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Cytokinový profil pupečníkové krve u novorozenců v riziku rozvoje T1D - monitoring buněčné autoreaktivity s využitím protein microarray

Zkrácený titul: Cytokiny pupečníkové krve a riziko vzniku T1D

Dizertační práce

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Cytokiny pupečníkové krve a riziko vzniku T1D

Obsah

Seznam použitých zkratek

ADA	Americká diabetologická společnost
AG	buňky po diabetogenní stimulaci
AIDA	Advanced Image Data Analyzer
BFU-E	Burst forming unit erythroid
CBMC	mononukleáry pupečníkové krve
CFSE	5,6-carboxylfluorescein diacetate succinimidyl ester
CFU	Colony forming unit
CFU-GEMM	Colony forming unit – granulocytes, erythrocytes, monocytes, macrophages
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
ČRDD	Český registr diabetických dětí
E. coli	Escherichia coli
ELISA	Enzyme-Linked Immuno-Sorbent Assay
ELISPOT	Enzyme-Linked Immuno-Sorbent Spot
FACS	průtoková cytometrie (Fluorescence-Activated Cell Sorting)
FCS	fetální telecí sérum
GAD	dekarboxyláza kys. glutamové
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte/Monocyte Colony-Stimulating Factor
GMP	Granulocyte monocyte progenitor
GRO	Growth Regulated Protein
GvHD	Graft versus Host Disease
HbA1c	glykovaný hemoglobin
HLA	lidský leukocytární antigen (human leukocyte antigen)
HRP	křenová peroxidáza
IA-2	tyrozinfosfatáza

IAA	protilátky proti inzulinu
IDDM	inzulin dependentní diabetes mellitus
IDS	Immunology of Diabetes Society
IFN	interferon
IL	interleukin
LT-HSC	Long-term hematopoietic stem cell
MCP	Monocyte Chemoattractant Protein
MEP	Megakaryocyte, erythrocyte progenitor
MHC	major histocompatibility complex
MIG	Monokine-induced by Interferon gamma
MPP	Multipotential progenitor
NK	1. negativní kontrola (nestimulované buňky); 2. natural killer
oGTT	test orální glukózové tolerance
PBMC	mononukleáry periferní krve
PCR	polymerázová řetězová reakce
PHA	fytohemaglutinin
PNC	penicilin
RANTES	Regulated upon Activation Normal T cell Expressed and Secreted
ST-HSC	Short-term hematopoietic stem cell
T1D	diabetes 1. typu
T1DR	novorozenci s diabetickými rodiči
T2D	diabetes 2. typu
Tc	cytotoxický T lymfocyt
TCR	T buněčný receptor
TGF	Transforming Growth Factor
Th	pomocný T lymfocyt (T-helper)
TNF	Tumor Necrosis Factor
WHO	Světová zdravotnická organizace

Cytokiny pupečníkové krve a riziko vzniku T1D

I. Souhrn

Diabetes 1. typu (T1D) je orgánově specifické Th1 autoimunitní onemocnění s celosvětově významně narůstajícím počtem nových případů. Také v České republice pozorujeme významný vzestup incidence T1D, a to zejména v nejmladší věkové skupině (0-4 roky). Hlavní úloha v rozvoji T1D je přisuzována T lymfocytům a jimi produkovaným cytokinům. Zatím neumíme rozpoznat časné známky buněčné autoreaktivity vedoucí k poškození pankreatických beta buněk a v současné době tak nelze tuto chorobu úspěšně vyléčit ani předejít jejímu vzniku.

Naivní imunitní systém novorozence, dosud nevystavený vlivům zevních faktorů, by mohl být využit jako důležitý model pro studium T1D patogeneze.

Vyšetřovali jsme pupečníkovou krev 22 novorozenců s rodičem trpícím diabetem (T1DR)

a 15 novorozenců s negativní rodinnou anamnézou. Pomocí proteinové microarray jsme v pupečníkové krvi stanovili produkci 23 cytokinů - před a po stimulaci diabetogenními autoantigeny.

V T1DR skupině jsme pozorovali nízkou bazální sekreci všech detekovaných cytokinů:

GM-CSF (p=0,025), GRO (p=0,002), GROalfa (p=0,027), IL1-alfa (p=0,051), IL-3 (p=0,008), IL-7 (p=0,027), IL-8 (p=0,042), MCP-3 (p=0,022), MIG (p=0,034) a RANTES (p=0,004).

V porovnání s bazálními hodnotami jsme u kontrol po stimulaci pozorovali pouze nižší hodnoty G-CSF (p=0,030) a GROalfa (p=0,041). U kontrol byl po stimulaci také pozorován signifikantní pokles G-CSF (p=0,030) a MCP-2 (p=0,009) v porovnání s T1DR. Zdá se, že imunitní systém T1DR novorozenců je méně zralý a více senzitivní v porovnání s novorozenci zdravých matek. Velmi zajímavý byl i vliv rizikového genotypu na výsledky protein microarray. T1DR s rizikovým genotypem měli vyšší bazální hladiny G-CSF

(p=0,038), GM-CSF (p=0,020) a GRO-alfa (p=0,033) a po stimulaci jsme u nich pozorovali sklon k reakci v Th1 směru – vzestup IL-2 (p=0,020), IFN-gama (p=0,001) v porovnání s T1DR v „nízkém riziku rozvoje T1D".

Srovnání cytokinového spektra secernovaného mononukleáry pupečníkové krve novorozenců (CBMC) a mononukleáry periferní krve (PBMC) jejich matek nám umožnilo ověřit možnost eventuální kontaminace pupečníkové krve mononukleáry mateřskými.

Zkoumali jsme mimo jiné i vliv délky diabetogenní stimulace a koncentrace glukózy v mediu na cytokinovou produkci CBMC, a to pomocí protein microarray a ELISPOTu. Pomocí ELISPOTu jsme také porovnávali produkci cytokinů po stimulaci diabetogenními autoantigeny a po stimulaci infekčním agens.

Protein microarray se jeví jako metoda, která by mohla přispět k dalšímu porozumění T1D patogeneze a mohla by tak být perspektivním prostředkem k odhalení imunitní odpovědi rizikové pro vznik T1D a to i u novorozenců.

II. Úvod

1. Diabetes 1. typu (T1D)

1.1 Epidemiologie

Diabetes mellitus je problémem nejen medicínským, ale i etickým, ekonomickým

a psychosociálním. V rozvinutých zemích postihuje diabetes až 5-6% populace. T1D představuje jedno z nejzávažnějších chronických onemocnění dětského věku - manifestuje se především u dětí, dospívajících a mladých dospělých. V roce 1989 byl u nás založen Český registr diabetických dětí (ČRDD), v roce jeho založení byla incidence T1D u dětí 0-14 let

7/100 000/rok. V této věkové skupině pak bylo v České republice v roce 2004 zachyceno

rekordních 306 případů T1D, incidence v tomto roce byla dokonce 20/100 000/rok,

a incidence do této doby rostla zejména v nejmladší věkové skupině (0-4 roky), ve které je

v České republice pozorován roční nárůst až o 6,3%. Od roku 2005 pak již dále nestoupá,

sledujeme spíše mírný pokles (v roce 2006 byla incidence T1D v dětské populaci 0-14 let

18,2/100 000/rok), nicméně po této plateau fázi můžeme pravděpodobně očekávat další

rapidní nárůst incidence, stejně jako tomu bylo v minulosti v některých skandinávských

zemích. Zároveň je patrné, že se diabetické děti manifestují čím dál dříve. Dnes je diabetes u

tříletých dětí dvakrát častější, než byl před patnácti lety u dětí pubertálních (zdroj: ČRDD).

[1-5]. .

1.2 Definice a klasifikace diabetu

Jako diabetes mellitus označujeme skupinu metabolických onemocnění charakterizovaných

hyperglykémií způsobenou poruchou inzulinové sekrece, biologické účinnosti inzulinu nebo

kombinací obou. Převážnou většinu tvoří dvě hlavní skupiny – T1D s absolutním

nedostatkem inzulinu a častější diabetes 2. typu (T2D) způsobený rezistencí vůči inzulinu

a jeho inadekvátní sekrecí. Ostatní formy diabetu jsou poměrně vzácné. T1D je imunitně

podmíněný a dříve byl také označován jako inzulin dependentní diabetes (IDDM). Rozdělení

diabetu dle etiologie (Tab.1) bylo vytvořeno Americkou diabetologickou společností (ADA)

[5-6].

Tab. 1: Klasifikace diabetu dle ADA

Etiologická klasifikace diabetu dle ADA
I. Diabetes I. typu A. imunitně podmíněný B. idiopatický
II. Diabetes II. typu
III. Jiné specifické typy
A. Geneticky podmíněné defekty beta buněk (MODY1-6, dysfunkce mitochondriální DNA a jiné)
B. Geneticky podmíněné defekty biologické účinnosti inzulínu (A typ inzulínové rezistence, leprechaunizmus, Rabsonův-Mendehallův syndrom, lipoatrofický diabetes a jiné)
C. Onemocnění exokrinního pankreatu (pankreatitis, trauma/pankreatektomie, tumory, cystická fibróza, hemochromatóza, fibrokalkulóza a jiné)
D. Endokrinopatie (akromegalie, Cushingův syndrom, glukagonom, feochromocytom, hypertyreóza, somatostatinom, aldosteronom a jiné)
E. Chemicky či léky indukovaný diabetes (vacor, pentamidin, kyselina nikotinová, glukokortikoidy, hormony štítné žlázy, diazoxid, antagonisté beta-adrenergních receptorů, thiazidy, dilantin, interferon alfa a jiné)
F. infekce (kongenitální rubeola, cytomegalovirus a jiné)
G. vzácné formy imunitně podmíněného diabetu (stiff-man syndrome, protilátky proti inzulinovému receptoru, jiné)
H. jiné geneticky podmíněné syndromy s občasným výskytem diabetes mellitus (Downův syndrom, Klinefelterův syndrom, Turnerův syndrom, Wolframův syndrom, Friedreichsova ataxie, Huntingtonova chorea, Lawrence-Moon-Biedelův syndrom, myotonická dystrofie, porfyrie, syndrom Prader-Willi a jiné)

1.3 Etiopatogeneze

T1D je považován za T-helper (Th) -1 autoimunitní onemocnění [5-9] charakterizované absolutním nedostatkem inzulínu, který je důsledkem autoimunitní zánětlivé destrukce pankreatických beta buněk ostrůvků – inzulitidy. Th1 lymfocyty infiltrují Langerhansovy

ostrůvky a jimi produkované cytokiny podporují selektivní destrukci beta buněk cytotoxickými (Tc) lymfocyty. Th1 lymfocyty navíc atrahují do místa zánětu makrofágy a interakcí s B lymfocyty spouští sekundárně tvorbu autoprotilátek, které nemají vlastní destruktivní účinek [5-10].

Interferon (IFN)-alfa [10-11] a IFN-gama [12] byly detekovány *in vivo* v Langerhansových ostrůvcích pacientů s čerstvým záchytem T1D. Th1 cytokinový profil s vysokou produkcí IFN-gama byl zjištěn v pre-diabetické fázi [13-15]. Nicméně krátce před vlastní manifestací diabetu, kdy zbývá jen velmi malé procento beta buněk, mizí Th1-like odpověď, která pak zůstává potlačena i u nově diagnostikovaných T1D pacientů [13, 16-18].

V poslední době je věnována značná pozornost také CD4+CD25+ T regulačním lymfocytům, jejichž defektní funkce by mohla mít vliv na rozvoj T1D [19,20]. Onemocnění má také silnou asociaci s polymorfizmy HLA molekul druhé třídy – DQA a DQB a je ovlivňována DRB geny, frekvence jednotlivých genotypů a haplotypů se pak liší mezi jednotlivými rasami a etniky. T1D je polygenně vázané onemocnění, na jeho rozvoj u geneticky predisponovaných jedinců mají tedy vliv i faktory zevního prostředí, za významné iniciátory autoimunitního procesu jsou považovány některé viry – např. enteroviry, ale i bovinní albumin kravského mléka, některé chemické látky a toxiny [4-7,21].

Autoimunitní inzulitida probíhá obvykle řadu měsíců až let, vede zpočátku k nedostatečné a posléze nulové produkci inzulinu, vedoucí k prvním klinickým projevům diabetu.

Zajímavým zjištěním je fakt, že zbytková sekrece inzulinu často přetrvává měsíce nebo roky po klinickém propuknutí T1D. Navíc nově diagnostikovaní dospělí pacienti s T1D mají zachovanou překvapivě vysokou produkci inzulinu (až 52 %) ve srovnání se zdravou dospělou populací, což souvisí nejspíše s inzulinovou rezistencí, která narůstá s věkem. Produkce inzulinu pak postupně klesá nebo zcela ustává během několika let. U dětských diabetiků je zbytková sekrece inzulinu při manifestaci T1D nižší, k manifestaci T1D je

nezbytná nekróza či apoptóza 70-80% beta buněk, a právě u dětí zpravidla dochází až k úplné

destrukci beta buněk, proto také pozorujeme u menších dětí při záchytu T1D rychleji se

rozvíjející symptomatologii [5-7, 22-24].

K vlastní manifestaci T1D obyčejně dochází v situacích spojených se zvýšenou potřebou

inzulinu, například při infekci či traumatu, ale pravé spouštěcí mechanizmy dosud nejsou

známy. [4, 11].

1.4 Klinický obraz a diagnostika

Pro manifestaci T1D je charakteristická hyperglykémie způsobená poruchou sekrece inzulinu,

která se projevuje častým močením (polyurií), nadměrnou žízní (polydipsií), nevysvětlitelným

hubnutím, neprospíváním, neostrým viděním. Nedojde-li k včasné diagnostice a léčbě,

dochází až k život ohrožujícímu metabolickému rozvratu s ketoacidózou, až

hyperglykemickému komatu [5-7]. Diagnostická kritéria jsou uvedena v tabulce (Tab. 2).

Tab. 2: Diagnostická kritéria diabetu mellitu

1. symptomy diabetu (polyurie, polydipsie, váhový úbytek) a běžná koncentrace glukózy $\geq$ 11,1 mmol/l (jako běžná koncentrace glukózy se považuje glykémie kdykoliv během dne, bez ohledu na dobu posledního jídla). NEBO
2. glykémie nalačno $\geq$ 7 mmol/l. (Lačnění je definováno jako nulový kalorický příjem alespoň po dobu 8 hodin). NEBO
3. Postprandiální glykémie 2 hodiny po podání glukózy při testu orální glukózové tolerance (oGTT) $\geq$ 11,1 mmol/l. (Test oGTT musí být prováděn podle kritérií WHO, s užitím 75g glukózy rozpuštěné ve vodě).

Doposud jedinou terapií je celoživotní podávání inzulinu – rekombinantní lidské inzuliny či inzulinová analoga v subkutánním podávání několikrát denně. Chronická hyperglykémie u špatně kompenzovaných diabetiků je pak spojena s dlouhodobými multiorgánovými komplikacemi (např. diabetická retinopatie, nefropatie, neuropatie, kardiovaskulární poruchy), které zvyšují morbiditu a mortalitu diabetiků. Pro hodnocení dlouhodobé kompenzace diabetiků lze využít hladinu glykovaného hemoglobinu (HbA1c), molekuly hemoglobinu v erytrocytech váží volnou glukózu, za vzniku HbA1c, čím vyšší je tedy dlouhodobě glykémie, tím vyšší je i hladina HbA1c v krvi [5-7].

Míru inzulinové sekrece můžeme stanovit pomocí vyšetření sérového C-peptidu, který se odštěpuje z molekuly inzulinového prekurzoru proinzulinu za vzniku biologicky aktivního inzulinu, a jehož hladina klesá se snižující se inzulinovou produkcí [5-7,23].

1.5 Prediktivní faktory

Jako markery destrukce beta buněk můžeme využít autoprotilátky namířené proti inzulinu IAA, dekarboxyláze kyseliny glutamové (GAD65) a tyrosinfosfatázám IA-2 a IA-2beta, které vznikají sekundárně a nepodílejí se na destrukci beta buněk, mají pouze prediktivní charakter Objevují se až tehdy, když je již většina beta buněk zničena cytotoxickými T lymfocyty, nelze je proto využít jako diagnostický marker, navíc někdy těsně před manifestací diabetu mizí. U dětí jsou pro predikci T1D nejvýznamnější IAA protilátky (přítomné u většiny dětí ve věku do 5 let v době manifestace T1D), které se u dospělých prakticky nevyskytují, nicméně jsou poměrně nestabilní a v praxi se velmi obtížně stanovují [5-7,24].

Míru genetického rizika rozvoje T1D můžeme stanovit na základě vyšetření polymorfizmů HLA II. třídy (viz Tab. 3) [21]).

Tab. 3: Genetické riziko rozvoje T1D založené na HLA-DQA1 a DQB1 genotypizaci

(volně převzato z [21])

Genetické riziko	Kritérium zařazení
velmi vysoké	DQA1*05-DQB1*0201/DQB1*0302 a současně nepřítomnost DRB1*0403
vysoké	DQA1*05-DQB1*0201 nebo DQB1*0302 a současně nepřítomnost DQB1*0602, 0301, 0603, DRB1*0403
průměrné	• zároveň nepřítomnost: DQB1*0302, DQA1*05-DQB1*0201, DQB1*0602, 0301, 0603 • přítomnost DQB1*0301/DQB1*0302 nebo DQB1*0302/DQB1*0603 • přítomnost DQB1*0302-DRB1*0403
nízké	DQB1*0301 nebo DQB1*0603 zároveň nepřítomnost DQB1*0302 a DQB1*0602
velmi nízké	DQB1*0602

Dosud však neexistuje vhodný prostředek k rozpoznání časných známek buněčné autoreaktivity vedoucí k destrukci beta buněk a rozvoji T1D.
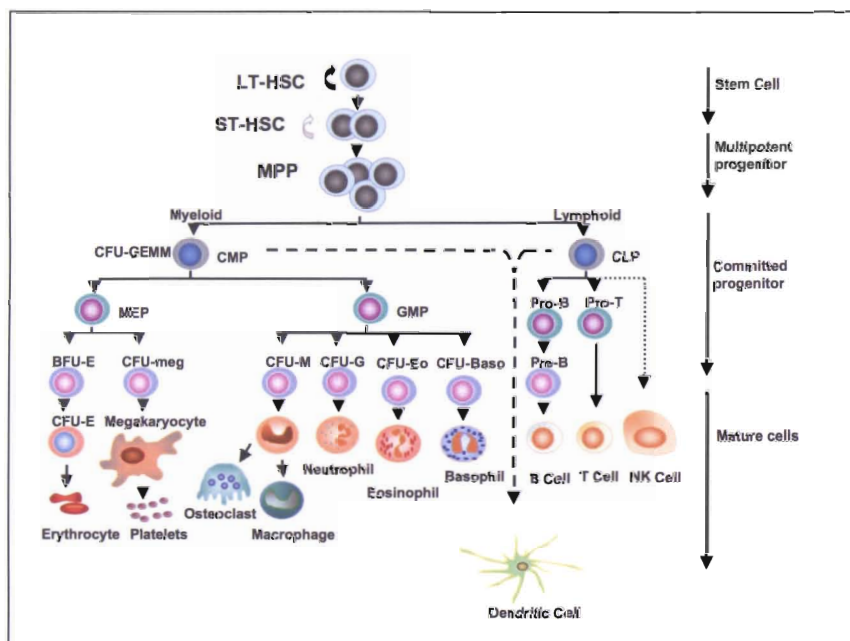
2. Vývoj T lymfocytů

Lymfocyty vznikají z pluripotentní kmenové buňky CD34, která se diferencuje a dává základ pro vznik dvou hlavních linií - lymfoidní a myeloidní. Z lymfoidní linie vznikají NK buňky, T a B lymfocyty. Z linie myeloidní se diferencují monocyty (ve tkáních pak jako makrofágy)

a granulocyty (neutrofily neboli polymorfonukleáry, eozinofily a bazofily se svou tkáňovou formou – žírnými buňkami), erytroblasty jakožto prekurzory erytrocytů, megakaryocyty dávající vznik trombocytům, a dále dendritické buňky vznikající buď přímo z myeloidního prekurzoru či monocytární linie [25-28]. (Viz schéma 1)

Schéma 1: Vývoj krevních buněk z kmenové buňky (zdroj: www.molmed.lu.se)

LT-HSC – Long-term hematopoietic stem cell, ST-HSC – Short-term hematopoietic stem cell, MPP – Multipotential progenitor, CFU-GEMM – Colony forming unit – granulocytes, erythrocytes, monocytes, macrophages, CMP – Common myeloid progenitor, CLP – Common lymphoid progenitor, MEP – Megakaryocyte, erythrocyte progenitor, GMP – Granulocyte monocyte progenitor, BFU-E Burst forming unit erythroid, CFU – Colony forming unit, E – erythroid, mega - megakaryocyte

Lymfocyty se objevují v krevním oběhu plodu relativně pozdě a stávají se plně funkčními až měsíce po narození. V průběhu těhotenství dochází k indukci autotolerance, pozitivní a negativní selekci, ale definitivně je specifická buněčná imunita nastartována až v raném dětství po kontaktu s faktory zevního prostředí. Velmi důležitá je eliminace vysokoafinních autoreaktivních T- a B-lymfocytů. Regulace tohoto procesu je významná zejména v období

dramatických změn vlastních struktur plodu a v období zvýšené apoptózy buněčných populací v průběhu prenatálního života [25-28].

Prekurzory lymfoidních buněk, progenitorových buněk T lymfocytů, se objevují ve žloutkovém váčku a játrech plodu kolem 7. gestačního týdne. Poté postupně přebírá funkci na tvorbě T lymfocytů kostní dřeň. V 15. týdnu těhotenství migrují progenitory T lymfocytů – prothymocyty, do vyvíjejícího se thymu (jeho základy je možné detekovat již od 6. týdne gestace, ale definitivní struktury nabývá až kolem 18. gestačního týdne). Prothymocyty nesou pan T lymfocytární znaky CD3 a CD5 a začínají exprimovat CD4, CD8 markery a T buněčný receptor (TCR). Po proběhnutí rekombinace genů kódujících TCR (nejprve geny pro TCRbeta a TCRdelta, poté proběhne přeskupování genů pro lehké řetězce TCRalfa a TCRgama), dochází k procesům negativní selekce (eliminace autoreaktivních klonů) a pozitivní selekce (eliminace buněk s nefunkčním TCR, neschopným rozeznávat MHC proteiny a vázat je s dostatečnou afinitou). Zhruba 98% prothymocytů tak podléhá v thymu apoptóze. Zbylé T buňky – zralé lymfocyty, opouštějí thymus a usidlují se v sekundárních lymfatických orgánech [25-28].

Na konci těhotenství je imunologická rovnováha více nakloněna ve prospěch CD4+ lymfocytů a poměr CD4+/CD8+ se pak mění v průběhu porodu. Jelikož dosud nedošlo k setkání s antigeny zevního prostředí, většina lymfocytů má zatím "naivní" charakter. Exprimují izoformy CD45, CD 45RA a CD45RB. Po stimulaci začnou novorozenecké T buňky exprimovat CD45RO, který nacházíme na paměťových buňkách. Velmi malé počty CD45RO pozitivních buněk v krevním oběhu donošených novorozenců poukazují na značně omezenou antigenem podmíněnou expanzi T buněk v období před narozením. Tyto "naivní" T lymfocyty mají také menší schopnost proliferace po polyklonální stimulaci. V porovnání s dospělými jedinci produkují menší množství cytokinů (IFN-gama, IL-4, IL-5), zejména lze pozorovat nedostatek cytokinů Th1 skupiny. Proto je snížena cytotoxická reaktivita namířená

proti buňkám infikovaným virem, typická pro novorozenecké období. Také interakce s B lymfocyty není v raném novorozeneckém období adekvátní. (T lymfocyty například v menší míře exprimují CD40L).

Zajímavé je, že pupečníková krev obsahuje i CD3-CD8+ lymfocyty vykazující NK aktivitu. Ty nikdy nebyly detekovány v periferní krvi dospělých a pravděpodobně reprezentují přechodné stádium mezi thymocyty a zralými T lymfocyty [26-28].

3. Modulace imunitní odpovědi v průběhu těhotenství

Modulace imunitní odpovědi v průběhu těhotenství mezi mateřským imunitním systémem

a imunitním systémem plodu umožňuje zdárný průběh těhotenství. Ten závisí na schopnosti relativně dlouhodobé tolerance alogenních struktur imunitním systémem matky, který suprimuje cytotoxickou Th1 buněčnou odpověď. Ta by vedla k odvrhnutí otcovských aloantigenů, které jsou součástí zárodečných buněk. Také rovnováha buněčné odpovědi Th1 versus Th2 je v těhotenství mírně převážena ve směru Th2. Stejně tak povšechná imunoreaktivita embrya je více směrována ve prospěch Th2.

Navíc placentární produkce cytokinů přispívá k modulaci mateřské imunitní reakce a brání "rejekci plodu" [26-32]. V lidském organizmu můžeme vysledovat placentární sekreci zejména následujících cytokinů: IFN-alfa, IFN-beta, nízké hladiny TNF-alfa, IFN-gama. Proto nacházíme zvýšené hladiny těchto cytokinů v pupečníkové krvi novorozenců při chorioamniotitidě. Vyšší hladiny některých cytokinů (IL-8) nacházíme u novorozenců matek, u kterých byla před porodem provedena indukce plicní zralosti plodu pomocí steroidů, nebo v pupečníkové krvi novorozenců narozených v endemických oblastech parazitárních onemocnění či tuberkulózy [26-32].

4. Metody detekce autoreaktivních T lymfocytů u T1D

Současné metody detekce T lymfocytů specifických pro antigeny beta buněk pankreatu u T1D nebyly dosud standardizovány. Většinou jsou založeny na stimulaci lymfocytů specifickými diabetogenními autoantigeny (GAD65, proinzulin, IA-2). Umožňují kvantifikaci antigen specifických T buněk a na základě fenotypické a cytokinové analýzy ozřejmení kvality jejich odpovědi na diabetogenní stimulaci [33-41].

4.1 Proliferační testy

- *Inkorporace ^3H thymidinu* - antigenem stimulované T lymfocyty proliferují a inkorporují tak do nově vytvořené DNA ^3H thymidin

- *Redukce intenzity CFSE* (5,6-carboxylfluorescein diacetate succinimidyl ester) - před stimulací jsou označeny všechny buňky CFSE. U proliferujících buněk fluorescence klesá. Míra fluorescence a povrchové markery T buněk se stanovují za pomoci průtokové cytometrie (FACS).

Tyto testy jsou poměrně jednoduché, vzhledem k nízké senzitivitě mají však spíše historický význam. Nejsou schopny detekovat efektorové T lymfocyty produkující cytokiny, které mají nízkou proliferační schopnost, či neproliferující chronicky stimulované T buňky náchylné k replikační senescenci [33, 34].

4.2 Stanovení aktivačních markerů, které exprimují antigenem stimulované T lymfocyty (zastoupení CD4+ buněk se znaky CD69+ a CD25+^{high} pomocí průtokové cytometrie (FACS). Při **stanovení intracelulárních cytokinů** jsou buňky kultivovány s antigenem za přítomnosti inhibitorů buněčné sekrece a cytokiny akumulované v buňkách jsou po permeabilizaci detekovány pomocí značených protilátek [33, 34].

4.3 Stanovení produkce cytokinů

- **ELISA** *(Enzyme-linked Immunosorbent Assay)* umožňuje kvantitativní stanovení produkce cytokinů. Podstatou je reakce antigenu s protil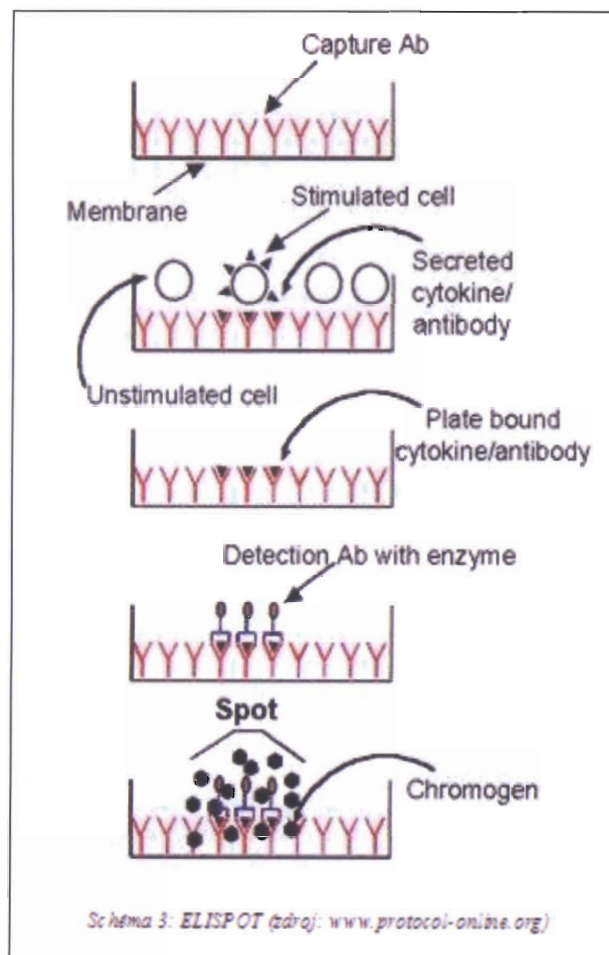átkou. Primární protilátka je zde navázána na pevné fázi (na dně jamky, většinou 96 jamkové destičky), po reakci s antigenem vzorku je nadbytečný antigen odstraněn a poté je přidána sekundární detekční protilátka s navázaným enzymovým substrátem a vzniklá barevná enzymatická reakce je následně změřena. Metoda je velmi jednoduchá, nicméně postrádá dostatečnou nepřináší informaci o frekvenci cytokiny produkujících buněk a neumožňuje rozlišení mezi malým počtem buněk produkujících velká kvanta cytokinů a velkým počtem buněk produkujících jen cytokiny jen v omezené míře.Výhodou je dostatečná specifita, senzitivita i reprodukovatelnost a umožnění detekce proteinů vyskytujících se ve vzorcích ve velmi nízkých koncentracích. Poměrné zrychlení a usnadnění metody nabízí také využití tzv. kitů Instant ELISa, kde jsou již standardy (vůči kterým se vztahují výsledky měření) předem naředěny a naneseny na dno jamek v prvních dvou sloupcích destičky [33,34].

Schéma 2: ELISA (zdroj: www. newenglandbiolabs.de)

Cytokiny pupečníkové krve a riziko vzniku T1D

- **ELISPOT** *(Enzyme-linked Immunosorbent Spot)* funguje na principu ELISy, ale detekuje přímo T lymfocyty produkující daný cytokin. Na pevné fázi jsou naneseny primární protilátky, po přidání nastimulovaných buněk produkujících hledaný cytokin a promytí jsou pak přidány detekční protilátky s navázaným enzymem, po navázání chromogenu lze pak zjistit počet tzv. spotů (míst, kde daný T lymfocyt produkoval daný cytokin), a to buď pod inverzním mikroskopem, či automaticky pomocí ELISPOT readeru, a provést kalkulaci procenta buněk produkujících daný cytokin. Tato metoda je vysoce senzitivní [33,34].

Schéma 3: ELISPOT (zdroj: www.protocol-online.org)

- *Protein microarray* je semikvantitativní metoda, která je dostatečně senzitivní, nepříliš drahá, která umožňuje simultánní detekci stovek proteinů v různých biologických vzorcích. Membrány z nitrocelulózy jsou potaženy primární protilátkou proti různým proteinům (např. cytokinům). Je-li ve zkoumaném vzorku obsažen hledaný protein (cytokin), váže se na přesně určené místo na membráně na navázanou primární protilátku. Poté přidáváme směs sekundárních protilátek značených biotinem. Signál je pak zesílen vazbou streptavidinu konjugovaného s křenovou peroxidázou (HRP) [33-35] (www.raybiotech.com).¨

Schéma 4: Protein microarray
(zdroj: www.raybiotech.com)

- *Antibody array* umožňuje simultánní kvantitativní stanovení koncentrace mnoha cytokinů. Spojuje v sobě vysokou senzitivitu a specifitu ELISy a celistvost a multiplexový formát arraye. Využívá se páru specifických protilátek proti cytokinům jako u standardní ELISY, primární protilátka nanesená v kvadruplikátu váže cytokin k pevné fázi, sekundární protilátka značená biotinem umožňuje detekci jednotlivých cytokinů po přidání komplexu značení Alexa555 se streptavi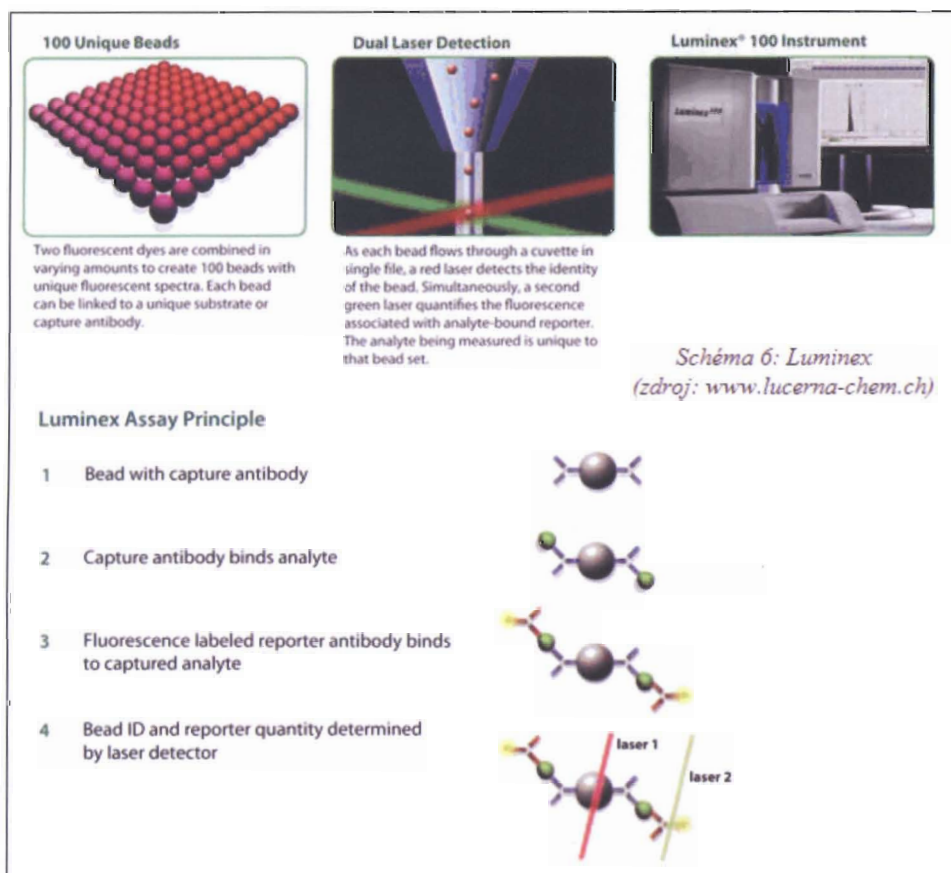dinem. Výsledný signál se detekuje pomocí laserového scanneru. Lze provést detekci až 64 vzorků zároveň. Antibody array je osmdesátkrát efektivnější než tradiční ELISA a lze ji využít i při limitovaných objemech vzorku. Stanovení jednotlivého cytokinu je při jejich simultánní detekci také méně nákladné (www.raybiotech.com).

- *Flow cytomix* je opět metoda na principu sendvičové imunoassaye. Na povrchu partikulí o různé velikosti jsou navázány protilátky proti jednotlivým cytokinům, po navázání cytokinu ze vzorku je přidána sekundární protilátka značená biotinem, na který se váže streptavidin s fykoertytrinem. Detekce je pak umožněna pomocí průtokové cytometrie. Jedná se o kvantitativní metodu, která umožňuje simultánní stanovení až 20 různých proteinů. Metoda je poměrně rychlá a senzitivní [36] (www.lucerna.chem.ch).

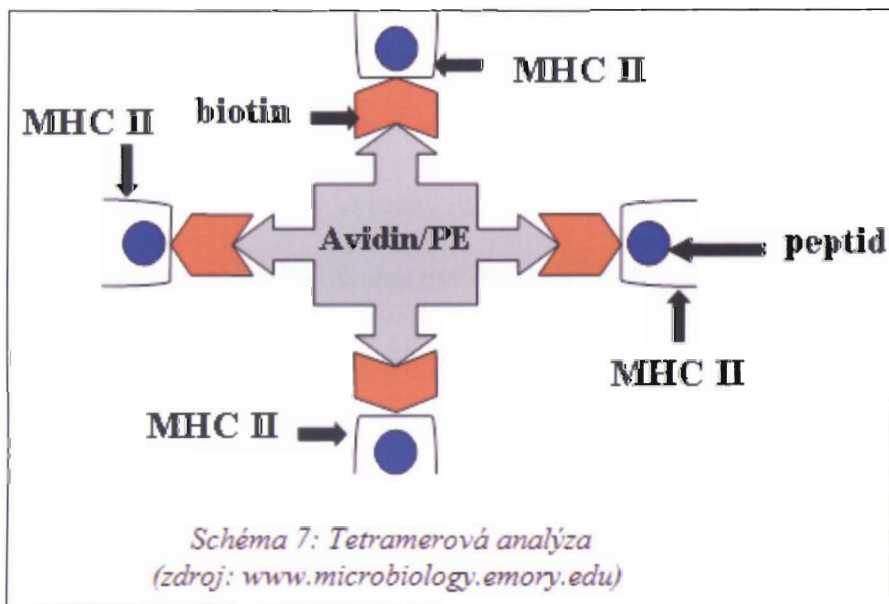*Schéma 5: Flowcytomix (zdroj: www.lucerna-chem.ch)*

Cytokiny pupečníkové krve a riziko vzniku T1D

- **_Luminex_** umožňuje kvantitativní stanovení až 100 cytokinů najednou i ve vzorcích o velmi malém objemu. Principem jsou partikule potažené protilátkou, uvnitř každé partikule je definovaná směs dvou fluorescenčních barev v různém poměru, který určuje specifitu partikule. Cytokin obsažený ve vzorku se váže na protilátku na povrchu partikule, poté je přidána fluorescenčně značená sekundární protilátka. Detekce pak probíhá v kyvetě, kudy procházejí jednotlivé partikule, a kde je po excitaci červeným laserem zjištěno spektrum specifické pro danou partikuli, poté je zeleným laserem detekována fluorescence způsobená vazbou sekundární protilátky. Tato metoda je vysoce senzitivní a relativně nenáročná na čas [37,38] (www.lucerna.chem.ch).

100 Unique Beads

Two fluorescent dyes are combined in varying amounts to create 100 beads with unique fluorescent spectra. Each bead can be linked to a unique substrate or capture antibody.

Dual Laser Detection

As each bead flows through a cuvette in single file, a red laser detects the identity of the bead. Simultaneously, a second green laser quantifies the fluorescence associated with analyte-bound reporter. The analyte being measured is unique to that bead set.

Luminex® 100 Instrument

Schéma 6: Luminex
(zdroj: www.lucerna-chem.ch)

Luminex Assay Principle

1 Bead with capture antibody

2 Capture antibody binds analyte

3 Fluorescence labeled reporter antibody binds to captured analyte

4 Bead ID and reporter quantity determined by laser detector

laser 1
laser 2

4.4 Specifická vazba TCR a MHC II.

Tetramerová analýza – tetramer vázaných rekombinantních MHC II. s navázaným specifickým peptidem, s kterými interagují antigen specifické T lymfocyty. Vazbou s tetramerem jsou T buňky fluorescenčně označeny a posléze detekovány pomocí průtokové cytometrie (FACS). Metoda umožňuje identifikaci i funkčně silentních specifických T buněk. Nicméně limitací zůstává poměrně nízká senzitivita [34,39].

Schéma 7: Tetramerová analýza
(zdroj: www.microbiology.emory.edu)

5. Cíle práce

Dle našeho nejlepšího vědomí, vyjma naše dosavadní studia, nikdo dosud nepublikoval práci zabývající se imunoreaktivitu proti autoantigenům u naivního imunitního systému novorozence. Nová zjištění by mohla přispět k dalšímu porozumění T1D patogeneze. Proto jsme se pomocí protein microarray rozhodli studovat produkci cytokinů a chemokinů (bazální a po stimulaci diabetogenními autoantigeny) mononukleáry pupečníkové krve.
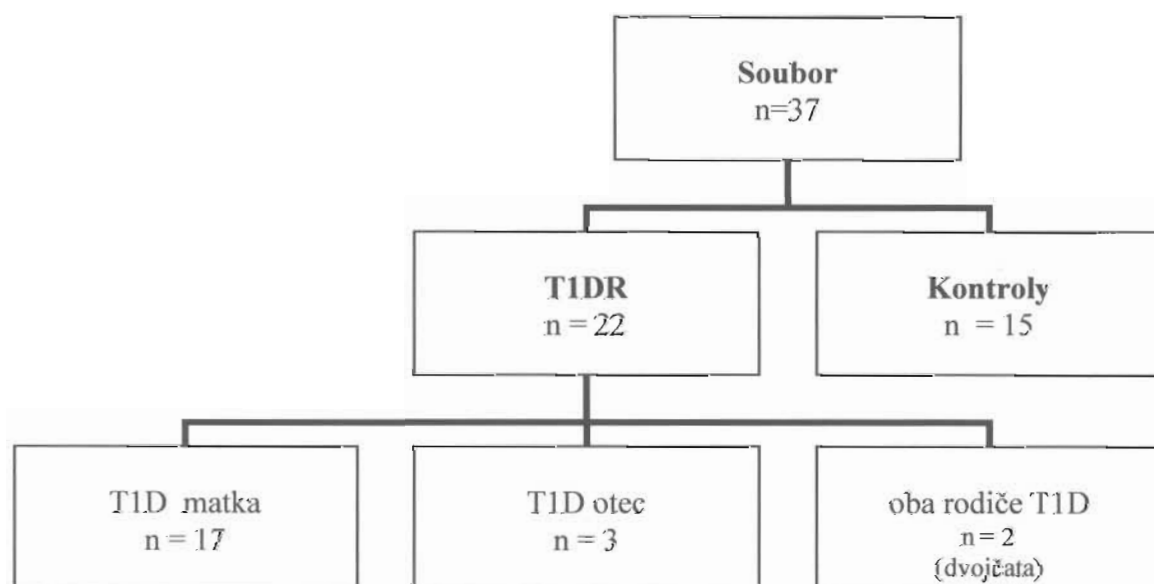
6. Metody

6.1 Soubor

Naše studie zahrnuje 22 novorozenců s diabetickým rodičem [s diabetickou matkou (17/22), s otcem diabetikem (3/22) a pár dvojčat s oběma rodiči diabetiky] a 15 novorozenců (kontrol) s negativní rodinnou anamnézou autoimunitních chorob (Diagram 1). Žádná z matek netrpěla jiným imunitně podmíněným onemocněním (jiné autoimunity, alergie, astma, imunodeficit). Všichni novorozenci se narodili v termínu a to z fyziologických těhotenství bez jakýchkoliv perinatálních komplikací. Četnost porodů *per vias naturales* a císařským řezem byla v obou skupinách shodná. Pro naši studii byly vybrány diabetické matky s dobrou compliance a kontrolou diabetu v průběhu celého těhotenství. Všechny byly sledovány na Interní klinice 2. lékařské fakulty UK v Praze, součástí kontrol bylo pravidelné stanovování glykovaného hemoglobinu (HbA1c) - s normálními výsledky. K ověření možnosti kontaminace pupečníkové krve mateřskými mononukleáry jsme krátce po porodu (do 4 hodin) odebrali vzorky periferní krve u 6 diabetických matek a 4 matek z kontrolní skupiny.

Diagram 1: Studovaný soubor

Naše studie byla schválena etickou komisí a všechny matky podepsaly před odběrem vzorků informovaný souhlas.

6.2 Izolace mononukleárních buněk a stimulace

Ve všech *in vitro* experimentech jsme používali mononukleární buňky pupečníkové krve (CBMC), mononukleáry periferní krve (PBMC) v případě matek. Odebírali jsme přibližně 5-8 ml pupečníkové krve a 10-15 ml mateřské periferní krve. CBMC (PBMC) jsme zizolovali z plné krve za použití Ficollové hustotní gradientové centrifugace (Amersham Biosciences, Uppsala, Švédsko). 2 x 10^6 čerstvě zizolovaných CBMC/PBMC jsme resuspendovali v 1 ml RPMI-1640 média doplněného 20% fetálním telecím sérem (FCS), L-glutaminem (10 ul/ml 200mM L-glutamin) a penicilin-streptomycinem (1 ul/ml PNC a 1 ug/l streptomycin; vše Sigma, St. Louis, U.S.A.) a kultivovali s diabetogenními autoantigeny.

Buňky jsme stimulovali směsí následujících autoantigenů: GAD65-peptidy (Dept. of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Švédsko); IA-2 a a.a. β proinzulinového řetězce (Sigma, St. Louis, U.S.A.). Všechny autoantigeny byly použity v koncentraci 1ug na 10^6 buněk (Tab. 4). Výběr a koncentrace autoantigenů, stejně tak množství testovaných PBMC, byly zvoleny na základě doporučení Immunology of Diabetes Society (IDS).

Tab. 4 Autoantigeny použité ke stimulaci CBMC/PBMC

autoantigen	mol. hmotnost
GAD a.a. 247-279 (NMYAMMIARFKMFPEVKEKGMAALPRLIAFTSEE-OH)	3823,7
GAD a.a. 509-528 (IPPSLRTLEDNEERMSRLSK-OH)	2371,7
GAD a.a. 524-543 (SRLSKVAPVIKARMMEYGTT-OH)	2238,7
IA-2 a.a. 853-872 SFYLK(Nleu)VQTQETRTLTQFHF	2489
a.a. **β proinzulin**ového řetězce 9-23 SHLVEALYLVCGERG	1645

Část buněk jsme využili pro pozitivní [CBMC/PBMC + 10 ug fytohemaglutininu (PHA; Sigma, St. Louis, U.S.A.) na 10^6 buněk] a negativní kontrolu (CBMC/ PBMC pouze v kultivačním médiu). Supernatant získaný po 72 hodinách stimulace (37° C, 5% CO_2) jsme zamrazili (- 20°C) a později využili pro protein microarray. Buňky pak byly použity při ELISPOTu.

Stimulovali jsme také CBMC novorozence s matkou diabetičkou a kontrolního novorozence za podmínek rozdílných koncentrací glukózy v médiu. V RPMI médiu je standardní koncentrace glukózy 11,1 mmol/l, přidáním glukózy, nebo naopak naředěním jsme docílili změn koncentrace. Buňky jsme pak vystavili stimulaci diabetogenními antigeny (koncentrace 1ug na 10^6 buněk, celková doba stimulace 72 hodin, 37° C, 5% CO_2) v médiu s různou koncentrací glukózy 8,11 mmol/l, 11,11 mmol/l, 16,11 mmol/l a 21 mmol/l po různou dobu (2, 24, či 72hod) (víz Tab. 5). K negativním kontrolám, za stejných podmínek složení média, nebyla přidána směs autoantigenů. Finální supernatanty byly zamraženy (- 20°C) a později zanalyzovány za použití protein microarray.

Tab. 5 Stimulace CMBC při různých koncentracích glukózy v médiu.

AG ano – stimulace autoantigeny, AG ne – negativní kontrola

vzorek	konc. glukosy 1 (mmol/l)	čas působení 1 (hod)	konc. glukosy 2 (mmol/l)	čas působení 2 (hod)	AG
DCBD 1	normal 11,11	72	-	-	ne
DCBD 2	normal 11,11	72	-	-	ano
DCBD 3	nízká 8,11	72	-	-	ne
DCBD 4	nízká 8,11	2	normal 11,11	70	ne
DCBD 5	nízká 8,11	2	normal 11,11	70	ano
DCBD 6	stř. vysoká 16,11	72	-	-	ne
DCBD 7	stř. vysoká 16,11	72	-	-	ano
DCBD 8	vysoká 21,11	2	normal 11,11	70	ne
DCBD 9	vysoká 21,11	2	normal 11,11	70	ano
DCBD 10	vysoká 21,11	24	normal 11,11	48	ne
DCBD 11	vysoká 21,11	24	normal 11,11	48	ano
DCBD 12	vysoká 21,11	72	-	-	ne

6.3 Protein microarray

Pro protein microarray jsme použili komerční kity a postupovali dle instrukcí výrobce (RayBiotech, Norcross, U.S.A.).

Chemiluminiscenci jsme detekovali pomocí Fuji LAS1000 imaging system (Fuji, Tokyo, Japonsko) a poté analyzovali AIDA softwarem (Advanced Image Data Analyzer, 3.28; Raytest Izotopenmessgeraete, Straubenhardt, Německo). Získané obrázky jsme pak editovali dle 8-bitové mapy stupňů šedi. Výsledky jsme dle doporučení výrobce stanovovali v procentech intenzity signálu. Membrány jsme porovnávali mezi sebou, hodnoty pozitivních kontrol jednotlivých membrán dosahovaly 100% intenzity, tudíž nebyla nezbytná další transformace.

Detekovali jsem produkci 23 cytokinů a chemokinů viz Tab. 6. Detekční limity jednotlivých cytokinů jsou volně dostupné na webových stránkách výrobce (www.raybiotech.com).

Tab. 6 Pomocí protein microarray jsme detekovali následujících 23 cytokinů a chemokinů

cytokin	celý název	detekční limit (pg/ml)
GM-CSF	Granulocyte/Macrophage Colony-Stimulating Factor	100
G-CSF	Granulocyte Colony-Stimulating Factor	2 000
GRO	Growth Regulated Protein	1 000
GROalfa	Growth Regulated Protein alfa	1 000
IL-1	Interleukin 1	1 000
IL-2	Interleukin 2	25
IL-3	Interleukin 3	100
IL-5	Interleukin 5	1
IL-6	Interleukin 6	1
IL-7	Interleukin 7	100
IL-8	Interleukin 8	1
IL-10	Interleukin 10	10
IL-13	Intetrleukin 13	100
IL-15	Interleukin 15	100
IFN-gama	Interferon gama	100
MCP-1	Monocyte Chemoattractant Protein 1	3
MCP-2	Monocyte Chemoattractant Protein 2	100
MCP-3	Monocyte Chemoattractant Protein 3	1 000
MIG	Monokine-induced by Interferon gama	1

RANTES	Regulated upon Activation Normal T cell Expressed and Secreted	2 000
TGF-beta	Transforming Growth Factor-beta	200
TNF-alfa	Tumor Necrosis Factor alfa	10
TNF-beta	Tumor Necrosis Factor beta	1 000

6.4 ELISPOT (Enzyme-linked Immuno-sorbent spot)

U jednoho z T1DR novorozenců jsme sledovali změny v produkci cytokínů CBMC po 3, 6

a 10 dnech diabetogenní stimulace (stimulace jednotlivými autoantigeny a směsí všech

autoantigenů v koncentraci 1ug na 10^6 buněk, $37\degree C$, 5% CO_2). Pomocí ELISPOTu (Mabtech

AB, Nacka Strand, Švédsko) jsme dle instrukcí výrobce stanovili produkci následujících

cytokinů: IL-10, 13 a IFN-gama.

Dále jsme ELISPOT využili ke stanovení produkce IL-6, IL-13 a IFN-gama u CBMC jednoho

T1DR novorozence a jednoho kontrolního novorozence. Jejich CBMC byly po 3 dny

stimulovány ($37\degree C$, 5% CO_2) jednak směsí diabetogenních autoantigenů (v koncentraci 1ug na

10^6 buněk), jednak referenčním kmenem *Escherichia coli* CCM 4751 (*E. coli*) z Národní

sbírky typových kultur ze Státního zdravotního ústavu (SZÚ) v koncentraci 10 ug na 2 mil

buněk (CFU = 10^7 buněk/ml).

Spoty byly odečítány a vyhodnoceny za použití LUCIA programu (Laboratory Imaging,

Praha, Česká republika).

6.5 Polymerázová řetězová reakce (PCR)

V Laboratoři molekulární genetiky při Pediatrické klinice, 2.LF UK a FNM byla provedena

kompletní HLA-DQA1 a DQB1 genotypizace ke stanovení genetického rizika rozvoje T1D

(Tab. 3) pomocí PCR za použití sekvence specifických primerů [21].

6.6 Statistika

Pro všechny testy byla zvolena hladina významnosti p=0,05. Výsledky byly zpracovány za použití statistického softwaru SPSS 14.0 a SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, U.S.A).

Bazální produkce

Cílem studie bylo zjistit, zda existuje rozdíl mezi bazální cytokinovou produkcí mononukleárů pupečníkové krve T1DR novorozenců a kontrol. Jelikož některé proměnné nevykazují normalitu rozdělení, byl k porovnání obou skupin použit Mann-Whitney U test.

Bazální produkce versus produkce po stimulaci

Byl použit Wilcoxonův test. Test byl uvažován pro každou skupinu zvlášť.

Rozdíl reaktivity po stimulaci u T1DR v porovnání s kontrolami

Vypočítali jsme rozdíly mezi bazálními hladinami a hladinami cytokinů po stimulaci a tyto rozdíly porovnali mezi oběma skupinami za použití neparametrického Mann-Whitney U testu.

T1DR dvojčata s oběma diabetickými rodiči:

Jelikož se jednalo o malou kohortu porovnávanou s nehomogenní skupinou ostatních T1DR novorozenců, nemohli jsme vypočítat hodnotu statistické významnosti. Pro porovnání byly vypočítány průměrné hodnoty všech cytokinů ve skupině T1DR bez dvojčat. Byl stanoven interval, ve kterém leží 95% jednotlivých hodnot pro každý cytokin u skupiny T1DR bez dvojčat (průměr +/- 2SD). K porovnání reaktivity po specifické stimulaci byl u dvojčat a v T1DR populaci bez dvojčat stanoven rozdíl mezi bazálními hladinami a hladinami cytokinů po stimulaci.

Sekrece cytokinů u matek v porovnání s jejich novorozenci:

Porovnali jsme bazální produkci cytokinů CBMC a mateřských PBMC za použití Wilcoxonova testu. Korelace mezi mateřskou produkcí regulačních cytokinů IL-10, TGF-beta

a Th2 cytokinu IL-13 a neonatálním cytokinovým profilem byla měřena pomocí Spearmanova koeficientu korelace.

Sekrece cytokinů u T1D matek v porovnání se zdravými matkami:

Mezi oběma skupinami matek byly porovnány hladiny cytokinů za použití Mann-Whitney U testu.

Sekrece cytokinů u T1DR novorozenců s nízkým versus vysokým genetickým rizikem rozvoje T1D:

Mann-Whitney U test a Wilcoxonův test byly použity ke stanovení rozdílu mezi bazální produkcí a produkcí cytokinů po stimulaci mezi oběma skupinami T1DR.
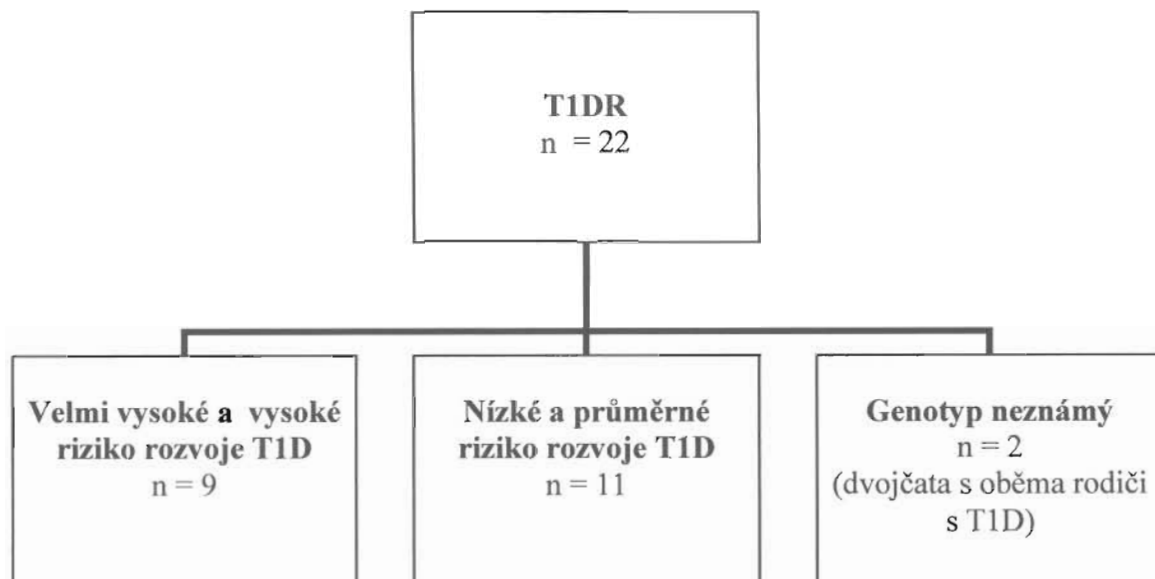
III.

1. Výsledky HLA typizace – stanovení „genetického rizika"

Devět T1DR novorozenců v naší studii vykazovalo genotyp „s velmi vysokým či vysokým rizikem rozvoje T1D" (Tab. 3). Zbytek T1DR populace nesl genotyp s „nízkým nebo průměrným rizikem rozvoje T1D". Bohužel dvojčata s oběma rodiči diabetiky nebyla vyšetřena, vzhledem k odmítnutí HLA-typizace rodiči. (Diagram 2)

Diagram 2: Výsledky HLA typizace v T1DR populaci – stanovení „genetického rizika"

rozvoje T1D

```
                    ┌─────────────────┐
                    │      T1DR       │
                    │     n = 22      │
                    └─────────────────┘
```

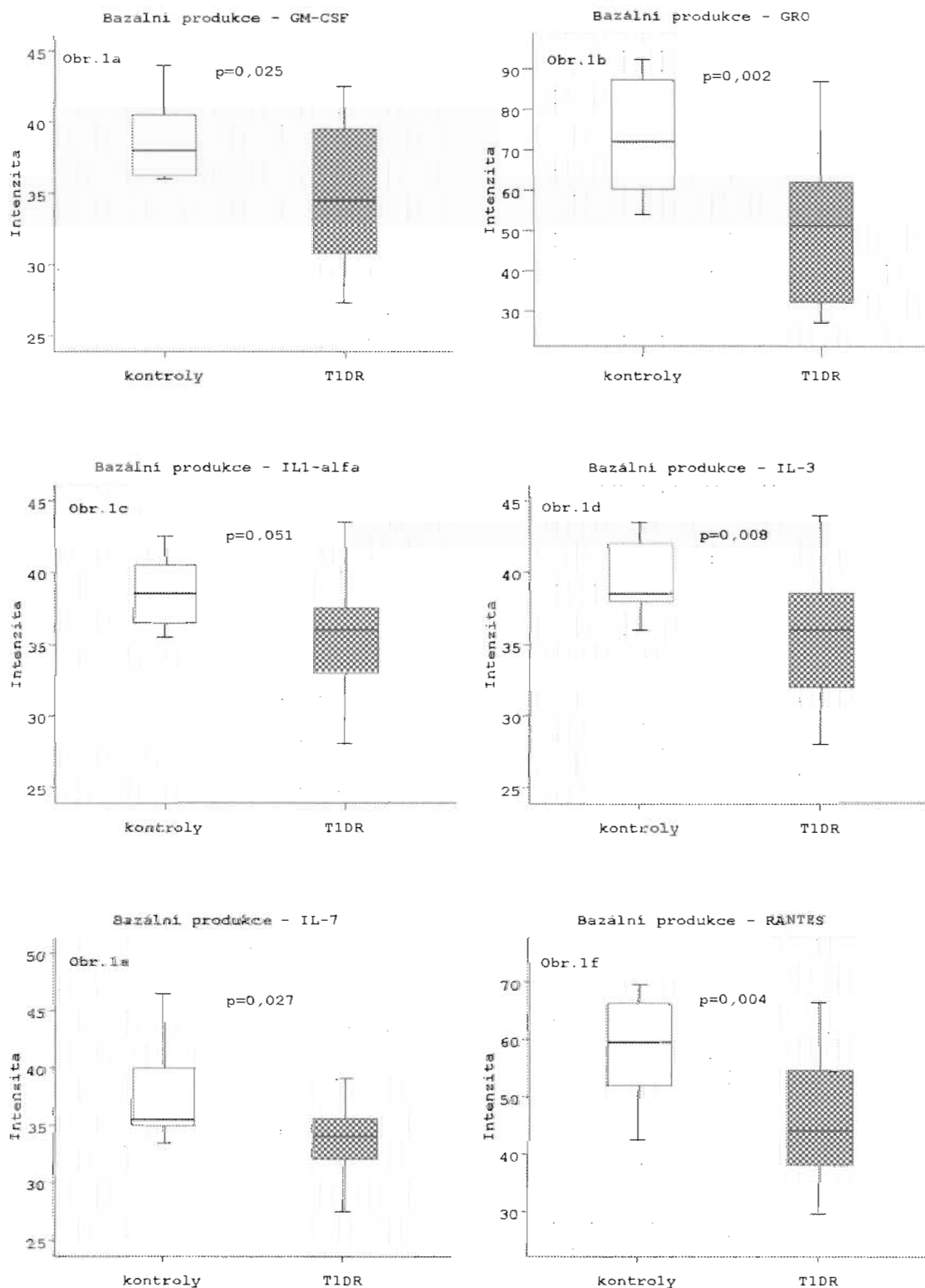| Velmi vysoké a vysoké riziko rozvoje T1D n = 9 | Nízké a průměrné riziko rozvoje T1D n = 11 | Genotyp neznámý n = 2 (dvojčata s oběma rodiči s T1D) |

2. Výsledky protein microarray – reakce na diabetogenní a polyklonální stimulaci

2.1 Bazální produkce

Ve skupině T1DR novorozenců jsme pozorovali nižší bazální produkci všech detekovaných cytokinů. Rozdíly byly statisticky signifikantní v případě následujících cytokinů: GM-CSF (p=0,025) (Obr. 1a), GRO (p=0,002) (Obr. 1b), GROalfa (p=0,027), IL1-alfa (p=0,051) (Obr. 1c), IL-3 (p=0,008) (Obr. 1d), IL-7 (p=0,027) (Obr. 1e), IL-8 (p=0,042), MCP-3 (p=0,022), MIG (p=0,034) a RANTES (p=0,004) (Obr. 1f).

Obr. 1 Bazální produkce: U T1DR jsme pozorovali nižší bazální produkci všech

detekovaných cytokinů, např. *1a* GM-CSF (p=0,025), *1b* GRO (p=0,002), *1c* IL1-alfa

(p=0,051), *1d* IL-3 (p=0,008), *1e* IL-7 (p=0,027), *1f* RANTES (p=0,004)

2.2 Bazální produkce versus produkce po stimulaci

U kontrolní skupiny jsme po stimulaci v porovnání s bazálními hodnotami pozorovali nižší

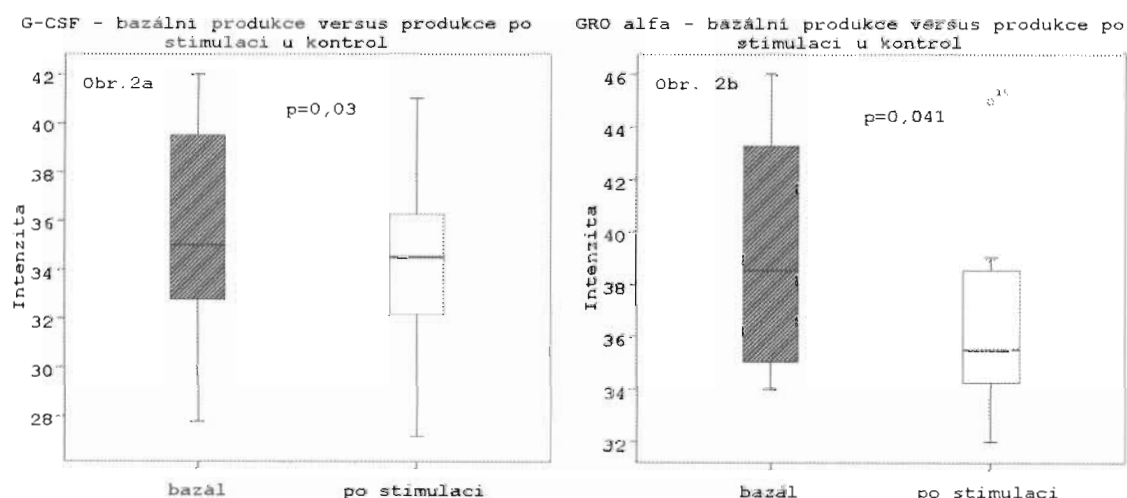hladiny výhradně u G-CSF (p=0,03) (Obr. 2a) a GROalfa (p=0,041) (Obr. 2b).

Obr. 2 Bazální produkce versus produkce po stimulaci

U kontrolní skupiny jsme po stimulaci v porovnání s bazálními hodnotami pozorovali nižší
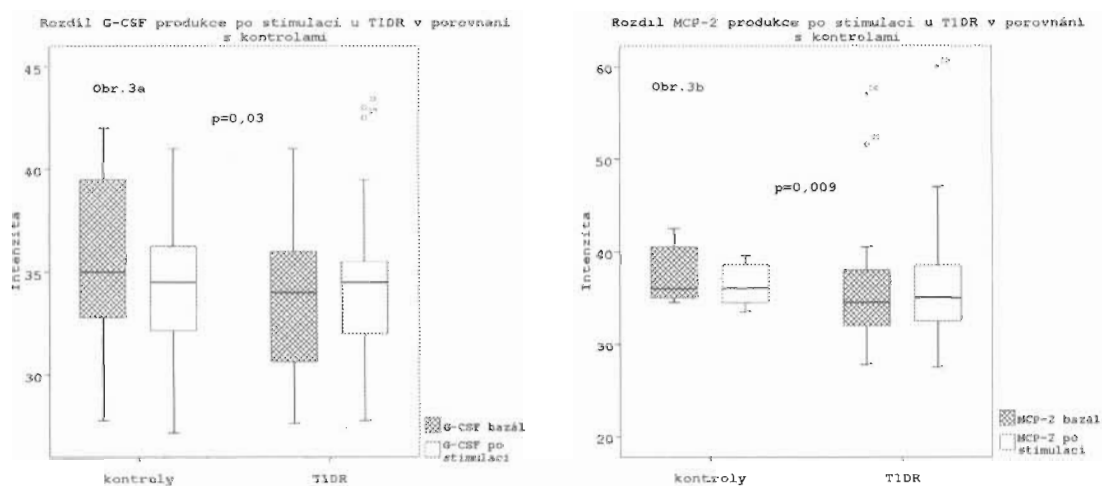
hladiny: *2a* G-CSF (p=0,03) and *2b* GROalfa (p=0,041)

2.3 Rozdíl reaktivity po stimulaci u T1DR v porovnání s kontrolami

V kontrolní skupině byl po stimulaci pozorován signifikantní pokles v produkci G-CSF na

rozdíl od vzestupu produkce cytokinů po stimulaci v T1DR skupině (p=0,030) (Obr. 3a). Dále

jsme u kontrol viděli postimulační pokles MCP-2, zatímco v T1DR skupině nenastala po

stimulaci téměř žádná změna v produkci tohoto cytokinu (p=0,009) (Obr. 3b).

Obr. 3 Rozdíl reaktivity po stimulaci u T1DR v porovnání s kontrolami: 3a v kontrolní skupině byl po stimulaci pozorován signifikantní pokles G-CSF narozdíl od vzestupu produkce cytokinů po stimulaci v T1DR skupině (p=0,030)

3b U kontrol došlo po stimulaci k poklesu MCP-2, zatímco v T1DR nenastala po stimulaci téměř žádná změna v produkci tohoto cytokinu.(p=0,009)

2.4 Sekrece cytokinů po nespecifické polyklonální stimulaci

Po polyklonální simulaci fytohemaglutininem byl pozorován vzestup v produkci všech sledovaných cytokinů, a to u obou skupin novorozenců. CBMC u T1DR byly trochu méně reaktivní, nicméně rozdíl mezi skupinami nebyl signifikantní.

2.5 Stimulace CBMC vystavených různé koncentraci glukózy v kultivačním médiu.

Nebyl pozorován žádný signifikantní rozdíl v produkci jednotlivých cytokinů, a to jak u T1DR novorozence, tak u kontrolních CBMC. Nicméně sloučíme-li jednotlivé cytokiny do skupin, poté lze po diabetogenní stimulaci u kontrol vysledovat snížení produkce cytokinů skupiny Th1 (IFN-gama, IL-2, TNF-beta) a růstových faktorů (IL-3, GCSF, GM-CSF) při vyšších koncentracích glukózy v médiu oproti buňkám kultivovaným ve standardním médiu.

U T1DR pak pozorujeme vzestup bazální produkce chemokinů (IL-8. MCP-1, 2, 3, MIG, RANTES) při vyšších koncentracích glukózy oproti standardu. Nicméně daná pozorování je třeba ověřit na větším souboru.

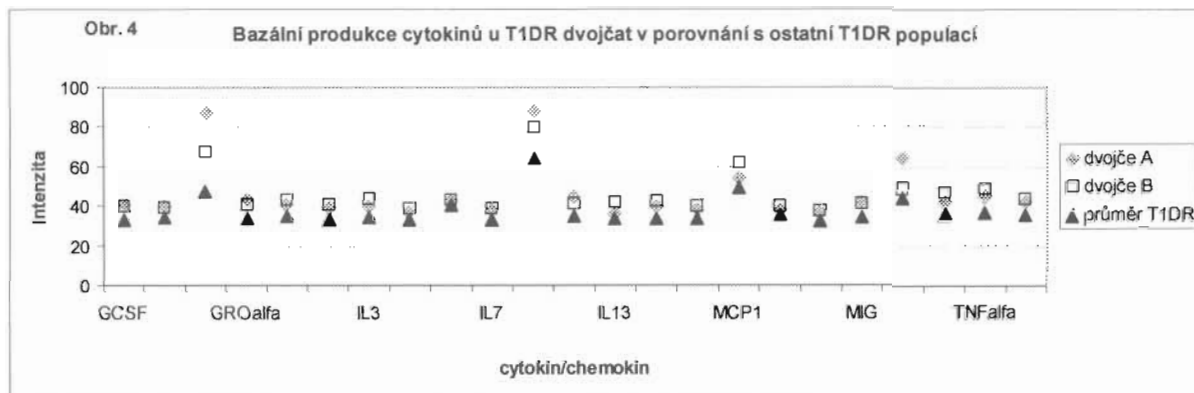## 2.6 Sekrece cytokinů u T1DR s diabetickou matkou versus T1D otcem

Nebyl pozorován statisticky významný rozdíl v hladinách cytokinů (bazálních i po stimulaci) ve skupině novorozenců s diabetickou matkou v porovnání s novorozenci s otcem diabetikem. Nicméně skupina novorozenců s diabetickou matkou byla podstatně větší.

2.7 T1DR dvojčata s oběma diabetickými rodiči

Jelikož byla porovnávána jen velmi malá kohorta s nehomogenní skupinou ostatních T1DR, následující unikátní výsledky nejsou statisticky významné, nicméně je považujeme za velmi zajímavé. Obě dvojčata s diabetickými rodiči měla vyšší bazální hladiny všech detekovaných cytokinů v porovnání s průměrnými hodnotami cytokinů u ostatních T1DR novorozenců (Obr. 4).

Po stimulaci jsme u dvojčat pozorovali vzestup v produkci IL-6 v porovnání s ostatní T1DR populací, kde byl pozorován spíše pokles. Naopak MCP-1 po stimulaci u dvojčat poklesl, zatímco u ostatních T1DR došlo k mírnému vzestupu produkce tohoto cytokinu.

Obr. 4 T1DR dvojčata s oběma diabetickými rodiči: Obě dvojčata měla vyšší bazální hladiny

všech detekovaných cytokinů v porovnání s průměrnými hodnotami cytokinů u ostatních

T1DR

Obr. 4 Bazální produkce cytokinů u T1DR dvojčat v porovnání s ostatní T1DR populací
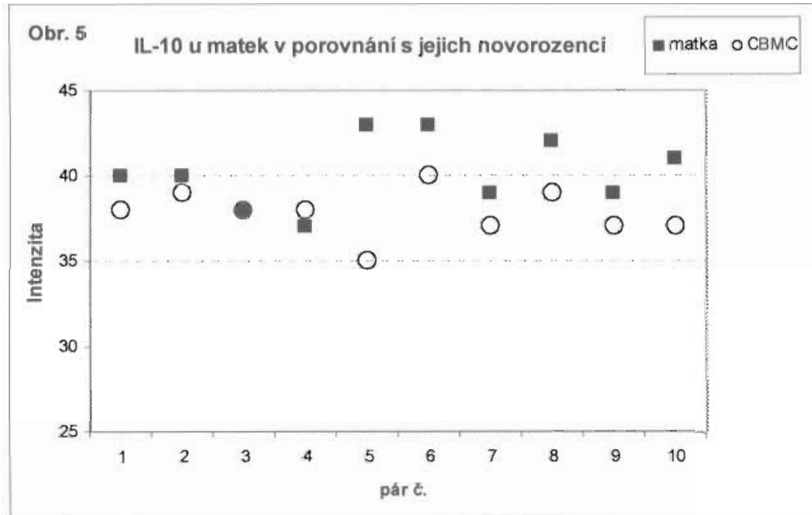
2.8 Sekrece cytokinů u matek v porovnání s jejich novorozenci

K vyloučení případné kontaminace mateřskými PBMC jsme porovnali bazální CBMC

produkci cytokinů s produkcí mateřských PBMC (získaných krátce po porodu). Bazální

produkce cytokinů byla vyšší u všech testovaných matek v porovnání s jejich novorozenci.

Rozdíl byl statisticky významný v případě IL-10 (p=0,010), GRO (p=0,010), GROalfa

(p=0,010), MCP-2 (p=0,020) a MCP-3 (p=0,023) (viz např. IL-10 - Obr. 5).

Neprokázali jsme žádnou korelaci mezi produkcí mateřského IL-10 a produkcí cytokinů u

novorozenců. Pozorovali jsme ale významnou pozitivní korelací mezi vysokými hladinami

TGF-beta u matek a vysokými hladinami IL-15 (r=0,886, p=0,019), IFN gama (r=0,841,

p=0,036) a TGF-beta (r=0,943, p=0,005) u jejich novorozenců. Pozitivně korelují i vysoké

mateřské hladiny IL-13 s novorozeneckým IL-7 (r=0,659, p=0,038) a negativně pak vysoké

IL-13 matek s nízkými GM-CSF a RANTES (r=-0,671, p=0,034, respektive r=-0,736,

p=0,015) novorozence.

Obr. 5 Sekrece cytokinů u matek v porovnání s jejich novorozenci:

bazální produkce cytokinů byla vyšší u všech testovaných matek v porovnání s jejich
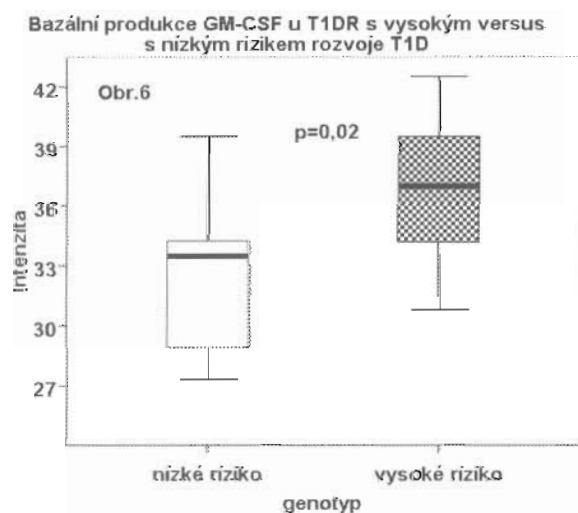
vlastními novorozenci – viz například IL-10 (p=0,010)

Obr. 5 IL-10 u matek v porovnání s jejich novorozenci   ■ matka o CBMC

2.9 Sekrece cytokinů u T1D matek v porovnání se zdravými matkami

V porovnání se zdravými matkami, bazální hladiny IL-6 (p=0,038) a IL-15 (p=0,019) se

signifikantně lišily od bazálních hladin těchto cytokinů u T1D matek. U zdravých matek jsme

pozorovali povšechně nízkou produkci všech detekovaných cytokinů. Imunologické spektrum

bylo mírně převáženo na stranu Th2 odpovědi a zejména IL-13 byl před stimulací jedním

z dominantních cytokinů. Po stimulaci byl pozorován vzestup v produkci IL-10. Diabetičky

měly také nízké bazální hodnoty, ale po stimulaci byl pozorován vzestup u většiny

detekovaných cytokinů – zejména IL-6, IL-10, IL-13 a IFN-gama. Nicméně tyto výsledky

nejsou statisticky signifikantní.

2.10 Sekrece cytokinů u T1DR novorozenců s nízkým versus vysokým genetickým rizikem rozvoje T1D. Ve skupině s vysokým rizikem jsme pozorovali signifikantně vyšší bazální hladiny G-CSF (p=0,038), GM-CSF (p=0,020) (Obr. 6) a GRO alfa (p=0,033) v porovnání s T1DR novorozenci s nízkým rizikem rozvoje T1D.

Obr. 6 Bazální produkce cytokinů u T1DR novorozenců s nízkým versus vysokým genetickým rizikem rozvoje T1D: Ve skupině s vysokým rizikem jsem pozorovali signifikantně vyšší bazální hladiny GM-CSF (p=0,020) v porovnání s T1DR novorozenci s nízkým rizikem rozvoje T1D
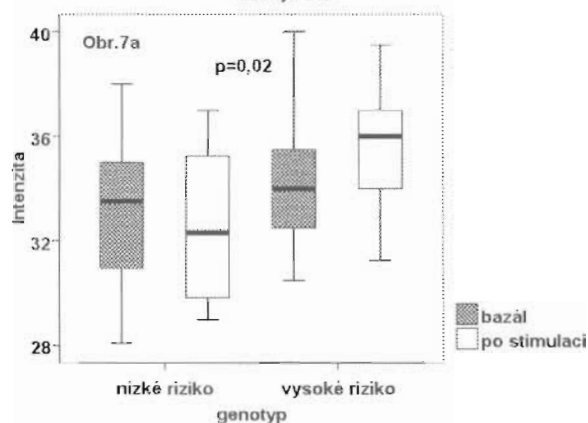

Novorozenci s vysokým genetickým rizikem vykazovali po stimulaci vzestup cytokinové produkce IL-2 (p=0,020), IFN-gama (p=0,001) (Obr. 7a, 7b) a MCP1 (p=0,046) v porovnání s T1DR v nízkém riziku rozvoje T1D.
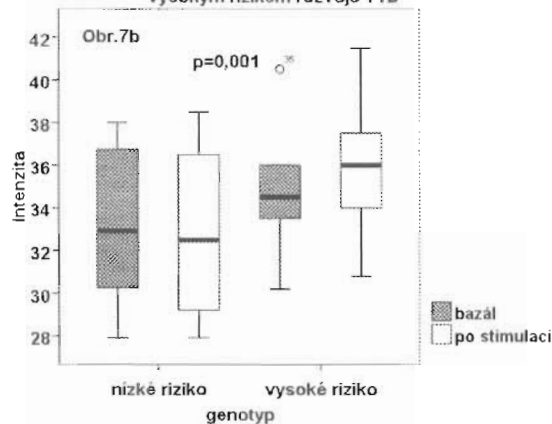
Obr. 7 Reakce na stimulaci u T1DR s nízkým versus s vysokým genetickým rizikem rozvoje

T1D

7a Novorozenci s vysokým genetickým rizikem vykazovali po stimulaci vzestup cytokinové

produkce IL-2 (0,020) a *7b* IFN-gama (0,001) v porovnání s T1DR v nízkém riziku rozvoje

T1D

3. Výsledky ELISPOT – vliv délky stimulace na produkci cytokinů, reakce na infekční agens

3.1 Porovnání produkce cytokinů při rozdílné délce diabetogenní stimulace

Produkce všech sledovaných cytokinů (IFN-gama, IL-10, IL-13) byla maximální po třech

dnech stimulace směsí diabetogenních autoantigenů, při delší stimulaci pak procento

produkujících buněk prudce klesá (Obr. 8). Naproti tomu v jamkách s nestimulovanými

buňkami (negativní kontrola) bylo dosaženo nejvyššího procenta buněk produkujících IL-10

a IFN-gama až po šesti dnech (s následným poklesem produkce po deseti dnech), zatímco

produkce IL-13 byla maximální po třech dnech, s delší dobou pak prudce klesá.

Při stimulaci jednotlivými autoantigeny byla detekována pouze malá procenta produkujících buněk, jako největší stimulant se přitom jevila celá molekula GAD65.

Obr. 8 Porovnání produkce cytokinů při různé délce diabetogenní stimulace

Produkce všech sledovaných cytokinů (IL-10, IL-13, IFN-gama) byla maximální po 3 dnech stimulace směsí autoantigenů.

3.2 Stimulace diabetogenními autoantigeny versus stimulace *E. coli*

Procento produkujících buněk se po stimulaci *E. coli* odlišovalo v porovnání s diabetogenní stimulaci i negativními kontrolami. CBMC T1DR novorozence také reagovaly nejvíce na diabetogenní stimulaci, vysoká byla i bazální produkce IL-6 a IL-13. CBMC kontrolního novorozence vykazovaly po stimulaci *E. coli* zvýšenou produkci IL-6, produkce ostatních cytokinů byla poměrně malá v porovnání s T1DR novorozencem (Obr. 9 a, b).

Cytokiny pupečníkové krve a riziko vzniku T1D

Obr. 9 Stimulace diabetogenními autoantigeny versus E. coli – rozdílná produkce cytokinů

9a T1DR reagují zejména na diabetogenní stimulaci, vysoká byla i bazální produkce IL-6

a IL-13.

9b U kontroly byla pozorována zvýšená produkce IL-6 jakožto reakce na stimulaci *E. coli*

AG – stimulace diabetogenními autoantigeny, NK – negativní kontrola, bazální produkce

nestimulovaných buněk, E. coli – buňky stimulované Escherichia coli

4. Diskuze

Vyvíjející se imunitní systém novorozence většinou nebývá vystaven vlivu zevních faktorů. Přesto existují studie poukazující na vliv nejen infekce, ale i například některých faktorů životního prostředí na cytokinové spektrum pupečníkové krve (například kouření u matky v průběhu těhotenství, znečištěné ovzduší) [42].

V našem souboru byl pozorován signifikantní rozdíl v imunitní odpovědi T1DR novorozenců v porovnání s kontrolní skupinou.

Porovnání cytokinové produkce u T1DR poukázalo na povšechně nízkou bazální produkci všech detekovaných cytokinů v této skupině novorozenců. Jejich reakce na diabetogenní stimulaci byla buďto nízká nebo zanedbatelná. Nicméně tyto výsledky nejsou statisticky signifikantní. Vzestup produkce po specifické stimulaci také nebyl pozorován. Vlivem nespecifické stimulace za použití fytohemaglutininu došlo u T1DR spíše jen k mírnému vzestupu cytokinové produkce na rozdíl od reakce kontrolní skupiny. Nicméně zde nebyl pozorován statisticky významný rozdíl mezi oběma skupinami. Zdá se, jako kdyby byl imunitní systém T1DR novorozenců méně zralý a více citlivý v porovnání s novorozenci zdravých matek.

Prakticky můžeme vyloučit vliv zevních faktorů, zejména hyperglykémie u matky, infekce a vlivy prostředí. Všechna těhotenství byla fyziologická a všechny vybrané T1D matky byly centralizovány v naší nemocnici, s velmi dobrou compliance a kontrolou diabetu. Četnost porodů *per vias naturales* a císařským řezem byla v obou skupinách shodná. Všechny matky žily v průběhu těhotenství ve velkých městech a byly nekuřačky. Žádná z nich netrpěla jiným imunitně zprostředkovaným onemocněním (jiná autoimunita, alergie, imunodeficit). Zdá se

také, že zde nehraje roli ani možná regulace-suprese mateřským imunitním systémem. Naopak jsme pozorovali spíše pozitivní vliv vysokých mateřských hladin regulačních cytokinů na cytokinové spektrum novorozence.

Devět z T1DR novorozenců bylo nositeli genotypu s „velmi vysokým nebo vysokým rizikem rozvoje T1D" , zatímco zbytek T1DR byl pouze v „nízkém nebo středním riziku rozvoje T1D". T1DR s rizikovým genotypem měli vyšší bazální hladiny G-CSF, GM-CSF a GRO-alfa a pozorovali jsme u nich inklinaci k postimulační reakci v Th1 směru – vzestup IL-2, IFN-gama po diabetogenní stimulaci. Dle dosud publikovaných prací byl Th1 cytokinový profil s vysokým IFNgama pozorován v prediabetické fázi [13-15]. Jelikož je T1D polygenním onemocněním, můžeme zatím pouze spekulovat o eventuálním budoucím rozvoji T1D v naší T1DR populaci, kterou hodláme dále sledovat. Předpokládali jsme, že genetické riziko rozvoje T1D u kontrol koreluje s populační incidencí rizikového genotypu, proto u kontrol HLA-typizace provedena nebyla.

Ve skupině T1DR s diabetickou matkou jsme nepozorovali žádný významný rozdíl v hladinách cytokinů, ať již bazálních či po stimulaci, v porovnání s novorozenci s otcem diabetikem. Nicméně počet novorozenců s diabetickým otcem byl velmi malý. Všechny matky, diabetičky, byly pravidelně kontrolovány v diabetologické ambulanci FN Motol a byly přijaty na Gynekologicko-porodnickou kliniku FN Motol několik dní před porodem. Bohužel je obtížné získat pupečníkovou krev novorozence s diabetickým otcem, neboť matka je většinou zcela zdráva, a tak porody nejsou zpravidla centralizovány.

Všechny diabetické matky z naší studie byly sledovány v průběhu celého těhotenství a jejich hladiny HbA1c v posledním trimestru gravidity byly normální. Chtěli jsme ověřit možný vliv hypo/hyperglykémie na CBMC a jejich cytokinovou produkci, proto jsme kultivovali CBMC T1DR a kontrolního novorozence za podmínek rozdílné koncentrace glukózy v kultivačním médiu po různě dlouhou dobu a provedli detekci cytokinové produkce (bazální i po

diabetogenní stimulaci) pomocí protein microarray. Nepozorovali jsme žádný signifikantní rozdíl v produkci jednotlivých cytokinů, a to jak u T1DR novorozence, tak u kontrolních CBMC. Po sloučení cytokinů do větších skupin, dle jejich převažující funkce (Th1, Th2, chemokiny, růstové faktory),, pak bylo možné po diabetogenní stimulaci u kontrol vysledovat snížení produkce cytokinů skupiny Th1 a růstových faktorů při vyšších koncentracích glukózy v médiu oproti standardnímu médiu. U T1DR pak pozorujeme vzestup bazální produkce chemokinů při vyšších koncentracích glukózy oproti standardu. Nicméně daná pozorování je třeba ověřit na větším souboru. Vliv případné hyperglykémie a „T1D prostředí *in utero*" na imunitní systém plodu a novorozence jsme tedy v našem případě považovali za zanedbatelný.

Měli jsme výjimečnou příležitost vyšetřit i cytokinové spektrum novorozenců, dvojčat s oběma rodiči diabetiky. Produkce cytokinů a chemokinů byla u obou dvojčat vyšší než u ostatních T1DR. Rozdíl v poststimulační reakci mezi dvojčaty a zbytkem T1DR nebyl signifikantní, T1DR skupina nebyla dostatečně homogenní a sledovaná kohorta nebyla dostatečně velká k validnimu statistickému zpracování. Můžeme tedy pouze spekulovat o tom, zda je cytokinový profil nalezený u dvojčat typem „rizikového vzorce" a je potřeba oba subjekty nadále sledovat. Vzhledem k nesouhlasu rodičů, nebyla u dvojčat bohužel provedena HLA-typizace, nicméně je známo, že děti s oběma rodiči diabetiky mají vyšší riziko rozvoje T1D v porovnání s dětmi se zdravými rodiči či pouze jedním rodičem diabetikem.

Výsledky pozorované u kontrol jsou v souladu s dosud publikovanými studiemi cytokinového spektra pupečníkové krve.

Naivní T lymfocyty mají malou proliferační schopnost po polyklonální stimulaci v porovnání s dospělými. Produkují také relativně malé množství cytokinů, zejména IL-5 [26-29]. Ač je imunitní systém novorozence nezralý, k diabetogenní stimulaci mononukleárů pupečníkové

krve stačilo 72 hodin stejně jako u mononukleárů periferní krve dospělých. Delší stimulace

dle našich výsledků ELISPOTu vedla naopak k postupnému snižování produkce sledovaných

cytokinů. Dále jsme pomocí ELISPOTu ověřili, zda se nejedná jen o nespecifickou reakci

naivních mononukleárů novorozence na diabetogenní stimulaci. Odpověď na diabetogenní

stimulus se lišily od reakce na stimulaci buněk *E. coli*, stejně tak byly odlišné výsledky

protein microarray po polyklonální stimulaci fytohemaglutininem v porovnání se stimulací

autoantigeny, a to jak u T1DR, tak u kontrol. Výsledky ELISPOTu jsou víceméně pilotní

a budeme na ně navazovat dalším výzkumem na rozsáhlejších souborech.

Dle našeho nejlepšího vědomí nikdo mimo našeho pracoviště dosud nepublikoval žádná data

týkající se imunoreaktivity vůči diabetogenním autoantigenům u novorozence. Tímto jsou

tedy naše výsledky bazální a poststimulační cytokinové odpovědi T1DR unikátní. Nové

poznatky by mohly také přispět k dalšímu pochopení patogeneze T1D.

Krátce po porodu jsme odebrali vzorky periferní krve 6 diabetických a 4 zdravých matek.

Chtěli jsme ověřit možnost zkreslení výsledků daného eventuální kontaminací pupečníkové

krve mateřskými mononukleáry. Ve všech případech byla ale bazální cytokinová produkce

odlišná od té novorozenecké. U zdravých matek jsme pozorovali povšechně nízkou produkci

všech detekovaných cytokinů. Bazální cytokinové spektrum bylo lehce převáženo na stranu

Th2 odpovědi, zejména IL-13 byl jedním z dominantních cytokinů. Toto je v souladu s dosud

publikovanými pozorováními – dominance Th2 buněčné odpovědi, nízké hladiny cytokinů,

vzestup Th1 cytokinů a IL-6 po nespecifické stimulaci či jako reakce na stres [29-32, 43-45].

Po stimulaci jsme pozorovali vzestup produkce IL-10. Diabetické matky měly také nízkou

bazální produkci cytokinů, ale po stimulaci byl pozorován vzestup u většiny detekovaných

cytokinů – IL-6, IL-10, IL-13 a IFN-gama. Zdá se, že by se mohlo jednat o známku

hyperreaktivity imunitního systému, který se stále snaží přetlačit vliv „autoimunitních Th1

cytokinů" a umožnit tak nekomplikovaný průběh vlastního těhotenství a porodu. Totéž bylo

pozorováno i v těhotenstvích žen s jinými autoimunitami – zřetelný posun těhotenské Th2 buněčné odpovědi ve prospěch Th1 odpovědi krátce po porodu. Při těhotenství je polarizace imunitní odpovědi více ve prospěch Th2, vyvažuje zvýrazněnou Th1 odpověď u Th1 mediovaných autoimunit (revmatoidní artritida, systémový lupus erythematosus, roztroušená skleróza). Tímto způsobem pak gravidita ovlivňuje (zmírňuje) příznaky choroby a často dochází i ke klinické remisi autoimunity [46-48].

Vysoké mateřské hladiny regulačního TGF-beta pozitivně korelovali s vysokými hladinami IL-15, IFN-gama a TGF-beta novorozence a nebyl pozorován žádný supresivní vliv vysokých hladin tohoto cytokinu u matek na cytokinový profil novorozence, jak by se dalo očekávat. Mateřské hladiny IL-10 neměly žádný významnější vliv na hladiny cytokinů u novorozence. Vysoký IL-13 u matek u novorozenců potlačoval bazální produkci GM-CSF a RANTES a naopak zvyšoval produkci hemopoetického růstového faktoru IL-7. Přesto nesmíme zapomínat na důležitost komplexní spolupráce jednotlivých cytokinů v rámci celé cytokinové sítě.

Bazální cytokinový profil pupečníkové krve byl také studován v případech perinatální infekce, chronického plicního postižení u nezralých novorozenců (zvýšené zánětlivé cytokiny), astmatu a alergie (Th2 odpověď) a pro účely transplantační imunologie (např. reakce štěpu proti hostiteli). Mononukleáry pupečníkové krve byly také podrobeny nespecifické stimulaci (fytohemaglutinin, bakteriální lipopolysacharid, enterotoxin) [49-51]. Je také popisována horší *in vitro* maturace a diferenciace T lymfocytů pupečníkové krve v Th2 prostředí a nižší exprese některých cytokinových a chemokinových receptorů, a tedy poměrně chabá indukce Th2 imunitní u T1DR novorozenců s „rizikovým" genotypem pro vznik T1D, což by mohlo eventuálně přispět k budoucímu rozvoji autoimunity u těchto jedinců [52].

Nikdo ale mimo naší studie dosud nepublikoval žádná data týkající se imunoreaktivity vůči autoantigenům u novorozenců.

IV.

1. Závěr

Naším výzkumem bychom chtěli přispět k nalezení vhodného modelu pro další studium patogeneze T1D, který by pak v budoucnosti mohl sloužit i v rámci programů predikce a časné diagnostiky, a to i u novorozenců. Nezbytné jsou další studie na rozsáhlejších souborech novorozenců. Zatím se zdá, že protein microarray by mohla být poměrně slibná metoda ke zjištění rizikového vzorce buněčné imunitní odpovědi T1D, a to i v raném věku, což je zásadní vzhledem k stále časnější manifestaci T1D.

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Cord Blood Cytokine Profile Detection in Neonates with T1D Parents – Monitoring of Cellular Auto-reactivity Using Protein Microarray

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Abstract

Type 1 diabetes (T1D) is a great medical challenge and its incidence rises rapidly. T lymphocytes and their cytokine production are supposed to play a major role in T1D development. So far, there is no potent tool to recognize the early signs of cellular auto-reactivity which leads to β-cell damage. The naïve immune system of the newborn (not yet influenced by external factors) can be used as an important model for T1D pathogenesis studies. Cord blood samples of 22 healthy neonates born at term to a diabetic parent (T1DR) and 15 newborns with no family history of any autoimmune disease (controls) were collected. Determination of 23 cytokines was performed before and after the stimulation with diabetogenic autoantigens using protein microarray. We observed lower basal production of all detected cytokines in the T1DR group – granulocyte/macrophage colony-stimulating factor (GM-CSF) ($P = 0.025$), growth regulated protein (GRO) ($P = 0.002$), GRO-α ($P = 0.027$), interleukin (IL)-1-α ($P = 0.051$), IL-3 ($P = 0.008$), IL-7 ($P = 0.027$), IL-8 ($P = 0.042$), monocyte chemoattractant proteins (MCP)-3 ($P = 0.022$), monokine-induced by IFN-γ (MIG) ($P = 0.034$) and regulated upon activation normal T-cell express sequence (RANTES) ($P = 0.004$). Exclusively lower post-stimulative levels of G-CSF ($P = 0.030$) and GRO-α ($P = 0.04$) were observed in controls in comparison with the basal levels. A significant post-stimulative decrease in G-CSF ($P = 0.030$) and MCP-2 ($P = 0.009$) levels was observed in controls in comparison with T1DR neonates. We also observed the interesting impact of the risky genotype on the protein microarray results. Protein microarray seems to be a useful tool to characterize a risk pattern of the immune response for T1D also in newborns.

Introduction

Type 1 diabetes (T1D) occurs mostly in children, teenagers and young adults. The prevalence represents 2% of the world population. The T1D incidence rises rapidly, mainly in the youngest. In the Czech population, an annual increase in incidence by 6.3% could be observed in the youngest age group (0–4 years) [1–4].

Type 1 diabetes is suggested to be a T helper 1 (Th1) autoimmune disease [5, 6] characterized by an absolute lack of insulin caused by the destruction of pancreatic β-islet cells due to the autoimmune inflammatory process – insulitis. Th1 lymphocytes are responsible for the

infiltration of Langerhans islets and cytokines are released and start supporting cytotoxic (Tc) lymphocytes-mediated destruction of β-islet cells [6, 7]. Due to this progressive damage, there is either insufficient or no production of insulin, which leads to the first clinical signs of diabetes. The manifestation of T1D usually occurs in situations linked with a higher need of insulin, e.g. infection or trauma but the real triggers are not yet known [4, 8].

Interferon (IFN)-α [7, 8] and IFN-γ [9] have been observed on human pancreatic islets of Langerhans *in vivo* in patients with recent-onset of T1D. Th1 cytokine profile with high IFN-γ secretion has been found during the pre-diabetic phase [10–12]. However, close to the

onset of T1D, when only few β-cells remain, the Th1-like response vanishes and remains suppressed in newly diagnosed T1D patients [10, 13–16].

Still there is no potent tool to recognize the early signs of cellular auto-reactivity which leads to β-cells damage and T1D development.

During the end of the pregnancy, the immunological balance is more in favour of CD4$^+$ lymphocytes and the CD4$^+$/CD8$^+$ proportion slightly changes in course of the labour. Most of the lymphocytes have the 'naïve' character as they have not yet been exposed to external antigens. They express the CD45 isoforms, CD45RA and CD45RB. By stimulation, T cells of newborns switch to the expression of CD45RO which is present in memory T cells. The low numbers of CD45RO cells in term newborns suggest that very little antigen-induced expansion occurs before birth. These 'naïve' T lymphocytes have lower ability to proliferate after poly-clonal stimulation. In comparison with the adults, they produce less cytokines (IFN-γ, interleukins IL-4 and IL-5). Especially the lack of Th1 subset cytokines can be observed as well as inadequate interaction with B lymphocytes. The immunological balance of the mother as well as of the foetus is slightly weighted in favour of Th2 [16–22]. Interestingly, cord blood also includes a subset of CD3$^-$ CD8$^+$ lymphocytes displaying NK activity that is undetectable in peripheral blood of adults. These cells are supposed to be representing the stage between thymocytes and mature T lymphocytes [16–22].

This far, according to our best knowledge, no one has obtained any data about the immunoreactivity against autoantigens in the naïve neonatal immune system. New findings could also play an important role in further understanding of the T1D pathogenesis. So we decided to study the cytokine and chemokine production (basal and also after the stimulation with diabetogenic autoanti-gens) of cord blood mononuclear cells (CBMC) using pro-tein microarray.

Protein microarray is a semi-quantitative technique, which is sufficiently sensitive and not too expensive and enables simultaneous detection of hundreds of proteins in different biological materials [23]. The nitrocellulose membranes are coated with primary antibodies against each cytokine in a distinct location. After the binding of cytokines contained in the super-natant, the biotin-labelled secondary antibodies 'cock-tail' is added to each membrane. The signal is then emphasized by horse radish peroxidase (HRP)-conju-gated streptavidin.

Patients and methods

Study subjects and ethics. This study included 22 newborns with a parent suffering from T1D (17 diabetic mothers,

three diabetic fathers and a couple of twins with both parents diabetics] and 15 newborns with no family history of any autoimmune disease as a control group. None of the mothers suffered from other immune-medi-ated diseases (other autoimmunity, allergy, asthma and immunodeficiency). All of the newborns were born at term (average 38th gestational week) after physiological pregnancies without any perinatal complication. The fre-quency of labour *per vias naturales* and Caesarean section was similar in both groups of mothers. Diabetic mothers with good compliance and control of diabetes during the whole pregnancy were chosen for our study. All of them were regularly checked by the diabetologist of the Department of Internal Medicine, 2nd Medical Faculty of Charles University, Prague, during the pregnancy (including HbA1c assessment with normal results). Peripheral blood samples of six T1D mothers and four healthy mothers were obtained shortly (within 4 h) after the labour for further studies to exclude the possibility of cord blood contamination with maternal mononuclear cells.

Ethical approval for this study was granted by the local ethics committee and informed consent was obtained.

Polymerase chain reaction. Complete HLA-DQA1 and DQB1 genotyping was carried out by polymerase chain reaction (PCR) with sequence-specific primers [24] to assess the genetic risk of T1D development (Table 1).

Cell isolation and stimulation. Cord blood mononuclear cells (CBMC) were used in all *in vitro* experiments, peripheral blood mononuclear cells (PBMC) in case of the mothers. It was obtained approximately 5–8 ml of cord blood, 10–15 ml of peripheral blood.

CBMC (PBMC respectively) were isolated from the whole blood by Ficoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). 2×10^6 freshly isolated CBMC PBMC were resuspended in 1 ml of RPMI-1640 medium supplemented with 20% fetal calf serum (FCS), L-glutamine (10 μl/ml 200 mM L-glu-tamine) and penicillin–streptomycin (1 μl/ml PNC and 1 μg/l streptomycin; all purchased from Sigma, St Louis, MO, USA) and cultivated with diabetogenic auto-antigens.

Cell cultures were stimulated with a mixture of the following autoantigens: GAD65 peptides amino acids (a.a.) 247–279 (NMYAMMIARFKMFPEVKEKGMAAL-PRLIAFTSEE–OH), molecular weight 3823.7; a.a. 509–528 (IPPSLRTLEDNEERMSRLSK–OH), molecular weight 2371.7; a.a. 524–543 (SRLSKVAPVIKARMMEYGTT–OH), molecular weight 2238.7 (all GAD65 peptides – Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden); IA-2 a.a. 853–872 SFYLK(Nleu)VQTQETRTLTQFHF, molecular weight 2489 and a.a. of β-proinsulin chain 9–23 SHLVEAL-YLVCGERG, molecular weight 1645 (Sigma, St Louis,

Table 1 Genetic risk of T1D development based on HLA-DQA1 and DQB1 genotyping.

Generic risk	Criteria
Very high	DQA1*05-DQB1*0201/DQB1*0302 but NO coincidence with DRB1*0403
High	DQA1*05-DQB1*0201 or DQB1*0302 but NO coincidence with DQB1*0602, 0301, 0603, DRB1*0403
Average	NO coincidence of following: DQB1*0302, DQA1*05-DQB1*0201, DQB1*0602, 0301, 0603
	DQB1*0301/DQB1*0302 or DQB1*0302/DQB1*0603
	DQB1*0302-DRB1*0403
Low	DQB1*0301 or DQB1*0603 but NO coincidence with DQB1*0302 and DQB1*0602
Very low	DQB1*0602

USA). Each autoantigen was used in the concentration of 1 μg per 10^6 cells.

Our experiments were completed with a positive control [PBMC or CBMC, respectively, +10 μg of phytohaemagglutinin (PHA; Sigma) per 10^6 cells] as well as a negative control (PBMC or CBMC in exclusive culture medium). Cell supernatant was harvested after 72 h of stimulation (37 °C, 5% CO_2), frozen ($-20°C$) and later used for a protein microarray analysis.

Protein microarray. A protein microarray analysis was performed using a commercially available array kit according to the instructions of the manufacturer (Ray-Biotech, Norcross, GA, USA).

Chemiluminescent signals were detected using the Fuji LAS1000 imaging system (Fujifilm, Tokyo, Japan) and then analysed using the Advanced Image Data Analyzer software (AIDA, 3.28; Raytest Izotopenmessgeraete, Straubenhardt, Germany). All the images were edited in the grey-scale 8-bit map. Results were obtained according to the instruction of the manufacturer in percentage of signal intensity. The membranes were compared together; the integral positive controls of each membrane reached the 100% of intensity so no other image transformation was necessary.

Production of the following 23 cytokines and chemokines was detected: granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), growth regulated protein (GRO), growth regulated protein-α (GRO-α), interleukins (IL)-1, -2, -3, -5, -6, -7, -8, -10, -13, -15, interferon-γ (IFN-γ), monocyte chemoattractant proteins (MCP)-1, -2, -3, monokine-induced by IFN-γ (MIG), regulated upon activation normal T-cell express sequence (RANTES), transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α) and tumours necrosis factor-β (TNF-β). Detection limits for cytokines are displayed on the manufacturer's website (http://www.raybiotech.com).

Statistics. A probability level of $P < 0.05$ was considered statistically significant in all tests that were carried out. The results were analysed using the statistical software SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

(1) *Basal production.* The aim was to find out if there was any difference of the basal cytokine levels in cord blood of the T1DR neonates in comparison with controls. Because the variables were not normally distributed, Mann–Whitney U-test was used for the comparison of the groups.

(2) *Basal production versus production after stimulation.* Non-parametric Wilcoxon signed rank test was used for the comparison. The test was made for each group separately.

(3) *Difference in the post-stimulative reactivity in the group of T1DR neonates in comparison with the control group.* The difference between basal and post-stimulative levels was calculated and the differences between the two groups were compared using non-parametric test – Mann–Whitney U-test.

(4) *T1DR twins with both parents diabetics.* Because of the small cohort being compared with the non-homogenous group of other T1DR newborns, the statistical significance could not be calculated. For the comparison, the mean of all cytokine values in T1DR without the twins was calculated. The interval for 95% of individual values for each cytokine in the T1DR newborns without twins (mean ± 2 SD) was determined.

To compare the reactivity after the specific stimulation, the difference between the basal and post-stimulative cytokine levels was assessed in the twins and in the rest of the T1DR population.

(5) *Cytokine secretion in mothers in comparison with their newborns.* The basal cytokine production of CBMC versus maternal PBMC was compared using Wilcoxon signed ranks test. Correlation between the maternal production of regulatory cytokines IL-10, TGF-β and Th2 cytokine IL-13 and neonatal cytokine profile was determined using Spearman correlation coefficient.

(6) *Cytokine secretion in T1D mothers in comparison with healthy mothers.* In the two groups of mothers, the cytokine levels were compared using Mann–Whitney U-test.

(7) *Cytokine secretion in T1DR neonates with low genetic risk of T1D development versus T1DR neonates with high risk of T1D development.* Mann–Whitney U-test and Wilcoxon signed ranks test were used to determine the difference of basal and post-stimulative cytokine production in the two groups of T1DR neonates.

Results

Basal production

We observed lower basal production of all detected cytokines in the group of T1DR newborns. The difference was statistically significant in following basal cytokine and chemokine levels: GM-CSF (P = 0.025) (Fig. 1A), GRO (P = 0.002) (Fig. 1B), GRO-α (P = 0.027), IL1-α (P = 0.051) (Fig. 1C), IL-3 (P = 0.008) (Fig. 1D), IL-7 (P = 0.027) (Fig. 1E), IL-8 (P = 0.042), MCP-3 (P = 0.022), MIG (P = 0.034) and RANTES (P = 0.004) (Fig. 1F).

Basal production versus production after stimulation

Exclusively lower levels of G-CSF (P = 0.03) (Fig. 2A) and GRO-α (P = 0.04) (Fig. 2B) were observed in the control group after stimulation in comparison with the basal levels.

Difference in the post-stimulative reactivity in the group of T1DR neonates in comparison with the control group

A significant decrease in G-CSF after stimulation was observed in the control group in contrast to a post-stimulative increase in the group of T1DR neonates (P = 0.030) (Fig. 3A). Further, the decrease in MCP-2 levels was seen after stimulation in the controls but there was hardly any change observed in the group of T1DR neonates (P = 0.009) (Fig. 3B).
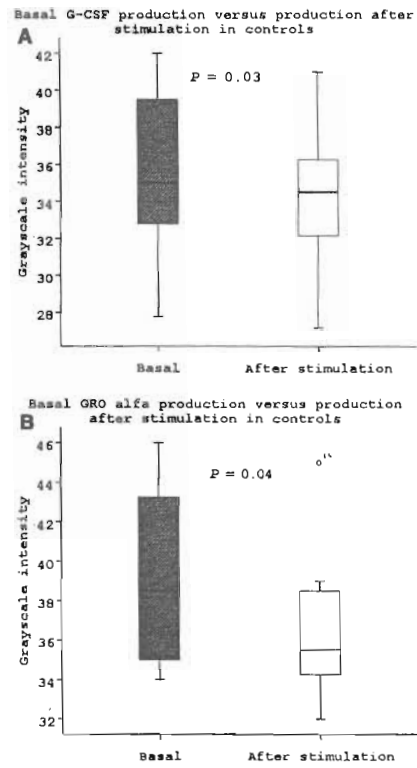
Figure 2 *Basal production versus production after stimulation.* We observed exclusively lower levels of following cytokines in the control group after stimulation in comparison with the basal levels: (A) G-CSF (P = 0.03) and (B) GRO-α (P = 0.04).
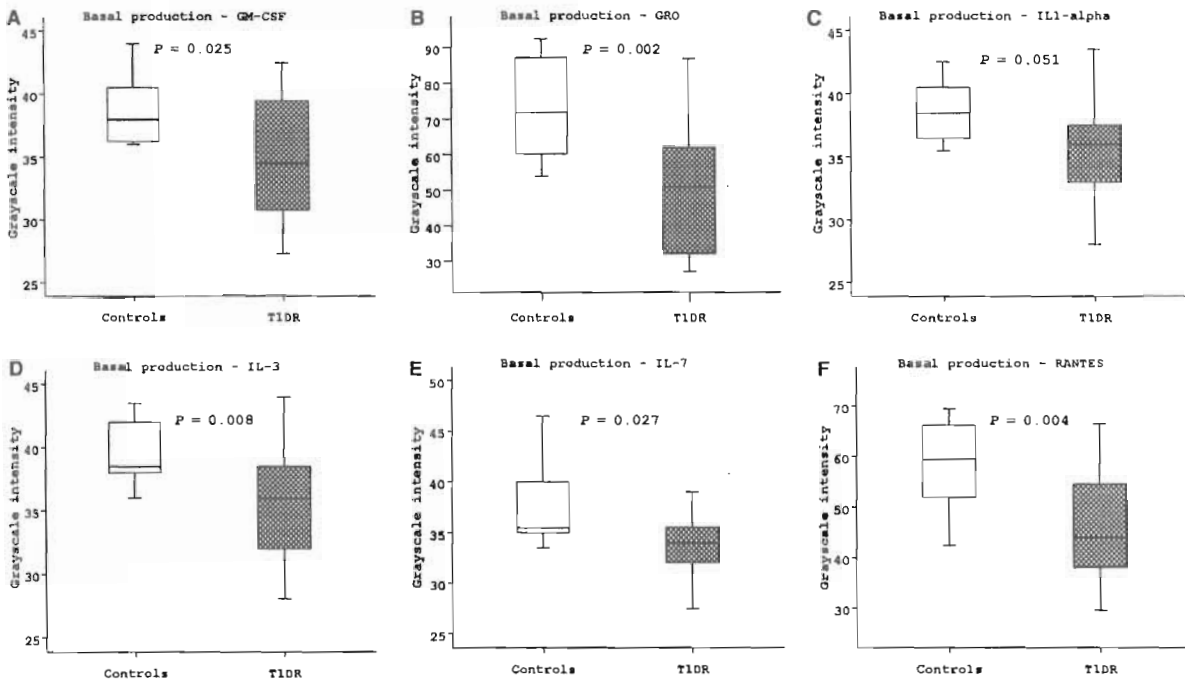

Figure 1 *Basal cytokine production.* We observed lower basal production of all detected cytokines: in the group of T1DR newborns – for example (A) GM-CSF (P = 0.025), (B) GRO (P = 0.002), (C) IL1-α (P = 0.051), (D) IL-3 (P = 0.008), (E) IL-7 (P = 0.027), (F) RANTES (P = 0.004).
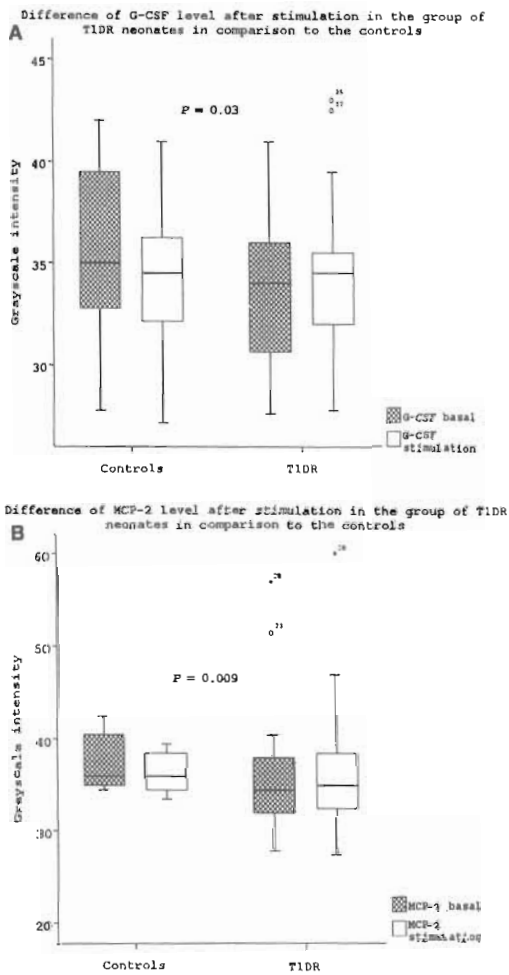
Figure 3 *Difference in the post-stimulative reactivity in the group of T1DR neonates in comparison with the control group.* (A) A significant decrease in G-CSF after stimulation was observed in the control group in contrast to a post-stimulative increase in the group of T1DR neonates (P = 0.030); (B) the decrease in MCP-2 levels was seen after stimulation in the controls but there was hardly any change observed in the group of T1DR neonates (P = 0.009).

Cytokine secretion after non-specific polyclonal stimulation

We observed an overall increase in cytokine production in both groups after the polyclonal stimulation with phytohaemagglutinin. The CBMC of T1DR newborns were slightly less reactive; however, there was no significant difference observed between the two groups.

There was no significant difference in the cytokine levels before and after stimulation in the group of newborns with T1D father in comparison with the newborns with a T1D mother. But the cohort of newborns with T1D mothers was much larger than the group with T1D fathers.

T1DR twins with both parents diabetics

Because of the small cohort being compared with the non-homogenous group of other T1DR newborns, the results are not statistically significant, but we consider these unique findings to be very interesting. Both of the twins (with both parents suffering from T1D) have got higher basal values of all detected cytokines compared with the mean value of each cytokine in the other T1DR newborns with just one diabetic parent (Fig. 4).
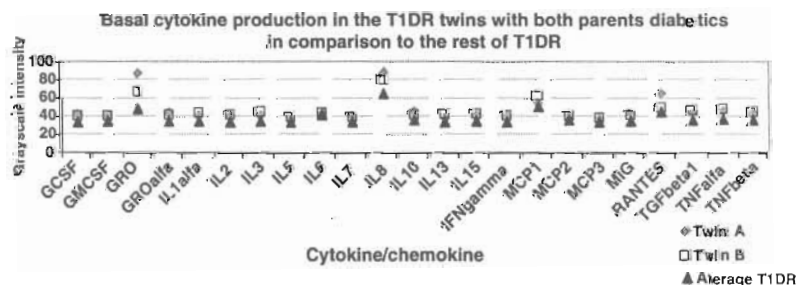
After the stimulation, an increase in IL-6 was observed in the twins in comparison with the average reaction of the rest of the T1DR population where the decrease could be seen. By contrast, MCP-1 decrease could be distinguished in the twins after the specific stimulation – the average reaction was a slight increase in the rest of the T1DR group.

Cytokine secretion in mothers in comparison with their newborns

We compared the basal cytokine production of CBMC with cytokine production of PBMC from the mother (shortly after delivery) to exclude a possible contamination with maternal PBMC. The basal cytokine production was higher in the mothers in comparison with their own neonates. We observed statistically significant difference in IL-10 (P = 0.010), GRO (P = 0.010), GRO-α (P = 0.010), MCP-2 (P = 0.020) and MCP-3 (P = 0.023) levels (see example of IL-10 below – Fig. 5)

There was not any significant correlation found between the IL-10 production in the mothers and the cytokine production in their own newborns. We observed a significant positive correlation among the high maternal TGF-β levels and high levels of IL-15 (r = 0.886,

Figure 4 *T1DR twins with both parents diabetis. Both of the twins (with both parents suffering from T1D) have got higher basal values of all detected cytokines compared with the average of each cytokine in other T1DR newborns with just one diabetic parent.*
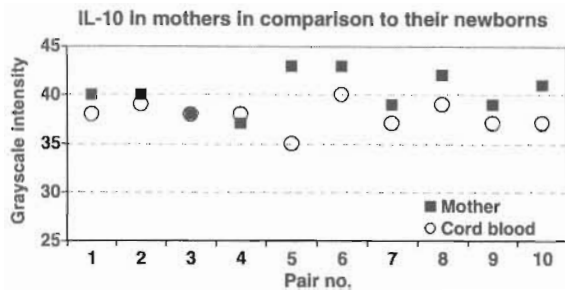
Figure 5 *Cytokine secretion in mothers in comparison with their newborns.* The basal cytokine levels in mothers were higher than in their own newborns. Example of IL-10 basal production ($P = 0.010$).

$P = 0.019$), IFN-γ ($r = 0.841$, $P = 0.036$) and TGF-β ($r = 0.943$, $P = 0.005$) in the neonates. Positive correlation was seen between the high IL-13 maternal levels and IL-7 neonatal levels ($r = 0.659$, $P = 0.038$) and negative correlation was observed among high IL-13 maternal levels and low neonatal GM-CSF and RANTES ($r = -0.671$, $P = 0.034$, $r = -0.736$, $P = 0.015$ respectively).

Cytokine secretion in T1D mothers in comparison with healthy mothers

The statistically significant difference of basal cytokine levels of IL-6 ($P = 0.038$) and IL-15 ($P = 0.019$) was observed in T1D mothers compared with healthy mothers. In healthy mothers, we observed an overall low production of all detected cytokines. The spectrum was weighted slightly in favour of Th2 and especially the secretion of IL-13 was dominant before stimulation. An increase in the production of IL-10 was observed after stimulation. Diabetic mothers had also a low basal secretion of cytokines but after the specific stimulation, we observed an increase in most of the detected cytokines – IL-6, IL-10, IL-13 and IFN-γ. Nevertheless these results are not statistically significant.

HLA genotyping

Nine of the T1DR subjects concerned in our study carried the 'very high or high risk of T1D development' genotype (Table 1). The rest of the T1DR population was at 'low or intermediate risk of T1D development'. Unfortunately, the twins were not HLA screened because of the refusal of their parents.

Cytokine secretion in T1DR neonates with low genetic risk of T1D development versus T1DR neonates with high risk of T1D development

We observed significantly higher basal levels of G-CSF ($P = 0.038$), GM-CSF ($P = 0.020$) (Fig. 6) and GRO-α

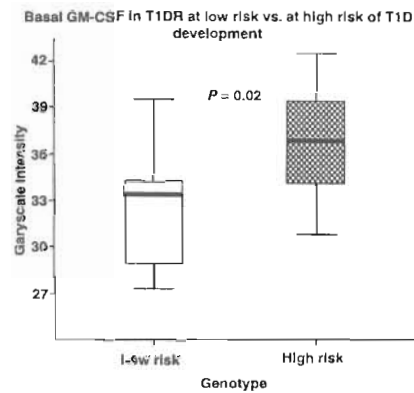
Figure 6 *Basal cytokine production in T1DR neonates with low genetic risk of T1D development versus T1DR neonates with high risk of T1D development.* We observed significantly higher basal levels of GM-CSF ($P = 0.020$) in T1DR at high risk in comparison with the group of T1DR with low genetic risk of T1D development.

(0.033) in the group of T1DR neonates at high risk in comparison with the group of T1DR neonates with the low genetic risk of T1D development.

Higher post-stimulative increase in IL-2 (0.020), IFN-γ (0.001) (Fig. 7A and B) and MCP1 (0.046) can be seen in the T1DR neonates at high risk compared with the group of T1DR neonates with the low genetic risk of T1D development.

Discussion

The developing immune system of the newborn has not yet been influenced by any external factors. Although, there are former studies showing that there is an effect of not only infection but also of some other environmental factors on the cytokine spectrum of the cord blood, e.g. maternal smoking or air pollution [25].

In this cohort of newborns, we observed a significant difference in the immune response between the group of T1DR newborns and the control group. Nevertheless, the cytokine spectrum in the group of T1DR neonates was not homogenous.

Comparing the cytokine production, an overall low basal production of all detected cytokines was seen in the T1DR newborns. The reaction to diabetogenic stimuli was either a low or default production of cytokines; nevertheless, these results are not statistically significant. We also have not seen any significant increase in cytokine secretion after the specific stimulation. The effect of the phytohaemagglutinin non-specific stimulation had a rather slight increase in comparison with the control group reaction. However, there was no significant difference between the two groups. It looks as if the immune system of the T1DR newborn is less mature than in controls and probably more vulnerable.
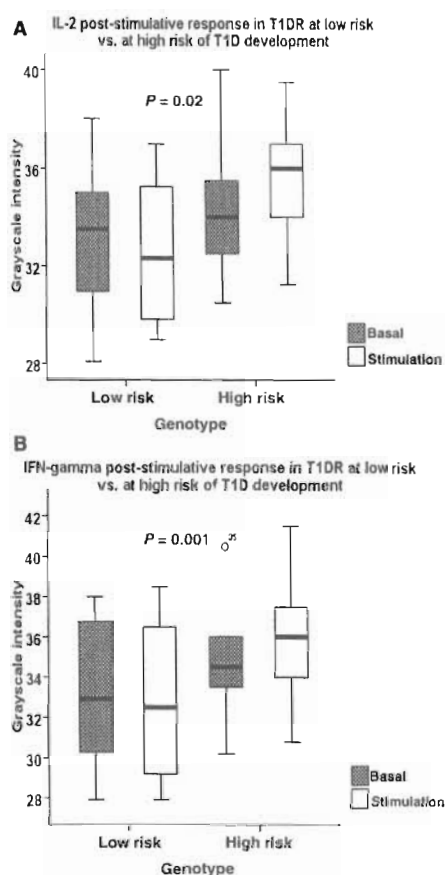
A

IL-2 post-stimulative response in T1DR at low risk
vs. at high risk of T1D development

B

IFN-gamma post-stimulative response in T1DR at low risk
vs. at high risk of T1D development

Figure 7 *Post-stimulative response in T1DR neonates with low genetic risk of T1D development versus T1DR neonates with high risk of T1D development.* (A) Significantly higher increase in IL-2 (0.020) and (B) IFN-γ (0.001) can be seen in T1DR neonates at high risk compared with the group of T1DR neonates with low genetic risk of T1D development after stimulation.

The influence of the external factors, especially maternal hyperglycaemia, infection and environmental factor bias, can be ruled out. All pregnancies were physiological and the diabetes of the mothers was well controlled, being centralized in our hospital; they were mainly living in big cities and all of the T1D mothers were non-smokers. None of the T1D mothers suffered from other immune-mediated diseases (other autoimmunity, allergy, immunodeficiency, etc.). The possible regulation–suppression by maternal immune system in T1DR also does not seem to play a role. On the contrary, we observed rather positive correlation among the high maternal regulatory cytokine levels and cytokine spectre in the newborn. There is the question if this immunological background can increase the risk of T1D development and that is why a long-term follow-up study of all subjects will be performed.

Nine of the T1DR subjects carried the 'very high or high risk of T1D development' genotype when the rest of the T1DR group was just at 'low or intermediate risk

of T1D development'. Interestingly, the T1DR neonates with the risky genotype had higher basal levels of cytokines G-CSF, GM-CSF and GRO-α and they also tend to react more in favour of Th1 – increase in IL-2, IFN-γ after diabetogenic stimulation. According to previously published papers, Th1 cytokine profile with high IFN-γ secretion has been found during the pre-diabetic phase [10–12]. But because T1D is known to be a polygenic disease, we can only speculate about the future T1D onset in our population. Further follow-up studies are needed on this issue. We assume that the genetic risk factors for T1D development in the controls should correlate with the incidence of 'risky' genotype in the whole population – so the genotyping was not performed in the control group.

We did not observe any significant difference among the cytokine levels before and after stimulation in the group of newborns with T1D father in comparison with the newborns with T1D mother but the number of subjects with T1D father was very small; so, we can only speculate on this issue. All of the T1D mothers were checked regularly by the diabetologist in our hospital and they were also admitted to the Department of Gynaecology and Obstetrics few days before the labour. But it is rather difficult to obtain cord blood of newborns with T1D father – there is not any special care and centralization of healthy pregnant women.

All of the mothers involved in this study were well controlled and their HbA1c levels were normal in the last 3 months of pregnancy so the possible influence of hyperglycaemia on the immune system of the foetus is rather small. So, we assume that there was not a big influence of the 'T1D environment *in utero*'.

We had the unique opportunity to study the couple of twins with both diabetic parents just as an interesting case presentation. The cytokine and chemokine production of the twins with both parents suffering from T1D were exceptional – higher basal levels can be observed in both of them in comparison with the rest of T1DR subjects. The reactivity after the stimulation was not homogenous in the T1DR group and the difference between the reaction of the twins and the rest of the T1DR population was not significant. Because of the small cohort being compared with the non-homogenous group of other T1DR newborns we do not have enough data on this issue, so we can just speculate if this 'twin pattern' could be a type of a 'risk pattern' in cytokine profile and the follow-up study is needed. Unfortunately, the twins were not genotyped because their parents refused the DNA testing but it is a known fact that the children with both T1D parents are at higher genetic risk of T1D development in comparison with the children with one T1D parent only.

The results found in the controls are in agreement with previous studies of CB cytokine spectre. The 'naïve'

T lymphocytes have a low proliferative ability after poly-clonal stimulation in comparison with the adults. They produced a very small amount of several cytokines; especially a lack of IL-5 was observed [16–19]. So far, according to our best knowledge no one has obtained any data about the immunoreactivity against diabetogenic autoantigens of the naïve neonatal immune system. Thus, our results of a significant difference in both basal and post-stimulative response of T1DR newborns are novel. New findings could also play an important role in further understanding the T1D pathogenesis.

Peripheral blood samples of six T1D mothers and four healthy mothers were obtained shortly after the labour to study a possible influence of false results caused by the contamination of the cord blood with maternal mononuclear cells. In all cases, the basal cytokine production was different in comparison with the protein microarray results of their own neonates. In healthy mothers, we observed an overall low production of all detected cytokines. The spectrum was weighted slightly in favour of a Th2-spectrum especially the IL-13 cytokine was dominant before stimulation. This is in agreement with studies published in the past – predominance of Th2 cell response, low cytokine levels, increase in Th1 cytokine levels and IL-6 caused by non-specific stimulation or stress [19–22, 26–28].

An increase in the production of IL-10 was observed after stimulation. T1D mothers had also a low basal secretion of cytokines but after the specific stimulation, we observed an increase in most of the detected cytokines – IL-6, IL-10, IL-13 and IFN-γ. It seems to be a sign of hyper-responsiveness of the immune system that is pushed to overweight the 'autoimmune' Th1 cytokines and to enable a successful pregnancy and delivery. This was also observed in other pregnancies of women with autoimmune diseases – the distinct shift from a Th2 cytokine bias during pregnancy towards a Th1 cytokine spectrum after delivery. The pregnancy polarizes the immune response towards a Th2 response, which may counter-balance the augmented Th1 response observed in Th1-mediated autoimmunities (rheumatoid arthritis, systemic lupus erythematosus and sclerosis multiplex). Thereby, pregnancy influences the signs and symptoms of the disease and a clinical remission could often be observed in pregnant women with autoimmune diseases [29–31].

A positive correlation was found between the high maternal levels of the regulatory cytokine TGF-β and high neonatal levels of IL-15, IFN-γ and TGF-β and we did not observe any suppressive impact of high levels of this cytokine in mothers on cytokine profile of their own newborns (which could be expected). Maternal IL-10 levels had no significant influence on neonatal cytokine levels. High maternal IL-13 levels probably tend to suppress the basal neonatal production of GM-CSF and RANTES

and it has a positive effect on haematopoietic growth factor IL-7 production. However, we should not forget the influence of co-operation within the whole cytokine net.

Basal cord blood cytokine profile was also studied in case of perinatal infection, chronic lung disease in premature infants (elevated inflammatory cytokines), asthma and allergy (Th2 response) and for the needs of transplantation immunology (e.g. GvHD). The mononuclear cells were also stimulated by non-specific activators (phytohaemogglutinin, bacterial lipopolysaccharide and enterotoxin) but no one has obtained any data about the human immunoreactivity against autoantigens in the newborn [32–34].

In our research, we would like to contribute in the way of finding a useful model for further T1D pathogenesis studies. In future, they could contribute in T1D prediction programme and early diagnostics including newborns. Further studies are necessary to be performed in larger cohorts of newborns but according to our findings protein microarray technique so far seems to be a useful tool to characterize a risk pattern of the immune response for T1D also in early life.

Acknowledgment

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CD 127⁻ and FoxP3⁺ Expression on CD25⁺CD4⁺ T Regulatory Cells upon Specific Diabetogeneic Stimulation in High-risk Relatives of Type 1 Diabetes Mellitus Patients

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Abstract

Abnormalities in CD4⁺CD25⁺ regulatory T cells (Treg) may contribute to type 1 diabetes (T1D) development. First-degree relatives of T1D patients are at increased risk especially when they carry certain HLA II haplotypes. Using two novel markers of CD4⁺CD25⁺ Treg (CD127⁻ and FoxP3⁺ respectively), we evaluated number and function of Treg after specific stimulation with diabetogeneic autoantigens in 11 high-risk (according to HLA-linked risk) relatives of T1D patients and 14 age-matched healthy controls using a cytokine secretion assay based on interferon-γ (IFN-γ) production. High-risk relatives of T1D patients had significantly lower pre- and post-stimulatory number of CD127⁻ Treg than that of healthy controls ($P < 0.05$). Labelling Treg with FoxP3⁺ demonstrated similar trend but did not reach statistical significance. Although the stimulation with diabetogenic autoantigens did not lead to a significant change in number of Treg in both groups, the defective function of Treg was performed by significantly higher activation of diabetogeneic T cells in high-risk relatives of T1D patients compared to healthy controls ($P \leq 0.02$). Individuals at increased HLA-associated genetic risk for T1D showed defects in Treg.

Introduction

Autoreactive subsets of T cells that escaped from negative selection in the thymus and the failure of peripheral tolerance mechanisms to control these potentially pathogenic T cells may lead to the breakdown of self-tolerance and autoimmune disease development [1–4]. Type 1 diabetes (T1D) represents a well-described model (with some known genetic background associations mainly with HLA molecules) of T-cell-mediated autoimmune destruction of insulin producing pancreatic β-cells [5, 6]. In the last decade, naturally occurring thymus-derived CD25^high CD4⁺ T regulatory cells (Treg) in humans have been described. They form about 2–4% of total CD4⁺ T cells in peripheral blood, have the ability of cell-contact dependent suppression of immune response and play an important role in maintaining immune homeostasis [2, 3, 7]. However, in humans about 10% of CD4⁺ T cells in peripheral blood can constitutively express CD25 [α chain of interleukin (IL)-2 receptor] and only those with high expression of CD25 have the regulatory properties [8, 9]. This fact introduced certain difficulties and confusion in distinguishing Treg from conventional non-regulatory CD25⁺CD4⁺ T cells which led to an intensive search for more characteristic markers [1–4, 10]. At present, intracellular forkhead/winged-helix family transcriptional repressor p3 (FoxP3) is supposed to be the most specific marker of Treg [3, 11]. FoxP3 expression correlates well with regulatory activity and number of Treg, it is exclusively expressed in CD25⁺CD4⁺ Treg and is considered as a key player for the development and function of Treg [2–4, 10]. FoxP3 repress the interleukin (IL)-2, IL-4 and interferon-γ (IFN-γ) gene's expression and interact with nuclear transcription factors of activated T cells (NF-kB, NFAT) that results in poor cytokine production and impared proliferation [10, 12–14].

Very recent studies have introduced a new surface marker of Treg: CD127⁻ (α chain of IL-7 receptor). A very strong correlation between the number of CD127⁻ and FoxP3⁺CD25⁺CD4⁺ T cells with similar suppressive

ability has been described [11, 15]. Downregulation of IL-7 receptor (CD127⁻) on Treg contrasts with the common expression of IL-7 receptor on non-regulatory T-cell subsets and shows the relative independency of Treg from IL-7 [15].

Many studies demonstrate associations with clinical manifestation of T1D, HLA genotype and pancreatic islets autoantibody/ies positivity in first-degree relatives within the Caucasian population [9, 16, 17]. Similar data about Treg in T1D relatives are still limited. In our previous study, we observed a significantly lower number of $CD25^{high}CD4^+$ Treg in siblings of children with T1D who were at increased HLA-associated genetic risk of T1D development [18]. We hypothesized that the low number of Treg can predispose these individuals to autoimmunity. In the present study, we focused on functional properties of Treg in these HLA-linked high-risk relatives of T1D patients using specific diabetogenic autoantigen stimulation and CD127 and FoxP3 as markers of $CD25^+CD4^+$ Treg.

Materials and methods

Study subjects. Heparinized blood samples were obtained from 11 healthy first-degree relatives of T1D patients (six females, five males, age 14–43, median age 23 years) followed Pediatric Department, University Hospital Motol, Prague and First Department of Pediatrics, University Hospital, Brno, Czech Republic. All of them carried at least one of the high-risk HLA haplotypes, i.e. DQA1*05–DQB1*0201 or DQA1*03–DQB1*0302 and had no protective alleles, i.e. DQB1*0602 and DRB1*0403 [9, 17] (Table 1). A complete HLA-DQA1 and HLA-DQB1 genotyping was performed by polymerase chain reaction (PCR) with sequence-specific primers and a stratification of HLA-linked genetic risk was performed [9]. Age-matched 14 healthy controls (six females, eight males, age 17–45, median age 26 years) were consecutively recruited from healthy blood donors with no family or personal history of T1D or any other autoimmune disease. None of them carried high-risk haplotype for T1D. Sera of subjects were examined by radioimmunoassay (RIA) (Solupharm, Brno, Czech Republic) for the presence of autoantibodies against islet antigens glutamic acid decarboxylase 65 (GADA) and

tyrosinephosphatase (IA-2A). Levels above 1 IU/ml for GADA as well as for IA-2A (above 2 standard deviations of normal) were considered positive. None of the tested subjects had positive autoantibodies. Blood samples of all study subjects were taken after signing the informed consent approved by the local Ethical Committee.

Stimulation assay. Peripheral blood mononuclear cells (PBMC) were obtained by Histopaque (Sigma-Aldrich, Prague, Czech Republic) gradient centrifugation of heparinized blood. Freshly isolated PBMC were resuspended in a complete culture medium containing X-Vivo 15 supplemented with 50 mg/l gentamycin, 2 mM L-glutamine and 10% heat-inactivated human AB-serum (all Sigma-Aldrich) in cell concentration 2×10^6 per ml. A mixture of the following synthetic autoantigens was used for stimulation: GAD65-peptides amino acids 247–279, a.a. 509–528; a.a. 524–543 (Dept. of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden); IA2 a.a. 853–872 and a.a. 9–23 of β proinsulin chain (Sigma, St Louis, MO, USA). Concentration of all autoantigens was 1 μg per 10^6 cells each. Insulin (Humulin R, Lilly France S.A.S., Fegersheim, France) at a concentration 5 U/ml was tested separately.

The selection and concentration of autoantigens as well as the amount of tested PBMC was made according to previous Immunology of Diabetes Society T-cell workshops, recommendations and also according to our experience [19–21]. After optimizing the length of autoantigen exposure (data not shown), we stimulated PBMC 72 h in 37 °C, 5% CO_2 atmosphere.

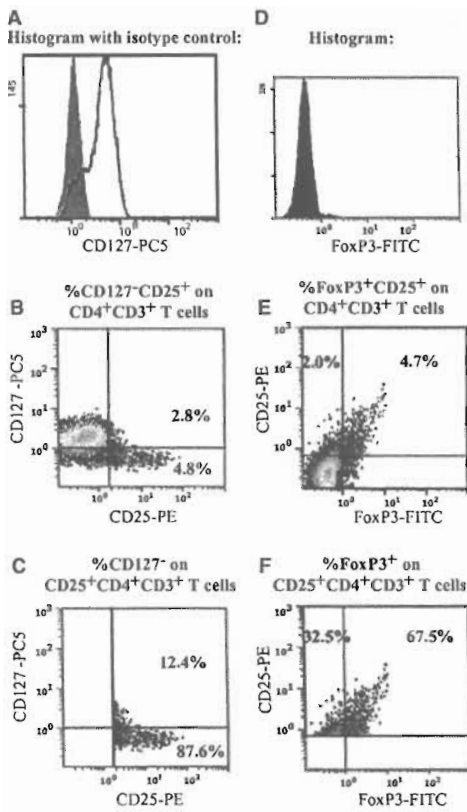Experiments were completed with negative controls, PBMC alone in complete medium.

Flow cytometry. Flow cytometric analysis of T-cell populations was preformed before and after stimulation using the following markers: anti-CD3, anti-CD4, anti-CD25, anti-CD127 labelled with fluorescein isorhiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanin 5 (PC5), or phycoerythrin-cyanin 7 (PC7) (Immunotech, Marseille, France). For intracellular detection of FoxP3, FITC-anti-human-FoxP3 staining set (e-Bioscience, San Diego, CA, USA) was used according to manufacturer's instructions. Cell activation was measured by surface expression of IFN-γ on activated $CD4^+$ T cells using the Secretion Assay Cell Detection Kit (Miltenyi Biotec, Bergish Gladbach, Germany) according to manufacturer's instructions. Samples were analysed by a 4-colour flow cytometry on a Cytomics™ FC 500 cytometer (Beckman Coulter, Miami, FL, USA). Data were analysed using the CXP Software (Beckman Coulter). Gating strategy is displayed on Fig. 1.

Statistical analyses. Non-parametric methods for statistical analysis were used due to asymmetric data distribution. Groups were compared with Mann–Whitney U-test; P-values of <0.05 were considered significant. For comparison of related data in each group separately,

Table 1 HLA-genotypes and other characteristics of tested subjects.

11 high-risk relatives of T1D patients (Age: median 23, sex: F/M = 6/5)

HLA-genotype	No. subjects	GADA-positive	IA-2A-positive
DQA1 *03/05, DQB1 *02/0302	6	0	0
DQA1 *03/03, DQB1 *0302/0302	5	0	0

Figure 1 Gating strategy for Treg. Analyses were performed on 4-coloured CytomicsTM FC 500 cytometer (Beckman Coulter, Miami, FL, USA). For analysis, PBMC were gated on lymphocytes (based on forward and side scatter and CD3$^+$). (A) CD127 negativity was determined with regard to a negative isotype control, (B) the percentage of CD127$^-$CD25$^+$ cells within all CD4$^+$CD3$^+$ T cells, (C) percentage of CD127$^-$ within only CD25$^+$CD4$^+$CD3$^+$ T cells, (D) FoxP3$^+$ was determined with regard to the negative isotype control, (E) the percentage of FoxP3$^+$CD25$^+$ cells within all CD4$^+$CD3$^+$ T cells and (F) the percentage of FoxP3$^+$ within only CD25$^+$CD4$^+$CD3$^+$ T cells.

Wilcoxon Signed Ranks Test and Friedman Test were used. Results are written in medians and inter-quartile range (IQR) of the first and the third quartile; P-values

of <0.05 are considered significant. All analyses were performed using the statistical software SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA).
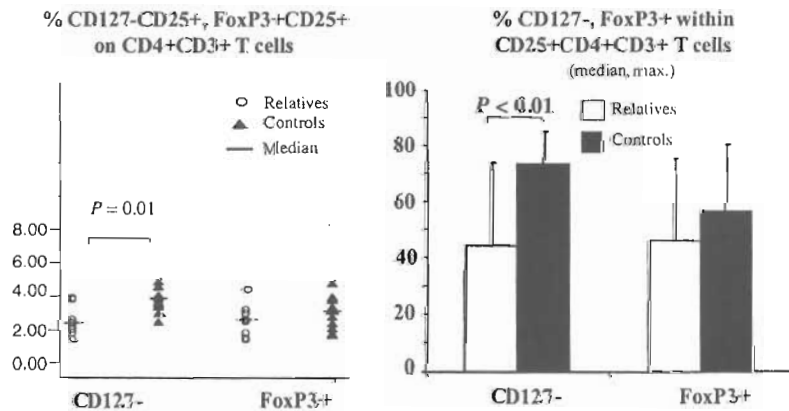
Results

High-risk relatives of T1D patients had low number of freshly isolated Treg

All 11 HLA-linked high-risk relatives of T1D patients without insulitis and 14 age-matched healthy controls were tested for the presence of Treg using a surface marker CD127 and an intracellular marker FoxP3. The number of Treg freshly isolated from peripheral blood was lower in a group of high-risk relatives of T1D patients compared to healthy controls. The relative frequency of CD127$^-$CD25$^+$ on CD4$^+$CD3$^+$ T cells had a median 2.4%; IQR 2.1–2.6% of all CD4$^+$CD3$^+$ T cells in high-risk relatives while healthy controls had a median 3.9%; IQR 3.4–4.4% (P = 0.01). The relative frequency of FoxP3$^+$CD25$^+$ on CD4$^+$CD3$^+$ T cells had a median 2.6%; IQR 2.0–2.9% in high-risk relatives, while healthy controls had a median 3.1%; IQR 2.3–3.7% (P = ns). The CD127$^-$ marker on CD25$^+$CD4$^+$CD3$^+$ T cells that should correspond to the percentage of Treg within the CD25$^+$CD4$^+$CD3$^+$ T cells was present in a median 44.4%; IQR: 38.7–57.6% in high-risk relatives and in a median 73.6%, IQR 63.8–76.0% in healthy controls (P < 0.01). FoxP3$^+$ was present in a median 46.3%, IQR 36.1–57.2% in high-risk relatives and in a median 57.2%, IQR 52.8–65.7% in healthy controls (P = ns) (Fig. 2).

The low number of Treg in high-risk relatives of T1D patients did not change after autoantigen stimulation

After 72-h in vitro stimulation assay with the mixture of previously defined diabetogenic peptides and the whole molecule of insulin, the relative frequencies of Treg in unstimulated and stimulated cell's cultures were

Figure 2 Relative frequencies of freshly isolated Treg. Significantly lower number of CD127$^-$ Treg in high-risk relatives compared to healthy controls was noted, lower number of FoxP3$^+$ Treg was not significant.

compared. The relative frequencies of Treg in each group separately (i.e. in high-risk relatives of T1D patients and in healthy controls) did not change significantly after autoantigen stimulation. Although in high-risk relatives of T1D patients the numbers of Treg remained lower during whole assay in comparison with healthy controls, the statistical significance was reached only for CD127⁻ Treg when cells were stimulated with the whole protein of insulin. This was even more obvious when the relative frequencies of CD127⁻ were studied within CD25⁺CD4⁺CD3⁺ Treg (Fig. 3). Labelling Treg with FoxP3⁺ demonstrated a similar trend but did not reach statistical significance at all. FoxP3 expression was measured only after stimulation with a mixture of diabetogenic peptide autoantigens due to the limited number of PBMCs for stimulation and cytometric determination (Fig. 4).

Diabetogeneic autoantigens led to strong Th1–type activation in high–risk relatives of T1D patients, but not in healthy controls

The unstimulated CD4⁺CD3⁺ lymphocytes of high-risk relatives of T1D patients demonstrated a significantly higher basal IFN-γ production after 72 h of *in vitro* culture: median 0.8%; IQR 0.7–0.9% compared to unstimulated healthy controls: median 0.4%; IQR 0.3–0.7% ($P = 0.02$). The significantly higher production of IFN-γ in this high-risk relatives of T1D patients was further enhanced after stimulation with a mixture of diabetogenic peptide autoantigens: median 1.2%; IQR 1.0–1.4% of IFN-γ⁺ CD4⁺CD3⁺ T cells compared to healthy controls: median 0.6%; IQR 0.4–1.0% of IFN-γ⁺ CD4⁺CD3⁺ T cells ($P < 0.01$); as well as after stimulation with the whole protein of insulin: median 1.7%; IQR 1.5–1.8% of IFN-γ⁺ CD4⁺CD3⁺ T cells in high-risk relatives versus median 0.5%; IQR 0.4–1.0% of IFN-γ⁺ CD4⁺CD3⁺ T cells in healthy controls ($P < 0.01$) (Fig. 5).

In each group separately, a significant increase of IFN-γ production in CD4⁺CD3⁺ T cells was observed in high-risk relatives of T1D patients after stimulation either with a mixture of diabetogenic peptides ($P = 0.017$) or with insulin ($P = 0.017$). In controls, stimulation with the same mixture of diabetogenic peptides did not lead to a significant increase of IFN-γ production in CD4⁺CD3⁺ T cells ($P = $ ns) but stimulation with insulin did ($P = 0.028$).
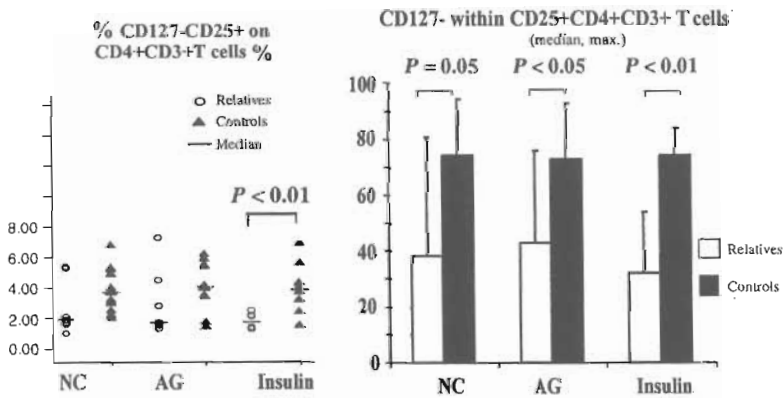

Figure 3 Relative frequencies of CD127⁻ Treg after stimulation. No significant change in CD127⁻ Treg was observed. Lower percentage of CD127⁻ Treg remained during the whole assay in high-risk relatives of T1D patients; relative frequencies of CD127⁻ related only to CD25⁺CD4⁺CD3⁺ T cells disclosed the statistical significance which is pointed. NC, negative control with unstimulated PBMC in culture medium; Ag, PBMC stimulated with mixture of diabetogeneic autoantigens; Insulin, PBMC stimulated with insulin.

Figure 4 Relative frequencies of FoxP3⁺ Treg after stimulation. No significant change in FoxP3⁺ Treg was observed. However, lower percentage of FoxP3⁺ Treg remained during the whole assay in high-risk relatives of T1D patients. This was more obvious when FoxP3⁺ was related only to CD25⁺CD4⁺CD3⁺ T cells. NC, negative control with unstimulated PBMC in culture medium; Ag, PBMC stimulated with mixture of diabetogeneic autoantigens.

% IFNg+ CD4+ on CD3+ T cells
(median, IQR, min., max.)

Figure 5 Relative frequencies of IFN-γ on CD4⁺ T cells. Significantly higher frequency of IFN-γ⁺ cell was observed after diabetogeneic stimulation in high-risk relatives of T1D patients. In high-risk relatives, stimulation of both (mixture of peptides autoantigens as well as whole molecule of insulin) statistically increase IFN-γ positivity. NC, negative control with unstimulated PBMC in culture medium; Ag, PBMC stimulated with mixture of diabetogeneic autoantigens; Insulin, PBMC stimulated with insulin.
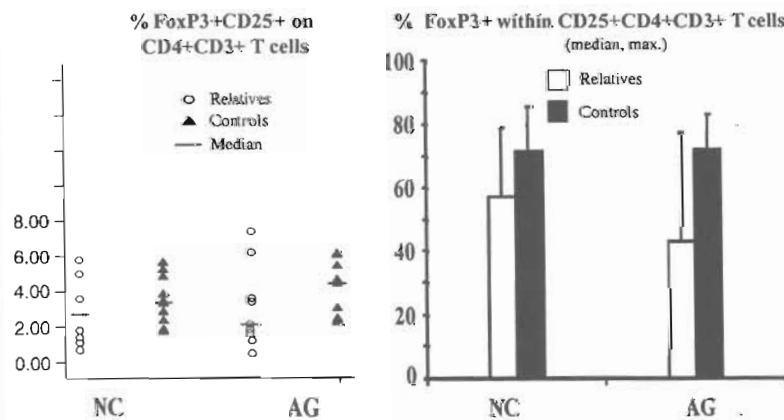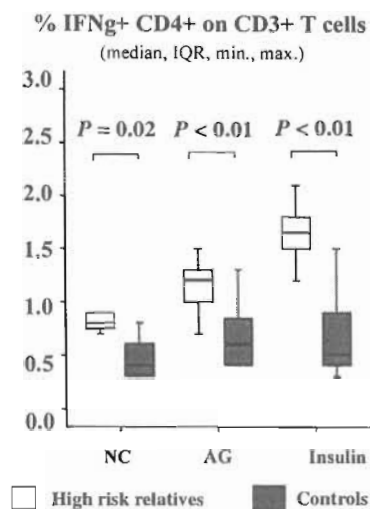
Discussion

Defects in Treg may significantly disturb the balance between activation and suppression of immune system as well as it may adversely affect the type of immune response (Th1 versus Th2) and thus contribute to β-cell destruction in the pancreas. Patients with T1D may have altered number and function of Treg [8, 18, 21–24]. There is less information about Treg in T1D patient's healthy relatives who have higher probability of T1D development according to their HLA-linked genetic risk [9, 16, 17]. In a previously published data, we demonstrated that healthy siblings of T1D patients showed a lower number of Treg when they carried a certain high-risk HLA-haplotype for T1D [18]. Other studies also demonstrate a lower number of Treg in patients with T1D or their relatives [23, 24]. On the other hand some studies did not reveal any significant differences in numbers of Treg [25–27]. However, in some of them HLA-linked genetic risk was not considered [25–27]. In addition, in most of these previous experiments (including our), percentage of Treg was based on the expression of CD25^high on CD4⁺CD3⁺ T lymphocytes. This marker has been proposed as insufficient for Treg determination as it can be broadly influenced by the gating strategy and thus limited in interpretation [28, 29]. Recently there is clear evidence that the regulatory functions of CD4⁺CD3⁺ T cells are associated with the intracellular presence of FoxP3⁺ [2, 3, 10, 11] as well as with a newly

introduced surface marker CD127⁻ [11, 15] on a subpopulation of CD25⁺CD4⁺CD3⁺ T cells. Previous observations described the highest suppressive ability in the subsets of CD127⁻ and FoxP3⁺CD25⁺CD4⁺ T cells; the level of FoxP3 expression positively correlates with the suppression rate [3, 10, 11, 15]. Microarray analysis of mRNA, flow cytometry and functional assays from individual T-cell subsets showed that CD127 was expressed at significantly lower levels in CD4⁺CD25^hi versus CD4⁺CD25⁻ T cells and inversely correlates with FoxP3⁺. It was suggested that FoxP3 interacts with a promoter of CD127 as a repressor [11].

In our present study, we used these two novel markers CD127 and FoxP3 to evaluate the presence and functional properties of Treg in a genetically defined group of high-risk relatives of T1D patients. Based on this determination we were able to reveal differences in number and function of Treg between high-risk relatives of T1D patients and age-matched healthy controls. We could observe lower numbers of Treg in the high-risk relatives group during the whole assay. However, only results regarding CD127⁻ as a marker of Treg reached statistical significance mainly when the percentage of CD127⁻ was related exclusively to CD25⁺CD4⁺CD3⁺ T-cell population. FoxP3⁺ Treg showed similar trend but without statistical significance. This can be due to the fact that not all FoxP3⁺ T cells are necessarily CD127⁻ T cells with regulatory ability. Also the regulatory activity may depend on the level of FoxP3 expression inside the cell as well as on isoforms of the protein [11, 12]. Specific diabetogenic stimulation did not significantly change number of Treg in any group.

In contrast, we observed a very strong Th1 response after autoantigen stimulation in high-risk relatives of T1D patients that was not seen in healthy controls. The presence of autoreactive T-cell subsets in relatives with high-HLA II-linked genetic risk may play its role. It is supposed that diabetogeneic autoreactive T cells express TCR that can recognize specific islet's autoantigens presented on certain HLA II molecules and produce Th1 spectrum of cytokines. The IFN-γ production was more pronounced when the whole protein of insulin rather then peptides were used as an antigen. This is in agreement with the fact that insulin is supposed to be a very potential primary autoantigen in T1D and the one in which the immunogenic adjustment can lead to prevention or acceleration of T1D development [21, 30, 31]. We think that the strong Th1 activation in the group of high-risk relative's may correspond with a poor immunosupressive function of Treg in this group. The underlying association, if any between the HLA type and the exact role of TCR in Treg remains unclear and requires further exploration [32, 33]. Our results can be controversial with other recently conducted studies that did not proved numerical changes in Treg [34]. However, there

should be notice our well-defined HLA-genetic background of the subjects and the fact that so far exist only a few human studies among neither T1D patients nor relatives using CD127$^-$ and FoxP3$^+$ as markers of Treg [34].

In summary, we have determined that CD127 is a reliable marker of Treg that is expressed at lower levels in high-risk relatives of T1D patients. These individuals are more reactive to diabetogenic antigen stimulation. All together this can increase the risk of clinical manifestation of T1D in future.

Acknowledgment

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Pediatric Diabetes

Original Article

Protein microarray analysis as a tool for monitoring cellular autoreactivity in type 1 diabetes patients and their relatives

Vrabelova Z, Kolouskova S, Böhmova K, Faresjö MK, Sumnik Z, Pechova M, Kverka M, Chudoba D, Zacharovova K, Stadlerova G, Pithova P, Hladikova M, Stechova K. Protein microarray analysis as a tool for monitoring cellular autoreactivity in type 1 diabetes patients and their relatives.
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Background: Autoreactive T cells have a crucial role in type 1 diabetes (T1D) pathogenesis.
Objectives: The aim of our study was to monitor the *in vitro* production of cytokines by peripheral blood mononuclear cells (PBMCs) after stimulation with diabetogenic autoantigens.
Subjects: Ten T1D patients (tested at the time of diagnosis and 6 and 12 months later), 10 first-degree relatives of the T1D patients, and 10 controls underwent the study.
Methods: PBMCs were stimulated with glutamic acid decarboxylase 65 (GAD65) amino acids (a.a.) 247–279, 509–528, and 524–543; proinsulin a.a. 9–23; and tyrosine phosphatase (islet antigen-2)/R2 a.a. 853–872. Interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-13, interferon (IFN)-γ, tumor necrosis factor β, transforming growth factor β1, and granulocyte colony-stimulating factor (GCSF) were analyzed by protein microarray.
Results: Differences in cytokine(s) poststimulatory and mainly in basal production were observed in all groups. The most prominent findings were in controls, the higher basal levels of IL-2, IL-4, IL-5, IL-13, and GCSF were observed when compared with relatives ($p < 0.05$, for all). After stimulation in controls, there was a significant decrease in IL-2, IL-13, GCSF, and IFN-γ ($p < 0.05$, for all). The group of relatives was the most variable in poststimulatory production. A strong correlation between cytokines production was found but groups differed in this aspect.
Conclusion: By multiplex analysis, it may be possible, for example, to define the risk immunological response pattern among relatives or to monitor the immune response in patients on immune modulation therapy.

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Type 1 diabetes (T1D) is a chronic disorder that results from the specific destruction of the insulin-producing pancreatic β cells by the immune system. The initial phase of T1D is clinically silent; the real triggers are not really known. The activated immune cells invade the pancreas and slowly destroy β cells until it becomes clinically evident in its consequences (hyperglycemia and ketoacidosis) (1–3).

The destruction of pancreatic β cells is T-cell dependent. The major role is played by the subset of CD4+ autoreactive T lymphocytes (T helper lymphocytes) that can recognize the autoantigens in the context of human leukocyte antigen (HLA) II molecules and then differentiate themselves into the T helper (Th) 1 cells. The production of Th1 cytokines [interferon (IFN)-γ and tumor necrosis factor (TNF)-β] leads to the activation of macrophages and CD8+ cytotoxic lymphocytes, and they then can invade the pancreatic islets and create the toxic environment. The death of β cells amplifies the inflammation.

The presence of antibodies alone is not sufficient to induce the destruction of β cells (1–4). On the contrary, the exceptional humoral immunity associated with the Th2 response after the antigen stimuli of Th0 naive lymphocyte is suppressed in T1D animal models. Thus, the cytokine profile typical of the Th2 response [interleukin (IL)-4, IL-5, and IL-13] seems to have a protective effect (1–5). Current studies also reveal the importance of the failure of regulatory mechanisms represented mainly by T regulatory cells. These cells are able to suppress proliferation and cytokine production from both CD4+ and CD8+ T cells in vitro in a cell-contact-dependent manner and by secretion of anti-inflammatory cytokines [for example, IL-10 and transforming growth factor (TGF) β)] (6).

We detected cytokines produced by peripheral blood mononuclear cells (PBMCs) after stimulation with diabetogenic autoantigens using a protein microarray. This method enables semiquantitative multiparameter analysis of one sample. In the case of cytokine detection, primary anticytokine antibodies are attached to the small membrane and visualization is made by secondary anticytokine antibodies, and the whole cytokine spectrum can bee seen at once (7). We tested five groups: the T1D patients at different times (at the diagnosis and 6 and 12 months later), their first-degree relatives, and the healthy controls. We evaluated the secretion of typical Th1, Th2, and Th3 cytokines, and we tested also one inflammatory cytokine (IL-6) and one cytokine from hematopoietic growth factors family (granulocyte colony-stimulating factor, GCSF).

Patients and methods

Subjects

Ten patients with recent onset T1D (mean age 13 yr, age range 3–18 yr, female/male 4/6), treated at the Paediatric Outpatient Departments of the University Hospital Motol, Prague, were included into the study. None of them was in the severe metabolic acidosis at the time of diagnosis or suffered from any other autoimmune disease or inflammation. The samples were collected in the morning within a week after the diagnosis and then the patients were retested 6 and 12 months later (marked as D1, D2, and D3, respectively).

Ten first-degree relatives of the T1D patients and 10 healthy controls (blood donors), with no personal history of any autoimmune disease, underwent this study as well. Informed consent, approved by local ethical committee, was obtained from all the tested subjects.

Subjects' characteristics. The complete HLA-DQA1 and HLA-DQB1 genotypings were carried out by polymerase chain reaction with sequence-specific primers in all subjects (data not shown) (8). Relatives and healthy controls were HLA risk, age, and sex matched.

The sera from all participants were examined by radioimmune assay (Solupharm, Brno, the Czech Republic) for the presence of autoantibodies against the islet antigens glutamic acid decarboxylase 65 (GAD65) and islet antigen-2 (IA-2). Positivity was considered to be above 1 IU/mL for GAD65 (GADA) as well as for IA-2 (IA-2A) (>99th percentile).

None of the relatives as well as the healthy controls was autoantibody(ies) positive.

Assays

PBMCs were prepared by Ficoll gradient centrifugation (Amersham Biosciences, Uppsala, Sweden), and 2×10^6 freshly isolated PBMCs were resuspended in 1 mL of RPMI-1640 medium supplemented with 20% fetal calf serum, L-glutamine (10 μL/mL, 200 mM), and penicillin (1 μL/mL)–streptomycin (1 μg/mL; all Sigma, St.Louis, MO, USA) and cultivated with autoantigens. In all cases, PBMCs were stimulated with a mixture of diabetogenic autoantigens, and if enough cells were available, autoantigens were tested also separately (2×10^6 PBMCs were necessary for each separate autoantigen). The concentration of all autoantigens was $1 \mu g/10^6$ PBMC each. The following autoantigens were used in a mixture and/or separately: GAD65 peptide amino acids (a.a.) 247–279 (NMY-AMMIARFKMFPEVKEKGMAALPRLIAFTSEE-OH), molecular weight 3823.7, marked GAD1; a.a. 509–528 (IPPSLRTLEDNEERMSRLSK-OH), molecular weight 2371.7, GAD2; a.a. 524–543 (SRLSK-VAPVIKARMMEYGTT-OH), molecular weight 2238.7, GAD3 (Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden); IA-2 a.a. 853–872 SFYLK(Nleu)VQT-QETRTLTQFHF, molecular weight 2489; and a.a. 9–23 SHLVEALYLVCGERG of β proinsulin chain, molecular weight 1645 (Sigma).

All experiments were completed with positive control [PBMC + 10 μg phytohemagglutinin (Sigma) per 10^6 PBMCs] as well as with a negative control (PBMC in exclusive culture medium). The medium was harvested after 72-h stimulation (37°C, 5% CO_2), frozen (−20°C), and later used for protein microarray analysis that was performed by a custom array kit according to the instructions by the manufacturer (RayBiotech, Norcross, GA, USA). The production of the following cytokines was assessed: IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, TNF-β, TGF-β1, and GCSF.

Detection was carried out using the Fuji LAS1000 imaging system. Chemiluminescent signals were analyzed using the AIDA software (Advanced Image Data Analyzer, 3.28; Raytest Izotopenmessgeraete, Straubenhardt, Germany). The detection limits according to the manufacturer's Web site (www.

Table 1. Detection limits for all tested parameters

Cytokine	Sensitivity (pg/mL)
IL-2	25
IFN-γ	100
TNF-β	1000
IL-4	1
IL-5	1
IL-13	100
IL-10	10
TGF-β1	200
IL-6	1
GCSF	2000

GCSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

raybiotech.com) are displayed in Table 1. The images were edited in the gray-scale 8-bit map. The results are expressed according to the instructions of the manufacturer in percentage of signal intensity. The membranes were compared together, the integral positive controls of each membrane reached the 100% of intensity; no other image transformation was necessary.

Statistics

The data were processed by SPSS software. For non-parametric data, Kruskal–Wallis test was used for comparison of three or more groups and the Mann–Whitney test was used for comparison of two groups. The Wilcoxon Signed Ranks test was used for comparison of signal intensities in each group (basal × poststimulatory response). For expression of correlation analysis, Spearman's coefficient was used.

Results

Th1 cytokines (IFN-γ and TNF-β)

Significantly higher production of TNF-β was observed in the D2 group in comparison with the relatives (p < 0.05).

After stimulation with the autoantigens mixture, we observed a decrease in IFN-γ production in the control group (p = 0.049) and in the D3 group (p = 0.048). The D3 group also had a decrease in TNF-β poststimulatory production (p = 0.018) (Fig. 1).

Th2 cytokines (IL-4, IL-5, and IL-13)

There was a higher basal production of IL-4, IL-5, and IL-13 within the control group when compared with the relatives (p < 0.05 for all three cytokines). The D2 group also had a higher IL-13 basal
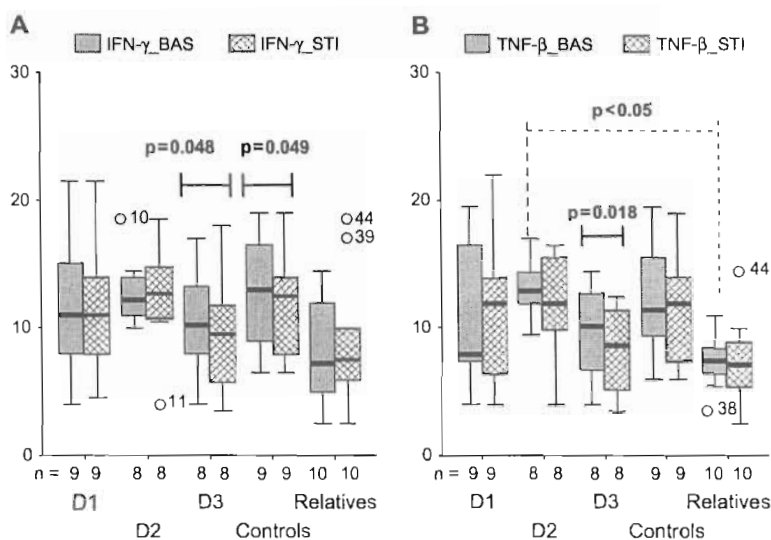
Fig. 1. Th1 cytokines in all groups together. (A) IFN-γ. (B) TNF-β. Results are displayed in percentage of spot intensity (basal and stimulated) and statistical significance is pointed. Data are expressed as median + range. Extreme values are shown. BAS, basal production; D1, type 1 diabetes patients at the time of diagnosis; D2, type 1 diabetes patients at 6 months later; D3, type 1 diabetes patients at 12 months later; IFN, interferon; STI, stimulation with autoantigens mixture; Th1, T helper 1 cells; TNF, tumor necrosis factor.

production when compared with their relatives (p < 0.05).

After stimulation, we observed a decrease in the IL-4 production in the D3 group (p = 0.025), whereas the production of IL-13 was suppressed in the control group (p = 0.035). The production of IL-5 was not significantly changed in all the groups (Fig. 2).

Th3 cytokines (IL-10 and TGF-β)

No difference in the overall basal production of IL-10 within the groups as well as in their poststimulatory response was observed. The controls had a higher basal production of TGF-β in comparison with the relatives (p < 0.05), whereas secretion of TGF-β was decreased by autoantigen stimulation in the D3 group (p = 0.049) (Fig. 3).

Other tested parameters (IL-2, IL-6, and GCSF)

A higher basal production of IL-2 and GCSF was seen in the group of controls, and it was statistically significant in comparison with the group of relatives (p < 0.05). Antigen-induced secretion of IL-2 and GCSF decreased (related to the basal levels) in the group of controls (p = 0.035 and 0.03, respectively) and IL-2 also decreased with statistical significance in the D3 group (p = 0.018).

Basal and poststimulatory IL-6 production was extremely variable in all groups. The tendency to decrease after stimulation was observed in the groups

of controls, relatives, and D3, while an increase was manifested in the D1 and D2 groups. Exclusively in the D3, a decrease was observed (p = 0.04) (Fig. 4).

Spearman's correlation analysis

To the section of statistics, when we supposed p < 0.01, the r_S should be above 0.8 for the following strong correlations.

In T1D patients, the basal production of IL-2, IL-4, IL-5, IL-13, GCSF, IFN-γ, TNF-β and TGF-β correlated well together (r_S > 0.8 for each pair). In this group, only basal TGF-β production did not strongly correlate with IL-13 and IFN-γ (r_S = 0.74 and 0.75, respectively), but after stimulation, the situation was slightly changed (r_S = 0.80 and 0.82, respectively). IL-6 and IL-10 showed no correlations; they were extremely variable. Situation is displayed in Fig. 5 and is expressed for D1, D2, and D3 all together as correlations did not differ within these groups.

In the control group, the strong correlations between IL-2, IL-4, IL-5, IL13, GCSF, IFN-γ, TNF-β and TGF-β were the same as observed in the group of T1D patients (r_S > 0.8 for each pair), and the relations remained even after stimulation. Moreover, basal production of TGF-β correlated with IL-13 and IFN-γ (r_S = 0.85 and 0.95, respectively) as well as by stimulation (r_S = 0.97 and 0.93, respectively). Furthermore, there was a correlation between basal IL-10 and IL-2, IL-4, IL-5, IL-13, and IL-6 (r_S > 0.8 for each pair). After stimulation, IL-10 correlated
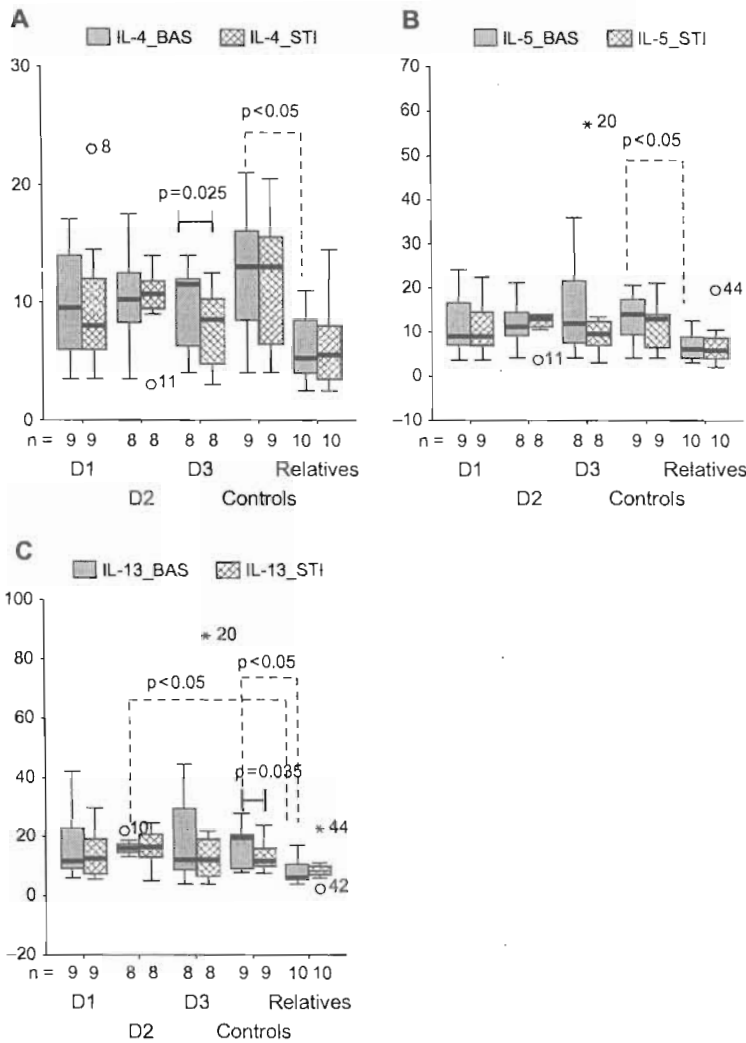
Fig. 2. Th2 cytokines in all groups together. (A) IL-4. (B) IL-5. (C)IL-13. Results are displayed in percentage of spot intensity (basal and stimulated) and statistical significance is pointed. Data are expressed as median + range. Extreme values are shown. BAS, basal production; D1, type 1 diabetes patients at the time of diagnosis; D2, type 1 diabetes patients at 6 months later; D3, type 1 diabetes patients at 12 months later; IL, interleukin; Th2, T helper 2 cells; STI, stimulation with autoantigens mixture. * indicates extreme values.

with IL-5, IL-13, IL-6, and GCSF and even with TGF-β ($r_S > 0.8$ for each pair). IL-6 also had correlations in basal production with IL-5, IL-13, and IL-10, and after stimulation IL-4, GCSF, IFN-γ, TNF-β, and TGF-β were added ($r_S > 0.8$ for each pair). Situation is displayed in Fig. 6.

In the group of relatives, we observed fewer correlations. Basal production of IL-2, IL-4, IL-5, and TGF-β correlated with each other ($r_S > 0.8$ for each pair) and then IFN-γ, TNF-β, GCSF, and IL-13 correlated with each other ($r_S > 0.8$ for each pair) but not together with IL-2, IL-4, IL-5, and TGF-β. After stimulation, the correlation among IL-2, IL-4, IL-5, and TGF-β remained and additional with IL-13 was noticed ($r_S > 0.8$ for each pair). IFN-γ and TNF-β only correlated to each other.

The basal production of TGF-β had a correlation only with IL-2, IL-4, and IL-5 ($r_S = 0.87$ and 0.86 and 0.87, respectively) and after stimulation also with IL-13 and GCSF ($r_S = 0.82$ and 0.80, respectively). There was no correlation of TGF-β with IFN-γ or TNF-β in basal or poststimulatory production ($r_S = 0.3$; 0.23, 0.52 and 0.72, respectively) and IL-6 and IL-10 correlated in basal and poststimulated production with each other. Situation is displayed in Fig. 7.

The stimulatory potential of autoantigens

The most potent autoantigen (highest spot intensities) was the GAD65 peptide (a.a. 509–528). In the relatives' group, we observed the strongest reaction
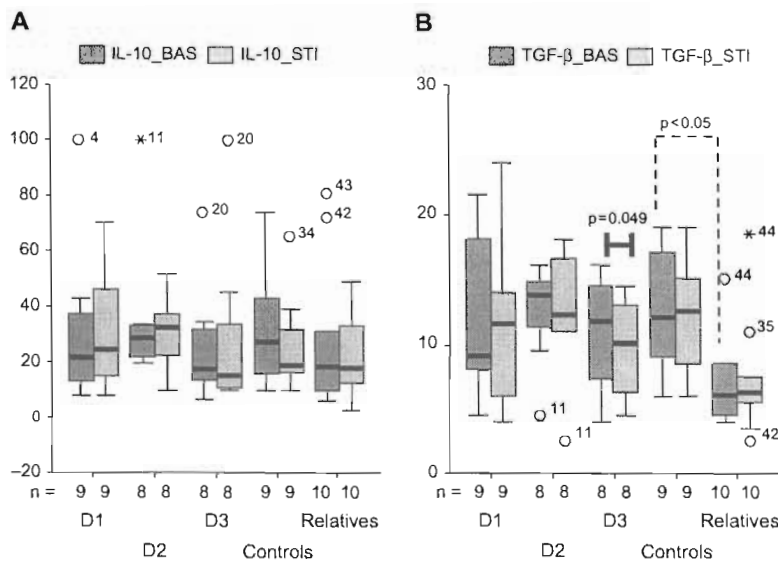
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Fig. 3. Th3 cytokines in all groups together. (A) IL-10. (B) TGF-β. Results are displayed in percentage of spot intensity (basal and stimulated) and statistical significance is pointed. Data are expressed as median + range. Extreme values are shown. Bas, basal production; D1, type 1 diabetes patients at the time of diagnosis; D2, type 1 diabetes patients at 6 months later; D3, type 1 diabetes patients at 12 months later; IL, interleukin; STI, stimulation with autoantigens mixture; Th3, T helper 3 cells; TGF, transforming growth factor. * indicates extreme values.

against the IA-2 peptide (5/10 tested). It was not possible to specify the most potential autoantigen in the controls because here the reaction was weak and proportional against single autoantigens (8/10 tested).

Discussion

Over the past two decades, several different systems have been used to study and monitor the autoreactive T cells in T1D patients: T-cell proliferation assay, cytokine-based assays including enzyme-linked immunosorbent spot (ELISPOT), approaches using flow cytometry etc. (9–14). Over the past decade, new genomic and proteomic technologies including protein and gene microarray assays for multiparameter analysis have become available (7, 15, 16). However, the progress and standardization of these autoreactive T-cell assays are quite slow and difficult as the levels of autoreactive T cells in circulation are very low (<1 in 100 000 of the total white blood cell population) (1–4, 9). To study the complex reactivity of PBMCs against diabetogenic autoantigens, we decided to use semi-quantitative detection of cytokines/chemokines by protein microarray. In our previous study, we compared protein microarray data by enzyme-linked immunosorbent assay and ELISPOT with good correlation (data not shown). However, for further exact quantification, we plan to use multiparameter technique (as, for example, Luminex). To avoid artifacts during the freezing, we worked with freshly isolated PBMCs.

The selected cultivation media contributed no important test interference. The stimulation by selected concentrations of autoantigens as well as the amount of tested PBMCs were performed according to the previous Immunology of Diabetes Society T-cell Workshops and recommendations and also according to our own experience (9, 17, 18). To adapt this test for clinical praxis, we used a mixture of autoantigens (sometimes not enough PBMCs are available for analysis with all autoantigens), but if it was possible, we also tested autoantigens separately (19).

The real benefit of this method could lie in the possibility to see the whole spectrum of cytokines as a unique combination with typical signs for each group, to observe and to analyze the reaction in the whole complex, and then to define the 'characteristic patterns' for each group and 'risk patterns' for individuals. We believe that for predicting the risk of T1D or for monitoring the efficacy of immunomodulation therapy, it is not so important to determine the levels of individual cytokines as to know how the cytokines cooperate together.

On the basis of our results, we were rather surprised that there were such significant differences even in the basal levels as we expected to see the changes mainly after stimulation. Upon this fact, we suggest that even the basal cytokine production and 'basal cytokine pattern' should be considered. In general, we could see the higher basal levels of all cytokines within the control group when compared with the all groups. In control group after stimulation, IL-2, IL-6, Th2 cytokines, and IFN-γ showed a tendency to decrease,
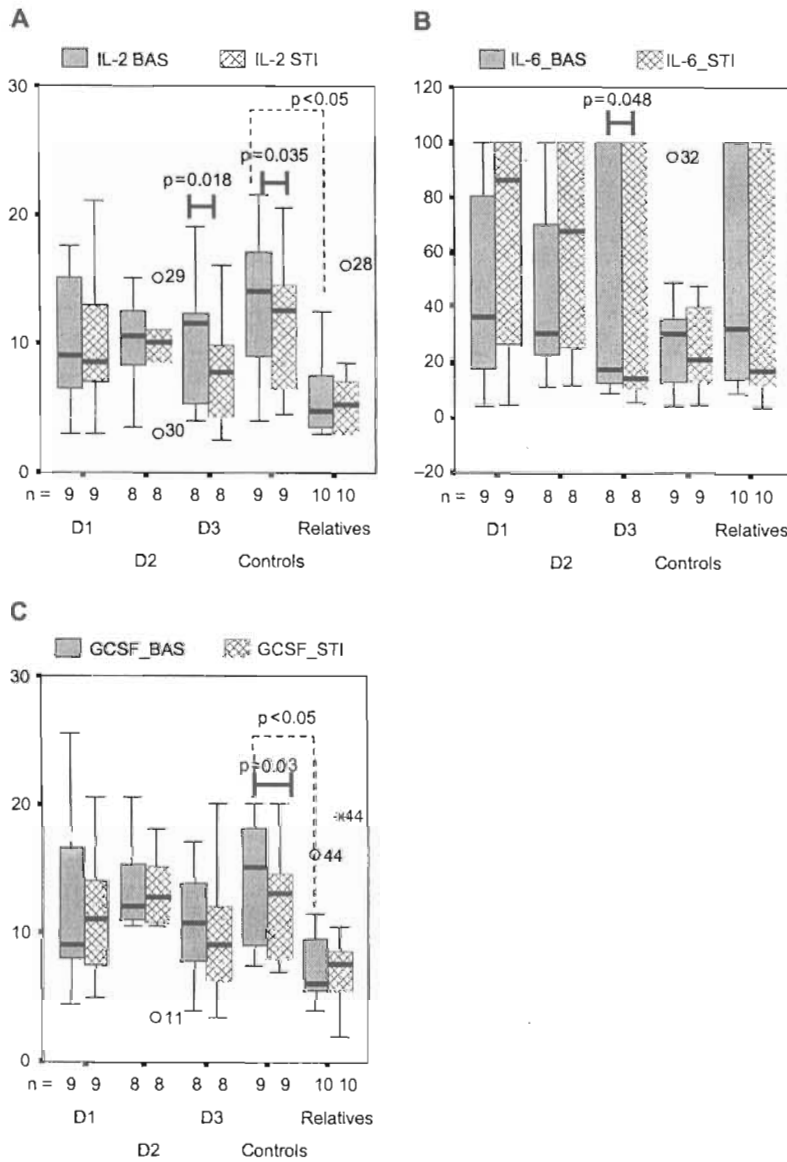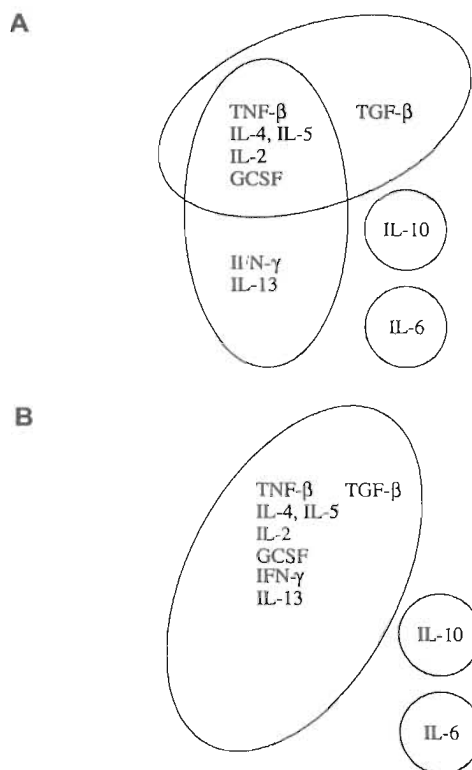
Fig. 4. IL-2, IL-6, and GCSF cytokines in all groups together. (A) IL-2. (B) IL-6. (C) GCSF. Results are displayed in percentage of spot intensity (basal and stimulated) and statistical significance is pointed. Data are expressed as median + range. Extreme values are shown. BAS, basal production; D1, type 1 diabetes patients at the time of diagnosis; D2, type 1 diabetes patients at 6 months later; D3, type 1 diabetes patients at 12 months later; GCSF, granulocyte colony-stimulating factor; IL, interleukin; STI, stimulation with autoantigens mixture. * indicates extreme values.

while the TGF-β showed a tendency to increase. Only some of these findings were significant. The most variable production of cytokines was within the group of relatives. The T1D patient groups at D1, D2 and D3 reacted in different way, however, without any 'specific pattern'. Only in D3, a tendency to decrease the inflammatory response by decrease in IL-6, IL-2, Th1, and Th2 cytokines but even in TGF-β was observed.

Karlsson Faresjo et al. showed that spontaneous and antigen-induced expression and secretion of cytokines (IFN-γ, IL-4, IL-10, and IL-13) is low at

the diagnosis of T1D (12). During the first month after diagnosis, one diabetogenic autoantigen (GAD65 a.a. 247–279) caused an increased ratio of IFN-γ/IL-4 messenger RNA expression and increased secretion of IFN-γ (12). The same authors showed that high-risk relatives had a high spontaneous ratio of IFN-γ/IL-4 compared with diabetic children as well as healthy controls. However, this spontaneous production decreased after stimulation with peptides of GAD65 and insulin and in contrast to an increased secretion of IL-4 (13). Arif et al. also showed that the quality of autoreactive T cells in patients with
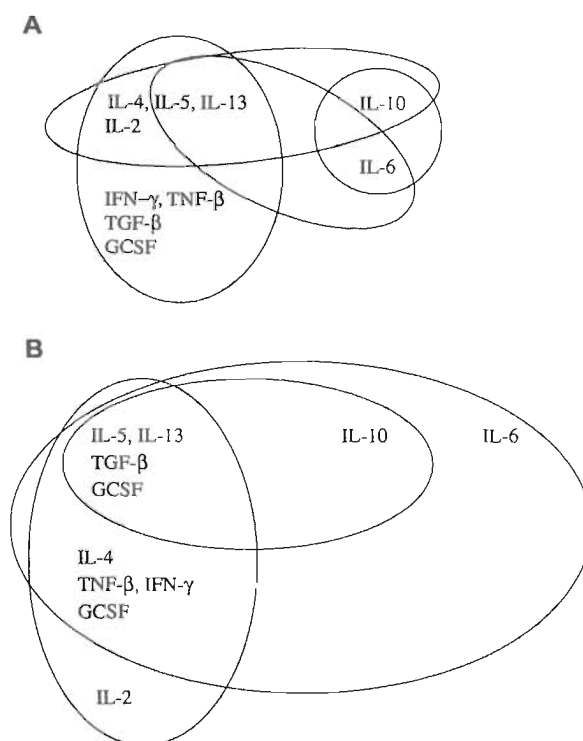
A

B

Fig. 5. (A) T1D patients: correlations in cytokine basal production. (B) T1D patients: correlations in stimulated cytokine production. Cytokines that produced was in correlation are displayed. In T1D patients correlations were *de facto* same for basal and for stimulated production within D1, D2 and D3 – so are displayed together as T1D group. GCSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; T1D, type 1 diabetes; TGF, transforming growth factor; TNF, tumor necrosis factor.

A

B

Fig. 6. (A) Controls: correlations in cytokine basal production. (B) Controls: correlations in cytokine-stimulated production. Cytokines that produced was in correlation are displayed. GCSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

T1D exhibits polarization toward a Th1 response. Furthermore, they demonstrated that the majority of non-diabetic, HLA-matched controls also manifest a response against islet peptides, but one that shows extreme T-regulatory cell bias (IL-10 secreting) (14). In general, these findings are in agreement with our results.

We also used the Spearman's analysis to reveal the relations between cytokines. There, we could see that the basal levels of cytokines in the control group were more or less in balance. The IL-2 and Th2 cytokine spectrum (IL-4, IL-5, and IL-13) strongly correlated with the Th1 cytokine spectrum (IFN-γ and TNF-β) and the Th3 cytokines (mainly TGF-β). The IL-10 production within the controls correlated with the Th2 cytokines and IL-6. The similar pattern could be seen even in the T1D patient's group, but with no correlation for Th3 cytokines with IL-13 and INF-γ (in basal production). In contrast, the situation was very different in the relatives group. The basal IL-2 and Th2 cytokine spectrum correlated with TGF-β, not with IL-10 and Th1 cytokines. The Th1 cytokine

production was not correlated with Th3 cytokines at all. After stimulation, the 'patterns' in all the groups remained rather the same. The most variable cytokines were IL-6 and IL-10. IL-6 and IL-10 did not correlate with any cytokine in the T1D group. In the group of controls, IL-6 and IL-10 correlated with the Th2 cytokines in the basal production and after stimulation they also correlated with Th1 and Th3 cytokines. In the relatives group, IL-6 correlated only with IL-10 and *vice versa*.

In the end, some cytokine preferences within the groups as well as some tendency to failure in cooperation of Th3 and Th1/Th2 response in the relatives and T1D groups could be seen. As could be expected, the most variable was the group of relatives. We suppose that there might be a correlation with genotype and antibody status, which however was not performed in this study because of the small numbers. Nevertheless, it would be interesting to focus this group to show all these relations.

We believe that the protein microarray approach and mainly quantitative multiparameter analysis can be very useful methodological tool in T1D research. However, there has to be considered the variety of data for analysis.
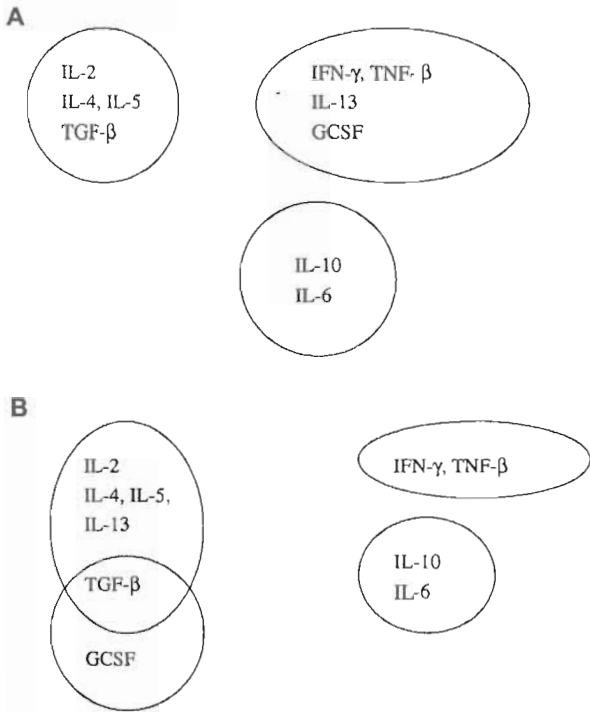
Vrabelova et al.

A

B

Fig. 7. (A) Relatives: correlations in cytokine basal production. (B) Relatives: correlations in cytokine-stimulated production. Cytokines that produced was in correlation are displayed. GCSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

Acknowledgements

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RESEARCH ARTICLE

High T-helper-1 cytokines but low T-helper-3 cytokines, inflammatory cytokines and chemokines in children with high risk of developing type 1 diabetes

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Abstract

Background Type 1 diabetes (T1D) is suggested to be of T-helper (Th)1-like origin. However, recent reports indicate a diminished interferon (IFN)-γ secretion at the onset of the disease. We hypothesize that there is a discrepancy in subsets of Th-cells between children with a high risk of developing T1D, children newly diagnosed with T1D and healthy children.

Methods Peripheral blood mononuclear cells (PBMC) were collected from children at high risk for T1D (islet cells antibodies [ICA] $\geq$20 IJDF-U), those newly diagnosed and healthy children carrying the HLA-risk gene *DQB1*0302* or *DQB1*0201* and *DQA1*0501*. Th1- (IFN-γ, tumour necrosis factor [TNF]-β, interleukin [IL]-2), Th2- (IL-4,-5,-13), Th3- (transforming growth factor [TGF-β], IL-10) and inflammatory associated cytokines (TNF-α, IL-1α,-6) and chemokines (monocyte chemoattractant protein [MCP]-1,-2,-3, Monokine unregulated by IFN-γ [MIG], Regulated on Activation, Normal T-cell Expressed and Secreted [RANTES], IL-7,-8,-15) were detected in cell-culture supernatants of PBMC, stimulated with glutamic acid decarboxylase 65 (GAD$_{65}$) and phytohaemagglutinin (PHA), by protein micro array and enzyme linked immunospot (ELISPOT) technique.

Results The Th1 cytokines IFN-γ and TNF-β, secreted both spontaneously and by GAD$_{65}$- and mitogen stimulation, were seen to a higher extent in high-risk children than in children newly diagnosed with T1D. In contrast, TNF-α and IL-6, classified as inflammatory cytokines, the chemokines RANTES, MCP-1 and IL-7 as well as the Th3 cytokines TGF-β and IL-10 were elevated in T1D children compared to high-risk children.

Conclusion High Th-1 cytokines were observed in children with high risk of developing T1D, whereas in children newly diagnosed with T1D Th3 cytokines, inflammatory cytokines and chemokines were increased. Thus, an inverse relation between Th1-like cells and markers of inflammation was shown between children with high risk and those newly diagnosed with T1D. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords type 1 diabetes; high-risk children; T-helper cells; cytokines; chemokines; protein micro array

Introduction

Type 1 diabetes (T1D) is an autoimmune disease suggested to be of T-helper (Th)1-like origin [1,2]. Cytotoxic actions of Th1-associated cytokines,

interferon (IFN)-α [3–5] and IFN-γ [6], have been observed on human islets *in vivo* in patients with recent-onset T1D. Studies of the peripheral immune system of patients with recent-onset T1D have shown significantly increased levels of interleukin (IL) -1α, -2, IFN-γ and tumour necrosis factor (TNF)-α [7,8]. The T-cell response against β-cell antigens has also shown an association with IFN-γ production in newly diagnosed T1D patients, suggesting a Th1-like phenotype of the T-cell lines [9]. We have previously observed a Th1-like dominated immune profile by high IFN-γ secretion during the pre-diabetic phase [10–12]. However, close to the onset of T1D, when only few β-cells remain, the Th1-like response vanishes and remains suppressed in newly diagnosed T1D patients [10,13–15]. Further, an immune-regulatory defect by reduced function of regulatory/suppressor T (T-reg) cells has been observed at diagnosis of T1D [16].

As part of their different effector capabilities, Th-cells express different sets of chemokine receptors, allowing them to migrate to different tissues. It has been shown that Th1-cells can be distinguished from Th2-cells by differences in chemokine synthesis [17]. Expression of monocyte chemoattractant protein (MCP)-1 in islets has been shown to increase concomitantly with the progression of insulitis in non-obese diabetic mice [18]. Monokine upregulated by IFN-γ (MIG) binds to the receptor CXCR3 on Th1-like cells, and has been found to be induced by IFN-γ in human islets [19]. Expression of Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) has been observed in pancreatic tissue from normal mice and may serve as protection from possible infectious agents because of the ability of islets to attract CCR5+ lymphocytes [20]. Interleukins such as IL-7, IL-8 and IL-15 are cytokines with a chemoattractant function. Interleukin-7 has a pivotal role in CD4+ T-cell homeostasis and stimulates the expression of CXCR4 on naive CD4+ T cells. IL-15 is a potent growth factor and activator of T cells and NK cells. IL-15 can also act as a T-cell chemoattractant and inducer of IFN-γ production by NK cells [21].

We hypothesize that there is a discrepancy in subsets of Th-cells at different stages of the disease process leading to T1D. The aim of this study was thus to investigate cytokines and chemokines in order to differentiate the subsets of Th-cells in high-risk children, children newly diagnosed with T1D and healthy children.

Materials and methods

Peripheral blood mononuclear cells from high-risk, newly diagnosed T1D and healthy children

The European Nicotinamide Diabetes Intervention Trial (ENDIT) included high-risk first-degree relatives of T1D patients receiving either nicotinamide or placebo [22]. More than 2000 first-degree relatives were screened in Sweden to identify individuals with as much as a 40% risk of developing the disease within 5 years ($\geq$20 islet cells antibodies IJDF units). Eight of 21 high-risk first-degree relatives included in Sweden were children (8–18 years, mean age 13 years, two female (F)/six male (M)) (Table 1). High-risk children were matched for age with eight children 4 days post-diagnosis of T1D (6–16 years, mean age 12 years, 4 F/4 M) and eight healthy children (7–15 years, mean age 11 years, 4 F/4 M) (Table 1). Blood samples from children with T1D were taken four days post-diagnosis at the Linköping University hospital, Linköping, Sweden. These T1D children were not participants of the *European Nicotinamide Diabetes Intervention* trial. The healthy children carried the HLA-risk gene DQB1*0302 or DQB1*0201 and DQA1*0501. None of the healthy children or their first-degree relatives had T1D or any other autoimmune disease and none had increased levels of glutamic acid decarboxylase (GADA) or tyrosinphosphatase (IA–2A) autoantibodies. Blood samples from children with T1D were taken when they visited the diabetes clinic, and blood samples from healthy children were taken at school, when possible during the morning hours to avoid time-of-day differences. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density-gradient centrifugation (Pharmacia Biotech, Sollentuna, Sweden) from sodium-heparinized venous blood samples. PBMC were cryopreserved in liquid nitrogen until use [10].

Table 1. Characteristics of high-risk, newly diagnosed T1D and healthy children

High-risk children							Newly diagnosed T1D children					Healthy children	
Age	Gender	N/P	Dev. T1D	C-peptide	GADA	IA-2A	Age	Gender	C-peptide	GADA	IA-2A	Age	Gender
8	F	N	Yes (6 mon)	0.57	183	1940	6	M	0.09	305	100	7	F
10	M	P	No	0.54	0	0	9	M	0.02	431	31	7	M
12	F	P	No	0.43	4120	6920	11	F	0.16	n.a.	n.a.	9	F
12	M	N	Yes (4 yrs)	0.46	860	3950	11	F	0.27	n.a.	n.a.	10	F
14	M	N	Yes (1 yr)	0.56	0	237	12	F	0.10	1650	1780	11	M
15	M	N	No	0.40	30 480	74	14	M	0.15	127	1010	13	M
15	M	P	No	0.05	139 440	0	16	F	0.38	n.a.	n.a.	14	M
18	M	P	No	0.98	13 560	3340	16	M	0.13	93	0	15	F

The individual characteristics of high-risk children, newly diagnosed T1D children and healthy children – age (years), gender (F = female/M = male), C-peptide (nmol/L), GAD_{65} autoantibodies (GADA, RA units/mL) and tyrosinphosphatase autoantibodies (IA-2A, RA units/mL) – plus for high-risk children treatment (N = nicotinamide/P = placebo) and development of T1D (Dev. T1D; months/years) after blood sampling. n.a. = not analysed

In vitro stimulation of PBMC

PBMC (1.5×10^6) (viability approximately 90% or more for each population) were diluted in 1500 μL AIM V research-grade serum-free medium (Gibco, Täby, Sweden) supplemented with 2 mM L-glutamine, 50 μg/L streptomycin sulphate, 10 μg/L gentamicin sulphate and 2×10^{-5} M 2-mercaptoethanol (Sigma, Stockholm, Sweden). PBMC were incubated in medium alone (spontaneous secretion) or with glutamic acid decarboxylase 65 ([GAD$_{65}$], DiamydTM, Diamyd Therapeutics AB, Stockholm, Sweden) and phytohaemagglutinin ([PHA], Sigma, Stockholm, Sweden) at a concentration of 5 μg/mL [15,23] at 37 °C, in a humidified atmosphere with 5% CO_2. The medium was harvested after 48 h stimulation and used for detection of cytokines and chemokines by protein micro array.

Protein micro array

Protein micro array was performed with a commercially available kit according to the manufacturer's instructions (RayBiotech, GA, USA), as previously shown [24]. Production of the following cytokines and chemokines was assessed: Granulocyte Colony Stimulating Factor (GCSF), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), Growth-Related Oncogene (GRO), GRO-α, IL-1α, -2, -3, -5, -6, -7, -8, -10, -13, -15, IFN-γ, MCP-1, -2, -3, MIG, RANTES, TGF-β1, TNF-α and TNF-β (kit no. H0108001). Detection was carried out using the Fuji LAS1000 imaging system. Chemiluminescent signals were analysed using the AIDA software (Advanced Image Data Analyzer 3.28, Raytest Izotopenmessgeraete, Straubenhardt, Germany). The intensity of spots (%) was calculated. Sensitivity for cytokines and chemokines (manufacturer's figures) are displayed in Table 2.

Stimulation of lymphocytes and enumeration of IL-4-secreting cells by enzyme linked immunospot (ELISPOT)

The enzyme linked immunospot (ELISPOT) technique was used for detection of low numbers of Th2-cytokine (IL-4)-secreting PBMC at the single cell level [10,11]. Aliquots of 100 000 PBMC/well were incubated in quadruplicate in medium alone (spontaneous secretion) or stimulated with GAD$_{65}$ (Diamyd), the synthetic peptide of GAD$_{65}$ a.a. 247–279 (NMYAMMIARFK MFPEVKEKGMAALPRLIAFTSE-OH) molecular weight 3823.7 (Dept of Medical and Physiological Chemistry, University of Uppsala, Sweden) and tyrosine phosphatase (IA-2, produced in *E. coli*, Åbo Akademi, Turku, Finland), all at the optimized concentration of 100 pg/mL, 10 mg/mL of ovalbumin (OVA, Sigma) and PHA at a concentration of 20 μg/mL [10]. In samples with a limited number of cells, the order of priority for stimulation with antigens was PHA, GAD$_{65}$, the GAD$_{65}$ peptide (a.a. 247–279), IA–2 and OVA.

Table 2. Cytokines & chemokines

Th1-ass. cytokines	Sensitivity (pg/mL)
IL-2	25
IFN-γ	100
TNF-β	1000

Th2-ass. cytokines	Sensitivity (pg/mL)
IL-5	1
IL-13	100

Th3-ass. cytokines	Sensitivity (pg/mL)
IL-10	10
TGF-β1	200

Chemokines	Sensitivity (pg/mL)
RANTES	2000
MCP-1	3
MCP-2	100
MCP-3	1000
MIG	1
IL-7	100
IL-8	1
IL-15	100
GRO	1000
GRO-α	1000

Inflammatory ass. cytokines	Sensitivity (pg/mL)
IL-1α	1000
IL-6	1
TNF-α	100

Sensitivity (pg/mL) of cytokines and chemokines, grouped according to association with subgroups of Th-cells.

Plates were blinded for identity to avoid any influence on the outcome of the observation. Plates were counted automatically, under manual supervision, using the AID ELISPOT Reader System (AID, Strasbourg, France). The median value of the quadruplicates was calculated for each stimulation and for spontaneous secretion. As a negative control, some wells on each plate were incubated exclusively with culture medium, without cells but otherwise treated as the other wells, whereas stimulation with PHA was used as a positive control. The laboratory of Faresjö participated in the first ELISPOT workshop as one of the core laboratories, and our Mabtech assay was judged to be sensitive and reproducible [25].

Autoantibodies

GADA and IA–2A were detected by radio immune assay, using *in vitro* transcribed and translated human ^{35}S–GAD$_{65}$ or ^{35}S–IA–2 as label [26]. For T1D and healthy children, the cut-off for positivity at the 98th percentile of 1-year-old Swedish children from the general population was >104 relative units/mL for GADA (N = 4400) and >36 relative units/mL for IA–2A (N = 4400). For high-risk children, the cut-off for positivity at the 98th percentile of 2–3-year-old Swedish children from the general population was >105.1 RA units/mL,

corresponding to 36.6 WHO units, for GADA and >30 RA units/mL (N = 4258), corresponding to 28.5 WHO units, for IA–2A (N = 4461).

C-peptide

C-peptide was determined with a radioimmunoassay technique based on the original assay developed by Heding [27]. The detection limit for the assay is 0.03 nmol/L, and the reference value among fasting healthy children and adolescents is 0.18–0.63 nmol/L.

Statistics

As the expression and secretion of immunological markers was not normally distributed (even after logarithmic transformation), two groups were compared by Mann–Whitney U-test and three or more groups using the Kruskal–Wallis test for unpaired observations. Post hoc comparisons of grouped immunological parameters (e.g. sum scores were calculated) were analysed with Wilcoxon signed-ranks test, with adjusted degrees of freedom to compensate for multiple comparisons. Spearman's rank correlation was used when comparing paired non-parametric variables. A probability level of <0.05 was considered to be statistically significant. Calculations were performed using the statistical package StatView 5.0.1 for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA).

Ethics

The study was approved by the Research Ethics Committee of the Faculty of Health Sciences, Linköping University.

Results

Th1-associated cytokines (IFN-γ, TNF-β, IL-2)

The typical Th1-like cytokine IFN-γ was seen to a higher extent spontaneously in high-risk children than in either diabetic ($p < 0.05$) or healthy ($p < 0.05$) children (Figure 1(a)). Furthermore, spontaneous secretion of TNF-β was found to be higher in high-risk ($p = 0.1$) and diabetic ($p < 0.05$) children than in healthy children (Figure 1(b)) and correlated to spontaneous secretion of IFN-γ ($r = 0.56$, $p < 0.01$).

GAD$_{65}$-induced IFN-γ was higher in the group of high-risk children than in diabetic ($p = 0.06$) or healthy ($p = 0.01$) children (Figure 1(a)). Further, TNF-β induced by GAD$_{65}$ was found to a higher extent in high-risk ($p < 0.05$) and T1D ($p < 0.01$) children than in healthy children (Figure 1(b)) and correlated positively with GAD$_{65}$-induced secretion of IFN-γ ($r = 0.63$, $p < 0.05$).

High-risk children showed a very high IFN-γ response by stimulation with PHA compared to T1D children ($p < 0.01$). Further, T1D children had a lower PHA-induced IFN-γ response than healthy children ($p = 0.09$).
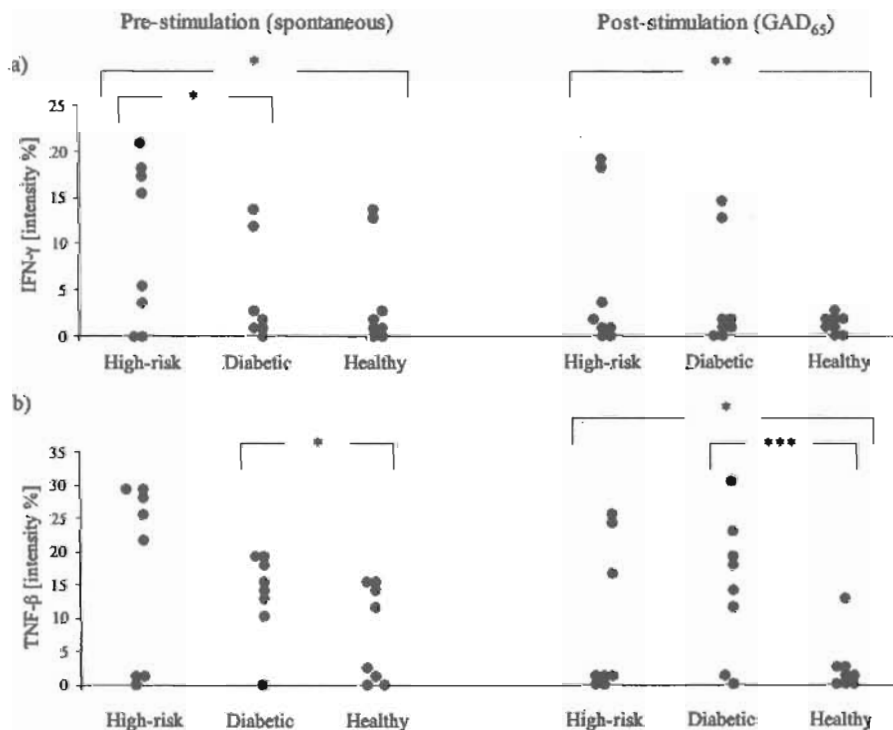

Figure 1. Secretion of the Th1-associated cytokines IFN-γ (a) and TNF-β (b) pre-stimulation (spontaneous) as well as post-stimulation (GAD65-induced). * $p < 0.05$, ** $p = 0.01$, *** $p < 0.01$

PHA induced secretion of TNF-β in all children without any differences between groups.

IL-2 was rarely detected in any of the studied children.

Th2-associated cytokines (IL-4, -5, -13)

High-risk children secreted higher levels of IL-5 by PHA stimulation compared to both diabetic ($p < 0.01$) and healthy children ($p < 0.01$), whereas PHA-induced IL-4 secretion was correlated to IL-13 in all children ($r = 0.49$, $p < 0.05$).

Th3-associated cytokines (TGF-β IL-10)

Spontaneous and GAD$_{65}$-induced TGF-β was significantly higher in newly diagnosed T1D children compared to high-risk ($p = 0.05$ and $p < 0.05$ respectively) and healthy children ($p < 0.05$ and $p < 0.01$ respectively) (Figure 2). High-risk children secreted less IL-10 both spontaneously compared to T1D children ($p = 0.06$) and by stimulation with PHA compared to healthy children ($p < 0.05$).

Chemokines (RANTES, MCP-1, -2, -3, MIG, IL-7, -8, -15, GRO, GRO-α)

The levels of both spontaneously secreted (Figure 3) and GAD$_{65}$-induced RANTES were significantly lower among high-risk children than healthy children ($p < 0.05$ and $p = 0.05$ respectively) and tended to compare with diabetic children ($p = 0.09$ and $p = 0.1$ respectively). Further, PHA-induced secretion of RANTES was significantly lower in high-risk children compared to diabetic ($p < 0.001$) and healthy ($p < 0.01$) children (Figure 3).

GAD$_{65}$-induced MCP-1 secretion was significantly higher in diabetic children than in high-risk ($p < 0.05$) or healthy ($p < 0.05$) children (Figure 4). Further, both diabetic ($p = 0.01$) and healthy ($p = 0.01$) children secreted higher levels of MCP-1 induced by PHA than high-risk children. Thus, spontaneous ($r = 0.78$, $p < 0.05$) as well as GAD$_{65}$ ($p = 0.51$, $p = 0.06$) or PHA ($r = 0.55$, $p = 0.01$)-induced MCP-1 was positively correlated to RANTES. No significant differences in secretion of MCP-2 and MCP-3 were observed between the groups of children studied.

MIG, induced by PHA, tended to be higher among high-risk children than in either diabetic or healthy children ($p = 0.06$ and $p = 0.06$ respectively) and was positively
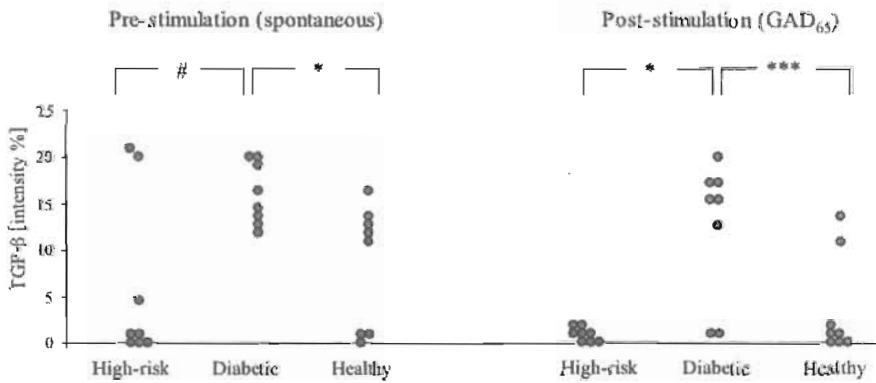

Figure 2. Secretion of the Th3-associated cytokine TGF-β pre-stimulation (spontaneous) and post-stimulation (GAD65-induced). # $p = 0.05$, * $p < 0.05$, *** $p < 0.01$
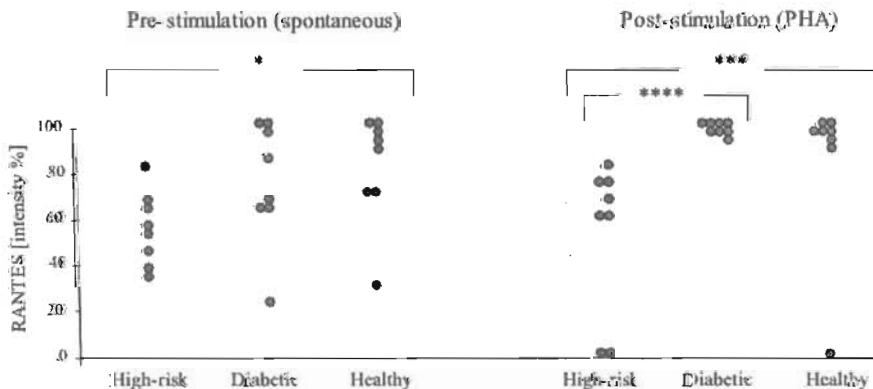

Figure 3. Secretion of the chemokine RANTES pre-stimulation (spontaneous) and post-stimulation (PHA-induced). * $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$
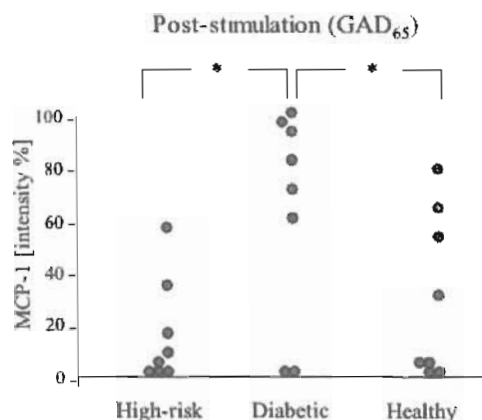
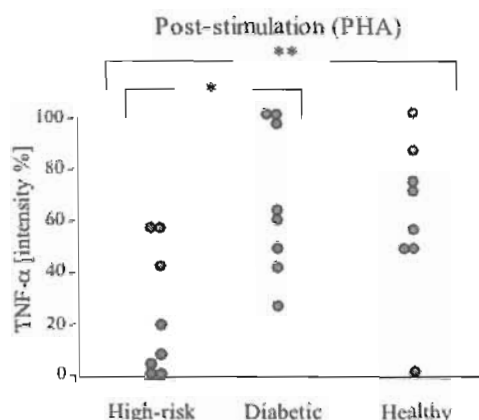Figure 4. Secretion of the chemokine MCP-1 induced by GAD65. $p < 0.05$

Figure 5. Secretion of the inflammatory cytokine TNF-α induced by PHA. * $p < 0.05$, ** $p = 0.01$

correlated to PHA-induced secretion of IFN-γ in diabetic children ($r = 0.85$, $p < 0.05$).

PHA-induced secretion of IL-7 tended to be higher in T1D children than high-risk children ($p = 0.06$). Spontaneously secreted RANTES was correlated to IL-7 among high-risk children ($r = 0.73$, $p = 0.05$), whereas both GAD$_{65}$- ($r = 0.44$, $p = 0.1$) and PHA-induced ($r = 0.41$, $p = 0.06$) IL-7 secretion was correlated to RANTES in all children. Spontaneous IL-7 secretion was inversely correlated to secretion of IL-4 in both high-risk and diabetic children ($r = -0.45$, $p < 0.05$).

A high level of IL-8 was detected in all children without differences between groups (data not shown), in contrast to IL-15, which was secreted only to a low extent in a few samples, equally between the groups.

Stimulation with PHA induced lower secretion of GRO in diabetic children compared to healthy children ($p < 0.05$), whereas spontaneously secreted GRO-α tended to be higher in children with T1D compared to healthy children ($p = 0.06$). GAD$_{65}$ induced equal secretion of GRO and GRO-α in all studied subjects.

Inflammatory associated cytokines (TNF-α, IL-1α, -6)

Spontaneous secretion of TNF-α ($p < 0.05$) as well as GAD$_{65}$- ($p = 0.09$) or PHA-induced TNF-α ($p < 0.05$) (Figure 5) was found to a higher extent in T1D children than in high-risk children. PHA-induced TNF-α was also found to a higher extent in healthy children than in high-risk children ($p = 0.01$) (Figure 5).

GAD$_{65}$-induced IL-6 tended to be higher in T1D children than in healthy children ($p = 0.08$). Secretion of IL-6 was correlated to TNF-α after stimulation with either GAD$_{65}$ ($r = 0.53$, $p = 0.06$) or PHA ($r = 0.55$, $p = 0.01$). No discrepancy was observed in secretion of IL-1α between the three studied groups.

Post hoc comparisons of grouped immunological parameters

Spontaneous secretion of following immunological parameters differed significantly between high-risk, T1D and healthy children: Th1 cytokines (IFN-γ, TNF-β), Th3 cytokine (TGF-β), inflammatory cytokine (TNF-α) and chemokine (RANTES). These Th1 cytokines were significantly lower in T1D ($p < 0.05$) and higher in high-risk children ($p < 0.05$), compared to the Th3 cytokine, inflammatory cytokine and chemokine (Figure 6(a)). Comparing all detectable immunological parameters still showed that Th1 cytokines (IFN-γ, TNF-β) were significantly lower among T1D children compared to Th3 cytokines (TGF-β, IL-10), inflammatory cytokines (TNF-α, IL-6) and chemokines (RANTES, MCP-1, MIG) ($p = 0.01$, data not shown).

GAD$_{65}$-induced secretion of the following immunological parameters differed significantly between high-risk, T1D and healthy children: Th1 cytokines (IFN-γ, TNF-β), Th3 cytokine (TGF-β), inflammatory cytokine (TNF-α) and chemokines (RANTES, MCP-1). These Th1 cytokines were significantly lower in T1D compared to the Th3 cytokine, inflammatory cytokine and chemokines ($p < 0.05$, Figure 6(b)).

PHA-induced secretion of the following immunological parameters differed significantly between high-risk, T1D and healthy children: Th1 cytokine (IFN-γ), Th3 cytokine (IL-10), inflammatory cytokine (TNF-α) and chemokines (RANTES, MCP-1). The Th1 cytokine was significantly lower in T1D ($p < 0.05$) and higher in high-risk children ($p < 0.05$), compared to the Th3 cytokine, inflammatory cytokine and chemokines. Comparing all detectable immunological parameters still showed that Th1 cytokines (IFN-γ, TNF-β) were significantly lower among T1D children ($p < 0.05$) and higher in high-risk children ($p < 0.05$), compared to the Th3 cytokines (TGF-β, IL-10), inflammatory cytokines (TNF-α, IL-6) and chemokines (RANTES, MCP-1, MIG) (data not shown).
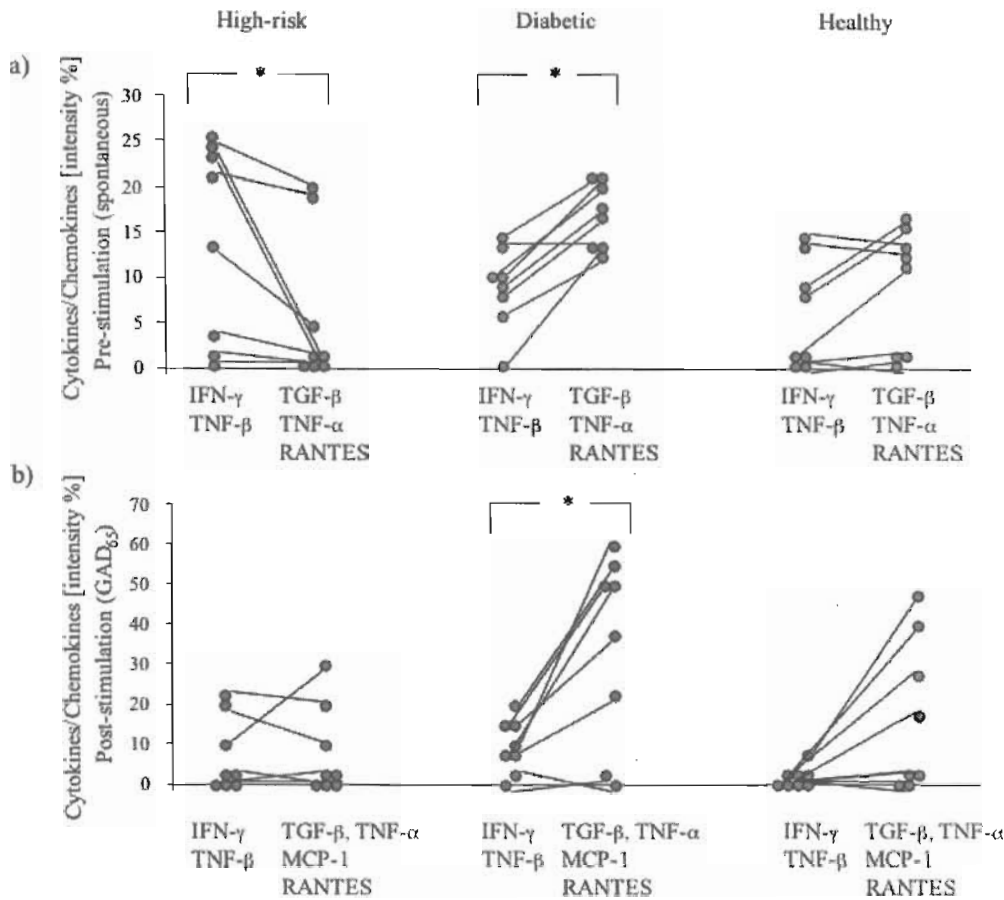
Figure 6. Post hoc comparison of spontaneous secretion of Th1 (IFN-γ, TNF-β) *versus* Th3 (TGF-β)/inflammatory marker (TNF-α)/chemokine (RANTES) (a) and GAD$_{65}$-induced comparison of Th1 (IFN-γ, TNF-β) *versus* Th3 (TGF-β)/inflammatory marker (TNF-α)/chemokines (MCP-1, RANTES) (b) on individual basis in high-risk, T1D and healthy children. * $p < 0.05$

Immunological markers in relation to C-peptide

The Th1-associated cytokines IFN-γ ($r = 0.66, p = 0.01$) and TNF-β ($r = 0.47, p = 0.07$), secreted spontaneously, correlated to C-peptide in both high-risk and T1D children, whereas the Th2-associated cytokine IL-13 correlated to C-peptide only in the high-risk children ($r = 0.59, p = 0.1$). Exclusively in newly diagnosed T1D children, spontaneously secreted IL-7 ($r = 0.73, p = 0.05$) (Figure 7) and IL-6 ($r = 0.67, p = 0.08$) tended to correlate with secretion of C-peptide.

Immunological markers not detected

GCSF, GM-CSF and IL-3 were not found in any child in the three studied groups.

Discussion

T1D has been associated with increased concentrations of Th1 cytokines, for example, IFN-α, IFN-γ, IL-2 and

Figure 7. Relation between C-peptide and spontaneous secretion of IL-7 ($r = 0.73, p = 0.05$)

TNF-β. Therefore, it has been suggested that T1D is a Th1-associated autoimmune disease. We found a high spontaneous secretion of IFN-γ and TNF-β in children with a high risk of developing T1D. The Th1-like profile was significantly higher in high-risk children than in newly diagnosed T1D children. This agrees with our previous observation of a Th1-like dominated immune profile by high IFN-γ secretion during the pre-diabetic

phase [10–12]. In high-risk individuals, the autoantigens GAD$_{65}$, IA–2 and heat shock protein as well as mitogen stimulation are found to induce prominent IFN-γ secretion [12]. Here, we observed that newly diagnosed T1D children secreted less autoantigen- and mitogen-induced IFN-γ and TNF-β than high-risk children. We and others have previously shown that close to the onset of T1D, when only few β-cells remain, the Th1-like response vanishes and remains suppressed in newly diagnosed T1D patients [12–15,28,29]. This observation agrees with previous investigations where T-cell reactivity to GAD$_{65}$ (a.a. 247–266 and 260–279) is shown to decrease at diabetes onset [30–32]. Diminished secretion of IFN-γ in newly diagnosed T1D patients has also been observed from in vitro mitogen stimulation [33,34]. Further, a disrupted ability to suppress T-cell proliferation during in vitro co-cultures of CD4 + CD25 + T cells in patients with recent-onset adult T1D has been found despite normal levels of this cell population [16]. This result is in line with our observation of a decreased secretion of the Th3-associated cytokines TGF-β and IL-10 in high-risk children, some of whom later developed T1D.

Compared to newly diagnosed T1D children, high-risk individuals responded with high secretion of IL-5 from mitogen stimulation. We have previously shown that healthy high-risk individuals seem to have an ability to change a Th1-like immune deviation into a more protective Th2-like response in the presence of diabetes-associated autoantigens [10,12]. Further, Th2 cytokines, for example, IL-5 and IL-13, show no relationship to multiple autoantibodies (GADA, IA–2A and islet cells antibodies [35].

There is increasing evidence that chemokines can play a role in the pathogenesis of T1D. It has been shown that Th1 cells can be distinguished from Th2 cells by their differences in chemokine synthesis [17]. Th1 cells have been associated with CCR5 and CXCR3, receptors for the chemokines RANTES and MIG, respectively. Delayed-type hypersensitivity-containing granulomas contain high levels of TNF-α and IFN-γ, and the ability of these cytokines to induce RANTES has been demonstrated in endothelial cells [36]. At diagnosis of T1D, but before insulin treatment, a reduced expression of the receptors CCR5 and CXCR3 has been observed [14]. We found that high-risk children, some of whom later developed T1D, secreted lower RANTES, both spontaneously and by GAD$_{65}$- and PHA stimulation compared to already diagnosed T1D children receiving insulin treatment. Recently, it has also been observed that two functional polymorphisms in the CCR5 gene cause decreased expression of the RANTES receptor on immunocompetent cells and are associated with increased risk of diabetic nephropathy in T1D [37].

Interleukin-7 has a pivotal role in CD4 T-cell homeostasis stimulating the expression of CXCR4 on naive CD4 T cells. We and others [38] have found a correlation between IL-7 and RANTES. This can possibly be explained by the fact that RANTES increase co-localization of surface molecules CD4 and CXCR4 on CD4 T cells [39].

The chemokine MCP-1 plays an important role in the development of local inflammation by attracting mono-cytes and lymphocytes [40], and expression of MCP-1 in islets has been shown to increase concomitantly with the progression of insulitis in non-obese diabetic mice [18]. We found MCP-1 induced by the autoantigen GAD$_{65}$ to a higher extent in newly diagnosed T1D children than in either high-risk or healthy children. Even though MCP-1 serum levels tend only to be higher in patients compared to control subjects [14], a high basal MCP-1 production by human islets is shown to correlate with poor clinical outcome following islet transplantation in T1D patients [41].

Both IFN-γ and MIG were high among high-risk children. MIG that binds to the receptor CXCR3 has been found to be induced by IFN-γ in human islets [19], explaining the positive correlation observed between MIG and IFN-γ in our cohort of newly diagnosed T1D children. MIG attracts monocytes and activated Th1- and NK cells. Thus, production of MIG by human islet cells can contribute to mononuclear, NK- and Th1 cell homing in early insulitis [19]. Human islet cells exposed to IFN-γ and also IL-1β show secretion of MIG and IL-15 [19]. In line with previous studies of human islets and other cell types, we found a low concentration of IL-15 from peripheral mononuclear cells [19,42]. However, picomolar amounts of IL-15 have been shown to be effective in maintaining NK cell survival, suggesting that even very low concentrations of this chemokine can be physiologically relevant.

TNF-α, classified as an inflammatory cytokine, is shown to induce IL-6. In type 2 diabetes (T2D), IL-6 is argued to be an important regulator of the acute phase response associated with insulin-resistant states [43], even though an independent role of IL-6 in T1D is still not proven [44]. Recently, it was shown that monocyte IL-6 in the resting state and IL-1β in activated monocytes were elevated in T1D patients (duration longer than one year) compared with control subjects [45]. In our cohort, both TNF-α and IL-6 were found to a higher extent in T1D children than in either high-risk or healthy children. Involvement of TNF-α in the damage of the insulin-producing cells has been observed in mice infected with coxsackie B4 and A7 viruses, indicating an immunity-related inflammatory process [46]. Further, it has been suggested that TNF-α plays a direct role in the metabolic syndrome, since T2D patients show a high concentration of TNF-α in plasma [47]. TNF-α, shown to impair insulin-stimulated rates of glucose storage in cultured human muscle cells, may indicate an effect on insulin signalling [48]. We speculate that the correlation between C-peptide and IL-6 as well as IL-7, observed only in children with recent-onset T1D, is a sign of an ongoing destruction of the remaining insulin-producing β-cells. This finding is in contrast to the correlation observed between C-peptide and the Th2 cytokine IL-13 seen exclusively in still healthy high-risk children. In fact, our previous finding of a diminished IFN-γ secretion associated with fasting C-peptide levels in T1D children suggests that factors related to β-cell function in T1D may modify T-cell function [28]. Thus,

T-cell responses detected at or after diagnosis may not reflect the pathogenic process leading to T1D.

Taken together, these findings show that protein micro array can be used for screening of possible immunological markers involved in the autoimmune process against the insulin-producing β-cells. This technique does not deliver exact concentrations but indicates higher or lower concentrations of secreted cytokines and chemokines. However, low secreted cytokines, especially IL-4, is better detected at low antigen concentration stimulation at the single level with the sensitive ELISPOT technique [10]. Thus, protein micro array is useful for screening and comparisons between, for example, children with high risk and those already diagnosed with T1D.

In conclusion, cytokines secreted by Th1-like cells (IFN-γ and TNF-β) were more pronounced in high-risk children, whereas in newly diagnosed T1D children, markers of inflammation (TNF-α and IL-6), chemokines associated with destructive insulitis (RANTES, MCP-1 and IL-7) and Th3 cytokines (TGF-β and IL-10) were elevated. Thus, an inverse relation observed between Th1-like cells and markers of inflammation was shown between children with high risk and those newly diagnosed with T1D. We speculate that the immunological process led by Th1-like cells precedes the clinical onset, followed by an increased activation of inflammatory cytokines and chemokines involved in the destruction of the remaining insulin-producing β-cells, but this needs to be confirmed in larger longitudinal cohorts before any conclusion can be drawn.

Acknowledgement

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SHORT COMMUNICATION

Anti-GAD65 reactive peripheral blood mononuclear cells in the pathogenesis of cystic fibrosis related diabetes mellitus

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Abstract

Objective: A role of autoreactive T cells for type 1 diabetes pathogenesis is considered crucial. In our pilot study we addressed if autoreactive mononuclear cells are present also in peripheral blood of patients with other specific forms of diabetes as cystic fibrosis related diabetes (CFRD).

Methods: Cellular immune responses to a known β-cell autoantigen (GAD65 and GAD65 derived peptides) were analysed by ELISPOT (IFN-γ) and by protein microarray analysis in four patients suffering from CFRD, in four cystic fibrosis (CF) patients without diabetes, in eight type 1 diabetes patients (without CF) and in four healthy controls.

Results: Response to the autoantigen GAD65 (protein and peptides) was observed in 7/8 patients suffering from CF and in all type 1 diabetes patients. Post-stimulation production of Th1 cytokines (IFN-γ, TNF-β) was observed in 2/4 CFRD, 1/4 CF patients and in 7/8 type 1 diabetes patients. All these patients carry prodiabetogenic HLA-DQ genotype. Th2- and Th3 type of cytokine pattern was observed in 2/4 CF patients. Production of IL-8 was observed in the third CFRD as well as in the third CF patient and in 1/8 type 1 diabetes patient and borderline production of this chemokine was also observed in 2/4 healthy controls. No reaction was observed in the other 2/4 healthy controls and in the fourth CFRD patient who carried a strongly protective genotype and did not produce autoantibodies. The most potent peptide of GAD65 was amino acids 509–528.

Conclusions: We consider our observations as a sign of a reaction directed against the self-antigen GAD65 that are closely connected to type 1 diabetes. In CF patients who do not develop diabetes autoreactive mechanisms are very probably efficiently suppressed by immune self-tolerance mechanisms. CFRD patients are a heterogenic group. To disclose those who may display features of autoimmune diabetes could have an impact for their therapy and prognosis.

Keywords: *Diabetes, cystic fibrosis, autoimmunity, cellular response, cytokines*

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disorder affecting the Caucasian population with an incidence of 1:2500. The current improvement in CF prognosis has resulted in co-morbidities such as diabetes mellitus, which has a great clinical impact (diabetes prevalence in CF patients is 12–34%) [1–4].

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Older age and pancreatic insufficiency are important factors contributing to the development of CFRD (cystic fibrosis related diabetes). Severe pulmonary infection, use of corticosteroids, supplemental nutrition as well as increased insulin clearance and increased glucose uptake from the gut in CF may also contribute [3,5–9].

A role of T cells and HLA-restricted self antigen recognition for type 1 diabetes pathogenesis is considered crucial [10–11] but nobody has addressed if autoreactive mononuclear cells are present in peripheral blood of patients with other specific forms of diabetes such as CFRD.

To assess cell responsiveness to a known diabetogenic autoantigen (GAD65 and GAD65 derived peptides) we studied post-stimulative cytokine release by protein microarray and enzyme linked immunospot (ELISPOT) [10–12].

Materials and methods

Subjects

Four patients suffering from CFRD (group A), four CF patients without diabetes (group B), four age, sex and HLA-matched healthy blood donors (group C) and eight type 1 diabetes patients (without CF; group

D) were analysed in our pilot study. CF patients data are summarised in Table I. Patients were selected from CF and CFRD patients who are treated in the Internal and Paediatric Outpatient Departments of the University Hospital Motol in Prague. None of the patients were treated with corticosteroids or any other diabetogenic drugs. All CFRD patients were insulinopenic (first phase of insulin response—FPIR—was under 1st percentile) and on insulin therapy.

Type 1 diabetes patients (group D) were selected from patients who were diagnosed in the Paediatric Department of the University Hospital Motol in Prague. These patients were matched for age and sex. All patients were carrying type 1 diabetes risk HLA-DQ genotype and by the time of type 1 diabetes diagnosis they were anti-GAD65 autoantibody positive. Their samples were collected 7 days after diagnosis.

Complete HLA-DQA1 and DQB1 genotyping were carried out by polymerase chain reactions with sequence-specific primers. CF and healthy subjects who were enrolled to this pilot study was selected to be of distinct HLA-DQ alleles (prodiabetogenic versus protective ones). Autoantibodies against GAD65 were measured by RIA (Solupharm kit, the Czech Republic) and positivity was considered above 1 IU/ml (above 99th percentile). Informed consent was obtained from all tested subjects.

Table I. Patients suffering from CF—characteristics and results of protein microarray.

Patient	Age (years), sex	CFRD (yes/no) If yes + diabetes duration (months)	Anti GAD auto-antibodies (positive/negative)	HLA-DQ genotype	Results of protein microarray		
					Strong positivity	Medium or low positivity	Strongest stimulator
1	22F	Yes/40	Positive	DQA1*03/05	IL-8, MCP-1	TNFβ, IFNγ	GAD65-2 similar to GAD65
				DQB1*02/0302			
2	22M	Yes/51	Positive	DQA1*02/03 DQB1*02/0302	IL-8	TNFβ, IFNγ	GAD65-2
3	23M	Yes/71	Negative	DQA1*01/02 DQB1*0303/0602	IL-8	--	GAD65-2
4	18M	Yes/32	Negative	DQA1*01/02 DQB1*0303/0602	--	--	--
5	15F	No	Negative	DQA1*01/01	IL6, IL 10, TGFβ1	IL8, GRO, MIG, MCP1, RANTES	GAD65
				DQB1*0602/0602			
6	11F	No	Negative	DQA1*03/05 DQB1*02/0302	TNFβ	IL8, IFNγ	GAD65-2
7	23F	No	Negative	DQA1*01/05	IL-8	MCP1, RANTES	GAD65
				DQB1*0301/0602			
8	13M	No	Negative	DQA1*02/03	IL6, IL10, TGFβ1	IL8, GROa, MCP1, MCP2, RANTES	GAD65-2
				DQB1*02/0302			

Strong positivity in protein microarray means signal with at least 50% of intensity of positive control which is an integral part of the commercial kit.

F, female; M, male.

Antigens

The following (auto)antigens were used: 10 μg/ml recombinant human Glutamic Acid Decarboxylase 65 (GAD, Diamyd Diagnostics AB, Stockholm, Sweden); 1 μg/ml each of the synthetic GAD65-peptides: Amino acids 247–279 (NMYAMMIARFKMFPEVKEKG-MAALPRLIAFTSEE-OH) molecular weight 3823.7 (marked GAD65-1); a.a.509–528 (IPPSLRTLEDN-EERMSRLSK-OH) molecular weight 2371.7, (GAD65-2) and a.a.524–543 (SRLSKVAPVIKARM-MEYGTT-OH) molecular weight 2238.7 (GAD65-3; Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden) and 10 μg/ml PHA (Sigma, St. Louis, USA).

Assays

Peripheral blood mononuclear cells (PBMC) were prepared and IFN-gamma ELISPOT was done according to current IDS (the Immunology of Diabetes Society) recommendation [11]. ELISPOT was performed by a commercially available IFN-gamma ELISPOT kit (Diaclone, USA).

Protein microarray: 1×10^6 thawed PBMC (twice washed in Earle's Balanced Salt Solution with 20% foetal calf serum (FCS); Sigma) per well were cultured at 37°C, 5% CO_2 in 1 ml of RPMI-1640 Medium supplemented with 10% FCS, L-glutamine (10 μl/ml, 200 mM L-glutamine) and penicillin-streptomycin (1 μl/ml PNC and 1 μg/l streptomycin; Sigma). Medium was harvested after 48 h stimulation and used for protein microarray analysis which was done by a commercially available kit according to instructions by the manufacturer (RayBiotech, Norcross, USA). Production of the following cytokines and chemokines was assessed: Granulocyte colony stimulating factor (GCSF), Granulocyte–Macrophage colony stimulating factor (GM-CSF), growth related oncogene (GRO), GRO-α, interleukin (IL) 1α,-2,-3,-5,-6,-7,-8,-10,-13,-15, interferon (IFN)-γ, Monocyte chemoattractant protein (MCP-1), MCP-2, MCP-3, Monokine induced by IFN-γ (MIG), regulated on activation, normal T-cell expressed and secreted (RANTES), transforming growth factor (TGF-β1), tumour necrosis factor TNF-α and TNF-β (kit No H0108001). Detection was done using the Fuji LAS1000 imaging system. Chemiluminescent signals were analysed using the AIDA software (Advanced Image Data Analyzer, 3.28; Raytest Izotopenmessgeraete, Straubenhardt, Germany). Detection limits for cytokines which production was observed (according to the manufacturer) are displayed in Table II. Strong positivity means signal with at least 50% of intensity of positive control which is an integral part of the commercial kit (see Figure 1).

Stimulated PBMC (6×10^5 cells per well) were used for ELISPOT. As a negative control, some wells on each plate were incubated exclusively with culture medium and FCS, without cells but otherwise treated as the other wells, whereas stimulation with PHA was used as a positive control. All tests were performed at least in duplicity. A positive response was scored when the number of spots were greater than the mean + 3 SD of the controls [12].

Results

Response to GAD65 was observed in 7/8 CF patients (Table I). Post-stimulation production of Th1 cytokines (IFNγ, TNFβ) was observed in 2/4 CFRD (group A) and in 1/4 CF patients (group B), all carrying the prodiabetogenic HLA-DQ genotype. IFNγ production was confirmed with ELISPOT in all persons.

Th2- and Th3 type cytokines were observed in 2/4 CF (group B) but in none of CFRD patients (group A).

One patient from group A (CFRD) and one from group B (CF only) as well as two healthy controls (group C) produce after specific stimulation chemokines (mainly IL-8) whereas in the two healthy controls only borderline IL-8 production was observed.

No reaction was registered in 2/4 healthy controls (group C) and in the fourth CFRD patient (group A) carrying a strongly protective genotype and without production of autoantibodies against GAD65.

The most potent epitope of GAD65 was a.a. 509–528. Strong IFNγ (analysed by protein microarray and confirmed with ELISPOT), IL-6, IL-8, and GRO mitogenic responses to PHA (control stimulator) were consistently high in all patients and controls. In contrast, few IFNγ spots per well (0–2 spots/well by ELISPOT) were found in negative control wells (medium alone) indicating low non-specific IFNγ production.

These data were compared to results achieved from type 1 diabetic patients (group D). Post-stimulation production of Th1 cytokines (IFN-γ, TNF-β) was observed in 7/8 type 1 diabetes patients. IFN-γ post-stimulation production had medium intensity, in the case of TNF-β we observed strong post-stimulation production. We detected also medium intensity production of other cytokines and chemokines (IL-1α, IL-2, IL-3, IL-6, IL-8, GCSF, MCP-2, MCP-3 and MIG) in these patients. One patient from group D produced after specific stimulation only chemokines (mainly IL-8). Among type 1 diabetic patients the most potent epitope of GAD65 was as well a.a. 509–528.

Discussion

Clinical course of CFRD differs from that in type 1 diabetes. CFRD patients rarely present in ketoacidosis and family history of diabetes is less common in CFRD than in either type 1 or type 2 diabetes [2]. CFRD is primarily an insulinopenic condition and there is, however, poor correlation between

Table II.   Detection limits for cytokines (chemokines) which were detected (according to the manufacturer).

Cytokine (chemokine)	Sensitivity (pg/ml)
GRO and GROa	1000
IFN-γ	100
IL-6	1
IL-8	1
IL-10	10
MCP-1	3
MCP-2	100
MIG	1
RANTES	2000
TGFβ1	200
TNFβ	1000

the number of islets lost and the degree of pancreatic fibrosis, implying that CFRD could not be simply explained by the degree of islet fibrotisation [13–14].

Autoimmune origin of CFRD has been contradictive [15–16]. Anyhow, recent reports at least partially suggest a role of autoimmune mechanisms in CFRD pathogenesis and T cell dysbalance in CF [17–18].

In our pilot study cytokine response to the type 1 diabetes related autoantigen GAD65 was found in almost all CF patients but this response differed between CF patients with and without diabetes (group A and B, respectively). Th1 polarisation of the response was observed in two CF patients from group A (who have diabetes, produce anti-GAD65 autoantibodies and have type 1 diabetes risk HLA-DQ genotype) and in one CF patient without diabetes at present (group B) but carrying the type 1 diabetes risk HLA-DQ genotype. We can speculate

that this patient may develop diabetes in the future. This Th1 response was very similar to the response observed in our recent onset type 1 diabetes patients (group D). On the contrary, the other three CF patients without diabetes (group B) displayed rather regular Th2- or Th3 cytokine pattern but higher chemokine production. Borderline production of chemokines was observed by GAD65 stimulation in healthy controls. We consider IL-8 production in our experiments as a non-specific irrelevant finding that may be due to high sensitivity of the kit to this chemokine.

Our results are in agreement with autoantibody status and type 1 diabetes risk genotype. Even though statistics cannot be applied on such a small study group, we suppose our observations to be a sign of a reaction directed against a self antigen in patients with CFRD. Pancreas is very often afflicted in CF patients and pancreatic autoantigens can be easily presented to local antigen presenting cells. In patients who will never develop diabetes the autoimmune mechanism is probably efficiently suppressed by self-tolerance mechanisms.

The strongest response was observed to GAD65-peptide 2 (a.a. 509–528). It has earlier been found that response to GAD65 in NOD mice is limited to a confined region (GAD65 a.a. 509–528 and 524–543) [19]. This response later spreads intramolecularly to additional determinants including GAD65-peptide 1 (a.a. 247–279). Our observation may be a sign of an early stage of the autoimmune process that is in some patients efficiently suppressed. GAD65-peptide 2 (a.a. 509–528) shares sequence homology with adenovirus, cytomegalovirus and Epstein-Barr virus. We can just speculate that the response observed against this epitope may be a sign of earlier infections in CF patients. GAD65-peptide 2 furthermore shares sequential homology with another important self antigen, proinsulin.

We consider CFRD patients to be a heterogenic group. To disclose those who may display some features of autoimmune diabetes could have an impact for their therapy and prognosis. Thus, it is necessary to examine larger cohort of patients (including disease onset) and to examine their response to other autoantigens (e.g. thyrosinphosphatase, proinsulin etc.) to verify if autoimmune cellular mechanism contributes to CFRD pathogenesis as well as to study immune regulatory mechanisms in these patients.
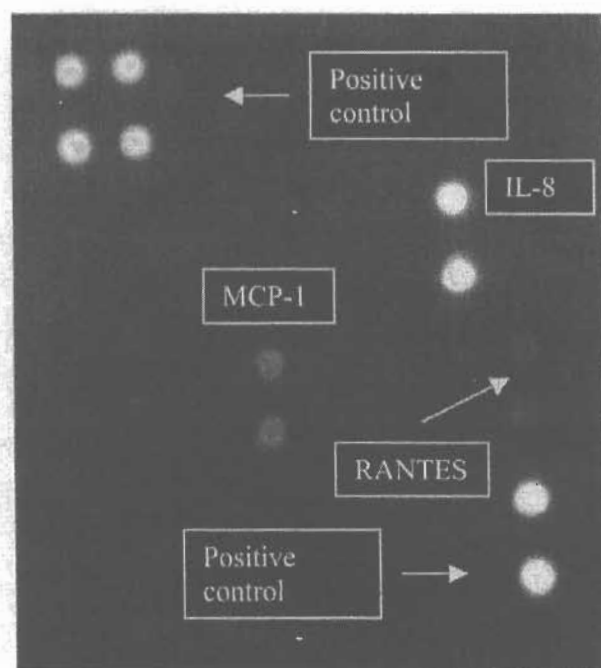
Acknowledgements

Figure 1.   Pat. No 7—example of results.

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