Univerzita Karlova v Praze

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DISERTAČNÍ PRÁCE

Doktorské studium biomedicíny - obor imunologie

Imunopatologie diabetu mellitu 1.typu - autoreaktivní versus regulační mechanismy

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V textu své disertační práce předkládám souhrn publikovaných prací doplněný širším výkladem týkajícím se problematiky imunopatologie diabetu 1.typu. Tématem jsem se zabývala v rámci svého postgraduálního studia v oboru "Imunologie" na 2.lékařské fakultě Univerzity Karlovy v Praze (2.LF UK) od roku 2003.

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SEZNAM POUŽITÝCH ZKRATEK

a.a.	peptidová sekvence - aminokyseliny (amino acid)
ADA	Americká diabetická asociace (American Diabetes Association)
Ag	antigen
APC	antigen prezentující buňka (antigen presenting cell)
CD	diferenciační znak (cluster of differentiation)
CTLA-4	antigen-4 cytotoxických T lymfocytů (cytotoxic T-lymphocyte antigen-4)
DC	dendritická buňka (dendritic cell)
DM	diabetes mellitus
DMSO	dimethylsulfoxid
EDTA	ethylen diamin tetraoctová kyselina (ethylene diamine tetraacetic acid)
FoxP3	transkripční represor (forkhead/winged-helix family transcriptional repressor p3)
GAD	dekarboxyláza kyseliny glutamové (glutamic acid decarboxylase)
GITR	glukokortikoidy indukovaný receptor pro tumor nekrotizující faktor (glucocorticoid-induced tumor necrosis factor receptor)
GM-CSF	granulocyto-makrofágové kolonie stimulující faktor (granulocyte-macrophage colony stimulating factor)
HLA	antigen lidských leukocytů (human leukocyte antigen)
Hsp	protein tepelného šoku (heat shock protein)
IA-2	ostrůvkový antigen (islet antigen)
ICAM	mezibuněčná adhezivní molekula (intercellular adhesion molecule)
ICOS	inducibilní pomocný stimulátor (inducible co-stimulator)
IDDM	inzulín dependentní diabetes mellitus
IDO	indol-amin 2,3 dioxygenáza
IEL	intraepiteliální lymfocyt
IFN	interferon
IL	interleukin
IPEX	syndrom imunodysregulage, polyendokrinopatie, enteropatie, X-vázaný
iTreg(s)	indukované regulační T lymfocyt(y) (inducible T regulatory cell(s))
i.v.GTT	nitrožilní, intravenózní glukózo-toleranční test
MHC	hlavní histokompatibilní komplex (major histocompatibility complex)
LFA	antigen spojený s lymfocytární funkcí (lymphocyte function-associated antigen)

LICOS	ligand inducibilního pomocného stimulátoru (ligand of inducible co-stimulator)
NFAT	nukleární faktor aktivovaných T lymfocytů
NF-kB	nukleární faktor kappa B
NK	přirozený zabíječ (natural killer)
NOD	linie neobézní diabetické myši (non-obese diabetic mouse)
nTreg(s)	přirozeně se vyskytující regulační T lymfocyt(y) (natural T regulatory cell(s))
oGTT	orální glukózo-toleranční test
PHA	fytohemaglutinin
PBMC(s)	mononukleární buňka(y) periferní krve (peripheral blood mononuclear cell(s))
SCID	těžký kombinovaný imunodeficit (severe combined immunodeficiency)
SNP	polymorfismy jednotlivých nukleotidů (single nucleotid polymorphism)
SOCS	supresor cytokinové signalizace (suppressor of cytokine signaling)
STAT	signálový přenašeč a transkripční aktivátor (signal transducers and activator of transcription)
T1D	diabetes 1.typu (type 1 diabetes)
Tc	cytotoxický T lymfocyt
TCR	receptor T lymfocytů (T cell receptor)
Teff	efektorový T lymfocyt
TGF	transformující růstový faktor (transforming growth factor)
Th	pomocný T lymfocyt
TNF	tumor nekrotizující faktor (tumor necrosis factor)
TNFR	receptor pro tumor nekrotizující faktor (tumor necrosis factor receptor)
Tr1	regulační T lymfocyt typ 1
Treg(s)	regulační T lymfocyt(y) (T regulatory cell(s))
TRAF	faktory asociované s receptorem pro tumor nekrotizující faktor (TNF receptor associated factors)
TSDR	pro regulační lymfocyty specifická demethylovaná oblast (Tregs specific demethylated region)

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1. Diabetes mellitus - obecný úvod

Diabetes mellitus je stále častěji nazýván "chorobou 21.století" [1]. V důsledku poruchy regulace metabolismu glukózy se onemocnění klinicky projevuje zvýšenou hladinou cukru v krvi (hyperglykémií) a dalšími metabolickými příznaky. Glukóza představuje hlavní energetický zdroj pro většinu buněk v lidském těle. K utilizaci glukózy dochází výhradně intracelulárně, pro její vstup do buněk je nezbytná přítomnost speciálních glukózových transportérů a inzulínu (výjimku představují neurony, červené krvinky a buňky střevní sliznice). Inzulín se tvoří v beta-buňkách endokrinní části slinivky břišní (pankreatu), jeho sekrece je regulována aktuální hladinou glukózy v krvi. Inzulín vazbou na inzulínové receptory zvyšuje počet glukózových receptorů na buněčné membráně a tím umožňuje vstup glukózy do buněk, mimoto má i celou řadu dalších účinků na metabolismus. Při nedostatku inzulínu, dochází ke hromadění glukózy extracelulárně a nedostatku glukózy intracelulárně, tzv."hladovění uprostřed nadbytku" [2]. Zvýšená hladina glukózy v krvi zvyšuje osmolaritu krve, vede k pocitu žízně, nadbytečnému pití (polydypsii) a močení (polyurii) s vylučováním glukózy do moče (glukosurie). Buňky jsou nuceny využívat jiné zdroje energie, především spalováním tuků (lipolýzou), dochází k hromadění ketolátek (intermediální produkty lipolýzy), které svou kyselou povahou snižují pH krve a vzniká metabolická acidóza, porucha homeostázy organismu. Neléčený absolutní nedostatek inzulínu končí smrtí. Při relativním nedostatku inzulínu a chronicky zvýšené hladině cukru v krvi se urychluje proces aterosklerózy, v důsledku mikroangiopatií vzniká diabetické poškození ledvin (nefropatie), sítnice (retinopatie), cév vyživujících nervy (neuropatie), atd. V čase se tak rozvíjí sekundární komplikace diabetu, které výrazně snižují kvalitu života [3].

Diabetes mellitus (DM) se dle etiologie dělí na: 1.typ, který je způsobený absolutním nedostatkem inzulínu při destrukci beta-buněk pankreatu, 2.typ, který je důsledkem relativního nedostatku inzulínu při inzulinorezistenci (nedostatek/nízká afinita inzulínových receptorů), ostatní vzácnější formy diabetu mellitu zahrnují různé, nejčastěji geneticky podmíněné, defekty a syndromy na receptorové či metabolické úrovni [1-3]. Rozdělení a diagnostická kritéria DM podle Americké diabetické asociace (ADA) jsou uvedena v tabulce č.1. Následující text disertační práce se týká výzkumu v oblasti DM 1.typu (T1D).

Tab.1 Rozdělení a diagnostická kritéria diabetu mellitu

Převzato od Americké diabetické asociace (ADA), 2007

I. Diabetes mellitus 1.typu *(absolutní nedostatek inzulínu)* • autoimunní (protilátky proti ostrůvkovým antigenům pozitivní) • idiopatický (protilátky proti ostrůvkovým antigenům negativní)	**Diagnostická kritéria diabetu**
II. Diabetes mellitus 2.typu *(relativní nedostatek inzulínu)*	**1. glykémie ≥ 11,1 mmol/l** (kdykoli během dne, bez ohledu na lačnění) NEBO **2. lačná glykémie ≥ 7,0 mmol/l** (tj.min. 8 hodin bez příjmu potravy a kalorických tekutin) NEBO **3. glykémie ve 120.minutě OGTT ≥ 11,1 mmol/l** (tj.při standardně provedeném orálním glukózo-tolerančním testu podle instrukcí WHO - Světové zdravotnické organizace)
III. Ostatní specifické formy diabetu • genetické defekty postihující vlastní působení inzulínu (receptory, iontové kanály, signalizační dráhy, tvorba a uvolnění inzulínu) • primární choroby exokrinní části pankreatu (nádory, fibrotizace) • endokrinopatie (nádory, paraneoplasie) • lékově podmíněný diabetes • infekčně podmíněný diabetes • vzácné formy imunologicky podmíněného diabetu (protilátky proti inzulínovému receptoru, inzulínu apod.) • genetické syndromy asociované s diabetem (Downův, Klinefelterův, Turnerův, Wolframův aj.)	
IV. Gestační diabetes mellitus *(relativní nedostatek inzulínu)*	

2. Diabetes mellitus 1.typu (T1D)

2.1 Incidence

Diabetes mellitus 1.typu (T1D) je autoimunitní choroba. Imunitní systém s porušenou regulací specificky ireverzibilně destruuje vlastní beta-buňky pankreatu produkující inzulín [4, 5]. Onemocnění začíná převážně v dětském věku, adolescenci nebo mladé dospělosti, s maximem výskytu mezi 12.- 15.rokem věku, s nutností doživotní substituce inzulínem, proto také starším označením bylo "juvenilní" DM, resp. inzulín-dependentní DM [1]. Incidence onemocnění rapidně narůstá a vykazuje trend k výskytu v mladším věku. Různé studie ukazují, že rozvoj autoimunitních onemocnění v zemích s vysokou životní úrovní je mnohem častější. Incidence T1D tak kolísá mezi 0,7/100.000 v Pákistánu a 50/100.000 ve Finsku [3]. Je předpovídáno, že celosvětová incidence T1D bude v roce 2010 až o 40% vyšší než byla v roce 1997 [3]. Česká republika patří k zemím se středně vysokou incidencí T1D, v letech 1990-1997 byla 10,1/100.000 dětí ve věku 0-14 let, v roce 2001 už 11,4/100.000 [1, 6, 7]. Meziroční nárůst incidence T1D v České republice patří k jedněm z nejvyšších a má dále vzestupný trend, cca o 4.3% za rok. Nejvyšší nárůst je pozorovaný ve skupině dětí od 0-4 let, kde tvoří 6,3% za rok [1, 7].

2.2 Klinické projevy a patogeneze

Začátek onemocnění je klinicky zcela němý. Dojde k prolomení imunologické tolerance a zahájení imunologické reakce proti vlastním beta-buňkám v Langerhansových ostrůvcích endokrinní části pankreatu. Samotný spouštěč není znám. Předpokládá se multifaktoriální etiologie u geneticky predisponovaného jedince [1, 3]. Pokusy na zvířecích modelech ukázaly, že naprosto nezbytná je přítomnost autoreaktivních T lymfocytů. Zvířatům (geneticky upraveným převážně myším liniím - NOD, SCID) byly transplantovány různé buněčné subpopulace za různých podmínek a bylo dokázáno, že pouze přenos T lymfocytů vedl k rozvoji onemocnění, zatímco B lymfocyty či jen samotné autoprotilátky neměly dostatečný vliv na vznik nemoci [4, 5]. Předpokládá se, že produkce autoprotilátek proti ostrůvkovým antigenům je až sekundárním projevem probíhající inzulitidy. Jejich detekce je však dosud teprve první možnou laboratorní známkou onemocnění.

S klesající masou beta-buněk se objevuje porucha glukózové tolerance, nejdříve v intravenózním a později v orálním glukózo-tolerančním testu. V případě intravenózního testu (i.v.GTT) je do žíly aplikováno určené množství glukózy a stanovuje se sekrece inzulínu a C-peptidu v 1. a 3.minutě. Sekrece inzulínu i C-peptinu je již snížená, i když hodnoty glukózy v krvi zatím nemusejí být zvýšené [8]. Při orálním testu (oGTT) je podána glukóza ústy a měří se hladina cukru v krvi s odstupem 60 a 120minut, hodnoty glykémie jsou již zvýšené [9].

Samotná klinická manifestace hyperglykémie s ketoacidózou se většinou projeví, až když je zničeno 80-90% beta-buněk [3]. Často k ní dochází po různě dlouhém období nejasných prodromů (únava, úbytek na váze, častější pití a močení) v období zvýšeného stresu, kdy je vyšší potřeba inzulínu (růstový spurt v pubertě, nemoc, psychický stres, apod.). Zbylé beta-buňky již nejsou schopny tuto potřebu pokrýt, jejich rezervy jsou vyčerpány [9].

Zbytková sekrece inzulínu přetrvává ještě různě dlouhé období, do zničení všech beta-buněk, poté klesá i titr autoprotilátek [10].

Názorně je patogeneze zachycena na obrázku č.1.

Obr.1 Schéma postupného klinického rozvoje diabetu mellitu 1.typu

1. **Klinicky němé prolomení imunologické tolerance**, autoreaktivita není potlačena regulačními mechanismy, vlastní spouštěcí moment není znám
2. **Začátek destrukce beta-buněk**, rozpadové produkty vedou k tvorbě autoprotilátek, v séru je možná jejich detekce (anti-GAD65, anti-IA-2), postupně se objevuje porucha glukózové tolerance, nejprve v i.v.GTT testu, poté v oGTT testu
3. **Klinická manifestace** onemocnění, hyperglykémie

2.3 Genetické pozadí

T1D je heterogenním polygenním onemocněním, s převážně non-familiárním výskytem. Pouze asi 5-10% nových jedinců s T1D jsou sourozenci diabetických pacientů, či děti diabetických rodičů. Průměrné riziko, že sourozenec diabetického dítěte onemocní T1D je asi 5%, tedy asi 25-50krát vyšší než v obecné populaci, kde je 1-2‰. [1]. Dosud bylo zjištěno kolem 20 s diabetem asociovaných genových oblastí, které jsou označovány IDDM 1-20. Nejvýznamnější genovou oblastí (s nejsilnější asociací s onemocněním) představuje oblast MHC-HLA II. třídy na chromozomu 6p21, tzv. IDDM1 geny. Zahrnují až 60% genetické vnímavosti pro onemocnění [3]. HLA molekuly II.třídy mají zásadní význam pro vazbu antigenu na antigen prezentujících buňkách a tím i aktivaci imunitní odpovědi. V souvislosti s diabetem jsou hlavně monitorovány lokusy HLA-DQ a HLA-DR. Heterodimer variabilních řetězců DQA1 a DQB1 molekuly HLA-DQ se podílí na genetickém riziku jako celek. V rámci genotypizace existují protektivní alely, jejichž nositel má velmi malou pravděpodobnost, že onemocní diabetem a rizikové alely, u kterých riziko onemocnění pro nositele dále narůstá při jejich kombinaci do rizikových haplotypů. Frekvence jednotlivých haplotypů a genotypů se liší mezi jednotlivými rasami i etnickými skupinami. Pro českou populaci v rámci hodnocení genetického rizika v Programu predikce diabetu 1.typu (při sledování HLA genotypu, pozitivity protilátek proti pankreatickým autoantigenům, i.v.GTT, aj.) byla stanovena následující genetická rizika (tab.2).

Tab.2 Genetické riziko pro rozvoj T1D na základě HLA II genotypu

(převzato z: "Predikce inzulin dependentního diabetes mellitus u dětských prvostupňových příbuzných diabetických pacientů", MUDr.Cinek O. a spol., Časopis lékařů českých 140, 2001, č.16)

Genetické riziko	Kritérium pro zařazení do kategorie	
1. silně zvýšené	DQ A1*05 - B1 0201/0302	současně DR B1 *0403 negativní
2. zvýšené	DQ A1*05- DQ B1 0201, nebo 0302, současně negativní DQ B1* 0301,*0602, *0603	současně DR B1 *0403 negativní
3. průměrné	a) přítomnost DQ B1* 0302-DR B1 *0403 b) DQ* 0301/0302 nebo * 0302/0603 c) současně negativní DQ B1* 0302, DQ A1 05 - B1 *0201, *0301, *0602, *0603	
4. snížené	přítomnost DQ B1*0603 nebo *0301	současně DQ B1 *0302 a *0602 negativní
5. velmi nízké	přítomnost DQ B1*0602, bez ohledu na další alely	

Příkladem dalších méně významných IDDM genů může být gen na chromozomu 11p5.5 (IDDM2), který má význam při navozování imunologické tolerance vůči vlastnímu inzulínu a proinzulínu. Tento lokus se podílí asi 10% na genetické vnímavosti k T1D. Za zmínku také stojí gen na chromozomu 2q33 (IDDM12), který leží v blízkosti 2 významných aktivačních molekul T lymfocytů: "cytotoxic-T lymphocyte-associated-protein 4" (CTLA4) a ko-aktivační molekuly CD28 [1,3].

2.4 Vliv zevního prostředí

Zkoumá se vliv celé řady faktorů. V čele stojí různé infekce, zejména enterovirové (coxsackie viry, echoviry) nebo herpesvirové (cytomegaloviry), které mohou vést k odkrytí kryptogenních antigenů nebo na základě antigenní podobnosti iniciovat autoimunitní zánět atd. Dále se věnuje pozornost vlivu potravy a životním podmínkám, zejména v časném období života, zkoumá se vliv kojení a bílkoviny kravského mléka na pozdější rozvoj onemocnění, očkování a celková životní úroveň, role vitamínu D a slunečního záření apod. [1, 3]. V naší práci jsme se faktory zevního prostředí nezabývali.

3. Imunopatologie diabetu mellitu 1.typu

3.1 Rozvoj autoimunitního zánětu

K rozvoji onemocnění dochází, jestliže se v periferii nacházejí autoreaktivní T lymfocyty, které rozpoznávají vlastní ostrůvkové antigeny beta-buněk, a navíc dojde k selhání regulační mechanismů, které je mají zabránit jejich klonální expanzi [11, 12].

Regulace imunitní odpovědi se v poslední době stává středem zájmu mnoha výzkumů, neboť přesné pochopení jejích principů může být klíčem k potlačení autoimunitního zánětu a tedy i k prevenci rozvoje autoimunitního onemocnění. Principy regulace imunitní odpovědi při zánětlivé reakci de facto úzce souvisí s principy periferní imunologické tolerance [22, 23]. Při tlumení autoimunitního zánětu je porucha imunitní regulace vyjádřena méně nápadně, o to však ve svých důsledcích zákeřněji. Imunitní systém je stimulován antigeny vlastních tkání a není zastaven.

Klíčovými hráči při zahájení zánětlivého procesu jsou antigen prezentující buňky a jejich interakce s T lymfocyty. Klíčovým principem pro ukončení zánětlivého procesu je suprese aktivovaných buněk. V případě reaktivity proti vlastním antigenům, je žádoucí spuštění supresivních mechanismů v samotném počátku zánětu, před vlastním rozvojem rozsáhlé

autoimunitní destrukce. K tomu slouží nástroje periferní imunologické tolerance, které mají své největší uplatnění při iniciálním kontaktu naivních T lymfocytů s antigen prezentujícími buňkami, kdy záleží, zda dojde k rozvoji zánětu, či nikoli [24]. Potlačení již jednou plně zahájené reakce je později mnohem náročnější [25].

3.2 Antigen prezentující buňky ("antigen presenting cells", APC)

Antigen prezentující buňky předkládají zpracovaný antigen T lymfocytům v podobě, v jaké ho jsou schopni rozeznat, aktivovat se a poté na něj adekvátně reagovat. Společně s aktivovanými T lymfocyty jsou APC jedinými buňkami, schopnými exprimovat HLA molekuly II.třídy, které váží extracelulární antigeny. Komplex HLA II s antigenem rozeznává TCR receptor s CD4 koreceptorem pomocného T (Th) lymfocytu. Komplex HLA I s antigenem rozeznává TCR receptor s CD8 koreceptorem cytotoxického T (Tc) lymfocytu [14, 15].

Rozdělení APC

Nejefektivnějšími APC jsou dendritické buňky (DC) odvozené od monocytů. Jejich nezralé formy ve tkáních fagocytují okolní materiál a po rozpoznání antigenu, při jeho vazbě na HLA II, migrují do přilehlých lymfatických uzlin a vyzrávají. Zralá DC exprimuje velké množství HLA I i HLA II molekul s antigenem, adhezivní a kostimulační molekuly, pro kontakt s lymfocyty. Dále secernuje chemokiny a cytokiny, které atrahují lymfocyty do uzliny a pomáhají při jejich aktivaci. Dalšími profesionálními APC jsou B lymfocyty a makrofágy, plně funkčními se však stávají až po aktivaci Th lymfocyty [14].

Interakce APC a T lymfocytů

Při kontaktu APC s T lymfocytem záleží na okolním cytokinovém prostředí (jak APC, tak T lymfocyt i ostatní přítomné buňky jsou schopné produkovat zánětlivé i protizánětlivé cytokiny), na kvalitě a délce kontaktu buněk daného adhezivními molekulami, na poměru aktivačních a inhibičních molekul na povrchu APC i T lymfocytu a na přítomnosti dalších buněk, které mohou interakci APC a T lymfocyt modifikovat (kompeticí o živiny, receptory, apod.). Výsledkem pak může být aktivace efektorových zánětlivých buněk, zejména $CD4^+$ pomocných T lymfocytů, které dále podporují rozvoj zánětlivé reakce, nebo indukce regulačních protizánětlivých lymfocytů, či vytvoření anergního klonu nebo dokonce apoptóza příliš aktivovaných lymfocytů (obr.3) [14, 15, 22-24]. Existuje celá řada experimentů, která zkoumá interakci na základně blokády jednotlivých molekul (obr.2).

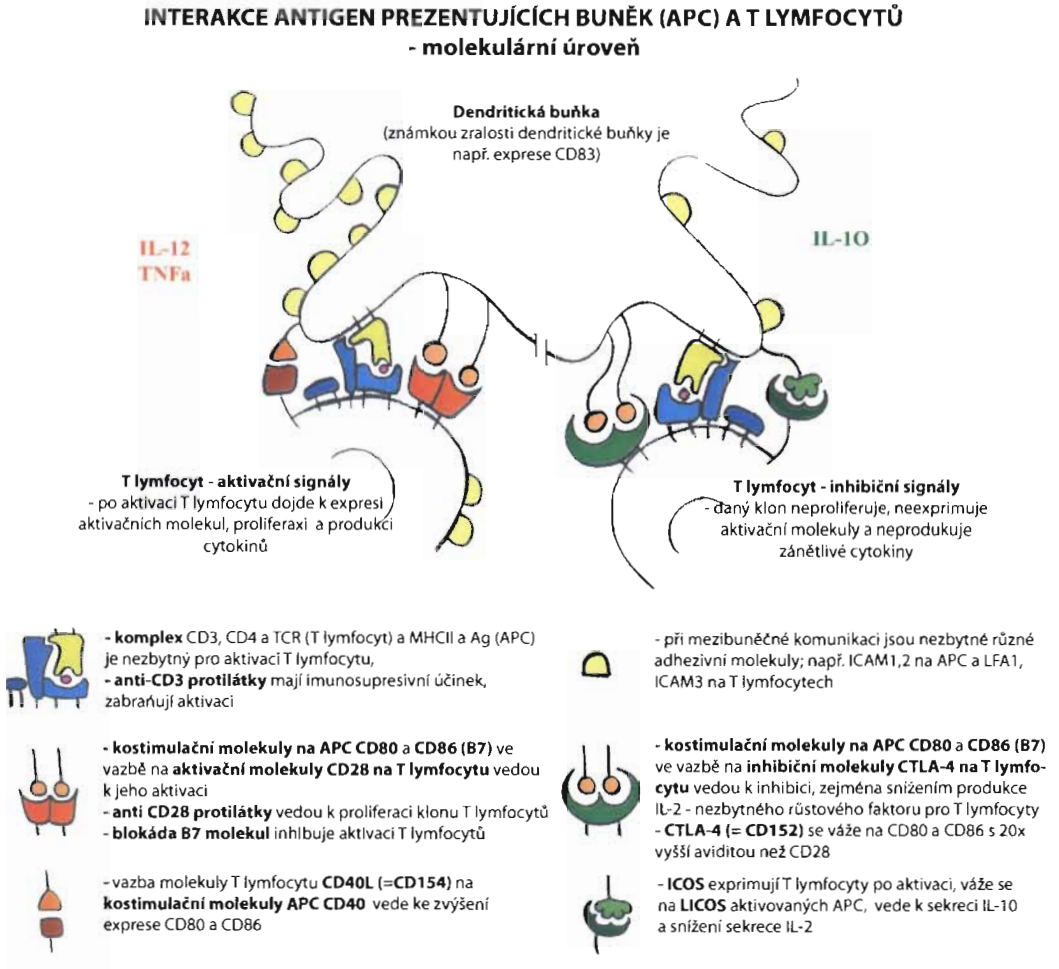
Obr.2 Interakce APC s T lymfocytem na molekulární úrovni:

Nezralá dendritická buňka se vyskytuje ve tkáni, fagocytuje okolní materiál, zpracovává ho v lysosomech a vyčkává na antigen, který vykazuje afinitu k MHC II. Jakmile se takový antigen objeví, ztrácí schopnost fagocytózy, migruje do kortexu nejbližší lymfatické uzliny a maturuje. Zralá dendritická buňka exprimuje vysoké množství MHC II (i MHC I) v kombinaci s antigenem a dále množství adhezivních molekul (ICAM 1,2, DC-sign=CD209, CD58), kostimulačních molekul (B7 = CD80 a CD86; CD40), cytokinů (IL-12, TNFα) a chemokinů (CCL21, CCL19, CCL18), které atrahují T lymfocyty do uzliny.

T lymfocyt pro interakci s dendritickou buňkou také potřebuje adhezivní molekuly (ICAM 3, LFA1, CD2) a rozpoznání komplexu MHC II s antigenem komplexem CD4 s TCR. Převládají-li na povrchu T lymfocytu aktivační signály přes molekuly CD28 a CD40L (=CD154), dochází k aktivaci a expresi aktivačních molekul (CD69, CD25, MHC II aj.), proliferaci klonu a přeměně v efektorové, zánětlivé cytokiny produkující T lymfocyty. Převládají-li na povrchu T lymfocytu inhibiční molekuly CTLA4 (= CD152) a ICOS, dochází k útlumu aktivace.

Při nedostatku kostimulačních molekul na nezralých dendritických buňkách nebo v protizánětlivém cytokinovém prostředí (s převahou IL-10, TGF-β) může vzniknout anergický nebo regulační T lymfocyt.

[odvozeno a upraveno z 14, 15]



APC in vitro

In vitro lze pomocí různých kultivačních protokolů získat zralé dendritické buňky, které prezentují zvolený antigen a produkují požadované cytokiny, které lze následně využít pro další pokusy, viz.dále [16].

3.3 Autoreaktivní T lymfocyty

Autoreaktivní klony T lymfocytů jsou takové, které se po setkání s vlastním antigenem patologicky aktivují. Jejich TCR receptor s koreceptory rozezná antigen ve vazbě na HLA molekuly. Po vazbě receptorů s ligandy dochází k převedení signálu a aktivaci T lymfocytu, která se projeví expresí aktivačních molekul a produkcí zánětlivého spektra cytokinů, tento klon dále proliferuje a rozvíjí se autoimunitní zánět [12]. Fakt, že určité HLA molekuly mohou vázat pankreatické autoantigeny snadněji než jiné, celou situaci potencuje.

Pro rozvoj T1D je zásadní aktivace pomocných $CD4^+$ T lymfocytů (Th), které, po kontaktu s antigen prezentující buňkou a spuštění signalizačních cest, začínají produkovat Th1 spektrum cytokinů: zejména interferon gama (IFNγ). Cytokinové spektrum Th1 podporuje buněčný typ imunitní odpovědi, přitahuje cytotoxické subpopulace autoreaktivních T lymfocytů: přirozené zabíječe (NKT buňky) a $CD8^+$ T lymfocyty, které navíc aktivuje v efektorové buňky. Tyto elementy pak infiltrují Langerhansovy ostrůvky v pankreatu a na úrovni buněčného kontaktu destruují beta-buňky, cytokinové prostředí a rozpadové produkty beta-buněk umocňují zánětlivý proces [5, 17-19].

Autoreaktivní T lymfocyty in vitro

In vitro lze autoreaktivní klony T lymfocytů vyhledávat pomocí stimulace specifickými autoantigeny a následnou detekcí produkce cytokinů či exprese aktivačních molekul. [20,21]

3.4 Periferní imunologická tolerance

V periferii každého jedince však výskyt klonů T lymfocytů s TCR receptory, které vykazují vysokou afinitu k vlastním antigenům, není žádnou výjimkou. Zejména, pokud jsou tyto antigeny nečekaně odkryty např. po nějakém infekčním inzultu apod. Dochází k tomu, jestliže takový klon nebyl zničen během svého vyzrávání v thymu (negativní selekcí), při selhání centrální imunologické tolerance. Za fyziologických podmínek se pak správně zapojují mechanismy periferní imunologické tolerance, které v samotném počátku zabrání klonální expanzi těchto potenciálně autoreaktivních T lymfocytů [11-13].

Mechanismy periferní imunologické tolerance

Mezi mechanismy periferní imunologické tolerance patří: klonální delece (v případě setkání se s vlastním antigenem a jeho rozpoznáním, se při nadměrné aktivaci spustí apoptóza daného buněčného klonu, např. přes Fas ligandy), klonální ignorace (v situaci, kdy je antigenu podprahové množství), klonální anergie (při nedostatku kostimulačních molekul nebo za přítomnosti protizánětlivého cytokinového spektra nedojde k aktivaci T lymfocytu) a posledním principem je vznik periferně indukovaných regulačních T lymfocytů, které samy aktivně začínají působit imunosupresivně [12, 13].

Existují místa v lidském těle, na rozhraní mezi vlastním (vnitřním) a cizím (vnějším), kde je vznik imunologické tolerance téměř pravidlem. Patří sem kožní a slizniční imunitní systém [22]. Prezentace antigenu ve slizničním prostředí probíhá v protizánětlivém cytokinovém prostředí (IL-4, IL-10, TGF-β) a antigen nemusí být prezentován pouze profesionálními APC, může tedy chybět dostatek kostimulačních signálů [22].

Regulační T lymfocyty (Tregs)

Regulační T lymfocyty (Tregs) jsou v ohnisku zájmu posledních 10-15 let. Koncept regulační buňky byl sice navržen již před více jak 30-ti lety, kdy bylo publikováno, že spojení T lymfocytů s antigenem, může navodit toleranci antigenu po přenesení do naivní myši, ale díky tehdejším dostupným technikám, nebylo možné tento jev dále studovat. Více se tedy o nich dozvídáme až nyní [22]. Postupně se ukazuje, že existuje celá rozrůstající se skupina jednotlivých podtypů Tregs, které jsou rámcově definovány na základě různého fenotypu (exprese povrchových a intracelulárních molekul, produkce cytokinů). Kandidátními povrchovými markery jsou "glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein" GITR, inhibiční molekula "cytotoxic-T lymphocyte-associated-protein 4" CTLA-4 (CD 152), adhezivní molekuly CD62L a αEβ7integrin (CD103), α-řetězec receptoru pro IL-2 (CD25), intracelulární molekula inhibičního transkripčního faktoru "forkhead/winged-helix family transcriptional repressor p3" FoxP3 a další. Jejich problematika je podrobněji popsána níže [22, 23]. Při snaze o přesnou klasifikaci Tregs ale narážíme na fakt, že některé znaky se u jednotlivých subtypů mohou překrývat (např. $FoxP3^+$) nebo mohou nabývat různých významů (např. $CD25^+$). Také záleží, v jakém kontextu a za jakých podmínek danou subpopulaci zkoumáme. To může být zdrojem určitých nejasností při orientaci mezi Tregs. Podobně jako lze imunitní odpověď dělit na nespecifickou a specifickou, lze Tregs rozdělit, v základním slova smyslu, na přirozeně se vyskytující a v periferii indukované regulační T lymfocyty [50, 53]. V obou těchto skupinách se pak vyskytují další podtypy regulačních T lymfocytů (tab.3).

Tab.3 Rozdělení Tregs

	Tregs	Mechanismus	Poznámka	Ref
Inducible	**CD4⁺ Th2**	IL-4 (méně IL-10)	Aktivace TCR CD4⁺ Th lymfo po setkání s Ag v komplexu HLA-II na APC vede k produkci Th2 cytokinového spektra, zejména IL-4	22,23
	CD4⁺ Th3	TGFb (méně IL-10)	Aktivace TCR CD4⁺ Th lymfo po setkání s Ag v komplexu HLA-II na APC vede k produkci Th3 cytokinového spektra, zejména TGFβ	22,23
	CD4⁺ Tr1	IL-10 (méně TGFb)	Aktivace TCR CD4⁺ Th lymfo po setkání s Ag v komplexu HLA-II na APC vede k produkci protizánětlivého cytokinového spektra, zejména IL-10	22,23
	CD8⁺ (CD28⁻)	IL-10, IL-4	Aktivace TCR CD8⁺ T lymfo po setkání s Ag v kompexu HLA-I vede k produkci protizánětlivých cytokinů	22,23
	CD8⁺γδ IEL	IL-10	TCRγδCD8⁺ T lymfo se přednostně vyskytují v epitelu a hrají významnou roli ve slizniční imunitě, aktivace po setkání s Ag v kombinaci s HLA-I molekulami vede k produkci protizánětlivého IL-10	22,23
Natural	**CD8⁺HLAE restricted**	suprese aktivovaných T lymfo a NK na bázi buněčného kontaktu	TCR receptor CD8⁺ T lymfo, který rozezná Ag v komplexu HLA-E molekul na aktivovaných T a B lymfo a DC, vede ke vzniku supresorového klonu, expresi inhibičních molekul CD94/NKG2A a C na NK a CD8⁺ buňkách, jejichž interakce s HLA-E vede k inhibici jejich cytotoxické aktivity	32
	NKT CD1 restricted	Th1/Th2 modulace, působení na DC, na bázi buněčného kontaktu a produkce cytokinů	TCR receptor váže glykolipid na CD1 molekulách (experimentálně a-galaktosylceramid), po aktivaci umí tvořit Th1 i Th2 spektrum i cytotoxické molekuly (perforin, granzym) v akutním stadiu, na počátku nespecifické odpovědi podporují aktivaci DC a tím nepřímo urychlují specifickou odpověď, opakovaná chronická aktivace stejným antigenem však naopak vede k supresi (produkcí IL-10 a působením na DC)	51, 52
	$CD4^+CD25^{high}$ nTregs	suprese aktivovaných T lymfo a snad i DC na bázi buněčného kontaktu	Polyklonální TCR receptor, k aktivaci stačí 10-100xnižší koncentrace Ag, po aktivaci neprodukuje IL-2 ani jiné zánětlivé cytokiny, na bázi buněčného kontaktu cytotoxicky zabíjí (granzym A) efektorové T lymfocyty, snad modulují i DC, přesný mechanismus není znám	50

3.5 Indukované regulační T lymfocyty ("adaptive", "inducible", iTregs)

Indukované regulační T lymfocyty vznikají v periferii po setkání s antigenem, jsou tedy antigenně specifické [22, 23].

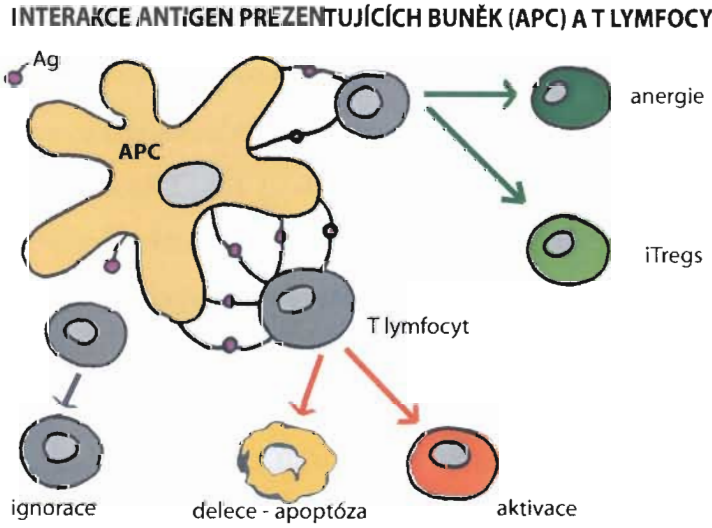
Zcela přesný způsob jejich vzniku není na molekulární a signalizační úrovni plně objasněn a je předmětem posledních výzkumů. Předpokládá se, že jsou výsledkem výše popsané interakce T lymfocytu s APC v určitém cytokinovém prostředí, v určitém čase a místě. Zásadní roli hraje charakter antigenu, který vedl k aktivaci APC, jeho množství, avidita k HLA a TCR, i místo, kde se objevil [22-24]. Jak již bylo řečeno, slizniční podání antigenu může vést k jiné imunitní odpovědi než systémové. Obecně lze říci, že za určitých podmínek, má každý antigen schopnost vyvolat tvorbu iTregs [22].

Interakce na receptorové úrovni, která odráží zralost a kvalitu APC a připravenost T lymfocytu, vede ke spuštění jednotlivých navzájem se ovlivňujících signalizačních kaskád, na jejichž konci je, v případě vzniku iTregs, indukce různě vyjádřeného supresivního fenotypu:

aktivace exprese regulačních transkripčních faktorů (FoxP3), zvýšení exprese inhibičních molekul (CTLA-4, aj.), produkce protizánětlivých cytokinů (IL-10, TGF-β, aj.), interakce s transkripčními faktory aktivovaných T lymfocytů (NF-κB, NFAT), která vede k inhibici produkce zánětlivých cytokinů a IL-2, se současným možným zvýšením exprese receptoru pro IL-2 (CD25), neboť IL-2 je obecně nezbytným pro přežití Tregs, aj. [22-24, 27-31]. Nezralé nebo neprofesionální APC díky absenci dostatečně kvalitních kostimulačních signálů vedou spíše k tvorbě anergních či regulačních klonů T lymfocytů a navození imunotolerance.

Obr.3 Interakce APC s T lymfocytem na buněčné úrovni

T lymfocyt, který v periferii rozpozná antigen ve vazbě na MHC I nebo MHC II a nedostane dostatek kostimulačních signálů se stane anergním nebo se přemění v iTreg, v opačném případě se stává efektorovým T lymfocytem nebo při výrazných signálech aktivace dochází k apoptóze. Dostatek kvalitních kostimulačních signálů může poskytnout pouze profesionální antigen prezentující buňka (zralé dendritické buňky, makrofágy, B lymfocyty). Nezralé dendritické buňky a ostatní buňky, které za určitých okolností dokáží exprimovat antigen, většinou dostatek kvalitních kostimulačních molekul postrádají [odvozeno a upraveno ze 14, 22, 23].



Rozdělení iTregs

V širším slova smyslu se mezi regulační populace řadí $CD4^+$ T lymfocyty reagující na setkání s autoantigenem Th2 a Th3 typem zánětlivé imunitní odpovědi. Th2 imunitní odpověď je charakterizována především produkcí IL-4, zatímco Th3 pak produkcí TGF-β [18, 26]. Je známo, že pouze samotné odchýlení imunitní odpovědi z Th1 typu na Th2 nebo Th3 typ může zabránit rozvoji autoimunitního diabetu [11, 12, 18, 26]. $CD4^+$ T lymfocyty, které produkují převážně protizánětlivý IL-10, jsou známé jako Tr1 typ [22]. Dále se mezi iTregs řadí $CD8^+$ T lymfocyty, které neexprimují kostimulační molekuly CD28 a po setkání s antigenem

produkují IL-4 a IL-10, a $CD8^+$ T lymfocyty se specifickým TCR receptorem γδ, které se vyskytují převážně ve sliznicích a po setkání s antigenem produkují IL-10 [22, 23]. Na pomezí mezi iTregs pak stojí cytotoxické $CD8^+$ T lymfocyty, jejichž TCR receptor reaguje na peptid prezentovaný molekulou HLA Ib třídy (HLA-E) a tlumí zánětlivou reakci převážně mezibuněčným kontaktem [32].

Mechanismus účinku iTregs

Mechanismus potlačení imunitní reakce iTregs spočívá převážně na produkci protizánětlivého cytokinového spektra, v případě výše zmíněných $CD8^+$ T lymfocytů dochází k buněčné cytotoxicitě. Nepřímo má svůj význam také v kompetice o vazbu na APC, živiny a růstové faktory [23].

IL-10 potlačuje Th1 zánětlivou odpověď přímo, snížením cytokinové produkce v aktivovaných Th1 lymfocytech (IL-2, TNFα, INFγ, GM-CSF, atd.), a nepřímo, znemožněním další aktivace Th lymfocytů inhibicí exprese HLA II a kostimulačních molekul na APC [33]. IL-10 aktivuje signalizační cestu STAT3 a také interaguje s transkripčním nukleárním faktorem aktivovaných T lymfocytů NF-κB [67, 68]. TGF-β vazbou na své receptory v závislosti na dávce snižuje cytokinovou produkci, buněčné dělení a indukuje apoptózu [24, 33]. Je popsána interakce signalizačních cest od receptoru TGF-β a CTLA-4, které společně vedou k aktivaci exprese FoxP3 [24]. IL-4 aktivuje signalizační cestu STAT6 a tím podporuje produkci Th2 zánětlivého spektra a inhibuje Th1 typ odpovědi [15].

Možnosti identifikace jednotlivých subtypů iTregs na molekulární úrovni

Možnost rozlišení iTregs pomocí kombinace povrchových a intracelulárních molekul je složitější neboť exprese dosud známých molekul se může u jednotlivých podtypů překrývat a zejména $CD4^+$ iTregs mohou splývat s $CD4^+CD25^+$ přirozeně se vyskytujícími nTregs, viz.dále. Nejvíce diskutovanými molekulami jsou α-řetězec povrchového receptoru pro IL-2 (CD25) a intracelulární transkripční faktor FoxP3. CD25 je molekula, která se na většině T lymfocytů vyskytuje ve větším množství až po aktivaci. Výjimku tvoří přirozeně se vyskytující Tregs, které exprimují CD25 ve vysoké hustotě konstitutivně, viz. níže [29]. Jednotlivé subpopulace iTregs mohou také exprimovat CD25 ve vysokém množství, ale existují i populace se schopností suprese, které jsou CD25 negativní [25]. FoxP3 je inhibiční transkripční faktor, který interaguje s nukleárními faktory aktivovaných T lymfocytů (NF-κB a NFAT), jeho exprese byla dosud doménou přirozeně se vyskytujících Tregs, viz.níže [30, 31]. Výzkumy ukazují, že jeho výskyt je možný i u iTregs, kde však jeho exprese není konstitutivní, ale fakultativní. Určitá $FoxP3^+$, většinou spojená s $CD25^+$, se může po aktivaci

přechodně objevit na různých subpopulacích T lymfocytů a způsobovat jejich sníženou odpověď na stimulaci, ale regulační efekt v podobě aktivní imunosuprese je spojen pouze s přetrvávající vysokou expresí FoxP3 [61]. Změna $FoxP3^-$ na $FoxP3^+$ po stimulaci antigenem, v kombinaci se schopností této populace tlumit imunitní reakci, může pomoci odkrýt populaci nově vzniklých iTregs ($CD4^+$ nebo $CD8^+$ a převážně $CD25^+$) [27, 61].

Další molekulou spojovanou s Tregs je povrchová exprese GITR [25, 28]. Jeho role je opět kontroverzní, jeho přítomnost je spojena jak s aktivací efektorových buněk, tak nespecificky s Tregs. Jeho ligandy se vyskytují na APC. Bylo zjištěno, že se na některých subpopulacích Tregs GITR vyskytuje ve zvýšené hustotě a následovala hypotéza, že jeho exprese je regulována FoxP3 [25]. Pokusy na myších modelech (linie $GITR^-/GITR^-$) však ukázaly, že přítomnost molekuly GITR není pro supresivní funkci nezbytná. Existuje více variant intracelulárních domén GITR a od nich se odvíjejících signalizačních cest. GITR převážně podporuje přežití Tregs (resp.zabraňuje jejich apoptóze) a aktivaci efektorových T lymfocytů při vazbě na APC neboť působí jako kostimulační molekula [25, 34]. Použití monoklonálních protilátek proti GITR může mít jak aktivační, tak inhibiční efekt, závisí na konkrétní imunitní interakci a charakteru použité protilátky [25, 35, 36].

Podobná ambivalence je spojená s povrchovou expresí ligandu pro L-selektin, molekuly CD62L, která se vyskytuje na naivních T lymfocytech a vazbou na adhezivní molekuly endotelu umožňuje vstup lymfocytu do uzliny. $CD62L^+$ je znakem naivních T lymfocytů, zatímco $CD62L^{low}$ je spojeno s lymfocyty, které se již setkaly s antigenem [37]. U obou variant byla pozorována supresní schopnost, nejčastěji u fenotypu $CD4^+CD25^+FoxP3^+CD62L^{high}$ - pravděpodobně přirozené Tregs [37-39]. Nicméně, v případě antigenně specifických iTregs bychom vzhledem k mechanismu jejich vzniku spíše čekali nízkou expresi CD62L [39].

Jasnější je exprese molekuly CTLA-4 (CD152), která je spojena s výhradně inhibiční funkcí a na Tregs se může vyskytovat ve vysokém množství [15]. Objevuje se na povrchu T lymfocytů do 24 hodin po aktivaci, po 96 hodinách klesá, soutěží s kostimulační molekulou CD28 o vazbu na molekuly CD80 a 86 na APC, které váže s vyšší afinitou [15, 40]. Signalizace přes CTLA-4 vede k inhibici produkce IL-2 a prozánětlivých cytokinů a inhibici proliferace [24, 39]. Účinek signalizace je potencován přítomností TGF-β, která vede k indukci exprese FoxP3 u iTregs. CTLA-4, stejně jako TGFβ a FoxP3 deficientní myši umírají na maligní autoimunitní a lymfoproliferativní nemoc [24].

Tyto a některé další molekuly spojované s označením regulačních subpopulací jsou uvedeny v tabulce č.5, většina z nich je opět spíše nespecifickými markery, které nabývají na významu

až po vzájemné kombinaci. Hypotézu, že by snad existoval rozdíl v kvantitativní genové expresi u jednotlivých subtypů iTregs, se zatím nepodařilo prokázat [24].

iTregs in vitro

In vitro lze specifické iTregs získat různými způsoby. Přidáním lymfocytů k maturovaným DC (po primingu specifickým antigenem) za přítomnosti IL-10 nebo TGF-β získáme specifické Tr1 nebo Th3 lymfocyty [27, 28, 42], které lze dále expandovat [43, 44]. Podávání upravených anti-CD3 monoklonálních protilátek vede ke zvýšení $CD8^+$ iTregs (suppopulace $CD8^+CD25^+FoxP3^+CTLA4^+$), apod. [45].

iTregs in vivo

In vivo se již v souladu s využitím principů regulačních mechanismů v případě T1D u lidí zkouší aplikace inzulínu s alterovanými peptidovými ligandami [46], podávání diabetogenních antigenů [47] nebo léčba anti-CD3 monoklonálními protilátkami [48]. Vše s cílem indukovat tvorbu iTregs a omezit či zabránit plnému rozvoji onemocnění.

3.6 Přirozeně se vyskytující regulační T lymfocyty ("natural"Tregs)

Přirozeně se vyskytujícím regulačním T lymfocytům bylo imunosupresivní působení "naprogramováno" již během jejich vyzrávání v thymu [50, 53]. Přirozeně exprimují geny spojené s potlačením rozvoje imunitní reakce, bez nutnosti indukce jejich transkripce [54]. Jejich počet v periferii je relativně stálý a nezávislý na přítomnosti antigenu. Jejich úkolem je suprimovat aktivované imunitní buňky, bez antigenní specificity. Dojde-li k poruše tvorby nTregs v thymu nebo k odstranění nTregs během několika prvních dní života, rozvíjí se multisystémové autoimunitní onemocnění [55]. Základní rozdíly mezi nTregs a iTregs jsou shrnuty v tabulce č. 4.

Tab. 4 Rozdíly mezi nTregs a iTregs

Vlastnosti	nTregs	iTreg
Vznik	thymus	periferie
Imunosupresní fenotyp	konstitutivně	fakultativně
TCR	polyklonální	antigenně specifický
Mechanismus suprese	buněčný kontakt (granzym A, perforin)	produkce cytokinů (IL-10, TGF-β)
Schopnost suprese	ano -vysoká	ano
Expanze *in vitro*	obtížná	lze vytvořit z naivních T lymfo
Typické znaky	$CD4^+CD25^{high}$ $FoxP3^+CD127^-$	$CD4^+/CD8^+$, $CD25^+/(CD25^-)$, $FoxP3^+/(FoxP3^-)$, CD127?
Ostatní znaky	$CD62L^+$, $GITR^+$, $CTLA\text{-}4^+$	$CD62L^-$, $GITR^+$, $CTLA\text{-}4^+$

Rozdělení nTregs

Pokud se hovoří o nTregs, v naprosté většině se myslí na populaci $CD4^+CD25^+$ nTregs, která tvoří 1-10% všech $CD4^+$ T lymfocytů [50]. V širším slova smyslu by však bylo možné zařadit

mezi nTregs populaci přirozených zabíječů s invariantním TCR receptorem Vα24- JαQ (NKT lymfocyty, $CD4^+$ nebo $CD4^-CD8^-$ dvojitě negativní), které mají také významný podíl na regulaci imunitní odpovědi a imunosupresi [51, 52].

Mechanismus účinku nTregs

$CD4^+CD25^+$ nTregs potlačují imunitní reakci na základě buněčného kontaktu. Cytokinová produkce je minoritní a *in vitro* nehraje v imunosupresi žádnou roli, jak bylo dokázáno při použití supernatantu z aktivovaných $CD4^+CD25^+$ nTregs [50, 53]. $CD4^+CD25^+$ nTregs mají polyklonální vysokoafinní αβ TCR receptor pro široké spektrum antigenů (k aktivaci stačí až 100x menší koncentrace antigenu oproti $CD25^-$ T lymfocytům). Jeho stimulace však nevede k proliferaci klonu a produkci zánětlivých cytokinů. Ani při kostimulaci přes molekulu CD28 nedochází k expresi IL-2 [50]. Aktivované $CD4^+CD25^+$ nTregs exprimují adhezivní molekuly a zejména cytotoxická granula s granzymem A, ale i perforinem [50, 53]. Rozeznávají aktivované imunitní buňky ($CD4^+$, $CD8^+$ T lymfocyty i APC), pravděpodobně pomocí aktivačních a možná i HLA molekul, a na nich pak uplatňují cytotoxický efekt [50, 53, 55]. Blokáda adhezivních molekul omezí supresivní schopnost [53].

NKT rozeznávají glykolipidy v kombinaci s CD1d molekulou na cílových buňkách, po aktivaci jsou schopné produkovat různé cytokiny, dvojitě negativní NKT spíše Th2 spektrum, $CD4^+$ NKT spíše Th1 spektrum [51], a cytotoxické molekuly [52]. Úkolem NKT je regulace imunitní odpovědi, v případě akutní aktivace, bezprostřední produkcí zánětlivých cytokinů, urychlují začátek zánětu, v případě chronické stimulace však působí spíše imunosupresivně [52]. Příkladem může být zabránění rozvoje T1D u NOD myší dvojitě negativními od thymu odvozeným NKT na základě produkce IL-4 a IL-10 [51].

Možnosti detekce $CD4^+CD25^+$ nTregs na molekulární úrovni

Za dosud nejspolehlivější marker pro $CD4^+CD25^+$ nTregs se považuje intracelulární exprese FoxP3, ale objevují se i další, např. receptor pro IL-7 (povrchová molekula CD127) a selektivně demethylovaný nekódující úsek FoxP3 v exonu 1 (TSDR; Tregs specifický demethylovaný region) [56-58]. Ostatní znaky Tregs, uvedené výše (GITR, CTLA-4, CD62L, aj.), nabývají svého významu ve vzájemné kombinaci, nejsou pro populaci $CD4^+CD25^+$ nTregs specifické, i když mohou pro ni mohou mít svůj specifický funkční význam, zejména při jejich vývoji a přežívání v periferii. Příkladem může být vysoká exprese GITR, která $CD4^+CD25^+$ nTregs může zajišťovat odolnost vůči apoptotickým signálům a vyvažovat tak jejich nízkou schopnost proliferace [25, 28].

Vysoká exprese α-řetězce receptoru pro IL-2, molekuly CD25, byla v roce 1995 prvním popsaným znakem Tregs [60]. Nejde však o specifický znak Tregs, vyskytuje se také na aktivovaných efektorových T lymfocytech. Jednotlivé populace lze odlišit pouze pomocí míry exprese, která je u $CD4^+CD25^+$ nTregs konstitutivně vysoká i v jejich neaktivovaném stavu [50, 60]. CD25 do jisté míry odráží zásadní význam IL-2 pro vývoj a přežití Tregs, její vysoká hustota vyvažuje fakt, že jedním z principů imunosuprese je i inhibice produkce IL-2, důležitého růstového faktoru T lymfocytů, tím umožňuje přežití receptorově lépe vybaveným Tregs [29, 50]. Ambivalence CD25, která způsobuje nepřesné odlišení Tregs od aktivovaných T lymfocytů vedla k hledání vhodnějších znaků.

Intracelulární transkripční faktor FoxP3, který kóduje transkripční represor "scurfin", je nyní považován za zlatý standard značení Tregs. Jeho stabilně vysoká exprese je spojovaná s imunosupresivním fenotypem a hyporesponzivním stavem buněk, jak bylo dokázáno při retrovirových transferech genu, a koreluje s regulační aktivitou [50,58, 61]. Zatímco při diferenciaci iTregs je dostatečná exprese FoxP3 až konečným výsledkem signalizačních kaskád po aktivaci, $CD4^+CD25^+$ nTregs exprimují FoxP3 konstitutivně [50, 54]. Nedávno bylo popsáno, že se u $CD4^+CD25^+$ nTregs, na rozdíl od konvenčních iTregs, stabilně vyskytuje selektivně demethylovaný nekódující region FoxP3 v exonu 1 (TSDR region), který u $CD4^+CD25^+$ iTregs zůstává i přes expresi FoxP3 trvale methylovaný [54]. Tento znak by mohl sloužit k lepšímu rozlišení fenotypu $CD4^+CD25^+FoxP3^+$ iTregs od nTregs. Dosud nebylo objasněno, které všechny geny FoxP3 kontroluje [50]. Byla popsána jeho interakce s transkripčními faktory aktivovaných T lymfocytů NF-kB a NFAT, které modulují expresi mnoha cytokinů (včetně IL-2) [30,31] a předpokládá se jeho vliv na expresi jednotlivých aktivačních a inhibičních molekul (např.CD25, GITR a jiné) [25]. Přítomnost FoxP3 je také důležitá pro vlastní vývoj $CD4^+CD25^+$ Tregs v thymu, kdy jeho mutace způsobují u lidí rozvoj IPEX syndromu (imunitní dysregulace, polyendokrinopatie, enteropatie, X-vázaného syndromu) [57-59]. Nevýhodou znaku je jeho intracelulární výskyt, který znemožňuje selektivní výběr populace pro její další použití.

Nízká exprese povrchové molekuly receptoru pro IL-7 (CD127) je dalším nedávno objeveným slibným znakem Tregs. Po aktivaci se objevuje ve vysokém množství na efektorových a paměťových T lymfocytech, které vyžadují pro své přežití IL-7 a tím se odlišují od Tregs, které jsou více než na IL-7 závislé na IL-2 [56, 57]. Byla vyslovena hypotéza o interakci FoxP3 s promotorem CD127, vysoká exprese FoxP3 dobře koreluje s nízkou expresí CD127 [57].

Problematika ostatních molekul je popsána výše a shrnuta v tabulce č.5.

$CD4^+CD25^+$ nTregs in vitro

In vitro lze u lidí $CD4^+CD25^+$ nTregs izolovat z mononukleárních buněk periferní krve pomocí magnetické separace, za použití $CD4^+CD25^+$ nebo $CD4^+CD25^+CD127^-$ speciálních kitů [63]. Také je možná jejich separace na průtokovém cytometru. Populaci lze použít k dalším *in vitro* testům. Existují protokoly k jejich expanzi, ta je však obtížná vzhledem k jejich základní vlastnosti snížené proliferace. Využívá se stimulace molekul CD28 a CD3, v přítomnosti IL-2, ale i tak je snazší expandovat spíše iTregs než nTregs [27, 50].

$CD4^+CD25^+$ nTregs in vivo

In vivo zatím u lidí kromě stanovení počtu nTregs a testech jejich funkce *in vitro* neexistují žádné jiné pokusy.

Tab.5 Molekulární znaky Tregs

Molekula	Výskyt	Ligand	Výskyt	Efekt	Poznámka	Ref
FoxP3	regulační T lymfo	nukleární faktory	buněčné jádro	regulátor genové exprese, interakce s nukleárními faktory aktivovaných T lymfo	specifický marker Tregs, konstitutivní exprese u nTregs, fakultativní exprese u iTregs	30, 31, 50
CD127	efektorové a paměťové T lymfo	IL-7	cytokin	receptor pro IL-7, nezbytný růstový faktor pro T lymfocyty	$CD127^-$ dobře koreluje s $FoxP3^+$, Tregs receptor pro IL-7 specificky neexprimují	56, 57, 62
CD25	T a B lymfo, makrofágy	IL-2	cytokin	receptor pro IL-2 (α-řetězec)	nespecifický marker Tregs, exprese CD25 je známkou aktivace lymfo, $CD25^+$ i $CD25^-$ populace mohou mít supresivní účinky, konstitutivně vysoká exprese je typická pro nTregs	50, 60
CTLA-4 (CD152)	T lymfo	CD80 a 86	APC	kompetice s kostimulační molekulou CD28 o stejný ligand, inhibice aktivačních signálů v T lymfo vede ke snížení produkce prozánětlivých cytokinů (zejména IL-2) a proliferace, podpora tvorby TGFβ, modulace Th1 v.s. Th2, inhibice exprese CD80 a 86 na APC, indukce IDO enzymu v APC, který vede ke katabolismu tryptofanu nezbytného pro T lymfo	specifický marker Tregs, T lymfo, které ho exprimují mají výhradně inhibiční efekt	15, 24, 39-41
CD28	T lymfo	CD80 a 86	APC	kostimulační molekula T lymfo, indukce produkce IL-2, proliferace	$CD28^-$ je známkou neschopnosti proliferace a aktivace, např. subpopulace iTregs $CD8^+CD28^-$	49
GITR	T,B, NK lymfo, zejména po aktivaci efektorových T lymfo se zvyšuje exprese, některé subpopulace Tregs exprimují konstitutivně	GITRL	APC	interakce s NF-κB a apoptotickými signály, umožněná přes TRAF ("TNF receptor associated factors")	nejde o specifický marker Tregs, i když na některých subpopulacích Tregs se vyskytuje ve vysokém množství, jde spíše o kostimulační molekulu s ambivalentním efektem	25, 34-36
CD62L	antigenně nezkušené naivní T lymfo	CD62	endotel	adheze pro homing T lymfo do uzliny	nejde o specifický marker Tregs, $CD62L^{high}$ značí naivní T lymfo, $CD62L^{low}$ zase antigenně zkušené, obě populace mohou mít supresivní účinek	37
Další molekuly spojované s Tregs:	CD45RA vs. CD45RO; OX40 (CD134); 4-1BB (CD137); β-řetězec IL-2R (CD122), $\alpha 4\beta 7$, TSDR region aj.					22, 28, 29, 54

4. Závěr

T1D je po svém propuknutí celoživotním systémovým metabolickým onemocněním. Ve snaze udržet normoglykémii jsou pacienti odkázáni na doživotní terapii inzulínem. Bohužel, ani při nejlepší spolupráci pacienta a lékaře, nemůže arteficielně dodávaný inzulín nahradit původní velmi jemnou regulaci sekrece inzulínu beta-buňkami podle aktuální glykémie, která odráží stav a potřeby celého organismu v každém okamžiku - reakce na psychické rozrušení, fyzickou zátěž, nemoc, růst apod. Proto je téměř každý pacient po letech léčby konfrontován s nepříjemnými sekundárními komplikacemi dlouhodobých kolísavých hyperglykémií - zejména diabetickou mikroangiopatií (nefropatie, retinopatie, neuropatie) a z ní pramenících důsledků (renální selhání a nutnost dialýzy, vysoký krevní tlak a ischemická choroba srdeční se srdečním selháním, slepota, diabetická noha s nutností amputace) [1-3]. Vezmeme-li v úvahu, že onemocnění začíná především v mladém věku a jeho incidence se zvyšuje, je jasné, proč se T1D věnuje tolik pozornosti. Snahou všech výzkumů k objasnění imunopatologie T1D je stanovit možnosti predikce a prevence rozvoje onemocnění nebo alespoň zpomalit jeho průběh. Vzhledem ke komplexnosti rozvoje T1D, jeho multifaktoriální etiologii, neznámému spouštěči a množství regulačních mechanismů, které se překrývají s fyziologickými mechanismy na obranu proti infekcím, je to úkol nadmíru náročný.
Z výzkumného hlediska možné imunoterapie je nejdůležitějším obdobím klinicky němá fáze prediabetu, kdy je již prolomena imunologická tolerance, dochází k destrukci beta-buněk a tvorbě protilátek, ale sekrece inzulínu je ještě relativně dostatečná. Vzhledem k absenci subjektivních potíží pacienta, je však téměř nemožné u většiny z nich toto období zachytit. Proto se velká část dosavadních klinických studií zaměřovala na pacienty s čerstvým záchytem T1D, u kterých se dá předpokládat ještě kolem 20% zbylých beta-buněk [47]. Imunoterapie v této skupině si klade za cíl zpomalit nebo zastavit jejich destrukci a snížit tak dávky arteficielně dodávaného inzulínu na minimum. Už jednou zničené beta-buňky však nemohou být z vlastních zdrojů obnoveny. Dosud provedené studie s podáváním různě upraveného inzulínu (alterované peptidové ligandy inzulínu nebo inzulín s různými nosiči pod kůži nebo přes nosní sliznici), vakcinace proinzulínem či ostrůvkovými nebo jinými antigeny (GAD65, Hsp60) ani užití jiných imunomodulačních léků (nikotinamid, ketotifen, cyklosporin, atd.), neměly ve většině případů očekávaný efekt. Na výsledky některých studií se zatím čeká (monoklonální anti-CD3 protilátky, dekosahexaenová kyselina DHA, vitamín D3 aj.) [47].

I přes výše zmíněné obtíže, se výzkum snaží zaměřit i na klinicky němé jedince ve fázi prediabetu. Jde především o skupinu příbuzných pacientů s T1D, u kterých lze určit genetické riziko, monitorovat přítomnost protilátek proti ostrůvkovým antigenům, která má vysoce prediktivní hodnotu pro rozvoj T1D, a hladiny glykémií [47]. Zůstává otázkou, jak nejlépe vytipovat "vysoce rizikové jedince", u kterých by bylo možné aplikovat imunoterapii (ať v podobě vakcinace k indukci imunotolerance či v podobě imunosupresiv), neboť nesprávně indikovaný jedinec by mohl být vážně poškozen. Nejnovější experimenty se zabývají především T lymfocytárními esejemi k vyhledávání autoreaktivních T lymfocytů po stimulaci ostrůvkovými antigeny pomocí produkce cytokinů a stanovením rizikových odpovědí.
Druhou velkou skupinu současných experimentů tvoří zkoumání populace Tregs, neboť existuje logická hypotéza, že jejich početní nebo funkční defekty mohou podpořit, ne-li přímo způsobit autoimunitní onemocnění. Problematika se zabývá rozdělením Tregs, optimalizací jejich značení, stanovením vhodných funkčních testů, eventuelně možnostmi jejich expanze a aplikace *in vivo*.
Jednou z dalších možností uplatnění znalosti imunopatologie T1D je i okruh pacientů po transplantaci Langerhansových ostrůvků, jejichž přijetí může ohrozit rejekce jak v důsledku allogenní povahy, tak i v důsledku antigenní homologie pankreatických autoantigenů.

II. PRAKTICKÁ ČÁST

Souhrn vlastní práce

1. Subjekty, hypotézy a cíle
    1.1 Část zaměřená na autoreaktivní T lymfocyty a cytokinovou produkci
    1.2 Část zaměřená na Tregs

2. Metodika
    a) Odběr biologického materiálu
    b) HLA II genotypizace
    c) Stanovení autoprotilátek proti ostrůvkovým antigenům
    d) Izolace PBMC
    e) T-buněčné eseje
    f) Proteinová array
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    h) Průtoková cytometrie

3. Výsledky
    3.1 Část zaměřená na autoreaktivní T lymfocyty a cytokinovou produkci
    3.2 Část zaměřená na Tregs

4. Diskuze
    4.1 Část zaměřená na autoreaktivní T lymfocyty a cytokinovou produkci
    4.2 Část zaměřená na Tregs

5. Závěr

V souhrnu vlastní práce předkládám a komentuji práce, jejichž originály jsou v příloze označeny čísly 1,3 a 5, tj. práce u kterých jsem hlavním autorem a v případě č.5 spoluautorem.

1. Subjekty, hypotézy a cíle:

Prováděli jsme klinický výzkum mezi pacienty s T1D, příbuznými pacientů s T1D a zdravými kontrolami, týkající se imunopatologie T1D, se zaměřením na období fáze "prediabetu". Cílem bylo zavedení jednotlivých metodik k objektivizaci dysregulace imunitního systému (autoreaktivní vs. regulační mechanismy), které by bylo možno použít ke zlepšení predikce T1D a eventuelně v budoucnosti i k prevenci tohoto onemocnění (imunomodulační terapií). Všem subjektům bylo stanoveno genetické riziko pro rozvoj T1D podle HLA II typizace (alely DQ, DR) pro možnost korelace se získanými výsledky a všem byly vyšetřovány autoprotilátky proti ostrůvkovým autoantigenům (anti -"glutamic acid decarboxylase" GAD65 a anti-"islet antigen" IA2).

1.1 Část zaměřená na autoreaktivní T- lymfocyty a cytokinovou produkci

Klinická data pocházejí převážně od dětských pacientů s T1D, kteří byli léčeni na Pediatrické klinice FN Motol, jejich prvostupňových příbuzných (rodiče, sourozenci) a zdravých kontrol. Tato část výzkumu byla zaměřena na monitorování imunitní odpovědi pomocí cytokinové produkce po stimulaci periferních mononukleárních buněk (PBMC) diabetogenními autoantigeny *in vitro* - zavedení metodiky vyhledávání autoreaktivních T lymfocytů. Předpokládali jsme, že pokud se v periferii testovaného subjektu nachází diabetogenní autoreaktivní T lymfocyty, rozpoznají jejich PBMC podané autoantigeny a zareagují změnou cytokinové produkce, kterou bude možno detekovat. K zachycení celého cytokinového spektra a zjištění typu imunitní odpovědi (rizikový Th1 vs. protektivní Th2, Th3) jsme použili metodu proteinové "array" (příloha 3).

U pacientů s čerstvým záchytem T1D nás zajímal typ imunitní odpovědi v čase diagnózy a s odstupem 6 a 12 měsíců, zda došlo k její změně. U zdravých příbuzných pacientů s T1D jsme pomocí stejné eseje také sledovali reaktivitu PBMC při diabetogenní stimulaci a vyhledávali rizikové odpovědi. Zdravé kontroly sloužili jako referenční skupina, u které jsme po diabetogenní stimulaci očekávali spíše minimální nebo protektivní (protizánětlivou) změnu v cytokinové produkci.

1.2 Část zaměřená na Tregs

Klinická data pocházejí od prvostupňových příbuzných dětských pacientů s T1D (rodiče, sourozenci) a zdravých kontrol. Pacienti byli léčeni na Pediatrické klinice FN Motol a 2.LF UK nebo na 1.dětské klinice FN Brno a LF MU. Dále byla využita data z Registru diabetických dětí a jejich sourozenců vedeném při Pediatrické klinice FN Motol a 2.LF UK v rámci Programu predikce diabetu 1.typu v České republice.

Tato část výzkumu se týkala stanovení počtu a testování funkce regulačních T lymfocytů, zejména $CD4^+CD25^+$ Tregs u prvostupňových příbuzných pacientů s T1D a zdravých kontrol. Zajímaly nás početní a funkční defekty této populace u příbuzných pacientů s T1D, kteří v závislosti na HLA II nesou různé riziko pro rozvoj onemocnění. V poslední práci (příloha 1) jsme záměrně testovali populaci $CD4^+CD25^+$ Tregs pouze u příbuzných nesoucích výhradně rizikové alely pro rozvoj T1D, u kterých jsme očekávali nejvíce patrné rozdíly v porovnání se zdravými kontrolami. Počátečním cílem bylo seznámení se s populací Tregs, zavedení metodiky jejich detekce na průtokovém cytometru, nejdříve pomocí molekuly CD25, později na základě objevení se nových znaků, i značením molekul FoxP3 a CD127 a jejich srovnáním. Dále jsme prováděli izolaci $CD4^+CD25^+$ Tregs metodou magnetické separace a testovali jejich funkci. V první práci (příloha 5) jsme jejich funkci testovali méně specificky, ale přímo, přidáváním magneticky selektované populace $CD4^+CD25^+$ Tregs v různém poměru k nespecificky stimulovaným PBMC (polyklonálním aktivátorem phytohemaglutininem (PHA) či ozářenými allogenními PMBC), abychom dokázali, zda je tato populace skutečně schopna tlumit zánětlivou reakci a to i při tak výrazném podnětu. Ve druhé práci (příloha 1) jsme funkci $CD4^+CD25^+$ Tregs testovali více specificky, ale nepřímo, měřením produkce IFNγ po stimulaci specifickými diabetogenními antigeny, současně za použití nejnovějších markerů k označení Tregs, FoxP3 a CD127. Také nás zajímalo, zda dojde ke změně exprese FoxP3 či CD127 po stimulaci v jednotlivých skupinách a jak se změní produkce IFNγ.

2. Metodika

Podrobně, včetně použitých chemikálií a jejich výrobců, je metodika popsána v jednotlivých publikacích. Zde stručně shrnuji:

a) Odběr biologického materiálu: Všem testovaným subjektům byla odebrána periferní krev k izolaci PBMC (Lithium-Heparin; 10-100ml), ke stanovení protilátek proti ostrůvkovým antigenům (Citrát; 1-2ml) a ke stanovení HLA II genotypu (EDTA; 4ml).

b) HLA II genotypizace (MUDr.Ondřej Cinek PhD., Laboratoř molekulární genetiky, Pediatrická klinika FN Motol a 2.LF UK):

Všem testovaným subjektům byl stanoven HLA II genotyp (alely DQ, DR) pomocí sekvenčně specifických primerů. Podle HLA DQ a DR alel byly subjekty rozděleny do skupin genetického rizika. Stratifikace byla upravena dle našich potřeb na 3 kategorie (z původních 5, které byly uvedeny v tab.2 v části 2.3 Teoretického úvodu): nositele rizikových alel a haplotypů, nositele protektivních alel a ostatní (tab.6).

Tab.6 Genetické riziko pro rozvoj T1D

(odvozeno a upraveno podle tab. 2)

Riziko	HLA - alely DQ, DR
1. vysoké	přítomnost DQ A1*05 - B1*0201 a/nebo *0302, současně nepřítomnost DQ B1* 0301,*0602, *0603 a DR B1*0403
2. střední	všechny ostatní
3. nízké	přítomnost DQ B1*0602

c) Stanovení autoprotilátek (Marta Pechová, Ústav biochemie FN Motol a 2.LF UK):
Hladiny protilátek proti ostrůvkovým antigenům (anti-GAD65 a anti-IA-2) byly stanoveny pomocí radioimunoeseje, za pozitivní byly považovány hodnoty nad 1 IU/ml (tj.nad 99.perc.).

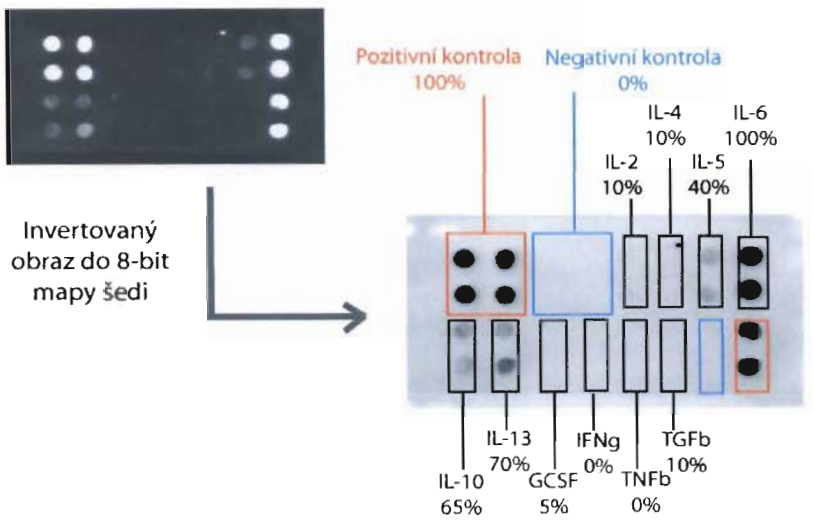
d) Izolace PBMC: Získání PMBC z periferní krve probíhalo podle standardních postupů pro hustotní gradientovou centrigufaci.

e) T-buněčné eseje: Vlastní stimulační eseje vycházely z doporučení "workshopů" Immunology of Diabetes Society [64, 65]. K čerstvě izolovaným PBMC (koncentrace $2x10^6$ buněk/ml) v kultivačním mediu s tepelně inaktivovaným bovinním sérem nebo u části pokusů

s AB lidským sérem byla přidána směs diabetogenních autoantigenů (specifické peptidové sekvence GAD65 - a.a.247-279, a.a. 509-528, a.a.524-543, IA2 a.a. 853-872, β-řetězce proinzulínu - a.a.9-23) a u části pokusů i samotný peptid inzulínu. U pozitivních kontrol jsme přidávali PHA a u části pokusů ozářené allogenní PMBC. Negativní kontroly tvořili samotné PMBC v kultivačním mediu. Kultivace trvala 72-96 hodin (37°C, 5% atmosféra CO2), poté byl zvlášť odebrán a zpracován supernatant a buňky. Pokud nedošlo k okamžitému zpracování, byl supernatant zamražen na -20°C a buňky po přidání DMSO (dimethylsulfoxid) na -72°C.

f) Proteinová array: Stanovení cytokinové produkce pomocí proteinové array je založeno na principu inkubace supernatantu na membráně, na které jsou na definovaných místech navázány primární anti-cytokinové protilátky. Po první vazebné fázi a následném promytí nastává fáze vazby sekundárních protilátek a jejich detekce chemiluminiscencí za použití chemiluminiscenčních činidel nebo fotoreakcí za použití fotoreaktivních činidel. Stanovovali jsme 10-(23) cytokinů. V místě navázání cytokinu na membránu je patrný spot - v případě chemiluminiscence světlý oproti černému pozadí, v případě fotoreaktivity tmavý oproti světlému pozadí. Jako kontroly jsou na membráně vždy pozitivní spoty (dosahující 100% intenzity) a negativní spoty (0% intenzity) a každý cytokin v dubletu. Hodnocení membrány bylo visuální, semikvantitativní, za pomoci digitálního zpracování obrazu a principů intenzity jasu a odstínů šedi jednotlivých spotů, průměr intenzity z dubletů (obr.4). Detekční limity koncentrace cytokinů byly stanoveny výrobcem (www.raybiotech.com) [66].

Obr.4 Ukázka hodnocení membrány "protein array"
(detekce sekundární protilátky chemiluminiscencí)

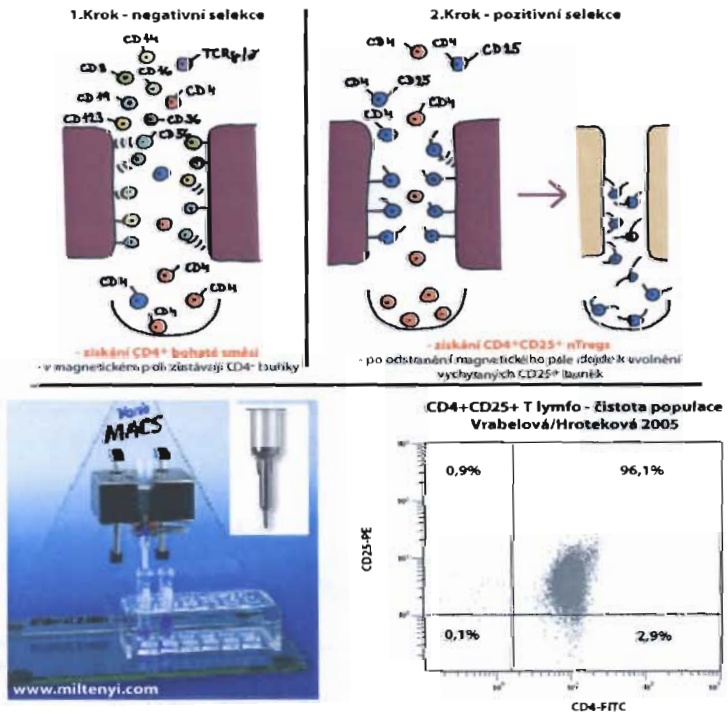


Detekční limity (pg/ml)	
IL-2	25
IL-4	1
IL-5	1
IL-6	1
IL-10	10
IL-13	100
GCSF	2000
IFNg	100
TNFb	1000
TGFb	200

g) Magnetická separace $CD4^+CD25^+$ Tregs a testy schopnosti imunosuprese: Separace $CD4^+CD25^+$ populace z PBMC probíhala ve dvou následných krocích v silném magnetickém poli pomocí protilátek s navázanými magnetickými částicemi. Prvním krokem je získání $CD4^+$ směsi, negativní selekcí, za použití koktejlu protilátek (proti CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, glykoforinu A). Buňky, které nesou některý z těchto znaků, zůstávají zachyceny v magnetickém poli a další separace probíhá se zbývající populací, která je převážně $CD4^+$. Ve druhém kroku se tato populace značí protilátkou CD25 a pozitivní selekcí (v magnetické poli zůstávají $CD25^+$ buňky) získáme po odstranění magnetického pole populaci $CD4^+CD25^+$ Tregs (obr.5). Čistota získané populace lze ověřit pomocí průtokové cytometrie. V našich pokusech dosahovala nad 90% a celkový počet buněk tvořil kolem 0,5-1% z původního počtu PBMC. Podrobný postup lze najít na www.miltenyi.com (63). Izolovaná populace byla použita k in vitro pokusům. PMBC byly přidávány k Tregs v různém poměru (1:1, 2:1, 4:1, atd.) a pomocí stimulace PHA (10ul/ml kultivačního media, 24 a 48 hod stimulace v 5% CO2, při 37°C) nebo ozářenými allogenními PBMC ($1x10^6$/ml, v poměru 1:1 k PBMC v kultivačním mediu, 24 a 48hod stimulace v 5%CO2, při 37°C) byla testována imunosupresní schopnost pomocí produkce IFNγ.

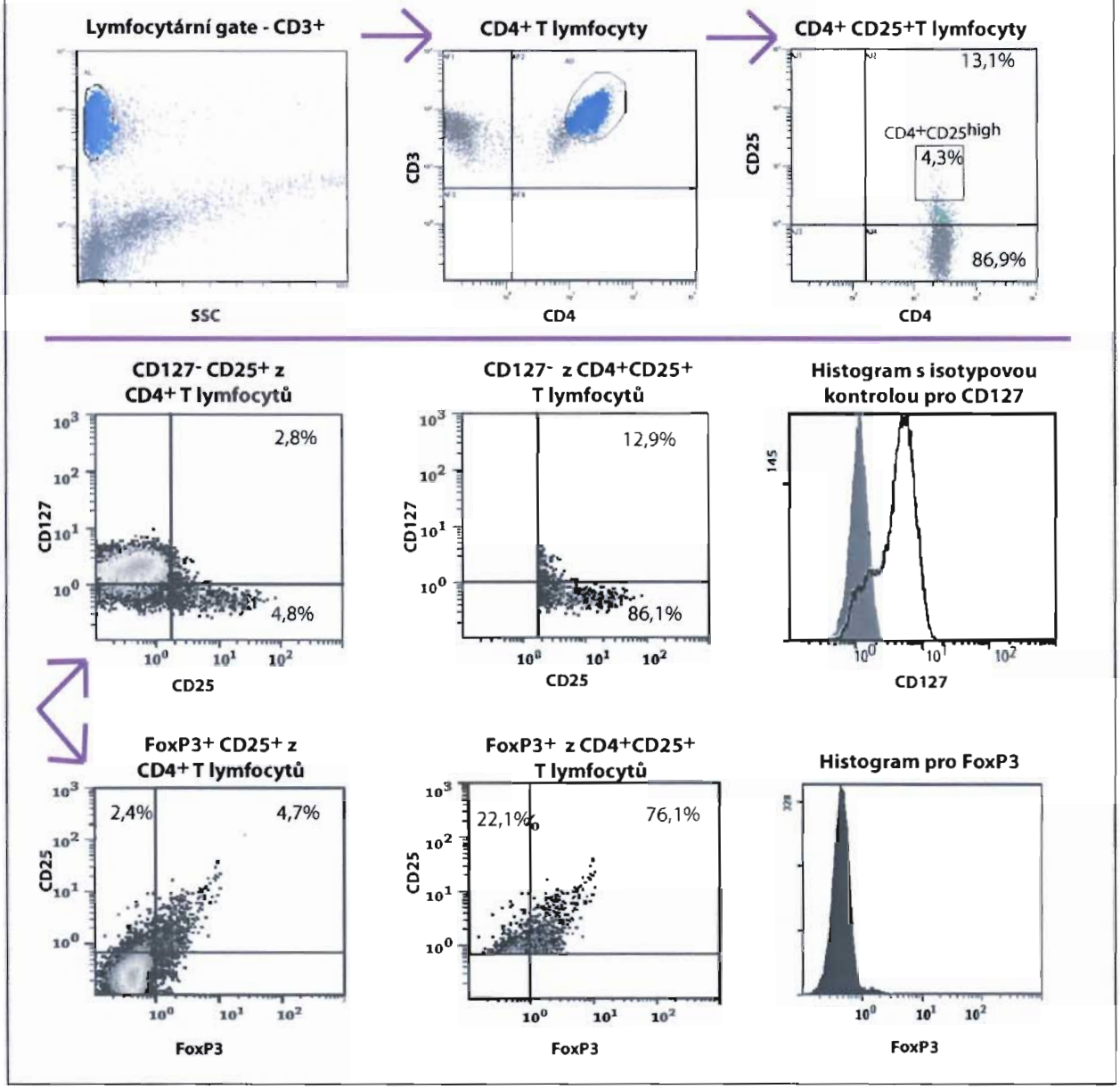
Obr.5 Magnetická separace $CD4^+CD25^+$ T lymfocytů

Princip magnetické separace $CD4^+CD25^+$ T lymfocytů s použitím "VarioMacs", s ukázkou čistoty získané populace na průtokovém cytometru



h) Průtoková cytometrie: Jednotlivé lymfocytární subpopulace jsme stanovovali za použití čtyř-barevné průtokové cytometrie a monoklonálních protilátek anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD127, anti-TCRα24, anti-TCRβ11, anti-FoxP3 a anti-IFNγ značených fluorescenčními barvami: fluorescein-isocyanát (FITC), phycoerythrin (PE), phycoerytrin-cyanin 5 (PC5), phycoerythrin-cyanin 7 (PC7). V případě detekce intracelulárního znaku FoxP3 a povrchové sekrece IFNγ jsme použili speciální kity a barvení dle návodu (www.miltenyi.com, www.e-bioscience.com). K vyhodnocení výsledků byl využit analyzační software cytometru. Gatovací strategie jsou znázorněny na obrázku č.6.

Obr.č.6 Ukázka gatovací strategie $CD4^+CD25^+$ Tregs ($CD127^-$, $FoxP3^+$)



3. Výsledky:

Podrobné výsledky, včetně číselných hodnot, grafů a použití statistických metod, jsou uvedeny v jednotlivých publikacích. Níže jsou shrnuty nejdůležitější zjištění.

3.1 Část zaměřená na autoreaktivní T- lymfocyty a cytokinovou produkci

Produkce cytokinů se v jednotlivých skupinách (příbuzní vs. zdravé kontroly vs. pacienti s T1D v době diagnózy, za 6 a 12 měsíců) lišila, a to již v jejich bazální sekreci (tj. po 72-96 hodinách kultivace v čistém kultivačním mediu). Po stimulaci docházelo ke změnám cytokinové produkce, spíše ve smyslu poklesu a stabilizace, která byla nejvíce patrná ve skupině příbuzných. Jen některé změny byly signifikantní (příloha 3).

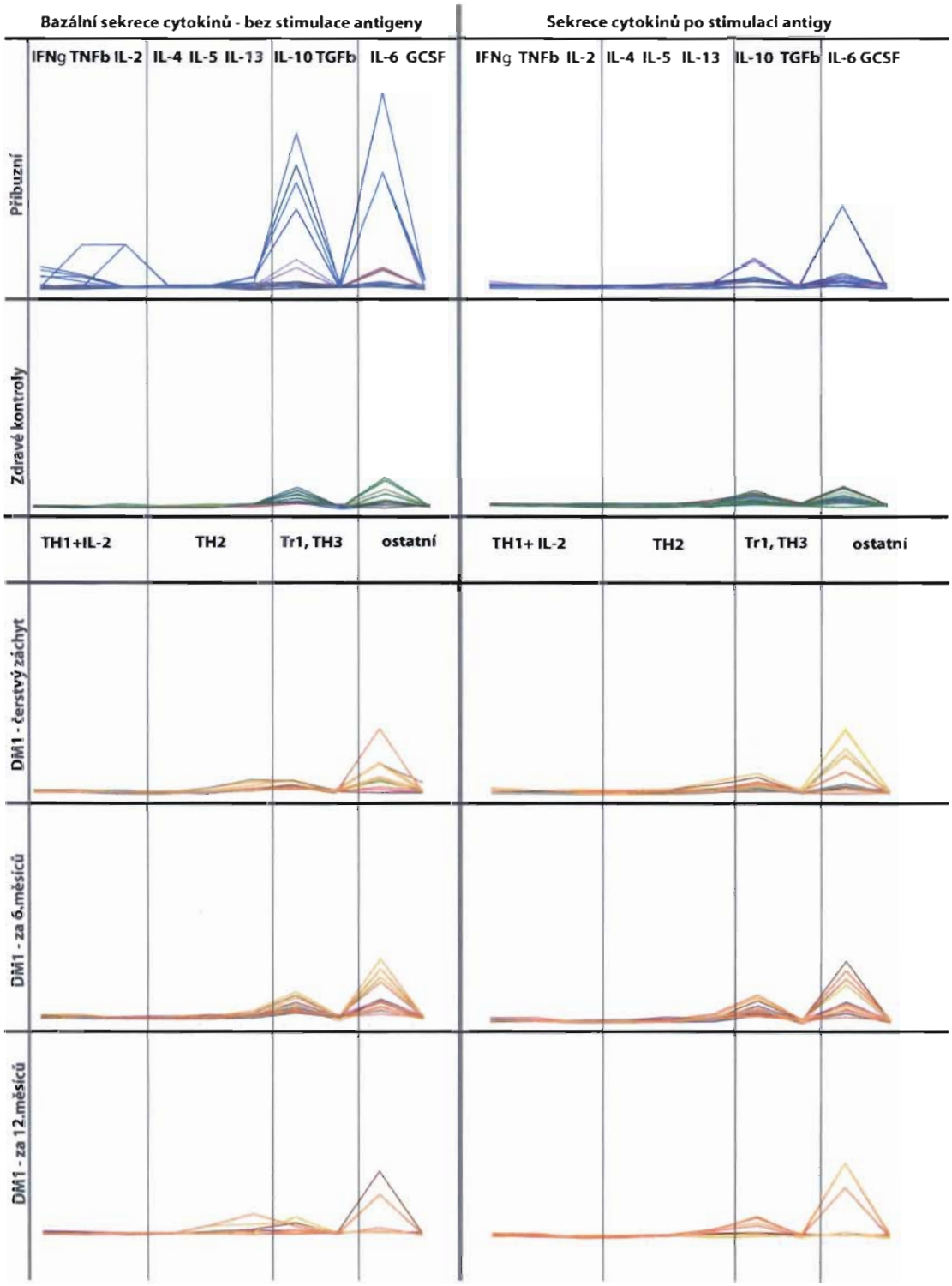
Skupina zdravých kontrol vs. příbuzných

Zdravé kontroly měly oproti příbuzným pacientů s T1D v nativním stavu signifikantně vyšší produkci cytokinů Th2 a Th3 spektra (IL-4, IL-5 a IL-13 a TGFβ), ale i IL-2 a GCSF. U zdravých příbuzných pacientů s T1D byla naopak, ve srovnání se zdravými kontrolami, signifikantně vyšší produkce obecně prozánětlivého cytokinu IL-6. Odchylky v produkci cytokinů z Th1 spektra (IFNγ a TNFβ) a IL-10 mezi zdravými kontrolami a příbuznými nebyly signifikantní. U příbuzných pacientů s T1D převládala v bazální produkci cytokinů velká variabilita, zatímco u zdravých kontrol nikoli. Tento fakt byl matematicky objektivizován pomocí Spearmanovy korelace. U příbuzných existovalo pouze částečné propojení mezi jednotlivými cytokinovými spektry, skupina cytokinů Th1 měla s většinou ostatních cytokinů nízký korelační koeficient. Oproti tomu, u zdravých kontrol byly korelační koeficienty jednotlivých cytokinů mezi sebou vysoké a skupina cytokinů z Th1 spektra přímo, nebo nepřímo souvisela se všemi ostatními cytokinovými spektry.

I přes pokles v cytokinové produkci po stimulaci autoantigeny, přetrvávala u zdravých kontrol v porovnání s příbuznými vyšší produkce cytokinů Th2, Th3 spektra i IL-2, GCSF a nižší produkce IL-6, ale tyto hodnoty nebyly signifikatní. U zdravých kontrol dále došlo po stimulaci k signifikantnímu poklesu IL-2, IFNγ, IL-13 a GCSF. U příbuzných pacientů s T1D se po stimulaci v produkci cytokinů neodehrála žádná statisticky významná změna. V této skupině se však snížila variabilita v cytokinové produkci (obr.7). Při provedení Spearmanovy analýzy po stimulaci, přetrvávaly ve skupině příbuzných nízké korelační koeficienty mezi cytokiny z Th1 spektra a všemi ostatními cytokiny. Oproti tomu, se u zdravých kontrol po stimulaci propojení mezi všemi cytokinovými spektry posílilo(obr.8)

Obr.7. Imunitní odpovědi v cytokinové produkci - schématické znázornění:

Jednotlivé nativní hodnoty intenzit spotů z membrán proteinové arraye jednotlivých cytokinů byly zaneseny do grafů, jednotlivé křivky patří jednotlivým pacientům v dané skupině, spojitý charakter křivek je zvolen pouze ilustrativně, neznamená skutečné zachycení vztahů mezi jednotlivými cytokiny, poměry velikostí jsou zachovány.



Skupina pacientů s T1D

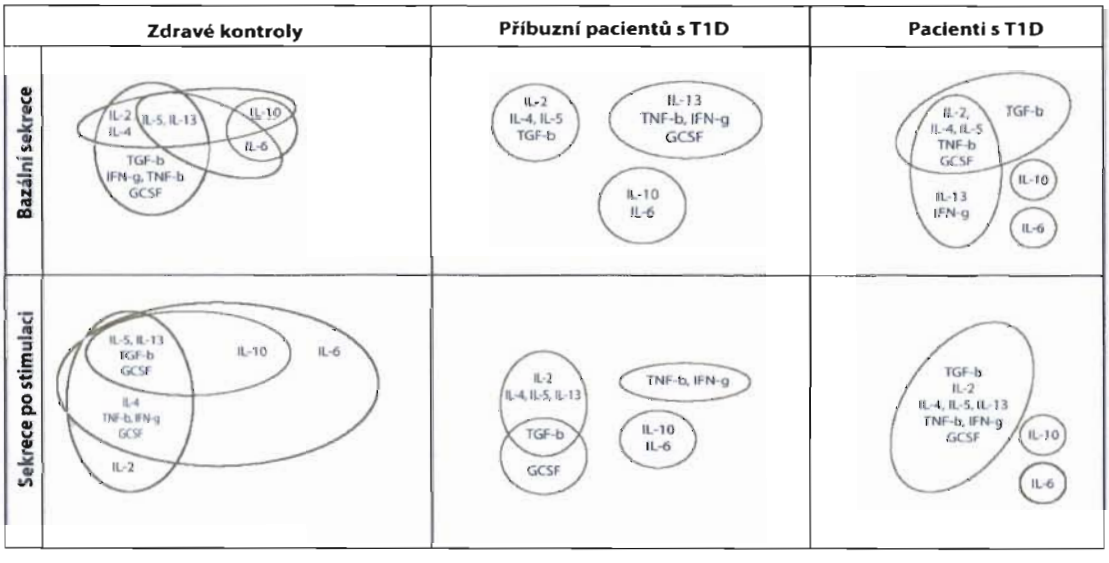
V bazální sekreci cytokinů skupiny pacientů s T1D, v období od stanovení diagnózy do 12-ti měsíců poté, reagovaly převážně bez statické významnosti mezi sebou. Při srovnání pacientů s T1D se zdravými kontrolami, nebyly v bazálních sekrecích cytokinů žádné signifikantní odchylky, ale u zdravých kontrol po celou dobu převažovala vyšší sekrece IL-2, IL-4 a nižší sekrece IL-6. Při srovnání pacientů s T1D a příbuzných pacientů s T1D se v bazální sekreci objevila pouze signifikantně vyšší produkce IL-13 a TNFβ u pacientů s T1D po 6-ti měsících od stanovení diagnózy, ostatní odchylky nebyly statisticky významné.

Po stimulaci diabetogenními autoantigeny nastala signifikatní změna pouze ve skupině pacientů s T1D za 12 měsíců od diagnózy. Došlo zde k poklesu sekrece IL-2, IL-4, INFγ a IL-6. Celková situace se mezi pacienty s T1D, zdravými kontrolami a příbuznými s T1D po stimulaci nezměnila.

Po provedení Spearmanovy analýzy u pacientů s T1D cytokinové spektrum Th1 korelovalo s Th2 i IL-2. Po stimulaci také s Th3 (TGFβ). Oproti tomu zde nebyl žádný vztah mezi IL-10 a IL-6 a ostatními cytokiny. Pacienti s T1D navíc byly jedinou skupinou, kde nebyla Spearmanovou analýzou prokázána spojitost mezi IL-10 a IL-6 a to jak v bazálním tak v postimulačním stavu.

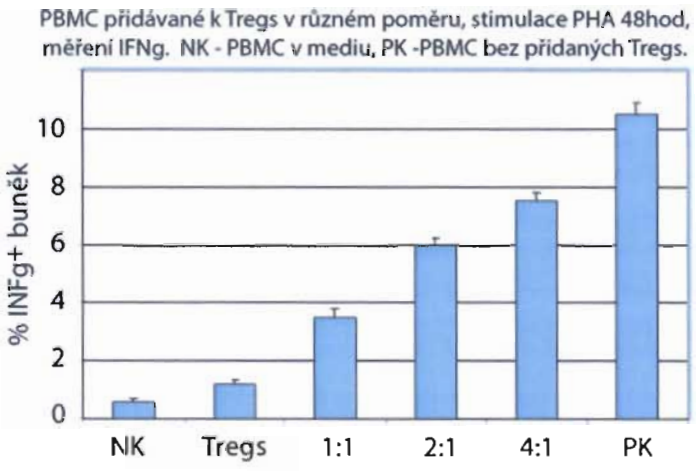
Jednotlivé grafy a statistické významnosti jsou zachyceny v publikaci. Na obrázku č.7 je pouze ilustrativní obrázek nativních hodnot, který dává představu o "vzorech" odpovědí jednotlivých skupin. Na obrázku č.8 jsou graficky znázorněná matematické korelace jednotlivých cytokinů.

Obr.8 Grafické znázornění Spearmanovy korelace mezi cytokiny a skupinami



3.2 Část zaměřená na Tregs

Obr.9. Imunosupresivní schopnost Tregs



Magnetickou separací se nám podařilo získat vysoce čistou populaci $CD4^+CD25^+$ Tregs, u které jsme, při pokusech *in vitro*, potvrdili na dávce závislou schopnost imunosuprese, jak při stimulaci PHA, tak při stimulaci ozářenými allogenními PBMC (obr.9). U příbuzných pacientů s T1D jsme prokázali početní i funkční defekty v $CD4^+CD25^+$ Tregs, v souvislosti s jejich HLA II genotypem. Pomocí znaků CD25, FoxP3 a CD127 a jejich srovnáním, jsme zmapovali populaci $CD4^+CD25^+$ Tregs.

V první práci (příloha 5) týkající se regulačních lymfocytů byla měřena frekvence $CD4^+CD25^+$ Tregs pouze pomocí exprese $CD25^{high}$ a dále frekvence NKT u sourozenců dětí s T1D. Potvrdili jsme signifikantně snížený počet $CD4^+CD25^+$ Tregs ve skupině sourozenců s rizikovým HLA II genotypem pro rozvoj T1D. Ve skupině sourozenců se standardním a nízkým genetickým rizikem pro rozvoj T1D nižší počty $CD4^+CD25^+$ Tregs, ve srovnání s počty u zdravých kontrol, nedosáhly statistické významnosti. Frekvence populace NKT buněk nebyla u sourozenců ve srovnání se zdravými kontrolami snížena v žádné skupině genetického HLA rizika.

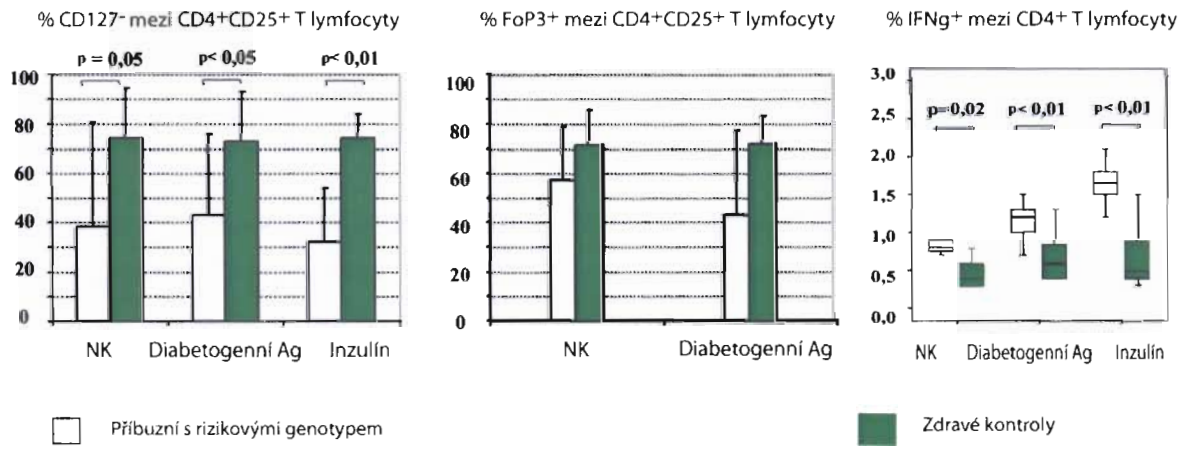
V následující práci (příloha 1) jsme se již zaměřili výhradně na populaci $CD4^+CD25^+$ Tregs u rizikových (tj.nesoucích výhradně rizikové alely pro rozvoj T1D) prvostupňových příbuzných pacientů s T1D v porovnání se zdravými kontrolami, za použití vhodnějšího intracelulárního markeru FoxP3 a nejnovějšího povrchového markeru CD127. Frekvenci $CD4^+CD25^+$ Tregs mezi PBMC jsme měřili u příbuzných i zdravých kontrol přímo a poté znovu po stimulaci diabetogenními antigeny a inzulinem (v porovnání s negativními kontrolami- nestimulovanými PBMC a pozitivními kontrolami - PBMC stimulovanými PHA). Současně jsme po stimulaci stanovovali produkci povrchového IFNγ u obou dvou skupin.

Potvrdili jsme přetrvávající nižší frekvence $CD4^+CD25^+$ Tregs u prvostupňových příbuzných s rizikovým HLA II genotypem, jak při použití znaku CD127, tak při použití znaku FoxP3. Statistická významnost však byla prokázána pouze při použití znaku CD127. Rozdíly mezi příbuznými s rizikovým genotypem a zdravými kontrolami se dále prohloubily, pokud byla frekvence $CD4^+CD25^+$ Tregs 127 nebo $FoxP3^+$ buněk vztažena pouze k populaci $CD25^+$

buněk. U zdravých kontrol buňky nesoucí znak CD25 byly ve většině případů $CD127^-$ a $FoxP3^+$, u rizikových příbuzných bylo procento $CD127^-$ a $FoxP3^+$ buněk mezi $CD25^+$ buňkami nižší. Ani ve skupině zdravých kontrol ani ve skupině rizikových příbuzných nedošlo v populaci $CD4^+CD25^+$ Tregs po 72-96 hodinách stimulace diabetogenními antigeny a peptidem inzulínu k signifikatní změně CD127 negativity nebo FoxP3 pozitivity. U příbuzných přetrvávaly nižší frekvence $CD4^+CD25^+$ Tregs, které opět dosáhly statistické významnosti pouze pro znak CD127. Oproti tomu, jsme u skupiny rizikových příbuzných po stimulaci, ve srovnání se zdravými kontrolami, při měření povrchové exprese IFNγ, prokázali signifikantně vyšší frekvence $IFN\gamma^+$ buněk. Rozdíl byl patrný již u negativních kontrol (samotné PBMC v kultivačním mediu), dále se zvýraznil po stimulaci směsí diabetogenních peptidů a nejvýraznějším se stal po stimulaci celým peptidem inzulínu (obr.10).

Obr.10 Frekvence Tregs a produkce IFNγ po stimulaci

% $CD127^-$ a $FoxP3^+$ mezi $CD4^+CD25^+$ T lymfocyty během stimulace inzulínem a směsí diabetogenních antigenů a následné vyjádření % IFNγ+ mezi CD4+ T lymfocyty. Doplněno negativními kontrolami (PBMC v kultivačním mediu). Srovnání skupiny příbuzných T1D pacientů s rizikovým genotypem pro rozvoj onemocnění a zdravých kontrol.



4. Diskuze

4.1 Část zaměřená na autoreaktivní T- lymfocyty a cytokinovou produkci

Multiparametrová analýza proteinovou "array" je výhodná z hlediska možnosti detekce kombinace různých cytokinů v supernatantu v jedné době [67]. Jde o semikvantitativní metodu, kdy se u pozitivity spotů, představujících jednotlivé cytokiny, můžeme vyjádřit k jejich intenzitě a kombinaci. Proteinová "array" je nejvíce limitována detekčními limity membrány pro koncentrace cytokinů. K přesnému stanovení množství jednotlivých cytokinů je vhodnější přístup například za pomocí ELISA, měřením exprese mRNA, použitím "Luminex Quatibody Array", apod. [73, 76]. Pro screeningové účely, k vytipování rizikových imunitních odpovědí po diabetogenní stimulaci, jsme považovali tuto metodu za velmi vhodnou. Při zavádění metodiky jsme se věnovali vypracování postupu odečtu výsledků z jednotlivých membrán. Nejnáročnější částí byla samotná analýza dat.

V naší práci jsme prokázali, že zdravé kontroly již v nativním stavu produkovaly více cytokinů z Th2 a Th3 spektra a že reagovaly na stimulaci diabetogenními autoantigeny. Po stimulaci však u nich došlo k signifikantnímu poklesu IFNγ, IL-13, IL-2 a GCSF, tedy k protektivní, protizánětlivé imunosupresní odpovědi. Po celou dobu měly, v porovnání s pacienty s T1D i s příbuznými, nižší produkci IL-6. Při matematické korelaci jednotlivé skupiny cytokinů (Th1,Th2, Th3) spolu dobře korelovaly, včetně IL-6 a IL-10. Domníváme se, že jde o projev fyziologické regulace imunitní odpovědi.

U žádné ze skupin pacientů s T1D ani u příbuzných pacientů s T1D jsme po stimulaci autoantigeny neprokázali reaktivitu Th1 typem imunitní odpovědi, ale popisujeme určitou míru dysregulace. Relativně nižší produkce některých cytokinů (IL-4, IFNγ, aj.) byla v souladu s výsledky ostatních [71,72]. Zajímavá byla trvale vysoká produkce IL-6 u obou těchto skupin v porovnání se zdravými kontrolami, která je v souladu s popisovanou vyšší hladinou IL-6 u chronických onemocnění, včetně autoimunitních (např.Crohnovy choroby) [68]. IL-6 je cytokin s pleiotropním imunomodulačním účinkem, zejména prozánětlivým - na počátku zánětu je jeho silným induktorem [68,69]. V souvislosti s časnějším rozvojem T1D je u žen publikován výskyt polymorfismu v promotoru pro IL-6 "174G>C SNP" a zkoumají se i polymorfismy další [69].

Dále pak pacienti s T1D a zejména příbuzní vykazovali výrazně vyšší variabilitu v bazální cytokinové sekreci a menší předvídatelnost v imunoreaktivitě, jejímž nepřímým důsledkem mohla být i statistická nevýznamnost změn při relativně malém počtu testovaných subjektů.

U obou dvou těchto skupin existovaly nízké korelační koeficienty, tedy určitá nepropojenost Th1 cytokinového spektra s ostatními protektivními (protizánětlivými) spektry imunitní odpovědi (Th2, Th3, IL-10 aj.). Navíc jsme při Spearmanově korelační analýze neprokázali, ani u pacientů s T1D ani u příbuzných, korelaci IL-6 s ostatními cytokiny. U skupiny pacientů s T1D byl tento fakt posílen absencí korelace IL-6 s IL-10, která byla jak u zdravých kontrol, tak u zdravých příbuzných zachována. U příbuzných IL-10 koreloval pouze s IL-6 a nikoli s ostatními cytokiny, zatímco u zdravých kontrol IL-10 se všemi ostatními cytokiny nepřímo souvisel. Účinky IL-10 a IL-6 se dají považovat za protikladné. IL-10 je silný protizánětlivý cytokin, který díky schopnosti interakce s transkripčním faktorem aktivovaných T lymfocytů NF-kB ovlivňuje imunitní aktivaci na počátku zánětu [68]. Za fyziologických podmínek se ale IL-6 a IL-10 navzájem modulují, protože po vazbě na své receptory na cílových buňkách ovlivňují stejnou signalizační cestu STAT3 a její regulační molekuly SOCS3, každý s jinou mírou účinnosti [68-70]. V případě vyšších hladin IL-6 u chronických zánětlivých onemocnění, bývají i vyšší hladiny STAT3 a SOCS3 [68]. Matematicky zjištěný nízký korelační koeficient mezi IL-6 a IL-10 u skupiny pacientů s T1D stejně jako nízké korelační koeficienty mezi IL-6, IL-10 a ostatními cytokiny u skupiny příbuzných by tak mohly potvrzovat poruchu regulace imunitní odpovědi u těchto skupin. Jsme si však vědomi relativně malého počtu dat, proto se tato domněnka neobjevila v diskuzi publikovaného článku (příloha 3).

Po provedení experimentů považujeme proteinovou array i nadále za vhodnou pro další pokusy na větším souboru pacientů k hledání "optických" (kombinace a intenzita spotů) i matematicky prokázaných závislostí v cytokinové síti. Je možné, že velký objem dat umožní odkrýt jednotlivé "vzory" odpovědí (viz.návrh na ilustračním obrázku č.7), z nichž některé bude možné považovat za rizikové a těmi se dále zabývat i v souvislosti s HLA II genotypem. Ten jsme nyní, vzhledem k malému počtu subjektů a rozmanitosti výsledků, do souvislostí s jednotlivými typy odpovědí nedávali. Stejně tak jsme se nevyjadřovali k pozitivitě protilátek proti ostrůvkovým antigenům, které jsou sice vysoce prediktivní pro rozvoj T1D [47], ale většina námi testovaných subjektů měla tyto protilátky negativní. Za přínosné považujeme i zjištění, že existují rozdíly již v bazální sekreci cytokinů. Celkové poznatky o komplexní cytokinové produkci po stimulaci diabetogenními autoantigeny u lidí, v souvislosti s T1D, jsou dosud omezené [78]. Častěji existují práce s užším zaměřením na hladiny jednotlivých cytokinů a jejich poměry (IL-4, IFNγ, IL-10, apod.), které však mohou zakrývat širší souvislosti [71-77].

4.2 Část zaměřená na Tregs

U autoimunitních onemocnění se předpokládá porucha regulace imunitního systému, mezi kterou patří i početní a funkční defekty Tregs [78-80]. Početní defekty lze prokázat pomocí průtokové cytometrie a vhodně zvolené kombinace jednotlivých znaků Tregs. U T1D existují rozporuplné práce, z nichž některé prokazují nízké počty Tregs [81, 82] a jiné nikoli [83-85]. Problém je především ve výběru subjektů a ve zvolených znacích pro definici populace Tregs. V porovnání s animálními modely tvoří humánní data menší část. Navíc některé práce jsou od pacientů s T1D [78], jiné od příbuzných pacientů s T1D [85] a míra genetického rizika není vždy zohledněna [78, 83, 84]. Průkaz funkčních defektů může být složitější, neboť může existovat porucha vývoje v thymu [58, 59], vzniku v periferii nebo snížená schopnost imunosupresní funkce [81, 83]. Metody průkazu funkčních defektů by se měly odvíjet v závislosti na zvolené regulační populaci (iTregs vs.nTregs).

Již z teoretického úvodu vyplývá, že výzkum v oblasti Tregs prochází v posledních letech neustálou změnou. Objevují se nové poznatky o vzniku, vývoji i funkci, mění se klasifikace a hledají se stále přesněji definující markery. Původní znak, podle kterého byla populace Tregs v roce 1995 definována, vysoká exprese molekuly CD25 [60], si vyžádal vzhledem ke své ambivalenci a obtížnému zvolení gatovací strategie na průtokovém cytometru revizi a byl nahrazen intracelulární molekulou FoxP3 [50]. V roce 2006 pak byly publikovány 2 práce, které uvedly další znak Tregs - povrchovou molekulu CD127, resp. že $CD127^{-/low}$ exprese může být novým markerem regulační populace. Práce srovnávali expresi FoxP3 a CD127 negativitu a oba jevy spolu dobře korelovaly [56,57]. Objevily se hypotézy o negativní interakci FoxP3 s promotorem pro CD127 [57]. Marker CD127 je v porovnání s FoxP3 vhodnější pro laboratorní účely vzhledem ke své lokalizaci na povrchu buněk a tím snadnějšímu značení a možnosti dalšího použití takto definovaných Tregs (využití magnetické separace k pokusům *in vitro*).

V naší první práci (příloha 5) jsme ke značení regulační populace použili povrchový znak CD25, neboť v době experimentů byl FoxP3 teprve diskutovaným a nově zaváděným markerem. Ve druhé práci (příloha 1) jsme již značili regulační populaci standardně pomocí FoxP3 a testovali jsme i nově zmíněný znak CD127. V první práci jsme prokázali početní defekty $CD4^+CD25^+$ Tregs ve skupině prvostupňových příbuzných, kteří byli nositeli rizikových alel pro rozvoj T1D. V počtech NKT nebyly signifikatní rozdíly. Proto jsme se v následující druhé práci, za využití dat z Registru diabetických dětí a jejich příbuzných vedeném při Pediatrické klinice FN Motol a 2.LF UK, zaměřili pouze na skupinu příbuzných nesoucích rizikové alely pro rozvoj T1D a použili nové, již zmíněné, markery pro značení

$CD4^+CD25^+$ Tregs. Opět jsme prokázali nižší počty $CD4^+CD25^+$ Tregs u skupiny příbuzných nesoucích rizikové alely pro rozvoj T1D v porovnání se zdravými kontrolami, ale signifikantní tentokrát byly jen výsledky vyjádřené pomocí míry negativity CD127. Při použití FoxP3 byly frekvence $CD4^+CD25^+$ Tregs také nižší, ale bez statistické významnosti. Tento ambivalentní výsledek mohl souviset s počtem testovaných subjektů, použitou metodou (čtyř-barevná průtoková cytometrie), rozdílnou metodikou barvení (intracelulární vs.povrchové), ale také s faktem, že ne všechny $FoxP3^+$ buňky musejí být nutně regulačními [61]. Regulační funkce může souviset s mírou a stabilitou exprese FoxP3 a jeho izoformou [30,57, 61]. Korelaci mezi FoxP3 pozitivitou a CD127 negativitou jsme vzhledem k relativně malé skupině čísel a zvolené metodice neprováděli.

Po stimulaci diabetogenními autoantigeny a inzulínem se populace $CD4^+CD25^+$ Tregs ani u jedné skupiny nezměnila, nedošlo ke změně exprese FoxP3 ani frekvence negativity CD127. Oproti tomu jsme ale prokázali, že ve skupině příbuzných s rizikovými HLA II alelami pro rozvoj T1D byla výrazně vyšší reaktivita PBMC reprezentovaná signifikantně vyšší produkcí IFNγ. Myslíme si, že může jít o projev poruchy regulace imunitní odpovědi v důsledku snížené supresní schopnosti Tregs. Tato data jsou v souladu s jinými studiemi, které spíše než jednoznačné početní defekty prokázali poruchu funkce Tregs [78, 81, 83].

Závěrem této části lze říci, že pokračování ve výzkumu Tregs u T1D považujeme za perspektivní. V budoucnosti bychom rádi využili vícebarevné průtokové cytometrie k lepšímu rozlišení $CD4^+CD25^+$ Tregs. Také bude nutno více zohlednit rozdíly mezi $CD4^+CD25^+$ iTregs a nTregs, neboť jak se ukazuje, obě populace mohou exprimovat FoxP3 a není jisté, jak je to se znakem CD127. Jednou ze slibných možností pro rozlišení se jeví použití demethylovaného regionu TSDR v exonu 1 pro FoxP3, který je typický pro nTregs (54).V případě predikce rozvoje T1D, mohou hrát významnější roli nTregs (nižší počet/porucha imunosupresní funkce), ale nelze vyloučit ani podíl poruchy vzniku iTregs v periferii, jejíž průkaz je obtížný. Dalo by se spekulovat, zda by *in vitro* poruchu vzniku iTregs pomohla odhalit například změna exprese FoxP3 a produkce protizánětlivých cytokinů po prezentaci diabetogenních autoantigenů "primingovanými" dendritickými buňkami. V případě prevence se zdají více perspektivní antigenně specifické iTregs, které lze získat *in vitro* za speciálních podmínek a použít k cílené imunosupresní terapii (viz.Teoretická část 3.5).

5.Závěr

V obou částech výzkumu jsme zvolenými metodami u jednotlivých skupin subjektů (pacienti s T1D, příbuzní pacientů s T1D, zdravé kontroly) prokázali odchylky v imunoreaktivitě i imunoregulaci. Výsledky u zdravých kontrol jsou v souladu s teoretickým úvodem o imunopatologii T1D. Imunitní buňky u zdravých kontrol rozpoznávaly vlastní diabetogenní antigeny, ale jejich imunitní odpověď hodnocená pomocí cytokinové produkce byla protizánětlivá, tlumivá. U zdravých kontrol jsme prokázali vyšší počty Tregs. Podobně, snížením cytokinové sekrece po 72-96 hodinové stimulaci autoantigeny, reagovali také dosud zdraví prvostupňoví příbuzní pacientů s T1D i pacienti s T1D. Může to být projev fyziologického nastavení imunitního systému reagovat při iniciaci imunitní odpovědi spíše imunosupresně a teprve, pokud stimulace trvá delší dobu (několik dní) a/nebo je intenzivní (prezentace antigenu kvalitními APC), rozvíjí se náležitá zánětlivá odpověď (15, 24, 25, 40, 61). Zajímavým se však stalo zjištění, že u zdravých příbuzných pacientů s T1D i u pacientů s T1D jsme pozorovali již odlišné nastavení bazální sekrece cytokinů a určitou dysregulaci jednotlivých cytokinových spekter v bazálním i postimulačním stavu. U skupiny příbuzných nesoucích rizikové alely pro rozvoj T1D jsme potvrdili také nižší počty a sníženou funkci Tregs. Myslíme si, že jde o projevy dysregulace imunitního systému, a ve svém důsledku o faktory, které souvisí s rozvojem T1D. V případě skupiny zdravých příbuzných pacientů s T1D je tato dispozice zatím kompenzována, v případě pacientů s T1D již mohlo dojít k selhání kompenzatorních mechanismů.

Získané a publikované výsledky, které jsou součástí této disertační práce, byly iniciálními dílčími kroky v pokračujícím výzkumu v oblasti T1D.

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IV. PŘÍLOHY - VLASTNÍ PUBLIKACE

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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 presenr 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 protecrive 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.

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

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 concenrration 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 instrucrions. Samples were analysed by a 4-colour flow cytometry on a Cytomics^{TM} 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,

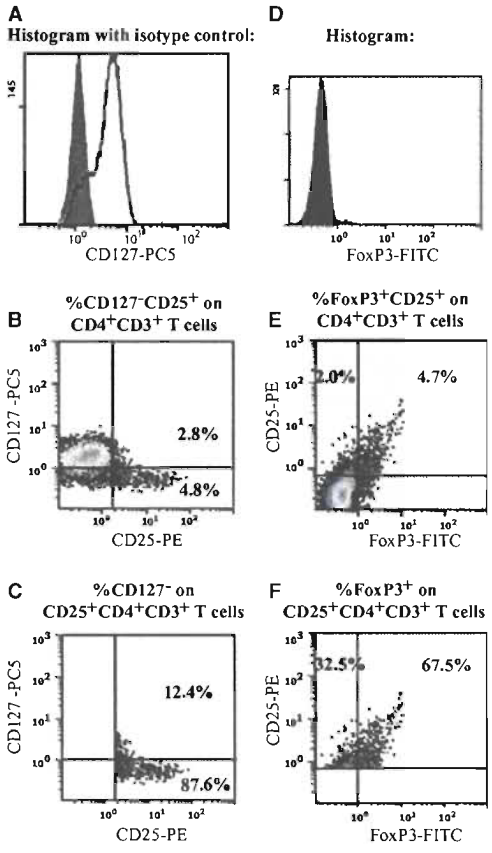

Figure 1 Gating strategy for Treg. Analyses were performed on 4-coloured CytomicsTM FC 500 cytometer (Beckman Coulter, Miami, FL, USA). For analysis, PBMC were gated on lymphocytes (based on forward and side scatter and CD3⁺). (A) CD127 negativity was determined with regard to a negative isotype control, (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 presenr 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

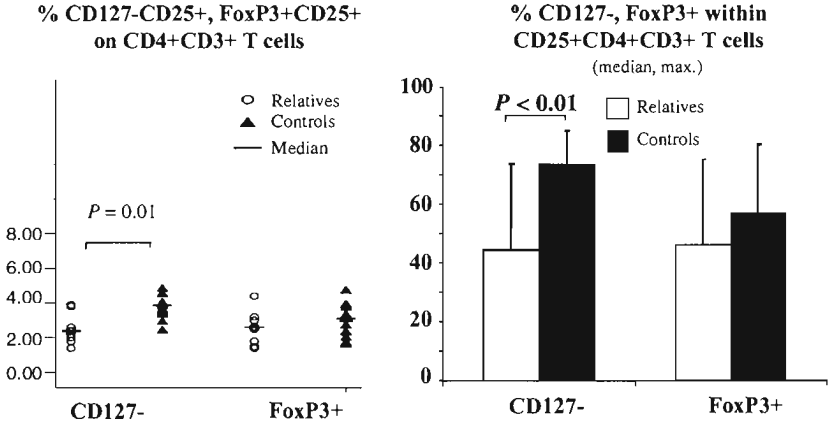

Figure 2 Relative frequencies of freshly isolated Treg. Significantly lower number of $CD127^{-}$ Treg in high-risk relatives compared to healthy controls was noted, lower number of $FoxP3^{+}$ Treg was not significant.

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).

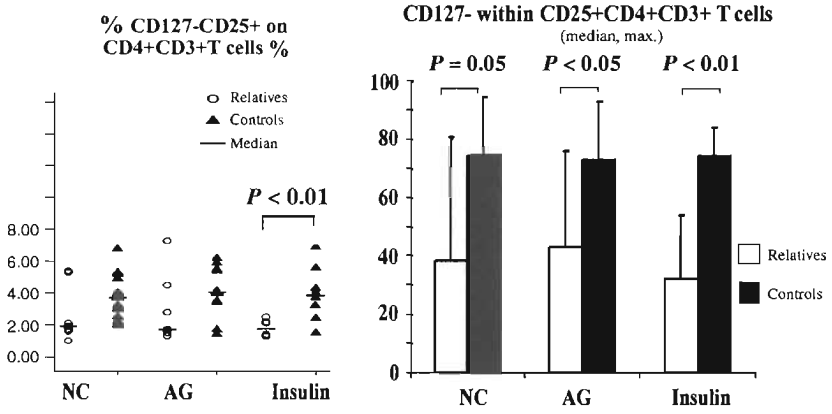

Figure 3 Relative frequencies of $CD127^-$ Treg after stimulation. No significant change in $CD127^-$ Treg was observed. Lower percentage of $CD127^-$ Treg remained during the whole assay in high-risk relatives of T1D patients; relative frequencies of $CD127^-$ related only to $CD25^+CD4^+CD3^+$ T cells 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 srimulated with insulin.

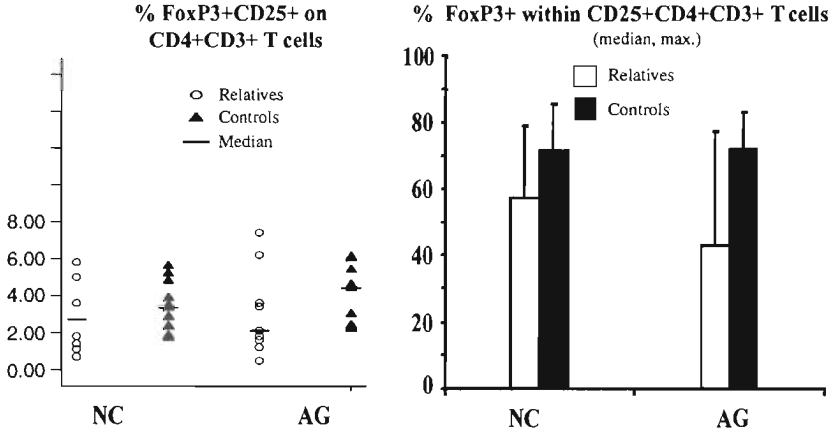

Figure 4 Relative frequencies of $FoxP3^+$ Treg after stimulation. No significant change in $FoxP3^+$ Treg was observed. However, lower percentage of $FoxP3^+$ Treg remained during the whole assay in high-risk relatives of T1D patients. This was more obvious when $FoxP3^+$ was related only to $CD25^+CD4^+CD3^+$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

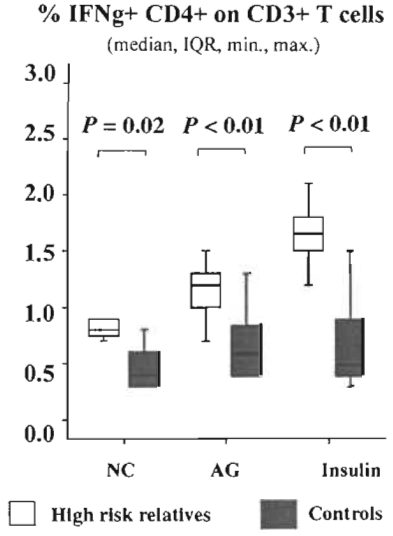

Figure 5 Relative frequencies of IFN-γ on CD4$^+$ T cells. Significantly higher frequency of IFN-γ^+ cell was observed after diabetogeneic stimulation in high-risk relatives of T1D patients. In high-risk relatives, stimulation of both (mixture of peptides autoantigens as well as whole molecule of insulin) statistically increase IFN-γ positivity. NC, negative control with unstimulated PBMC in culture medium; Ag, PBMC stimulated with mixture of diabetogeneic autoantigens; Insulin, PBMC srimulated 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 pancteas. 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 auroreactive 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]. Howevet, 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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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 polyclonal 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 autoantigens) of cord blood mononuclear cells (CBMC) using protein 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 supernatant, the biotin-labelled secondary antibodies 'cocktail' is added to each membrane. The signal is then emphasized by horse radish peroxidase (HRP)-conjugated 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-mediated 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 frequency 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-glutamine) and penicillin–streptomycin (1 μl/ml PNC and 1 μg/l streptomycin; all purchased from Sigma, St Louis, MO, USA) and cultivated with diabetogenic autoantigens.

Cell cultures were stimulated with a mixture of the following autoantigens: GAD65 peptides amino acids (a.a.) 247–279 (NMYAMMIARFKMFPEVKEKGMAALPRLIAFTSEE–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 SHLVEALYLVCGERG, molecular weight 1645 (Sigma, St Louis,

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

Genetic 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 (RayBiotech, 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 ro 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).

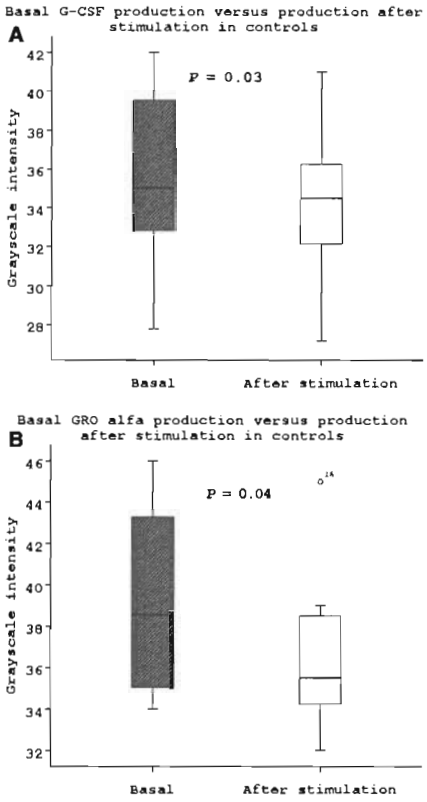

Figure 2 *Basal production versus production after stimulation.* We observed exclusively lower levels of following cytokines in the control group after stimulation in comparison with the basal levels: (A) G-CSF (P = 0.03) and (B) GRO-α (P = 0.04).

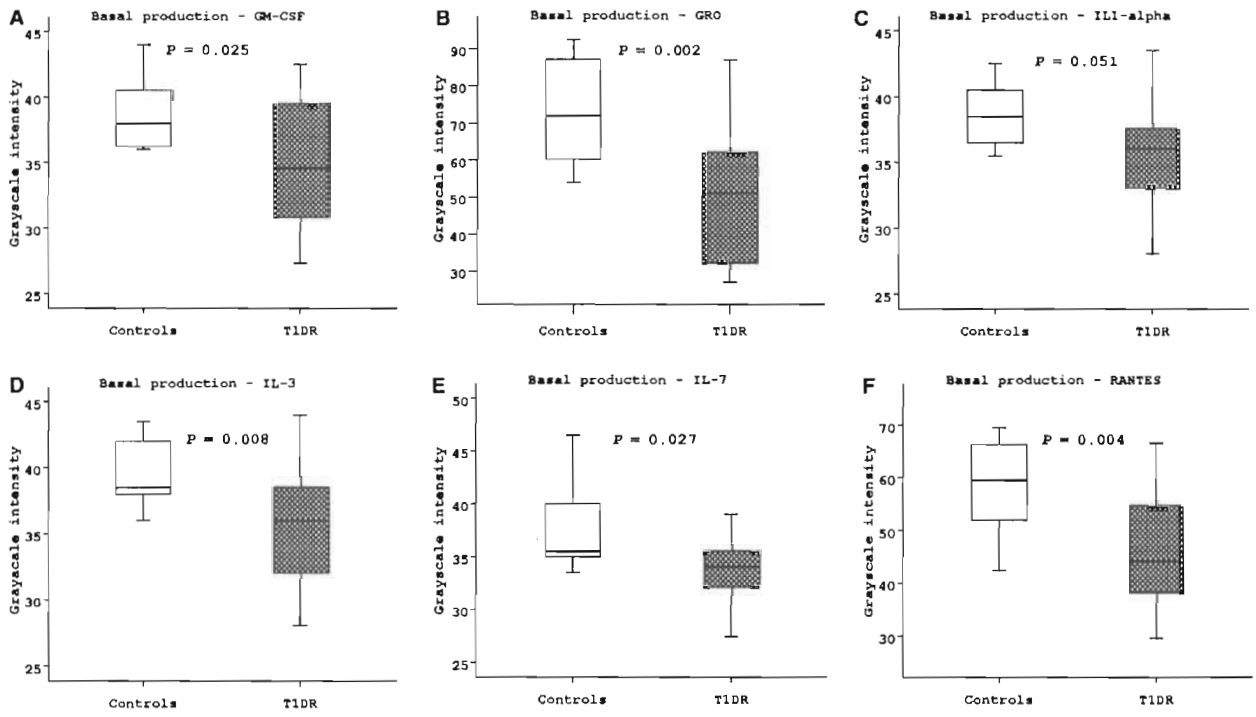

Figure 1 *Basal cytokine production.* We observed lower basal production of all detected cytokines: in the group of T1DR newborns – for example (A) GM-CSF (P = 0.025), (B) GRO (P = 0.002), (C) IL1-α (P = 0.051), (D) IL-3 (P = 0.008), (E) IL-7 (P = 0.027), (F) RANTES (P = 0.004).

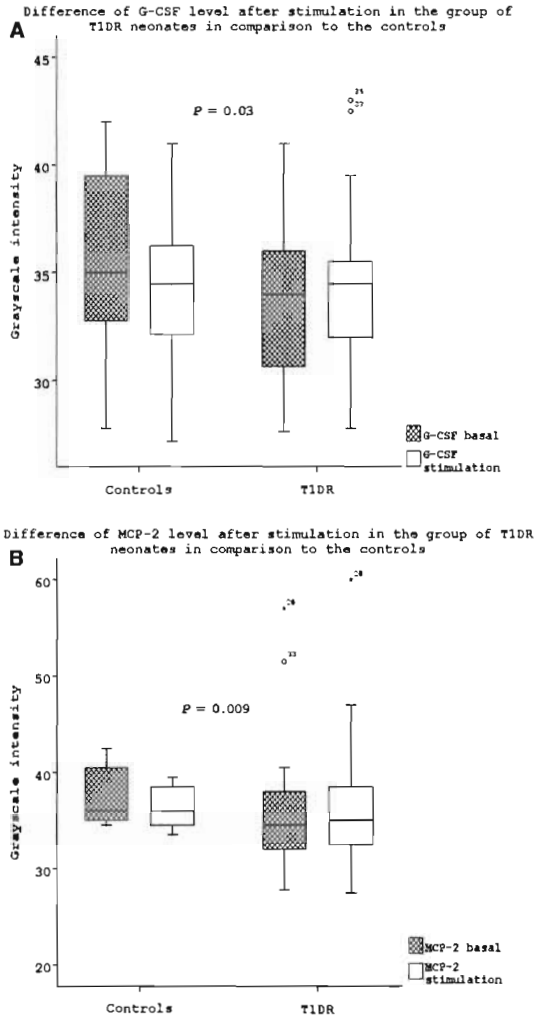

Figure 3 *Difference in the post-stimulative reactivity in the group of T1DR neonates in comparison with the control group.* (A) A significant decrease in G-CSF after stimulation was observed in the control group in contrast to a post-stimulative increase in the group of T1DR neonates (P = 0.030); (B) 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,

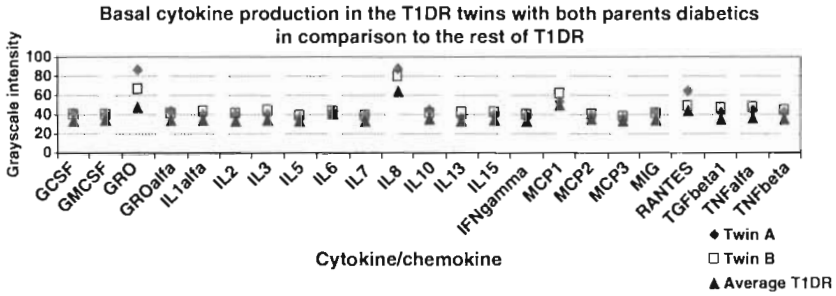

Figure 4 *T1DR twins with both parents diabetics.* Both of the twins (with both parents suffering from T1D) have got higher basal values of all detected cytokines compared with the average of each cytokine in other T1DR newborns with just one diabetic parent.

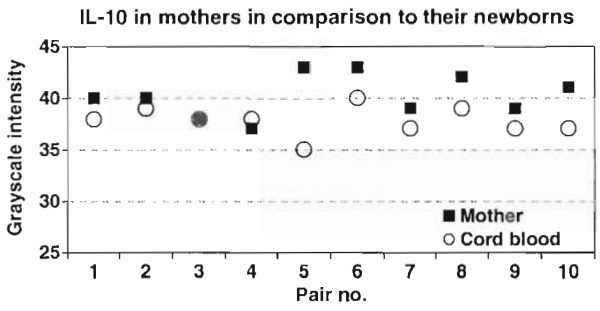

Figure 5 *Cytokine secretion in mothers in comparison with their newborns.* The basal cytokine levels in mothers were higher than in their own newborns. Example of IL-10 basal production (P = 0.010).

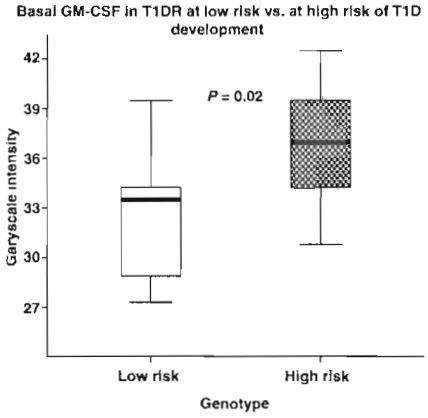

Figure 6 *Basal cytokine production in T1DR neonates with low genetic risk of T1D development versus T1DR neonates with high risk of T1D development.* We observed significantly higher basal levels of GM-CSF (P = 0.020) in T1DR at high risk in comparison with the group of T1DR with low genetic risk of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

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 (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.

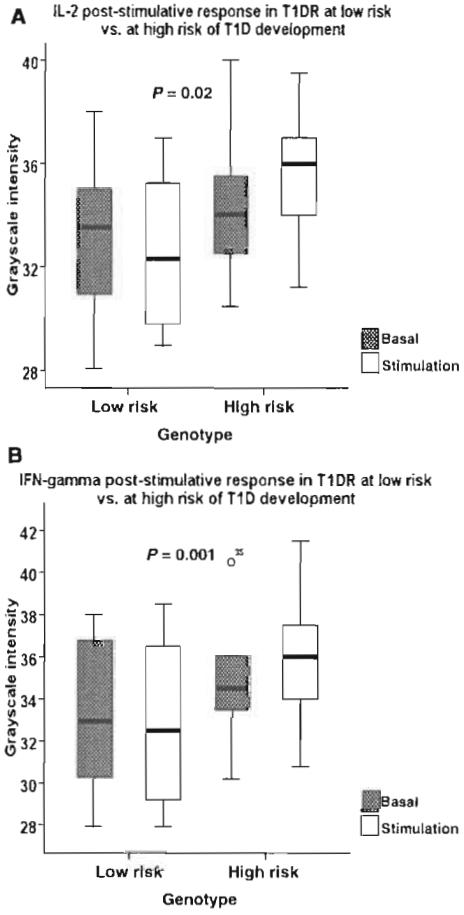

Figure 7 *Post-stimulative response in T1DR neonates with low genetic risk of T1D development versus T1DR neonates with high risk of T1D development.* (A) Significantly higher increase in IL-2 (0.020) and (B) IFN-γ (0.001) can be seen in T1DR neonates at high risk compared with the group of T1DR neonates 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'. Intetestingly, 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 aftet diabetogenic stimulation. According to previously published papets, 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 newbotns 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 ptolifetative ability aftet polyclonal stimulation in comparison with the adults. They ptoduced 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 newbotns are novel. New findings could also play an important role in futther 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 factot IL-7 production. However, we should not forget the influence of co-operation within the whole cytokine net.

Basal cotd blood cytokine profile was also studied in case of petinatal 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 mononucleat 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.

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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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Key words: autoantigen – autoreactive T cells – cytokine – protein microarray – T1D

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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 μg/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.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.

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.

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

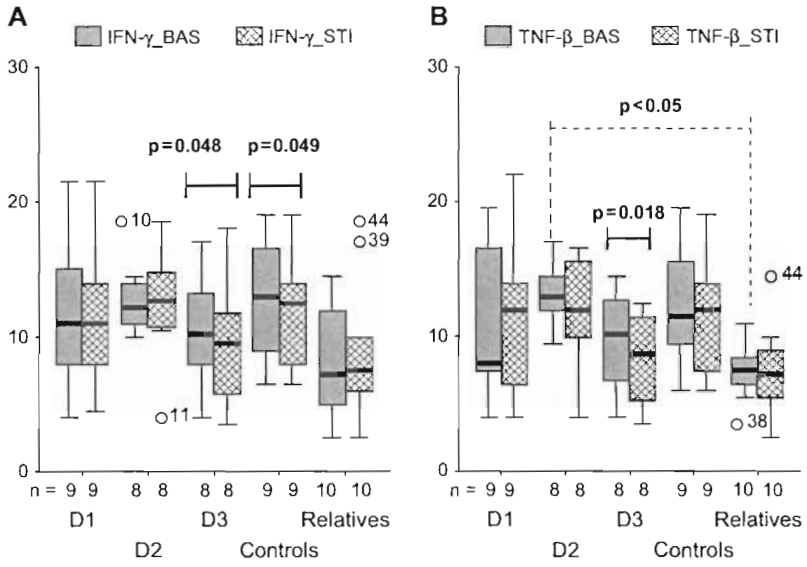

Fig. 1. Th1 cytokines in all groups together. (A) IFN-γ. (B) TNF-β. Results are displayed in percentage of spot intensity (basal and stimulated) and statistical significance is pointed. Data are expressed as median + range. Extreme values are shown. BAS, basal production; D1, type 1 diabetes patients 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

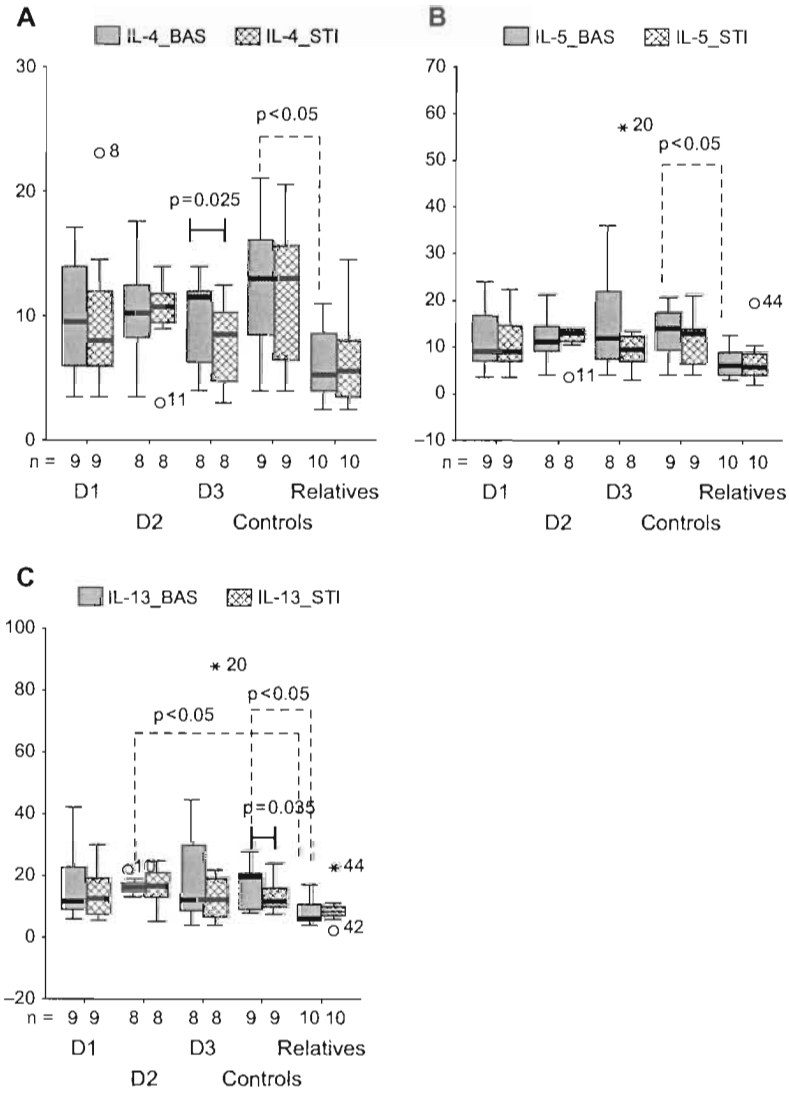

Fig. 2. Th2 cytokines in all groups together. (A) IL-4. (B) IL-5. (C)IL-13. Results are displayed in percentage of spot intensity (basal and stimulated) and statistical significance is pointed. Data are expressed as median + range. Extreme values are shown. BAS, basal production; D1, type 1 diabetes 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

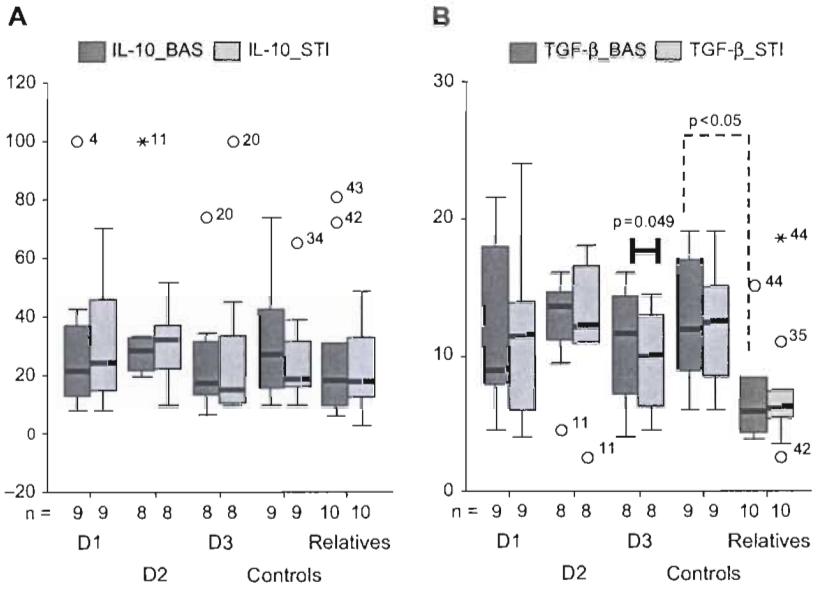

Fig. 3. Th3 cytokines in all groups together. (A) IL-10. (B) TGF-β. Results are displayed in percentage of spot intensity (basal and stimulated) and statistical significance is pointed. Data are expressed as median + range. Extreme values are shown. Bas, basal production; D1, type 1 diabetes patients 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,

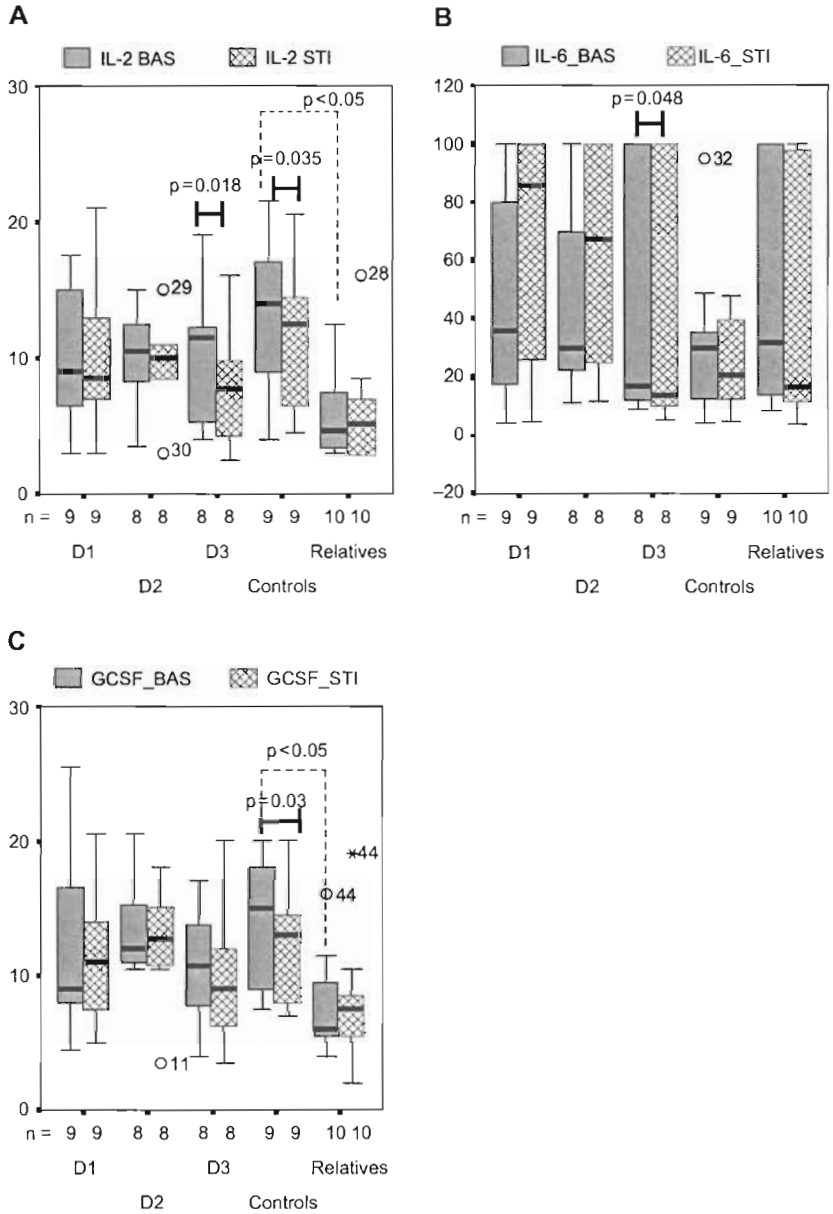

Fig. 4. IL-2, IL-6, and GCSF cytokines in all groups together. (A) IL-2. (B) IL-6. (C) GCSF. Results are displayed in percentage of spot intensity (basal and stimulated) and statistical significance is pointed. Data are expressed as median + range. Extreme values are shown. BAS, basal production; D1, 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

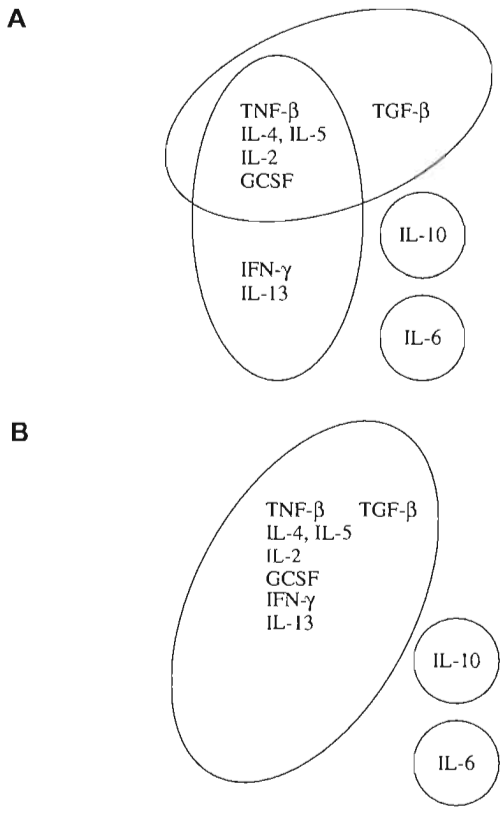

Fig. 5. (A) T1D patients: correlations in cytokine basal production. (B) T1D patients: correlations in stimulated cytokine production. Cytokines that produced was in correlation are displayed. In T1D patients correlations were *de facto* same for basal and for stimulated production within D1, D2 and 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.

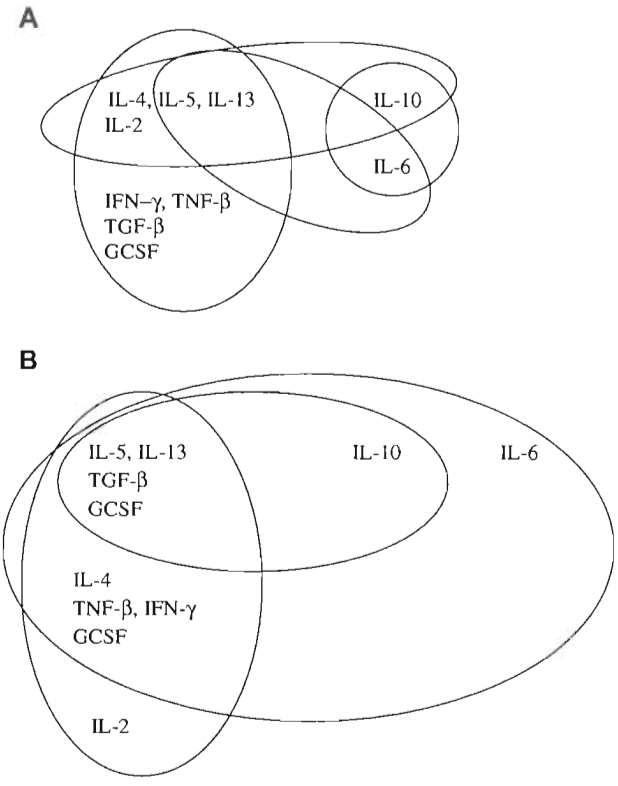

Fig. 6. (A) Controls: correlations in cytokine basal production. (B) Controls: correlations in cytokine-stimulated production. Cytokines that produced was in correlation are displayed. GCSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF,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

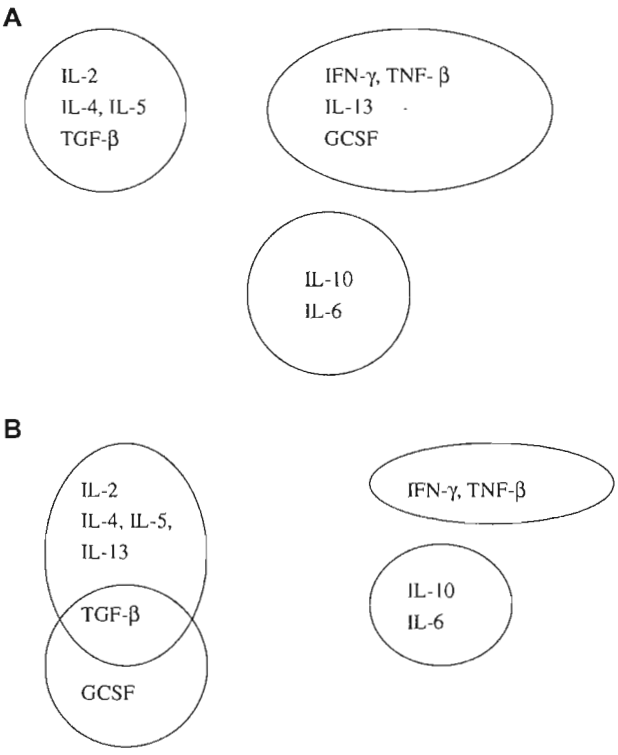

Fig. 7. (A) Relatives: correlations in cytokine basal production. (B) Relatives: correlations in cytokine-stimulated production. Cytokines that produced was in correlation are displayed. GCSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF,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

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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 TID, 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.

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 TID 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	30480	74	14	M	0.15	127	1010	13	M
15	M	P	No	0.05	139440	0	16	F	0.38	n.a.	n.a.	14	M
18	M	P	No	0.98	13560	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

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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.

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.

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.

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 ($p = 0.09$).

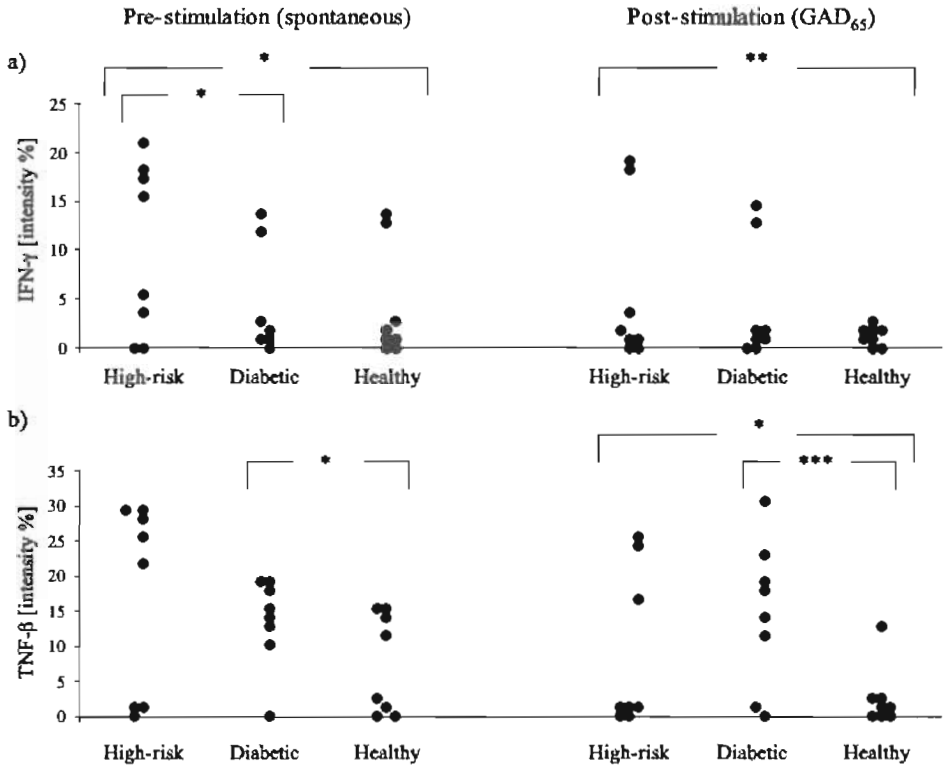

Figure 1. Secretion of the Th1-associated cytokines IFN-γ (a) and TNF-β (b) pre-stimulation (spontaneous) as well as post-stimulation (GAD65-induced). * $p < 0.05$, ** $p = 0.01$, *** $p < 0.01$

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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

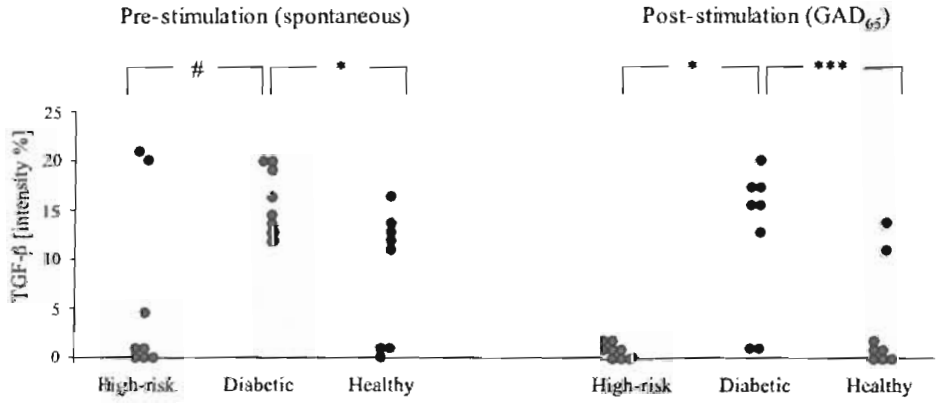

Figure 2. Secretion of the Th3-associated cytokine TGF-β pre-stimulation (spontaneous) and post-stimulation (GAD65-induced). # $p = 0.05$, * $p < 0.05$, *** $p < 0.01$

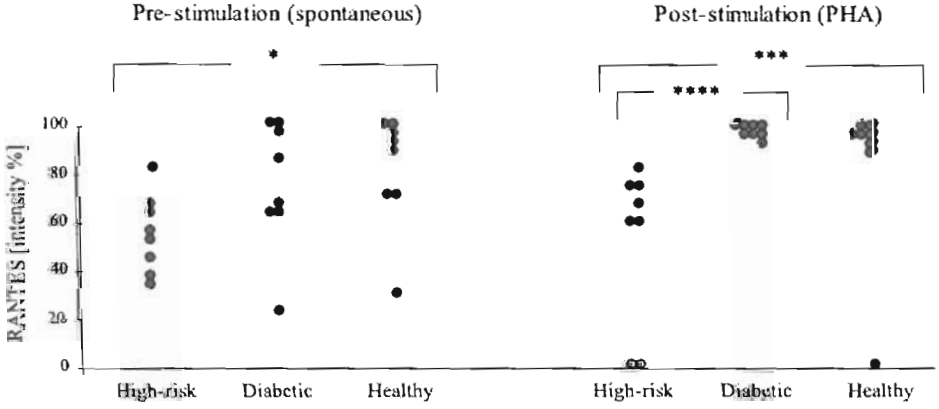

Figure 3. Secretion of the chemokine RANTES pre-stimulation (spontaneous) and post-stimulation (PHA-induced). * $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$

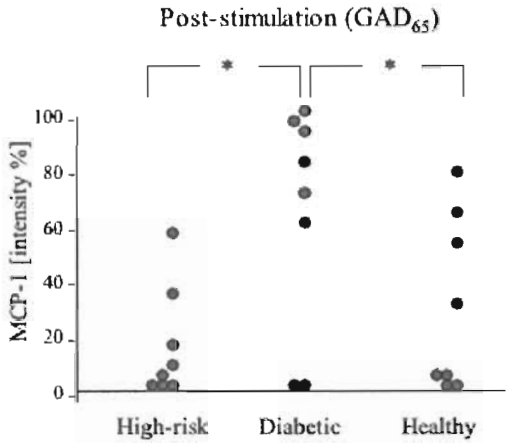

Figure 4. Secretion of the chemokine MCP-1 induced by GAD65. * $p < 0.05$

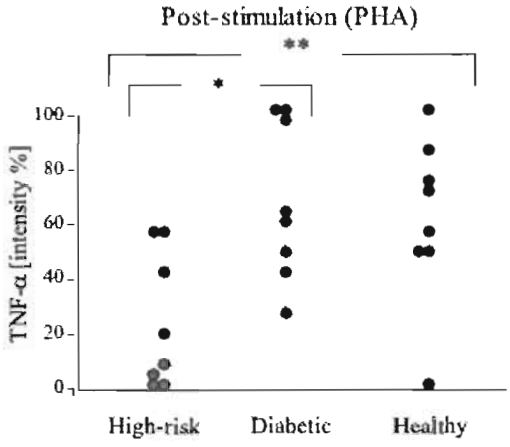

Figure 5. Secretion of the inflammatory cytokine TNF-α induced by PHA. * $p < 0.05$, ** $p = 0.01$

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).

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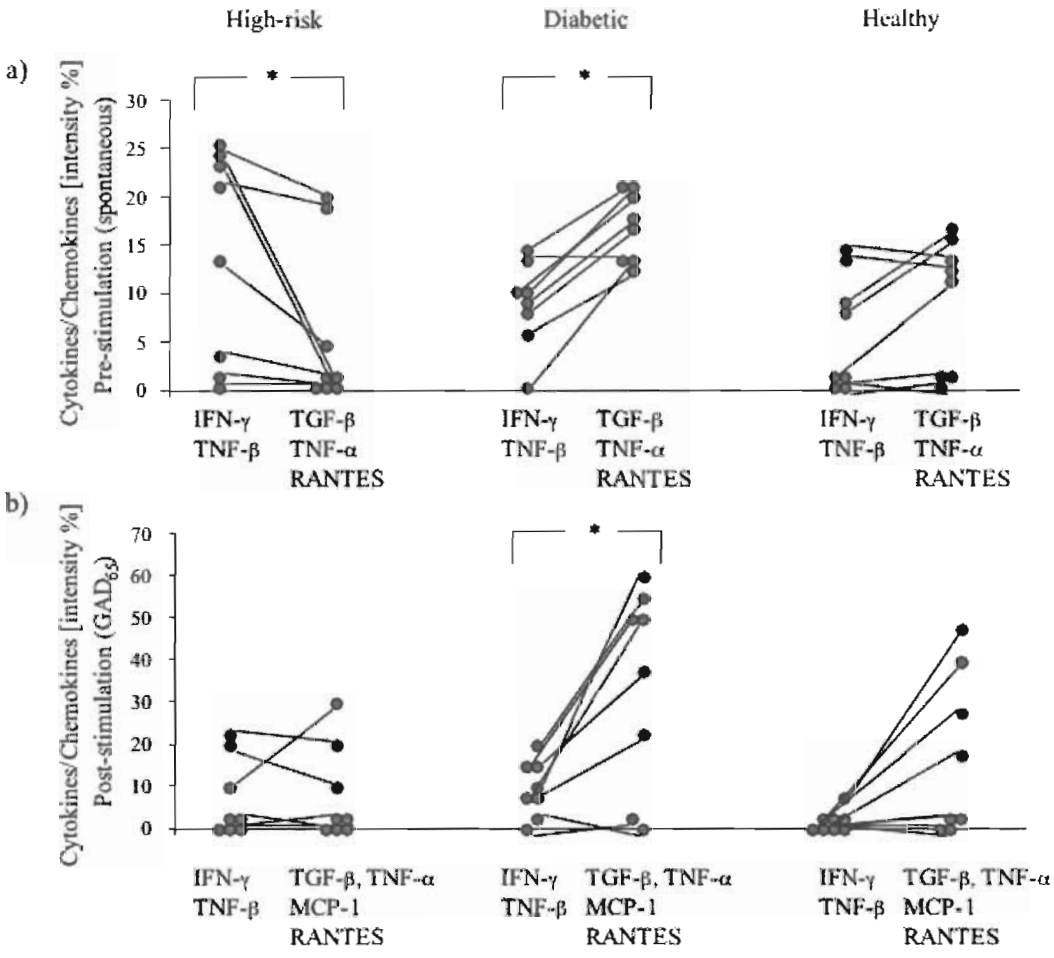

Figure 6. Post hoc comparison of spontaneous secretion of Th1 (IFN-γ, TNF-β) *versus* Th3 (TGF-β)/inflammatory marker (TNF-α)/chemokine (RANTES) (a) and GAD_{65}-induced comparison of Th1 (IFN-γ, TNF-β) *versus* Th3 (TGF-β)/inflammatory marker (TNF-α)/chemokines (MCP-1, RANTES) (b) on individual basis 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.

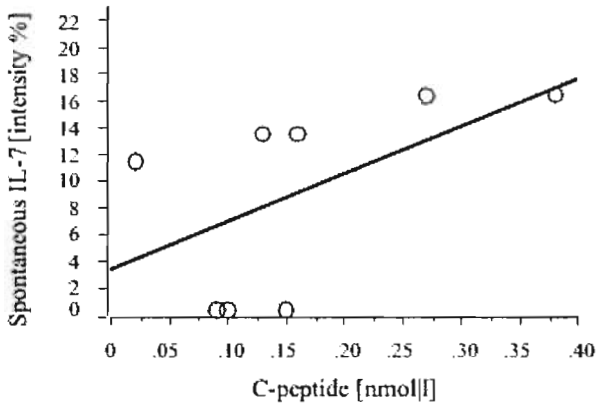

Figure 7. Relation between C-peptide and spontaneous secretion of IL-7 ($r = 0.73$, $p = 0.05$)

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 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 monocytes 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 TID.

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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Immune Regulatory T Cells in Siblings of Children Suffering from Type 1 Diabetes Mellitus

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Abstract

Patients with type 1 diabetes are suffering from defects in immune regulatory cells. Their siblings may be at increased risk of type 1 diabetes especially if they are carriers of certain human leucocyte antigen (HLA) alleles. In a prospective non-randomized study, we intended to evaluate 31 healthy siblings of paediatric patients with type 1 diabetes and explore immune regulatory populations of $CD4^+CD25^+$ T cells and natural killer (NK) T cells. Tested siblings of type 1 diabetes patients were stratified according to the HLA-associated risk of possible diabetes development. Immune regulatory function of $CD4^+CD25^+$ T cells was tested *in vitro*. Significant differences in $CD4^+CD25^+$ but not in NK T cells have been identified. Siblings of type 1 diabetes patients carrying high risk HLA alleles (DQA1*05, DQB1*0201, DQB1*0302) had significantly lower number of immune regulatory $CD4^+CD25^+$ T cells than the age-matched healthy controls or siblings carrying low-risk HLA alleles (DQB1*0301, DQB1*0603, DQB1*0602). Regulatory function of $CD4^+CD25^+$ T cells demonstrated a dose-escalation effect. In siblings of type 1 diabetes patients, the defect in immune regulatory $CD4^+CD25^+$ T cells exists in association with genetic HLA-linked risk for type 1 diabetes.

Introduction

Type 1 diabetes (T1D) arises from a breakdown of tolerance to islet antigens resulting in uncontrolled T-cell-mediated autoimmune destruction of insulin-producing β-cells in the pancreas. Autoreactive subsets of $CD4^+$ T-helper (Th) lymphocytes recognize self-antigens and after activation preferentially produce Th1 cytokine spectrum that initiate the autoimmune process. For further development, the presence of autoreactive $CD8^+$ cytotoxic T (Tc) lymphocytes is necessary as well [1]. To prevent reactivity against self-tissues, autoreactive T cells can be controlled through active suppression by different types of regulatory T cells [2–4]. Sakaguchi has demonstrated an immune regulatory role of $CD4^+CD25^+$ T cells (Tregs) naturally occurring in peripheral blood [5]. Elimination of these cells early in life results in development of various T-cell-mediated autoimmune diseases while reconstitution of $CD4^+CD25^+$ T-cell population prevents such pathological effects [2–6]. Recent data describe immune regulatory subpopulation of human $CD4^+$ T cells as cells with high expression of CD25 ($CD4^+CD25^{high}$) and same functional characteristics as $CD4^+CD25^+$ regulatory cells in mice [7]. Other putative markers include cell-surface expression of $CD62L^+$, $CD45RB^{low}$, glucocorticoid-induced tumour necrosis factor receptor (GITR), overexpression of CTLA-4 and intracellular expression of transcriptional repressor FoxP3 (forkhead box P3) [2, 4, 8]. The suppression mechanisms of regulatory cells are indirect through secretion of anti-inflammatory cytokines such as interleukin 4 (IL-4), IL-10, transforming growth factor β (TGF-β) or direct through cell–cell contact, for example via the perforin pathway [2–4, 8]. Evidence that natural Tregs are antigen specific is still limited [4].

Recent studies demonstrate multiple defects in T-cell regulation of T1D individuals [1, 7, 9, 10]. In contrary, limited information exists about siblings of T1D patients that may have an increased risk of T1D. In this work, we focused on immune regulatory populations of $CD4^+CD25^+$ T cells and natural killer T cells (NKT) in siblings of children and adolescents with T1D. We were particularly interested in revealing potential defects in these regulatory T-cell populations in healthy

individuals, especially siblings of T1D patients who may be at an increased genetic risk of developing T1D. Early identification of at risk individuals may lead to an early therapeutic intervention prior to complete destruction of insulin-producing cells.

Materials and methods

Study subjects. Heparinized blood samples were obtained from 31 healthy siblings (10 females, 21 males of age 1–20, median age 13 years) of children with T1D followed at the 1st Department of Pediatrics, University Hospital Brno. Healthy controls (16 females, 20 males of age 1–17, median age 10 years) were consecurively recruited from healthy children and adolescents undergoing minor surgery with no family or personal history of T1D or any other autoimmune disease. Blood samples of all study subjects were taken after signing the informed consent approved by the Ethical Committee of the University Hospital Brno, Brno, Czech Republic. A complete human leucocyte antigen (HLA)-DQA1 and HLA-DQB1 genotyping was carried out by polymerase chain reaction (PCR) with sequence-specific primers in all T1D siblings. A stratification of HLA-linked genetic risk was performed according to the T1D prediction programme of the Czech Republic [11] and divided into three groups with high, standard or low risk (Table 1). Sera of all T1D siblings 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 (more than 2 standard deviations) were considered positive. GAD65 and IA-2 autoantibodies were screened every 12 months in a high-risk group and every 24 months in a standard-risk group. T1D siblings with positive autoantibodies had a standard intravenous glucose tolerance test (ivGTT) and the first phase of insulin response (FPIR) was assessed as described previously [12, 13]. FPIR levels above the fifth percentile were considered normal [12].

Flow cytometry. Flow cytometric determination of the lymphocyte populations from whole blood was performed by the following cell matkers: anti-CD4, anti-CD8, anti-CD3, anti-CD25, anti-TCRα24 and anti-TCRβ11 labelled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanin 5 (PC5) or phycoerythrin-cyanin 7 (PC7) (Immunotech, Matseille, France). Samples were analysed by a four-colour flow cytometry on a Cytomics^{TM} FC 500 cytometer (Beckman Coulter, Miami, FL, USA). Data were analysed using the CXP Software (Beckman Coulter).

Immunomagnetic cell sorting. Immunomagnetic sorting of $CD4^+CD25^+$ T cells was performed from peripheral blood mononuclear cells (PBMC) obrained by Histopaque (Sigma-Aldrich, Prague, Czech Republic) gradient centrifugation of the whole blood by rhe $CD4^+CD25^+$ Human Regulatory T Cell Isolation Kit in two steps according to the manufacturer's insttuctions on a VarioMACS (Miltenyi Biotec, Bergish Gladbach, Germany). Briefly, the first step immunomagnetically eliminated major cell populations except $CD4^+$ T cells that were labelled in the second step with anti-CD25 and positively selected by magnetic beads. The purity of $CD4^+CD25^+$ Tregs was more than 97%.

$CD4^+CD25^+$ Tregs testing in vitro. $CD4^+CD25^+$ Tregs were used alone or were mixed in different ratios (1:1–1:4) with autologous $CD4^+CD25^-$ T cells to test their ability to suppress reactivity of lymphocytes to different antigenic stimuli such as irradiated allogeneic PBMC (1:1 ratio to tested $CD4^+CD25^-$ T cells) or phytohaemagglutinin (PHA) 10 μg/ml (Sigma-Aldrich, Prague, Czech Republic). Cells were cultured at 37 °C and 5% CO_2 atmosphere in a complete media (CM) containing X-VIVO 10, 50 mg/l gentamycin, 2 mM L-glutamine, 25 mg/ml HEPES (BioWhittaker, Walkersville, MD, USA) and 10% heat-inactivated human AB-serum

Table 1 Sibling characteristics and an estimated human leucocyte antigen (HLA)-linked genetic risk of type 1 diabetes

HLA characteristics	No. of subjects	HLA	Autoantibodies positive cases	Impaired ivGTT
High risk	8	DQA1*05 DQB1*0201 DQB1*0302	1	0
Standard risk	11	Other than high or low risk	2	1
Low risk	12	DQB1*0301 DQB1*0603 DQB1*0602	1	0
Age	1–20, median age 13 years			
Sex	9 females, 18 males			

ivGTT, intravenous glucose tolerance test.

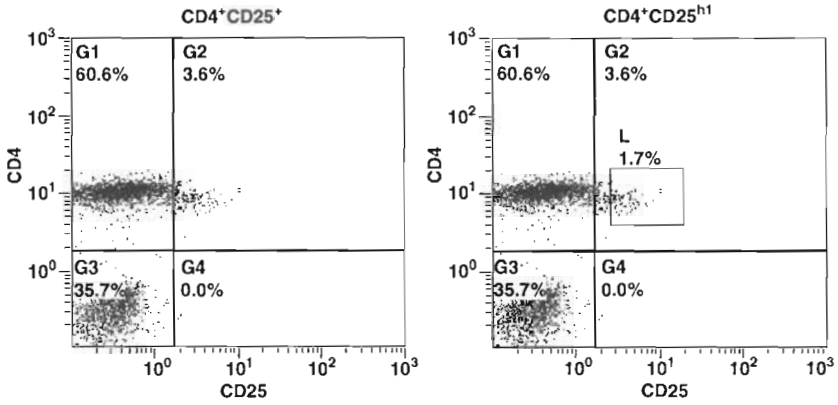

Figure 1 Gating strategy for identification of $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ cells. Cells were gated based on CD3 and CD4 positivity, expression of CD25, and divided into $CD4^+CD25^-$ or $CD4^+CD25^+$ cells. Cells with expression of CD25 exceeding that of $CD4^-CD25^+$ cells within PBMC (predominantly activated B cells) were considered $CD4^+CD25^{hi}$ cells. Due to a smaller size in the forward scatter channel (data not shown) $CD4^+CD25^{hi}$ cells showed a slightly lower CD4 expression then the rest of $CD4^+$ cells.

(Sigma-Aldrich). Cell concentration was 1.0×10^6 cells/ml CM, usually 0.5×10^6 of $CD4^+CD25^-$ cells were tested. Cell activation to the antigen was measured by the production of interferon gamma (IFN-γ) on the surface of activated $CD3^+$ T cells using the Secretion Assay Cell Detection Kit (Miltenyi Biotec) according to the manufacturer's instructions by flow cytometry. To exclude dead irradiated PBMC, labelling with propidium iodide was used. As a positive control, PBMC with no $CD4^+CD25^+$ Tregs were used. As a negative control, unstimulated PBMC were tested.

Statistical analysis. The four age-matched groups (control, low , standard and high risk) were compared using Kruskal–Wallis test due to asymmetric data distribution. Any results with P-value of less than 0.05 were considered significant. For *in vitro* testing of $CD4^+CD25^+$ Tregs, a descriptive statistic of means and standard deviation was used. All analyses were done using Statistica for Windows 7.1 and Mictosoft Office Excel 2003.

Results

HLA-linked risk, autoantibodies and ivGTT in siblings of T1D patients

HLA-linked risk was evaluated in 31 siblings of T1D patients. There were eight, 11, 12 subjects in high-, standard- and low-risk groups respectively. In five subjects the presence of GAD65 and/or IA-2 autoantibodies was detected. One subject from the standard-risk group was repeatedly positive for GAD65 autoantibodies and had FPIR lesser than first percentile in the ivGTT. The remaining four autoantibody-positive subjects had FPIR values above fifth percentile which was considered normal.

$CD4^+CD25^+$ T cells

$CD4^+CD25^+$ cells as well as $CD4^+CD25^{hi}$ cells were evaluated by flow cytometry in siblings of T1D patients. Gating strategy is demonstrated in Fig. 1. Study subjects were divided into three groups based on a predicted HLA-linked risk of T1D and the evaluation of $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ T-cell populations were performed for each group. A statistically significant decrease of both $CD4^+CD25^{hi}$ T cells and $CD4^+CD25^+$ was noticed in the high-risk group in comparison to healthy age-matched controls ($P < 0.0001$ and <0.0001 respectively) as well as to the low-risk group ($P = 0.049$ and 0.0014 respectively) (see Fig. 2). Standard-risk group had a significant decrease of $CD4^+CD25^{hi}$ T cells ($P = 0.011$) but not $CD4^+CD25^+$ T cells ($P = 0.36$) in comparison to healthy controls. No significant difference was detected between standard- and high-risk groups or standard- and low-risk groups.

NKT cells

NKT cells defined as CD3-positive, TCR-α 24-positive and TCR-β 11-positive cells were examined in all siblings of T1D patients and compared with age-matched healthy controls. No difference was detected among the risk groups of siblings or between siblings of T1D patients and healthy controls (Fig. 3).

Regulatory effect of $CD4^+CD25^+$ T cells

Immune regulatory $CD4^+CD25^+$ T cells were isolated by magnetic separation from PBMC of healthy donors. Sorted population of $CD4^+CD25^+$ T cells contained approximately 0.5–1% of the original number of PBMC with more then 97% purity of $CD4^+CD25^+$ T cells. Autologous PBMC were activated either by PHA or by irradiated allogeneic PBMC and Tregs were added in different ratios to demonstrate their regulatory effect based on IFN-γ production. Tregs were able to suppress activation of PBMC in a dose-dependent fashion (Fig. 4).

Discussion

A deficiency in the number or function of Tregs can contribute to the onset of T1D as previously documented by several studies in the NOD mice as well as in humans

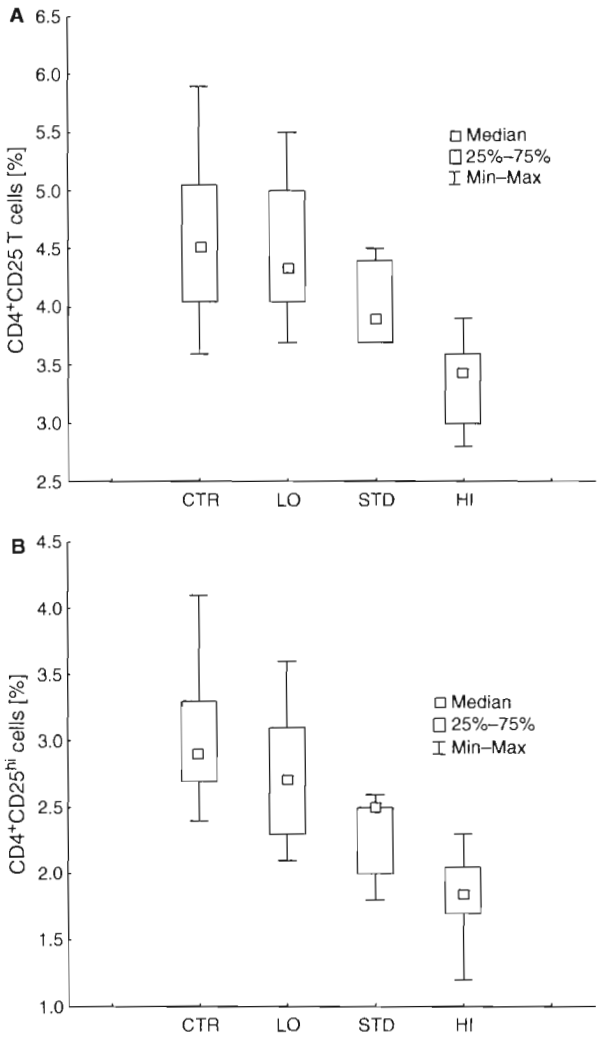

Figure 2 Percentage of (A) $CD4^+CD25^+$ T cells and (B) $CD4^+CD25^{hi}$ cells in siblings with high (HI), standard (STD) and low (LO) human leucocyte antigen (HLA)-linked risk in comparison to healthy controls (CTR).

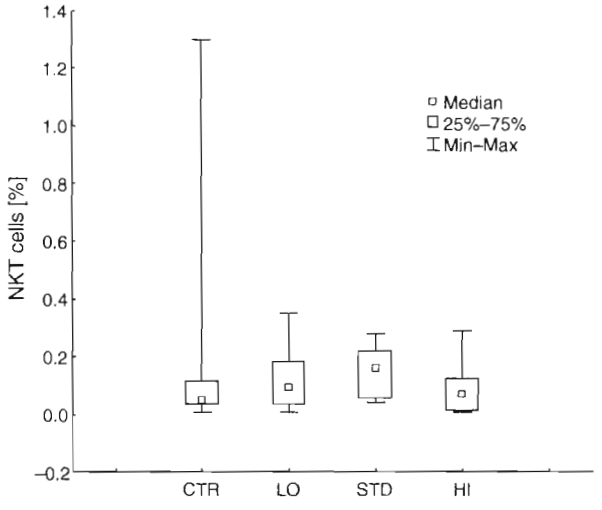

Figure 3 Percentage of natural killer T (NKT) cells in siblings with high (HI), standard (STD) and low (LO) human leucocyte antigen (HLA)-linked risk in comparison to healthy controls (CTR).

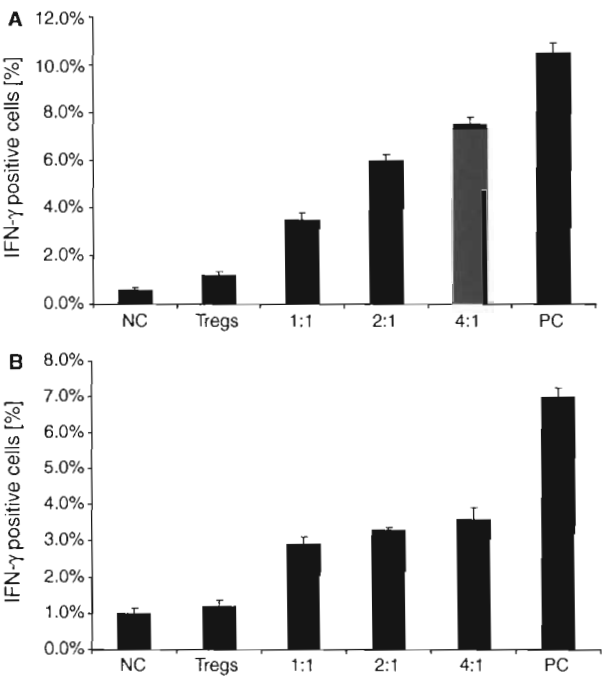

Figure 4 Dose-dependent suppression with $CD4^+CD25^+$ T cells. $CD4^+CD25^+$ Tregs were added to activated peripheral blood mononuclear cells (PBMC) in a dose-dependent manner in a ratio 1:1, 2:1 and 4:1 (PBMC:Tregs), non-activated PBMC were used as a negative control (NC) and activated PBMC without addition of Tregs were used as a positive control (PC). Interferon gamma (IFN-γ) production was measured after 48 h. Data represent at least three independent experiments: (A) activation of PBMC by phytohaemagglutinin and (B) activation of PBMC by irradiated allogeneic PBMC.

[1–3, 6]. In children with T1D and their healthy siblings the data are rather limited. Kukreja *et al.* [14] were able to demonstrate defects affecting both the $CD4^+CD25^+$ T cells and NKT cells in patients with T1D as well as defects in NKT cells in 12 siblings of those patients. Unfortunately, $CD4^+CD25^+$ T cells in siblings of patients with diabetes were not studied in that study. In our previous study, we were able to demonstrate defects in T1D children only in $CD4^+CD25^+$ T cells but not in NKT cells [15]. Here, we focused on siblings of T1D patients for whom, to our knowledge, no data are available.

Siblings of T1D patients can be also stratified based on predicted genetic HLA-linked risk for the development of T1D in the future. That stratification has been previously described for the Czech population [11]. For the purpose of our study, we divided the subject population into three groups with high, standard and low risk of T1D. We were able to demonstrate a close association between HLA-linked risks and the

$CD4^+CD25^+$ T cell population, i.e. the higher the genetic risk the lower the $CD4^+CD25^+$ Tregs population. As expected, the differences were more pronounced in $CD4^+CD25^{hi}$ cells that mainly contain Tregs cells rather then the general population of $CD4^+CD25^+$ T cells that contains regulatory as well as recently activated T cells. The standard-risk group revealed a significant decrease of $CD4^+CD25^{hi}$ T cells but not $CD4^+CD25^+$ in comparison to healthy controls. This can be explained by rather insufficient classification of the standard-risk group that is defined by exclusion of HLA alleles for high- or low-risk groups. In general, regardless HLA type, siblings of patients with T1D have an increased risk of T1D [11, 14] and one of the mechanisms can involve diminished numbers of Tregs. We were able to demonstrate decreased number of Tregs in this group of healthy siblings of T1D patients.

We completed our study with a set of expetiments that document the regulatory effect of immune magnetically isolated $CD4^+CD25^+$ T cells from peripheral blood. Despite a strong activation by mitogen or allogeneic PBMC, the suppressive effect of $CD4^+CD25^+$ T cells was obvious in a dose-dependent mannet. These data ate in agreement with previously published results describing regulatory effect of $CD4^+CD25^+$ T cells [3, 7, 10].

In conclusion, the defect in immune regulatory $CD4^+CD25^+$ T cells has been described in siblings of paediatric patients with T1D. This defect is associated with an incteased HLA-associated risk of T1D and thus confirms the hypothesis that an autoimmune mechanism that, in general, leads to target organ destruction, is initiated well before the clinical manifestation of the disease appears. Further research and a careful long-term immunological monitoring of regulatory T cells in individuals at risk of T1D based on HLA-linked risk can be helpful in considering an early possible intervention to prevent a complete destruction of target organ or tissue.

Acknowledgment

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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 DQB1*02/0302	IL-8, MCP-1	TNFβ, IFNγ	GAD65-2 similar to GAD65
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 DQB1*0602/0602	IL6, IL-10, TGFβ1	IL8, GRO, MIG, MCP1, RANTES	GAD65
6	11F	No	Negative	DQA1*03/05 DQB1*02/0302	TNFβ	IL8, IFNγ	GAD65-2
7	23F	No	Negative	DQA1*01/05 DQB1*0301/0602	IL-8	MCP1, RANTES	GAD65
8	13M	No	Negative	DQA1*02/03 DQB1*02/0302	IL6, IL10, TGFβ1	IL8, GROa, MCP1, MCP2, RANTES	GAD65-2

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

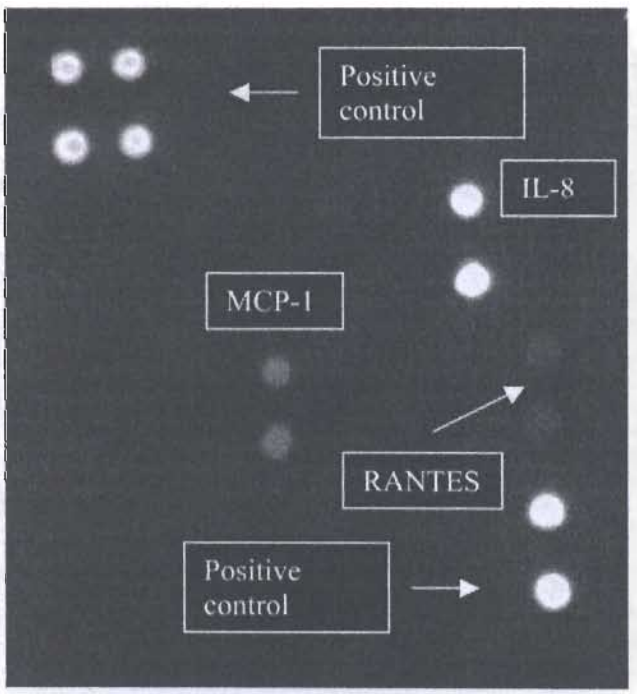

Figure 1. Pat. No 7—example of results.

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.

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