Charles University in Prague

Faculty of Science

Molecular and Cellular Biology, Genetics and Virology

Ing. Mgr. Ivan Štěpánek

Immunotherapy of HPV16 - associated cancers and regulation of antitumour immune response

Imunoterapie nádorů asociovaných s virem HPV16 a regulace protinádorové imunitní odpovědi

Dissertation

Supervisor: RNDr. Milan Reiniš, CSc.

Prague, 2013

I declare that this dissertation is my own work and I cited all used information sources and literature. The PhD thesis does not contain any material which to a substantial extent has been accepted for the award of any other degree of the university or other institute of higher learning.

In Prague, 14th February 2013

Acknowledgements

I would like to express gratitude to my supervisor RNDr. Milan Reiniš, CSc. for the continual guidance and help during the manuscripts writing. I am grateful to my colleagues from the Laboratory of Tumour Immunology (Institute of Molecular Genetics, Prague) for creating of friendly environment and help with performing experiments. Furthermore, I am thankful to the members of Flow Cytometry and Genomics Core Facility for the technical support and valuable advices. Finally, I acknowledge the financial support provided by grants 301/07/1410 and 301/10/2174 from the Grant Agency of the Czech Republic, by A500520605 from the Grant Agency of the Academy of Sciences of the Czech Republic and in part by grant No. AVOZ50520514 awarded by the Academy of Sciences of the Czech Republic and by grant of the Clinigene project EU-FP6-NOE No. 018933 which made this research possible.

List of abbreviations

mAb = monoclonal antibody

5AC = 5-azacytidine

Ag = antigen

APC = antigen presenting cells

APM = antigen presenting machinery

CpG ODN = oligodeoxynucleotide containing CpG motif

CTL = cytotoxic T lymphocytes

DC = dendritic cells

DEREG mice = transgenic mice with depletion of Treg

DNA = deoxyribonucleic acid

DNMT = DNA methyl transferases

ds = double stranded

Flt3L = FMS-like tyrosine kinase 3 ligand

α-GalCer = α-galactosylceramide

GM-CSF = granulocyte-macrophage colony-stimulating factor

HDAC = histone deacetylases

HPV = human papillomavirus

HSP = heat shock proteins

IFN = interferon

LC = Langerhans cells

MHC = major histocompatibility complex

NK = natural killer cells

NKT = natural killer T cell

NLR = NOD-like receptors

ODN = oligodeoxynucleotide

PAMP = pathogen-associated molecular pattern

Rb = retinoblastoma

RNA = ribonucleic acid

TAA = tumour associated antigen

TCR = T cell receptor

TEIPP = T-cell epitopes associated with impaired peptide processing

TLR = Toll-like receptors

Treg = T regulatory cells

TSA = trichostatin A

Contents:

Introduction

The idea of possible neoplasia recognition and eradication by the immune system is not new. One of the first documented pieces of evidence concerning the role of immune system activation in tumour therapy was observed by William Bradley Coley in the late nineteenth century. He noticed that patients' tumours occasionally shrank when the tumours became infected (severe septicaemia/erysipelas) [Coley 1910]. However, since that observation until the end of the twentieth century the question whether the immune system is able to eliminate tumour cells remained very controversial. In the 1970s Zinkernagel and Doherty validated the hypothesis that $CD8^+$ T cell recognition and destruction of cells is MHC class I-dependent [Zinkernagel and Doherty, 1974 a/b]. This brought more arguments for the supporters of the theory that tumour growth control by the immune system. Although it referred to the viral antigens (Ags), it suggested the mechanism of how tumour Ags (in the context of MHC class I molecules) could be recognized by cytotoxic lymphocytes. Besides that, the discovery of dendritic cells (DC), leukocyte population with unique ability to present both self and non-self Ags, was another finding which contributed to the clarification of the mechanisms of anti-tumour immunity [Steinman and Cohn, 1973]. Later, many tumour-associated or -specific Ags were identified. One of the tumour-specific Ags described were proteins derived from human papillomavirus 16 (HPV 16), the etiological agent of cervical cancer [reviewed by Frazer et al., 2011]. Together with the DC discovery it created the theoretical background for the development of dendritic cell- or peptide-based anti-tumour vaccines. Despite the growing knowledge in the field of tumour immunology, clinically efficient peptide-based therapeutic vaccine has been so far formulated for precarcinogenic lesions [Kenter et al., 2009].

Besides the role in the protection against neoplasia, development of the immune system can also contribute to the acceleration of tumour growth. Certain leukocyte populations such as Treg (regulatory T cells) or NKT cells can regulate immune response and thus contribute to cancer progression/regression. Tumour cells or tumour microenvironment in general can influence immune cells and also inhibit efficient anti-tumour response. One of the major obstacles during the tumour immunotherapy is posed by the loss of the presentation of the cell surface tumour Ag. It is often mediated by the downregulation of MHC class I molecules on tumour cells and represents a common

mechanism by which tumour cells can escape anti-tumour immunity [summarized by Reinis 2010]. However, epigenetically silenced expression of MHC class I molecules can be reversed by epigenetic modifiers such as inhibitors of DNA methyltransferases (DNMT) or histone deacetylases (HDAC) [Khan et al., 2008; Manning et al., 2008; Setiadi et al., 2008; Simova et al., 2011]. This thesis focuses on the potential of the DC-based vaccines against HPV-16-associated tumours with a different MHC class I expression, on the combination of cancer immunotherapy with the treatment using epigenetic modifiers, with special attention paid to their effects on DC, and, finally, on the impacts of the anti-CD25 antibody (used for Treg elimination) on Treg and NKT cells, as well as on tumour progression. The experimental part was performed in the Laboratory of Tumour Immunology (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague) and the thesis is based on four original articles (see the list of my publications).

Aims

The subject of the thesis is experimental immunotherapy of HPV16-associated MHC class I-deficient or -positive tumours in the murine model and regulation of anti-tumour immune response with special attention paid to the effects of epigenetic agents. The main objectives were following:

1) to evaluate the efficacy of DC-based vaccines in a murine model of MHC class I-deficient tumours (TC-1/A9) expressing E6/E7 oncogenes derived from HPV16, especially:
   a) the effect of different types of CpG ODN (oligodeoxynucleotide containing CpG motif) on murine DC maturation
   b) the number of specific $CD8^+$ T cells after immunization with DC pulsed with peptides containing CD8 or CD4 epitopes
   c) the efficiency of DC pulsed with peptides containing CD8 or CD4 epitopes to inhibit tumour growth

2) to analyze how epigenetic modifier 5-azacytidine can influence tumour cell interactions with the immune system and their sensitivity to immunotherapy, especially:
   a) the tumour inhibitory effect of the combinatorial therapy of 5-azacytidine (5AC) and CpG ODN
   b) the impact of depletion of selected lymphocyte population on the therapeutic effect of 5AC and CpG ODN
   c) the tumour inhibitory effect of the combinatorial therapy of 5AC and cellular TC-1 tumour vaccine producing IL-12 (TC-1/IL-12)
   d) the effect of epigenetic modifier (5AC) on MHC class I expression of MHC class I deficient tumours (TC-1/A9)
   e) the effect of epigenetic modifier (5AC) on the expression of MHC class I or genes of the antigen presentation machinery (APM) in the MHC class I-deficient tumours
   f) the methylation status of APM regulatory sequences

3) to determine the effect of epigenetic modifiers (5AC, TSA) on DC, especially:
   a) toxicity of 5AC or TSA
   b) expression of MHC and costimulatory molecules on mature DC
   c) expression/ production of cytokines by mature DC
   d) priming capacity of DC treated by 5AC or TSA
   e) ability of 5AC or TSA treated DC to stimulate Th lymphocyte

4) to determine the effect of PC61 antibody on Treg or activated NKT cells, and its implication for immunotherapy of the experimental murine model of HPV16-associated tumours, especially:
   a) the efficiency of Treg depletion after anti-CD25 Ab treatment (PC61) or after diphtheria toxin (DT) application into transgenic DEREG mice
   b) the number of NKT cells after stimulation with α-GalCer (α-galactosylceramide) and their activation status, production of cytokines and ability to polarize Th response in the presence or absence of Treg
   c) the influence of Treg depletion and NKT cell activation on TC-1 tumour growth

Literature review

1. Dendritic cells in tumour immunotherapy

Discovery

Dendritic cells (DC) of the skin were observed already in 1868 by Paul Langerhans. There was an extensive discussion about their function. In 1973 Ralph Steinman and Zanvil Cohn identified spleen DC as a new class of white blood cells with a number of distinctive features and functions [Steinman and Cohn, 1973]. They initiated a series of experiments leading to the description of immunostimulatory properties of DC. DC were also discovered in non-lymphoid organs and their role in the organ rejection after transplantations was noticed.

Features

DC represent a rare leukocyte subset with the unique ability to present Ags to T lymphocytes and thus initiate specific primary T cell response and permit establishment of immunological memory. DC originate from BM precursors. Immature DC quickly respond to environmental changes, termed „danger signals“ (such as bacterial/viral products or heat shock proteins) and differentiate extensively into immunogenic mature DC. DC thereby link the innate immune response to the adaptive immunity. The ability of immature DC to activate T lymphocytes is limited, but they are more potent in Ag internalization in comparison to mature DC. DC can “alert“ lymphocytes to the presence of injury or infection. DC were characterized by Hart in 1997 by:

- the ability to stimulate primary T cell response
- the ability to regulate membrane processes at 37°C *in vitro*
- the ability to migrate in the tissues and to search for T lymphocytes
- active endocytosis (immature DC)
- spontaneous clustering with T cells *in vitro* at 37°C
- differences from macrophages or monocytes in cytochemical parameters and surface markers

- expression of certain surface markers depending on the level of maturation
- other attributes such as dendritic-like appearance or high density of MHC II on the surface (B lymphocytes and fibroblasts can show similar characteristics, but follicular DC are excluded by this criterion)

Subpopulations of dendritic cells

DC can be classified into subpopulations based on their phenotypes, anatomical locations or functions. DC are usually classified into two main subpopulations termed classical (or conventional) DC and plasmacytoid DC (pDC) [Boonstra et al., 2003].

Classical dendritic cells (cDC)

DC were originally defined by "dendritic" morphology and unique capacity for Ag presentation and T cell priming capacity. Later, these classical or conventional DC can be phenotypically defined in the mouse blood as CD11c/ CD11b positive cells negative for CD45RA. Conversely pDC lack expression of integrin CD11b, have low expression of CD11c and are positive for CD45RA [Wu and Liu, 2007]. Classical DC can be further classified into two subsets, $CD8^{-}/CD11b^{+}$ and $CD8^{+}/CD103^{+}$ DC in mice [Reizis 2012]. The $CD8^{-}$ classical DC subtypes are more efficient in MHC class II-restricted Ag presentation to $CD4^{+}$ T cells, but the $CD8^{+}$ subpopulation of classical DC is far more effective in MHC class I presentation of exogenous Ags to $CD8^{+}$ T cells (also termed as cross-presentation) [Schnorrer et al., 2006; Pooley et al., 2001; Iyoda et al., 2002].

Classical DC are rare in mouse blood, highly migratory and they can move from the tissues to the T cell and B cell zones of lymphoid organs via afferent lymphatics and high endothelial venules. They are short-lived and replaced by blood-borne precursors. DC are also distinct from Langerhans cells (DC found in the epidermis), which are not replaced by blood-borne cells at steady state [Geismann et al., 2010]. Recently, transcription factor Zbtb46 was described to be specifically expressed in all classical DC (in both humans and mice) and it supports the notion that classical DC constitute a unique immune cell lineage [Meredith et al., 2012; Satpathy et al., 2012].

Plasmacytoid dendritic cells (pDC)

Plasmacytoid DC are lymphocyte-like cells that specialize in type I interferon production, but they possess weak ability to stimulate T cells [Boonstra et al., 2003; Cella

et al., 1999; Siegal et al., 1999]. They are relatively long lived. Mouse plasmacytoid DC partially carry immunoglobulin IgH D-J rearrangements and express Rag gene. As opposed to classical DC, plasmacytoid DC differentiate directly in the bone marrow from the common DC precursors [Reizis et al., 2011].

Monocyte-derived dendritic cells (MoDC)

Monocytes (Gr-1$^+$ monocytes in mice or CD14$^+$ monocytes in humans) possess the ability to differentiate into DC. Conversion of monocytes to DC occurs in the presence of GM-CSF and LPS [Steinman 2012]. GM-CSF and IL-4 is commonly used *ex vivo* to prepare human DC cultures from monocytes or peripheral blood mononuclear cells. These monocyte-derived DC are used as therapeutic anti-tumour vaccines (pulsed with tumour Ag) or they can be used as the powerful tool for the study of human DC differentiation and maturation processes.

Antigen internalization, processing and presentation

DC are termed as professional APC (APC) for their ability to efficiently stimulate Ag specific T lymphocyte response. They can uptake Ag from their environment, which is further processed and presented on their surface for the T cell recognition. After Ag internalization, DC can migrate, mature and interact with T cells and stimulate them. Functional changes during DC maturation leads to transition from Ag-capturing cell to APC. DC form congregates with T cells, with high proportion of CD4$^+$ T cells. DC also activate cytotoxic CD8$^+$ T lymphocytes, both with the help of CD4$^+$ T cells and independently on them. DC with the higher expression of the MHC and costimulatory molecules, termed mature DC, are more efficient stimulators of T cells. High phagocytic and proteolytical activity is typical for the macrophages. By contrast, phagocytic or pinocytic ability of DC is relatively limited but still sufficient for the Ag uptake, which is further processed for T cell activation; the rate of the Ag internalization in B cell in comparison to DC is negligible [Hart 1997]. Phagocytosis of bacteria or cells was also described [Fossum et al., 1986; Inaba et al., 1993; Alfaro et al., 2011; Newman et al., 2001]. DC precursors contain phagocytic activity and quickly migrate to afferent lymphatic vessels, where the ability to phagocytose is reduced.

DC present Ags in the context of MHC to CD4$^+$ and CD8$^+$ T lymphocytes. These cells receive from DC activatory or inhibitory signals (depending on the affinity to MHC

molecules, depending on the expression of the costimulatory, coinhibitory or adhesive molecules, cytokines) and initiate following immune response.

Ag internalization

Rate of Ag internalization is dependent on the DC maturation state [Garrett et al., 2000]. Immature DC are able to internalize large amount of Ag. By contrast, mature DC are efficient in T cell stimulation but Ag internalization is limited. Exogenous Ag can be internalized by pinocytosis, phagocytosis or receptor mediated phagocytosis [Villadangos and Schnorrer, 2007]. Some Ag can also bind directly the MHC molecules without being processed by DC [Hart 1997].

Ag processing

Endogenous Ag can be degraded by intracellular proteases into peptides. These peptides bind TAP molecules and are delivered through the ER membrane to the MHC class I molecules before their move to the cell surface (marked as conventional pathway). Several alternative pathways can be used for MHC class I Ag processing. DC can obtain external Ag by pinocytosis and release them into the cytosol for the classical TAP-dependent MHC class I presentation. Optionally, peptides can be replaced directly in the MHC molecules, especially in the environment with the high concentration of Ag. Other possibility involves chaperons (Hsp96) or transporters capturing Ag and transporting them through DC membrane into endoplasmic reticulum, where the Ags are released and bind into the complex of MHC class I molecules [Lanzavecchia 1996]. Presentation of endogenous Ag is predominant for the MHC class I molecules but exogenous Ag can be also presented (cross-presentation) [Bevan 1976a, Bevan 1976b]. Exogenous Ag has to undergo proteolysis before they become part of the MHC class II complexes. Vesicles of endocytic-lysosomal pathway containing exogenous peptides fuse with the MHC class II containing vesicles. Dissociation of the invariant chain from the MHC class II complex due to the lower pH is followed by binding of suitable peptides to MHC class II molecules [Germain et al., 1993; Hausmann et al., 1999]. Enzymatic degradation proteins to peptides and their association with MHC class II molecules probably take part in lysosomes containing acid pH. DC are able to present soluble Ag to specific T lymphocytes. Antigenic material from the solid Ag can be also processed probably without Ag

internalization by serum proteases, surface peptidases (e.g. CD13) or other leukocytes can contribute to the Ag digesting [De Bruijn et al., 1992; Falo et al., 1992].

Toll-like receptors

In the 90s of the last century Toll receptor, which helps fruit flies defense against yeast and fungal infections, was described [Lemaitre et al., 1996]. This fact led to the discovery of the „Toll-like receptors“ (TLR) in humans and other vertebrates. Up to now, at least 12 type of TLRs in human [Trinchieri et al., 2007] and 13 types of TLRs in the murine system [Kawai et al., 2007] have been identified. These receptors are able to recognize characteristic microbial products and „endogenous danger signals“ e.g. LPS, lipoproteins, flagellin from the bacterial flagellum, bacterial DNA, ds viral RNA. That is the reason why TLR represent one of the most important receptors recognizing structures generally termed as pathogen-associated molecular patterns, shortly PAMP. Most of the TLRs can be found on the cell surface, but TLR9 is localized to endosomes [Latz et al., 2004]. Mouse TLR9 is expressed in pDC precursors, B cells [Kadowaki et al., 2001; Hornung et al., 2002], conventional DC [Uchijima et al., 2005] and macrophages [Sweet et al., 2002].

TLR9 ligands as dendritic cell maturation inducing agents:

Bacterial DNA, CpG motif, oligodeoxynucleotides

To become fully functional APC, DC have to undergo a maturation process that can be triggered by several pathways. DC can sense „danger signals“ by numerous distinct mechanisms including TLR, helicases and NLR [Palucka, Bancherau 2012]. TLR9, an important member of the TLR family, is the intracelular receptor responsible for the recognition of bacterial DNA and oligodeoxynucleotides with unmethylated CpG motifs (CpG ODN) [Hemmi et al., 2000]. TLR9 is expressed in DC and DC maturation can be triggered after DC activation via TLR9. Mature DC can further present Ag to T lymphocytes and stimulate both specific and unspecific immune response.

Bacterial extracts

More than 100 years ago William Coley successfully implemented the use of bacterial extracts for the tumour treatment [Wiemann B., Starnes C. O.1994]. Bacterial

extracts of mycobacterium bacillus Calmette Guerin (BCG) are standard therapy for the bladder cancers. Surprisingly, one of the active components of the extract is DNA. It has been suggested that palindromic sequences with CpG motifs were responsible for the DNA immunostimulatory properties [Yamamoto et al., 1992; Kuramoto et al., 1992]. Bacterial DNA is active in single- and in double-stranded form and it was able to activate NK and B cells, induce B cell proliferation, Ig secretion as well as DC maturation [Krieg 2002].

CpG motif

Vertebrate and bacterial DNA differs in the content and methylation status of the CpG dinucleotides. Probability of occurrence and methylation of the CpG dinucleotides is about 75 % lower in the vertebrate DNA than it could be predicted from the random arrangement of bases and this motif (unmethylated CpG) can represent a danger signal for some of the vertebrates. Nucleotides in the proximity of the vertebrate CpG are not random; usually CpG motif is preceded by C and followed by G. This kind of CpG motif does not represent a strong immunostimulatory capacity [Krieg 2002]. CpG dinucleotides are not methylated in bacterial DNA but about 70 % of the CpG vertebrate sequences are methylated. Methylation of the CpG can block the immunostimulatory effect [Krieg et al., 1995]. As a result of testing of hundreds of sequences, the motif XCGX was identified as the DNA structure responsible for the immunostimulatory capacity, where X marks each base except the C and Y is each base except the G [Krieg 2002]. Optimal immunostimulatory sequence is species specific, for example sequence GACGTT was identified as the one of the most powerful motifs for the stimulation of the murine immune system [Rankin et al., 2001; Yi et al., 1998]. Bacterial unmethylated DNA can be replaced with synthetic oligodeoxynucletides (ODN). It was empirically determined that CpG motif is in the optimal ODN duplicated or triplicated, other copy has no or low influence on the activity ODN. Most of the ODN with the significant activity in the murine system are 15-25 base long nucleotides. CpG motifs should be separated by at least two bases to reach the optimal activity. ODN containing methylated C in the position 5 lose the ability to bind to TLR9 and thereby its immunostimulatory effects. 2- keto, 3-imino and 4-amino groups of C and groups in the position 1 (amino), 2 (imino) and 6 (keto) in G are important for the recognition of CpG ODN by TLR9 [Kandimalla et al., 2001]. Immunomodulatory capacity of the CpG ODN was described during the experiments with the antisense ODN. In these studies, some antisense ODN or control ODN with no homology to described stimulatory

sequences were able to activate immune system [Tamaka et al., 1992; Branda et al., 1993; Pisetsky et al., 1994]. Several independent studies have demonstrated that the dependence on CpG motifs for TLR9 activation is restricted to synthetic phosphorothioate ODN and that natural unmodified phosphodiester ODN activate TLR9 in a sequence-independent manner [Latz et al., 2004; Yasuda et al., 2005; Haas et al., 2008; Wagner, 2008; Ashman et al., 2011].

Modifications of oligodeoxynucleotides backbone

Phosphodiester backbone of DNA is rapidly degraded inside the lymphocytes [Zhao et al., 1993]. Therefore ODN with the modified backbones are frequently used. Phosphorothioate backbone in which one atom of oxygen is replaced by sulphur is highly resistant to nuclease hydrolysis, has increased ability to activate B cells, but increases unspecific binding to proteins and limits the ability of NK cell and macrophages activation in comparison to ODN with phosphodiester backbone [Krieg 2002]. ODN with phosphorothioate backbone have higher affinity to cell membrane and exhibit enhanced cellular uptake [Zhao et al., 1993; Zhao et al., 1994; Crooke 1998].

CpG oligodeoxynucleotides (CpG ODN 1826, CpG ODN 1585)

When the immunostimulatory properties of bacterial DNA were described, there was a strong effort to replace it by short synthetic ODN. Hundreds of CpG ODN were synthesized and their efficiency evaluated. Ballas in 2001 suggested sequences marked as CpG ODN 1826 and CpG ODN 1585 as one of the most powerful immunostimulatory ODN in the murine system [Ballas et al., 2001].

CpG ODN 1826 contains two CpG motifs within the phosphorothiote backbone. CpG ODN 1826 was optimized to stimulate mouse B cells and to induce production of Th1 cytokines [Ballas et al., 2001]. Immunostimulatory properties are based on stimulation of maturation and cytokine production by macrophages, DC, B and NK cells [Krieg 2002, Ballas et al., 2001].. CpG ODN 1826 was successfully tested in the several murine tumour models and CpG ODN 1826 alone exhibited anti-tumour effects as well as an adjuvant in peptide or cell vaccines [Blazar et al., 2001; Reinis et al., 2006; Davila et al., 2000; Sandler et al., 2003]. CpG ODN 1826 can trigger DC maturation which leads to increase expression of MHC class II and costimulatory molecules (CD40, CD80 and CD86),

cytokines (IL-10 and IL-12), and these mature DC are potent in T cell stimulation [Takenaka et al., 2007; Brunner et al., 2000].

CpG ODN 1585 is a CpG ODN with phosphorothioate backbone with two CpG motifs with addition of poly G motif, which should enhance the cellular uptake and substantially improve its ability to activate NK cells [Ballas et al., 1996; Kimura et al., 1994]. CpG ODN 1585 is very efficient in NK cell activation; induces expression of IL-12, IFNγ and TNFα by spleen cells [Ballas et al., 2001; Blazar et al., 2001]. It was successfully applied in the experimental therapy of MHC class I deficient tumours [Ballas et al., 2001; Reinis et al., 2006].

Mature dendritic cells

Dendritic cell maturation

The term „mature DC“ was originally used to describe the ability of some DC to induce proliferation of naive T lymphocytes. Nowadays mature DC are usually described as DC with higher expression of MHC and costimulatory molecules (in the murine system commonly defined as MHC class II^{high}/ $CD86^{high}$ double-positive DC). However, there is not always a correlation between the higher expression of MHC/ costimulatory molecules on DC and their immunostimulatory potential [Villadangos and Schnorrer, 2007]. Differentiation and activation of DC precursors in the fully competent APC is a regulated process. As discussed above, DC maturation can be triggered by different “danger” signals, e.g. by PAMP, such as DNA with CpG motifs, flagellin, LPS), as well as by inflammatory mediators (TNFα), cytokines or increased temperature. Maturation also depends on the type of DC. Plasmacytoid DC produce large amount of type I interferon and they are weak stimulators of T cells. By contrast, conventional DC are important producers of IL-12 and they are extremely efficient in T cell stimulation. Mature DC express large amount of MHC, costimulatory and coinhibitory molecules, CD1d and increase their cytokine production (IL-2, IL-4, IL-10, IL-12p70, IL-13, IL-16, IL-23, TNFα and β, IFNγ). In the absence of the maturation stimuli, the expression of cytokines, MHC and costimulatory molecules retain low and such DC can induce anergy or tolerance of the T lymphocytes. DC potent in T cell stimulation can also receive negative signal and become ineffective „exhausted“ APC [Doyen et al., 2003; Kajino 2007]. In this way, T cell multiactivation and possible incorrect autoreactive function of APC is regulated.

Cytokines produced by mature dendritic cells

DC produce during maturation cytokines (i.a. IL-6, IL-10, IL-12 and IL-23), which can modulate immune response.

IL-6 is produced by DC. Langerhans cells (LC) and DC are main sources of IL-6 in the mouse lymph nodes [Hart 1997]. IL-6 mediates systemic inflammatory response, promotes Th2 type response and stimulates B cells.

IL-10 has an important role in the regulation of the inflammatory processes. It is produced by monocytes, Th2 cells and mature DC. IL-10 regulates Th1 response and macrophages activity, stimulate B cells to plasmocytes, take part in the induction of exhausted DC [Kajino 2007].

IL-12 is a proinflammatory cytokine, heterodimer, consisting of two subunits (smaller IL-12p35 and inducible IL-12p40). DC, macrophages and B cells are important source of IL-12 in the organism; they coordinate secretion of IL-12p35 and IL-12p40 to produce functional IL-12 [Vignali and Kuchroo, 2012]. Subunit IL-12p40 is shared with heterodimeric IL-23. IL-12p40 can be secreted in excess and can inhibit function of IL-12p70 by competition [Gately et al., 1998]. Production of IL-12 correlates with the ability of DC to activate T cells, leads to Th1 response and stimulates NK cells [Brunner et al., 2000; Vignali and Kuchroo, 2012].

IL-23 is mainly proinflammatory cytokine with the key role in the development of the Th17 subset of helper T cells [Aggarwal et al., 2003]. IL-23 is a heterodimer, consisted of IL-12p40 (share with IL-12) and IL-23p19 subunits. IL-23 is produced by activated DC and macrophages.

MHC class II glycoproteins on the cell surface of dendritic cells

MHC class II glycoproteins are transmembrane heterodimers with the extracelular N-terminal polymorfic domains. These domains form groove which binds mainly processed extracelular peptides. The groove is open and thus can bind longer peptides, 13-25 amino acids. MHC class II molecules can be found on antigen-presenting cells and lymphocytes. Mature DC express high level of MHC class II molecules by contrast to immature DC and together with CD86 are two commonly used markers of mature DC. Peptides are retained in the MHC class II complexes for long term (1-2 days) [Puré et al., 1990]. Peptides in the context of MHC class II molecules are presented to the $CD4^+$ T helper lymphocytes. Lipid and glycolipid Ag can be presented in the context of CD1

molecules to NKT cells. Role of Ag internalization, processing and presentation in the context of MHC class II molecules is mentioned in the homonymous chapter.

B7 family of costimulatory/ coinhibitory molecules on the cell surface of dendritic cells

Costimulatory molecules contribute (together with cytokines and Ag binded to MHC molecules) to the efficient communication of DC with the cells of the immune system, e.g. T cells. Activation of Ag specific T lymphocytes without costimulation can lead to anergy or to the cell death of T cells [Schwartz et al., 1990; Guinan et al., 1994]. The B7-CD28 molecules are probably one of the most intensively studied receptor-ligand systems in the field of immunology. Interactions of molecules of B7 family molecules with molecules of CD28 family play important role in the regulation of T cell activation.

CD80 (B7-1), CD86 (B7-2)

CD80 and CD86 are costimulatory molecules from the B7 family, which are expressed also on DC. They interact with two ligands, CD28 and CTLA-4. CD80 and CD86 bind to different part of CD28 (CD28 is expressed on the most of the T cells) and provide strong costimulatory signal to T cells [Coyle and Gutierrez-Ramos, 2001]. Second ligand of the CD80/86 is CTLA-4. It is expressed on the activated T lymphocytes and provides an alternative negative signal [Gribben et al., 1995]. Differentiation of T cells as the response to CD80 (preferentially to Th1) and to CD86 (preferentially to Th2) binding was described by Kuchroo et al. 1995. CD80/CD86 are not found on resting blood DC, but they are expressed after DC activation [McLellan et al., 1995; Hart et al., 1993]. Cultivation of isolated DC in cell culture system leads to the relatively fast upregulation of CD80 and CD86. MHC class II crosslinking can provide feedback loop signal which can lead to increased expression of CD80/CD86. By contrast, IL-10 regulates expression of CD80/CD86 on the spleen DC and inhibits mix leukocyte reaction [Kawamura et al., 1995; Ruffner et al., 2009; Alba Soto et al., 2010; Zhu et al., 2003; Emmanouilides et al., 1996].

CD274 (B7-H1, PD-L1)

Expression of CD274 is increased on activated DC, monocytes, B and T cells and on the cells of some non-lymphoid tissues (lung, heart, placenta, and pancreas) [Greenwald et al., 2005]. PD-1 was identified as the receptor for CD274. Activation of the inhibitory receptor PD-1, which is expressed on the surface of T cells, represents important role in the

regulation of the inflammation. PD-1 and CD274 negatively regulates $CD8^+$ T cell response. CD274 regulate peripheral T cell tolerance and it is used by tumour cells to escape from the T lymphocyte response (tumours can inhibit T cell response by induction of the coinhibitory molecules e.g. CD274 on the DC cell surface).

CD275 (B7-H2, ICOS-L)

CD275 is constitutively expressed on DC, macrophages, T and B cells and on some non-lymphoid tissues (lung, liver, kidney, testicles) [Greenwald et al., 2005]. Receptor of CD275 is ICOS. Increased expression of ICOS was described on activated T lymphocytes and NK cells. Stimulation via CD28 or ICOS leads to production of Th1 and Th2 cytokines although ICOS does not induce expression of IL-2. ICOS is more potent in the induction of IL-10 production, a cytokine important for the suppressive function of Treg [van Berkel et al., 2006].

Dendritic cell – T cell interactions

DC are more potent stimulators of T cell response in comparison to B cells. DC immunization stimulates strong immune response mediated by T helper (Th) and cytotoxic T lymphocytes (CTL). Ag presentation in the context of MHC class I or class II molecules is decisive for the initiation of the $CD8^+$ or $CD4^+$ T cell response, respectively. Secretion of cytokines and presence of costimulatory molecules are other important factors for the initiation of the T cell response. Differences in these factor contributions influence both $CD4^+$ and $CD8^+$ T lymphocyte response. DC are able to stimulate both Th1 and Th2 clones. Mature DC produce IL-12, which can be important factor for DC to polarize $CD4^+$ T cell response to Th1. Th polarization is also dependent on the route of DC application. Subcutaneous DC application induces Th1 response. By contrast, intravenous DC administration induce antibody response [Hart 1997]. Immature DC can be tolerogenic, inhibit T cell response [Dhodapkar et al., 2001] and it constitutes the obstacle in the tumour immunotherapy for which T cell response is critical.

DC-T cell interaction is stabilized by adherent molecules. CD54 (ICAM-1) occurs in lower density on the cell surface of resting DC, but it‘s expression is increased after the signalization from the T lymphocytes or during the DC maturation [Starling et al., 1995, Hartmann et al., 1999; Radhakrishnan et al., 2007]. CD54 stabilizes DC-T cell clusters. CD50 (ICAM-3), which is constitutively expressed on the DC in the high quantity, can

provide initial adhesive interaction with CD11a/CD18 (LFA-1) on the T lymphocytes [Starling et al., 1995]. Participation of other adhesive molecules such as CD58, LFA-3, VCAM is also possible.

Ex vivo generation of dendritic cells

Several methods for *ex vivo* cultivation of DC have been described. Procedures differ mainly in medium content, time of the cultivation, maturation agents used, origin of the progenitors, purity, yield and the composition of the final population, reviewed in 2007 by Lutz and Rössner. In general, after cocultivation of progenitors in the suitable mixture of factors and cytokines, it is possible to obtain large amount of heterogeneous cells (morphologically and functionally, which are rich of DC).

The generation of DC from the bone marrow precursors has developed to one of the standard and valuable tools for studying DC biology in murine system. APC can be differentiated from the bone marrow precursors by cocultivation with GM-CSF (critical factor) while IL-4 can be supplemented as a cofactor supporting DC differentiation from precursors. IL-4 also reduces expression of CD14 and suppresses macrophages differentiation [Lauener et al., 1990; Jansen et al., 1989]. For the generation of immunogenic DC high dose of GM-CSF is required (more than 200 U/ML). As mentioned above there are plenty of limiting factors which can influence DC cultures. For example, FCS batch is very critical factor for the quality of DC. Bacterial quality plastic results in fewer adherent macrophages and limit spontaneous DC maturation. Using tissue culture treated plastic, high density of bone marrow precursors; shear forces during centrifugation, passaging or pipetting contribute to the spontaneous maturation of DC [Lutz and Rössner, 2007].

Data presented in this dissertation were mainly received from the culture of the murine bone marrow precursors cultivated for seven days in complete medium supplemented with GM-CSF and IL-4, as described by Lutz et al. 1999 and with minor modification published by Indrova et al. in 2004 and Stepanek et al. 2011. GM-CSF is a cytokine commonly used in the majority of the protocols for DC preparations. Bone marrow-derived DC prepared in the presence of GM-CSF do not correspond with any of the lymphoid-organ-resident DC subsets found *in vivo* but DC resemble monocyte-derived DC [Villadangos et al., 2007].

Alternatively, it is possible to replace GM-CSF with Flt3L, which stimulates differentiation of precursors into both conventional ($CD8^+$ and $CD8^-$ DC) and plasmacytoid DC. This mixture of APC more closely resembles DC in classical lymphoid tissue [Liu et al., 2010]. Despite of using of GM-CSF for the DC preparations, it was shown that mice deficient in GM-CSF or GM-CSF receptor have quite normal number of DC in the steady state [Steinman 2012]. By contrast, mice homozygous for the Flt3L knock-out allele exhibit reduce number of DC, NK cells, lymphoid and myeloid progenitors [McKenna et al., 2000]. For the studies presented in this thesis, for the last 24 hours APC were cultivated with CpG ODN 1826 (ligand of TLR9) to obtain fully mature DC in terms of expression of MHC and costimulatory molecules, cytokines [Lutz and Schuler, 2002].

Dendritic cell-based anti-tumour vaccines

DC play an irreplaceable role in anti-tumour immunity. Function and number of DC are important factors in the control of tumour progression. Tumour cells themselves are poor antigen-presenting cells. DC as the APC are able to capture and present tumour derived Ag to T cells in tumour draining lymph nodes. This results in generation of specific CTL that can contribute to tumour rejection. Mouse models demonstrate that generation of protective anti-tumour immunity depends on the presentation of tumour Ags by DC [Palucka and Banchereau, 2012]. Vaccination strategies use unique abilities of DC to efficiently stimulate specific T cell response and to link innate and adaptive response. The aim of the DC vaccination is to induce tumour-specific effector T cells which can reduce tumour mass specifically and induce immunological memory to control tumour relapse.

Defects in DC function were described in tumour-bearing animals by mixed lymphocyte reaction or by limited ability of DC to stimulate T lymphocytes. This malfunction was associated with reduced ability to present Ag by MHC molecules. Supernatant from the tumour cells inhibited DC generation from the bone marrow precursors but it did not influence the function of already mature DC [Gabrilovich et al., 1996]. It was documented that only small number of DC infiltrate prostate cancer and these DC had phenotype without the activation markers. Similar situation was described at the breast and colon cancers [Hart 1997]. Based on the fact, that insufficient Ag presentation by DC is responsible for the defects in anti-tumour immunity, the concept of application of

ex vivo generated DC as the cell vaccines was designed. Cultured DC can be delivered in the high numbers and they are able to effectively present Ag in the presence of all necessary signals for activation of interacting cells (costimulation, cytokine production). T cell responses against TAA are often weak and it was described that *in vitro* generated DC pulsed by TAA are potent immunotherapeutic agents [Palucka and Banchereau, 2012]. Vaccination by DC pulsed with tumour Ag can protect mice against tumour challenge or can be successfully used for the tumour immunotherapy in some tumour animal models [Timmerman and Levy, 1999; Vulink et al., 2008; Apetoh et al., 2012]. In human DC clinical trials situation is less „optimistic“ because of the clinical response of the vaccination is reduced only to a small number of oncological patients. There could be several reasons for this insufficiency of efficiency:

- patients usually diagnosed in the late stage of disease and induced immune response is not possible to cope with the large tumour mass
- unknown tumour specific Ag
- proper function of the patient's immune system can be compromised by chemotherapy/ radiotherapy
- cancer cells or tumour suppressive environment can limit function of effector cells
- suboptimal dose of DC, nonoptimal state or type of DC
- DC vaccines alone are not able to induce sufficient anti-tumour response and it should be combined with other therapeutic approaches

All reasons mentioned above create enormous unexplored areas in the DC biology to study.

2. HPV16-associated cervical cancer

Biology of human papillomaviruses

Human papillomaviruses (HPVs) belong to a family of small non-enveloped DNA viruses (Papillomaviridae) consisting of over 100 related genetic types. HPVs do not infect any other species, nor do any of the animal papillomaviruses infect humans. The approximately 8-kb papillomavirus genome is encapsulated by 360 copies of the major capsid protein L1 and the variable number of molecules of L2. The HPV genome additionally encodes six nonstructural proteins (E1, E2, E4-E7) that regulate viral gene

replication and transcription and also alter the growth characteristics of the infected epithelial cells [Frazer et al., 2011]. For some HPV types (collectively known as high-risk types, e.g. HPV16 and HPV18), these proteins also induce instability in the genomes of the infected cells [Frazer et al., 2011]. HPV16 and HPV18 are responsible for the development of about 70% of cervical cancers and only proliferating keratinocytes can support replication of the viral episome [Bhat et al., 2011]. HPV infect keratinocytes in the basal layer of the epithelium through microwounds (figure 1). The viral genome does not contain DNA polymerase and its replication is in synchrony and reliant on cellular DNA replication. After the cell division, daughter cells migrate away from the basal layer and undergo differentiation. Differentiation of the HPV positive cell induce productive phase of the viral cell cycle.

Figure 1. Model of the HPV-mediated progression to neoplasia in the cervical epithelium [Frazer et al., 2011]

E6 and E7 proteins

E6 and E7 are the primary transforming viral proteins. Expression of these viral non-structural proteins E6/E7 delays exit from the cell cycle and differentiation. Transformation of keratinocytes to a malignant phenotype generally involves integration of the viral genome into the host DNA [Bhat et al., 2011]. In this process, genes encoding early viral proteins E1 and E2 are lost [Romanczuk 1992], allowing upregulation of E6 and E7 oncoproteins. A primary target of E7 is the retinoblastoma (Rb) family of proteins that control the activity of E2F transcription factors, which are key regulators of S phase genes [Moody and Laimins, 2010]. Inactivation of Rb is important for the tumour progression but it leads to increased levels of p53. E6 proteins have evolved to target p53 for the degradation [Moody and Laimins, 2010]. Besides that there were described multiple mechanisms how E6 and E7 proteins interfere with the cell cycle or intracellular processes through e.g. inducing telomerase expression, interaction with HDAC, cyclins, cyclin-dependent kinase inhibitors, induce genomic instability [Moody and Laimins, 2010]. Although E6 and E7 proteins are able to immortalize cell lines, transfected cells are not tumorigenic in the mouse model and require e.g. expression of additional oncogene, such as v-ras or v-fos.

HPV and cervical cancer

HPV infection initiates more than 99% of cervical cancers; it is necessary but not sufficient for progression to cancer [Moody and Laimins, 2010]. HPV can be sexually transmitted [Bhat et al., 2011] and cervical cancer is the second largest cause of deaths in women worldwide. Although most infections resolve within 1-2 years of acquisition [Insinga et al., 2011], viral persistence can induce precancerous lesions termed cervical intraepithelial neoplasia (CIN). Most of the HPV-related cancers develop into the squamous cell carcinomas [Bhat et al., 2011]. Recently introduced prophylactic vaccines (Gardasil and Cervarix) can prevent the initial infection by two high-risk types, HPV 16 and HPV 18, which are responsible for about 70% of cervical cancers [Moody and Laimins, 2010]. Furthermore Gardasil prevents the infection of HPV 6 and HPV 11, which cause most of the genital warts. But these prophylactic vaccines are not efficient against established HPV infections and vaccines capable to treat persistent HPV infection would be of benefit. Efforts to develop immunotherapeutic vaccines have been hampered by the relative non-immunogenicity of the HPV infection, by immunoregulatory processes in the skin, and the subversion of immune response induction and immune effector functions by HPV proteins [Bhat et al., 2011].

Immunotherapy of the cervical cancer

Effect of the most preventive vaccines is based on the induction of neutralizing antibodies (in case of HPV frequently against L1 capsid protein). By contrast, resolution of HPV infection and therapeutic vaccines require induction of robust cell immunity (figure 2) mediated especially by proper activated DC which are able to induce $CD4^+$ and $CD8^+$ T cell responses that are associated with the regression of established (pre)malignant lesions, including those induced by high risk HPV [Melief and van der Burg, 2008; Frazer et al., 2011].

In patients with naturally regressing premalignant lesions of HPV, the measured Ag responses were $CD4^+$ T cell responses. Only occasionally HPV specific $CD8^+$ T cells were detected [Frazer et al., 2011]. The observed slow clearance of the HPV infection and weak immune responses to viral protein is likely a consequence of the non-lytic nature of HPV infection and delay in the induction of inflammatory responses [Frazer et al., 2011]. Data obtained from the transplantation of the murine E6 or E7 expressing transgenic skin also

demonstrated sufficient HPV E7 Ag presentation by dermal DC [Frazer et al., 2011] although an absence of the local inflammation inhibited generation and/or prevented effector function of the specific effector T cells. Vaccines aimed at the treatment of the established disease require long-lived Ag presentation by appropriately activated DC [Melief and van der Burg, 2008].

Figure 2. A model for local regulation of induced immune responses to HPV in skin [Frazer et al., 2011]. HPV proteins (red) produced in keratinocytes (pale blue) are taken by skin APC (gray) to the local lymph node to prime cytotoxic effector, memory effector and helper T (Th) cells. Effector cells return to the skin to kill HPV-expressing keratinocytes. In the absence of inflammation, APC inhibit induction of CTL in response to skin-derived negative regulatory signals.

Therapeutic peptide vaccines

Several approaches as vaccination with recombinant viral vectors, recombinant proteins with adjuvant, Ag-loaded DC, DNA encoded tumour Ag or synthetic peptides has been utilized in anti-tumour therapeutic vaccination. It has been suggested that direct injection of protein Ag would be more efficient than using DNA vaccines, since viral vectors suffer from the competition of the possible viral Ag with the inserted Ag and, further, can contain the intrinsic genes that suppress immune responses [Melief and van der Burg, 2008]. Vaccines based on the short synthetic peptides containing exact MHC class I binding epitopes have been a popular approach for the therapeutic cancer vaccination. Despite of the ability to establish protection against transplantable tumours or viruses, administration of the short peptide-based vaccines led to deletion of Ag specific T cells, tumour outgrowth and to immunological tolerance. It was suggested that short peptides can be exogenously loaded on the MHC class I of cells including nonprofessional Ag presenting cells, immature DC, T or B cells [Bijker et al., 2008; Melief and van Burg 2008], and that the lack of the surface costimulatory molecules on these cells required for the appropriate CTL generation can shift immune response to tolerance [Melief and van der Burg, 2008; van Hall and van Burg 2012]. Tolerance induced by vaccination with short peptides containing $CD8^+$ T cell epitope can be overcome by loading these peptides directly onto DC vaccines [Toes et al., 1998], prolongation of the peptide sequence to the length that require processing by professional APC [Bijker et al., 2007] or using adjuvants. It turned out that some of the first successful short peptide vaccines contain $CD4^+$ T cell

epitopes or they are longer than minimal MHC class I binding sequence. Activated $CD4^+$ T cells deliver help for the $CD8^+$ T cells by fully activating DC by CD40-CD40L interaction as well as by production of IL-2 [van Hall and van Burg, 2012]. Injection of the mixture of peptides containing both CD8 and CD4 epitopes increased magnitude of specific CTL response and it was able to confer protection of mice against otherwise lethal tumour challenge [Melief and van der Burg, 2008]. Vaccination of mice/ rabbits with long peptide (containing CD8 and CD4 epitopes), but not with short minimal peptide (containing CD8 epitope), were able to eradicate palpable HPV 16-associated tumours [Zwaveling et al., 2002] or they were able to abrogate latent papillomavirus infection and control the growth of establish warts [Vambutas et al., 2005]. Another significant improvement in the immunogenicity of the peptide vaccine was achieved by joining of minimal CD8 epitope with the T helper epitope into a single peptide [Shirai et al., 2004]. In addition, T cell responses induced by peptide conjugation with TLR ligand vastly exceeded the responses following the application of the mixed components [Khan et al., 2007]. Long peptides over minimal short peptide containing the same $CD8^+$ T cell epitope increased duration of Ag presentation in the draining lymph node [Bijker et al., 2008].

Clinical trials of peptide vaccines

Detection of malignant or premalignant cervical cytological changes by Papanicolaou smear screening, with the appropriate surgical treatment, is currently the most widely used method for reducing the risk of death from cervical cancer [Bhat et al., 2011]. Because of known tumour specific viral Ags it could be suggested that immunotherapy of HPV associated cancer should be in principle easier than tumours expressing modified self proteins. Although promising animal studies were performed (demonstrated $CD8^+$ T cell- mediated vaccine effect [Chen et al., 1991]), despite many clinical trials only recently any translation to clinical efficiency was shown. Only small study from the LUMC Leiden has shown convincing evidence of the therapeutic effect. Immunization with the long peptides from E6 and E7 together with an adjuvant enhanced the rate of clearance of HPV- associated vulvar intraepithelial neoplasia with the complete response reaching almost 50 % [Kenter et al., 2009]. So far three major classes of immunotherapy have undergone trials: adjuvanted protein or peptide vaccines, recombinant viral vectors and polynucleotide vaccines; the most trials have focused on the E6 or E7 proteins of HPV16 [Frazer et al., 2011].

TC-1 model system of the HPV-associated cancers

Transplantable tumours engineered to express papillomavirus nonstructural proteins can be used as a murine model for HPV16-associated cancers. TC-1 tumour cell line used in such studies was derived from primary lung epithelial cells of C57BL/6 mice cotransformed with HPV-16 E6 and E7 and c-Ha-ras oncogene [Lin et al., 1996]. MHC class I-deficient cell line derived from the parental TC-1 cell line marked as TC-1/A9 [Smahel et al., 2003], was obtained from the selected TC-1 tumour developed in immunized mice.

3. Cancer immunosurveillance and immunoediting

Since one of the first documented observation of using patient's immune system against the cancer published by William B. Coley in the nineteenth century, it was very controversial if our immunity can play a significant role in the tumour therapy. Paul Ehrlich was perhaps the first one who hypothesized protective effects of immunity in long-lived organisms, but his theory could not be experimentally tested because of little of knowledge of the immune system [Ehrlich 1900]. More than 50 years later, Burnet and Thomas formulated concept of immunosurveillance by which innate and adaptive immune systems identify and eliminate self cells that have become transformed through spontaneous, chemically or virally induced genetic alterations [Burnet 1957]. However, subsequent studies provided little support for this hypothesis, e.g. experiments showing that cancer susceptibility of immunocompetent mice (to both spontaneous and carcinogen-induced tumours) was similar to that of nude mice that had major but not total immunodeficiency [Schreiber et al., 2011]. By contrast, later identification of the tumour associated Ags, ability of DC to present tumour Ags and analyses of cancer incidence in a range of immunodeficient knockout mice ($RAG^{-/-}$, $STAT1^{-/-}$, $perforin^{-/-}$, $IFN\gamma^{-/-}$, $IFNR\gamma^{-/-}$) reassessed the role of the immunity in the cancer control [Parish 2003]. In 1990s, immunological function of TLR (which are able to sense „danger signals") was described and „danger signal" theory by Polly Matzinger [Matzinger 1994] replaced already overcome the self-non-self model. Theory of „danger signal" is based on the fact that

antigen-presenting cells respond to "danger signals" as the signs of abnormal state and activate our immune system. In the late 20th century it was apparent (by discovery of immunoregulatory cell populations, tumour immune escape mechanisms and regulatory role of the tumour environment) that tumour immunosurveillance theory needs to be revised. In 2001, it was described that the immune system does not only protect the host against tumour formation but also shape tumour immunogenicity and it has become the basis of the cancer immunoediting hypothesis [Shankaran et al., 2001; Dunn et al., 2002]. It was postulated that it comprises of three distinct phases: „elimination“, „equilibrium“ and „escape“. Elimination phase is an updated version of cancer immunosurveillance, in which immune system detects and destroys cancer cells before tumours become clinically apparent [Schreiber et al., 2011]. In the equilibrium phase, the host immune system and tumour cell variants that survived the elimination phase enter into a dynamic equilibrium. It is probably the longest of three phases and may occur over a period of many years in humans [Dun et al., 2004]. In the escape phase, tumour cells avoid recognition of the immune system and occur as the growing, visible tumours. Progression from the tumour escape to equilibrium can occur because of changes in the tumour cell population selected by the immune system or/and because of the cancer-induced suppression or the immune system deterioration. Tumour cell escape can be caused by many different mechanisms; one of the most important mechanisms by which the tumour cells are able to escape from the CTL-mediated immune response is the lost of the tumour Ag on the tumour cell surface, which can be caused by:

- lack of the Ag expression
- loss of MHC class I proteins that present Ags to tumour-specific T cells
- loss of the Ag processing function that is necessary for the proper Ag presentation by MHC class I molecules

4. MHC class I-deficient tumours

MHC class I antigen processing and presentation

MHC class I molecules are responsible for the presentation of the protein fragments of mostly intracellular Ags at the cell surface (figure 3). Presentation of exogenous Ags by MHC class I molecules is termed crosspresentation and it reaches high efficiency in $CD8^+$

DC [Den Haan et al., 2000]. Proper expression of MHC class I molecules is therefore necessary for the presentation of Ags to CTL. The majority of antigenic peptides are generated upon ubiquitination of mainly intracellular proteins followed by their degradation via multicatalytic proteasome complex yielding precursor peptides with MHC class I compatible correct C-terminus, but extended N-terminus [Bukur et al., 2012]. Proteasome complex efficiency and specificity can be modified by converting it into the immunoproteasome by substitution of parts of the proteasome by proteasome activator (PA) 28 and IFNγ-inducible proteasomal β subunits (the low molecular weight proteins LMP2 and LMP7, and MECL-1). Immunoproteasome is more active than the 26S proteasome under the immune stress or IFNγ exposure, enabling the cells to handle expanded pool of substrates and prevent the aggregation of proteins, resulting in the production of more peptides [Neefjes et al., 2011]. MHC class I molecules sample and present the results. These peptides can be further trimmed by different peptidases and translocated via heterodimeric TAP1/TAP2 (transporter associated with Ag processing) into the lumen of the endoplasmic reticulum (ER). The complex of TAP, β2-microglobulin, MHC class I molecules together with chaperons (tapasin, ERp57 and calreticulin) is called peptide loading complex and serves as the platform for the efficient peptide loading onto MHC class I molecules [Neefjes et al., 2011]. After the binding to peptide of sufficient affinity, MHC class I complex (MHC class I molecule+peptide+β2-microglobulin) is released from the chaperons and can pass through trans-Golgi to the cell surface. Under physiological conditions MHC class I components of APM are constitutively expressed in all adult nucleated cells with the exception of immune privileged organs and their expression could by regulated by different cytokines such as IL-10 as their negative regulator and, by contrast, IFNγ as the inducer [Bukur et al., 2012].

Figure 3. MHC class I antigen presentation pathway [Neefjes et al., 2011]

MHC class I-deficient tumours

In general, the MHC class I status of tumours during the immunotherapy is often disregarded. MHC class I downregulation is one of the important tumour-escape mechanisms from the immunosurveillance and it can negatively influence results of the anti-tumour immunotherapy. Analysis of large banks of human tumour specimens has

shown that between 40-90% of human tumours display total or selective allelic losses of HLA class I proteins [Dunn et al., 2004], which can be caused by the loss of HLA haplotype, specific loci or alleles. The MHC class I presentation pathway can be also disrupted as a consequence of mutation and/or dysregulation of one or several genes encoded by antigen processing machinery components, frequently resulting in complete loss, or downregulation of MHC class surface molecules [Seliger, 2005], see Table I. Mechanisms underlying altered expression of APM component can be caused by structural alterations, epigenetic modifications, and transcriptional/posttranscriptional alternations of genes of the MHC class I antigen processing machinery. Inactivating mutations of HLA class I proteins or β2-microglobulin by contrast to mutations of other components of APM like *TAP1*, *TAP2*, *TPN* and the proteasomal subunits only rarely occur in tumours [Bukur et al., 2012]. Epigenetic silencing (methylation or histone deacetylation) of promoters of both MHC class I molecules or other components of APM such as *β2-microglobulin*, *TAP1*, *TAP2* as well as *LMP2* or *LMP7* is associated with impaired MHC class I surface expression and this can be reversed by epigenetic modifiers treatment, such as by application of HDAC or DNMT inhibitors [Khan et al., 2008; Manning et al., 2008; Setiadi et al., 2008; Reinis, 2010; Bukur et al., 2012]. Further, transcriptional/ posttranscriptional coordinated downregulation of TAP1, TAP2, LMP2 and LMP7 molecules was described in various types of cancers [Bukur et al., 2012; Reinis, 2010], which leads to decrease in the MHC class I surface expression. This kind of downregulation is often cytokine (IFNγ, TNFα) reparable, reviewed by Bubenik and Vonka in 2003, but in melanomas, resistance to IFNγ caused by deletion of the *JAK2* gene [Selinger, 2005] was described.

Table I. Mechanisms of MHC class I downregulation [Seliger, 2005]

Mechanism	APM components
Structural alternations	Total, allelic, or locus-specific loss of MHC class I heavy chain
	β2-microglobulin deletion, rearrangements or mutations
	TAP mutations
Dysregulation	Methylation and acetylation
	Transcriptional downregulation
	Posttranscriptional downregulation
	Deficient IFNγ signal transduction

Immunotherapy of MHC class I-deficient tumours

Despite of the MHC class I molecule deficiencies and the resulting resistance of tumours to CTL, in some experimental tumour systems, C57BL/6 mice were capable of being immunized against MHC class I-deficient tumours [Bubenik and Vonka, 2003]. This response was dependent on NK cells, NK-mediated antibody-dependent cellular cytotoxicity, $CD4^+$ T cells or lymphokine-activated killer cells (LAK). At the cytokine level, IFNγ seems to be a crucial factor for the effective anti-tumour response, it is capable to both restore MHC class I expression on the tumour cells and to antagonize TGF-β in the tumour microenvironment [Lou et al., 2005; Smahel et al., 2003; Jabrane-Terrat et al., 1990; Lou et al., 2008; Lorenzi et al., 2012]. Loss of the MHC class I expression hinders tumours rejection by specific cellular immune responses; although the MHC class I deficient tumours are not completely invisible to the specific cytotoxic lymphocytes. Such cancer cells were shown to be able to present tumour specific self-proteins, marked as TEIPP (T cell epitopes associated with impaired Ag processing) which can be recognized by specific CTL and can protect mice against the challenge with the TAP-deficient RMA-S tumor cells [van Hall et al., 2006]. Futhermore, Chambers et al. published in 2007 results showing that immunization with TAP1 -/- DC (presenting self peptides associated with dysregulated Ag processing) protects mice from the outgrowth of TAP2-deficient RMA-S lymphoma cells or TAP1-deficient MCA fibrosarcoma cells. Cytokine therapy and gene therapy using tumour cells transfected with the immunomodulatory cytokine genes (*IL-2, IL-12, GM-CSF* or *IFNγ*) could also inhibit the growth of MHC class I-deficient tumours [review by Bubenik and Vonka, 2003]. Both direct and indirect effects of cytokines is responsible for the therapeutical effect e.g. IL-12, in addition to enhancing DC function, IL-12 can be responsible for the anti-angiogenic properties, stimulation of IFNγ production by NK cells and stimulation of the TNFα production. IL-2 can enhance production of IFNγ by T and NK cells [Bubenik and Vonka, 2003]. Other activators of the immune system, namely unmethylated CpG ODN or β-galactosylceramide, can be efficient as well as peptide, RNA or DNA vaccines [reviewed by Reinis, 2010]. Another perspective approach to the development of the therapeutic protocol aiming to upregulation of MHC class I molecules could be induction of demethylation or hyperacetylation of MHC class I promoters or other molecules involved in MHC class I Ag processing suppressed by

epigenetic silencing. Global DNA demethylation of melanoma cells upregulated expression of tumour Ags (MAGE-1) [de Smet et al., 1996; Weber et al., 1994], restored HLA expression and CTL response to tumour cells [Serrano et al., 2001]. Treatment of breast carcinoma cells with HDAC and DNMT inhibitors displayed synergistic, anti-neoplastic effect [Primeau et al., 2003]. Except of epigenetic modifiers, differentiation agents like retinoic acid [Vertuani et al., 2003] or effects of radiotherapy [Reits et al., 2006] can reverse MHC class I expression and can efficiently stimulate anti-tumour immune response.

5. Epigenetic modifications, dendritic cells and tumour therapy

Epigenetics studies heritable changes caused by mechanisms other than changes in the DNA sequence. Alterations in the expression of genes in tumours are not always due to mutagenesis. They may also be caused by transcriptional regulation by epigenetic mechanisms. Epigenetic regulations in the genetic information of the tumour or immune cells can contribute to the activation or inhibition of the immune system [Leggatt and Gabrielli, 2012]. One of the possible epigenetic modifications can be histone tails acetylation or DNA methylation. Both of them serve to regulate gene expression and remain through cell divisions. Only these two epigenetic alterations, relevant for the experimental part, will be discussed.

DNA methyl transferases and their inhibitors

DNA methylation is an important regulator of gene transcription. Transfer of methyl group from the S-adenosyl-L-methionine to the position 5 in the cytosin is usually termed as DNA methylation. Spontaneous deamination of 5-methylcytosine into thymine occurs and CpG sequences are therefore underrepresented in the mammalian genomes, although in adult somatic tissues, DNA methylation typically occurs in a CpG dinucleotide context which can be found in some CpG rich promoter regions and are usually associated with transcriptionally silent genes. By contrast, aberrant DNA methylation is commonly associated with cancers and is considered as one of the cause of their hallmarks [Jones and Baylin, 2002; Hanahan and Weinberg, 2011].

Gene expression is regulated by DNA methylation both directly and indirectly. Direct regulation takes place in prevention of direct binding of transcriptional factors to DNA due to methylated cytosine [Comb and Goodman, 1990; Prendergast et al., 1991]. Indirect regulation by methylated CpG sequence occurs due to interaction with protein containing methyl DNA binding domains (MBD) which can be associated with corepressors or HDAC which subsequently inactivates gene transcription [Nan et al., 1998]. Methyl-CpG-binding proteins can also interact with SUMO ligases or PIAS proteins which can reverse inhibition of gene transcription [Lyst et al., 2006].

DNMT are enzymes responsible for the proper preservation of DNA methylation. DNMT 1 is the most abundant DNMT in murine cells, it has higher affinity for the hemimethylated DNA, keeping the methylation patern of CpG sequences during DNA replication and therefore is described as „maintenance DNMT“. DNMT 3a and DNMT 3b, which can methylate both hemimethylated and unmethylated CpG sites, are marked as „de novo DNMT“. Nucleoside based DNMT inhibitors such 5AC, 2'-deoxy-5-azacytidine and zebularine are well established and they have already been tested in clinical or preclinical studies [Yoo and Jones, 2006]. They are metabolized by kinases that convert the nucleosides into nucleotides for incorporation into DNA and/or RNA. 5AC is a cytidine analogue, inhibitor of DNMT for the first time synthesized in the Institute of Organic Chemistry and Biochemistry in Prague in 1964 [Sorm et al., 1964]. After incorporation into DNA, it covalently binds DNMT and causes hypomethylation of DNA besides direct cytotoxic effect. 5AC (under the trade name Vidaza) is used for the treatment of the myelodysplastic syndrome and has been used in a number of clinical trials for both non-solid and solid tumour therapy [http://www.clinicaltrials.gov/] although it´s efficiency in the treatment of the nonsolid tumour was limited. Currently there is available also a number of non-nucleoside based DNMT inhibitors such MG98, RG108, epigallocatechin-3-gallate (EGCG) or procaine. These drugs might not be as robust inhibitors as the nucleoside analogues. Although they are usually low molecular weight molecules with direct binding to the catalytic site of the enzyme without necessity to be incorporated into DNA and therefore they have large potential for the DNMT inhibitors development [Yoo and Jones, 2006]. MG98 is antisense oligodeoxynucleotide which inhibits translation of mRNA of DNMT1, RG108 was designed to fit into the pocket of the active site of DNMT1 and epigallocatechin-3-gallate (EGCG) is active component of the green tea extract [http://ncit.nci.nih.gov, NCI thesaurus version 13.02d, Suzuki et al., 2010].

Histone deacetylases and their inhibitors

Histone tail acetylation is involved in activating gene expression by making chromatin structures less compact and it is one of the markers of constitutive heterochromatin. Acetylation and deacetylation of lysine residues of histones tails is mediated by histone acetyltransferases (HAT) and histone deacetylases (HDAC). The balance between histone acetylation and deacetylation is well regulated, but the balance is often disrupted in diseases such as in cancer. Histone acetylation affects chromatin structure, which can impact gene transcription. HDAC can be divided into four subgroups by the sequence identity and domain organization. Classical HDAC (class I, II and IV) share sequence similarity and are dependent on Zn^{2+} with cooperation with histidine-aspartate residues for enzymatic activity, whereas the class III sirtuins act through a distinct NAD^{+} -dependent mechanism [Shakespear et al., 2011]. Class I HDAC are homologous to the yeast protein Rpd3 and include HDAC 1, 2, 3 and 8. They are ubiquitinously expressed and usually localized in nucleus. HDAC of the class II include HDAC 4, 5, 6, 7, 9 and 10. Class II enzymes are larger proteins (120-130kDa) related to the yeast Hda1 protein. They are localized in both nucleus and cytoplasma which suggest also their possible non histone substrate deacetylation. Sirtuins as the class III of HDAC share homology with the yeast SIR2 protein, they are not dependent on Zn^{2+}, react by different mechanism and are insensitive to the inhibition by large set of HDAC inhibitor such trichostatin A (TSA) or derivates of hydroxamic acid [Emanuele et al., 2008].

Inhibition of HDAC leads to global histone tail acetylation [Galasinski et al., 2002; Glaser et al., 2003; Peart et al., 2005; McFarland et al., 2012] and can be used to restore expression of some epigenetically silenced genes in neoplasias [Ferrara et al., 2001; Lin et al., 1998; Glaros et al., 2007]. Furthermore, treatment by HDAC inhibitors can induce expression of proapoptotic genes, and consequently the tumour cell differentiation, growth arrest or apoptosis [Stimson et al., 2009]. Therefore inhibitors of HDAC represent promising anti-tumour therapeutic agents. Now-days there are many of described inhibitors of HDAC in clinical trials for the treatment of especially non-solid tumours including leukemias, lymphomas, melanomas, lung carcinomas or prostate cancers (http://www.clinicaltrials.gov/). TSA, the anti-fungal antibiotic product of Streptomyces hygroscopicus [Tsuji et al., 1976], active in nanomolar concentration [Vigushin et al., 2001], is usually selected as the „gold standard“ for the inhibition of HDAC. TSA blocks

the binding site of HDAC of class I and II, in this way it causes histone hyperacetylation and reactivates epigenetically silenced genes in tumours.

Epigenetic changes during anti-tumour immune response

Dysregulation in the epigenetic landscape is one of the hallmarks of cancer and it represents one of the molecular mechanisms by which cancer cells can escape from the immune response. Tumour immune escape mechanisms, such as the reversible loss of the expression of tumour Ags or downregulation of MHC class I molecules (discussed above), defective Ag processing and presentation, down-regulation of NKG2D ligand or defective death receptor pathway can be reversed by treatment of inhibitors of HDAC or DNMT [Tomasi et al., 2008; Manning et al., 2008]. Deficiency in the MHC class I molecules can be caused by direct downregulation of MHC class I expression and/or by defects in expression of MHC class I antigen processing machinery components (Tap1, Tap2, Lmp7, Tapasin) and these tumors are resistant to effector mediated cytolysis [Tomasi et al., 2008]. Decreased expression of these molecules, associated with decreased acetylation and increased methylation in the promoter regions of these genes, can be reversed by treatment with epigenetic modifiers [Manning et al., 2008].

Epigenetic changes play a role during many immunologically important events, including DC maturation. So far there have been few results describing epigenetic changes during DC maturation published, mostly obtained by using inhibitors of HDAC reporting changes in expression of costimulatory molecules and cytokines and shift in the balance between induced Th1 an Th2 responses towards Th2 [Jung et al., 2009; Kim et al., 2010; Brogdon et al., 2007; Nencioni et al., 2007; Bode et al., 2007; Bosisio et al., 2008]. It was described that TSA inhibited induction of IL-12p40 by DC after stimulation of TLR. HDAC inhibition increased acetylation and binding of transcription factors NF-κB (RelA)/ IRF-1 to the *IL-12p40* locus was abrogated [Bode et al., 2007]. Nencioni et al. in 2007 reported that HDAC inhibition in TLR ligand (poly I:C)-matured monocyte-derived DC disrupted nuclear translocation of IRF-3, IRF-8 and NF-κB (RelA), inhibited production of cytokines (TNFα, IL-6, IL-10 and IL-12) and decreased the cell surface expression of selected costimulatory molecules (CD40, CD80 and CD83). It was shown that treatment with HDAC inhibitors during the DC maturation led to the inhibition of specific set of genes (such as costimulatory molecule *CD40*, Th1 cytokine *IL-12p40*, Th1 attracting

cytokines *MIG* and *IP10*) which controlled Th1 effector cells activation and migration [Brogdon et al., 2007].

6. Immunoregulatory populations of lymphocytes

In the tumour escape phase, tumour cells themselves can employ (except the changes that occur directly on the tumour cells) immunoevasive strategies either e.g. through the production of immunosuppressive cytokines, such as TGF-β and IL-10, or by function of cells with immunosuppressive/ regulatory activities, such as Treg, myeloid suppressor cells, NKT cells or immature DC. A number of these immunosuppressive/ regulatory cell populations have been described but their interrelations and co-operation are still not fully understood.

T regulatory cells

Treg can be defined as the $CD4^+$, $CD25^+$ T cells that constitutively express transcription factor FoxP3. They are one of the important leukocyte populations with the immunosuppressive properties. When stimulated, they inhibit the function of tumour specific CTL by expressing coinhibitory molecules such as CTLA-4, PD-1 and PD-L1; by producing the immunosuppressive cytokines IL-10 and TGF-β; and by consuming IL-2, a cytokine that is critical for maintaining proper functionality of CTL, from the environment [Schreiber et al., 2011]. The amount of Treg correlates with the tumour size in some cases and can be used as the prognostic marker [Kordasti et al., 2007; Suzuki et al., 2013; Kashimura et al., 2012; Chen et al., 2011]. Analysis of melanoma, ovarian and colon cancers showed that the ratio and the distribution patterns of intratumoral $CD8^+$ T cells and Treg were critical determinant of prognosis [Schreiber et al., 2011]. Recent studies proved that $FoxP3^+$ Treg are often responsible for the failure of naive murine hosts to eliminate transplanted tumours [Klages et al., 2010; Teng et al., 2010]. These results documented that depletion of Treg by using an administration of diphtheria toxin to transgenic mice expressing diphtheria toxin receptor under the *FoxP3* promoter (DEREG) enabled mice to reject tumours that grew progressively in control mice. Furthermore, depletion of Treg enhanced the effect of the administered tumour vaccines [Klages et al., 2010; Mattarollo et al., 2012]. Alternatively to using transgenic mice model for the depletion of Treg it is

possible to apply anti-CD25 mAb or low dose of cyclofosfamide. But these approaches, which have been intensively studied for their possible clinical applications, are less specific in comparison to DEREG mice model and can influence other cell populations.

NKT cells

NKT cells are a heterogeneous subpopulation of T lymphocytes which share properties of both T cells and NK cells. NKT cells recognize the lipid Ags presented usually in the context of non-polymorphic CD1d molecule on the surface of APC. Invariant NKT cells (iNKT) are a subset of CD1d restricted NKT cells which recognize α-GalCer. In mice iNKT cells express mainly TCRs comprising an invariant Vα14Jα18 α-chain paired with a limited Vβ chain repertoire (Vβ2, Vβ7, Vβ8.1, Vβ8.2 or Vβ8.3) [Benlagha et al., 2001; Matsuda et al., 2001]. In addition to iNKT cells, there is another population of CD1d-restricted T cells, marked as "type II" NKT cells. They express diverse TCR chains and do not recognize α-GalCer but other lipid Ags [Brennan et al., 2013]. Relatively little is known about "type II" NKT in comparison to iNKT and we will be focused on iNKT in the experimental part of this thesis.

Increased amount of the Treg or immature myeloid cells caused by tumour environment is one of mechanism of the tumour immunosuppression. NKT cell activation can regulate function of Treg, immature myeloid cells and other leukocyte populations mainly due to the production of large set of cytokines as IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IL-21, IFNγ, GM-CSF and TNFα [Coquet et al., 2008]. Activated NKT cells are able to mediate anti-tumor cytotoxicity to $CD1d^+$ tumour cells directly or even indirect effect via production of IL-2 and further activation of NK cells was described by Metelitsa et al. in 2001.

Processing and presentation of lipid antigens

CD1d glycoprotein, a molecule related to the MHC class I molecules, is involved in the presentation of lipid Ags to the T cells and it is expressed on APC. The level of its expression correlates with the APC ability to stimulate NKT cells. TLR-dependent signalling events in DC are able to enhance the expression of CD1d and increase the activation of iNKT cells [Salio et al., 2007; Paget et al., 2007]. NKT cells can be activated by immature DC which is different from conventional T cell activation [Fujii et al., 2010]. For the efficient CD1d lipid presentation, there are also required molecules involved in

CD1 trafficking and lipid Ag uptake [Brunner et al., 2000; Barral and Brenner, 2007]. CD1d heavy chains are assembled in endoplasmic reticulum, where they bind β2 microglobulin and chaperons ERp57, calnexin, calreticulin. Tyrosine motifs on the CD1d molecules binds AP2/ AP3 proteins which mediates internalization from the plasma membrane or target CD1d molecules to the late endosomes and lysosomes respectively. Saposins, membrane-perturbing proteins, transfer lipids between membranes, mediate loading of lipids onto CD1d molecules in lysosomes. Mice deficient in saposins have impaired presentation of CD1d restricted exogenous Ags [Zhou et al., 2004].

Presentation of lipid antigens to NKT cells

In contrast to humans, mice lack the pool of CD1 molecules (CD1a-e) and harbour only highly homologous group (95%) of two genes, CD1d1 and CD1d2. Expression patterns of CD1d1 and CD1d2 are distinct, CD1D1 expression can be detected in B cells, T cells, macrophages and DC but CD1d2 was detected at low level on thymocytes and $CD4^+$ T cells. Despite their sequence similarity, expression of the CD1d2 is not sufficient for the development of NKT cells compared to CD1d1. Two mechanisms of NKT cell activation by DC have been proposed; exogenous lipid Ags can be presented on the DC surface in the context of CD1d or even in the absence of cognate endogenous lipid Ags by production of IL-12 in the combination with presentation of self lipid Ags [Barral and Brenner, 2007].
α-GalCer, as the potent endogenous lipid Ag to stimulate murine NKT cells, is widely used. NKT cells after stimulation produce a large amount of IFNγ and IL-4, and become CTL [Barral and Brenner, 2007], able to kill $CD1d^{high}$ target cell via CD95/CD178-dependent mechanism [Wingender et al., 2010]. Activated NKT cells also upregulate CD40L, OX40 which in turn with produced IFNγ can stimulate DC maturation and thus serve as the positive feedback loop. Another feedback is that mature DC produce IL-12, IL-18 and type I interferons which stimulate NKT cells. These facts support a model in which an important function of NKT cells is to „jump start“ the expansion of specific immune responses. NKT cell stimulation by α-GalCer or adoptive transfer of NKT cells increased the capacity of $CD8\alpha^+$ DC subpopulation to induce type 1 immune responses which is favour of the cancer immunotherapy [Joyee et al., 2010]. NKT cell stimulation has been shown to be the potent immunotherapeutic approach in the mouse tumour models (B16 melanoma and lung cancer) and number of clinical trials focused on NKT cell activation in cancer patients has been carried out. Direct cytotoxicity of NKT cells is not

required for the tumour rejection, as the tumours that lack CD1d expression can be rejected in the wild type animals. It is probable that NKT cell produce Th1 cytokines, which favour NK cell activation and inhibit angiogenesis, support tumour elimination. Also upregulation of CD70 and OX40L by mature DC is important for costimulating NKT cells and for promoting Ag-specific $CD8^+$ T-cell responses [Cerundolo et al., 2009].

NKT cell-based immunotherapy

α-GalCer-loaded DC transduced with tumour specific Ag activated iNKT cells and were shown to break the tolerance in the tolerogenic murine tumour model [Ko et al., 2007]. B cells loaded with α-GalCer and tumour MHC class I binding peptide can offer both preventive and therapeutic anti-tumor immunity against ovalbumin-transfected B16 melanoma. B cells directly primed CTL, the α-GalCer and the peptide were presented on the same cell [Chung et al., 2006]. DNA vaccine with α-GalCer administered at the prime phase significantly improved therapeutic and preventive effects against an E7-expressing tumour model (TC-1) in vaccinated mice [Kim et al., 2010]. Immunization with α-GalCer-loaded DC pulsed by tumour antigens demonstrated potent anti-tumour responses in the B16 murine melanoma model [Petersen et al., 2010]. It also showed that depletion of Treg before vaccine administration has a therapeutic effect and indicates that it is possible to improve this type of vaccine by further immunomodulatory agents. Timing of NKT cells activation is important because mature DC reduce their ability to uptake Ags and it was shown that simultaneous administration of α-GalCer and Ag was optimal to get a maximum number of specific T-cells.

Although activation of NKT cells can be exploited in the anti-cancer vaccination the NKT cells can also inhibit anti-tumour response. Production of IL-2 is a suggested mechanism of induction of Treg and tolerance by mainly $CD4^+$ subpopulation of NKT cells [La Cava et al., 2006]. This is in accordance with the conclusion of experiments published by Terabe et al. in 2000 and Moodycliffe et al. in 2000, in which they showed NKT cell-mediated inhibition of the immune response to a number of experimental tumours. Besides, the therapeutic administration of the immunostimulatory CpG ODN was more efficient in CD1d deficient mice than in wild-type one [Sfondrini et al., 2002].

Publication I

Induction of protective immunity against MHC class I-deficient, HPV16-associated tumours with peptide and dendritic cell-based vaccines.

Reinis M, Stepanek I, Simova J, Bieblova J, Pribylova H, Indrova M, Bubenik J.

Int J Oncol. 2010; 36: 545-51

Contribution of the author of the thesis:

I carried out all experimental work performed on DC; DC-based vaccine preparation, DC immunization experiments, analysis of the immune responses after vaccination by tetramer staining and the evaluation of the results. I participated in the experimental work design and in the preparation of the manuscript.

Publication II

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

Símová J, Polláková V, Indrová M, Mikyšková R, Bieblová J, Stěpánek I, Bubeník J, Reiniš M.

Br J Cancer. 2011; 105: 1533-41

Contribution of the author of the thesis:

I participated in the analysis of spleen cell responses to 5-azacytidine and measured production of cytokines (IL-12 and IFNγ) by spleen cells by ELISA.

Publication III

Effects of 5-azacytidine and trichostatin A on dendritic cell maturation.

Stepanek I, Indrova M, Bieblova J, Fucikova J, Spisek R, Bubenik J, Reinis M.

J Biol Regul Homeost Agents. 2011; 25: 517-29

Contribution of the author of the thesis:

I carried out all experimental work performed on mouse DC. I participated in the study design and the preparation of the manuscript.

Publication IV

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

Rosalia RA*, Stěpánek I*, Polláková V, Símová J, Bieblová J, Indrová M, Moravcová S, Přibylová H, Bontkes HJ, Bubeník J, Sparwasser T, Reiniš M.

Immunobiology. 2013; 218: 851-59

*/ these authors contributed equally to the work

Contribution of the author of the thesis:

I carried out most of the experimental work performed on DEREG mice including analysis of Treg depletion after diphteria toxin application, determination of the NKT response in DEREG mice after Treg depletion, occassionally I collected the blood samples and measured cytokines levels (IFNγ, IL-2, IL-4, IL-12) by ELISA. I participated in the manuscript preparation.

Discussion

MHC class I downregulation on tumour cells constitutes an obstacle for immunotherapy. Peptide- or DC-based vaccines provide a promising approach to tumour immunotherapy, but so far there are only few reports on this topic regarding their efficacy against MHC class I-deficient tumours. Cancer cells can reduce surface Ag presentation by deficiency in the Ag processing and presentation pathway caused by the defects in the transporter associated with Ag processing (TAP), tapasin, or in the proteasome. In spite of an impaired MHC class I Ag presentation it is still possible to induce protective immunity against MHC class I-deficient tumours [Levitsky et al., 1994]. This is in accordance with our conclusions from experiments performed on different models compared to those used by Levitsky et al., who used B78H1 and B16F10 tumours. In our experiments we utilized the TC-1 tumour cell line and its MHC class I-deficient subpopulation TC-1/A9. These cancer cells express non-self HPV16-derived E6 and E7 proteins. Immunodominant epitopes, especially in the E7 protein, are well characterized. This makes these cells an excellent experimental model for testing vaccines against tumours with a distinct MHC class I expression.

Vaccination with a short peptide containing only $CD8^+$ T cell epitope actually accelerated tumour growth of TC-1/A9 tumours, but it provided protection against parental TC-1 cells. It was suggested that short peptides can be exogenously loaded on the MHC class I molecules of cells, including nonprofessional Ag presenting cells, immature DC, T or B cells [Bijker et al., 2008; Melief and van Burg 2008], and the lack of surface costimulatory molecules on these cells required for appropriate CTL generation can shift the immune response to tolerance [Melief 2011, van Hall and van Burg 2012]. Addition of CD4 epitope to minimal peptides containing CD8 epitope is able to increase the efficiency and eradicate even palpable HPV16-associated tumours [Zwaveling et al., 2002]. Peptides harbouring CD4 epitope in addition to CD8 epitope were more efficient in our tumour protection experiments, since they induced a higher numbers of specific CTL which produced large amounts of IFNγ, as compared to the short peptides harbouring just the CTL epitope. It was shown by Bennett et al. in 1997 that induction of cytotoxic lymphocytes by cross-priming requires help of $CD4^+$ T cells and that this interaction is effective only when both the helper and CTL epitopes are recognized on the same APC.

Longer peptide used in our studies contained an overlapping T helper and cytotoxic T cell epitope and we could not evaluate the necessity for the presentation of both determinants on the same cell. Other possibilities of how to potentiate the CTL response without the necessity for the $CD4^+$ T cell help include an application of anti-CD40, anti-CTLA-4 mAb [Ito et al., 2000] or application of type I interferon [Ontiveros et al., 2011]. Tolerance induced by vaccination with short peptides containing $CD8^+$ T cell determinants can also be overcome by loading these peptides directly onto DC vaccines [Toes et al., 1998], prolongation of the peptide sequence to the length that requires processing by professional APC [Bijker et al., 2007], or using adjuvants.

In our setting, peptide-pulsed DC efficiently presented Ags, provided sufficient costimulatory and cytokine signals for T cell activation [Lutz and Schuler, 2002], induced sufficient CTL response and protected mice against the challenge of TC-1/A9 cells. Thus, the tumour growth acceleration with the vaccination by the short peptide was overcome as described previously. Data from the studies mentioned above are in agreement with our results, and conclusions from the experiments using MHC class I-deficient tumours suggested the validity of the discussed principles for immunotherapy of MHC class I-deficient tumours as well. Indeed, our experiments revealed that vaccines inducing specific immunity against E7 protein could prevent or inhibit growth of MHC class I-deficient tumours, although the mechanisms may be different from those that play role in the development of protective immunity against the parental, MHC class I-positive tumours.

CpG ODN, the efficient experimentally used adjuvants, were selected for our peptide vaccination studies mentioned above. Especially the CpG ODN 1826 was successfully used in a number of murine studies [Krieg 2002]. CpG ODN 1826 was optimized to stimulate mouse B cells and to induce production of Th1 cytokines [Ballas et al., 2001]. In our experiments, it efficiently matured DC and protected mice against tumour challenge, which was in line with the results published by Takenaka et al. in 2007; Brunner et al. in 2000 and Ballas et al. in 2001. By contrast, CpG ODN 1585 was optimized to activate NK cells [Krieg 2002] but it was not able to directly mature DC [Reinis et al., 2010], and therefore its superior efficiency in combination with peptide vaccination was not expected. We could not explain its potential and mechanism of action by the number of specific CTL or their production of IFNγ. However, an experiment performed by Klebanoff et al. in 2009 demonstrated the importance of IFNγ. Its release by

effector cells facilitated expression of MHC class I molecules on responding tumour cells, leading to recognition and subsequent tumour lysis.

Apart from approaches leading to the development of vaccines targeting MHC class I- deficient neoplasia, the induction of MHC class I expression by epigenetic modifiers and combination with established immunotherapy could be an alternative to the former one. Epigenetic agents (HDAC and DNMT inhibitors) are able to reverse DNA hypermethylation or histone hypoacetylation and in this way induce expression of the silenced genes. This could also be the case of molecules involved in the MHC class I antigen processing machinery and thus induce presentation of Ags in the context of MHC. We reported that 5AC was able to induce expression of MHC class I molecules on the TC-1/A9 tumour cells of lung epithelia origin both *in vitro* and *in vivo* [Manning et al., 2008; Simova et al., 2011]. Re-expression of MHC class I molecules by effects of epigenetic modifiers was reported by Serrano et al. and Nie et al. in 2001, who demonstrated demethylation of the genes encoding MHC class I heavy chains and restoration of CTL response. In our model, expression of MHC molecules correlated with the level of promoter demethylation and with the mRNA level of genes of MHC class I APM (*TAP1, TAP2, LMP2, LMP7* and *TAPBP*). These data are in agreement with the results from previous studies on epigenetic changes and antigen processing machinery by Manning et al., 2008; Seliger et al., 2006; Setiadi et al., 2007; Setiadi et al., 2008. Not only antigen processing machinery, but other silenced molecules could be the target of epigenetic therapy, such as costimulatory molecules CD40, CD80, CD86 [Tomasi et al., 2006], and thus enhance immunogenicity of neoplasias. Moreover, 5AC increased expression of the components of IFNγ signaling pathway such as STAT-1, IRF-1 and IRF-8, which suggests involvement of IFNγ in MHC class I upregulation and/ or in the immune response mechanism [Pollakova, unpublished].

5AC and 5-aza-2´-deoxycytidine as cytostatic compounds and inhibitors of DNMT have been approved for clinical use in the treatment of myelodysplastic syndromes [Mai and Altucci, 2009; http://www.fda.gov/, 2012]. We demonstrated that chemotherapy with 5AC reversed silencing of MHC class I Ag presentation and increased sensitivity of tumours to unspecific immune intervention by administration of CpG ODN 1826 or IL-12-producing cellular vaccine. Interestingly, the level of surface MHC class I molecules after *in vivo* application of 5AC was higher compared to *in vitro* treatment of TC-1/A9 cells, which suggests an additional indirect effect of 5AC on MHC class I expression *in vivo*. *In*

vivo cell depletion study revealed a major role of NK 1.1^+ cells in mice during the combined treatment. It was previously shown that the NK 1.1^+ cell population was able to control the early phases of tumour growth of the parental MHC class positive TC-1 cell line [Simova et al., 2004]. Data document the role of the innate immunity against neoplasias regardless of their MHC class I status. $CD8^+$ cell dependence of the therapeutic effect was not observed after 5AC administration, but only after combined therapy with CpG ODN 1826, which proved induction of a $CD8^+$ cell-dependent mechanism in the protective immune responses.

Other immunomodulatory effects of epigenetic modifiers can be mediated by influencing other components of the immune system and could not been monitored by tumour growth inhibition or cell-depletion studies. Thus, we tested possible adverse effects of 5AC treatment in our experimental setting. The total number of spleen cells and IFNγ-producing splenocytes was decreased after chemotherapy with 5AC, but the proliferative capacity and the proportion of spleen cell populations remained unaffected. 5AC also did not alter the percentage of activated T, B and NK cells induced by CpG ODN 1826.

In another set of experiments, we decided to monitor the effects of 5AC and another epigenetic modifier, TSA, on DC function with the focus on their maturation. There is still lack of data on the effect of DNMT inhibitors on DC function. On the other hand, it was reported that HDAC inhibitors such as TSA, besides their anti-tumor properties, have anti-inflammatory effects in a number of pathological conditions such as autoimmune manifestations of graft-versus-host disease, systemic lupus erythematosus, hepatitis, experimental autoimmune encephalomyelitis, rheumatoid arthritis and colitis [Reddy et al., 2004; Leng et al., 2006; Camelo et al., 2005; Chung et al., 2003; Glauben et al., 2006; Leoni et al., 2002; Reilly et al., 2004]. Later, it was documented that inhibitors of HDAC were able to block expression of costimulatory molecules and production of IL-12 by murine bone marrow-derived or human monocyte-derived DC and to inhibit DC-drived Th1 responses [Bode et al., 2007; Nencioni et al., 2007; Brogdon et al., 2007]. According to our experiments, treatments by both 5AC and TSA blocked the production of IL-12 by mature DC. Furthermore, incubation of DC with TSA reduced the amount of surface MHC and costimulatory molecules CD40 and CD86 on the mature DC. Concentrations and incubation time of the inhibitors were selected to maximize their biological effects and reduce direct cytotoxicity. No effect of 5AC or TSA on the cell viability was observed by MTT test or trypan blue staining. Hoechst 33258 staining revealed moderate cytotoxicity

of inhibitors, but the results suggest that the inhibitory effects cannot be attributed to their toxicity only. Despite the low toxicity of the inhibitors, both epigenetic agents blocked DC proliferation. Cell yields of the treated DC reached about 50 % of the control samples. Cell cycle arrest in sub-G1 phase by 5AC was described in acute myeloid leukaemia cell lines [Hollenbach et al., 2010] and a similar effect could play a role in our DC cell culture system as indicated by the cell yields of DC and cell viability assays.

Because 5AC has to be incorporated into DNA to be functional as DNMT inhibitor [Christman 2002] and possesses very low stability in solution [IARC 1990], proper timing of the inhibitor application is required. DC are one of the most important sources of IL-12 in the organism, and therefore, in the second step, kinetics of IL-12 production by mature, CpG ODN-treated, DC was analyzed. Production of IL-12 by immature DC was not determined; the level of IL-12 was below the detection limit of our assay (ELISA). Interestingly, the effects of TSA on IL-12 production manifested themselves after 24 hour and appeared faster in comparison to 5AC, whose effects were apparent after 48 hour of cocultivation. This was probably caused by the necessity of 5AC to be incorporated into DNA for the proper inhibitory function [Christman 2002], which can happen only once per cell division. Epigenetic modifiers decreased expression of mRNA corresponding to the larger subunit of IL-12 (IL-12p40) in mature DC. The level of IL-12p40 correlated with the level of secreted IL-12 and it seems that the expression of the inducible IL-12p40 subunit was a limiting factor for the IL-12 production in our setting. However, it was shown that the excess of IL-12p40 caused homodimerization, and the excess of homodimer can antagonize the effect of IL-12 by competition [Gately et al., 1998]. Thus the changes in the IL-12 level do not always correlate with the biological effect of IL-12. In contrast to IL-12p40, the level of smaller subunit IL-12p35 was not affected by the presence of epigenetic modifiers. Production/expression of other important cytokines such as IL-6, IL-10, IL-23p19, IFNγ and TNFα by DC was also decreased and regulated in a similar manner as IL-12, which suggests a shared regulatory mechanism. Experiments from the cytokine studies were performed on LPS- and CpG ODN 1826-matured DC derived from either murine bone marrow or human monocytes. This fact corroborates the conclusion that the results are neither species nor maturation stimuli specific. The evidence on the effect of DNMT on DC maturation was mentioned by Borden in his review of 2007. However, his raw data showing upregulation of IL-12 produced by DC after the treatment with 5-aza-2´-deoxycytidine has not been published so far. Despite the inhibitory effect of 5AC on

cytokine production, 5AC-treated DC cocultivated with specific T cells did not change their ability to induce T cell response or to polarize T helper cells. Furthermore, TSA additionally blocked expression of MHC class II and costimulatory molecules (CD86 and CD40) on the surface of mature DC. This DC phenotype was associated with decreased ability to stimulate naive specific CD4 or CD8 T cells and their production of IFNγ. Splenocytes cocultivated with TSA-treated mature DC downregulated expression of RORγt and increased expression of FoxP3. This data suggests tolerogenic properties of DC and possible induction of Treg. This is in line with the experiments showing that pharmacologically modulated or immature DC can induce Treg or tolerogenic signals in their environment [Cobbold et al., 2010]. 5-aza-2´-deoxycytidine, DNMT inhibitor and 5AC analogue, has been described to induce differentiation of myeloid suppressor cells towards DC and thus convert the immunosuppressive population into an Ag-presenting one, which was able to protect mice against tumour challenge [Daurkin et al., 2010]. This conclusion does not contradict our data indicating that 5AC does not interfere with the DC differentiation, as we did not notice any marked changes in the expression of CD11c or maturation markers (CD86 and MHC class II) on DC after 5AC treatment.

In another experimental part, we focused on the problem of Treg cell elimination by specific anti-CD25 antibody in order to overcome immune suppression and to increase the efficacy of immunotherapy. Treg play a critical role in ensuring tolerance, but their presence can be counterproductive to the attempts of inducing anti-tumour immunity [Nishikawa and Sakaguchi, 2010]. We focused on depletion of Treg by anti-CD25 mAb and its possible additive effect on tumour immunotherapy targeting NKT cells with their activation ligand α-GalCer. This assumption was supported by data from two recent studies. Petersen et al. in 2010 showed potentiation of anti-B16 tumour response to immunization with DC loaded with α-GalCer. Additionally, direct administration of α-GalCer combined with anti-CD25 (PC61) increased the therapeutic effects of α-GalCer in a BALB/c metastatic mammary model (4T1) [Hong et al., 2010]. However, in our system, a synergistic/additive effect of the combination treatments with the anti-CD25 antibody (PC61) and α-GalCer was not observed. The possible explanation of the discrepancies of our data with the published literature could be the difference in the experimental setting, mouse strains or cancer cell lines. We tried to determine in more detail if anti-CD25 antibody (PC61) interferes with the NKT cell stimulation. We used DEREG mice expressing diphtheria toxin receptor under the *FoxP3* promoter. This system

allows specific Treg depletion [Lahl et al., 2007]. By comparison of results obtained in DEREG mice to the results from the PC61 antibody-treated wild type mice we could distinguish the specific effects of Treg depletion from further effects of PC61 mAb. CD25 is also expressed on the surface of activated human NKT cells and NKT cells can be regulated by IL-2 [Bessoles et al., 2008]. We observed similar findings on mouse NKT, which was also previously shown by Kim et al. in 2006. However, in comparison to human NKT, the expression of CD25 was observed only on a small fraction of NKT cells and this CD25 positive population was induced by α-GalCer. This data proved that PC61 antibody was able to target murine NKT cells. Activation of NKT cells was analyzed 72 hours after stimulation by downregulation of activation marker CD69 [Ikarashi et al., 2006]. PC61 mAb abrogated activation of NKT cells, and inhibited their proliferation and IFNγ production by activated NKT cells. Decrease in IFNγ production reduced the expression level of Tbet, transcriptional factor typically expressed in Th1 cells [Szabo et al., 2000; Wei et al., 2007], which indicates limited T helper polarization towards the Th1 response. Expression of Th2 transcription factor Gata-4 as well as IL-4 was not affected. These data clearly demonstrate that anti-CD25 mAb treatment targeting Treg can be controversial and its efficacy is dependent on the particular treatment setting.

Taken together, our results support the data that peptide vaccines effective against MHC class-I positive tumours might not be efficient against tumours with defects in MHC class I presentation and further improvements of protocols as addition of T helper epitopes or using peptide-pulsed DC are desirable for the induction of the efficient immune response against MHC class I-deficient tumours. We have concluded that the potential 5AC adverse effects on the immune system, especially on DC, were not an obstacle for an effective combination treatment with immunotherapy against MHC class I-positive or -deficient tumours. Besides that, our data indicate that epigenetic changes (induced e.g. by 5AC or TSA) play a role in many immunological important events including DC maturation, and therefore it is important to know the consequences of chemotherapy on the function of DC. Additionally, understanding of mechanisms behind DC maturation is beneficial for the possible clinical application. Since chemo- or immunotherapy can induce negative regulators of the immune responses (Treg), the therapeutic efficacy could be increased by combining the treatment with anti-immunosuppressive treatments such as depletion of Treg. Although using mAb targeting CD25 can deplete Treg, direct effects on effector cell populations (NKT cells) have been observed. Therefore, other possibilities for

Treg modulation, e.g. application of low doses of cyclophosphamide [Motoyoshi et al., 2006] or 1-methyl-tryptophan [Feunou et al., 2007; Chen et al., 2008] should be considered.

Conclusion

The MHC class I status of tumour cells during immunotherapy is often underestimated. It represents one of important tumour escape mechanisms and thus can contribute to the failure of most of the cancer clinical trials that are usually based on the induction of CTL responses. The results presented in this dissertation thesis were obtained from the TC-1 tumour cell model of cervical cancer associated with HPV16 and its derived MHC class I-deficient cell line TC-1/A9. Immunization of tumour-bearing individuals by peptides or DC vaccines represents one of the most common anti-cancer immune interventions developed in the past 30 years. However, there are a very limited number of studies on this topic concerning the MHC class I molecule level on tumour cells. Immunization with a peptide containing only a short $CD8^+$ T cell epitope protected mice against the challenge with parental MHC class I-positive tumours but not against less immunogenic MHC class I-deficient TC-1/A9 tumours. By contrast, immunization with longer peptides harbouring $CD4^+$ and $CD8^+$ T cell epitopes was efficient both in MHC class I-positive and deficient tumours. In the next set of experiments, peptide vaccines were substituted by peptide-pulsed DC to avoid a free peptide application, which could cause tolerance. Administration of such DC presenting the same short peptide increased the protection against the MHC class-I deficient tumours.

Epigenetic changes in the promoters of genes involved in the MHC class I Ag presentation can result in decreased expression of the cell surface MHC molecules on tumour cells. Thus, epigenetic modifiers can restore an expression of the MHC class I molecules and make tumours visible to the $CD8^+$ effector cells. 5AC, an inhibitor of DNMT, is utilized for the therapy of non-solid tumours and limits DNA methylation. Treatment of MHC class I-deficient tumours (TC-1/A9) by 5AC increased demethylation and expression of genes involved in the MHC class I Ag presentation (*Tap1, Tap2, Lmp2, Lmp7*) and sensitized neoplasia to immunotherapy. The immunotherapeutic effect was at least partially mediated by $CD8^+$ cells.

Besides the epigenetic changes on the tumour cells, we were interested in the indirect immunomodulatory effects of chemotherapy with epigenetic agents on cells of the immune system. We focused on the DC as the critical components of initiation of adaptive immune responses. In our study the effects of 5AC and TSA (as the epigenetic agent capable to increase histone acetylation) were tested on murine DC or human monocyte-

derived DC. Both agents decreased the expression/production of selected cytokines (IL-6, IL-10, IL-12, IL-23, IFNγ) by mature DC. Moreover, TSA but not 5AC significantly reduced expression of costimulatory molecule CD40 and the capacity of mature DC to stimulate proliferation of the $CD4^+$ and $CD8^+$ T lymphocytes.

Tumour cells can escape from the immune response not only by changes in the cancer cells, but also by influencing, expanding and/or activating immunoregulatory cell populations, such as Treg. We investigated whether the depletion of Treg by commonly used anti-CD25 mAb (PC61) can inhibit tumour growth and be helpful during the NKT cell-targeted immunotherapy by administration of α-GalCer in the tumour-bearing organism. Unexpectedly, depletion of Treg had no additive effect on the NKT cell activation on the tumour growth. Further studies showed that PC61 antibody not only depleted Treg, but also inhibited activated NKT cells. PC61 limited proliferation of NKT cells after activation, decreased the production of IFNγ by activated NKT cells and was associated with downregulation of the early surface activation marker CD69.

Results obtained from the projects involved in this dissertation are important for optimization of vaccination and immunotherapeutic strategies that take into the account the MHC class I status of neoplasia. Our findings suggest that the efficiency of peptide vaccines against MHC class I-deficient tumours can be increased by peptides harbouring CD4 epitopes or by longer peptides requiring DC processing. In addition, the treatment of MHC class I-deficient tumour by epigenetic agents sensitized neoplasia towards the immunotherapy using of CpG ODN or IL-12-producing cellular vaccine. We reported the modulation of immune responses in several experimental settings. Application of anti-CD25 mAb (PC61) impaired the NKT cell activation and treatment by 5AC and TSA interfered with DC maturation. 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.

References

Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem. 2003;278(3):1910-4.

Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2001;2(8):675-80.

Alba Soto CD, Solana ME, Poncini CV, Pino-Martinez AM, Tekiel V, González-Cappa SM. Dendritic cells devoid of IL-10 induce protective immunity against the protozoan parasite Trypanosoma cruzi. Vaccine. 2010;28(46):7407-13.

Alfaro C, Suarez N, Oñate C, Perez-Gracia JL, Martinez-Forero I, Hervas-Stubbs S, Rodriguez I, Perez G, Bolaños E, Palazon A, Sanmamed MF, Morales-Kastresana A, Gonzalez A, Melero I. Dendritic cells take up and present antigens from viable and apoptotic polymorphonuclear leukocytes. PLoS One. 2011;6(12):e29300.

Ashman RF, Goeken JA, Latz E, Lenert P. Optimal oligonucleotide sequences for TLR9 inhibitory activity in human cells: lack of correlation with TLR9 binding. Int Immunol. 2011;23(3):203-14.

Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol. 1996;157(5):1840-5.

Ballas ZK, Krieg AM, Warren T, Rasmussen W, Davis HL, Waldschmidt M, Weiner GJ. Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. J Immunol. 2001;167(9):4878-86.

Barral DC, Brenner MB. CD1 antigen presentation: how it works. Nat Rev Immunol. 2007;7(12):929-41.

Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. J Exp Med. 2000;191(11):1895-903.

Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J Exp Med. 1997;186(1):65-70.

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.

Bevan MJ. Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. J Immunol. 1976;117(6):2233-8.

Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med. 1976;143(5):1283-8.

Bhat P, Mattarollo SR, Gosmann C, Frazer IH, Leggatt GR. Regulation of immune responses to HPV infection and during HPV-directed immunotherapy. Immunol Rev. 2011;239(1):85-98.

Bijker MS, van den Eeden SJ, Franken KL, Melief CJ, van der Burg SH, Offringa R. Superior induction of anti-tumor CTL immunity by extended peptide vaccines involves prolonged, DC-focused antigen presentation. Eur J Immunol. 2008;38(4):1033-42.

Bijker MS, van den Eeden SJ, Franken KL, Melief CJ, Offringa R, van der Burg SH. CD8+ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. J Immunol. 2007;179(8):5033-40.

Blazar BR, Krieg AM, Taylor PA. Synthetic unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides are potent stimulators of antileukemia responses in naive and bone marrow transplant recipients. Blood. 2001;98(4):1217-25.

Boccardo E. HPV-mediated genome instability: at the roots of cervical carcinogenesis. Cytogenet Genome Res. 2010;128(1-3):57-65.

Bode KA, Schroder K, Hume DA, Ravasi T, Heeg K, Sweet MJ, Dalpke AH. Histone deacetylase inhibitors decrease Toll-like receptor-mediated activation of proinflammatory gene expression by impairing transcription factor recruitment. Immunology. 2007;122(4):596-606.

Boonstra A, Asselin-Paturel C, Gilliet M, Crain C, Trinchieri G, Liu YJ, O'Garra A. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T heper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. J Exp Med. 2003;197(1):101-9.

Borden EC. Augmentation of effects of interferon-stimulated genes by reversal of epigenetic silencing: potential application to melanoma. Cytokine Growth Factor Rev. 2007;18(5-6):491-501.

Bosisio D, Vulcano M, Del Prete A, Sironi M, Salvi V, Salogni L, Riboldi E, Leoni F, Dinarello CA, Girolomoni G, Sozzani S. Blocking TH17-polarizing cytokines by histone deacetylase inhibitors in vitro and in vivo. J Leukoc Biol. 2008;84(6):1540-8.

Branda RF, Moore AL, Mathews L, McCormack JJ, Zon G. Immune stimulation by an antisense oligomer complementary to the rev gene of HIV-1. Biochem Pharmacol. 1993;45(10):2037-43.

Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat Rev Immunol. 2013;13(2):101-17.

Brogdon JL, Xu Y, Szabo SJ, An S, Buxton F, Cohen D, Huang Q. Histone deacetylase activities are required for innate immune cell control of Th1 but not Th2 effector cell function. Blood. 2007;109(3):1123-30.

Brunner C, Seiderer J, Schlamp A, Bidlingmaier M, Eigler A, Haimerl W, Lehr HA, Krieg AM, Hartmann G, Endres S. Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo. J Immunol. 2000;165(11):6278-86.

Bubeník J, Vonka V. MHC class I status of tumours and design of immunotherapeutic strategies. Immunol Lett. 2003;90(2-3):177-8.

Bukur J, Jasinski S, Seliger B. The role of classical and non-classical HLA class I antigens in human tumors. Semin Cancer Biol. 2012;22(4):350-8.

Burnet M. Cancer; a biological approach. I. The processes of control. Br Med J. 1957;1:779–786.

Camelo S, Iglesias AH, Hwang D, Due B, Ryu H, Smith K, Gray SG, Imitola J, Duran G, Assaf B, Langley B, Khoury SJ, Stephanopoulos G, De Girolami U, Ratan RR, Ferrante RJ, Dangond F. Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. J Neuroimmunol. 2005;164(1-2):10-21.

Cella M, Jarrossay D, Facchetti F, Alebardi O, Nakajima H, Lanzavecchia A, Colonna M. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med. 1999;5(8):919-23.

Cerundolo V, Silk JD, Masri SH, Salio M: Harnessing invariant NKT cells in vaccination strategies. Nat Rev Immunol. 2009;9:28-38

Chambers B, Grufman P, Fredriksson V, Andersson K, Roseboom M, Laban S, Camps M, Wolpert EZ, Wiertz EJ, Offringa R, Ljunggren HG, van Hall T. Induction of protective CTL immunity against peptide transporter TAP-deficient tumors through dendritic cell vaccination. Cancer Res. 2007;67(18):8450-5.

Chen W, Liang X, Peterson AJ, Munn DH, Blazar BR. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J Immunol. 2008;181(8):5396-404.

Chen KJ, Lin SZ, Zhou L, Xie HY, Zhou WH, Taki-Eldin A, Zheng SS. Selective recruitment of regulatory T cell through CCR6-CCL20 in hepatocellular carcinoma fosters tumor progression and predicts poor prognosis. PLoS One. 2011;6(9):e24671.

Chen LP, Thomas EK, Hu SL, Hellström I, Hellström KE. Human papillomavirus type 16 nucleoprotein E7 is a tumor rejection antigen. Proc Natl Acad Sci U S A. 1991;88(1):110-4.

Christman JK. 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. Oncogene.

2002;21(35):5483-95.

Chung Y, Kim BS, Kim YJ, Ko HJ, Ko SY, Kim DH, Kang CY: CD1d-restricted T cells license B cells to generate long-lasting cytotoxic antitumor immunity in vivo. Cancer Res. 2006;66:6843-50

Chung YL, Lee MY, Wang AJ, Yao LF. A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. Mol Ther. 2003;8(5):707-17.

Cobbold SP, Adams E, Nolan KF, Regateiro FS, Waldmann H. Connecting the mechanisms of T-cell regulation: dendritic cells as the missing link. Immunol Rev. 2010;236:203-18.

Coley WB. The Treatment of Inoperable Sarcoma by Bacterial Toxins (the Mixed Toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). Proc R Soc Med. 1910;3(Surg Sect):1-48.

Comb M, Goodman HM. CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res. 1990;18(13):3975-82.

Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, Berzins SP, Smyth MJ, Godfrey DI. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. Proc Natl Acad Sci U S A. 2008;105(32):11287-92.

Coyle AJ, Gutierrez-Ramos JC. The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. Nat Immunol. 2001;2(3):203-9.

Crooke RM. In vitro cellular uptake, distribution, and metabolism of oligonucleotides. In Antisense Research and Application, ed. ST Crooke, Berlin: Springer. 1998;103-40

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.

Davila E, Celis E. Repeated administration of cytosine-phosphorothiolated guanine-containing oligonucleotides together with peptide/protein immunization results in enhanced CTL responses with anti-tumor activity. J Immunol. 2000;165(1):539-47.

De Bruijn ML, Nieland JD, Harding CV, Melief CJ. Processing and presentation of intact hen egg-white lysozyme by dendritic cells. Eur J Immunol. 1992;22(9):2347-52.

Den Haan JM, Lehar SM, Bevan MJ. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med. 2000;192(12):1685-96.

De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci U S A. 1996;93(14):7149-53.

Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med. 2001;193(2):233-8.

Doyen V, Rubio M, Braun D, Nakajima T, Abe J, Saito H, Delespesse G, Sarfati M. Thrombospondin 1 is an autocrine negative regulator of human dendritic cell activation. J Exp Med. 2003;198(8):1277-83.

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, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol. 2004;22:329-60.

Ehrlich P. On immunity with special reference to cell life. Proc R Soc Lond 1900;66:424-448.

Emanuele S, Lauricella M, Tesoriere G. Histone deacetylase inhibitors: apoptotic effects and clinical implications (Review). Int J Oncol. 2008;33(4):637-46.

Emmanouilides CE, Luo J, Baldwin G, Buckley D, Lau P, Lopez E, Tabibzadeh S, Yu J, Wolin M, Rigor R, Territo M, Black AC. Murine IL-10 fails to reduce GVHD despite inhibition of alloreactivity in vitro. Bone Marrow Transplant. 1996;18(2):369-75.

Falo LD Jr, Colarusso LJ, Benacerraf B, Rock KL. Serum proteases alter the antigenicity of peptides presented by class I major histocompatibility complex molecules. Proc Natl Acad Sci U S A. 1992;89(17):8347-50.

Ferrara FF, Fazi F, Bianchini A, Padula F, Gelmetti V, Minucci S, Mancini M, Pelicci PG, Lo Coco F, Nervi C. Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. Cancer Res. 2001;61(1):2-7.

Feunou P, Vanwetswinkel S, Gaudray F, Goldman M, Matthys P, Braun MY. Foxp3+CD25+ T regulatory cells stimulate IFN-gamma-independent CD152-mediated activation of tryptophan catabolism that provides dendritic cells with immune regulatory activity in mice unresponsive to staphylococcal enterotoxin B. J Immunol. 2007;179(2):910-7.

Fossum S, Rolstad B. The roles of interdigitating cells and natural killer cells in the rapid rejection of allogeneic lymphocytes. Eur J Immunol. 1986;16(4):440-50.

Frazer IH, Leggatt GR, Mattarollo SR. Prevention and treatment of papillomavirus-related cancers through immunization. Annu Rev Immunol. 2011;29:111-38.

Fujii S, Motohashi S, Shimizu K, Nakayama T, Yoshiga Y, Taniguchi M: Adjuvant activity mediated by iNKT cells. Semin Immunol. 2010;22:97-102

Gabrilovich DI, Nadaf S, Corak J, Berzofsky JA, Carbone DP. Dendritic cells in antitumor immune responses. II. Dendritic cells grown from bone marrow precursors, but not mature

DC from tumor-bearing mice, are effective antigen carriers in the therapy of established tumors. Cell Immunol. 1996;170(1):111-9.

Galasinski SC, Louie DF, Gloor KK, Resing KA, Ahn NG. Global regulation of post-translational modifications on core histones. J Biol Chem. 2002;277(4):2579-88.

Garrett WS, Chen LM, Kroschewski R, Ebersold M, Turley S, Trombetta S, Galán JE, Mellman I. Developmental control of endocytosis in dendritic cells by Cdc42. Cell. 2000;102(3):325-34.

Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U, Presky DH. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol. 1998;16:495-521.

Germain RN, Rinker AG Jr. Peptide binding inhibits protein aggregation of invariant-chain free class II dimers and promotes surface expression of occupied molecules. Nature. 1993;363(6431):725-8.

Glaros S, Cirrincione GM, Muchardt C, Kleer CG, Michael CW, Reisman D. The reversible epigenetic silencing of BRM: implications for clinical targeted therapy. Oncogene. 2007;26(49):7058-66.

Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther. 2003;2(2):151-63.

Glauben R, Batra A, Fedke I, Zeitz M, Lehr HA, Leoni F, Mascagni P, Fantuzzi G, Dinarello CA, Siegmund B. Histone hyperacetylation is associated with amelioration of experimental colitis in mice. J Immunol. 2006;176(8):5015-22.

Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol. 2005;23:515-48.

Gribben JG, Freeman GJ, Boussiotis VA, Rennert P, Jellis CL, Greenfield E, Barber M, Restivo VA Jr, Ke X, Gray GS, et al. CTLA4 mediates antigen-specific apoptosis of human T cells. Proc Natl Acad Sci U S A. 1995;92(3):811-5.

Guinan EC, Gribben JG, Boussiotis VA, Freeman GJ, Nadler LM. Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. Blood. 1994;84(10):3261-82.

Haas T, Metzger J, Schmitz F, Heit A, Muller T, Latz E, Wagner H, (2008) The DNA sugar backbone 20 deoxyribose determines toll-like receptor 9 activation. Immunity 28: 315–323

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

Hart DN, Starling GC, Calder VL, Fernando NS. B7/BB-1 is a leucocyte differentiation antigen on human dendritic cells induced by activation. Immunology. 1993;79(4):616-20.

Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc Natl Acad Sci U S A. 1999;96(16):9305-10.

Hausmann DH, Yu B, Hausmann S, Wucherpfennig KW. pH-dependent peptide binding properties of the type I diabetes-associated I-Ag7 molecule: rapid release of CLIP at an endosomal pH. J Exp Med. 1999;189(11):1723-34.

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA. Nature. 2000;408(6813):740-5.

Henri S, Poulin LF, Tamoutounour S, Ardouin L, Guilliams M, de Bovis B, Devilard E, Viret C, Azukizawa H, Kissenpfennig A, Malissen B. CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. J Exp Med. 2010;207(1):189-206.

Hollenbach PW, Nguyen AN, Brady H, Williams M, Ning Y, Richard N, Krushel L, Aukerman SL, Heise C, MacBeth KJ. A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. PLoS One. 2010;5(2):e9001.

Hong H, Gu Y, Zhang H, Simon AK, Chen X, Wu C, Xu XN, Jiang S. 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. 2010;159(1):93-9.

Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, Endres S, Hartmann G. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. 2002;168(9):4531-7.

IARC. Azacitidine. In Pharmaceutical Drugs. Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 50. Lyon, France: International Agency for Research on Cancer. IARC. 1990;50: 47-63.

Ikarashi Y, Iizuka A, Koshidaka Y, Heike Y, Takaue Y, Yoshida M, Kronenberg M, Wakasugi H. Phenotypical and functional alterations during the expansion phase of invariant Valpha14 natural killer T (Valpha14i NKT) cells in mice primed with alpha-galactosylceramide. Immunology. 2005;116(1):30-7.

Inaba K, Inaba M, Naito M, Steinman RM. Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. J Exp Med. 1993;178(2):479-88.

Indrová M, Reinis M, Bubeník J, Jandlová T, Bieblová J, Vonka V, Velek J. Immunogenicity of dendritic cell-based HPV16 E6/E7 peptide vaccines: CTL activation and protective effects. Folia Biol (Praha). 2004;50(6):184-93.

Insinga RP, Perez G, Wheeler CM, Koutsky LA, Garland SM, Leodolter S, Joura EA, Ferris DG, Steben M, Hernandez-Avila M, Brown DR, Elbasha E, Muñoz N, Paavonen J, Haupt RM; FUTURE I Investigators. Incident cervical HPV infections in young women: transition probabilities for CIN and infection clearance. Cancer Epidemiol Biomarkers Prev. 2011;20(2):287-96.

Insinga RP, Perez G, Wheeler CM, Koutsky LA, Garland SM, Leodolter S, Joura EA, Ferris DG, Steben M, Brown DR, Elbasha EH, Paavonen J, Haupt RM; FUTURE I Investigators. Incidence, duration, and reappearance of type-specific cervical human papillomavirus infections in young women. Cancer Epidemiol Biomarkers Prev. 2010;19(6):1585-94.

Ito D, Ogasawara K, Iwabuchi K, Inuyama Y, Onoé K. Induction of CTL responses by simultaneous administration of liposomal peptide vaccine with anti-CD40 and anti-CTLA-4 mAb. J Immunol. 2000;164(3):1230-5.

Iyoda T, Shimoyama S, Liu K, Omatsu Y, Akiyama Y, Maeda Y, Takahara K, Steinman RM, Inaba K. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. J Exp Med. 2002;195(10):1289-302.

Jabrane-Ferrat N, Calvo F, Faille A, Lagabrielle JF, Boisson N, Quillet A, Fradelizi D. Recombinant gamma interferon provokes resistance of human breast cancer cells to spontaneous and IL-2 activated non-MHC restricted cytotoxicity. Br J Cancer. 1990;61(4):558-62.

Jansen JH, Wientjens GJ, Fibbe WE, Willemze R, Kluin-Nelemans HC. Inhibition of human macrophage colony formation by interleukin 4. J Exp Med. 1989;170(2):577-82.

Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet. 2002;3(6):415-28.

Joyee AG, Uzonna J, Yang X: Invariant NKT cells preferentially modulate the function of CD8 alpha+ dendritic cell subset in inducing type 1 immunity against infection. J Immunol. 2010 15;184:2095-106

Jung ID, Lee JS, Jeong YI, Lee CM, Chang JH, Jeong SK, Chun SH, Park WS, Han J, Shin YK, Park YM. Apicidin, the histone deacetylase inhibitor, suppresses Th1 polarization of murine bone marrow-derived dendritic cells. Int J Immunopathol Pharmacol. 2009;22(2):501-15.

Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu YJ. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med. 2001;194(6):863-9.

Kashimura S, Saze Z, Terashima M, Soeta N, Ohtani S, Osuka F, Kogure M, Gotoh M. CD83(+) dendritic cells and Foxp3(+) regulatory T cells in primary lesions and regional lymph nodes are inversely correlated with prognosis of gastric cancer. Gastric Cancer. 2012;15(2):144-53.

Kajino K, Nakamura I, Bamba H, Sawai T, Ogasawara K. Involvement of IL-10 in exhaustion of myeloid dendritic cells and rescue by CD40 stimulation. Immunology. 2007;120(1):28-37.

Kandimalla ER, Yu D, Zhao Q, Agrawal S. Effect of chemical modifications of cytosine and guanine in a CpG-motif of oligonucleotides: structure-immunostimulatory activity relationships. Bioorg Med Chem. 2001;9(3):807-13.

Kawai T, Akira S. TLR signaling. Semin Immunol. 2007;19(1):24-32.

Kawamura T, Furue M. Comparative analysis of B7-1 and B7-2 expression in Langerhans cells: differential regulation by T helper type 1 and T helper type 2 cytokines. Eur J Immunol. 1995;25(7):1913-7.

Khan S, Bijker MS, Weterings JJ, Tanke HJ, Adema GJ, van Hall T, Drijfhout JW, Melief CJ, Overkleeft HS, van der Marel GA, Filippov DV, van der Burg SH, Ossendorp F. Distinct uptake mechanisms but similar intracellular processing of two different toll-like receptor ligand-peptide conjugates in dendritic cells. J Biol Chem. 2007;282(29):21145-59.

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(5):647-54.

Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, Essahsah F, Fathers LM, Offringa R, Drijfhout JW, Wafelman AR, Oostendorp J, Fleuren GJ, van der Burg SH, Melief CJ. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med. 2009;361(19):1838-47.

Kim D, Hung CF, Wu TC, Park YM: DNA vaccine with α-galactosylceramide at prime phase enhances anti-tumor immunity after boosting with antigen-expressing dendritic cells. Vaccine. 2010;28:7297-305

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 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.

Kimura Y, Sonehara K, Kuramoto E, Makino T, Yamamoto S, Yamamoto T, Kataoka T, Tokunaga T. Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. J Biochem. 1994;116(5):991-4.

Klages K, Mayer CT, Lahl K, Loddenkemper C, Teng MW, Ngiow SF, Smyth MJ, Hamann A, Huehn J, Sparwasser T. Selective depletion of Foxp3+ regulatory T cells improves effective therapeutic vaccination against established melanoma. Cancer Res. 2010;70(20):7788-99.

Klebanoff CA, Yu Z, Hwang LN, Palmer DC, Gattinoni L, Restifo NP. Programming tumor-reactive effector memory CD8+ T cells in vitro obviates the requirement for in vivo vaccination. Blood. 2009;114(9):1776-83.

Ko HJ, Kim YJ, Kim YS, Chang WS, Ko SY, Chang SY, Sakaguchi S, Kang CY: A combination of chemoimmunotherapies can efficiently break self-tolerance and induce antitumor immunity in a tolerogenic murine tumor model. Cancer Res. 2007;67:7477-86

Kordasti SY, Ingram W, Hayden J, Darling D, Barber L, Afzali B, Lombardi G, Wlodarski MW, Maciejewski JP, Farzaneh F, Mufti GJ. CD4+CD25high Foxp3+ regulatory T cells in myelodysplastic syndrome (MDS). Blood. 2007;110(3):847-50.

Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature. 1995;374(6522):546-9.

Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol. 2002;20:709-60.

Kuramoto E, Yano O, Kimura Y, Baba M, Makino T, Yamamoto S, Yamamoto T, Kataoka T, Tokunaga T. Oligonucleotide sequences required for natural killer cell activation. Jpn J Cancer Res. 1992;83(11):1128-31.

La Cava A, Van Kaer L, Fu-Dong-Shi: CD4+CD25+ Tregs and NKT cells: regulators regulating regulators. Trends Immunol. 2006;27:322-7

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.

Lanzavecchia A. Mechanisms of antigen uptake for presentation. Curr Opin Immunol. 1996;8(3):348-54.

Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, Lien E, Nilsen NJ, Espevik T, Golenbock DT. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol. 2004;5(2):190-8.

Latz E, Verma A, Visintin A, Gong M, Sirois CM, Klein DC, Monks BG, McKnight CJ, Lamphier MS, Duprex WP, Espevik T, Golenbock DT. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. Nat Immunol. 2007;8(7):772-9.

Lauener RP, Goyert SM, Geha RS, Vercelli D. Interleukin 4 down-regulates the expression of CD14 in normal human monocytes. Eur J Immunol. 1990;20(11):2375-81.

Leggatt GR, Gabrielli B. Histone deacetylase inhibitors in the generation of the anti-tumour immune response. Immunol Cell Biol. 2012;90(1):33-8.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 1996;86(6):973-83.

Leng C, Gries M, Ziegler J, Lokshin A, Mascagni P, Lentzsch S, Mapara MY. Reduction of graft-versus-host disease by histone deacetylase inhibitor suberonylanilide hydroxamic acid is associated with modulation of inflammatory cytokine milieu and involves inhibition of STAT1. Exp Hematol. 2006;34(6):776-87.

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. The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. Proc Natl Acad Sci U S A. 2002;99(5):2995-3000.

Levitsky HI, Lazenby A, Hayashi RJ, Pardoll DM. In vivo priming of two distinct antitumor effector populations: the role of MHC class I expression. J Exp Med. 1994;179(4):1215-24.

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 istocompatibility class II presentation of tumor antigen. Cancer Res. 1996;56(1):21-6.

Lin RJ, Nagy L, Inoue S, Shao W, Miller WH Jr, Evans RM. Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature. 1998;391(6669):811-4.

Liu K, Nussenzweig MC. Origin and development of dendritic cells. Immunol Rev. 2010;234(1):45-54.

Lorenzi S, Forloni M, Cifaldi L, Antonucci C, Citti A, Boldrini R, Pezzullo M, Castellano A, Russo V, van der Bruggen P, Giacomini P, Locatelli F, Fruci D. IRF1 and NF-kB restore MHC class I-restricted tumor antigen processing and presentation to cytotoxic T cells in aggressive neuroblastoma. PLoS One. 2012;7(10):e46928.

Lou Y, Vitalis TZ, Basha G, Cai B, Chen SS, Choi KB, Jeffries AP, Elliott WM, Atkins D, Seliger B, Jefferies WA. Restoration of the expression of transporters associated with antigen processing in lung carcinoma increases tumor-specific immune responses and survival. Cancer Res. 2005;65(17):7926-33.

Lou Y, Basha G, Seipp RP, Cai B, Chen SS, Moise AR, Jeffries AP, Gopaul RS, Vitalis TZ, Jefferies WA. Combining the antigen processing components TAP and Tapasin elicits enhanced tumor-free survival. Clin Cancer Res. 2008;14(5):1494-501.

Lutz MB, Kukutsch N, Ogilvie AL, Rössner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods. 1999;223(1):77-92.

Lutz MB and Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol. 2002;23(9):445-9.

Lutz MB, Rössner S. Factors influencing the generation of murine dendritic cells from bone marrow: the special role of fetal calf serum. Immunobiology. 2007;212(9-10):855-62.

Lyst MJ, Nan X, Stancheva I. Regulation of MBD1-mediated transcriptional repression by SUMO and PIAS proteins. EMBO J. 2006;25(22):5317-28.

Mai A and Altucci L. Epi-drugs to fight cancer: from chemistry to cancer treatment, the road ahead. Int J Biochem Cell Biol. 2009;41(1):199-213.

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(2):218-27.

Mattarollo SR, Steegh K, Li M, Duret H, Foong Ngiow S, Smyth MJ. Transient Foxp3(+) regulatory T-cell depletion enhances therapeutic anticancer vaccination targeting the immune-stimulatory properties of NKT cells. Immunol Cell Biol. 2013;91(1):105-14.

Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol. 1994;12:991-1045.

Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, Koezuka Y, Kronenberg M. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J Exp Med. 2000;192(5):741-54.

McFarland KN, Das S, Sun TT, Leyfer D, Xia E, Sangrey GR, Kuhn A, Luthi-Carter R, Clark TW, Sadri-Vakili G, Cha JH. Genome-wide histone acetylation is altered in a transgenic mouse model of Huntington's disease. PLoS One. 2012;7(7):e41423.

McKenna HJ, Stocking KL, Miller RE, Brasel K, De Smedt T, Maraskovsky E, Maliszewski CR, Lynch DH, Smith J, Pulendran B, Roux ER, Teepe M, Lyman SD, Peschon JJ. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood. 2000;95(11):3489-97.

McLellan AD, Starling GC, Williams LA, Hock BD, Hart DN. Activation of human peripheral blood dendritic cells induces the CD86 co-stimulatory molecule. Eur J Immunol. 1995;25(7):2064-8.

Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. Nat Rev Cancer. 2008;8(5):351-60.

Meredith MM, Liu K, Darrasse-Jeze G, Kamphorst AO, Schreiber HA, Guermonprez P, Idoyaga J, Cheong C, Yao KH, Niec RE, Nussenzweig MC. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. J Exp Med. 2012;209(6):1153-65.

Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, Perussia B, Kronenberg M, Seeger RC. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. J Immunol. 2001;167(6):3114-22.

Moody CA, Laimins LA. Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer. 2010;10(8):550-60.

Moodycliffe AM, Nghiem D, Clydesdale G, Ullrich SE. Immune suppression and skin cancer development: regulation by NKT cells. Nat Immunol. 2000;1(6):521-5.

Motoyoshi Y, Kaminoda K, Saitoh O, Hamasaki K, Nakao K, Ishii N, Nagayama Y, Eguchi K. Different mechanisms for anti-tumor effects of low- and high-dose cyclophosphamide. Oncol Rep. 2006;16(1):141-6.

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature. 1998;393(6683):386-9.

Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol. 2011;11(12):823-36.

Newman SL, Holly A. Candida albicans is phagocytosed, killed, and processed for antigen presentation by human dendritic cells. Infect Immun. 2001;69(11):6813-22.

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.

Nie Y, Yang G, Song Y, Zhao X, So C, Liao J, Wang LD, Yang CS. DNA hypermethylation is a mechanism for loss of expression of the HLA class I genesin human esophageal squamous cell carcinomas. Carcinogenesis. 2001;22(10):1615-23.

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

Ontiveros F, Wilson EB, Livingstone AM. Type I interferon supports primary CD8+ T-cell responses to peptide-pulsed dendritic cells in the absence of CD4+ T-cell help. Immunology. 2011;132(4):549-58.

Paget C, Mallevaey T, Speak AO, Torres D, Fontaine J, Sheehan KC, Capron M, Ryffel B, Faveeuw C, Leite de Moraes M, Platt F, Trottein F: Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. Immunity. 2007;27:597-609

Parish CR. Cancer immunotherapy: the past, the present and the future. Immunol Cell Biol. 2003;81(2):106-13.

Peart MJ, Smyth GK, van Laar RK, Bowtell DD, Richon VM, Marks PA, Holloway AJ, Johnstone RW. Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. Proc Natl Acad Sci U S A. 2005;102(10):3697-702.

Petersen TR, Sika-Paotonu D, Knight DA, Dickgreber N, Farrand KJ, Ronchese F, Hermans IF. Potent anti-tumor responses to immunization with dendritic cells loaded with tumor tissue and an NKT cell ligand. Immunol Cell Biol. 2010;88(5):596-604.

Pisetsky DS, Reich CF. Stimulation of murine lymphocyte proliferation by a phosphorothioate oligonucleotide with antisense activity for herpes simplex virus. Life Sci. 1994;54(2):101-7.

Pooley JL, Heath WR, Shortman K. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. J Immunol. 2001;166(9):5327-30.

Prendergast GC, Lawe D, Ziff EB. Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell. 1991;65(3):395-407.

Primeau M, Gagnon J, Momparler RL. Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. Int J Cancer. 2003;103(2):177-84.

Puré E, Inaba K, Crowley MT, Tardelli L, Witmer-Pack MD, Ruberti G, Fathman G,

Rankin R, Pontarollo R, Ioannou X, Krieg AM, Hecker R, Babiuk LA, van Drunen Littel-van den Hurk S. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. Antisense Nucleic Acid Drug Dev. 2001;11(5):333-40.

Reddy P, Maeda Y, Hotary K, Liu C, Reznikov LL, Dinarello CA, Ferrara JL. Histone deacetylase inhibitor suberoylanilide hydroxamic acid reduces acute graft-versus-host disease and preserves graft-versus-leukemia effect. Proc Natl Acad Sci U S A. 2004;101(11):3921-6.

Reilly CM, Mishra N, Miller JM, Joshi D, Ruiz P, Richon VM, Marks PA, Gilkeson GS. Modulation of renal disease in MRL/lpr mice by suberoylanilide hydroxamic acid. J Immunol. 2004;173(6):4171-8.

Reiniš M. Immunotherapy of MHC class I-deficient tumors. Future Oncol. 2010;6(10):1577-89.

Reinis M, Símová J, Bubeník J. Inhibitory effects of unmethylated CpG oligodeoxynucleotides on MHC class I-deficient and -proficient HPV16-associated tumours. Int J Cancer. 2006;118(7):1836-42.

Reizis B. Classical dendritic cells as a unique immune cell lineage. J Exp Med. 2012;209(6):1053-6.

Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V. Plasmacytoid dendritic cells: recent progress and open questions. Annu Rev Immunol. 2011;29:163-83.

Romanczuk H, Howley PM. Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. Proc Natl Acad Sci U S A. 1992;89(7):3159-63.

Ruffner MA, Kim SH, Bianco NR, Francisco LM, Sharpe AH, Robbins PD. B7-1/2, but not PD-L1/2 molecules, are required on IL-10-treated tolerogenic DC and DC-derived exosomes for in vivo function. Eur J Immunol. 2009;39(11):3084-90.

Salio M, Silk JD, Cerundolo V. Recent advances in processing and presentation of CD1 bound lipid antigens. Curr Opin Immunol. 2010;22(1):81-8.

Salio M, Speak AO, Shepherd D, Polzella P, Illarionov PA, Veerapen N, Besra GS, Platt FM, Cerundolo V: Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation. Proc Natl Acad Sci U S A. 2007;104:20490-5

Sandler AD, Chihara H, Kobayashi G, Zhu X, Miller MA, Scott DL, Krieg AM. CpG oligonucleotides enhance the tumor antigen-specific immune response of a granulocyte macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma. Cancer Res. 2003;63(2):394-9.

Satpathy AT, KC W, Albring JC, Edelson BT, Kretzer NM, Bhattacharya D, Murphy TL, Murphy KM. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. J Exp Med. 2012;209(6):1135-52.

Schnorrer P, Behrens GM, Wilson NS, Pooley JL, Smith CM, El-Sukkari D, Davey G, Kupresanin F, Li M, Maraskovsky E, Belz GT, Carbone FR, Shortman K, Heath WR, Villadangos JA. The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. Proc Natl Acad Sci U S A. 2006;103(28):10729-34.

Schreiber TH, Podack ER. A critical analysis of the tumour immunosurveillance controversy for 3-MCA-induced sarcomas. Br J Cancer. 2009;101(3):381-6.

Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011;331(6024):1565-70.

Schwartz RH. A cell culture model for T lymphocyte clonal anergy. Science. 1990;248(4961):1349-56.

Seliger B. Strategies of tumor immune evasion. BioDrugs. 2005;19(6):347-54.

Seliger B, Ritz U, Ferrone S. Molecular mechanisms of HLA class I antigen abnormalities following viral infection and transformation. Int J Cancer. 2006;118(1):129-38.

Serrano A, Tanzarella S, Lionello I, Mendez R, Traversari C, Ruiz-Cabello F,Garrido F. 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. 2001;94(2):243-51.

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(22):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(23):9601-7.

Sfondrini L, Besusso D, Zoia MT, Rodolfo M, Invernizzi AM, Taniguchi M, Nakayama T, Colombo MP, Ménard S, Balsari A. Absence of the CD1 molecule up-regulates antitumor activity induced by CpG oligodeoxynucleotides in mice. J Immunol. 2002;169(1):151-8.

Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ. Histone deacetylases as regulators of inflammation and immunity. Trends Immunol. 2011;32(7):335-43.

Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001;410(6832):1107-11.

Shirai M, Pendleton CD, Ahlers J, Takeshita T, Newman M, Berzofsky JA. Helper-cytotoxic T lymphocyte (CTL) determinant linkage required for priming of anti-HIV CD8+ CTL in vivo with peptide vaccine constructs. J Immunol. 1994;152(2):549-56.

Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, Liu YJ. The nature of the principal type 1 interferon-producing cells in human blood. Science. 1999;284(5421):1835-7.

Símová J, Bubeník J, Bieblová J, Jandlová T. The role of NK1.1+ cells in the protection against MHC class I+ HPV16-associated tumours. Folia Biol (Praha). 2004;50(6):200-2.

Símová J, Polláková V, Indrová M, Mikyšková R, Bieblová J, Stěpánek 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.

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(11-12):1125-36.

Sorm F, Pískala A, Cihák A, Veselý J. 5-Azacytidine, a new, highly effective cancerostatic. Experientia. 1964;20(4):202-3.

Starling GC, McLellan AD, Egner W, Sorg RV, Fawcett J, Simmons DL, Hart DN. Intercellular adhesion molecule-3 is the predominant co-stimulatory ligand for leukocyte function antigen-1 on human blood dendritic cells. Eur J Immunol. 1995;25(9):2528-32.

Steinman RM. Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. J Exp Med. 1990;172(5):1459-69.

Steinman RM. Decisions about dendritic cells: past, present, and future. Annu Rev Immunol. 2012;30:1-22.

Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med. 1973;137(5):1142-62.

Stepanek I, Indrova M, Bieblova J, Fucikova J, Spisek R, Bubenik J, Reinis M. Effects of 5-azacytidine and trichostatin A on dendritic cell maturation. J Biol Regul Homeost Agents. 2011;25(4):517-29.

Stimson L, Wood V, Khan O, Fotheringham S, La Thangue NB. HDAC inhibitor-based therapies and haematological malignancy. Ann Oncol. 2009;20(8):1293-302.

Stresemann C, Brueckner B, Musch T, Stopper H, Lyko F. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. Cancer Res. 2006;66(5):2794-800.

Suzuki H, Onishi H, Morisaki T, Tanaka M, Katano M. Intratumoral FOXP3+VEGFR2+ regulatory T cells are predictive markers for recurrence and survival in patients with colorectal cancer. Clin Immunol. 2013;146(1):26-33.

Suzuki T, Tanaka R, Hamada S, Nakagawa H, Miyata N. Design, synthesis, inhibitory activity, and binding mode study of novel DNA methyltransferase 1 inhibitors. Bioorg Med Chem Lett. 2010;20(3):1124-7.

Sweet MJ, Campbell CC, Sester DP, Xu D, McDonald RC, Stacey KJ, Hume DA, Liew FY. Colony-stimulating factor-1 suppresses responses to CpG DNA and expression of toll-like receptor 9 but enhances responses to lipopolysaccharide in murine macrophages. J Immunol. 2002;168(1):392-9.

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100(6):655-69.

Takenaka S, Safroneeva E, Xing Z, Gauldie J. Dendritic cells derived from murine colonic mucosa have unique functional and phenotypic characteristics. J Immunol. 2007;178(12):7984-93.

Tanaka T, Chu CC, Paul WE. An antisense oligonucleotide complementary to a sequence in I gamma 2b increases gamma 2b germline transcripts, stimulates B cell DNA synthesis, and inhibits immunoglobulin secretion. J Exp Med. 1992;175(2):597-607.

Teng MW, Ngiow SF, von Scheidt B, McLaughlin N, Sparwasser T, Smyth MJ. Conditional regulatory T-cell depletion releases adaptive immunity preventing carcinogenesis and suppressing established tumor growth. Cancer Res. 2010;70(20):7800-9.

Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, Carbone DP, Paul WE, Berzofsky JA. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat Immunol. 2000;1(6):515-20.

Toes RE, van der Voort EI, Schoenberger SP, Drijfhout JW, van Bloois L, Storm G, Kast WM, Offringa R, Melief CJ. Enhancement of tumor outgrowth through CTL tolerization after peptide vaccination is avoided by peptide presentation on dendritic cells. J Immunol. 1998;160(9):4449-56.

Tomasi TB, Magner WJ, Khan AN. Epigenetic regulation of immune escape genes in cancer. Cancer Immunol Immunother. 2006;55(10):1159-84.

Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol. 2007;7(3):179-90.

Tsuji N, Kobayashi M, Nagashima K, Wakisaka Y, Koizumi K. A new antifungal antibiotic, trichostatin. J Antibiot (Tokyo). 1976;29(1):1-6.

Uchijima M, Nagata T, Aoshi T, Koide Y. IFN-gamma overcomes low responsiveness of myeloid dendritic cells to CpG DNA. Immunol Cell Biol. 2005;83(1):92-5.

Vambutas A, DeVoti J, Nouri M, Drijfhout JW, Lipford GB, Bonagura VR, van der Burg SH, Melief CJ. Therapeutic vaccination with papillomavirus E6 and E7 long peptides results in the control of both established virus-induced lesions and latently infected sites in a pre-clinical cottontail rabbit papillomavirus model. Vaccine. 2005;23(45):5271-80.

van Berkel ME, Oosterwegel MA. CD28 and ICOS: similar or separate costimulators of T cells? Immunol Lett. 2006;105(2):115-22.

van Hall T, van der Burg SH. Mechanisms of peptide vaccination in mouse models: tolerance, immunity, and hyperreactivity. Adv Immunol. 2012;114:51-76.

van Hall T, Wolpert EZ, van Veelen P, Laban S, van der Veer M, Roseboom M, Bres S, Grufman P, de Ru A, Meiring H, de Jong A, Franken K, Teixeira A, Valentijn R, Drijfhout JW, Koning F, Camps M, Ossendorp F, Kärre K, Ljunggren HG,Melief CJ, Offringa R. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. Nat Med. 2006;12(4):417-24.

Vignali DA, Kuchroo VK. IL-12 family cytokines: immunological playmakers. Nat Immunol. 2012;13(8):722-8.

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.

Villadangos JA, Schnorrer P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol. 2007;7(7):543-55.

Wagner H. The sweetness of the DNA backbone drives Toll-like receptor 9. Curr Opin Immunol. 2008;20(4):396-400.

Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J, Rosenberg SA. Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. Cancer Res. 1994;54(7):1766-71.

Wei J, Duramad O, Perng OA, Reiner SL, Liu YJ, Qin FX. 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. 2007;104(46):18169-74.

Wiemann B, Starnes CO. Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. Pharmacol Ther. 1994;64(3):529-64.

Wingender G, Krebs P, Beutler B, Kronenberg M: Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. J Immunol. 2010;185:2721-9

Woodman CB, Collins SI, Young LS. The natural history of cervical HPV infection: unresolved issues. Nat Rev Cancer. 2007;7(1):11-22.

Wu L, Liu YJ. Development of dendritic-cell lineages. Immunity. 2007;26(6):741-50.

Yamamoto S, Yamamoto T, Shimada S, Kuramoto E, Yano O, Kataoka T, Tokunaga T. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol Immunol. 1992;36(9):983-97.

Yasuda K, Yu P, Kirschning CJ, Schlatter B, Schmitz F, Heit A, Bauer S, Hochrein H, Wagner H. Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. J Immunol. 2005;174(10):6129-36.

Yi AK, Chang M, Peckham DW, Krieg AM, Ashman RF. CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. J Immunol. 1998;160(12):5898-906.

Yoo CB, Jones PA. Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov. 2006;5(1):37-50.

Zhao Q, Matson S, Herrera CJ, Fisher E, Yu H, Krieg AM. Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. Antisense Res Dev. 1993;3(1):53-66.

Zhao Q, Waldschmidt T, Fisher E, Herrera CJ, Krieg AM. Stage-specific oligonucleotide uptake in murine bone marrow B-cell precursors. Blood. 1994;84(11):3660-6.

Zhou D, Cantu C 3rd, Sagiv Y, Schrantz N, Kulkarni AB, Qi X, Mahuran DJ, Morales CR, Grabowski GA, Benlagha K, Savage P, Bendelac A, Teyton L. Editing of CD1d-bound lipid antigens by endosomal lipid transfer proteins. Science. 2004;303(5657):523-7.

Zhu M, Wei MF, Liu F, Shi HF, Wang G. Interleukin-10 modified dendritic cells induce allo-hyporesponsiveness and prolong small intestine allograft survival. World J Gastroenterol. 2003;9(11):2509-12.

Zinkernagel RM, Doherty PC. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. Nature. 1974;251(5475):547-8. a

Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature. 1974;248(450):701-2. b

Zwaveling S, Ferreira Mota SC, Nouta J, Johnson M, Lipford GB, Offringa R, van der Burg SH, Melief CJ. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol. 2002;169(1):350-8.

Project-related list of own publications

Reinis M, Stepanek I, Simova J, Bieblova J, Pribylova H, Indrova M, Bubenik J. Induction of protective immunity against MHC class I-deficient, HPV16-associated tumours with peptide and dendritic cell-based vaccines. Int J Oncol. 2010;36(3):545-51. (IF_{2011}= 2.399)

Rosalia RA, Stěpánek I, Polláková V, Símová 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. (IF_{2011}= 3.205)

Símová J, Polláková V, Indrová M, Mikyšková R, Bieblová J, Stěpánek 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. (IF_{2011}= 5.042)

Stepanek I, Indrova M, Bieblova J, Fucikova J, Spisek R, Bubenik J, Reinis M. Effects of 5-azacytidine and trichostatin A on dendritic cell maturation. J Biol Regul Homeost Agents. 2011;25(4):517-29. (IF_{2011}= 5.183)