell proliferation in vitro more efficiently than CY-induced MDSC. When stimulated with IFNγ, the suppressive capacity of CY-MDSC increased but did not reach the suppressive capacity of TU-MDSC. The less immunosuppressive capacity found in vitro corresponds to the more differentiated (monocytic) phenotype and lower expression of immunosuppressive genes. Despite the difference in the immunosuppressive capacity detected in vitro, both CY-MDSC and TU-MDSC significantly accelerated the growth of TC-1 tumors in vivo. As the suppressive capacity of MDSC can be induced by IFNγ and perhaps also by many other stimuli,[39] we can understand that the immunosuppressive capacity of CY and tumor-induced MDSC can be, after induction in the organism, comparable.

We have also analyzed MDSC from TC-1 tumor-bearing mice treated with CY. This setting imitates the situation when a cancer patient undergoes CY therapy. Our results show that spleens from mice bearing large TC-1 tumor treated with CY consist of higher percentage of MDSC when compared with CY-treated or TC-1 tumor-bearing mice. Thus, dual accumulation probably occurs in this setting. The phenotypic and functional analysis of the CYTU-MDSC group documents that these cells resemble a mixture of CY-MDSC and TU-MDSC, as their phenotype and function can be, in most experiments, placed between those of CY-MDSC and TU-MDSC.

As it has been shown that ATRA had a potent activity in the differentiation of MDSC from tumor-bearing mice in vitro,[21,22] we also evaluated the ability of ATRA to alter CY-induced MDSC. After in vitro ATRA treatment of CY-MDSC, the percentage of $CD11b^+/Gr\text{-}1^+$ cells decreased, together with the increase in F4/80 and CD11c expression accompanied by reduced levels of immunosuppressive genes including ROS. Nefedova et al[23] reported that ATRA-induced differentiation of tumor-induced MDSC primarily by neutralization of ROS and this differentiation was joined with decreased percentage of $CD11b^+/Gr\text{-}1^+$ cells and increased F4/80 and CD11c expression. On the basis of the successful results in vitro, we performed additional experiments to find out whether MDSC can also be modulated in vivo by ATRA administration after CY injection. Spleens of mice administered

with CY and subsequently with ATRA contained lower percentages of CD11b$^+$/Gr-1$^+$ cells when compared with CY-only–treated mice. This decrease was accompanied by the reduction of relative expression of immunosuppressive genes and lower VEGF production. Previous reports have shown that treatment of cancer patients and tumor-bearing mice with ATRA resulted in a substantial decrease of these cells and improvement of immune responses.[24,25] Our results demonstrated for the first time that CY-induced MDSC were also susceptible to the modulation with ATRA. It was shown recently that ATRA was able to restore antitumor immune response of HPV therapeutic vaccine in large TC-1 tumors by the suppression of MDSC and Tregs.[42,43] Thus, we performed experiments investigating whether ATRA can similarly improve the antitumor effect of CY. The chemotherapy with CY resulted in strong tumor-inhibitory effect and the subsequent therapy with ATRA significantly enhanced the observed antitumor effects of CY. These results supported our idea that ATRA could be a suitable agent for combined chemoimmunotherapy.

As we have documented previously,[33] immunotherapy with IL-12 was effective for the treatment of TC-1 tumors after chemotherapy with a CY derivative, Racemic chlorobromofosfamide-4A. We have also reported that strong accumulation of MDSC observed in the spleens of mice after chemotherapy was abolished by adjuvant IL-12 vaccination.[34] These findings were corroborated by other reports.[44–46] Medina-Echeverz et al[46] showed that combination of CY and IL-12 eliminated intratumoral MDSC. Although the antitumor effect of IL-12 has been extensively studied during the past decades, the direct effect of IL-12 on MDSC has not been fully determined yet. Steding et al[31] showed that IL-12 induced in vivo and in vitro maturation of MDSC and decreased levels of NOS and IFNγ RNA. In this paper, we have focused on the in vitro analysis of IL-12 effect on the spleen cells from CY-treated animals. We have found that after long-term in vitro cultivation of spleen cells from CY-treated mice or TC-1 tumor-bearing in the presence of IL-12, decrease in percentage of CD11b$^+$/Gr-1$^+$ cells accompanied by the increase in percentage of CD86$^+$/MHCII$^+$ cells was observed. Both types of spleen MDSC cultivated in vitro in the presence of IL-12 exhibited also reduced levels of immunosuppressive genes together with significant decrease in VEGF production. Spleen cells from TC-1 tumor-bearing mice treated with CY were also analyzed and a similar modulatory effect as in CY-MDSC or TU-MDSC was found. As MDSC in our mouse model (C57Bl/6) do not have receptor for IL-12, indirect effect of IL-12 must be considered. As MDSC (CD11b$^+$ cells) were separated during cultivation from the rest of spleen cells (CD11b$^-$ cells) using Transwell inserts, the modulatory effect of IL-12 could not be generated by cell-to-cell contact mechanisms, but rather mediated by cytokines produced by CD11b$^-$ spleen cells. However, precise indirect mechanism of the IL-12 effect on MDSC should be clarified.

Collectively, in this paper we have documented that MDSC accumulating after CY administration have the suppressive ability but they are distinct when compared with TC-1 tumor-induced MDSC. We have also demonstrated that CY-induced MDSC could be a suitable target for immunotherapeutic approaches and that the modulation of MDSC (either with ATRA or IL-12) during chemotherapy of tumors can enhance the antitumor effect of the applied chemotherapeutic agent. These findings may have important implications for the development of immunotherapeutic strategies.

ACKNOWLEDGMENTS

The authors are grateful to Mrs Renáta Turečková for skillful technical assistance and to Dr Šárka Takáčová for editorial help.

CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

Supported by grants from the Czech Science Foundation Nos. GAP301/11/P220, GA301/09/1024, GA301/07/1410, by grant of the Clinigene project EU-FP6-NOE No. 018933, and, in part, by Grant No. AV0Z50520514 awarded by the Academy of Sciences of the Czech Republic. PhD student (Veronika Polláková) was supported in part by the Faculty of Science, Charles University, Prague.

All authors have declared there are no financial conflicts of interest in regards to this work.

REFERENCES

1. Gabrilovich DI, Bronte V, Chen SH, et al. The terminology issue for myeloid-derived suppressor cells. *Cancer Res.* 2007; 67:425.
2. Bronte V, Apolloni E, Cabrelle A, et al. Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. *Blood.* 2000;96: 3838–3846.
3. Kusmartsev S, Gabrilovich DI. Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J Leukoc Biol.* 2003;74:186–196.
4. Serafini P, De Santo C, Marigo I, et al. Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol Immunother.* 2004;53:64–72.
5. Bronte V, Serafini P, Apolloni E, et al. Tumor-induced immune dysfunctions caused by myeloid suppressor cells. *J Immunother.* 2001;24:431–446.
6. Huang B, Pan PY, Li Q, et al. Gr-1 + CD115 + immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res.* 2006;66:1123–1131.
7. Suzuki E, Kapoor V, Jassar AS, et al. Gemcitabine selectively eliminates splenic gr-1 + /CD11b + myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res.* 2005;11:6713–6721.
8. Kusmartsev S, Gabrilovich DI. Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer. *Cancer Metastasis Rev.* 2006;25:323–331.
9. Emadi A, Jones RJ, Brodsky RA. Cyclophosphamide and cancer: golden anniversary. *Nat Rev Clin Oncol.* 2009;611:638–647.
10. Lake RA, Robinson BWS. Immunotherapy and chemotherapy—a practical partnership. *Nat Rev Cancer.* 2005;5:397–405.
11. Bass KK, Mastrangelo MJ. Immunopotentiation with low-dose cyclophosphamide in the active specific immunotherapy of cancer. *Cancer Immunol Immunother.* 1998;47:1–12.
12. Wada S, Yoshimura K, Hipkiss EL, et al. Cyclophosphamide augments antitumor immunity: studies in an autochthonous prostate cancer model. *Cancer Res.* 2009;69:4309–4318.
13. Shimizu J, Yamasaki S, Sakaguchi S. Induction of tumor immunity by removing CD25 + CD4 + T cells: a common basis between tumor immunity and autoimmunity. *J Immunol.* 1999;163:5211–5218.
14. Motoyoshi Y, Kaminoda K, Saitoh O, et al. Different mechanisms for anti-tumor effects of low- and high-dose cyclophosphamide. *Oncol Rep.* 2006;16:141–146.
15. Angulo I, de las Heras FG, García-Bustos JF, et al. Nitric oxide-producing CD11b(+)Ly-6G(Gr-1)(+)CD31(ER-MP12)(+) cells in the spleen of cyclophosphamide-treated mice: implications for T-cell responses in immunosuppressed mice. *Blood.* 2000;95:212–220.

16. Peláez B, Campillo JA, López-Asenjo JA, et al. Cyclophosphamide induces the development of early myeloid cells suppressing tumor cell growth by a nitric oxide-dependent mechanism. *Immunology*. 2001;166:6608–6615.
17. Diaz-Montero CM, Salem ML, Nishimura MI, et al. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol Immunother*. 2009;58:49–59.
18. Shimizu J, Yamasaki S, Takahashi T, et al. Stimulation of CD25 + CD4 + regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol*. 2002;3:135–142.
19. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10: 942–949.
20. Indrová M, Bieblová J, Rossowska J, et al. HPV 16-associated tumours IL-12 can repair the absence of cytotoxic and proliferative response of tumour infiltrating cells after chemotherapy. *Int J Oncol*. 2009;34:173–179.
21. Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients. A mechanism of immunosuppression in cancer. *J Immunol*. 2001;166:678–689.
22. Gabrilovich DI, Velders M, Sotomayor E, et al. Mechanism of immune dysfunction in cancer mediated by immature Gr-1 + myeloid cells. *J Immunol*. 2001;166:5398–5406.
23. Nefedova Y, Fishman M, Sherman S, et al. Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. *Cancer Res*. 2007;67:11021–11028.
24. Kusmartsev S, Cheng F, Yu B, et al. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res*. 2003; 63:4441–4449.
25. Mirza N, Fishman M, Fricke I, et al. All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res*. 2006;66:9299–9307.
26. Gervais A, Leveque J, Bouet-Toussaint F, et al. Dendritic cells are defective in breast cancer patients: a potential role for polyamine in this immunodeficiency. *Breast Cancer Res*. 2005;7:326–335.
27. Brunda MJ, Luistro L, Rumennik L, et al. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med*. 1993;4:1223–1230.
28. Nanni P, Rossi I, De Giovanni C, et al. Interleukin 12 gene therapy of MHC-negative murine melanoma metastases. *Cancer Res*. 1998;58:1225–1230.
29. Vagliani M, Rodolfo M, Cavallo F, et al. Interleukin 12 potentiates the curative effect of a vaccine based on interleukin 2-transduced tumor cells. *Cancer Res*. 1996;56:467–470.
30. Cavallo F, Signorelli P, Giovarelli M, et al. Antitumor efficacy of adenocarcinoma cells engineered to produce interleukin 12 (IL-12) or other cytokines compared with exogenous IL-12. *J Natl Cancer Inst*. 1997;89:1049–1058.
31. Steding CE, Wu ST, Zhang Y, et al. The role of interleukin-12 on modulating myeloid-derived suppressor cells, increasing overall survival and reducing metastasis. *Immunology*. 2011;133:221–238.
32. Indrová M, Bubeník J, Mikyšková R, et al. Chemoimmunotherapy in mice carrying HPV-16 associated, MHC class I and MHC class I tumours: effects of CBM-4A potentiated with IL-2, IL-12, GM-CSF and genetically modified tumour vaccines. *Int J Oncol*. 2003;22:691–695.
33. Indrová M, Bieblova J, Jandlová T, et al. Chemotherapy, IL-12 gene therapy and combined adjuvant therapy of HPV16-associated MHC class I-proficient and -deficient tumours. *Int J Oncol*. 2006;28:253–259.
34. Indrová M, Bieblová J, Bubeník J, et al. IL-12 immunotherapy of minimal residual disease in murine models of HPV16-associated tumours: induction of immune responses, cytokine production and kinetics of immune cell subsets. *Int J Oncol*. 2008;32:499–507.
35. Mikyšková R, Bubeník J, Mendoza L, et al. Local cytokine treatment of HPV16-associated tumours results in inhibition of their lung metastases. *Clin Exp Metastasis*. 2000;18:581–587.
36. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res*. 1996;56:21–26.
37. Manning J, Indrová M, Lubyová B, et al. Induction of MHC class I molecule cell surface expression and epigenetic activation of antigen-processing machinery components in a murine model for human papilloma virus 16-associated tumours. *Immunology*. 2008;123:218–227.
38. Youn JI, Gabrilovich DI. The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. *Eur J Immunol*. 2010;401: 2969–2975.
39. Greifenberg V, Ribechini E, Rössner S, et al. Myeloid-derived suppressor cell activation by combined LPS and IFN-gamma treatment impairs DC development. *Eur J Immunol*. 2009;10: 2865–2876.
40. Youn JI, Nagaraj S, Collazo M, et al. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol*. 2008;181:5791–5802.
41. Movahedi K, Guilliams M, Van den Bossche J, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood*. 2008;111:4233–4244.
42. Song X, Ye D, Liu B, et al. Combination of all-trans retinoic acid and a human papillomavirus therapeutic vaccine suppresses the number and function of immature myeloid cells and enhances antitumor immunity. *Cancer Sci*. 2009;100:334–340.
43. Song X, Guo W, Cui J, et al. A tritherapy combination of a fusion protein vaccine with immune-modulating doses of sequential chemotherapies in an optimized regimen completely eradicates large tumors in mice. *Int J Cancer*. 2011;128: 1129–1138.
44. Malvicini M, Rizzo M, Alaniz L, et al. A novel synergistic combination of cyclophosphamide and gene transfer of interleukin-12 eradicates colorectal carcinoma in mice. *Clin Cancer Res*. 2009;15:7256–7265.
45. Malvicini M, Ingolotti M, Piccioni F, et al. Reversal of gastrointestinal carcinoma-induced immunosuppression and induction of antitumoural immunity by a combination of cyclophosphamide and gene transfer of IL-12. *Mol Oncol*. 2011;5:242–255.
46. Medina-Echeverz J, Fioravanti J, Zabala M, et al. Successful colon cancer eradication after chemoimmunotherapy is associated with profound phenotypic change of intratumoral myeloid cells. *J Immunol*. 2011;186:807–815.

6.4. Publikácia IV

DNA demetylačné činidlo 5-azacytidin inhibuje myeloidné supresorové bunky akumulované počas rastu nádoru a po liečbe cyklofosfamidom.

DNA demethylating agent 5-azacytidine inhibits myeloid-derived suppressor cells induced by tumor growth and cyclophosphamide treatment.

Romana Mikyšková, Marie Indrová, Veronika Vlková, Jana Bieblová, Jana Šímová, Zuzana Paračková, Elzbieta Pajtasz-Piasecka, Joanna Rossowska a Milan Reiniš

Myeloidné supresorové bunky sú schopné sprostredkovať imunosupresiu. Tieto bunky sa akumulujú v mikroprostredí nádoru, v lymfoidných orgánoch a krvi počas rastu nádoru. Ich mobilizácia bola tiež popísaná pri terapii s cyklofosfamidom. Bolo popísané, že MDSC indukujú poruchy T buniek cez produkciu napríklad TGF-β, ROS, NO a najmä Arg-1 (Kusmartsev and Gabrilovich, 2006). Arg-1 je marker imunosupresívneho prostredia a hlavným producentom Arg-1 v mikroprostredí nádoru sú MDSC. Touto zvýšenou expresiou Arg-1 indukujú anergiu T-buniek depléciou L-arginínu, čo narúša proliferáciu T buniek a produkciu cytokínov (Rodriguez et al., 2007). Inhibícia Arg-1 môže znovu obnoviť správnu funkciu T buniek a indukovať protinádorovú odpoveď (Rodriguez et al., 2004). 5-azacytidin je inhibítor DNA metyltransferáz a zaraďuje sa medzi protinádorové činidlá. V predkladanej práci sme skúmali efekt 5-azacytidinu na MDSC akumulované v mikroprostredí nádoru a slezine počas rastu nádoru a terapie nádorov TRAMP-C2 a TC-1/A9 s cyklofosfamidom. Percento MDSC kleslo v mikroprostredí nádoru a v slezinách myší s nádorom TRAMP-C2 a TC-1/A9 po ošetrení 5-azacytidinom. Zmeny boli asociované s nižšou expresiou Arg-1 v mikroprostredí nádoru a slezinách myší s nádorom TRAMP-C2 a TC-1/A9. Ošetrenie nádorov CY zapríčinilo ďalšiu akumuláciu MDSC v mikroprostredí nádoru a slezine. Táto akumulácia bola inhibovaná ošetrením 5-azacytidinom. Kombinácia CY a 5-azacytidinu viedla ku zvýšenej inhibícii rastu nádoru. *In vitro* kultivácia MDSC izolovaných zo slezín cyklofosfamidom ošetrených myší alebo izolovaných zo slezín myší s nádorom v prítomnosti 5-azacytidinu znižovala percento MDSC, čo bolo asociované s nižšou expresiou Arg-1, nižšou produkciou VEGF a nižšou supresívnou kapacitou. Naše výsledky naznačujú, že 5-azacytidin okrem priameho protinádorového efektu, znižuje percento MDSC akumulovaných v mikroprostredí nádoru a slezine počas rastu nádoru a chemoterapie s cyklofosfamidom. Táto skutočnosť môže byť prospešná pre výsledok danej chemoterapie.

Prínos autora dizertačnej práce k danej publikácii:

Mojou úlohou pri vypracovaní predkladanej publikácie bolo monitorovanie relatívnej expresie Arg-1 akumulovanej v mikroprostredí nádoru a slezine po terapii s CY a u myeloidných supresorových buniek akumulovaných v mikroprostredí nádoru a slezine počas rastu nádoru TC-1/A9 a TRAMP-C2. Ďalšou úlohou bolo zistiť účinok terapie s epigenetickým činidlom 5-azacytidinom na relatívnu expresiu Arg-1 myeloidnými supresorovými bunkami v mikroprostredí nádoru a slezine po terapii s CY a myeloidnými supresorovými bunkami akumulovanými v mikroprostredí nádoru a slezine počas rastu nádoru TC-1/A9 a TRAMP-C2. Tiež som sledovala zmeny v relatívnej expresii Arg-1 po *in vitro* kultivácii TU-MDSC a CY-MDSC s 5-azacytidinom.

Epub ahead of print January 3, 2014 - doi:10.1189/jlb.0813435

JLB

Article

DNA demethylating agent 5-azacytidine inhibits myeloid-derived suppressor cells induced by tumor growth and cyclophosphamide treatment

Romana Mikyšková,[,1] Marie Indrová,*[,1] Veronika Vlková,* Jana Bieblová,* Jana Šímová,* Zuzana Paračková,* Elzbieta Pajtasz-Piasecka,[†] Joanna Rossowska,[†] and Milan Reiniš*[,2]*

*Department of Tumor Imunology, Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, Prague, Czech Republic; and [†]Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland

RECEIVED AUGUST 9, 2013; REVISED NOVEMBER 27, 2013; ACCEPTED DECEMBER 13, 2013. DOI: 10.1189/jlb.0813435

ABSTRACT

MDSCs represent one of the key players mediating immunosuppression. These cells accumulate in the TME, lymphoid organs, and blood during tumor growth. Their mobilization was also reported after CY therapy. DNMTi 5AC has been intensively studied as an antitumor agent. In this study, we examined, using two different murine tumor models, the modulatory effects of 5AC on TU-MDSCs and CY-MDSCs tumor growth and CY therapy. Indeed, the percentage of MDSCs in the TME and spleens of 5AC-treated mice bearing TRAMP-C2 or TC-1/A9 tumors was found decreased. The changes in the MDSC percentage were accompanied by a decrease in the Arg-1 gene expression, both in the TME and spleens. CY treatment of the tumors resulted in additional MDSC accumulation in the TME and spleens. This accumulation was subsequently inhibited by 5AC treatment. A combination of CY with 5AC led to the highest tumor growth inhibition. Furthermore, in vitro cultivation of spleen MDSCs in the presence of 5AC reduced the percentage of MDSCs. This reduction was associated with an increased percentage of CD11c$^+$ and CD86$^+$/MHCII$^+$ cells. The observed modulatory effect on MDSCs correlated with a reduction of the Arg-1 gene expression, VEGF production, and loss of suppressive capacity. Similar, albeit weaker effects were observed when MDSCs from the spleens of tumor-bearing animals were cultivated with 5AC. Our findings indicate that beside the direct antitumor effect, 5AC can reduce the percentage of MDSCs accumulating in the TME and spleens during tumor growth and CY chemotherapy, which can be beneficial for the outcome of cancer therapy. *J. Leukoc. Biol.* 95: 000–000; 2014.

Introduction

Tumor progression is supported by chronic inflammatory conditions that developed in the TME and is characterized by long-term secretion of various inflammatory soluble factors (especially GM-CSF, G-CSF, IL-6, IL-10, VEGF, IL-1β, or others) and leukocyte infiltration. Among leukocyte-infiltrating tumors, MDSCs represent one of the key players mediating immunosuppression [1–4]. MDSCs are a heterogeneous population of undifferentiated cells characterized in mice by markers of monocytes (CD11b) and neutrophils (Gr-1). Recently, two main different subsets of MDSCs—monocytic (Ly6G$^-$Ly6C^{High}) and granulocytic (Ly6G$^+$Ly6C^{Low})—have been described [5, 6]. The main feature of these cells is their ability to suppress T cell responses in antigen-specific or nonspecific manners [7] and NK cells. Several mechanisms responsible for the MDSC-mediated immunosuppression have been described. MDSCs have been linked to the induction of T cell dysfunction through production of TGF-β, ROS, NO, and especially Arg-1 [8]. Both granulocytic and monocytic MDSCs express high levels of Arg-1 and thereby, induce T cell anergy by depleting L-arginine, which impairs T cell proliferation and cytokine production [9]. Increased numbers of MDSCs in the peripheral blood of renal cell carcinoma patients correlated with low L-arginine and high ornithine levels in plasma and profound T cell dysfunction [10]. Inhibition of Arg-1 restores T cell function in vitro and induces an antitumor response in vivo [11].

Abbreviations: 5AC=5-azacytidine, Arg-1=arginase 1, ATCC=American Type Culture Collection, CY=cyclophosphamide, CY-MDSC=cyclophosphamide-induced myeloid-derived suppressor cells, DAC=2′-deoxy-5-azacytidine, DNMTi=DNA methyltransferase inhibitor, Gr-1=granulocyte receptor-1, HPV=human papillomavirus, MDSC=myeloid-derived suppressor cell, qRT-PCR=quantitative RT-PCR, TIL=tumor-infiltrating cell, TME=tumor microenvironment, TRAMP-C2=transgenic adenocarcinoma mouse prostate C2, Treg=regulatory T cell, TU-MDSC=tumor-induced myeloid-derived suppressor cell

1. These authors contributed equally to this work.
2. Correspondence: Department of Tumor Imunology, Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, v. v. i., Vídeňská 1083, Prague 4, 142 20, Czech Republic. E-mail: milan.reinis@img.cas.cz

In healthy mice, $CD11b^+/Gr\text{-}1^+$ cells can be detected in sizeable numbers in the bone marrow; a small number of these cells (<4%) can be found in the blood and spleen [12]. Disturbances in cytokine homeostasis induced by tumor growth, infection, inflammation, or stress reaction can alter the equilibrium of this population, leading to its accumulation in lymphoid organs and blood [13]. It has been suggested that different protumorigenic and proinflammatory cytokines, such as G-CSF, GM-CSF, IL-6, IL-10, IL-1, and VEGF, can mobilize MDSC recruitment from bone marrow hematopoietic precursors and induce their immunosuppressive phenotype [2].

MDSC mobilization was also reported upon administration of DNA-alkylating agent CY [14–17]. CY is a widely used antineoplastic drug, used alone or in combination with other products [18]. We have reported recently that although CY-MDSCs and TU-MDSCs accelerated tumor growth, their phenotype and function differed. Compared with TU-MDSC, the CY-MDSC subpopulation consisted particularly of monocyte-like cells; it was accompanied by lower Arg-1 gene expression and lower T cell proliferation suppression. The phenotype and function of MDSCs obtained from mice bearing TC-1 tumors treated with CY were, in general, found to lie between CY-MDSCs and TU-MDSCs [17].

As MDSCs were identified as key players mediating immunosuppression, several pharmacological approaches, which involve their elimination or modulation, are currently being explored in tumor-bearing hosts [19]. Among them, a promising approach in cancer immunotherapy targeting MDSCs is to promote their differentiation into mature myeloid cells that no longer possess the suppressive activity.

DNMTi are cytostatic agents with epigenetic effects; among them, the most widely used are 5AC or DAC. They have emerged recently as potent therapies for the preleukemic hematological disease, myelodysplastic syndrome, and for established leukemia [20, 21], leading to U.S. Food and Drug Administration approval for patients with myelodysplastic syndrome [22]. They represent a promising group of anti-tumor therapeutics that display pleiotropic activities as a result of their ability to induce reexpression of genes down-regulated by DNA methylation. Beside direct antitumor effects, epigenetic agents can also influence tumor cell interactions with the immune system [23]. It has been shown that treatment with 5AC restores expression of the MHC class I molecules on MHC class I-deficient tumor cells and renders the tumor cells susceptible to the attack of cytotoxic T lymphocytes [24, 25]. In general, DNMTi therapy can display not only primary effects on tumor cells but also positive effects on antitumor immune response and resulting immunotherapy [26, 27]. Furthermore, the effects of 5AC on murine bone marrow-derived and human monocyte-derived DC maturation were also documented in our laboratory [28]. Notably, the most striking effect was inhibition of cytokine production, a phenomenon described previously for the histone deacetylase inhibitors [29].

Several chemotherapeutic agents have been described to affect MDSC cellularity and activity [19, 30–33]. However, the effects of epigenetic agents on MDSCs have not been studied in detail. So far, it has been demonstrated that tumor-infiltrated CD11b myeloid cells cultured in vitro can be differentiated in the presence of the DNA-demethylating agent DAC into mature tumor-derived APCs [34]. In another recent paper, low-dose DAC decreased pro-tumor MDSCs in mice bearing B16 tumors [35]. On the other hand, epigenetic agent Trichostatin A increased the number of $CD11b^+/Gr\text{-}1^+$ cells in bone marrow and spleens [36]. In this study, we examined the modulatory effects of 5AC on MDSCs accumulating in the TME and spleens during tumor growth and CY therapy of TC-1/A9 and TRAMP-C2 tumors. Indeed, the percentage and activity of MDSCs in the TME and spleens of mice bearing TRAMP-C2 or TC-1/A9 tumors treated with 5AC or a subsequent CY and 5AC combination were found decreased. The modulatory effect of 5AC was also verified by cultivation of isolated MDSCs from the spleens of CY-treated or tumor-bearing mice. After in vitro cultivation of CY-MDSCs in the presence of 5AC, the percentage and activity of MDSCs were lower. Similar but weaker effects were observed when MDSCs from the spleens of tumor-bearing animals were cultivated with 5AC.

MATERIALS AND METHODS

Mice

C57BL/6 male mice, 6–8 weeks old, were obtained from AnLab (Prague, Czech Republic). Experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics (Prague, Czech Republic).

Tumor cell lines

The TC-1 tumor cell line (obtained from ATCC, Manassas, VA, USA) was developed by cotransfection of murine C57BL/6 lung cells with HPV16 E6/E7 genes and activated (G12V) Ha-ras plasmid DNA [37]. TC-1/A9 cells, a MHC class I-deficient variant of TC-1 tumor cells, were obtained after transplantation of TC-1 cells into a DNA vaccine-immunized mouse [38]. TRAMP-C2 tumor cells (obtained from ATCC), MHC class I-deficient, were established from a heterogeneous, 32-week tumor of the TRAMP model [39]. TC-1/A9 and TC-1 cells were maintained in RPMI-1640 medium (Sigma-Aldrich GmbH, Steinheim, Germany), supplemented with 10% FCS (PAN Biotech GmbH, Aidenbach, Germany), 2 mM L-glutamine, and antibiotics; TRAMP-C2 cells were maintained in DMEM (Sigma-Aldrich), supplemented with 5% FCS, NuSerum IV (5%; BD Biosciences, Bedford, MA, USA), 0.005 mg/ml insulin (Sigma-Aldrich), DHEA (10 nM; Sigma-Aldrich), and antibiotics. Both cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Therapeutic experiments

The therapeutic schemes were designed for treatment of early growing or established tumors. In the early tumor therapy setting, TC-1/A9 (5×10^4 cells) or TRAMP-C2 (1×10^6 cells) tumor cells were s.c.-transplanted on Day 0. Starting on Day 1, 100 μg 5AC (Sigma-Aldrich) was administered repeatedly, twice/week, for 3 weeks in the vicinity of the tumor cell challenge site or peritumorally, when the growing tumors appeared. Mice were observed twice/week, and the size of the tumors was recorded.

For treatment of established tumors, TC-1/A9 (5×10^4 cells) or TRAMP-C2 (1×10^6 cells) tumor cells were s.c.-transplanted on Day 0. When the tumors reached ~0.5 cm in diameter, 100 μg 5AC was peritumorally administered repeatedly for 2 (TC-1/A9) or 3 weeks (TRAMP-C2), twice/week. Mice were observed twice/week, and the size of the tumors was recorded. Two days after the last 5AC treatment, mice (pool of three mice) from each group were killed, and their spleens and tumors were excised and used for analysis. The size of the tumors on the day of analysis was recorded.

To induce the model of established tumors treated with CY and 5AC (combined therapy), TC-1/A9 (5×10^4 cells) or TRAMP-C2 (1×10^6 cells) tumor cells were transplanted on Day 0. When the tumors reached the size of ~0.5 cm in diameter, mice received a single i.p. dose of CY (Endoxan; Baxter Oncology GmbH, Halle, Germany; 200 mg/kg) and then were peritumorally treated repeatedly every other day with 100 μg 5AC. Two days after the last 5AC (i.e., 7 days after CY) treatment, mice were killed; their spleens and tumors were excised and used for analysis. The size of the tumors on the day of analysis was recorded.

To investigate the direct effect of i.p. 5AC administration on spleen CY-MDSCs and TU-MDSCs in vivo, mice, 7 days after CY administration, or mice bearing large TC-1/A9 tumors were injected with one i.p. dose of 5AC (100 μg). FACS analysis was performed 72 h after 5AC administration.

TME analysis

The growing tumors were excised, and the tumor tissue was cut into small pieces and dissociated with the gentleMACS dissociator using the Tumor Dissociation Kit (Miltenyi Biotec, Auburn, CA, USA) and then by a protocol for preparation of single-cell suspensions, using gentleMACS C tubes (Miltenyi Biotec). The percentage of tumor-infiltrating MDSCs ($CD11b^+/Gr\text{-}1^+$) was analyzed by FACS analysis of $CD45^+$-gated TILs.

The relative expression of the Arg-1 gene was analyzed by qRT-PCR in tumor homogenates prepared with the gentleMACS dissociator using the protocol for homogenization of tissue for total RNA isolation by gentleMACS M tubes (Miltenyi Biotec).

Flow cytometry

To determine the percentage of $CD11b^+/Gr\text{-}1^+$ cells, FITC rat anti-mouse CD11b (M1/70) and allophycocyanin or PE rat anti-mouse Ly-6G and Ly-6C (Gr-1; RB6-8C5) were used. Expression of CD11c, MHC class II, CD86, and F4/80 was analyzed using the following antibodies: allophycocyanin anti-CD11c (HL3), FITC anti-MHCII (AF6-120.1), PE anti-CD86 (GL1; RB6-8C5), and PE anti-F4/80 (BM8). For analysis of TILs, allophycocyanin rat anti-mouse CD45 (30-F11) was used to set the CD45 gate. Relevant isotype controls, FITC, allophycocyanin, and PE-labeled antibody of irrelevant specificity were used. All products were purchased from BD PharMingen (San Diego, CA, USA). Apoptotic cells were determined by the Apoptosis Assay Kit-FITC (Exbio, Prague, Czech Republic). Dead cells were determined by PI (Sigma-Aldrich). FACS was performed using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo 7.6.5 software.

qRT-PCR

Total RNA was extracted with the High Pure RNA Isolation Kit (Roche, Basel, Switzerland). The amount of 1 μg RNA was reverse-transcribed to cDNA using random hexamer primers from the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA, USA) in a 20-μl reaction volume at 42°C for 30 min. Quantification of PCR products was performed in 10 μl LightCycler 480 SYBR Green I Master mix (Roche), using a real-time PCR LightCycler (Roche). DNA was denatured at 95°C for 2 min; 45 cycles of denaturation at 95°C for 25 s, annealing at 60°C for 45 s, elongation at 72°C for 1 min, and incubation at 80°C for 5 s followed. cDNAs were amplified with specific primers for β-actin and Arg-1, as described elsewhere [24].

The PCR primer sequences were as follows: Arg-1, 5′ CTCCAAGCCAAAGTCCTTAGAG (forward) and AGGAGCTGTCATTAGGGACATC (reverse); β-actin, 5′ CCAGAGCAAGAGAGGTATCC (forward) and GAGTCCATCACAATGCCTGT (reverse). Fold changes in transcript levels were calculated using comparative threshold values standardized to β-actin, used as the endogenous reference gene control. The relative expression of the Arg-1 gene in the control groups was normalized to one. All samples were run in biological triplicates.

Proliferation assay

For proliferation assay, autologous splenocytes were resuspended at the concentration of 10^7 cells/ml in PBS, supplemented with 5% FCS, and labeled with 2.5 μM CFSE (Sigma-Aldrich) by incubation for 5 min at 37°C in 5% CO_2. Labeling was quenched with cold RPMI-1640 medium and supplemented with 10% FCS, and the cells were washed. After CFSE staining, splenocytes were stimulated with 1.0 μg/ml anti-CD3 (clone 145-2C11) and 1.0 μg/ml anti-CD28 (clone 37.51) antibodies (BD PharMingen) and cultured alone or admixed with TU-MDSCs or CY-MDSCs, cultivated previously alone or with 5AC for 4 days. Cells were gated for $CD4^+$ and $CD8^+$ populations using PE anti-CD4 (GK1.5) and PE anti-CD8 (53-6.7) antibodies (BD PharMingen), and CFSE dilution was analyzed by flow cytometry.

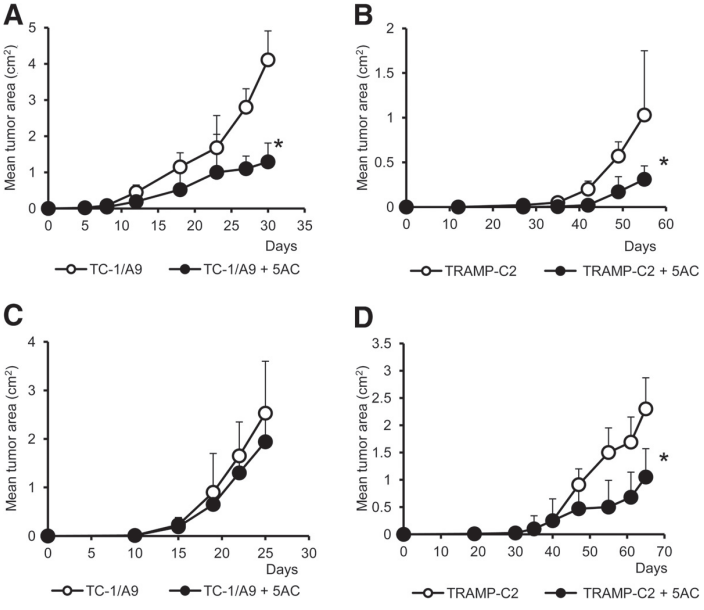


Figure 1. Tumor inhibitory effect of 5AC on early and established TC-1/A9 and TRAMP-C2 tumors. (A) TC-1/A9 and (B) TRAMP-C2 tumor cells were transplanted s.c. on Day 0. Starting on Day 1, 100 μg 5AC was administered repeatedly, twice/week for 3 weeks. Significant inhibition of tumor growth (*$P\leq0.05$, determined by ANOVA) was observed in TC-1/A9 and TRAMP-C2 early tumors treated with 5AC compared with untreated controls. Eight mice/group were used for the experiments. (C) TC-1/A9 and (D) TRAMP-C2 tumors cells were transplanted on Day 0. When the tumors reached ~0.5 cm in diameter, 100 μg 5AC was repeatedly, peritumorally administered for 2 (TC-1/A9) or 3 weeks (TRAMP-C2), twice/week. Significant inhibition of tumor growth was observed only in the case of established TRAMP-C2 tumors treated with 5AC compared with untreated controls. Six mice/group were used for the experiments. All data are representative of $n = 3$ independent experiments.

Immunohistochemical analyses

For detection of Gr-1$^+$ cells, tumor cryosections were fixed in 4% PFA and incubated overnight with rat anti-Gr-1 antibodies (BD PharMingen). The slides were subsequently washed with PBS and incubated (1 h) with goat anti-rat antibody, conjugated with Alexa Fluor 488 (Molecular Probes, Life Technologies, Carlsbad, CA, USA). Thereafter, slides were washed and counterstained with PI (2 μg/ml). The fluorophore-labeled tissue sections were analyzed using a Bio-Rad MRC 1024 scanning confocal fluorescence microscope, equipped with LaserSharp software. Cryosections from three distinct parts of a tumor from each individual mouse were analyzed. The intensity of Gr-1$^+$ cell influx into tumor tissue was presented using the scale + to +++++, where + is very few lymphocytes in tumor tissue, and +++++ is the most intensive infiltration [40, 41].

Modulation of MDSCs with 5AC in vitro

CD11b$^+$ cells from the spleens of CY-treated (CY-MDSC) or TC-1 tumor-bearing (~2 cm in diameter; TU-MDSC) mice were isolated using anti-mouse CD11b (macrophage-1α) antibodies (Miltenyi Biotec) conjugated to magnetic beads, in accordance with the manufacturer's instructions, as described previously [17]. Cell separation was performed with autoMACS (Miltenyi Biotec). The purity of cells was verified by FACS, and the percentage of CD11b$^+$/Gr-1$^+$ cells achieved 86–91%. CY-MDSCs and TU-MDSCs were cultivated in vitro for 4 days in the presence of 3 μM 5AC and 10 ng/ml GM-CSF (R&D Systems, Minneapolis, MN, USA) in ultralow cluster six-well plates (Corning Costar, Corning, NY, USA). Control samples were cultivated without the presence of 5AC. After cultivation, cells were analyzed by FACS for the percentage of CD11b$^+$/Gr-1$^+$, CD11c$^+$, F4/80$^+$, and CD86$^+$/MHCII$^+$ cells. Relative expression of the Arg-1 gene was analyzed by qRT-PCR. The percentage of apoptotic cells was analyzed by FACS. VEGF production in supernatants from cultured cells was analyzed by ELISA. Cells were also used in a CFSE proliferation-based assay.

All cultivations of MDSCs were performed in RPMI-1640 medium, supplemented with 10% FCS, 2 mM L-glutamine, antibiotics, and 2-ME in a humidified atmosphere with 5% CO_2 at 37°C.

ELISA assay

For VEGF production, supernatants from cultivated cells were analyzed with ELISA kits (BD Biosciences), according to the manufacturer's instructions.

Statistical analyses

For statistical analyses of in vitro experiments, Student's *t*-test was used. For evaluation of in vivo experiments, ANOVA from the Number Cruncher Statistical System (Kaysville, UT, USA) statistical package was used. SDs are indicated in the figures.

RESULTS

5AC inhibits growth of early TC-1/A9 and TRAMP-C2 tumors but of TRAMP-C2-established tumors only

First, we demonstrated the anti-tumor effects of DNMTi 5AC using two etiologically different mouse tumor models: TC-1/A9 and TRAMP-C2. As expected, 5AC inhibited growth of TC-1/A9 and TRAMP-C2 tumors when the treatment started at early stages of tumor progression (Day 1 after tumor cell inoculation). Significant inhibition of tumor growth ($P<0.05$) was observed in TC-1/A9 and TRAMP-C2 early tumors treated with 5AC compared with untreated controls (**Fig. 1A** and **B**). In the second set of the experiments performed with established tumors, the treatment started when the tumors reached ~0.5 cm in diameter. A significant tumor inhibitory effect was observed only on TRAMP-C2 ($P<0.05$) but not on TC-1/A9 tumors (Fig. 1C and D). The TC-1/A9 data are in agreement with our previous experiments [25]. This therapeutic setting was

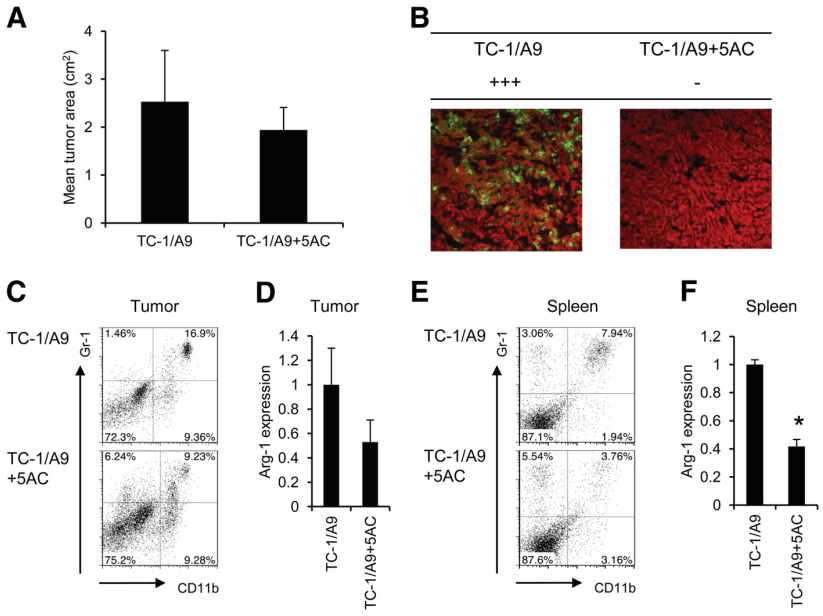


Figure 2. Peritumoral treatment of established TC-1/A9 tumors with 5AC led to inhibition of MDSCs in the TMEs and spleens of treated mice. Tumors and spleens from mice (pool of three mice from each group) with established TC-1/A9 tumors treated with 5AC in the experiment (Fig. 1C) were analyzed 24 h after the last 5AC injection. The size of tumors on the day of analysis is given (A). (B) Fluorescence staining of Gr-1$^+$ cells in the tumors revealed that although there was no tumor-inhibitory effect of 5AC, the number of Gr-1$^+$ cells in the TMEs of 5AC-treated mice decreased remarkably. The selected pictures show typically stained sections from the edge of the tumors. (C) FACS analysis of CD45$^+$-gated TILs showed a decreased percentage of CD11b$^+$/Gr-1$^+$ in TMEs of 5AC-treated mice accompanied by a nonsignificant decrease in the expression of the Arg-1 gene in TMEs (D). (E) Peritumoral treatment with 5AC led to a decrease in the percentage of CD11b$^+$/Gr-1$^+$ in the spleens of 5AC-treated mice associated with a significant (*$P<0.05$) decrease in the expression of the Arg-1 gene in the spleens (F). All data are representative of $n = 3$ independent experiments. Error bars indicate SD of triplicate wells. Differences were considered significant compared with untreated control (*$P<0.05$) using the Student's *t*-test.

used further in the experiments focused on the modulation of MDSCs accumulating in TMEs and in the spleens of mice bearing TC-1/A9 and TRAMP-C2 tumors.

Peritumoral treatment of established TC-1/A9 or TRAMP-C2 tumors with 5AC led to the inhibition of MDSCs in the TMEs and spleens of treated mice

Tumors and spleens explanted from mice with established TC-1/A9 tumors treated with 5AC, according to the aforementioned therapeutic setting (Fig. 1C), were analyzed 24 h after the last 5AC injection; the size of tumors on the day of analysis is given in **Fig. 2A**. Detection of Gr-1$^+$ cells was done in tumor cryosections to visualize these cells in the tumor tissue. Although no significant tumor-inhibitory effect was detected, fluorescence staining of Gr-1$^+$ cells in the tumors revealed a remarkable decrease of their percentage in the group of mice treated with 5AC (Fig. 2B). These results were in agreement with FACS analysis of CD45$^+$-gated TILs performed in parallel specimens. Tumors were cut into small pieces and dissociated with gentleMACS dissociator using the Tumor Dissociation Kit and protocol for preparation of single-cell suspensions. FACS analysis of CD45$^+$-gated TILs showed a decreased percentage of CD11b$^+$/Gr-1$^+$ MDSCs in TMEs of 5AC-treated mice compared with control, untreated mice (Fig. 2C). This effect was associated with a noticeable but not significant decrease in the total Arg-1 gene expression in TMEs (Fig. 2D). The expression of the Arg-1 gene was analyzed by qRT-PCR in tumor homogenates prepared with the gentleMACS dissociator using the protocol for homogenization of the tissue for total RNA isolation. To exclude the possibility that the changes in the Arg-1 gene expression could be generated by Arg-1 in tumor cells, the relative expression of the Arg-1 gene in tumor cells, cultured alone or in the medium supplemented with 5AC, was also tested but was negligible compared with that in the explanted tumor (data not shown). Peritumoral treatment with 5AC also led to a decreased percentage of CD11b$^+$/Gr-1$^+$ in the spleens (Fig. 2E) of 5AC-treated mice. These findings were accompanied by a significant ($P<0.05$) decrease in the expression of the Arg-1 gene in the spleens (Fig. 2F) compared with untreated mice. Similar analysis of TMEs and spleens was performed using TRAMP-C2 tumor cells (**Fig. 3**). The size of tumors on the day of analysis is given in Fig. 3A. Similarly, as in TC-1/A9 cells, fluorescence staining of Gr-1$^+$ cells in the tumors revealed a remarkable reduction of Gr-1$^+$ cells in the TMEs of 5AC-treated mice (Fig. 3B), which was confirmed by FACS analysis of CD45$^+$-gated TILs (Fig. 3C) and was associated with a noticeable but not significant decrease in the Arg-1 gene expression in TMEs (Fig. 3D). Peritumoral treatment with 5AC also led to reduction of the percentage of CD11b$^+$/Gr-1$^+$ cells, as well as to a significant ($P<0.05$) decrease in the Arg-1 gene expression in the spleens of 5AC-treated mice compared with the untreated controls (Figs. 3E and F). It is noteworthy that the accumulation of CD11b$^+$/Gr-1$^+$ cells in the spleens of mice bearing TRAMP-C2 tumors was not detected; their percentage was comparable with untreated, control, healthy mice (data not shown). Despite that, the percentage decreased after 5AC administration.

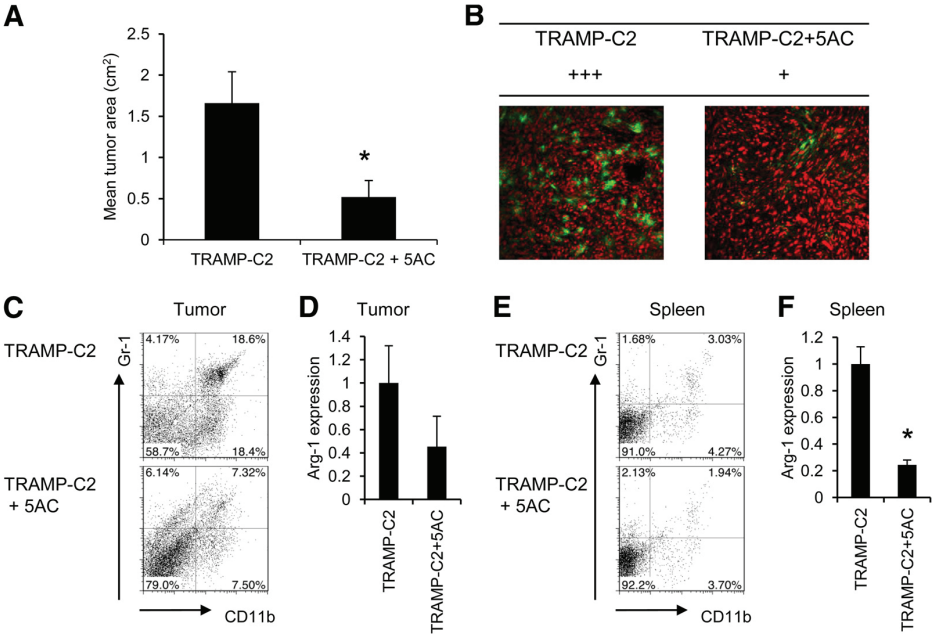


Figure 3. Peritumoral treatment of established TRAMP-C2 tumors with 5AC led to inhibition of MDSCs in the TMEs and spleens of treated mice. Tumors and spleens from mice (pool of three representative mice from each group) with established TRAMP-C2 treated with 5AC in the experiment (Fig. 1D) were analyzed 24 h after the last 5AC treatment. The size of tumors on the day of analysis is given (A). (B) Fluorescence staining of Gr-1$^+$ cells in the tumors revealed a remarkable decrease of Gr-1$^+$ cells in the TMEs of 5AC-treated mice. (C) FACS analysis of CD45$^+$-gated TILs showed a decreased percentage of CD11b$^+$/Gr-1$^+$ in the TMEs of 5AC-treated mice, accompanied by a nonsignificant decrease in the expression of the Arg-1 gene in TMEs (D). (E) Peritumoral treatment with 5AC also resulted in a decreased percentage of CD11b$^+$/Gr-1$^+$ cells in the spleens of 5AC-treated mice associated with a significant decrease in the Arg-1 expression in the spleens (F). All data are representative of $n = 3$ independent experiments. Error bars indicate SD of triplicate wells. Differences were considered significant compared with untreated control (*$P<0.05$) using the Student's t-test.

I.p. administration of 5AC decreased the percentage of CD11b$^+$/Gr-1$^+$ cells in the spleens of mice treated with CY or bearing TC-1/A9 tumors

To analyze the impact of the route of 5AC administration on the spleen CY-MDSCs or TU-MDSCs, mice were injected with a single i.p. dose of 5AC, 7 days after CY administration. In parallel, a single i.p. dose of 5AC was injected into mice bearing large TC-1/A9 tumors. FACS analysis showed that in both therapeutic settings, the percentage of CD11b$^+$/Gr-1$^+$ cells in the spleens of mice treated with one i.p. dose of 5AC decreased compared with the control group without 5AC (**Fig. 4**).

Peritumoral treatment of established TC-1/A9 and TRAMP-C2 tumors with 5AC led to reduced numbers of MDSCs accumulating in TMEs and spleens in CY-treated mice

We have documented previously that the percentage of MDSCs accumulating in the spleens during tumor growth can be increased additionally by CY administration [17]. Here, we show that the additional accumulation of MDSCs also occurs in the TMEs of growing TC-1/A9 and TRAMP-C2 tumors. CY administration increased the percentage of CD11b$^+$/Gr-1$^+$ cells and the relative expression of the Arg-1 gene (**Figs. 5D** and **6D**). To find out whether 5AC is able to inhibit the noticed, additional MDSC accumulation induced by CY treatment, we administered the CY-pretreated TC-1/A9- and TRAMP-C2-established tumors with 5AC. As shown in Fig. 5, treatment of TC-1/A9 tumors with a subsequent combination of CY + 5AC displayed the highest but not significant antitumor effect compared with monotherapies (Fig. 5A and B). Administration of CY resulted in an increase in the CD11b$^+$/Gr-1$^+$ cell percentage in the TMEs. This accumulation was inhibited by 5AC administration, as detected by FACS analysis (Fig. 5C). The effect was associated with similar changes in the Arg-1 gene expression detected by qRT-PCR (Fig. 5D). A situation analogical to TMEs was observed in the spleens (Figs. 5E and F). After CY administration, the percentage of CD11b$^+$/Gr-1$^+$ cells in the spleens of mice increased. This accumulation was inhibited by 5AC administration (Fig. 5E). The effect was associated with similar changes ($P<0.05$ compared with CY therapy only) in the Arg-1 gene expression detected by qRT-PCR (Fig. 5F). Parallel experiments combining CY + 5AC were also performed using the etiologically different TRAMP-C2 model. As can be seen in Fig. 6, treatment with a subsequent combination of CY + 5AC displayed a significant ($P<0.05$ compared with untreated control mice) anti-tumor effect when compared with untreated control mice (Fig. 6A and B). After CY administration, the percentage of CD11b$^+$/Gr-1$^+$ cells in the TME increased. This accumulation was inhibited by 5AC administration, as detected by FACS analysis (Fig. 6C) and was also accompanied by similar changes in the expression of the Arg-1 gene detected by qRT-PCR (Fig. 6D). Similarly, after CY administration, the CD11b$^+$/Gr-1$^+$ cell percentage in the spleens of mice increased. This accumulation was inhibited by 5AC administration (Fig. 6E), and the effect was associated with a significant ($P<0.05$ compared with CY therapy only) decrease in the Arg-1 gene expression detected by qRT-PCR (Fig. 6F).

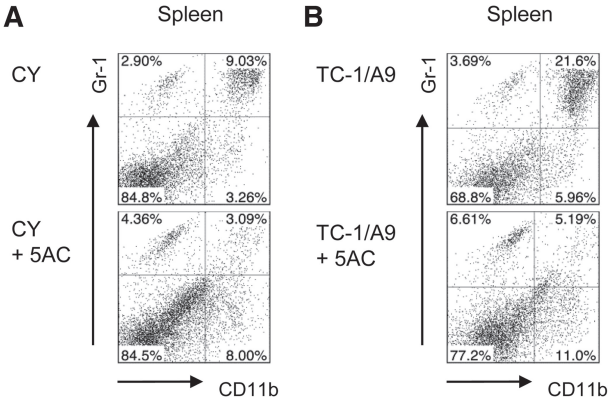


Figure 4. I.p. administration of 5AC decreased the percentage of CD11b$^+$/Gr-1$^+$ cells in the spleens of mice treated with CY or bearing TC-1/A9 tumors. (A) Mice, 7 days after CY administration (200 mg/kg i.p.), were injected with one i.p. dose of 5AC (100 μg). FACS analysis was performed 72 h after 5AC administration and showed that the percentage of CD11b$^+$/Gr-1$^+$ cells in the spleens of mice treated with one i.p. dose of 5AC decreased compared with the control group without 5AC (data are representative of $n=2$ independent experiments). (B) Mice bearing large TC-1/A9 tumors were injected with one i.p. dose of 5AC (100 μg). FACS analysis performed 72 h after 5AC administration showed the percentage of CD11b$^+$/Gr-1$^+$ cells in the spleens of mice treated with one i.p. dose of 5AC decreased compared with the control group without 5AC (data are representative of $n=2$ independent experiments).

Percentages of CY-MDSCs and TU-MDSCs decreased after their cultivation with 5AC in vitro

We investigated further the 5AC impact on MDSCs obtained by magnetic separation of CD11b$^+$ cells from the spleens of mice, 7 days after CY administration, or from the spleens of tumor-bearing mice. CY-MDSCs or TU-MDSCs were isolated by CD11b$^+$ immunomagnetic beads and cultivated with 10 ng/ml GM-CSF and 3 μM 5AC for 4 days. The concentration of 5AC was selected after cultivation of CY-MDSCs with different doses of 5AC (3 μM, 1.5 μM, 0.6 μM, and 0.3 μM) as the lowest concentration that was able to reduce significantly the CD11b$^+$/Gr-1$^+$ cell percentage in the CY-MDSC population (61.6±14.6% in controls vs. 34.1±10.2% in 3 μM 5AC; $P<0.05$). To verify the optimal dose, the toxicity of 5AC was also tested; although the significant toxicity noticed in the 3 μM 5AC (61.7±10.5% in controls vs. 30.9±22.8% in 3 μM 5AC; $P<0.05$) decreased to nonsignificant levels at lower doses (50.4±22.8% in 1.5 μM 5AC; 58.5±19.8% in 0.6 μM 5AC; and 60.3±9.8 in 0.3 μM 5AC; $P>0.05$ in all doses), the ability to reduce the CD11b$^+$/Gr-1$^+$ population disappeared (43.9±11.2% in 1.5 μM 5AC; 48.4±22.9% in 0.6 μM 5AC; and 55.6±14.0% in 0.3 μM 5AC; $P>0.05$ in all doses). After cultivation of CY-MDSCs in the presence of 3 μM 5AC, the percentage of CD11b$^+$/Gr-1$^+$ cells decreased significantly ($P<0.05$ compared with the control group without 5AC supplementation; **Fig. 7A**), and the cells lost the Gr-1 marker, but they retained their CD11b$^+$ phenotype (data not shown). The

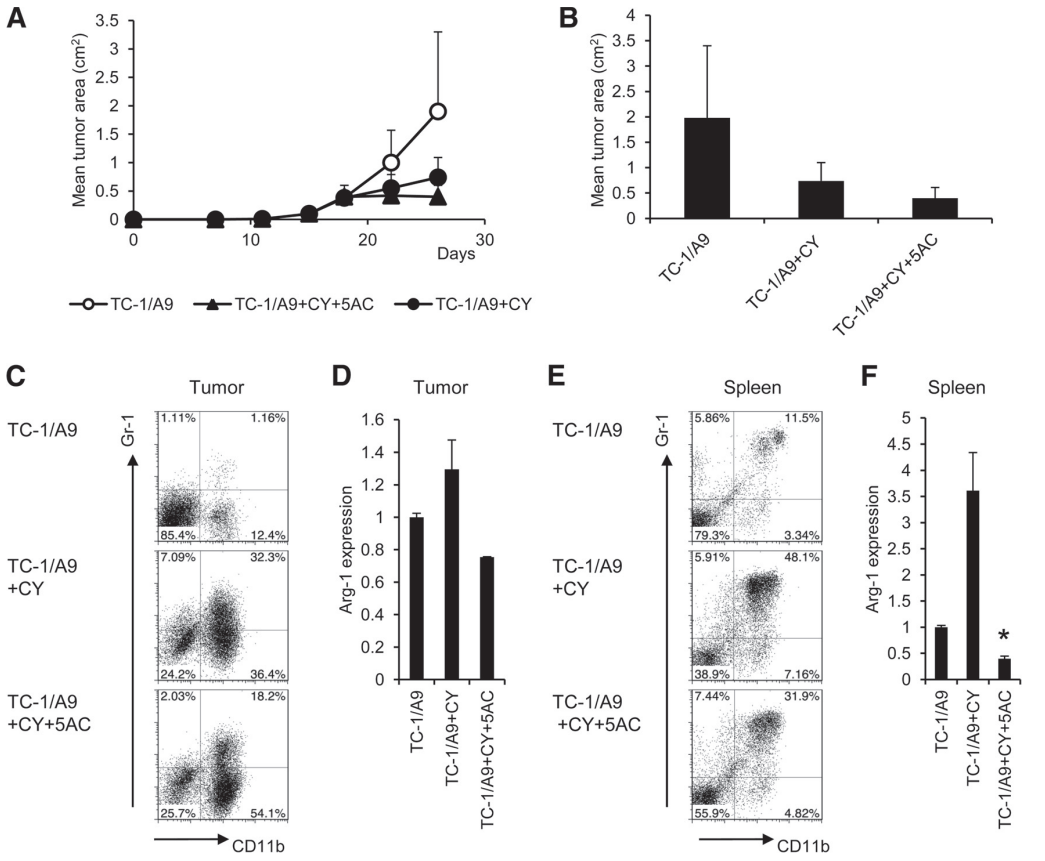


Figure 5. Peritumoral treatment of established TC-1/A9 tumors with a subsequent CY and 5AC combination led to a decreased number of MDSCs accumulating in TMEs and spleens of mice after CY administration. TC-1/A9 cells were transplanted on Day 0. When the tumors reached the size of ~0.5 cm in diameter, mice received one i.p. dose of CY (200 mg/kg) and were then treated twice/week repeatedly with 100 μg 5AC. Growth curves (A) and size of tumors on the day of analysis are given (B). Six mice/group were used for the experiment. Tumors and spleens from each group of mice (pool of three mice/group) were analyzed, 7 days after CY administration (24 h after the last 5AC injection). (C) After CY administration, the $CD11b^+/Gr\text{-}1^+$ cell percentage in the TMEs increased. This accumulation was inhibited by 5AC administration (FACS analysis). (D) The effect was accompanied by a nonsignificant reduction in the Arg-1 expression detected by qRT-PCR. (E) After CY administration, the $CD11b^+/Gr\text{-}1^+$ cell percentage in the spleens of mice increased. This accumulation was inhibited by 5AC administration (FACS analysis). (F) The effect was accompanied by changes in the Arg-1 gene expression, detected by qRT-PCR. All data are representative of $n = 3$ independent experiments. Error bars indicate SD of triplicate wells. Differences were considered significant compared with untreated control (*$P<0.05$) using the Student's t-test.

$CD11c^+$ marker was up-regulated ($P<0.05$), and the percentage of $CD86^+/MHCII^+$ cells also increased ($P<0.05$; Fig. 7A). The percentage of $F4/80^+$ was not increased significantly ($P>0.05$). This finding was accompanied by lower expression of the immunosuppressive Arg-1 gene ($P<0.05$), as well as lower VEGF production ($P<0.05$; Fig. 7B). Cultivation with 5AC increased the percentage of apoptotic cells in the cell cultures compared with the control group without 5AC (Fig. 7D). After in vitro cultivation of TU-MDSCs with 5AC, the results had a similar profile as in CY-MDSCs, but the sensitivity of TU-MDSCs to the 5AC treatment was weaker compared with CY-MDSCs. FACS analysis revealed that 5AC nonsignificantly lowered the percentage of $CD11b^+/Gr\text{-}1^+$ cells in cell cultures compared with the control group without 5AC (Fig. 7A). Furthermore, the percentage of $CD11c^+$ and $CD86^+/MHCII^+$ cells in the cell cultures, supplemented with 5AC, increased slightly ($P>0.05$) compared with the control group without 5AC (Fig. 7A). The increase in the percentage of $F4/80^+$ cells was not significant. The observed changes in the proportion of distinct lymphocyte subpopulations were accompanied by a significant ($P<0.05$) decrease in the Arg-1 gene expression detected by qRT-PCR and slightly lower VEGF production (Fig. 7C). Analysis of apoptosis showed a similar, but weaker, effect after cultivation of TU-MDSCs with 5AC (Fig. 7E). The capacity of TU-MDSCs and CY-MDSCs, cultivated 4 days with or without 5AC, to suppress $CD8^+$ and $CD4^+$ T cell proliferation was also analyzed by a CFSE-based proliferation assay. It was found that TU-MDSCs or CY-MDSCs, cultivated previously with 5AC, were not able to suppress T cell proliferation (**Fig. 8A** and **B**), whereas control TU-MDSCs cultivated only with GM-CSF retained part of their suppressive capacity. As we have seen previously, control CY-MDSCs displayed only a minor suppressive capacity on $CD4^+$ cells, and their suppressive effect on $CD8^+$ cell proliferation was negligible, as CY-MDSCs generally have substantially lower suppressive capacity than TU-MDSCs [17].

DISCUSSION

Inhibition of immune suppression occurring during tumor progression can be crucial for successful cancer immunother-

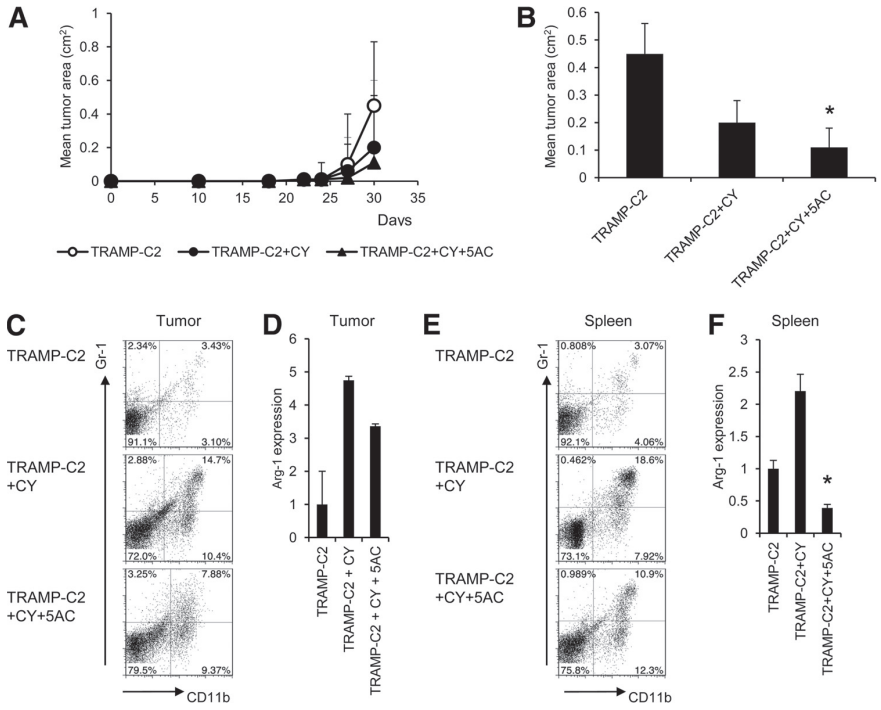


Figure 6. Peritumoral treatment of established TRAMP-C2 tumors with subsequent CY and 5AC combination reduced the number of MDSCs accumulating in TMEs and spleens of mice after CY administration. TRAMP-C2 cells were transplanted on Day 0. When the tumors reached the size of ~0.5 cm in diameter, mice received one i.p. dose of CY (200 mg/kg) and then were repeatedly treated twice/week with 100 μg 5AC. The growth curves (A) and size of tumors on the day of analysis are given (B). Six mice/group were used for the experiment. Tumors and spleens from each group of mice (pool of three mice/group) were analyzed 7 days after CY administration (24 h after the last 5AC injection). (C) After CY administration, the Gr-1$^+$/CD11b$^+$ cell percentage in the TMEs was increased. This accumulation was inhibited by 5AC administration (FACS analysis; CD45$^+$-gated TILs). (D) The effect was associated with a nonsignificant reduction in the Arg-1 gene expression detected by qRT-PCR. (E) After CY administration, the CD11b$^+$/Gr-1$^+$ cell percentage in the spleens of mice increased. This accumulation was inhibited by 5AC administration (FACS analysis). (D) The effect was accompanied by similar changes in the Arg-1 gene expression detected by qRT-PCR. All data are representative of $n = 3$ independent experiments. Error bars indicate SD of triplicate wells. Differences were considered significant compared with untreated control (*$P<0.05$) using the Student's *t*-test.

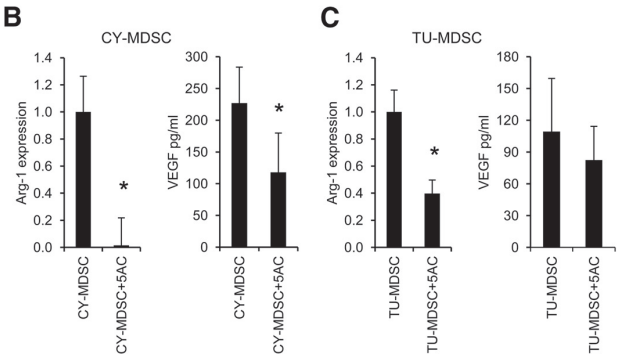
apy. One of the promising approaches in cancer immunotherapy is targeting/inhibiting MDSCs by their elimination or promotion of their differentiation. It has been reported that elimination of MDSCs can be achieved by some chemotherapeutic agents, such as gemcitabine or 5-fluorouracil [30, 42]. Noteworthy, some cytostatic agents, such as paclitaxel, were proven to promote differentiation of MDSCs [43]. Our study was specifically designed to target the MDSC population accumulating in TMEs and spleens of CY-treated TC-1/A9 and TRAMP-C2 tumor-bearing mice using a cytostatic agent with epigenetic effects of 5AC.

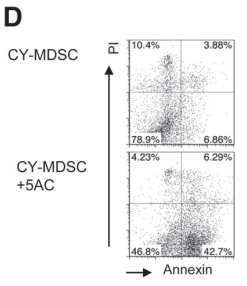
5AC is a chemotherapeutic agent displaying antitumor effects, mainly against hematologic cancers. We have reported previously that 5AC had an antitumor effect when used for treatment of early growing but not established murine HPV-associated TC-1/A9 tumors [25]. In this study, we have shown that 5AC is also effective for treatment of early and contrary to TC-1/A9, established TRAMP-C2 cells of adenocarcinoma mouse prostate model. Besides direct antitumor effects, 5AC can also influence tumor cell interactions with the immune system by its effects on tumor cells or immunocytes. Our previous studies revealed that the treatment with 5AC restores expression of the MHC class I molecules on MHC class I-deficient tumor cells and renders the tumor cells susceptible to the attack of CTLs [24, 25]. On the other hand, its administration can induce Tregs by demethylating promoter regions of the forkhead box P3 gene, a crucial transcription factor for Tregs [44]. We have found that peritumoral treatment of established tumors with 5AC led to the decrease in the percentage of CD11b$^+$/Gr-1$^+$ cells in the TME and spleens of mice bearing TC-1/A9 or TRAMP-C2 tumors. The changes in the MDSC percentage were associated with reduced expression of the Arg-1 gene in TMEs and in the spleens. Arg-1 was selected as a marker of immunosuppressive environment, as MDSCs are major producers of Arg-1 [10], although we cannot exclude their production by other immunosuppressive cell populations as well (tumor-associated macrophages). Indeed, in all our experiments, we found a good correlation between MDSC cellularity and Arg-1 RNA proportion in the TME or spleen RNA specimens. We can also claim that the decrease of the CD11b$^+$/Gr-1$^+$ cell percentage was not caused by the reduced tumor size after the treatment with 5AC, as in the case of the established TC-1/A9 tumor, no tumor inhibitory effect was found. Our results are in accord with the findings obtained by Triozzi et al. [35]. These authors used low-dose DAC treatment for mice bearing B16 tumors and found decreased numbers of protumor MDSCs.

As it emerged from our previous experiments, CY administration induces MDSC accumulation in the spleens of nontumor-bearing mice or their additional accumulation in the spleens of tumor-bearing mice [17]. In this study, we have found that such MDSC mobilization can also be found in the TMEs of CY-treated tumors in TC-1/A9 and TRAMP-C2 tumors similarly. Therefore, we raised the question of whether

A

	CD11b$^+$/Gr-1$^+$ (%)		CD11c$^+$ (%)		CD86$^+$/MHCII$^+$ (%)		F4/80$^+$ (%)	
CY-MDSC	61.6	14.6	16.18	6.2	5.2	0.7	43.9	23.4
CY-MDSC+5AC	34.1	10.2*	35.3	13.4*	15.3	6.4*	58.5	18.8
TU-MDSC	57.2	9.3	43.0	33.2	7.8	1.4	68.5	7.2
TU-MDSC+5AC	24.7	21.9	57.2	33.0	12.7	3.3	56.6	20.3





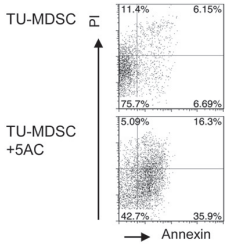


Figure 7. The percentage of CY-MDSCs and TU-MDSCs decreased after their cultivation with 5AC in vitro. (A) CY-MDSCs were isolated by CD11b$^+$ immunomagnetic beads from the spleens of mice, 7 days after CY administration (200 mg/kg, i.p.). TU-MDSCs were isolated by CD11b+ immunomagnetic beads from the spleens of mice bearing TC-1/A9 tumors (~2 cm in diameter). Cells were subsequently cultivated with 10 ng/ml GM-CSF and 3 μM 5AC for 4 days. FACS analysis of cultured CY-MDSCs revealed that 5AC decreased the percentage of CD11b$^+$/Gr-1$^+$ cells significantly in cell cultures compared with the control group without 5AC. FACS analysis revealed further that the percentage of CD11c$^+$ and CD86$^+$/MHCII$^+$ cells in cell cultures supplemented with 5AC increased significantly compared with the control group without 5AC; the increase in the percentage of F4/80$^+$ cells was not significant (data are cumulative from n=6 independent experiments). For TU-MDSCs, the differences between 5AC-treated vs. untreated populations were not significant (data of n=3 independent experiments). Both percentage and SD values are presented. (B) and (C) 5AC treatment of cell cultures was associated with a significant decrease in the expression of the Arg-1 gene detected by qRT-PCR (data are representative of n=3 independent experiments) and a decrease in VEGF production (significant for CY-MDSCs only; data are representative of n=3 independent experiments). (D) FACS analysis revealed that 5AC increased the percentage of apoptotic cells in the cell cultures compared with the control group cultivated without 5AC (data are representative of n=2 independent experiments). (E) FACS analysis of cultured TU-MDSCs revealed that 5AC increased the percentage of apoptotic cells in the cell cultures compared with the control group without 5AC (data are representative of n=2 independent experiments). Differences were considered significant compared with untreated control (*P<0.05) using the Student's t-test.

5AC can affect CY-MDSCs by its administration following CY treatment of established TC-1/A9 and TRAMP-C2 tumors. As we expected, CY treatment of the established tumors led to additional accumulation of CD11b$^+$/Gr-1$^+$ cells in the spleens and TMEs. This accumulation was subsequently inhibited by 5AC treatment. The subsequent combination of CY with 5AC led to the most effective tumor growth inhibition (TRAMP-C2) compared with monotherapies. We can speculate that instead of a direct cytostatic effect, this inhibition of tumor growth resulted from the elimination of CY-MDSCs, as they are considered to be the most potent effectors of tumor-induced T cell unresponsiveness.

Several mechanisms have been proposed to be responsible for the reduced levels of MDSCs, such as blockage of MDSC generation, stimulation of MDSC differentiation, or induction of MDSC apoptosis [19, 43]. The mechanisms responsible for MDSC modulation by DNMTi are not fully understood. To cast more light on the mechanism of effect of 5AC on MDSCs, we isolated CY-MDSCs and TU-MDSCs and cultivated them in the presence of 5AC. After cultivation, we observed a remarkable decrease in the percentage of CD11b$^+$/Gr-1$^+$ cells associated with the decrease of Arg-1 and VEGF production. Importantly, a loss of suppressive capacity after MDSC cultivation with 5AC was also detected. The decrease in the percentage of CD11b$^+$/Gr-1$^+$ cells was associated with increased percentages of CD11c$^+$ and CD86$^+$/MHCII$^+$ cells, suggesting partial differentiation of MDSCs toward DCs. In this experiment, TU-MDSCs seemed to be less susceptible to 5AC compared with CY-MDSCs, as the effects were weaker in this population. The difference may be caused by the fact that CY-MDSCs consist of a higher percentage of the monocytic (Ly6G$^-$Ly6C^{High}) population when compared with TU-MDSCs, whereas the percentage of the granulocytic (Ly6G$^+$Ly6C^{Low}) population was higher in TU-MDSCs [17]. These results are in correspondence with the results of Daurkin et al. [34], who demonstrated that tumor-infiltrated CD11b myeloid cells can be differentiated in vitro into mature tumor-derived APCs in the presence of DAC. On the other hand, our data do not indicate that MDSC elimination would be associated with a massive rise of DCs in general. Therefore, we also analyzed induction of apoptosis of CY-MDSCs or TU-MDSCs after their cultivation with 5AC, and we found that 5AC increased significantly the percentage of apoptotic cells in the cell cultures compared with untreated controls. The effect was again weaker in the case of TU-MDSCs. We therefore suggest the combination of apoptosis induction with differentiation of the remaining cells into APCs as the mechanism of the modulatory effects of 5AC

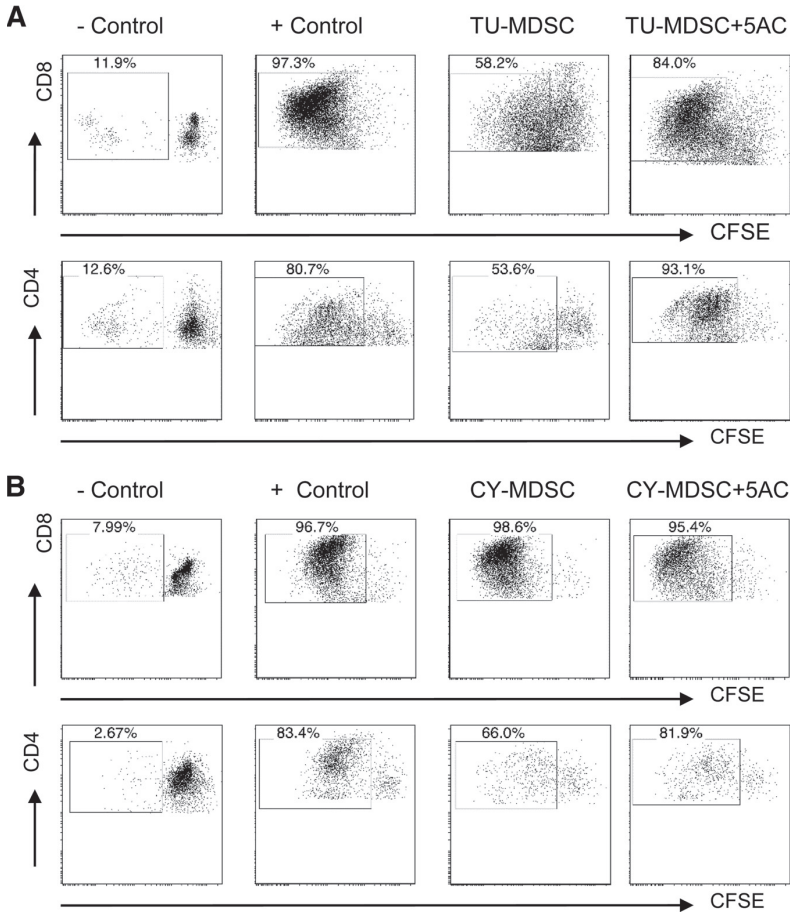


Figure 8. TU-MDSCs and CY-MDSCs cultivated for 4 days with 5AC did not suppress proliferation of autologous T cells. TU-MDSCs and CY-MDSCs were cultivated for 4 days with 5AC and then used in a CFSE-based proliferation assay. In an autologous, CFSE-based proliferation assay, on Day 4 of MDSC coculture with autologous splenocytes, T cell proliferation was measured by CFSE dilution. (A) Representative density plots showed that TU-MDSCs precultivated with 5AC did not suppress proliferation of autologous $CD4^+$ and $CD8^+$ T cells when compared with control TU-MDSCs cultivated without 5AC. (B) Representative density plots showed that CY precultivated with 5AC did not suppress proliferation of autologous $CD4^+$ and $CD8^+$ T cells. Control CY-MDSCs, cultivated without 5AC, displayed a minor suppressive effect (data are representative of $n=2$ independent experiments).

on MDSCs. This effect could also be dose-dependent, similarly as with other chemotherapeutic agents [33, 43].

Taken collectively, our results indicate that the epigenetic modifier 5AC is a promising cytostatic agent that affects not only tumor cells but also targets MDSCs, accumulating in growing tumors or in tumors treated with CY. This could be beneficial for the outcome of cancer chemotherapy, given that higher levels of MDSCs have been reported in patients with advanced cancer who also received cytostatic therapy with CY [16].

AUTHORSHIP

R.M. and M.I. designed and performed the study, collected and analyzed data, and wrote the paper, V.V., J.B., J.Š., Z.P., E.P-P., and J.R. performed the study and analyzed the data. M.R. designed the study, analyzed data, and wrote the paper.

ACKNOWLEDGMENTS

This work was supported by grants from the Czech Science Foundation (Nos. GAP301/11/P220 and P301/10/2174) and, in part, by grant RVO 68378050, awarded by the Academy of Sciences of the Czech Republic. Support was also obtained from the Ministry of Science and Higher Education Republic of Poland (grant N N401 316239) and by the Polish-Czech Republic Joint Research Project (2012–2014). Ph.D. student V.V. was, in part, supported by the Faculty of Science, Charles University, Prague. The authors are grateful to Mrs. Renáta Turečková for skilful technical assistance and to Dr. Šárka Takáčová for editorial help.

DISCLOSURES

The authors declare no conflict of interest.

REFERENCES

1. Gabrilovich, D. I., Ostrand-Rosenberg, S., Bronte, V. (2012) Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol.* **22,** 253–268.
2. Gabrilovich, D. I., Nagaraj, S. (2009) Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* **9,** 162–174.
3. Umansky, V., Sevko, A. (2012) Tumor microenvironment and myeloid-derived suppressor cells. *Cancer Microenvironment* **61,** 275–282.
4. Sevko, A., Umansky, V. (2013) Myeloid-derived suppressor cells interact with tumors in terms of myelopoiesis, tumorigenesis and immunosuppression: thick as thieves. *J. Cancer* **4,** 3–11.
5. Movahedi, K., Guilliams, M., Van den Bossche, J., Van den Bergh, R., Gysemans, C., Beschin, A., De Baetselier, P., Van Ginderachter, J. A. (2008) Identification of discrete tumor-induced myeloid-derived sup-

pressor cell subpopulations with distinct T cell-suppressive activity. *Blood* **111,** 4233–4244.
6. Youn, J. I., Nagaraj, S., Collazo, M., Gabrilovich, D. I. (2008) Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J. Immunol.* **181,** 5791–5802.
7. Kusmartsev, S., Gabrilovich, D. I. (2003) Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J. Leukoc. Biol.* **74,** 186–196.
8. Kusmartsev, S., Gabrilovich, D. I. (2006) Role of immature myeloid cells in mechanisms of immune evasion in cancer. *Cancer Immunol. Immunother.* **55,** 237–245.
9. Rodriguez, P. C., Quiceno, D. G., Ochoa, A. C. (2007) L-Arginine availability regulates T-lymphocyte cell-cycle progression. *Blood* **109,** 1568–1573.
10. Zea, A. H., Rodriguez, P. C., Atkins, M. B., Hernandez, C., Signoretti, S., Zabalet, J., McDermott, D., Quiceno, D., Youmans, A., O'Neill, A., Mier, J., Ochoa, A.C. (2005) Arginase producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res.* **65,** 3044–3048.
11. Rodriguez, P. C., Quiceno, D. G., Zabaleta, J., Ortiz, B., Zea, A. H., Piazuelo, M. B., Delgado, A., Correa, P., Brayer, J., Sotomayor, E. M., Antonia, S., Ochoa, J. B., Ochoa, A. C. (2004) Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.*. **64,** 5839–5849.
12. Bronte, V., Apolloni, E., Cabrelle, A., Ronca, R., Serafini, P., Zamboni, P., Restifo, N. P., Zanovello, P. (2000) Identification of a CD11b+/Gr-1+/CD31+ myeloid progenitor capable of activating or suppressing CD8+ T cells. *Blood* **96,** 3838–3846.
13. Serafini, P., De Santo, C., Marigo, I., Cingarlini, S., Dolcetti, L., Gallina, G., Zanovello, P., Bronte, V. (2004) Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol. Immunother.* **53,** 64–72.
14. Angulo, I., de las Heras, F. G., García-Bustos, J. F., Gargallo, D., Muñoz-Fernández, M. A., Fresno, M. (2000) Nitric oxide-producing CD11b(+)Ly-6G(Gr-1)(+)CD31(ER-MP12)(+) cells in the spleen of cyclophosphamide-treated mice: implications for T-cell responses in immunosuppressed mice. *Blood* **95,** 212–220.
15. Peláez, B., Campillo, J. A., López-Asenjo, J. A., Subiza, J. L. (2001) Cyclophosphamide induces the development of early myeloid cells suppressing tumor cell growth by a nitric oxide-dependent mechanism. *Immunology* **166,** 6608–6615.
16. Diaz-Montero, C. M., Salem, M. L., Nishimura, M. I., Garrett-Mayer, E., Cole, D. J., Montero, A. J. (2009) Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol. Immunother.* **58,** 49–59.
17. Mikyšková, R., Indrová, M., Polláková, V., Bieblová, J., Šímová, J., Reiniš, M. (2012) Cyclophosphamide-induced myeloid-derived suppressor cell population is immunosuppressive but not identical to myeloid-derived suppressor cells induced by growing TC-1 tumours. *J. Immunotherapy* **35,** 376–384.
18. Emadi, A., Jones, R. J., Brodsky, R. A. (2009) Cyclophosphamide and cancer: golden anniversary. *Nat. Rev. Clin. Oncol.* **611,** 638–647.
19. Ugel, S., Delpozzo, F., Desantis, G. (2009) Therapeutic targeting of myeloid-derived suppressor cells. *Curr. Opin. Pharmacol.* **9,** 470–481.
20. Blum, W., Klisovic, R. B., Hackanson, B., Liu, Z., Liu, S., Devine, H., Vukosavljevic, T., Huynh, L., Lozanski, G., Kefauver, C., Plass, C., Devine, S. M., Heerema, N. A., Murgo, A., Chan, K. K., Grever, M. R., Byrd, J. C., Marcucci, G. (2007) Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. *J. Clin. Oncol.* **25,** 3884–3891.
21. Cashen, A. F., Schiller, G. J., O'Donnell, M. R., Dipersio, J. F. (2010) Multicenter phase II study of decitabine for the first-line treatment of older patients with acute myeloid leukemia. *J. Clin. Oncol.* **28,** 556–561.
22. Kantarjian, H, Issa, J. P., Rosenfeld, C. S., Bennett, J. M., Albitar, M., DiPersio, J., Klimek, V., Slack, J., de Castro, C., Ravandi, F, Helmer III, R., Shen, L., Nimer, S. D., Leavitt, R., Raza, A., Saba, H. (2006) Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer* **106,** 1794–1803.
23. Tomasi, T. B., Magner, W. J., Khan, A. N. (2006) Epigenetic regulation of immune escape genes in cancer. *Cancer Immunol. Immunother.* **55,** 1159–1184.
24. Manning, J., Indrová, M., Lubyová B, Přibylová, H., Bieblová, J., Hejnar, J., Šímová, J., Jandlová, T., Bubeník, J., Reiniš, M. (2008) Induction of MHC class I molecule cell surface expression and epigenetic activation of antigen-processing machinery components in a murine model for human papilloma virus 16-associated tumours. *Immunology* **123,** 218–227.
25. Šímová, J., Polláková, V., Indrová, M., Mikyšková, R., Bieblová, J., Štěpánek, I., Bubeník, J., Reiniš, M. (2011) Immunotherapy augments the effect of 5-azacytidine on HPV16-associated tumours with different MHC class I-expression status. *Brit. J. Cancer* **105,** 1533–1541.
26. Setiadi, A. F., David, M. D., Seipp, R. P., Hartikainen, J. A., Gopaul, R., Jefferies, W. A. (2007) Epigenetic control of the immune escape mechanisms in malignant carcinomas. *Mol. Cell. Biol.* **27,** 7886–7894.
27. Reiniš, M. (2010) Immunotherapy of MHC class I-deficient tumors. *Future Oncol.* **6,** 1577–1589.
28. Štěpánek, I., Indrová, M., Bieblová, J., Fučiková, J., Spíšek, R., Bubeník, J., Reiniš, M. (2011) Effects of unmethylated CpG oligodeoxynucleotides, TNFα, and epigenetic agents on dendritic cell maturation. *J. Biol. Regul. Homeost. Agents* **25,** 517–529.
29. Nencioni, A., Beck, J., Werth, D., Grünebach, F., Patrone, F., Ballestrero, A., Brossart, P. (2007) Histone deacetylase inhibitors affect dendritic cell differentiation and immunogenicity. *Clin. Cancer Res.* **13,** 3933–3941.
30. Suzuki, E., Kapoor, V., Jassar, A. S., Kaiser, L. R., Albelda, S. M. (2005) Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin. Cancer Res.* **11,** 6713–6721.
31. Ghiringhelli, F., Bruchard, M., Apetoh, L. (2013) Immune effects of 5-fluorouracil: ambivalence matters. *Oncoimmunology* **2,** e23139.
32. Kodumudi, K. N., Woan, K., Gilvary, D. L., Sahakian, E., Wei, S., Djeu, J. Y. (2010) A novel chemoimmunomodulating property of docetaxel: suppression of myeloid-derived suppressor cells in tumor bearers. *Clin. Cancer Res.* **16,** 4583–4594.
33. Sevko, A., Michels, T., Vrohlings, M., Umansky, L., Beckhove, P., Kato, M., Shurin, G. V., Shurin, M. R., Umansky, V. (2013) Antitumor effect of paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model. *J. Immunol.* **190,** 2464–2471.
34. Daurkin, I., Eruslanov, E., Vieweg, J., Kusmartsev, S. (2010) Generation of antigen-presenting cells from tumor-infiltrated CD11b myeloid cells with DNA demethylating agent 5-aza-2′-deoxycytidine. *Cancer Immunol. Immunother.* **59,** 697–706.
35. Triozzi, P. L., Aldrich, W., Achberger, S., Ponnazhagan, S., Alcazar. O., Saunthararajah, Y. (2012) Differential effects of low-dose decitabine on immune effector and suppressor responses in melanoma-bearing mice. *Cancer Immunol. Immunother.* **61,** 1441–1450.
36. Rosborough, B. R., Castellaneta, A., Natarajan, S., Thomson, A. W., Turnquist. H. R. (2012) Histone deacetylase inhibition facilitates GM-CSF-mediated expansion of myeloid-derived suppressor cells in vitro and in vivo. *J. Leukoc. Biol.* **91,** 701–709.
37. Lin, K. Y., Guarnieri, F. G., Staveley-O'Carroll, K. F., Levitsky, H. I., August, J. T., Pardoll, D. M., Wu, T. C. (1996) Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res.* **56,** 21–26.
38. Šmahel, M., Šíma, P., Ludvíková, V., Marinov, I., Pokorná D., Vonka, V. (2003) Immunisation with modified HPV16 E7 genes against mouse oncogenic TC-1 cell sublines with downregulated expression of MHC class I molecules. *Vaccine* **21,** 1125–1136.
39. Foster, B. A., Gingrich, J. R., Kwon, E. D., Madias, C., Greenberg, N. M. (1997) Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. *Cancer Res.* **57,** 3325–3330.
40. Indrová, M., Bieblová, J., Bubeník, J., Reiniš, M. (2008) IL-12 immunotherapy of minimal residual disease in murine models of HPV16-associated tumours: induction of immune responses, cytokine production and kinetics of immune cell subsets. *Int. J. Oncol.* **32,** 499–507.
41. Rossowska, J., Pajtasz-Piasecka, E., Rycœnik, O., Wojas, J., Krawczenko, A., Szyda, A., Duœ, D. (2011) Generation of antitumor response by IL-2-transduced JAWS II dendritic cells. *Immunobiology* **216,** 1074–1084.
42. Vincent, J., Mignot, G., Chalmin, F., Ladoire, S., Bruchard, M., Chevriaux, A., Martin, F., Apetoh, L., Rébé, C., Ghiringhelli, F. (2010) 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res.* **70,** 3052–3061.
43. Michels, T., Shurin, G. V., Naiditch, H., Sevko, A., Umansky, V., Shurin, M. R. (2012) Paclitaxel promotes differentiation of myeloid-derived suppressor cells into dendritic cells in vitro in a TLR4-independent manner. *J. Immunotoxicol.* **9,** 292–300.
44. Sánchez-Abarca, L. I., Gutierrez-Cosio, S., Santamaría, C., Caballero-Velazquez, T., Blanco, B., Herrero-Sánchez, C., García, J. L., Carrancio, S., Hernández-Campo, P., González, F. J., Flores, T., Ciudad, L., Ballestar, E., Del Cañizo, C., San Miguel, J. F., Pérez-Simon, J. A. (2010) Immunomodulatory effect of 5-azacytidine (5-azaC): potential role in the transplantation setting. *Blood* **115,** 107–121.

KEY WORDS:
immunosuppression · microenvironment · arginase-1

6.5. Publikácia V

Po podaní protilátky anti-CD25 mAb dôjde k narušeniu produkcie IFN-γ indukovanej α-galaktosylceramidom.

Administration of anti-CD25 mAb leads to impaired α-galactosylceramide-mediated induction of IFN-γ production in a murine model.

Rodney A. Rosalia, Ivan Štěpánek, Veronika Polláková (Vlková), Jana Šímová, Jana Bieblová, Marie Indrová, Simona Moravcová, Hana Přibylová, Hetty J. Bontkes, Jan Bubeník, Tim Sparwasser a Milan Reiniš

$CD4^+CD25^+FoxP3^+$ regulačné T-lymfocyty (Treg) a CD1d-rozpoznávajúce invariantné NKT-lymfocyty (iNKT) sú typy buniek, ktoré regulujú imunitné reakcie. Hlavnou funkciou iNKT buniek po ich aktivácii je regulácia imunitnej odpovede cez produkciu cytokínov. iNKT bunky majú kapacitu zvýšiť imunitu hostiteľa voči mikroorganizmom a nádorovým ochoreniam. Receptor na iNKT bunkách viaže ligand glycosfingolipid α-galaktosylceramid (α-GalCer) prezentovaný na antigén prezentujúcich bunkách exprimujúcich CD1d a tak aktivuje iNKT bunky. Aktivácia iNKT buniek s α–GalCer vedie v konečnom dôsledku k aktivácii cytotoxických T-lymfocytov, NK buniek a dendritických buniek. Regulačné T-lymfocyty prednostne ochraňujú organizmus pred autoimunitnými ochoreniami, ale tiež prispieva k znižovaniu protinádorovej imunitnej odpovede. Deplécia alebo inaktivácia regulačných T-lymfocytov použitím špecifickej protilátky anti-CD25 v kombinácii s imunostimuláciou by mohla byť dobrou metódou v protinádorovej imunoterapii. Avšak, molekula CD25 nie je exprimovaná výhradne na regulačných T-lymfocytoch, ale tiež aj na aktivovaných efektoroch, ako T a B bunkách, NK a NKT bunkách, ktoré môžu byť tiež cieľom protilátky anti-CD25. Preto môžeme predpokladať, že k efektu špecifickej protilátky anti-CD25 tiež prispieva aj jej interakcia s efektorovými bunkami. Na základe tejto skutočnosti sme sa rozhodli sledovať detailne, či protilátka anti-CD25 (PC61) zasahuje do stimulácie NKT buniek. V predkladanej práci sme preto sledovali efekt protilátky anti-CD25 mAb PC61 na α–GalCer sprostredkovanú aktiváciu iNKT buniek a tiež účinnosť kombinácie protilátky PC61 a α–GalCer proti nádorom TC-1. Inými slovami v danej práci sme hodnotili dopad protilátky PC61, ktorú sme použili na inaktiváciu regulačných T-lymfocytov v myšacom modeli, na efektorovú funkciu iNKT buniek po vystavení α–GalCer, čo je ligand NKT bunkového receptora s terapeutickým potenciálom.

Hodnotili sme aditívny/synergistický efekt kombinácie PC61 s α–GalCer v protinádorovej imunoterapii. Zistili sme, že α-galaktosylceramidom aktivovaná imunitná odpoveď u zvierat, ktoré boli ošetrené protilátkou PC61, vykazuje porušenú produkciu IFN-γ. Museli sme zistiť, či tento výsledok môže byť spôsobený priamou inaktiváciou regulačných T-lymfocytov protilátkou PC61 alebo efektom protilátky anti-CD25 na $CD25^+$ populáciu efektorových buniek, napríklad na aktivované iNKT bunky. CD25 je možné detekovať na povrchu aktivovaných myšacích a ľudských NKT buniek (Kim et al., 2006; Bessoles et al., 2008) a CD25 je antigénom, ktorý je rozpoznaný protilátkou PC61. Aby sme podrobne preskúmali efekt deplécie regulačných T-lymfocytov pomocou protilátky anti-CD25 mAb na aktiváciu iNKT buniek, aktiváciu iNKT buniek sme sledovali aj v transgénnom modeli myší DEREG, v ktorých depléciu $FoxP3^+$ regulačných T-lymfocytov dosiahneme pomocou podania diphtheria toxínu. Tento systém spôsobuje špecifickú depléciu regulačných T-lymfocytov (Lahl et al., 2007). Pri porovnaní výsledkov získaných prácou s myšami DEREG s výsledkami získanými prácou s protilátkou PC61, ktorou sme ošetrili štandardný typ myší, sme mohli vidieť efekt špecifickej deplécie Treg na aktiváciu NKT buniek od ďalších efektov protilátky PC61. Nebola pozorovaná žiadna inhibícia produkcie IFN-γ po podaní α-galaktosylceramidu u myší DEREG, v porovnaní s produkciou u štandardných myší ošetrených s protilátkou anti-CD25. Môžeme teda povedať, že negatívny efekt tohto ošetrenia na hladinu imunitnej odpovede by mohol byť spôsobený skôr elimináciou $CD25^+$ efektorových buniek ako inaktiváciou/depléciou regulačných T-lymfocytov. Naše dáta teda limitujú použitie protilátky anti-CD25 v štúdiách zameraných na interakciu regulačných T-lymfocytov s efektorovými bunkami.

Prínos autora dizertačnej práce k danej publikácii:

Mojou úlohou pri vypracovaní predkladanej publikácie bolo monitorovanie relatívnej expresie génov kódujúcich IFN-γ, IL-4, T-bet a GATA-3 po ošetrení kontrolných a DEREG myší protilátkou PC61 a α-galaktosylceramidom.

Immunobiology 218 (2013) 851–859

Contents lists available at SciVerse ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Administration of anti-CD25 mAb leads to impaired α-galactosylceramide-mediated induction of IFN-γ production in a murine model

Rodney A. Rosalia[a,b,1], Ivan Štěpánek[a,1], Veronika Polláková[a], Jana Šímová[a], Jana Bieblová[a], Marie Indrová[a], Simona Moravcová[a], Hana Přibylová[a], Hetty J. Bontkes[b], Jan Bubeník[a], Tim Sparwasser[c], Milan Reiniš[a,*]

[a] Department of Tumour Immunology of the Institute of Molecular Genetics, v. v. i., Academy of Sciences of the Czech Republic, Prague 4, Czech Republic
[b] Department of Pathology of the VU University Medical Center, Cancer Center Amsterdam, Amsterdam, The Netherlands
[c] Institute of Infection Immunology, TWINCORE, Centre for Experimental and Clinical Infection Research, Feodor-Lynen-Straße 7, 30625 Hannover, Germany

ARTICLE INFO

Article history:
Received 3 November 2011
Received in revised form 4 September 2012
Accepted 17 October 2012
Available online 26 October 2012

Keywords:
Cancer immunotherapy
DEREG mice
Interferon γ
Natural killer T cells
PC61 monoclonal antibody
T regulatory cells

ABSTRACT

$CD4^+CD25^+Foxp3^+$ T regulatory cells (Tregs) and CD1d-restricted invariant natural killer T (iNKT) cells are two cell types that are known to regulate immune reactions. Depletion or inactivation of Tregs using specific anti-CD25 antibodies in combination with immunostimulation is an attractive modality especially in anti-tumour immunotherapy. However, CD25 is not expressed exclusively on Tregs but also on subpopulations of activated lymphocytes. Therefore, the modulatory effects of the specific anti-CD25 antibodies can also be partially attributed to their interactions with the effector cells. Here, the effector functions of iNKT cells were analysed in combination with anti-CD25 mAb PC61. Upon PC61 administration, α-galactosylceramide (α-GalCer)-mediated activation of iNKT cells resulted in decreased IFN-γ but not IL-4 production. In order to determine whether mutual interactions between Tregs and iNKT cells take place, we compared IFNγ production after α-GalCer administration in anti-CD25-treated and "depletion of regulatory T cell" (DEREG) mice. Since no profound effects on IFNγ induction were observed in DEREG mice, deficient in $FoxP3^+$ Tregs, our results indicate that the anti-CD25 antibody acts directly on $CD25^+$ effector cells. *In vivo* experiments demonstrated that although both α-GalCer and PC61 administration inhibited TC-1 tumour growth in mice, no additive/synergic effects were observed when these substances were used in combination therapy.

Introduction

Invariant natural killer T cells (iNKT) and $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) are two populations of T lymphocytes that regulate adaptive and innate immune responses. NKT cells are lipid-antigen reactive T cells sharing some characteristics with NK cells. In C57BL/6 (BL/6) mice CD1d-restricted iNKT cells were characterized as T cells expressing NK1.1 together with CD3 and a semi-invariant Vα14-Jα18 chain coupled with either Vβ8.2, 7, or 2 (Godfrey et al. 2004; Kronenberg 2005; Fujii et al. 2003). The main function of iNKT cells is to regulate immune responses through the production of a wide variety of cytokines upon activation. Owing to the broad spectrum of cytokines, iNKT cells have the capacity to enhance host immunity to microorganisms and cancer as well as to prevent autoimmunity. The iNKT cell T receptor ligand glycosphingolipid α-galactosylceramide (α-GalCer) is presented by CD1d-expressing antigen-presenting cells (APC) and potently activates iNKT cells. Activation of iNKT cells with α-GalCer leads to potent downstream activation of cytotoxic T lymphocytes (CTL), natural killer cells (NK), and dendritic cells (DC), but can also induce iNKT cell anergy in mice (Silk et al. 2004; Burdin et al. 1999; Parekh et al. 2005; Seino et al. 2005). Activation of these cells is crucial to the protective anti-tumour and anti-microbial immunity mediated by α-GalCer.

Tregs function under steady state conditions to prevent autoimmunity and, importantly, their increase contributes to suppressing anti-tumour immune responses (Sakaguchi et al. 2006; Thompson and Powrie 2004; Miyara and Sakaguchi 2007; Nishikawa and Sakaguchi 2010). Typically, Tregs comprise 5–10% of murine peripheral $CD4^+$ T cells and express Foxp3, CD25, and CD62L

Abbreviations: Tregs, T regulatory cells; iNKT cells, invariant natural killer T cells; α-GalCer, α-galactosylceramide; IFN-γ, interferon γ; IL-4, interleukin 4; APC, antigen-presenting cells; BL/6 mice, C57BL/6 mice; DEREG mice, depletion of the regulatory Treg mice; DT, diphtheria toxin; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PE, phycoerythrin; FITC, fluorescein isothiocyanate; APC, Allophycocyanin; Cy7, cyanine dye; TDLN, tumour-draining lymph nodes.

* Corresponding author at: Institute of Molecular Genetics, v. v. i., Academy of Sciences of the Czech Republic, Department of Tumour Immunology, Vídeňská 1083, 142 20 Prague 4, Czech Republic. Tel.: +420 241063461; fax: +420 224310955.
E-mail address: reinis@img.cas.cz (M. Reiniš).
[1] These authors contributed equally to the work.

0171-2985/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.imbio.2012.10.012

(Sakaguchi et al. 2006; Seddiki et al. 2006; Taylor et al. 2004). Tregs elimination with specific anti-CD25 antibodies has become a widely investigated and attractive modality to augment immune response when used in combination with immunotherapy or vaccination, especially in anti-tumour immunotherapy, and several preparations have already been developed to be used in humans (Schabowsky et al. 2007). However, CD25, α-chain of the IL-2 receptor, is not a specific marker expressed exclusively on the Treg cells. CD25 is transiently expressed on activated effectors, such as T and B cells, but also NK and NKT cells, both in humans and mice (Thèze et al. 1996; Kim et al. 2006), which can also be targeted with an anti-CD25 antibody, such as PC61 in mice (Ko et al. 2005).

The objective of this study was to analyse the impact of $CD25^+$ cell targeting on iNKT cell activation in mice. Previously we have reported, using the TC-1 murine model for HPV16-associated tumours, that treatment with the Tregs targeting anti-CD25 mAb (PC61) resulted in inhibition of tumour growth including significantly lower recurrences after surgery (Símová et al. 2006), yet the growth of established tumours was not affected. Using the same model, we have also shown therapeutic effects of iNKT cell activation as a treatment of minimal residual tumour disease after surgery or chemotherapy (Símová et al. 2010). Here, we investigated the effects of anti-CD25 mAb PC61 on subsequent α-GalCer-mediated iNKT cell activation, as well as the efficacy of combined PC61 and α-GalCer treatment against TC-1 tumours. To discriminate between Treg depletion and direct anti-CD25 mAb effects on iNKT cell activation, we have performed further analysis of iNKT cell activation in transgenic DEREG mice, in which effective $FoxP3^+$ Treg depletion could be achieved by diphtheria toxin injection (Lahl et al. 2007).

Materials and methods

Mice

BL/6 mice were purchased from AnLab Co., Prague, Czech Republic and maintained in the animal facility of the Institute of Molecular Genetics, Prague. All mice were housed at least 1 week before starting the experimental schedule. Male mice, aged 8–12 weeks at the initiation of the experiments, were used. DEREG ("depletion of the regulatory Treg") mice, BL/6 background transgenic mice expressing a diphtheria toxin (DT) receptor fused with a green fluorescent protein (GFP) under the control of the *foxp3* gene locus (Lahl et al. 2007), originating from Twincore, Hannover, were bred at the Institute of Molecular Genetics. Selective depletion of the $FoxP3^+$ cells was performed by injection of 1 μg DT for three consecutive days as described previously (Baru et al. 2010). Experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics, Prague.

Reagents

α-GalCer (kindly provided by Dr Shigeyuki Yamano, Pharmaceutical Research Laboratories, Kirin Brewery, Gunma, Japan or purchased from Axxora, San Diego, CA) was reconstituted in MilliQ water to a final concentration of 200 μg/ml and further diluted with phosphate-buffered saline (PBS) before use. The vehicle used was PBS supplemented with 0.5% polysorbate-20. Diphtheria toxin was obtained from Merck, NJ. The anti-TCRβ-PeCy5 (H57-597), anti-CD4-FITC (RM4-4), anti-CD8α-FITC (LY-2), anti-CD25-PE (PC61) or anti-CD25-PE (7D4), anti-CD3ε-APC (145-2C11), anti-CD45RB-PE/Cy7 or -APC (C363.16A), anti-CD62L-APC (MEL-4), anti-NK1.1 (NKR-P1B), anti-CD44-FITC (IM7) and anti-CD69-FITC (H1.2F3) antibodies were purchased from BD Pharmingen, San Diego, CA. Anti CD19-BV421 (6D5), anti CD25-APC (3C7) were purchased from BioLegend, San Diego, USA. The isotype rat IgG1 antibody (R3–34) was purchased from Sigma Aldrich, St. Louis, MO, and the anti-FoxP3-PE-Cy5 (FJK-16s) mAb was purchased from eBiosciences, San Diego, CA. The anti-CD25 mAb (PC61) used to inactivate $CD4^+CD25^+$ Tregs *in vivo* (Malek et al. 1983) was obtained from Exbio, Prague, Czech Republic. Purified rat IgG mAb (Sigma-Aldrich) was used as isotype control in all experiments. RPMI 1640 tissue culture medium supplemented with 2 mM glutamine was further supplemented with 10% heat-inactivated FCS and antibiotics. The α-GalCer-loaded CD1d (α-GalCer/CD1d) tetramer conjugated with PE was obtained from ProImmune, Oxford, UK.

Flow cytometry

Single-cell suspensions from tumour-draining lymph nodes (TDLN) or spleens were prepared by homogenization through a nylon mesh. Erythrocytes were osmotically lysed using Tris–NH_4Cl lysis buffer and lymphocytes were incubated with flow cytometry antibodies of choice. iNKT cells were identified as $CD3^+NK1.1^+$ or $CD3^+CD19$ α-GalCer/CD1d $tetramer^+$ whereas Tregs were defined as $CD4^+CD25^+$ or $CD25^+Foxp3^+$ and further analysed for $CD62L^+$ expression. Intracellular staining for Foxp3 was performed using Mouse Foxp3 Buffer Set (eBiosciences) according to the manufacturer's protocol. Expression of markers was measured with a LSRII device (BD Biosciences), and the acquired data were analysed using FlowJo software (Tree Star, Ashland, OR).

Tumour cell lines and monitoring of tumour growth

The TC-1 tumour cell line was established by transformation of primary BL/6 mouse lung cells with HPV16 E6/E7 and activated Ha-ras DNA (Lin et al. 1996). Mice were challenged with 5×10^4 TC-1 cells (s.c.). Mice in selected groups were treated with a single dose of 0.3 mg PC61 or rat IgG isotype control (administered i.p. 3 days before TC-1 cell transplantation) and on day 6 after tumour cell implantation, 10 μg α-GalCer or vehicle control was injected i.p. Tumour growth was further followed to examine the impact of different treatments on tumour growth. For analysis of the anti-CD25 effects on α-GalCer NKT cell activation in tumour-bearing mice, mice were injected with 1×10^6 TC-1 tumour cells and when tumours reached a size of 1.0 cm^2 mice were treated as described below. Blood samples were collected and sera stored for further analysis.

Measurement of cytokines

The concentrations of IFN-γ and IL-4 in sera were measured using BD Opt EIA ELISA kits (BD Biosciences) according to the manufacturer's protocol.

Reverse transcriptase quantitative PCR

Total RNA was extracted from splenocytes using RNeasy Mini Kit (Qiagen, Hilden, Germany) and 1 μg of RNA was transcribed into cDNA using the GeneAmp® RNA PCR kit (Applied Biosystems, Foster City, CA) utilizing a combination of random hexamers and oligo(dT) as primers. PCR primers were purchased from Generi Biotech, Hradec Kralove, Czech Republic. Real-time PCR was performed using FastStart SYBR Green Master (Roche, Penzberg, Germany) as described by the manufacturer and an LC480 cycler (Roche). The sequences of specific primers were: T-bet 5′-CAACAACCCCTTTGCCAAAG-3′ and 5′-TCCCCCAAGCAGTTGACAGT-3′, GATA-3 5′-CTGTGGGCTGTACTACAAGCTTCA-3′ and 5′-ACCCATGGCGGTGACCATGC-3′, IL-4 5′-GAGAGATCATCGGCATTTTGA-3′, 5′-TCTGTGGTGTTCTTCGTTGC-3′, IFNγ 5′-ATCTGGAGGAACTGGCAAAA-3′, 5′-TTCAAGACTTCAAAGAGTCTGAGG-3′, β-actin 5′-CCAGAGCAAGAGAGGTATCC-3′ and

5′-GAGTCCATCACAATGCCTGT-3′. Samples were analysed in triplicate and their relative gene expression was determined by normalizing expression of each target gene to β-actin expression and presented as a fold change related to the results from untreated controls. PCR efficiencies were calculated from amplification curves (Ramakers et al. 2003).

Statistical analysis

Statistical significance of cytokine, qPCR and flow cytometry assays was determined using two-sided Student's *t*-test for paired or unpaired data; two-tailed analysis was applied as determined by *F*-test, as appropriate. For statistical analysis of *in vivo* differences in tumour growth between treatment groups, single factor ANOVA, Bonferroni multiple-comparison test from the Number Cruncher Statistical System (Kaysville, UT) statistical package was applied. Differences between experimental and control samples with $P < 0.05$ were considered to be statistically significant.

Results

Efficacy of anti-CD25 mAb administration and Treg elimination and effects of α-GalCer treatment on Treg frequencies

Residual expression of the $CD4^+CD25^+$ population in regional lymph nodes or spleen was about 0.3% (drop from 3.9% in the lymph nodes and 2% in the spleens, respectively) after PC61 treatment. To test the $CD4^+CD25^+$ cell elimination and possible differential effects of PC61 on distinct sets of $CD25^+$ cells, characterized by the CD62L expression, spleen cells were analysed on day 6 after PC61 administration by FACS (Fig. 1a). In the spleens from PC61-treated animals a significant reduction of $CD4^+CD25^+CD62L^+$ as well as $CD4^+CD25^+CD62L^-$ cell frequencies was observed, as compared to control groups. Similar results were obtained in tumour draining lymph nodes (data not shown). As can be seen in Fig. 1b, a significant reduction of $CD4^+CD25^+CD62L^+$ as well as $CD4^+CD25^+CD62L^-$ populations was observed in DEREG mice after DT treatment, although in both populations more cells persisted, as compared to the PC61 treatments. To assess whether these populations really represent mostly $CD25^+CD4^+$ cells that persisted, and not repopulated cells, we have measured $CD4^+CD25^+$ population immediately after three DT doses when the $FoxP3^+$ cell population depletion was maximal (more than 98%); at this time point the $CD4^+CD25^+$ population dropped to 30%. This finding suggests that both subpopulations comprise $FoxP3^+$ Tregs and, on the other hand, anti-CD25 targets not only Tregs. Further, α-GalCer treatment had no significant effect on Treg frequencies in LN ($CD25^+FoxP3^+$ cell populations) while the decrease was observed in spleen (Fig. 1c and d). PC61 treatment, as well as $FoxP3^+$ cell depletion in DEREG mice resulted in the Treg cell decrease that was not further influenced by the α-GalCer treatments.

PC61 treatment inhibited NKT cell expansion after α-GalCer treatment, as well as CD69 downregulation on primed NKT cells

As can be seen in Fig. 2, PC61 administration 3 days prior to the α-GalCer treatment abrogated α-GalCer-induced expansion of NKT cells, while DT-induced Treg depletion in DEREG mice had no such effect. Further, it has been shown that murine Vα14i NKT cells expressed the early activation marker CD69 that is downregulated upon their priming with α-GalCer (Ikarashi et al. 2006). Our data, demonstrating that PC61 administration inhibited this decrease in mice, suggest that NKT cell priming is blocked by the anti-CD25 antibody (Fig. 3a). Control experiment on DEREG mice revealed no significant effect of the $FoxP3^+$ cell depletion on these processes (Fig. 3b). We have also demonstrated the increase of the $CD25^+$ subpopulation within NKT cells from spleen in α-GalCer-treated mice (Fig. 3c). Surprisingly, apart from the Treg cell population, we did not observe long lasting significant elimination of the $CD25^+$ population after the PC61 *in vivo* treatment in our setting. Unfortunately, we could not determine the portion of the $CD25^+$ NKT cells immediately or 24 h after α-GalCer treatments when the cytokine levels were measured due to the NKT cell T receptor downregulation so we could not exclude that the $CD25^+$ population represented activated cells that appeared later after activation.

Impaired induction of IFN-γ production upon α-GalCer treatment in the PC61-treated mice

Animals were challenged with 1×10^6 TC-1 tumour cells on day 0. On day 28, when the tumour reached 1.0 cm^2, animals were injected with either 0.5 mg PC61 or rat IgG isotype control followed by a single injection of 1 μg α-GalCer or vehicle on day 31 (Fig. 4a and b). As an α-GalCer-mediated activation of the immune system readout, cytokine responses (IFN-γ, IL-4) were measured in the blood serum 7 days before start of the treatment (day 21) and 2 h and 24 h after α-GalCer administration. Similar experiments were performed in naïve tumour-free animals. Cytokine levels were below the detection limit in both naïve and tumour-bearing animals before start of the treatment (not shown) as well as in animals treated with the vehicle, rat IgG isotype control or PC61. As previously reported, α-GalCer administration resulted in increased IL-4 and IFN-γ serum levels. Interestingly, we observed decreased levels of IFN-γ but not of IL-4 in animals pre-treated with PC61 compared to animals treated with IgG isotype at both time points analysed (2 h and 24 h). Naïve tumour-free PC61-treated animals showed the same defective IFNγ response to α-GalCer (not shown).

PC61 administration, not $FoxP3^+$ cell depletion, inhibited IFNγ production in α-GalCer treated mice

To investigate whether the Treg elimination or rather direct effect of the antibody on effector cells was crucial for the IFNγ and IL-4 production regulation upon iNKT cell activation, we compared the impacts of PC61 administration to the effects of DT-induced $FoxP3^+$ cell depletion in DEREG mice. Since the defects in α-GalCer-induced IFNγ responses upon PC61 were observed both in tumour-free and tumour-bearing mice, this set of experiments was performed on the tumour free-mice only. As can be seen in Fig. 4c, only PC61 administration in contrast to specific Treg depletion (DEREG mice) decreased the IFNγ levels in the blood of α-GalCer treated animals. This result strongly suggests that the direct PC61 effects on effector cells are responsible for the IFNγ inhibition rather than disturbed interactions with Tregs.

Similar results were obtained by the analysis of cytokine expression in the spleens at the RNA level. qRT-PCR showed induction of both IL-4 and IFNγ specific mRNA in the spleens of the α-GalCer-treated wild type and Treg-depleted animals, and only IFNγ mRNA was inhibited upon the PC61 administration (Fig. 5). These results were in agreement and correlated with the expression levels of Th1 or Th2 cell transcription factor T-bet and GATA-3, respectively. The treatment with α-GalCer induced significant upregulation of T-bet mRNA transcripts in splenocytes in comparison to IgG isotype and/or vehicle-treated animals. Spleen cells from the animals pre-treated with PC61 but not spleen cells from FoxP3 cell-depleted animals followed by α-GalCer showed impaired induction of T-bet expression compared to animals treated with the IgG isotype and α-GalCer. The expression of GATA-3 was not significantly hampered (Fig. 5).

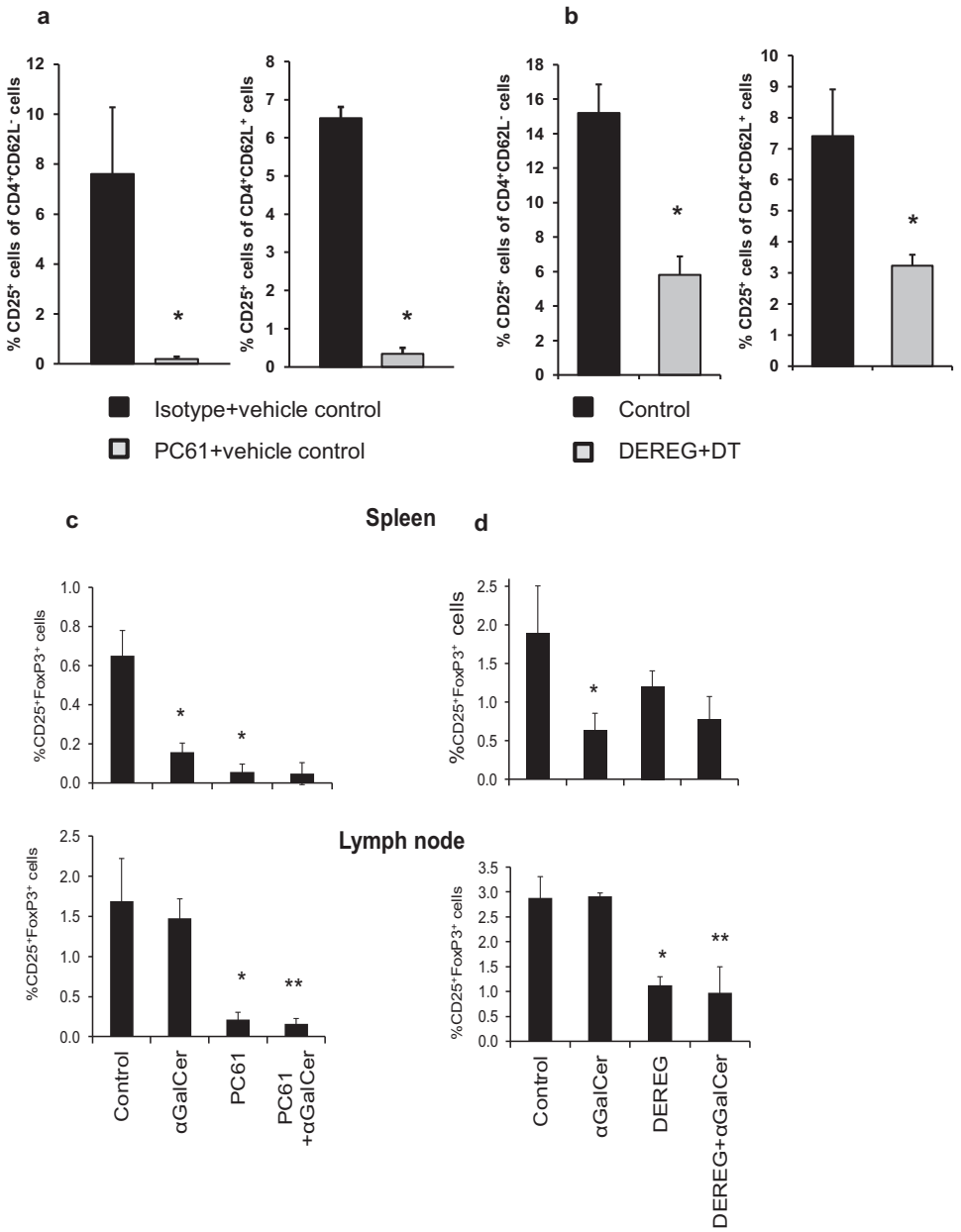


Fig. 1. Efficacy of $CD4^+CD25^+CD62L^-$ and $CD4^+CD25^+CD62L^+$ cell elimination and effects of α-GalCer treatment on Treg frequencies. Splenocytes from PC61-treated BL/6 mice were stained with anti-CD4, anti-CD25 (clone 7D4), and anti-CD62L and analysed by flow cytometry for the presence of $CD25^+$ cells on day 6 post treatment. (a) The percentages of $CD25^+$ cells among $CD4^+CD62L^-$ and $CD4^+CD62L^+$ splenocytes from both PC61 and IgG isotype control-treated groups are shown. Percentages are presented as the mean (± SD) of three or four individual mice. $^*P < 0.05$ vs. IgG isotype or control, two-sided Student's *t*-test. (b) Same analysis performed on control and DT-treated DEREG mice. (c) BL/6 mice were injected once i.p. with anti-CD25 mAb (0.5 mg PC61) or IgG isotype control, followed by either vehicle or α-GalCer (1 μg) treatment after 3 days. Single-cell suspensions were obtained from spleens and lymph nodes, harvested 3 days after vehicle control or α-GalCer treatment. Cells were stained with anti-CD25-PE (7D4), and anti-FoxP3-PE-Cy5 followed by flow cytometric analysis for the presence of $CD25^+Foxp3^+$ cells. (d) Analogical experiment using non-transgenic controls or DEREG mice. All groups of mice were injected on three consecutive days i.p. with 1 μg DT, followed by either vehicle or α-GalCer (1 μg) treatment after 24 h. Single-cell suspensions were obtained from spleens and lymph nodes, harvested 3 days after vehicle control or α-GalCer treatment. All experiments were repeated three times with similar results. $^*P < 0.05$ vs. control, $^{**}P < 0.05$ vs. α-GalCer treated group, two-sided Student's *t*-test.

No synergistic anti-tumour effect upon combination therapy using α-GalCer and PC61

Since our analysis revealed that anti-CD25 antibody can inhibit IFNγ production upon α-GalCer treatment, it was of interest to investigate the efficacy of combination therapy using the TC-1 tumour model (Fig. 6). As expected, both PC61 and α-GalCer treatments as monotherapy partially inhibited the tumour growth. However, no synergistic effect was observed in combination therapy.

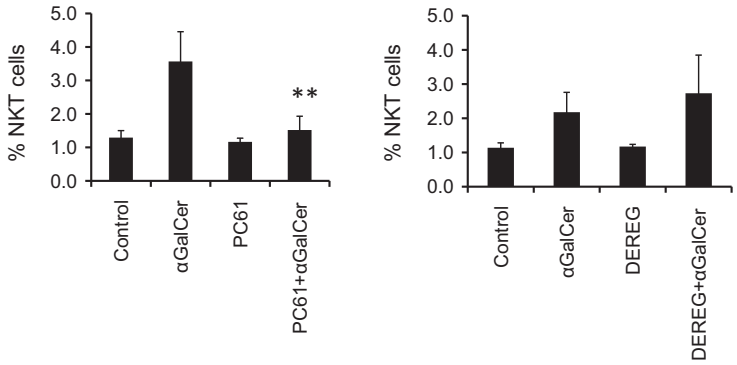


Fig. 2. PC61 but not Treg cell depletion abrogates NKT cell expansion after α-GalCer *in vivo* administration. *In vivo* experiment was performed in the same setting as that presented in Fig. 1. NKT cell population was determined in spleen as $CD3^+CD19^+$ α-GalCer/CD1d $tetramer^+$ cell population. Percentages are presented as the mean (± SD) of three individual mice. $^{**}P < 0.05$ vs. α-GalCer treated group, two-sided Student's *t*-test. Experiments were repeated three times yielding similar results.

Discussion

In this study, we evaluated the impacts of PC61, a prototypic anti-CD25 mAb commonly used to inactivate Tregs in murine models, *in vivo* administration on iNKT effector functions upon administration of α-GalCer, NKT cell T receptor ligand with a therapeutic potential. Subsequently, we investigated potential PC61 and α-GalCer additive/synergistic effects when used in combination anti-tumour immunotherapy.

We have evaluated the effects of the α-GalCer-mediated NKT cell activation on Tregs. Administration of α-GalCer did not increase the percentages of spleen or TDLN-residing Tregs or on the intracellular Foxp3 expression. This finding was in agreement with the recently published data using murine tumour models

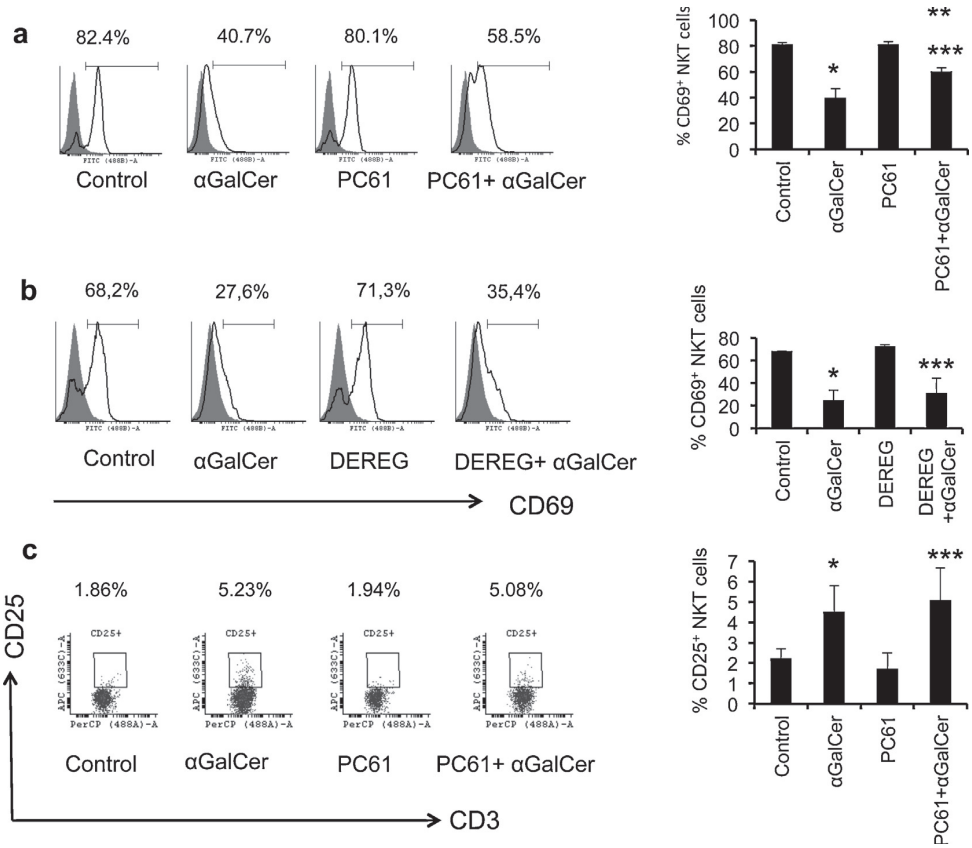


Fig. 3. NKT cell phenotype following PC61 and α-GalCer treatment. BL6 or DEREG mice were pretreated with PC61 (0.3 mg) or IgG isotype 3 days prior to α-GalCer (1 μg) or vehicle control administration (a) or with DT on three consecutive days before α-GalCer administration, 24 h after the last DT dose (b) and CD69 expression on spleen NKT cells was analysed after 3 days. (c) CD25 expression on NKT cells from mice treated with PC61 and α-GalCer. Experiments were repeated two or three times with similar results. Percentages are presented as the mean (± SD) of three individual mice. $^{*}P < 0.05$ vs. control, $^{**}P < 0.05$ vs. α-GalCer treated group, $^{***}P < 0.05$ vs. PC61 or DEREG group two-sided Student's *t*-test.

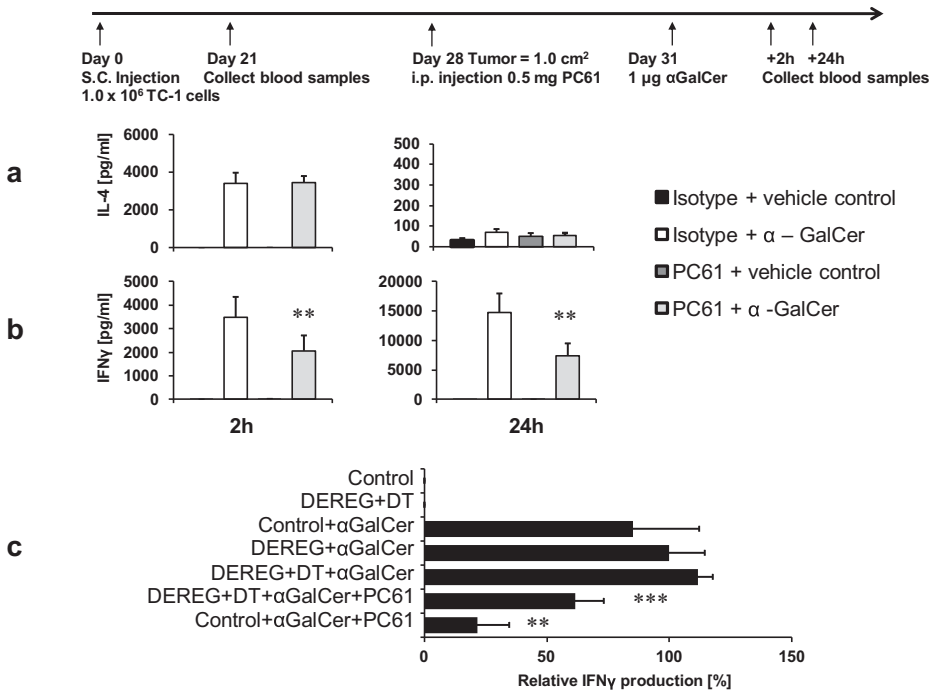


Fig. 4. Cytokine production following PC61 and α-GalCer treatment. Tumour-bearing animals (tumour size ≈ 1.0 cm^2) were pretreated with PC61 (0.5 mg) or IgG isotype 3 days prior to α-GalCer (1 μg) or vehicle control administration (a and b). Two hours (for peak IL-4 production) or 24 h (for peak IFNγ production) after injection, blood samples were collected and the levels of IL-4 (a) and IFNγ (b) in serum were measured by ELISA. Each column represents the means (± SD) of three independent experiments. $^{**}P < 0.05$ vs. IgG isotype + α-GalCer, two-sided Student's *t*-test. (c) Identical experiment repeated in tumour naïve control and DEREG mice treated with DT. Blood samples were collected 24 h (for peak IFNγ production) after injection of α-GalCer, and the levels of IFNγ in serum were measured by ELISA. Relative IFNγ production levels are related to the level found in α-GalCer-treated DEREG mice (define as 100%). $^{**}P < 0.05$ vs. IgG isotype + α-GalCer, and $^{***}P < 0.05$ vs. DEREG + DT + α-GalCer, two-sided Student's *t*-test. The experiments were repeated three times with similar results.

(Petersen et al. 2010) but distinct from the results obtained in some autoimmune disease models, in which activated iNKT cells play a regulatory role (Liu et al. 2005; Ly et al. 2006). We observed decreased cellularity of Treg cells in spleens from α-GalCer-treated animals, as compared to untreated animals. We have not explained this phenomenon but we hypothesized that the effect of the i.p. administration in the spleen vicinity can take place.

PC61 efficiently decreased the number of $CD4^+CD25^+CD62L^+$ Tregs. In addition, PC61 also inhibited and/or depleted activated $CD4^+CD25^+CD62L^-$ cells, which is in agreement with previous findings that this antibody can inhibit expansion of tumour antigen-specific T cells (Curtin et al. 2008) and demonstrates the capacity of the anti-CD25 antibody to target effector T cells. However, depletion of $CD4^+CD25^+CD62L^-$ cells seen in PC61-treated mice was also observed in DT-treated DEREG mice, suggesting that in both CD62-positive and negative $CD4^+CD25^+$ spleen cell subpopulations FoxP3-positive Tregs prevailed.

Tregs have been described to suppress proliferation, cytokine release and cytotoxic activity of iNKT cells by cell-contact-dependent mechanisms (La Cava et al. 2006). On the other hand, as human α-GalCer-activated iNKT cells express CD25 and their function can be regulated by IL-2 (Bessoles et al. 2008), administration of anti-CD25 antibodies can also affect directly activated iNKT cells, besides disturbing Tregs function. We have documented that anti-CD25 mAb administration prior to the α-GalCer treatment inhibits NKT cell expansion, as well as CD69 downregulation. In control experiments using DEREG mice we excluded the role of the Treg cell depletion in this inhibition. CD25 expression has been documented on a NKT cell subpopulation of the B6 mice origin (Kim et al. 2006). We demonstrated a small fraction of $CD25^+$ cells on spleen NKT cells which was increased in spleens from α-GalCer-treated animals. However, we cannot exclude that anti-CD25 mAb inhibitory effects on the α-GalCer-mediated activation can at least partially be attributed to the effects of other than NKT $CD25^+$ populations of activated effector cells.

We demonstrated that α-GalCer-mediated priming of the immune response in animals pre-treated with PC61 showed defective IFN-γ production, while no difference was observed in IL-4 production. This finding was independent of tumour-related suppressive factors as similar results were observed in either naïve or tumour-bearing animals. Further, the analysis of Th1 and Th2 immune response master regulators T-bet and GATA-3 transcription (Szabo et al. 2000; Wei et al. 2007) in spleen cells as the *T-bet* but not *GATA3* expression by α-GalCer was decreased upon anti-CD25 treatment.

To assess whether the described effects on α-GalCer-mediated iNKT-activation of immune response could be attributed to direct Treg inactivation by PC61 or to the anti-CD25 antibody effects on other $CD25^+$ effector cell populations including activated iNKT cells, we repeated the experiments in DEREG mice. As expected, DT treatment of DEREG mice induced depletion of $FoxP3^+$ cells and the residual population of the $CD25^+$ cells was higher, as compared to their proportion after the anti-CD25 treatment. Since no inhibition of α-GalCer induction of IFNγ production was observed in $FoxP3^+$ cell-depleted mice, as compared to the anti-CD25 mAb-treated mice, we have concluded that the negative effects of this treatment on the immune response levels can be attributed rather to the elimination of $CD25^+$ effector cells than to the Treg depletion/inactivation. As the above-mentioned studies that suggested a role for Tregs in iNKT-cell mediated alleviation of autoimmune

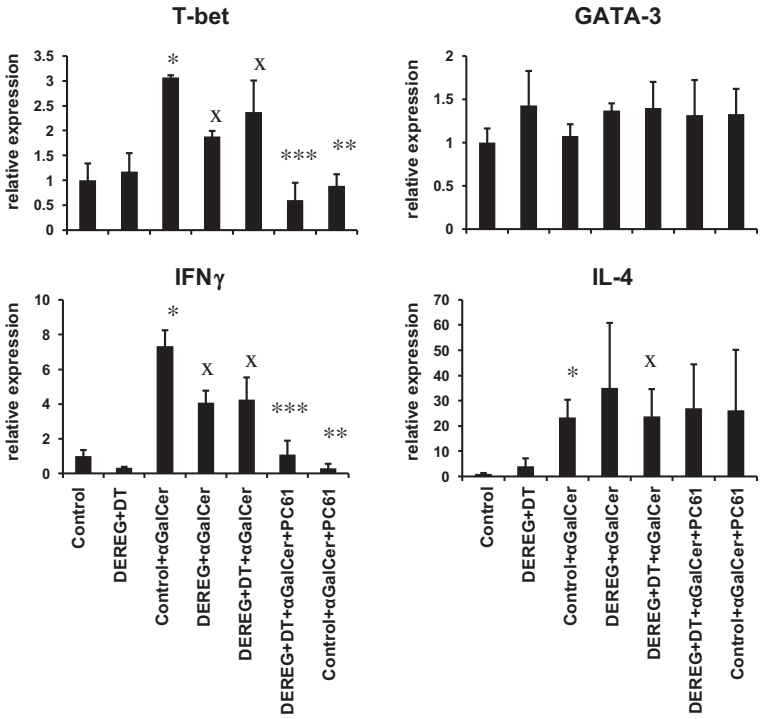


Fig. 5. IFNγ, IL-4, T-bet and GATA-3 mRNA expression following PC61 and α-GalCer treatment in control and DEREG mice. Total splenic RNA from naïve tumour-free animals were isolated and analysed independently following 24 h of stimulation with α-GalCer, with or without pre-treatment with PC61. The mRNA expression of IFNγ, T-bet, IL-4 and GATA-3 is shown as the relative value normalized to β-actin expression and related to the values from untreated controls; $n = 3–4$, mean (± SD). $^{*}P < 0.05$ vs. IgG isotype + vehicle control, $^{x}P < 0.05$ vs. DEREG + DT, $^{**}P < 0.05$ vs. IgG isotype + α-GalCer, and $^{***}P < 0.05$ vs. DEREG + DT + α-GalCer, two-sided Student's *t*-test. The experiments were repeated three times with similar results.

diseases were based on anti-CD25 antibody treatments, we cannot exclude that also in these models the effects could have been partially attributed to direct targeting of effector (activated iNKT) cells. It is of interest why IFNγ but not IL-4 production was decreased in anti-CD25-treated animals. NKT cells are the main source of IFNγ and IL-4 at the early phase after the α-GalCer-mediated activation (Crowe et al. 2003; Moreno et al. 2008). IL-4 induction is very rapid reaching its peak approximately 2 h after NKT cell stimulation while IFNγ production is delayed several hours reaching a plateau 12–24 h after activation (Miyamoto et al. 2001; Yu et al. 2005a). It has been shown, using a murine model of murine cytomegalovirus-mediated NKT cell activation, that CD25 upregulation appeared by 20 h, peaked 1.5 days post-infection and correlated with IFNγ production by NKT cells which peaked at day 1.5 post-infection (Wesley et al. 2008). Thus it is possible that IFNγ producing cells are preferential targets for anti-CD25 antibody, as compared to the IL-4 producing cells.

Our results are in agreement with previous findings that, in some settings, the efficacy of immunotherapy or resistance to infection were not augmented but rather impaired (Curtin et al. 2008; Couper et al. 2009) and strongly support the idea that the beneficial $CD25^+$ cell depletion effects on the efficacy of immunotherapy might be controversial and proper timing or dosing of anti-CD25 mAb administration is of particular importance. Indeed, Curtin et al. (2008) have shown in an intracranial glioblastoma BL/6 murine model that $CD25^+$ cell depletion 15 days after tumour implantation inhibited the tumour growth, but treatment 24 days after tumour implantation inhibited clonal expansion of effector cells and blocked T cell-dependent tumour regression. On the other hand, $CD25^+$ cell population depletion induced potent immune responses even against established immunogenic tumours heavily infiltrated with $CD4^+$ $CD25^+$ cells, probably because of their high immunogenicity and a potential of $CD8^+$ cells to mediate anti-tumour immunity without the help of activated effector CD4 cells (Yu et al. 2005b).

Various reports have shown that α-GalCer successfully protected against tumour challenge and inhibited tumour growth in mice (Nakagawa et al. 2000; Chang et al. 2007). Previously, we have demonstrated the tumour inhibitory effects of both iNKT cell activation and $CD25^+$ cell depletion including a setting of the treatment of minimal residual tumour disease, using the TC-1 model for HPV16-associated tumours (Símová et al. 2006, 2010). Thus the question on potential augmentation of the treatment efficacy by combination therapy arose and it was of interest to see whether anti-CD25 positive or negative effects on NKT cell-mediated induction of anti-tumour immunity prevail. In this study, we did not see any additive/synergic effects of anti-GalCer PC61mAb combined treatment. This is in agreement with the *in vitro* data discussed above, as well as with our previous preliminary result showing no synergy of the PC61 treatment combined with another iNKT cell T receptor ligand, β-galactosylceramide (Símová et al. 2010). Our study was performed on BL/6 mice and the results may be dependent on the particular mouse strain used and may have been different on distinct tumour or mouse models used. It has been recently shown that the treatment with PC61 before *Toxoplasmosa gondii* infection eliminated most of Treg cells in BALB/c mice while in C57BL/6J, other activated cell subsets were targeted (Tenorio et al. 2011). This finding coincides with another study in which anti-CD25 antibody was capable to deplete effector T cell populations, to inhibit IFNγ production, as well as to reduce weight loss and liver pathology in the inflammatory phase of *T. gondii* infection in a C57BL murine model (Couper et al. 2009).

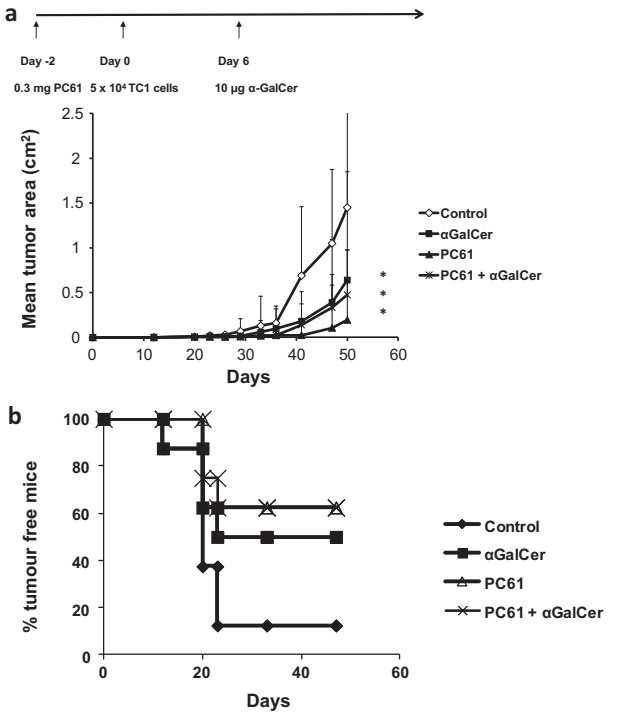


Fig. 6. Tumour growth after subcutaneous implantation of TC-1 cells in mice treated with PC61 and/or α-GalCer. A single dose of 0.3 mg PC61 or rat IgG isotype control was administered i.p. 3 days before TC-1 implantation. TC-1 tumour cells (5×10^4 cells) were implanted subcutaneously. Six days after implantation, 10 μg α-GalCer or vehicle control was injected i.p. to animals pre-treated with PC61 or isotype control. (a) Tumour growth was followed by measuring the area of each tumour every 3–4 days. Average values for each group are shown ($n = 8$ in each group). (b) Kaplan–Meier graph presenting the percentage of the tumour-free animals at different time-points. Similar results were obtained in two independent experiments. $^*P < 0.05$ vs. IgG isotype + vehicle control.

Recently, two studies focused on anti-CD25$^+$ cell depletion and iNKT cell activation have been performed in tumour models. In the first one, administration of PC61 increased the therapeutic effects of α-GalCer in a BALB/c metastatic murine mammary model 4T1 (Hong et al. 2010). Our opposite findings may probably be explained by the different mouse strain and tumour model used. The beneficial effects of the combination therapy were seen in the later stages of the tumour growth and also in a percentage of lung metastases. Potent anti-B16 tumour response was observed in another recent study, after combination of the CD25$^+$ cell depletion and immunization with dendritic cells loaded with tumour tissue and anti-GalCer (Petersen et al. 2010). We can hypothesize that the observed difference in the therapeutic outcome, as compared to our results, could be explained by different therapeutic setting when mice were challenged with the tumour cells 9 days after PC61 administration and 7 days after DC immunization. However, also in this experiment, a mild decrease of IFNγ production in the serum upon PC61-treatment was observed. Since no induction of iNKT cell activation upon PC61 treatment and no accumulation of Tregs upon α-GalCer treatments were observed, iNKT cell activation and Treg cell depletion increase the vaccination efficacy independently.

Collectively, our data demonstrate direct inhibitory effects of the anti-CD25 mAb on iNKT cell-mediated immune response, which may result in the lack of additive effect or synergy in induction of antitumour immunity based on iNKT cell activation and Treg cell depletion. The results underscore the necessity of proper timing in anti-CD25 treatments to avoid the detrimental effects of the treatment on effector cells. They also strongly suggest that alternative methods of Treg targeting, such as low doses of cyclophosphamide (Motoyoshi et al. 2006; Ohkura et al. 2011), may be more appropriate than anti-CD25 usage. Indeed, a strong potential to improve therapeutic vaccination against established melanoma by selective depletion of FoxP3$^+$ cells has been demonstrated in DEREG mice (Klages et al. 2010). Our data also document the limitations of the use of anti-CD25 antibodies in the studies focused on Treg interactions with effector cells.

Acknowledgements

We are grateful to Dr. T.C. Wu, who kindly provided the TC-1 cells. This work was supported by grant Nos. 301/07/1410 and 301/10/2174 from the Grant Agency of the Czech Republic and grant No. AV0Z50520514 from the Academy of Sciences of the Czech Republic. The Dutch Cancer Society granted R.R. a student fellowship for this work. R.R. would like to thank Mr. R. Gomes Casseres, Mr. S. Martina and Mr. Gregory Elias for their generous financial support. The authors would like to thank Dr. S. Yamano (KIRIN Brewery, Gunma, Japan) for providing KRN7000 (α-GalCer). Authors are grateful to Ms. Renata Tureckova and Ms. Marie Maleckova for technical assistance and Dr. Sarka Takacova for editorial help.

References

Baru, A.M., Hartl, A., Lahl, K., Krishnaswamy, J.K., Fehrenbach, H., Yildirim, A.O., et al., 2010. Selective depletion of Foxp3$^+$ Treg during sensitization phase aggravates experimental allergic airway inflammation. Eur. J. Immunol. 40, 2259–2266.

Bessoles, S., Fouret, F., Dudal, S., Besra, G.S., Sanchez, F., Lafont, V., 2008. IL-2 triggers specific signaling pathways in human NKT cells leading to the production of pro- and anti-inflammatory cytokines. J. Leukoc. Biol. 84, 224–233.

Burdin, N., Brossay, L., Kronenberg, M., 1999. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. Eur. J. Immunol. 29, 2014–2025.

Chang, Y.J., Huang, J.R., Tsai, Y.C., Hung, J.T., Wu, D., Fujio, M., et al., 2007. Potent immune-modulating and anticancer effects of NKT cell stimulatory glycolipids. Proc. Natl. Acad. Sci. U.S.A. 25, 10299–10304.

Couper, K.N., Lanthier, P.A., Perona-Wright, G., Kummer, L.W., Chen, W., Smiley, S.T., et al., 2009. Anti-CD25 antibody-mediated depletion of effector T cell populations enhances susceptibility of mice to acute but not chronic *Toxoplasma gondii* infection. J. Immunol. 182, 3985–3994.

Crowe, N.Y., Uldrich, A.P., Kyparissoudis, K., Hammond, K.J., Hayakawa, Y., Sidobre, S., et al., 2003. Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. J. Immunol. 171, 4020–4027.

Curtin, J.F., Candolfi, M., Fakhouri, T.M., Liu, C., Alden, A., Edwards, M., et al., 2008. Treg depletion inhibits efficacy of cancer immunotherapy: implications for clinical trials. PLoS ONE 4, 1983.

Fujii, S., Shimizu, K., Smith, C., Bonifaz, L., Steinman, R.M., 2003. Activation of natural killer T cells by α-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. J. Exp. Med. 198, 267–279.

Godfrey, D.I., MacDonald, H.R., Kronenberg, M., Smyth, M.J., Van Kaer, L., 2004. NKT cells: what's in a name? Nat. Rev. Immunol. 4, 231–237.

Hong, H., Gu, Y., Zhang, H., Simon, A.K., Chen, X., Wu, C., et al., 2010. Depletion of CD4$^+$CD25$^+$ regulatory T cells enhances natural killer T cell-mediated anti-tumour immunity in a murine mammary breast cancer model. Clin. Exp. Immunol. 159, 93–99.

Ikarashi, Y., Iizuka, A., Koshidaka, Y., Heike, Y., Takaue, Y., Yoshida, M., et al., 2006. Phenotypical and functional alterations during the expansion phase of invariant Valpha14 natural killer T (Valpha14i NKT) cells in mice primed with alpha-galactosylceramide. Immunology 116, 30–37.

Kim, H.Y., Kim, S., Chung, D.H., 2006. FcgammaRIII engagement provides activating signals to NKT cells in antibody-induced joint inflammation. J. Clin. Invest. 116, 2484–2492.

Klages, K., Mayer, C.T., Lahl, K., Loddenkemper, C., Teng, M.W., Ngiow, S.F., et al., 2010. Selective depletion of Foxp3$^+$ regulatory T cells improves effective therapeutic vaccination against established melanoma. Cancer Res. 70, 7788–7799.

Ko, K., Yamazaki, S., Nakamura, K., Nishioka, T., Hirota, K., Yamaguchi, T., et al., 2005. Treatment of advanced tumours with agonistic anti-GITR mAb and its effects on tumour-infiltrating Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells. J. Exp. Med. 202, 885–891.

Kronenberg, M., 2005. Toward an understanding of NKT cell biology: progress and paradoxes. Annu. Rev. Immunol. 23, 877–900.

La Cava, A., Van Kaer, L., Fu Dong, S., 2006. CD4$^+$CD25$^+$ Tregs and NKT cells: regulators regulating regulators. Trends Immunol. 7, 322–327.

Lahl, K., Loddenkemper, C., Drouin, C., Freyer, J., Arnason, J., Eberl, G., et al., 2007. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J. Exp. Med. 204, 57–63.
Lin, K.Y., Guarnieri, F.G., Staveley-O'Carroll, K.F., Levitsky, H.I., August, J.T., Pardoll, D.M., et al., 1996. Treatment of established tumours with a novel vaccine that enhances major histocompatibility class II presentation of tumour antigen. Cancer Res. 56, 21–26.
Liu, R., La Cava, A., Bai, X.F., Jee, Y., Price, M., Campagnolo, D.I., et al., 2005. Cooperation of invariant NKT cells and CD4+CD25+ T regulatory cells in the prevention of autoimmune myasthenia. J. Immunol. 12, 7898–7904.
Ly, D., Mi, Q.S., Hussain, S., Delovitch, T.L., 2006. Protection from type 1 diabetes by invariant NK T cells requires the activity of CD4+CD25+ regulatory T cells. J. Immunol. 6, 3695–3704.
Malek, T.R., Robb, R.J., Shevach, M.J., 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor–ligand complex. Proc. Natl. Acad. Sci. U.S.A. 80, 5694–5698.
Miyamoto, K., Miyake, S., Yamamura, T., 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing Th2 bias of natural killer T cells. Nature 413, 531–534.
Miyara, M., Sakaguchi, S., 2007. Natural regulatory T cells: mechanisms of suppression. Trends Mol. Med. 13, 108–116.
Moreno, M., Molling, J.W., von Mensdorff-Pouilly, S., Verheijen, R.H., von Blomberg, B.M., van den Eertwegh, A.J., et al., 2008. In vitro expanded human invariant natural killer T-cells promote functional activity of natural killer cells. Clin. Immunol. 1, 145–154.
Motoyoshi, Y., Kaminoda, K., Saitoh, O., Hamasaki, K., Nakao, K., Ishii, N., et al., 2006. Different mechanisms for anti-tumour effects of low- and high-dose cyclophosphamide. Oncol. Rep. 16, 141–146.
Nakagawa, R., Serizawa, I., Motoki, K., Sato, M., Ueno, H., Iijima, R., et al., 2000. Antitumor activity of alpha-galactosylceramide. KRN7000, in mice with the melanoma B16 hepatic metastasis and immunohistological study of tumour infiltrating cells. Oncol. Res. 2, 51–58.
Nishikawa, H., Sakaguchi, S., 2010. Regulatory T cells in tumour immunity. Int. J. Cancer 127, 759–767.
Ohkura, N., Hamaguchi, M., Sakaguchi, S., 2011. FOXP3+ regulatory T cells: control of FOXP3 expression by pharmacological agents. Trends Pharmacol. Sci. 32, 158–166.
Parekh, V.V., Wilson, M.T., Olivares-Villagómez, D., Singh, A.K., Wu, L., Wang, C.R., et al., 2005. Glycolipid antigen induces long-term natural killer T cell anergy in mice. J. Clin. Invest. 115, 2572–2583.
Petersen, T.R., Sika-Paotonu, D., Knight, D.A., Dickgreber, N., Farrand, K.J., Ronchese, F., et al., 2010. Potent anti-tumour responses to immunization with dendritic cells loaded with tumour tissue and an NKT cell ligand. Immunol. Cell Biol. 88, 596–604.
Ramakers, C., Ruijter, J.M., Deprez, R.H., Moorman, A.F., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–66.
Sakaguchi, S., Setoguchi, R., Yagi, H., Nomura, T., 2006. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in self-tolerance and autoimmune disease. Curr. Top. Microbiol. Immunol. 305, 51–66.
Schabowsky, R.H., Madireddi, S., Sharma, R., Yolcu, E.S., Shirwan, H., 2007. Targeting CD4+CD25+FoxP3+ regulatory T-cells for the augmentation of cancer immunotherapy. Curr. Opin. Investig. Drugs 8, 1002–1008.
Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., et al., 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J. Exp. Med. 7, 1693–1700.
Seino, K., Fujii, S., Harada, M., Motohashi, S., Nakayama, T., Fujisawa, T., et al., 2005. Valpha14 NKT cell-mediated anti-tumour responses and their clinical application. Springer Semin. Immunopathol. 1, 65–74.
Silk, J.D., Hermans, I.F., Gileadi, U., Chong, T.W., Shepherd, D., Salio, M., et al., 2004. Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. J. Clin. Invest. 114, 1800–1811.
Símová, J., Bubeník, J., Bieblová, J., Rosalia, R.A., Fric, J., Reinis, M., 2006. Depletion of T(reg) cells inhibits minimal residual disease after surgery of HPV16-associated tumours. Int. J. Oncol. 6, 1567–1571.
Símová, J., Indrová, M., Bieblová, J., Mikysková, R., Bubeník, J., Reinis, M., 2010. Therapy for minimal residual tumour disease: beta-galactosylceramide inhibits the growth of recurrent HPV16-associated neoplasms after surgery and chemotherapy. Int. J. Cancer 126, 2997–3004.
Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., Glimcher, L.H., 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 6, 655–669.
Taylor, P.A., Panoskaltsis-Mortari, A., Swedin, J.M., Lucas, P.J., Gress, R.E., Levine, B.L., et al., 2004. L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. Blood 12, 3804–3812.
Tenorio, E.P., Fernández, J., Olguín, J.E., Saavedra, R., 2011. Depletion with PC61 mAb before *Toxoplasma gondii* infection eliminates mainly Tregs in BALB/c mice, but activated cells in C57BL/6J mice. FEMS Immunol. Med. Microbiol. 62, 362–367.
Thèze, J., Alzari, P.M., Bertoglio, J., 1996. Interleukin 2 and its receptors: recent advances and new immunological functions. Immunol. Today 17, 481–486.
Thompson, C., Powrie, F., 2004. Regulatory T cells. Curr. Opin. Pharmacol. 4, 408–414.
Wei, J., Duramad, O., Perng, O.A., Reiner, S.L., Liu, Y.J., Qin, F.X., 2007. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. Proc. Natl. Acad. Sci. U.S.A. 46, 18169–18174.
Wesley, J.D., Tessmer, M.S., Chaukos, D., Brossay, L., 2008. NK cell-like behavior of Valpha14i NK T cells during MCMV infection. PLoS Pathog 4e, 1000106.
Yu, K.O., Im, J.S., Molano, A., Dutronc, Y., Illarionov, P.A., Forestier, C., et al., 2005a. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. Proc. Natl. Acad. Sci. U.S.A. 102, 3383–3388.
Yu, P., Lee, Y., Liu, W., Krausz, T., Chong, A., Schreiber, H., et al., 2005b. Intratumor depletion of CD4+ cells unmasks tumour immunogenicity leading to the rejection of late-stage tumours. J. Exp. Med. 201, 779–791.

7. Diskusia

Nádorové bunky vyvinuli mechanizmus, akými môžu unikať imunitnému dohľadu a ktorými unikajú pred rozpoznaním bunkami imunitného systému. Medzi mechanizmy imunosupresie patrí napríklad znížená tvorba nádorových antigénov, indukcia expresie inhibičných molekúl, produkcia inhibičných cytokínov, zvýšená tvorba indolamín 2,3-dioxygenázy a nereagovanie na apoptotické signály alebo zvýšená hladina komponentov s imunosupresívnym účinkom, ako sú regulačné T-lymfocyty, myeloidné supresorové bunky alebo nezrelé dendritické bunky. Spracovanie a prezentácia antigénu je podstatnou zložkou v imunitnom dohľade (Setiadi et al., 2005). Rozpoznanie nádorových buniek bunkami efektorovými sa deje pomocou spracovania a prezentácie endogénnych nádorových antigénov v komplexe s molekulami MHC I. Zníženie expresie MHC I na povrchu nádorových buniek je bežný spôsob, ktorým nádorové bunky unikajú imunitnému dohľadu (Garrido et al., 1997; Bubeník, 2003; Reiniš, 2010; Seliger, 2012). Expresia týchto génov môže byť obnovená pomocou IFN-γ (Gabathuler et al., 1994; Seliger et al., 2000) alebo pomocou inhibítorov DNA metyltransferáz, 5-aza-2'-deoxycytidinu (DAC) alebo 5-azacytidinu (5-azaC; 5AC). Hlavným cieľom práce bolo detailne popísať úlohu epigenetických regulácií, najmä metylácie DNA v reverzibilných mechanizmoch, ktorými nádorové bunky unikajú špecifickej imunite a tiež monitorovanie génov s imunosupresívnym účinkom v nádorových a imunitných bunkách počas rastu a liečby nádoru.

Bolo popísané, že IFN-γ zvyšuje expresiu génov antigén-prezentujúcej mašinérie tým, že zvyšuje acetyláciu histónu 3. O účinku IFN-γ na zmenu metylačného statusu promótorových oblastí génov antigén-prezentujúcej mašinérie veľa informácií zatiaľ nie je. Bolo zistené, že indukcia expresie indolamín 2,3-dioxygenázy (IDO)-1 je spojená s DNA demetyláciou oblasti promótora génu pre *IDO-1* (Xue et al., 2012). Ošetrenie TAP-1 deficientných buniek pomocou IFN-γ zvyšuje acetyláciu histónu 3 a aktívnu transkripciu génu *TAP-1*. Setiadi a kol. (Setiadi et al., 2007) takto popísali nový mechanizmus, ktorým IFN-γ zvyšuje expresiu génu *TAP-1*. Christova a kol. (Christova et al., 2007) popísali zmeny v štruktúre chromatínu celého lokusu MHC I, ktorá bola indukovaná IFN-γ. V tejto oblasti sa nachádzajú gény rodiny *TAP* a aj *LMP*. Súčasné poznatky v danej problematike sme obohatili o dáta, ktoré dokazujú, že IFN-γ môže plniť úlohu epigenetického agensu a navodzuje demetyláciu DNA mnohých génov, s dôrazom kladeným najmä na gény antigén-prezentujúcej mašinérie. Našou prácou sme prispeli zistením, že DNA demetylácia

sprostredkovaná IFN-γ je závislá na signalizácii cez JAK/STAT dráhu, pretože inhibítor Janusových kináz blokoval demetyláciu DNA a indukciu expresie MHC I na povrchu buniek. Ďalej DNA demetylácia sprostredkovaná IFN-γ je dynamickejšia v porovnaní s demetyláciou DNA indukovanou pomocou inhibítora DNA metyltransferáz 5-azacytidinom, čo naznačuje že proces demetylácie je aktívny a nezávislý od replikácie DNA na rozdiel od DNA demetylácie indukovanej 5-azacytidinom, ktorá je od replikácie DNA závislá a vyžaduje inkorporáciu liečiva do DNA a blokuje metyláciu nascentného reťazca DNA kvôli inhibícii metyltransferáz (Creusot et al., 1982). Bolo popísané, že aj ďalší cytokín dokáže navodiť demetyláciu DNA, konkrétne TGF-β spôsobil aktívnu demetyláciu DNA a tak obnovil expresiu tumor-supresorového génu $p15^{ink4b}$ (Thillainadesan et al., 2012). Nakoniec DNA demetylácia sprostredkovaná IFN-γ je asociovaná s acetyláciou histónu H3 v oblasti promótorov génov antigén-prezentujúcej mašinérie.

Epigenetické mechanizmy v regulácii prezentácie antigénu nádorovými bunkami boli tiež analyzované v experimentoch s inhibítormi DNA metyltransferáz. Zhodnotili sme efekt inhibítora DNA metyltransferáz 5-azacytidinu na MHC I deficientné a pozitívne nádory. Optimalizovali sme terapeutický protokol založený na kombinácii imunoterapie experimentálnych nádorov v myšiach s inhibítorom DNA metyltransferáz. Výsledky naznačujú, že pre maximálny terapeutický výsledok, zvýšená citlivosť nádorových buniek k imunitnému systému po chemoterapii pomocou epigenetického agensu spôsobená zvýšením expresie MHC I na nádorových bunkách, by mohla byť kombinovaná s aktiváciou imunitnej odpovede pomocou imunoterapie. Zvýšenie expresie MHC I pravdepodobne nie je jediným dôvodom, prečo sa nádorové bunky stanú citlivejšími k eliminácii imunitným systémom, vzhľadom na to, že 5-azacytidin ovplyvní expresiu množstva génov. Bolo tiež popísané, že gén *IRF8* býva často epigeneticky umlčaný pri nádorových ochoreniach a inhibítory DNA metyltransferáz dokážu zvýšiť citlivosť nádorových buniek k apoptóze cez zvýšenú hladinu IRF8 (Yamashita et al., 2010). Pri nádoroch explantovaných zo zvierat ošetrených 5-azacytidinom sme pozorovali zvýšenú povrchovú expresiu MHC I na povrchu nádorových buniek, ktorá bola asociovaná so zvýšenou expresiou génov antigén-prezentujúcej mašinérie a génov dráhy IFN-γ (*STAT1, IRF1* a *IRF8*). Toto zvýšenie pri nádoroch explantovaných zo zvierat ošetrených 5-azacytidinom korešpondovalo s DNA demetyláciou promótorových oblastí génov antigén-prezentujúcej mašinérie. Naše neopublikované výsledky tiež naznačujú zvýšenie expresie

faktorov IRF vplyvom 5-azacytidinu a demetyláciu DNA v promótorových oblastiach génov *IRF1* a *IRF8* sprostredkovanú IFN-γ a 5-azacytidinom.

Únik nádorových buniek môže byť sprostredkovaný aj tvorbou imunosupresívneho stavu v rámci mikroprostredia nádoru (Radoja et al., 2000; Radoja and Frey, 2000). Nádorové bunky sú schopné produkovať imunosupresívne faktory a tieto majú ďalší vplyv na funkciu imunitného systému (Chambers et al., 2003). Sú to napríklad imunosupresívne faktory ako vaskulárny endoteliálny rastový faktor (VEGF), transformujúci rastový faktor (TGF-β), galectin alebo indolamín 2,3-dioxygenáza (IDO) (Vesely et al., 2011). Regulačné T-lymfocyty a myeloidné supresorové bunky sú dve hlavné imunosupresívne populácie buniek, ktoré majú významnú úlohu v inhibícii ochrannej protinádorovej odpovede (Schreiber et al., 2011). Nádorom indukovaná imunosupresia patrí medzi kritické mechanizmy, akými nádory unikajú imunitnému dohľadu. V ďalšej časti práce sme sa preto zaoberali monitorovaním imunosupresívneho účinku mikroprostredia nádorov a jeho ovplyvnením chemoterapiou s dôrazom na účinky 5-azacytidinu. Myeloidné supresorové bunky majú tiež významnú úlohu v úniku nádorových buniek imunitnému systému a veľmi prispievajú k nádorom indukovanej imunosupresii. Bolo popísané, že MDSC indukujú poruchy T buniek cez produkciu napríklad TGF-β, ROS, NO a najmä Arg-1 (Kusmartsev and Gabrilovich, 2006). Arg-1 je marker imunosupresívneho prostredia a hlavným producentom Arg-1 sú MDSC. Touto zvýšenou expresiou Arg-1 indukujú anergiu T buniek depléciou L-arginínu, čo narúša proliferáciu T buniek a produkciu cytokínov (Rodriguez et al., 2007). Inhibícia Arg-1 môže znovu obnoviť správnu funkciu T buniek a indukovať protinádorovú odpoveď (Rodriguez et al., 2004). V predkladanej práci sme sa najskôr snažili preskúmať podrobne fenotyp a mechanizmus akumulácie MDSC po chemoterapii s CY (úloha prozápalových cytokínov) a následne identifikovať možnú imunoterapiu s cieľom zoslabiť indukovanú imunosupresiu. V práci sme porovnali fenotyp a funkciu akumulovaných MDSC v slezine po terapii s CY (CY-MDSC) s tými, kde sú MDSC akumulované počas rastu nádoru TC-1 (TU-MDSC). Do porovnania sme začlenili aj MDSC, ktoré sú akumulované počas rastu nádoru TC-1 pri ošetrení s CY, čo podporuje ich ďalšiu akumuláciu v slezine (CYTU-MDSC). MDSC akumulované po podaní CY vykazujú viac monocytárny fenotyp ako MDSC akumulované rastúcim nádorom TC-1 a keď porovnáme ich fenotyp s ich nižšiou expresiou imunosupresívnych génov, vykazujú CY-MDSC menšie supresívne vlastnosti. Fenotyp a funkcia CYTU-MDSC populácie boli medzi populáciami CY-MDSC a TU-MDSC. Podporili sme využitie induktora diferenciácie, kyseliny ATRA alebo imunostimulačného cytokinu IL-12 pre upravenie

akumulácie MDSC po chemoterapii s CY. Takáto modulácia MDSC kyselinou ATRA alebo cytokínom IL-12 počas chemoterapie nádorov môže zvýšiť protinádorový efekt daného chemoterapeutického agensu. Ďalšie pokusy zaoberajúce sa MDSC priniesli nové poznatky o imunomodulačných vlastnostiach 5-azacytidinu. Sľubný antagonista akumulácie MDSC, chemoterapeutické činidlo 5-azacytidin, bol úspešne použitý v terapeutických experimentoch pri kombinovanej chemoterapii a imunoterapii. Naše výsledky naznačujú, že 5-azacytidin okrem priameho protinádorového efektu, znižuje percento MDSC akumulovaných v mikroprostredí nádoru a slezine počas rastu nádoru a chemoterapie s cyklofosfamidom. Táto skutočnosť môže byť prospešná pre výsledok danej chemoterapie.

Zdá sa, že 5-azacytidin môže ovplyvniť imunosupresiu rôznymi spôsobmi. Zatiaľ čo tým, že potlačí MDSC by mal prispieť k potlačeniu imunosupresie, svojím vplyvom na expresiu génu *FoxP3* môže aktivovať regulačné T-lymfocyty a tým imunosupresiu indukovať (Lal et al., 2009).

V posledných rokoch sa objavuje čoraz viac štúdií, ktoré zaznamenali zvýšenú hladinu regulačných T-lymfocytov pri nádorových ochoreniach (Facciabene et al., 2012; Whiteside et al., 2012) a táto skutočnosť je často zodpovedná za slabú protinádorovú efektorovú odpoveď a tak je ohrozená a znížená protinádorová imunita (Elkord et al., 2010; Nishikawa and Sakaguchi, 2010). Deplécia alebo inaktivácia regulačných T-lymfocytov použitím špecifickej protilátky v kombinácii s imunostimuláciou by mohla byť dobrou metódou v protinádorovej imunoterapii a tiež v kombinácii s terapiou inhibítormi DNA metyltransferáz. Preto sme sa v ďalšej časti práce venovali deplécii regulačných T-lymfocytov pomocou protilátky anti-CD25 mAb PC61 a jej ďalšiemu efektu v imunoterapii nádorov, ktorý zacieľuje NKT bunky po ich aktivácii pomocou ligandu α-galaktosylceramidu. Sledovali sme efekt protilátky anti-CD25 mAb PC61 na α-galaktosylceramidom sprostredkovanú aktiváciu iNKT buniek a tiež účinnosť kombinácie protilátky PC61 a α –GalCer proti nádorom TC-1. Zistili sme, že α-galaktosylceramidom aktivovaná imunitná odpoveď po ošetrení s protilátkou PC61, vykazuje porušenú produkciu IFN-γ. Negatívny efekt tohto ošetrenia na hladinu imunitnej odpovede by mohol byť spôsobený skôr elimináciou $CD25^+$ efektorových buniek ako inaktiváciou/depléciou regulačných T-lymfocytov. CD25 je možné detekovať na povrchu aktivovaných myšacích a ľudských NKT buniek (Bessoles et al., 2008; Kim et al., 2006) a CD25 je tiež antigénom pre protilátku PC61. Protilátka PC61 ruší aktiváciu NKT buniek a inhibuje ich proliferáciu a produkciu IFN-γ aktivovanými NKT bunkami. Naše dáta limitujú použitie protilátky

anti-CD25 v štúdiách zameraných na interakciu regulačných T-lymfocytov s efektorovými bunkami.

V našej práci sme priniesli nové informácie o zmene metylačného statusu promótorových oblastí génov antigén-prezentujúcej mašinérie účinkom IFN-γ. Súčasné poznatky v danej problematike sme obohatili o dáta, ktoré naznačujú, že IFN-γ môže plniť úlohu epigenetického agensu a navodzuje demetyláciu DNA mnohých génov, s dôrazom kladeným najmä na gény antigén-prezentujúcej mašinérie. DNA demetylácia sprostredkovaná IFN-γ je závislá na signalizácii cez JAK/STAT dráhu, je dynamickejšia v porovnaní s demetyláciou DNA indukovanou pomocou inhibítora DNA metyltransferáz 5-azacytidinom a v neposlednom rade je asociovaná s acetyláciou histónu H3 v oblasti promótorov génov antigén-prezentujúcej mašinérie. Zhodnotili sme efekt inhibítora DNA metyltransferáz 5-azacytidinu na MHC I deficientné a pozitívne nádory. Optimalizovali sme terapeutický protokol založený na kombinácii imunoterapie s inhibítorom DNA metyltransferáz a získali sme nové poznatky o imunomodulačných schopnostiach 5-azacytidinu. Podporili sme využitie induktora diferenciácie, kyseliny ATRA alebo imunostimulačného cytokinu IL-12 pre upravenie akumulácie MDSC po chemoterapii CY. Takáto modulácia MDSC kyselinou ATRA alebo cytokínom IL-12 počas chemoterapie nádorov môže zvýšiť protinádorový efekt daného chemoterapeutického agensu. Naše výsledky ďalej naznačujú, že aj 5-azacytidin znižuje percento MDSC akumulovaných v mikroprostredí nádoru a slezine počas rastu nádoru a chemoterapie s cyklofosfamidom. Táto skutočnosť môže byť prospešná pre výsledok danej chemoterapie. Keďže chemo- a imunoterapia môže indukovať negatívnych regulátorov imunitného systému (regulačné T-lymfocyty), je cieľom ustanoviť vhodnú terapiu, ktorá by kombinovala liečbu s ošetrením, ktoré má anti-imunosupresívne vlastnosti, ako napríklad deplécia regulačných T-lymfocytov. Aj napriek tomu, že použitie protilátky proti CD25 inhibuje regulačné T-lymfocyty, pozorovali sme priamy účinok na populáciu efektorových, IFN-γ produkujúcich buniek (NKT bunky). Naše dáta limitujú použitie protilátky anti-CD25 v štúdiách zameraných na interakciu regulačných T-lymfocytov s efektorovými bunkami.

8. Záver

Celkovo výsledky projektu, ktoré boli zahrnuté do predkladanej dizertačnej práce sú dôležité pre lepšie pochopenie mechanizmov, ktorými nádorové bunky unikajú špecifickej imunite a pre optimalizáciu kombinovaných chemo-imunoterapeutických stratégií berúcich ohľad na status MHC I na neopláziách. Dokumentujeme, že ošetrenie MHC I deficientných nádorov IFN-γ indukuje demetyláciu DNA promótorových oblastí génov dôležitých pre prezentáciu antigénu a tiež použitie epigenetických modifikátorov môže obnoviť expresiu MHC I a tak môžu zviditeľniť nádory pre imunitný systém. Naše dáta poskytujú informácie o chemoterapii pomocou diferenciačných liečiv, prednostne pri použití v kombinácii s ďalšími liečivami pre dosiahnutie nízkeho imunosupresívneho rozsahu mikroprostredia nádoru. Dáta poskytujú dôkazy, že mimo známych cieľov epigenetických agensov alebo imunoregulačných protilátok musia byť zvážené aj nešpecifické alebo nepriame účinky počas terapie.

Z výsledkov vyplýva:

1. IFN-γ môže plniť úlohu epigenetického agensu, ktorý zvyšuje expresiu génov potrebných pre prezentáciu antigénu cez DNA demetyláciu.
2. Aplikácia epigenetických agensov zlepšila odpoveď MHC I deficientných nádorov k imunoterapii s CpG ODN alebo s IL-12 produkujúcou bunkovou vakcínou, imunoterapeutický efekt bol aspoň čiastočne sprostredkovaný CD8+ bunkami.
3. Využitie kyseliny ATRA alebo IL-12 pre dosiahnutie zmien v akumulácii MDSC po chemoterapii s CY s dôrazom kladeným na preukázanie ich protinádorového efektu.
4. Epigenetický modifikátor 5AC je sľubným cytostatickým agensom, ktorý ovplyvňuje MDSC akumulujúce sa počas rastu nádorov alebo pri liečbe nádorov s CY a 5AC je schopný redukovať percento MDSC akumulovaných v mikroprostredí nádoru a slezine počas rastu nádoru a chemoterapie s CY.
5. Podanie protilátky proti CD25 (PC61) používanej pri deplécii regulačných T-lymfocytov narušilo aktiváciu NKT buniek.

9. Zoznam citovanej literatúry

Alimonti J, Zhang QJ, Gabathuler R, Reid G, Chen SS, Jefferies WA. TAP expression provides a general method for improving the recognition of malignant cells in vivo. *Nat. Biotechnol.* 2000;**18**(5):515–20.

Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J. Immunol.* 2001;**166**(1):678–89.

Angulo I, de las Heras FG, García-Bustos JF, Gargallo D, Muñoz-Fernández MA, Fresno M. Nitric oxide-producing CD11b(+)Ly-6G(Gr-1)(+)CD31(ER-MP12)(+) cells in the spleen of cyclophosphamide-treated mice: implications for T-cell responses in immunosuppressed mice. *Blood* 2000;**95**(1):212–20.

Antequera F, Bird A. Number of CpG islands and genes in human and mouse. *Proc. Natl. Acad. Sci. USA* 1993;**90**(24):11995–9.

Antequera F. Structure, function and evolution of CpG island promoters. *Cell Mol. Life Sci.* 2003;**60**(8):1647–58.

Araujo FD, Knox JD, Szyf M, Price GB, Zannis-Hadjopoulos M. Concurrent replication and methylation at mammalian origins of replication. *Mol. Cell Biol.* 1998;**18**(6):3475–82.

Bailey JA, Carrel L, Chakravarti A, Eichler EE. Molecular evidence for a relationship between LINE-1 elements and X chromosome inactivation: the Lyon repeat hypothesis. *Proc. Natl. Acad. Sci. USA* 2000;**97**(12):6634–9.

Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. *Nat. Rev. Immunol.* 2002;**2**(6):401–9.

Bartlett NW, Buttigieg K, Kotenko SV, Smith GL. Murine interferon lambdas (type III interferons) exhibit potent antiviral activity in vivo in a poxvirus infection model. *J. Gen. Virol.* 2005;**86**(Pt 6):1589–96.

Bass KK, Mastrangelo MJ. Immunopotentiation with low-dose cyclophosphamide in the active specific immunotherapy of cancer. *Cancer Immunol. Immunother.* 1998;**47**(1):1–12.

Baylin SB. DNA methylation and gene silencing in cancer. *Nat. Clin. Pract. Oncol.* 2005;**2**(Suppl 1):S4–11.

Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet.* 2000;**16**(4):168–74.

Berger SL. Histone modifications in transcriptional regulation. *Curr. Opin. Genet. Dev.* 2002;**12**(2):142–8.

Bernstein BE, Kamal M, Lindblad-Toh K, Bekiranov S, Bailey DK, Huebert DJ, McMahon S, Karlsson EK, Kulbokas EJ 3rd, Gingeras TR, Schreiber SL, Lander ES. Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell* 2005;**120**(2):169–81.

Bessoles S, Fouret F, Dudal S, Besra GS, Sanchez F, Lafont V. IL-2 triggers specific signaling pathways in human NKT cells leading to the production of pro- and anti-inflammatory cytokines. *J. Leukoc. Biol.* 2008;**84**(1):224–33.

Bestor TH, Ingram VM . Two DNA methyltransferases from murine erythroleukemia cells: Purification, sequence specificity, and mode of interaction with DNA. *Proc. Natl. Acad. Sci. USA* 1983;**80**:5559–63.

Bird A, Taggart M, Frommer M, Miller OJ, Macleod D. A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. *Cell* 1985;**40**(1):91–9.

Bird A. DNA methylation patterns and epigenetic memory. *Genes & Dev.* 2002;**16**:6–21.

Bird AP. DNA methylation and the frequency of CpG in animal DNA. *Nucleic Acids Research* 1980;**8**(7):1499–504.

Bock C, Paulsen M, Tierling S, Mikeska T, Lengauer T, Walter J. CpG island methylation in human lymphocytes is highly correlated with DNA sequence, repeats, and predicted DNA structure. *PLoS Genet.* 2006;**2**(3):e26.

Borden EC, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR, Stark GR. Interferons at age 50: past, current and future impact on biomedicine. *Nat. Rev. Drug Discov.* 2007;**6**(12):975–90.

Bourc'his D, Bestor TH. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature* 2004;**431**(7004):96–9.

Bronte V., Serafini P., Apolloni E., Zanovello P. Tumor-induced immune dysfunctions caused by myeloid suppressor cells. *J. Immunother.* 2001;**24**:431–46.

Brown CJ, Willard HF. The human X-inactivation centre is not required for maintenance of X-chromosome inactivation. *Nature* 1994;**368**(6467):154–6.

Bubenik J. Tumour MHC class I downregulation and immunotherapy (Review). *Oncol. Rep.* 2003;**10**:2005–8.

Carninci P, Sandelin A, Lenhard B, Katayama S, Shimokawa K, Ponjavic J, Semple CA, Taylor MS, Engström PG, Frith MC, Forrest AR, Alkema WB, Tan SL,Plessy C, Kodzius R, Ravasi T, Kasukawa T, Fukuda S, Kanamori-Katayama M, Kitazume Y, Kawaji H, Kai C, Nakamura M, Konno H, Nakano K, Mottagui-Tabar S, Arner P, Chesi A, Gustincich S, Persichetti F, Suzuki H, Grimmond SM, Wells CA, Orlando V, Wahlestedt C, Liu ET, Harbers M, Kawai J, Bajic VB,Hume DA, Hayashizaki Y. Genome-wide analysis of mammalian promoter architecture and evolution. *Nat. Genet.* 2006;**38**(6):626–35.

Cohen NM, Kenigsberg E, Tanay A. Primate CpG islands are maintained by heterogeneous evolutionary regimes involving minim al selection. *Cell* 2011;**145**(5):773–86.

Collins M, Ling V, Carreno BM. The B7 family of immune-regulatory ligands. *Genome Biology* 2005;**6**:223.

Cortellino S, Xu J, Sannai M, Moore R, Caretti E, Cigliano A, Le Coz M, Devarajan K, Wessels A, Soprano D, Abramowitz LK, Bartolomei MS, Rambow F, Bassi MR, Bruno T, Fanciulli M, Renner C, Klein-Szanto AJ, Matsumoto Y, Kobi D, Davidson I, Alberti C, Larue L, Bellacosa A. Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. *Cell* 2011;**146**(1):67–79.

Coulondre C, Miller JH, Farabaugh PJ, Gilbert W. Molecular basis of base substitution hotspots in Escherichia coli. *Nature* 1978;**274**(5673):775–80.

Creusot F, Acs G, Christman JK. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. *J. Biol. Chem.* 1982;**257**:2041–8.

Csankovszki G, Panning B, Bates B, Pehrson JR, Jaenisch R. Conditional deletion of Xist disrupts histone macroH2A localization but not maintenance of X inactivation. *Nat. Genet.* 1999;**22**(4):323–4.

Curiel TJ, Wei S, Dong H, Alvarez X, Cheng P, Mottram P, Krzysiek R, Knutson KL, Daniel B, Zimmermann MC, David O, Burow M, Gordon A, Dhurandhar N,Myers L, Berggren R, Hemminki A, Alvarez RD, Emilie D, Curiel DT, Chen L, Zou W. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nature Med* 2003;**9**:562–67.

Daurkin I, Eruslanov E, Vieweg J, Kusmartsev S. Generation of antigen-presenting cells from tumor-infiltrated CD11b myeloid cells with DNA demethylating agent 5-aza-2'-deoxycytidine. *Cancer Immunol. Immunother.* 2010;**59**(5):697–706.

Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol. Immunother.* 2009;**58**(1):49–59.

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat. Immunol.* 2002;**3**(11):991–8.

Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat. Rev. Immunol.* 2006;**6**(11):836–48.

Dunn GP, Sheehan KC, Old LJ, Schreiber RD. IFN unresponsiveness in LNCaP cells due to the lack of JAK1 gene expression. *Cancer Res.* 2005;**65**(8):3447–53.

Eberharter A, Becker PB. Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. *EMBO Rep.* 2002;**3**(3):224–9.

Eberharter A, Ferreira R, Becker P. Dynamic chromatin: concerted nucleosome remodelling and acetylation. *Biol. Chem.* 2005;**386**(8):745–51.

Eccleston A, Cesari F, Skipper M. Transcription and epigenetics. *Nature* 2013;**502**(7472):461.

Eccleston A, DeWitt N, Gunter Ch, Marte B, Nath D. Introduction Epigenetics. *Nature* 2007;**447**:395.

Elkord E, Alcantar-Orozco EM, Dovedi SJ, Tran DQ, Hawkins RE, Gilham DE. T regulatory cells in cancer: recent advances and therapeutic potential. *Expert. Opin. Biol. Ther.* 2010; **10**(11):1573–86.

Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. *Nat. Rev. Cancer* 2006;**6**(4):259–69.

Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002;**21**:5427–40.

Esteller M. Epigenetic changes in cancer. *F1000 Biol. Rep.* 2011;**3**:9.

Esteller M. Epigenetics in cancer. *N. Engl. J. Med.* 2008;**358**:1148–59.

Estève PO, Chin HG, Pradhan S. Human maintenance DNA (cytosine-5)-methyltransferase and p53 modulate expression of p53-repressedpromoters. *Proc. Natl. Acad. Sci. U S A* 2005; **102**(4):1000–5.

Facciabene A, Motz GT, Coukos G. T-regulatory cells: key players in tumor immune escape and angiogenesis. *Cancer Res.* 2012;**72**(9):2162–71.

Fatemi M, Hermann A, Pradhan S, Jeltsch A. The activity of the murine DNA methyltransferase Dnmt1 is controlled by interaction of the catalytic domain with the N-terminal part of the enzyme leading to an allosteric activation of the enzyme after binding to methylated DNA. *J. Mol. Biol.* 2001;**309**(5):1189–99.

Fujita N, Watanabe S, Ichimura T, Tsuruzoe S, Shinkai Y, Tachibana M, Chiba T, Nakao M. Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation- based transcriptional repression. *J. Biol. Chem.* 2003;**278**(26):24132–8.

Fuks F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat. Genet.* 2000;**24**(1):88–91.

Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J. Biol. Chem.* 2003;**278**(6):4035–40.

Fuks F, Burgers WA, Godin N, Kasai M, Kouzarides T. Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. *EMBO J.* 2001;**20**(10):2536–44.

Fulda S, Debatin KM. 5-Aza-20-deoxycytidine and IFN-gamma cooperate to sensitize for TRAIL-induced apoptosis by upregulating caspase-8. *Oncogene* 2006;**25**:5125–33.

Gabathuler R, Reid G, Kolaitis G, Driscoll J, Jefferies WA. Comparison of cell lines deficient in antigen presentation reveals a functional role for TAP-1 alone in antigen processing. *J. Exp. Med.* 1994;**180**:1415–25.

Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nature Reviews Immunology* 2009;**9**:162–74.

Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. *J. Immunol.* 2001;**166**(9):5398–406.

Gabrilovich, D. I., V. Bronte, S. H. Chen, M. P. Colombo, A. Ochoa, S. Ostrand-Rosenberg, and H. Schreiber. The terminology issue for myeloid-derived suppressor cells. *Cancer Res.* 2007;**67**:425; author reply 426.

Garcia-Lora A, Martinez M, Algarra I, Gaforio JJ, Garrido F. MHC class I-deficient metastatic tumor variants immunoselected by T lymphocytes originate from the coordinated downregulation of APM components. *Int. J. Cancer* 2003;**106**:521–7.

Gardiner-Garden M, Frommer M. CpG Islands in vertebrate genomes. *Journal of Molecular Biology* 1987;**196**(2):261–282.

Garrido F, Cabrera T, Aptsiauri N. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy (Review). *Int. J. Cancer* 2010;**127**:249–56.

Garrido F, Ruiz-Cabello F, Cabrera T, Perez-Villar JJ, Lopez-Botet M, Duggan-Keen M, Stern PL. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol. Today* 1997;**18**:89–95.

Geiman TM, Sankpal UT, Robertson AK, Zhao Y, Zhao Y, Robertson KD. DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system. *Biochem. Biophys. Res. Commun.* 2004;**318**(2):544–55.

Glozak MA, Seto E. Histone deacetylases and cancer. *Oncogene* 2007;**26**(37):5420–32.

Goll MG, Kirpekar F, Maggert KA, Yoder JA, Hsieh CL, Zhang X, Golic KG, Jacobsen SE, Bestor TH. Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science* 2006;**311**(5759):395–8.

Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. *Annu. Rev. Biochem.* 2005;**74**:481–514.

Grant PA. A tale of histone modifications. *Genome Biol.* 2001;**2**(4):REVIEWS0003.

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA. Epigenetic reprogramming in mouse primordial germ cells. *Mech. Dev.* 2002;**117**(1-2):15–23.

Hajkova P: Epigenetic reprogramming in mouse germ cells; Dissertation, Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I , publiziert am 05.03.2004, 121. urn:nbn:de:kobv:11-10021220

Hata K, Okano M, Lei H, Li E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development* 2002;**129**(8):1983–93.

Hayakawa Y, Takeda K, Yagita H, Smyth MJ, van Kaer L, Okumura K, Saiki I. IFN-γ–mediated inhibition of tumor angiogenesis by natural killer T-cell ligand, α-galactosylceramide. *Blood* 2002;**100**(5): 1728–33.

Hedges DJ, Batzer MA. From the margins of the genome: mobile elements shape primate evolution. *Bioessays* 2005;**27**(8):785–94.

Hellebrekers DM, Castermans K, Viré E, Dings RP, Hoebers NT, Mayo KH, Oude Egbrink MG, Molema G, Fuks F, van Engeland M, Griffioen AW. Epigenetic regulation of tumor endothelial cell anergy: silencing of intercellular adhesion molecule-1 by histone modifications. *Cancer Res.* 2006;**66**(22):10770–7.

Hendrich B, Bird A. Identification and Characterization of a Family of Mammalian Methyl-CpG Binding Proteins. *Mol. Cell. Biol.* 1998;**18**(11):6538–47.

Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA* 1996;**93**(18):9821–6.

Hitt MM, Wu TL, Cohen G, Linn S. De novo and maintenance DNA methylation by a mouse plasmacytoma cell DNA methyltransferase. *J. Biol. Chem.* 1988;**263**(9):4392–9.

Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;**299**(5609):1057–61.

Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, Divino CM, Chen SH. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res.* 2006;**66**:1123–31.

Huang M, Sharma S, Mao JT, Dubinett SM. Non-small cell lung cancer-derived soluble mediators and prostaglandin E2 enhance peripheral blood lymphocyte IL-10 transcription and protein production. *J. Immunol.* 1996;**157**(12):5512–20.

Chambers WH, Rabinowich H, Herberman RB. Mechanisms of Immunosuppression. In: Kufe DW, Pollock RE, Weichselbaum RR, et al., editors. Holland-Frei *Cancer Medicine* **6th** edition. Hamilton (ON): BC Decker; 2003. Available from: http://www.ncbi.nlm.nih.gov/books/NBK12565/

Chen ZX, Riggs AD. DNA methylation and demethylation in mammals. *J. Biol. Chem.* 2011;**286**(21):18347–53.

Christova R, Jones T, Wu PJ, Bolzer A, Costa-Pereira AP, Watling D, Kerr IM, Sheer D. P-STAT1 mediates higher-order chromatin remodelling of the human MHC in response to IFNgamma. *J. Cell Sci.* 2007;**120**(Pt 18):3262–70.

Chuang LS, Ian HI, Koh TW, Ng HH, Xu G, Li BF. Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* 1997;**277**(5334):1996–2000.

Iwase S, Lan F, Bayliss P, de la Torre-Ubieta L, Huarte M, Qi HH, Whetstine JR, Bonni A, Roberts TM, Shi Y. The X-linked mental retardation gene SMCX/JARID1C defines a family of histone H3 lysine 4 demethylases. *Cell* 2007;**128**(6):1077–88.

Jäger E, Ringhoffer M, Karbach J, Arand M, Oesch F, Knuth A. Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and CD8+ cytotoxic-T-cell responses: evidence for immunoselection of antigen-loss variants in vivo. *Int. J. Cancer.* 1996;**66**(4):470–6.

Jeltsch A, Nellen W; Lyko F. Two substrates are better than one: dual specificities for Dnmt2 methyltransferases. *Trends Biochem. Sci.* 2006;**31**(6):306–8.

Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007;**128**:683–92.

Jones PA, Laird PW. Cancer-epigenetics comes of age. *Nature Genetics* 1999;**21**:163–7.

Kalkhoven E. CBP and p300: HATs for different occasions. *Biochemical Pharmacology* 2004;**68**(6):1145–55.

Kelly TL, Trasler JM. Reproductive epigenetics. *Clin. Genet.* 2004;**65**(4):247–60.

Khan AN, Gregorie CJ, Tomasi TB. Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. *Cancer Immunol. Immunother.* 2008;**57:**647–54.

Khong HT, Wang QJ, Rosenberg SA. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. *J. Immunother.* 2004;**27**(3):184–90.

Kim ES, Lee JK. Histone deacetylase inhibitors decrease the antigen presenting activity of murine bone marrow derived dendritic cells. *Cell. Immunol.* 2010;**262**(1):52–7.

Kim HY, Kim S, Chung DH. FcgammaRIII engagement provides activating signals to NKT cells in antibody-induced joint inflammation. *J. Clin. Invest.* 2006;**116**(9):2484–92.

Kim TH, Barrera LO, Zheng M, Qu C, Singer MA, Richmond TA, Wu Y, Green RD, Ren B. A high-resolution map of active promoters in the human genome. *Nature* 2005; **436**(7052): 876–80.

Klenova EM, Morse HC 3rd, Ohlsson R, Lobanenkov VV. The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. *Semin. Cancer Biol.* 2002;**12**(5):399–414.

Kohli RM, Zhang Y. TET enzymes, TDG and the dynamics of DNA demethylation. *Nature* 2013;**502**(7472):472–9.

Kondo E, Gu Z, Horii A, Fukushige S. The thymine DNA glycosylase MBD4 represses transcription and is associated with methylated p16(INK4a) and hMLH1 genes. *Mol. Cell Biol.* 2005;**25**(11):4388–96.

Krueger F, Kreck B, Franke A, Andrews SR. DNA methylome analysis using short bisulfite sequencing data. *Nat. Methods* 2012;**9**(2):145–51.

Kuang DM, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, Zheng L. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J. Exp. Med.* 2009;**206**(6):1327–37.

Kusmartsev S, Gabrilovich DI. Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer. *Cancer Metastasis* Rev. 2006;**25**:323–31.

Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE, Bromberg JS. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J. Immunol.* 2009;**182**(1):259–73.

Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, Hamann A, Wagner H, Huehn J, Sparwasser T. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* 2007;**204**(1):57–63.

Lahtz C, Pfeifer GP. Epigenetic changes of DNA repair genes in cancer. *J. Mol. Cell Biol.* 2011;**3**(1):51–8.

Laird PW. Early detection: The power and the promise of DNA methylation markers. *Nature Reviews Cancer* 2003;**3**:253–66.

Laird PW. Cancer epigenetics. *Hum. Mol. Genet.* 2005;**14**(Spec No 1):R65–76.

Lake RA, Robinson BWS. Immunotherapy and chemotherapy — a practical partnership. *Nature Reviews Cancer* 2005;**5**:397–405.

Legube G, Trouche D. Regulating histone acetyltransferases and deacetylases. *EMBO Rep.* 2003;**4**(10):944–7.

Levine AJ. p53, the Cellular Gatekeeper for Growth and Division. *Cell* 1997;**88**(3):323–331.

Li E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nature Reviews Genetics* 2002;**3**:662–73.

Ling Y, Sankpal UT, Robertson AK, McNally JG, Karpova T, Robertson KD. Modification of de novo DNA methyltransferase 3a (Dnmt3a) by SUMO-1 modulates its interaction with histonedeacetylases (HDACs) and its capacity to repress transcription. *Nucleic Acids Res.* 2004;**32**(2):598–610.

Liu Y, Zeng B, Zhang Z, Zhang Y, Yang R. B7-H1 on myeloid-derived suppressor cells in immune suppression by a mouse model of ovarian cancer. *Clin. Immunol.* 2008;**129**:471–81.

Liu WM, Maraia RJ, Rubin CM, Schmid CW. Alu transcripts: cytoplasmic localisation and regulation by DNA methylation. *Nucleic Acids Res.* 1994;**22**(6):1087–95.

Lund AH, van Lohuizen M. Epigenetics and cancer. *Genes & Dev.* 2004;**18**:2315–35.

Lyko F, Brown R. DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. *J. Natl. Cancer Inst.* 2005;**97**(20):1498–506.

Lyon MF. X-chromosome inactivation: a repeat hypothesis. *Cytogenet. Cell Genet.* 1998;**80**(1–4):133–7.

Maksakova IA, Goyal P, Bullwinkel J, Brown JP, Bilenky M, Mager DL, Singh PB, Lorincz MC. H3K9me3-binding proteins are dispensable for SETDB1/H3K9me3-dependent retroviral silencing. *Epigenetics Chromatin* 2011;**4**(1):12.

Manning J, Indrova M, Lubyova B, Pribylova H, Bieblova J, Hejnar J, Simova J, Jandlova T, Bubenik J, Reinis M. Induction of MHC class I molecule cell surface expression and epigenetic activation of antigen-processing machinery components in a murine model for human papilloma virus 16-associated tumours. *Immunology* 2008;**123:**218–27.

Martínez-Lorenzo MJ, Alava MA, Gamen S, Kim KJ, Chuntharapai A, Piñeiro A, Naval J, Anel A. Involvement of APO2 ligand/TRAIL in activation-induced death of Jurkat and human peripheral blood T cells. *Eur. J. Immunol.* 1998;**28**(9):2714–25.

Maxam AM, Gilbert W. A new method for sequencing DNA. *Proc. Nati. Acad. Sci. USA* 1977;**74**(2):560–4.

McDermott DF, Atkins MB. PD-1 as a potential target in cancer therapy. *Cancer Med.* 2013;**2**(5):662–73.

Miller OJ, Schnedl W, Allen J, Erlanger BF. 5-Methylcytosine localised in mammalian constitutive heterochromatin. *Nature* 1974;**251**(5476):636–7.

Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SI. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* 2001;**2 92**(5514):110–3.

Nencioni A, Beck J, Werth D, Grünebach F, Patrone F, Ballestrero A, Brossart P. Histone deacetylase inhibitors affect dendritic cell differentiation and immunogenicity. *Clin. Cancer Res.* 2007;**13**(13):3933–41.

Ng HH, Zhang Y, Hendrich B, Johnson CA, Turner BM, Erdjument-Bromage H, Tempst P, Reinberg D, Bird A. MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat. Genet.* 1999;**23**(1):58–61.

Niehrs C. Active DNA demethylation and DNA repair. *Differentiation* 2009;**77**:1–11.

Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. *Int. J. Cancer* 2010;**127**(4):759–67.

Norris DP, Patel D, Kay GF, Penny GD, Brockdorff N, Sheardown SA, Rastan S. Evidence that random and imprinted Xist expression is controlled by preemptive methylati on. *Cell* 1994;**77**(1):41–51.

O'Connell J, Bennett MW, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: cancer as a site of immune privilege. *Immunol. Today* 1999;**20**(1):46–52.

O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. *J. Exp. Med.* 1996;**184**(3):1075–82.

Okano M, Xie S, Li E. Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. *Nucl. Acids Res.* 1998;**26**(11):2536–40.

Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nature Reviews Cancer* 2012;**12**:252–64.

Park JJ, Omiya R, Matsumura Y, Sakoda Y, Kuramasu A, Augustine MM, Yao S, Tsushima F, Narazaki H, Anand S, Liu Y, Strome SE, Chen L, Tamada K. B7-H1/CD80 interaction is required for the induction and maintenance of peripheral T-cell tolerance. *Blood* 2010;**116**(8):1291–8.

Paulsen M, Tierling S, Walter J. DNA methylation and the mammalian genome. In: Tost J. *Epigenetics* Caister academic press, Norfolk, UK; 2008.

Peláez B, Campillo JA, López-Asenjo JA, Subiza JL. Cyclophosphamide induces the development of early myeloid cells suppressing tumor cell growth by a nitric oxide-dependent mechanism. *J. Immunol.* 2001;**166**(11):6608–15.

Peña PV, Davrazou F, Shi X, Walter KL, Verkhusha VV, Gozani O, Zhao R, Kutateladze TG. Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. *Nature* 2006;**442**(7098):100–3.

Petronzelli F, Riccio A, Markham GD, Seeholzer SH, Stoerker J, Genuardi M, Yeung AT, Matsumoto Y, Bellacosa A. Biphasic kinetics of the human DNA repair protein MED1 (MBD4), a mismatch-specific DNA N-glycosylase. *J. Biol. Chem.* 2000;**275**(42):32422–9.

Piccolo FM, Fisher AG. Getting rid of DNA methylation. *Trends Cell Biol.* 2014;**24**(2):136–43.

Plass C, Pfister SM, Lindroth AM, Bogatyrova O, Claus R, Lichter P. Mutations in regulators of the epigenome and their connections to global chromatin patterns in cancer. *Nat. Rev. Genet.* 2013;**14**(11):765–80.

Pradhan S, Bacolla A, Wells RD, Roberts RJ. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. *J. Biol. Chem.* 1999;**274**(46):33002–10.

Radoja S, Frey AB. Cancer-induced Defective Cytotoxic T Lymphocyte Effector Function: Another Mechanism How Antigenic Tumors Escape Immune-mediated Killing. *Molecular Medicine* 2000;**6**(6):465–79.

Radoja S, Rao TD, Hillman D, Frey AB. Mice Bearing Late-Stage Tumors Have Normal Functional Systemic T Cell Responses In Vitro and In Vivo. *The Journal of Immunology* 2000;**164**(5):2619–28.

Radoja S, Saio M, Frey AB. CD8+ tumor-infiltrating lymphocytes are primed for Fas-mediated activation-induced cell death but are not apoptotic in situ. *J. Immunol.* 2001;**166**(10):6074–83.

Raizis AM, Schmitt F, Jost JP. A Bisulfite Method of 5-Methylcytosine Mapping That Minimizes Template Degradation. *Analytical Biochemistry*1995;**226**(1):161–6.

Reiniš M. Immunotherapy of MHC class I-deficient tumors. *Future Oncol.* 2010;**6**:1577–89.

Restifo NP, Marincola FM, Kawakami Y, Taubenberger J, Yannelli JR, Rosenberg SA. Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. *J. Natl. Cancer Inst.* 1996;**88**(2):100–8.

Ritz U, Seliger B. The transporter associated with antigen processing (TAP): structural integrity, expression, function, and its clinical relevance. *Mol. Med.* 2001;**7**(3):149–58.

Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2 F-responsive promoters. *Nat. Genet.* 2000a;**25**(3):338–42.

Robertson KD, Keyomarsi K, Gonzales FA, Velicescu M, Jones PA. Differential mRNA expression of the human DNA methyltransferases (DNMTs) 1, 3a and 3b during the G(0)/G(1) to S phase transition in normal and tumor cells. *Nucleic Acids Res.* 2000b;**28**(10):2108–13.

Robertson KD. DNA methylation, methyltransferases, and cancer. *Oncogene* 2001;**20**:3139–55.

Rodriguez PC, Quiceno DG, Ochoa AC. L-Arginine availability regulates T-lymphocyte cell-cycle progression. *Blood* 2007;**109**:1568–73.

Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, Delgado A, Correa P, Brayer J, Sotomayor EM, Antonia S, Ochoa JB, Ochoa AC. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* 2004;**64**(16):5839–49.

Rollins RA, Haghighi F, Edwards JR, Das R, Zhang MQ, Ju J, Bestor TH. Large-scale structure of genomic methylation patterns. *Genome Res.* 2006;**16**(2):157–63.

Rothlein, R. D. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *Journal of Immunology* 1986;**137**(4):1270–4.

Sado T, Fenner MH, Tan SS, Tam P, Shioda T, Li E. X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation. *Dev. Biol.* 2000;**225**(2):294–303.

Saito Y, Friedman JM, Jones PA. The role of microRNAs in human cancer. In: Tost J. *Epigenetics* Caister academic press, Norfolk, UK; 2008.

Santos-Rosa H, Schneider R, Bannister AJ, Sherriff J, Bernstein BE, Emre NC, Schreiber SL, Mellor J, Kouzarides T. Active genes are tri-methylated at K4 of histone H3. *Nature* 2002;**419**(6905):407–11.

Seliger B, Wollscheid U, Momburg F, Blankenstein T, Huber C. Coordinate downregulation of multiple MHC class I antigen processing genes in chemicalinduced murine tumor cell lines of distinct origin. *Tissue Antigens* 2000;**56**:327–36.

Seliger B, Maeurer MJ, Ferrone S. TAP off--tumors on. *Immunol. Today.* 1997;**18**(6):292–9.

Seliger B. Novel insights into the molecular mechanisms of HLA class I abnormalities. *Cancer Immunol. Immunother.* 2012;**61**:249–54.

Setiadi AF, David MD, Seipp RP, Hartikainen JA, Gopaul R, Jefferies WA. Epigenetic control of the immune escape mechanisms in malignant carcinomas. *Mol. Cell. Biol.* 2007;**27:**7886–94.

Setiadi AF, Omilusik K, David MD, Seipp RP, Hartikainen J, Gopaul R, Choi KB, Jefferies WA. Epigenetic enhancement of antigen processing and presentation promotes immune recognition of tumors. *Cancer Res* 2008;**68:**9601–7.

Setiadi AF, David MD, Chen SS, Hiscott J, Jefferies WA. Identification of mechanisms underlying transporter associated with antigen processing deficiency in metastatic murine carcinomas. *Cancer Res.* 2005;**65**(16):7485–92.

Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of Apoptosis and Bcl-2 Expression by Prostaglandin E2 in Human Colon Cancer Cells. *Cancer Res.* 1998;**58**:362–6.

Shi L, Chen S, Yang L, Li Y. The role of PD-1 and PD-L1 in T-cell immune suppression in patients with hematological malignancies. *Journal of Hematology & Oncology* 2013;**6**:74.

Schreiber RD, Old LJ, Smyth MJ. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. *Science* 2011;**331**(6024):1565–70.

Schulz WA. L1 retrotransposons in human cancers. *J. Biomed. Biotechnol.* 2006;**2006**(1): Article ID 83672:1–12.

Sigalotti L, Coral S, Fratta E, Lamaj E, Danielli R, Di Giacomo AM, Altomonte M, Maio M. Epigenetic modulation of solid tumors as a novel approach for cancer immunotherapy. *Semin. Oncol.* 2005;**32**:473–8.

Sims JK, Magazinnik T, Houston SI, Wu S, Rice JC. Histone modifications and epigenetics. In: Tost J. *Epigenetics* Caister academic press, Norfolk, UK; 2008.

Sims RJ 3rd, Nishioka K, Reinberg D. Histone lysine methylation: a signature for chromatin function. *Trends Genet.* 2003;**19**(11):629–39.

Steding CE, Wu ST, Zhang Y, Jeng MH, Elzey BD, Kao C. The role of interleukin-12 on modulating myeloid-derived suppressor cells, increasing overall survival and reducing metastasis. *Immunology* 2011;**133**(2):221–38.

Struhl K. Histone acetylation and transcriptional regulatory mechanisms. *Genes & Dev.* 1998;**12**:599–606.

Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG, Baylin SB. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat. Genet.* 2002;**31**(2):141–9.

Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *PNAS.* 2002;**99**(6):3740–5.

Takeshima H, Suetake I, Shimahara H, Ura K, Tate S, Tajima S. Distinct DNA methylation activity of Dnmt3a and Dnmt3b towards naked and nucleosomal DNA. *J. Biochem.* 2006;**139**(3):503–15.

Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T, Tohyama M. Tumor Necrosis Factor Induces Bcl-2 and Bcl-x Expression through NFκB Activation in Primary Hippocampal Neurons. *J. Biol. Chem.* 1999;**274**:8531–8.

Teperek-Tkacz M, Pasque V, Gentsch G, Ferguson-Smith AC. Epigenetic reprogramming: is deamination key to active DNA demethylation? *Reproduction* 2011;**142**(5):621–32.

Thillainadesan G, Chitilian JM, Isovic M, Ablack JN, Mymryk JS, Tini M, Torchia J. TGF-β-dependent active demethylation and expression of the p15ink4b tumor suppressor are impaired by the ZNF217/CoREST complex. *Mol. Cell* 2012;**46**(5):636–49.

Tomasi TB, Magner WJ, Khan AN. Epigenetic regulation of immune escape genes in cancer. *Cancer Immunol. Immunother.* 2006;**55**:1159–84.

Tost J. Methods for the genome-wide and gene-specific analysis of DNA methylation levels and patterns. In: Tost J. *Epigenetics* Caister academic press, Norfolk, UK; 2008.

Van Emburgh BO, Robertson KD. DNA methyltransferases and methyl-CpG binding proteins as multifunctional regulators of chromatin structure and development in mammalian cells. In: Tost J. *Epigenetics* Caister academic press, Norfolk, UK; 2008.

Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural Innate and Adaptive Immunity to Cancer. *Annu. Rev. Immunol.* 2011;**29**:235–71.

Vigushin DM, Ali S, Pace PE, Mirsaidi N, Ito K, Adcock I, Coombes RC. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo. *Clin. Cancer Res.* 2001;**7**(4):971–6.

Vogel MC, Papadopoulos T, Müller-Hermelink HK, Drahovsky D, Pfeifer GP. Intracellular distribution of DNA methyltransferase during the cell cycle. *FEBS Lett.* 1988;**236**(1):9–13.

Vogelstein B, Lane D,Levine AJ. Surfing the p53 network. *Nature* 2000;**408**:307–310.

Weaver JR, Bartolomei MS. Chromatin regulators of genomic imprinting. *Biochim. Biophys. Acta.* 2014;**1839**(3):169–77.

Whiteside TL, Schuler P, Schilling B. Induced and natural regulatory T cells in human cancer. Expert. *Opin. Biol. Ther.* 2012;**12**(10):1383–97.

Wutz A, Jaenisch R. A Shift from Reversible to Irreversible X Inactivation Is Triggered during ES Cell Differentiation. *Molecular cell* 2000;**5**(4):695–705.

Xue ZT, Sjögren HO, Salford LG, Widegren B. An epigenetic mechanism for high, synergistic expression of indoleamine 2,3-dioxygenase 1 (IDO1) by combined treatment with zebularine and IFN-γ: potential therapeutic use in autoimmune diseases. *Mol. Immunol.* 2012;**51**(2):101–11.

Yamashita M, Toyota M, Suzuki H, Nojima M, Yamamoto E, Kamimae S, Watanabe Y, Kai M, Akashi H, Maruyama R, Sasaki Y, Yamano H, Sugai T, Shinomura Y, Imai K, Tokino T, Itoh F. DNA methylation of interferon regulatory factors in gastric cancer and noncancerous gastric mucosae. *Cancer Sci.* 2010;**101**(7):1708–16.

Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-α-activated vascular endothelium under flow. *Blood* 2005;**106**(2):584–92.

Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* 1997;**13**(8):335–40.

Yokochi T, Robertson KD. Preferential methylation of unmethylated DNA by Mammalian de novo DNA methyltransferase Dnmt3a. *J. Biol. Chem.* 2002;**277**(14):11735–45.

Yoo ChB, Jones PA. Epigenetic therapy of cancer: past, present and future. *Nature Reviews Drug Discovery* 2006;**5**:37–50.

Yu Z. Non-coding RNAs in gene regulation. In: Tost J. *Epigenetics* Caister academic press, Norfolk, UK; 2008.

Zaidi MR, Merlino G. The Two Faces of Interferon-γ in Cancer. *Clin. Cancer Res.* 2011;**17**(19):1–7.

Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, McDermott D, Quiceno D, Youmans A, O'Neill A, Mier J, Ochoa AC. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res.* 2005;**65**(8):3044–8.

Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A, Reinberg D. Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. *Genes Dev.* 1999;**13**(15):1924–35.

Zheng L, Fisher G, Miller RE, Peschon J, Lynch DH, Lenardo MJ. Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature* 1995;**377**(6547):348–51.

Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. *Nature Reviews Immunology* 2008;**8**:467–77.

10. Zoznam vlastných publikácií

Šímová J*, Polláková (Vlková) V*, Indrová M, Mikyšková R, Bieblová J, Štěpánek I, Bubeník J, Reiniš M. Immunotherapy augments the effect of 5-azacytidine on HPV16-associated tumours with different MHC class I-expression status. *Br. J. Cancer* 2011;**105**(10):1533–41. (IF2012= 5.082)
(*These authors contributed equally to this work.)

Mikyšková R, Indrová M, Vlková V, Bieblová J, Šímová J, Paračková Z, Pajtasz-Piasecka E, Rossowska J, Reiniš M. DNA demethylating agent 5-azacytidine inhibits myeloid-derived suppressor cells induced by tumor growth and cyclophosphamide treatment. *J. Leukoc. Biol.* 2014;**95**(5):1–11. (IF2012= 4.568)

Mikyšková R, Indrová M, Polláková (Vlková) V, Bieblová J, Šímová J, Reiniš M. Cyclophosphamide-induced myeloid-derived suppressor cell population is immunosuppressive but not identical to myeloid-derived suppressor cells induced by growing TC-1 tumors. *J. Immunother.* 2012;**35**(5):374–84. (IF2012= 3.463)

Rosalia RA, Štěpánek I, Polláková (Vlková) V, Šímová J, Bieblová J, Indrová M, Moravcová S, Přibylová H, Bontkes HJ, Bubeník J, Sparwasser T, Reiniš M. Administration of anti-CD25 mAb leads to impaired α-galactosylceramide-mediated induction of IFN-γ production in a murine model. *Immunobiology* 2013;**218**(6):851–9. (IF2012= 2.814)

Veronika Vlková, Ivan Štěpánek, Veronika Hrušková, Filip Šenigl, Veronika Mayerová, Martin Šrámek, Jana Šimová, Jana Bieblová, Marie Indrová, Tomáš Hejhal, Nicolas Derian, David Klatzmann, Adrien Six and Milan Reiniš. Epigenetic regulations in the IFNγ signalling pathway: IFNγ-mediated MHC class I upregulation on tumour cells is associated with DNA demethylation of antigen-presenting machinery genes. In revision in *International Journal of Cancer* (IF2012= 6.198)