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Mgr. Lucia Straňavová

Role paměťových T lymfocytů v transplantační imunitě

The role of memory T cells in transplant immunity

Disertační práce

Školitel: prof. MUDr. Ondřej Viklický, CSc.

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Poděkování

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Abstrakt

Paměťové T lymfocyty představují specifickou subpopulaci buněk, které vznikají během prvního setkání s antigenem, aby u reinfekcí byla sekundární reakce imunitního systému rychlejší a efektivnější. V transplantační imunitě mohou tyto buňky představovat potenciální riziko pro přežití štěpu. Kromě dárcovsky-specifických paměťových T buněk, které přímo ohrožují transplantovaný orgán, mohou pomocí zkřížené reaktivity, tzv. heterologní imunity, ohrozit zdraví a funkčnost štěpu i virově specifické paměťové buňky.

V této práci jsme se zaměřili na dárcovsky-specifické a CMV-specifické paměťové/efektorové T lymfocyty. Zajímal nás vliv imunosupresivní terapie na frekvenci CMV specifických paměťových/efektorových T buněk. Zjistili jsme, že použitá imunosuprese, profylaxe ani délka dialýzy výrazně neovlivňuje počet CMV-reaktivních buněk, jejich počet byl 6 měsíců po transplantaci stejný jako před ní.

Dále jsme se zabývali zkříženou reaktivitou mezi CMV a antigeny dárce, tzv. heterologní imunitou, kterou jsme ověřili analýzou repertoáru T receptorů β (TCR-β) pomocí sekvenování nové generace (NGS) u CMV a dárcovsky-reaktivních T buněk. Funkční zkříženě reagující T buněčné klony (sdílející stejnou TCR-β sekvenci) jsme pak nalezli jak v periferní krvi pacientů před transplantací, tak i v potransplantační biopsii štěpu.

Také nás zajímal vliv dialyzační léčby na imunitní paměť. Dlouhodobá dialyzační terapie je často asociovaná s přítomností špatně definovaných poruch imunitního systému. Zjistili jsme, že dlouhodobá dialyzační léčba má vliv na cirkulující B lymfocyty marginální zóny, nicméně virově-reaktivní T buňky, obdobně jako ostatní subpopulace T a B lymfocytů a dendritických buněk nebyly ovlivněny předchozí dialýzou.

Abstract

Memory T cells represent a specific subpopulation of cells formed during the first encounter with antigen. The main role of these cells is to elicit faster and more effective secondary response during reinfections. In transplant immunity, they may affect graft survival directly with donor-specific memory T cells or with cross-reactive virus-specific memory T cells.

In this study, we focused on donor-specific and CMV-specific memory/effector T cells. We were interested in the effect of immunosuppressive therapy on the frequency of these cells in periphery. We found that the immunosuppression, prophylaxis and length of dialysis did not significantly affect the number of CMV-reactive cells 6 months after transplantation.

We were also interested in the cross-reactivity between CMV and donor antigens, so-called heterologous immunity, which we verified by analyzing the TCR-β repertoire using next-generation sequencing (NGS) in CMV and donor-reactive T cells. Functional cross-reactive T cell clones (shared the same TCR-β sequence) were then found both in the peripheral blood of pre-transplant patients and in the post-transplant graft biopsy.

We were also interested if long-term dialysis treatment affects immune memory. Dialysis therapy is often associated with the presence of poorly defined immune system disorders. We found that long-term dialysis treatment affects circulating marginal zone B cells, however, virus-reactive T cells, like other subpopulations of T and B lymphocytes and dendritic cells (DC), were not affected by previous dialysis.

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1 Seznam zkratek

ADCC	Cytotoxická reakce závislá na protilátkách
CMV	Cytomegalovirus
ELISPOT	Enzyme-linked immunospot assay
FACS	Průtoková cytometrie
FKBP	F506-vazebným protein
GvH	Reakce štěpu proti hostiteli
Gzmb	Granzym B
HLA	Hlavní histokompatibilní komplex
IE-1	Časný iniciační protein 1
IgG	Imunoglobulin G
LCMV	Virus lymfatické choriomeningitidy
Pp65	Phosphoprotein 65
PRA	Panel reaktivní protilátky
Prf1	Perforin 1
PTP	Protein tyrosin fosfatázová rodina
rATG	Králičí anti-thymocytární globulin
SCID	Těžká kombinovaná imunodeficience
TCM	Centrální paměťové T lymfocyty
TCR	T-buněčný receptor
TEM	Efektorové paměťové T lymfocyty
TEMRA	Terminálně diferencované efektorové paměťové T lymfocyty
TF	Transkripční faktor
TPM	Periferní paměťové T lymfocyty
TRM	Tkáňově rezidentní paměťové T lymfocyty

2 Literární přehled

2.1 Úvod

Paměťové T lymfocyty umožňují po opětovném setkání s antigenem velice rychlou a efektivní sekundární odpověď. V transplantační imunitě se mohou paměťové buňky podílet na zvýšení rizika odhojení transplantovaného orgánu pomocí: i) dárcovsky-specifických paměťových T lymfocytů, ii) zkříženou reakcí mezi patogen-specifickými paměťovými T buňkami a alloantigeny transplantovaného štěpu, iii) homeostatickou proliferací reziduálních paměťových buněk po lymfopenii způsobené imunosupresivní léčbou.

Míra rizika odhojení štěpu se odhaduje především stanovením humorální sensitizace, pomocí panel reaktivních (PRA) a anti-HLA specifických protilátek. Tento odhad však nemusí být dostatečný, jelikož rejekce se vyskytuje i u pacientů, kteří mají hladiny PRA a anti-HLA protilátek nízké nebo nulové. Studium celulární sensitizace, pomocí virově- i dárcovsky-specifických paměťových T lymfocytů, a faktorů, které je ovlivňují (depleční léčba, dlouhodobá dialýza) by mohlo pomoci zpřesnit predikci rejekce.

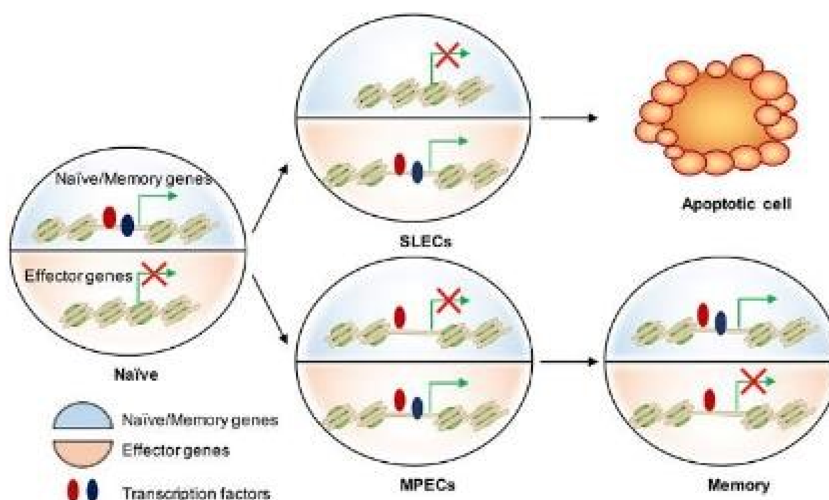
2.2 Vlastnosti paměťových T lymfocytů

2.2.1 Dlouhá životnost paměťových T lymfocytů

Ve srovnání s jinými subpopulacemi T lymfocytů (naivní, efektorové), které žijí v řádech dnů, paměťové T lymfocyty můžeme najít v lidském těle i několik let po setkání s daným antigenem. Jejich prodloužený *life span* je, kromě jiného, možný i díky vysoké expresi anti-apoptotických proteinů (Bcl-2, Bcl-X_L), které brání uvolnění cytochromu c (pro-apoptotický protein) z mitochondrií a tím blokují apoptózu. Produkce těchto proteinů umožňuje paměťovým buňkám přežít i poté, co byl daný antigen eliminován, a tudíž chybějí signály pro jejich přežití a proliferaci (1,2).

2.2.2 Rychlá odpovídavost na antigen

Paměťové T lymfocyty odpovídají na stimulaci antigenem výrazně rychleji, než naivní T buňky (3–5). Bylo prokázáno, že naivní T lymfocyty odpovídají na antigen do 5-7 dnů, naproti tomu paměťové buňky po opětovném setkání reagovaly za 1-3 dny (6). Možným vysvětlením této rychlé odpovídavosti je, že genový lokus pro cytokiny a ostatní efektorové molekuly je u paměťových buněk fixovaný v aktivním stavu pomocí epigenetických modifikací (Obr. 01) (7).

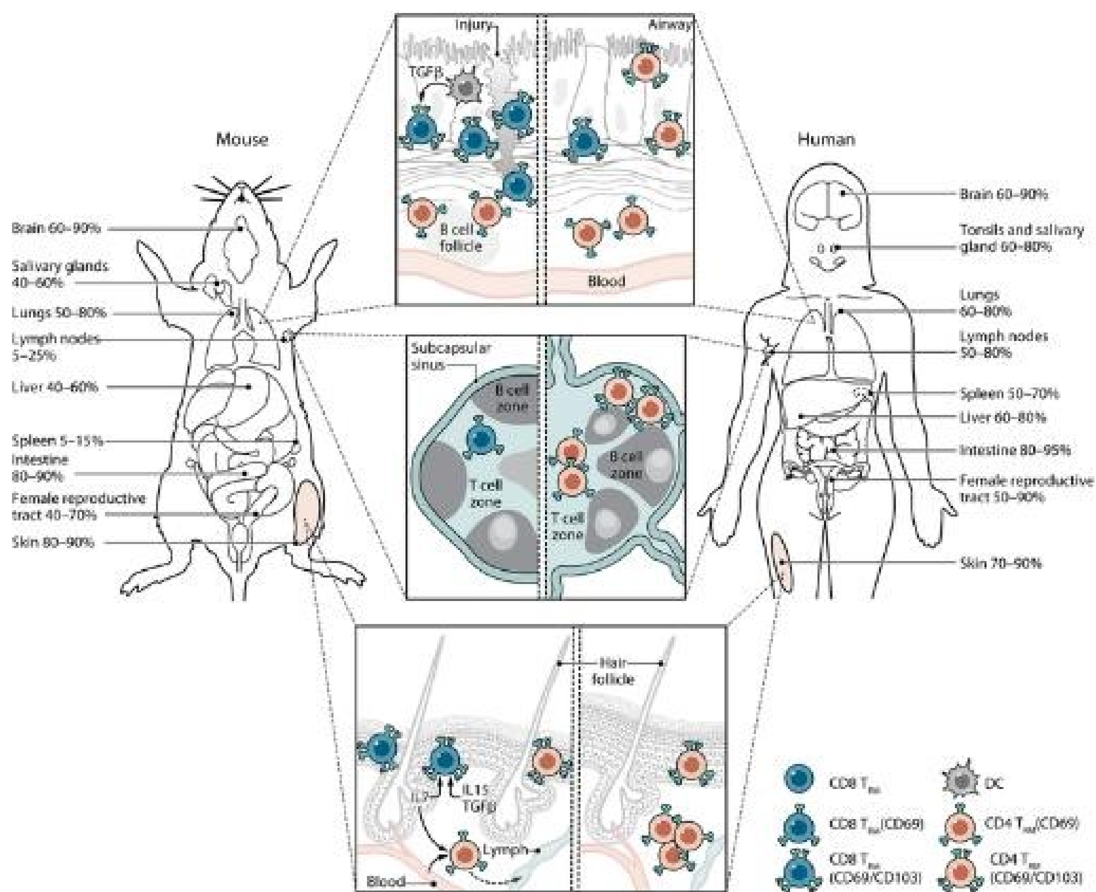


Obr. 1: *Epigenetická regulace časné diferenciace T lymfocytů během akutní infekce, převzato z Chen et al. (2018)* (8)*. Geny T lymfocytů jsou připraveny na iniciaci transkripce pomocí transkripčních faktorů. Celý proces je regulován buď epigeneticky (červeně) nebo transkripčně (modře). Když je aktivní transkripce genů pro naivní nebo paměťové buňky, tak je zároveň inaktivována transkripce pro geny efektorových buněk.*

Aby byla u $CD8^+$ T lymfocytů podpořena exprese povrchových markerů typických pro paměťový fenotyp (CD62L, CD27, CXCR3) je důležitá demetylace promotorů a acetylace histonů (8–11). Demetylace je důležitá i pro následné udržení paměťového fenotypu a rychlou dostupnost těchto buněk u reinfekcí (12). Při studiu vakcinace proti žluté zimnici se ukázalo, že místa zásadní pro tvorbu efektorových cytotoxických molekul Granzymu B (Gzmb) a Perforinu 1 (Prf1) zůstala v demetylovaném stavu minimálně 12 let po podání vakcíny (13).

2.2.3 Migrace do tkání

Paměťové T lymfocyty mohou teoreticky migrovat do jakýchkoliv tkání v závislosti na expresi adhezních molekul a cytokinových receptorů a lokálně zde působit proti antigenu (Obr. 2) (14,15).



Obr. 2: Kompartment necirkulujících tkáňově rezidentních paměťových T lymfocytů (CD69$^{+/-}$CD103) u myší a lidí, převzato z Szabo et al. (2019). Procenta udávají frekvenci T buněk v jednotlivých tkáních (15).

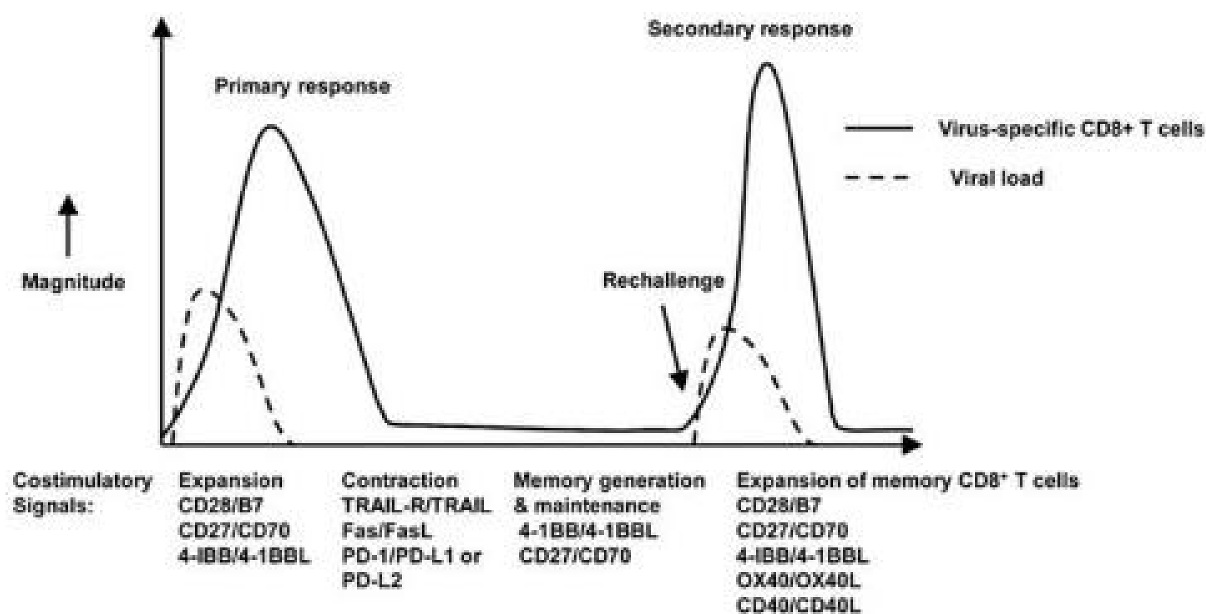
Ty paměťové T lymfocyty, které necirkulují v periférii, ale jsou trvale usídlené ve tkáních nelymfoidního původu se nazývají tkáňově rezidentní paměťové T buňky (T_{RM}). Jejich hlavní funkcí je zabezpečit lokální imunitní odpověď a zefektivnit tak eliminaci patogenů přímo ve tkáních (16). Přestože odhojení transplantovaného štěpu je multifaktoriální proces, zdá se, že T_{RM} můžou hrát v tomto procesu důležitou roli. Při studiu T_{RM} u nefrektomií transplantovaných štěpů bylo zjištěno, že tyto buňky byly

přítomny ve všech studovaných vzorcích. U štěpů, které selhaly časně po transplantaci (< 1 měsíc) převládaly dárcovské T_{RM}, a u později selhaných štěpů převládaly naopak T_{RM} od příjemce. Všechny T_{RM} exprimovaly vysokou koncentraci efektorových molekul uplatňujících se v rejekci štěpu (IFN-γ, TNF-α, Granzym B) (17).

2.2.4 Nižší nároky na kostimulaci

Při aktivaci virus specifických paměťových T buněk se kostimulační signál, uplatňuje v nižší míře než u ostatních subpopulací (18,19). Na obrázku č. 3 jsou zobrazeny kostimulační molekuly uplatňující se v jednotlivých fázích imunitní aktivace.

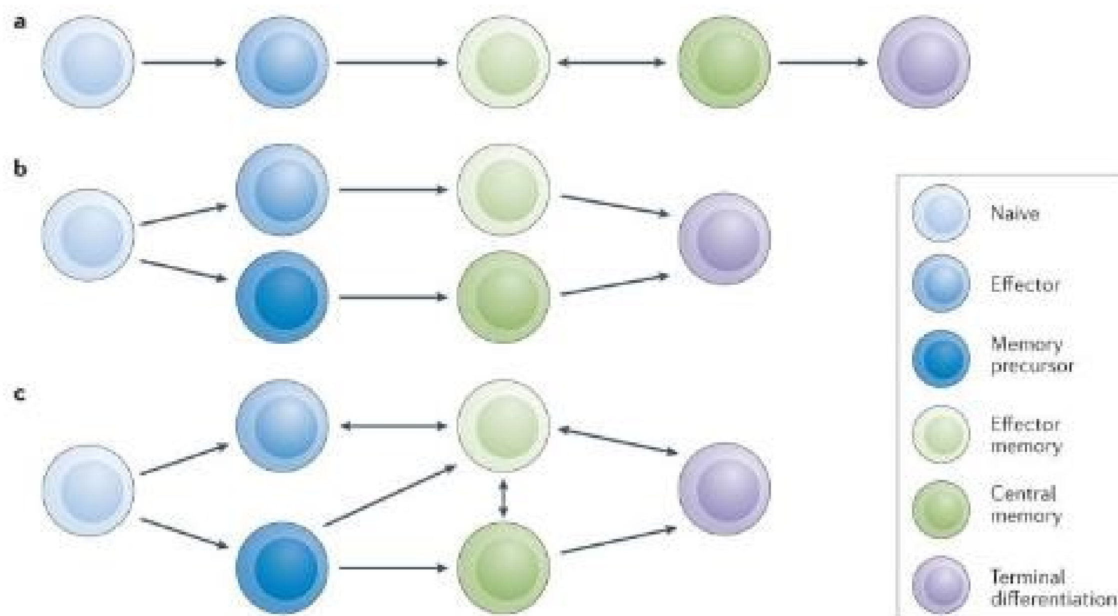
V mnoha starších studiích bylo uváděno, že k aktivaci paměťových T lymfocytů dochází i bez přítomnosti kostimulačního signálu. Tato tvrzení vycházela z několika *in vitro* a *in vivo* studií provedených na LCMV-specifických (virus lymfatické choriomeningitidy) paměťových $CD8^+$ T buňkách. Časem se však ukázalo, že LCMV se replikuje mnohem rychleji a extenzivněji než ostatní viry a antigenní prezentace tak zůstává v organizmu po delší dobu a s větší intenzitou, což umožní vznik silného TCR signálu, který již nepotřebuje dodatečnou kostimulaci (20–24).



Obr. 3: *Kostimulační molekuly uplatňující se během primární a sekundární fáze imunitní odpovědi, převzato z Duttagupta et al. (2009). Kostimulační molekuly mohou podpořit tvorbu a udržování paměťových virově-specifických* $CD8^+$ *T buněk a také jejich aktivaci během reinfekcí* (18).

2.3 Tvorba paměťových T lymfocytů

Jsou popsány dva modely popisující vznik a uchovávání paměťových T buněk: sekvenční (lineární) a paralelní (divergentní) (Obr. 4) (25).



Obr. 4: *Plasticita paměťových T lymfocytů, převzato z Espinosa et al. (2016). Sekvenční model diferenciace (a) je progrese diferenciace z naivních do efektorových a pak do paměťových subpopulací. Paralelní model diferenciace (b) vzniká, když se aktivovaný efektorový T lymfocyt rozdělí na 2 dceřiné buňky, ze kterých se stanou buď efektorové, nebo paměťové T buňky. Kombinovaná cesta T buněčného vývoje (c) spojuje oba modely diferenciace dohromady* (25).

U sekvenčního (lineárního) modelu naivní T lymfocyt po setkání s konkrétním antigenem projde procesem klonální expanze a získá své efektorové funkce ještě předtím, než se diferencuje do jednoho ze dvou základních fenotypů paměťových buněk: centrálního nebo efektorového (25).

Model paralelní (divergentní) diferenciace spočívá v tom, že aktivovaný T lymfocyt se po rozeznání antigenu rozdělí na dvě dceřiné buňky s odlišným fenotypem a funkcí: efektorovou a paměťovou (26,27). Asymetrické T buněčné dělení vychází z buněčné polarizace rodičovských buněk u jejich prvního dělení. Imunologická synapse koordinuje asymetrické buněčné dělení a paralelní diferenciaci tím, že

stabilizuje a prodlouží specifické vysokoafinitní interakce mezi TCR a HLA. To pak může vést k asymetrické segregaci proteinů umožňujících diverzifikaci T buněk během buněčného dělení (28,29).

2.3.1 Regulace diferenciace paměťových a efektorových T lymfocytů

K tomu, aby se z naivního T lymfocytu stala krátce žijící efektorová buňka, nebo dlouho žijící paměťová buňka jsou potřeba 3 druhy signálů: první je rozeznání antigenního peptidu v kontextu HLA I/II ($CD8^+$/ $CD4^+$) pomocí T-buněčného receptoru (TCR), druhý signál je kostimulační, a třetí je zprostředkován cytokiny (8).

Na úrovni všech tří signálu jsou přítomny regulační faktory, které podpoří diferenciaci buď směrem k paměťovému, nebo efektorovému fenotypu (Obr. 5). Tyto signály pak vedou k intracelulární aktivaci příslušného transkripčního faktoru (TF), který podpoří diferenciaci jednoho z fenotypů.

Zdali je indukce jednotlivých faktorů stochastická (náhodná), nebo je ovlivněna specifickým externím signálem prozatím není zcela známo. Ukazuje se, že tuto diferenciaci může také ovlivnit extracelulární koncentrace cytokinů: IL2, IL12 a IL27 podporují formaci efektorového fenotypu během akutních fází bakteriální nebo virové infekce a interferony typu I a II společně s IL15, IL10 a IL21 naopak podporují diferenciaci paměťových buněk (8).

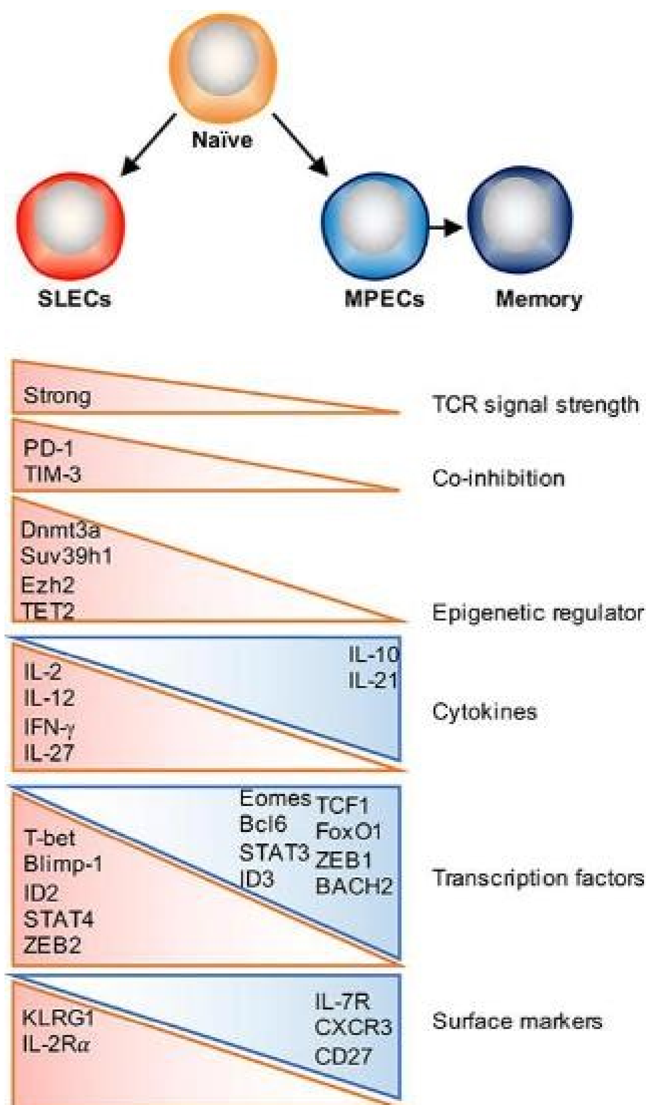
Také rovnováha mezi kostimulačními a koinhibičními signály je důležitá pro efektorovou T buněčnou aktivaci a expanzi. Vysoká exprese kostimulačních molekul (CD28, 4-1BB, CD27 a OX40) společně s nízkou expresí koinhihičního PD-1 podporují formaci paměťových buněk a následnou sekundární odpověď (8).

Signalizace přes TCR je jedním z prvních signálů, které pomáhají utvářet T buněčnou paměť. Síla a kvalita TCR signalizace, která je determinována: i) afinitou TCR pro komplex peptid-HLA (pHLA), ii) dávkou antigenu prezentovaného na APC a iii) délkou a načasováním interakce TCR-pHLA, přispívá k vytváření funkčního paměťového fenotypu T lymfocytů.

Signalizace přes TCR se nenachází pouze ve stavu „vypnuto/zapnuto“ ale je to souhrn signálů s rozdílnou intenzitou (30). Během infekce je efektorová buňka vystavena aktivně se replikujícímu patogenu, je proto důležité, aby se vyselektovaly T lymfocyty s nejvyšší afinitou pro daný antigen, z nich se pak stanou efektorové T lymfocyty, které mají vysokou schopnost proliferace. Naopak u re-infekcí je důležité najít kompromis mezi správnou mírou specificity a diverzity. Selekční tlak působí i na

patogen a podporuje zvýšenou mutagenicitu jeho epitopů, aby nebyl rozeznán imunitním systémem hostitele. V tomto případě by byla vysoká specificita TCR spíše na škodu, protože u opětovného setkání patogen pravděpodobně vypadá jinak než na začátku (31).

Afinita TCR se ukazuje být klíčová nejen u infekcí, ale i u orgánových transplantací, kde je jedním z důležitých faktorů vzniku následné rejekce. Na myším modelu transplantace kůže byl otestován vliv vysoko- i nízko-afinitních TCR na vznik rejekce a ukázalo se, že u rejekce je vyšší podíl T lymfocytů s vysoko-afinitním TCR a jejich eliminace/blokace by mohla být jedním z aspektů k navození transplantační tolerance (32).



Obr. 5: *Faktory regulující osud buněčné diferenciace efektorových a paměťových T lymfocytů, převzato z Chen et al. (2018). V procesu rozhodování, který z fenotypů se bude dál vyvíjet, se účastní několik faktorů: síla TCR signalizace, kostimulace vs koinhibice, cytokiny, transkripční faktory a epigenetické regulátory* (8).

2.4 Subpopulace paměťových T lymfocytů

Podle místa působení a rozdílné exprese povrchových markerů (tab. 1) rozdělujeme paměťové T lymfocyty na centrální (T_{CM}) a efektorové (T_{EM}).

2.4.1 Centrální paměťové T lymfocyty (T_{CM})

T_{CM} migrují do sekundárních lymfoidních orgánů, jsou dlouho žijící, zachovávají si vysokou proliferační kapacitu a mohou velice efektivně amplifikovat sekundární imunitní odpověď po opětovném setkání s daným antigenem. Na svém povrchu exprimují „*homing*“ molekuly $45RO^{+}CCR7^{+}$ $CD62L^{+}$. Povrchový marker CD62L (L-selektín) a CCR7 (chemokín) napomáhají „*homingu*“ T lymfocytů do sekundárních lymfatických orgánů přes endoteliální venuly.

2.4.2 Efektorové paměťové T lymfocyty (T_{EM})

T_{EM} na svém povrchu neexprimují „*homing*“ molekuly, protože jejich místo určení jsou periferní tkáně (33,34). Jejich základní fenotypizace je $45RO^{+}CCR7^{-}CD62$-. Tyto lymfocyty cirkulují v periferii, žijí krátce a mají omezenou proliferační kapacitu. Rychle se infiltrují do místa zánětu, kde vykonávají své efektorové funkce, zároveň však mají omezenou schopnost expanze a amplifikace (33).

2.4.2.1 Subpopulace T_{EM} (tab. 1):

a) Terminálně diferencované efektorové paměťové T lymfocyty (T_{EMRA}) jsou koncovým stadiem efektorové diferenciace a na svém povrchu re-exprimují CD45RA (35). CD45 je členem protein tyrosin fosfatázové rodiny (PTP), který se exprimuje na všech diferencovaných hematopoetických buňkách (kromě erytrocytů a plasmatických buněk), izoforma CD45 RA obsahuje jen A proteinový region, který je typický pro naivní T lymfocyty a T_{EMRA} buňky ho znovu re-exprimují.

b) Tkáňově rezidentní paměťové T buňky (T_{RM}), které necirkulují v periferii, ale jsou lokalizované ve tkáních. Jsou typické svou expresí CD69 (časný marker aktivace), αEβ7 integrin (CD103) a také několik tkáňově specifických chemokinových receptorů které se exprimují podle lokalizace v konkrétní tkáni (36,37).

c) Periferní paměťové T lymfocyty (T_{PM}) jsou charakteristické expresí Cx3Cr1 a specifickou migrační dráhou. T_{PM} se vyskytují ve vysoké koncentraci v periferní cirkulaci. T_{PM} mohou migrovat do tkání a i zpět do lymfatických uzlin přes aferentní cévy (38).

Subpopulace	Fenotyp	Funkce	Lokace	Transkripční faktor	Reference
T_{EM}	$CCR7^{LO}/CD62^{LO}$ $CX3CR^{HI}/CD27^{LO}$ $CD127^{HI}$ $CD27^{-}/CD45RA^{-}$	++cytotoxicita +-proliferace	Cirkulace	$Tbet^{HI}$ $Blimp1^{HI}/ID2^{HI}/STAT4^{HI}$	(39–44)
T_{CM}	$CCR7^{HI}/CD62^{HI}$ $CX3CR^{LO}/CD27^{HI}$ $CD127^{HI}$ $CD27^{+}/CD45RA^{-}$	+-cytotoxicita ++proliferace	Cirkulace Lymfatické uzliny	$Tbet^{LO}$ $Eomes^{HI}/TCF1^{HI}/BCL6^{HI}/$ $STAT3^{HI}/ID3^{HI}$	(39–42,45,46)
T_{EMRA}	$CCR7^{-}/CD27^{-}/CD45RA^{+}$ $CD127^{LO}$	++cytotoxicita +-proliferace	Cirkulace		(47)
T_{RM}	$CD69^{HI}/CD103^{HI}/CD49a^{HI}$ (závislé od tkáně) $CXCR3^{HI}/KLRG1^{LO}/CCR7^{LO}/$ $CD62L^{LO}$, $CD127^{HI}$, $Cx3Cr1^{LO/INT}$	Kontrola prostředí (alarm) +proliferace	Rezidence v tkáních	$KLF2^{-/LO}/Eomes^{-/LO}$ $Tbet^{LO}/TCF1^{LO}$ $Hobit^{HI}/Blimp1^{HI}$	(38,48–50)
T_{PM}	$CCR7^{+/-}/CD62L^{+/-}/CD127^{HI}$ $Cx3Cr1^{INT}/CD27^{HI}$	+cytotoxicita +proliferace	Cirkulace Lymfatické uzliny Rezidence v tkáních	$Tbet^{+/-}$	(38,51,52)
Ostatní	$CD27^{LO}/CD43^{LO}$ $KLRG1^{HI}$, $CD127^{LO}$	++cytotoxicita +-proliferace	Rezidence v tkáních	$Tbet^{HI}/Eomes^{LO}$	(52,53)

Tab 1: *Heterogenita paměťových $CD8^{+}$ T lymfocytů, přeloženo z Martin and Badovinac 2018* (14).

2.5 Detekce antigen-specifických paměťových T lymfocytů

Pro detekci antigen-specifických buněk v těle pacienta můžeme použít 2 základní metody- průtokovou cytometrii (FACS) a ELISPOT. Použití obou metod sebou nese jisté výhody a zároveň i nevýhody.

U průtokové cytometrie se antigen-specifické buňky detekují pomocí multimerů (tetramery, pentamery, dextramery), intracelulárního barvení cytokinů nebo kvantifikací buněčné proliferace. Výhodou této metody je, že známe přesnou fenotypizaci analyzované subpopulace buněk. Mezi limitace analýzy buněk pomocí FACSu patří u multimerního barvení znalost epitopů a jejich restrikčních HLA molekul, což může být omezující při diagnostice v klinické praxi. U alogenních transplantací mohou hrát důležitou roli alloantigen specifické paměťové buňky, které mohou negativně ovlivnit přežití transplantovaného štěpu (54–56). Jejich kvantifikace by tedy mohla být přínosná pro použití v klinické praxi (57).

Pomoci by v tomto případě mohla druhá metoda- ELISPOT, která není limitovaná HLA restrikcí a přesnou znalostí epitopů dárce. Také vysoká reprodukovatelnost výsledků mezi laboratořemi může naplnit svůj potenciál ve využití v klinické praxi (58–60). ELISPOTem detekujeme živé buňky, které po stimulaci produkující IFN-γ. Stimulujícím antigenem mohou být virové peptidy (CMV, EBV, BKV) nebo buňky dárce. U této metody neznáme přesnou fenotypizaci analyzovaných buněk, známe pouze počet buněk reagujících na přítomnost antigenu (61).

2.6 Paměťové T lymfocyty u orgánových transplantací

Tato disertační práce je zaměřená na dárcovsky- specifické a CMV- specifické paměťové T lymfocyty. Oba druhy buněk mohou ovlivnit potransplantační fungování štěpu tím, že mohou interagovat s antigeny štěpu ať již přímo (dárcovsky- specifické) nebo zkříženě (CMV- specifické).

2.6.1 Alloreaktivní paměťové T lymfocyty (dárcovsky-reaktivní T lymfocyty)

Cirkulující alloreaktivní paměťové buňky byly již několikrát spojeny s klinickým a subklinickým poškozením transplantovaného orgánu (62,63). Jejich přítomnost u pacientů před transplantací může mít několik možných vysvětlení: těhotenství, krevní transfuze, heterologní imunita, duálně receptorové T lymfocyty nebo homeostatická proliferace (54,64–66).

2.6.1.1 Zdroje alloreaktivních paměťových T lymfocytů

2.6.1.1.1 Duálně-receptorové T lymfocyty

Standardně T lymfocyt díky alelické exkurzi exprimuje na svém povrchu pouze jeden alfa a jeden beta řetězec TCR. Nicméně již byla dokumentována existence 2 rozdílných TCR na jednom T lymfocytu. Již v roce 1993 Padovan et al. ukázal, že až 30% lidských T lymfocytů exprimuje druhý alfa a beta řetězec na úrovni mRNA a až 8% lidských T lymfocytů exprimuje druhý alfa a beta řetězec na úrovni na úrovni proteinu (67).

Každý TCR může rozeznat jak specifický patogen, tak i alloantigen, oba mohou koexistovat na tom samém T lymfocytu, tudíž po setkání s patogenem se T lymfocyt aktivuje a diferencuje do paměťového fenotypu a následné rozeznání alloantigenu je pak efektivnější. Praktický vliv těchto T lymfocytů na přežití štěpu byl ověřen na myším modelu reakce štěpu proti hostiteli (GvH), kde duální $CD4^+$ T buňky byly ve větším množství nalezeny u allorektivního T buněčného kompartmentu (68).

2.6.1.1.2 Heterologní imunita

Dalším způsobem generování paměťových T buněk je jejich tvorba během infekcí jako součást heterologní imunity. Heterologní imunita je velice dobře popsána jak u lidí, tak i na experimentálních zvířecích modelech (69–71).

Heterologní imunita může zvyšovat přirozenou odolnost vůči infekcím i účinnost očkování (72). Na druhé straně může přispět ke vzniku většího množství alloreaktivních buněk.

Během infekcí, jsou virové peptidy prezentované na endogenních HLA a následně pak rozeznány pomocí TCR na povrchu T lymfocytů. To pak spustí jejich efektorovou diferenciaci, aktivaci a expanzi. Část paměťových virově specifických T buněk, může zkříženě reagovat s alloantigenem a ovlivnit tak funkčnost transplantovaného orgánu. Ve studii Stranavova et al. (2019) byly $CD8^+$ zkříženě reagující klony nalezeny nejen v periferní krvi u příjemců transplantované ledviny, ale i v potransplantační biopsii, což dokazuje potenciální klinický význam těchto buněk (72).

Přítomnost patogenem-indukovaných paměťových alloreaktivních T lymfocytů představuje bariéru transplantační tolerance (71,73). Ukazuje se, že patogenem vyvolaná zkřížená reaktivita je pravděpodobně častější než původně vzniklé allospecifické T lymfocyty (74,75). Ve studii Amir at al. (2010) autoři testovali reaktivitu T buněk proti panelu HLA-typizovaných buněk a zjistili, že 80% testovaných $CD8^+$ T buněčných linii a 45% patogen-specifických T buněk vykazuje alloreaktivitu proti alespoň 1 HLA alele (76).

2.6.1.1.3 Homeostatická proliferace

Paměťové T buňky mohou být také generovány homeostatickou proliferací, která často nastává po lymfopenii jako následek imunosupresivní léčby, dlouhodobé infekce, nádorového onemocnění, těžkých kombinovaných imunodeficiencí (SCID), nebo transplantací (77).

Naivní $CD4^+$ a $CD8^+$ alloreaktivní prekurzory T buněk mohou v prostředí s IL7 a IL15 proliferovat a diferencovat přímo do paměťového fenotypu. Paměťové T lymfocyty produkované během lymfopenií indukované homeostatické proliferace se liší od konvenčních paměťových T lymfocytů. Vznikají tak, že naivní T lymfocyty rozeznají vlastní peptid prezentovaný na HLA neaktivní antigen prezentující buňky (APC) s afinitou, která je mnohem nižší než v případě rozeznávání cizích peptidů, ale zároveň je vyšší než afinita potřebná pro thymickou pozitivní selekci.

Homeostatická proliferace naivních buněk do paměťového fenotypu není spojena se zvýšenou expresí aktivačních markerů, jakými jsou např. CD25, CD69, nebo CD45RB, je pro ně ale typická zvýšená exprese CD122 (receptor pro IL7 a IL15) a CD132 (cytokinový receptor). Také se ukazuje, že kostimulace přes CD28 a dostupnost IL2 jsou mnohem víc důležité pro antigen-specifické paměťové T lymfocyty než pro ty diferencované homeostatickou proliferací (78).

Myší modely ukázaly, že homeostatická proliferace může být překážkou v navození tolerance transplantovaného orgánu (79). Studie naznačují, že v lymfopenickém prostředí může homeostatická proliferace přispět k expanzi paměťových buněk, které brání navození tolerance, a to jak proliferací reziduálních paměťových buněk, tak i konverzí naivních buněk do paměťového fenotypu. Paměťové T lymfocyty proliferují v procesu homeostatické proliferace po depleci výrazně rychleji než naivní T buňky. Tyto data naznačují, že homeostatická proliferace může přispět

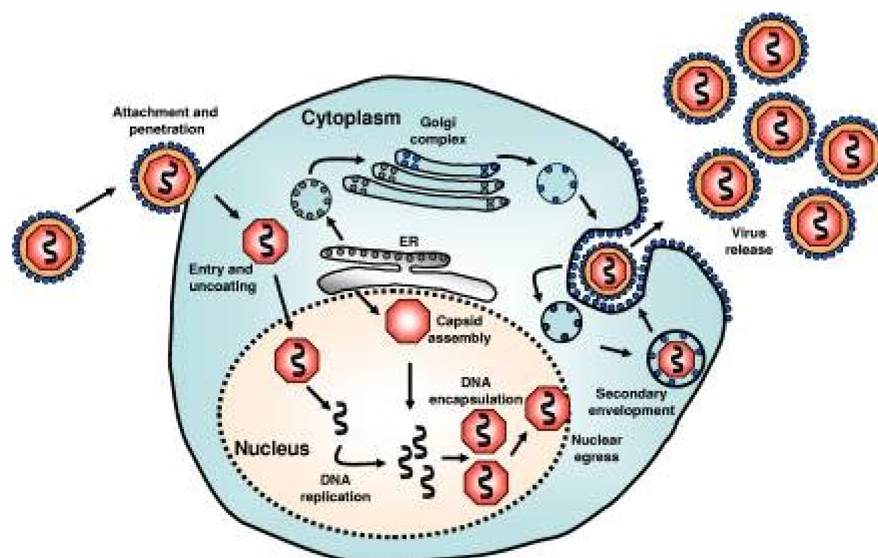
k rozvoji alloreaktivity v případě, že již existující alloreaktivní T lymfocyty jsou expandovány, nebo převedeny do paměťového fenotypu (79).

2.6.2 CMV-reaktivní paměťové T lymfocyty

Další subpopulace paměťových T lymfocytů, kterou se tato disertační práce zabývá jsou CMV-reaktivní paměťové T buňky, které vznikly během CMV primoinfekce a v kontextu allogenních transplantací mohou u imunosuprimovaných pacientů negativně ovlivnit funkčnost a přežití transplantovaného štěpu.

2.6.2.1 Cytomegalovirus

Lidský cytomegalovirus (CMV) je ubiquitinní herpesvirus beta typ 5 s rozsáhlým genomem ~235 kb (80). Virion obsahuje dvou- řetězcovou lineární DNA, která je uložena v nukleokapsidě obalené virovými proteiny a glykoproteiny (81). Tyto proteiny a glykoproteiny pomáhají viru při vstupu do hostitelské buňky. Tam mohou vstoupit buď přímou fúzí s cytoplasmatickou membránou nebo pomocí endocytózy. Fúze virového obalu s buněčnou membránou umožní uvolnění nukleokapsid do cytoplasmy, ty jsou pak přemístěny do jádra, kde se uvolňuje virová DNA. Následně pak dochází k replikaci viru a zapouzdření nově vzniklého virionu do kapsidy, která je dále transportována do cytoplasmy a po přidání sekundárního glykolipidového obalu je virion uvolněn z buňky exocytózou (Obr. 6) (82).



Obr 6: Životní cyklus lidského CMV, přeloženo z Crough et al. (2009) (82).

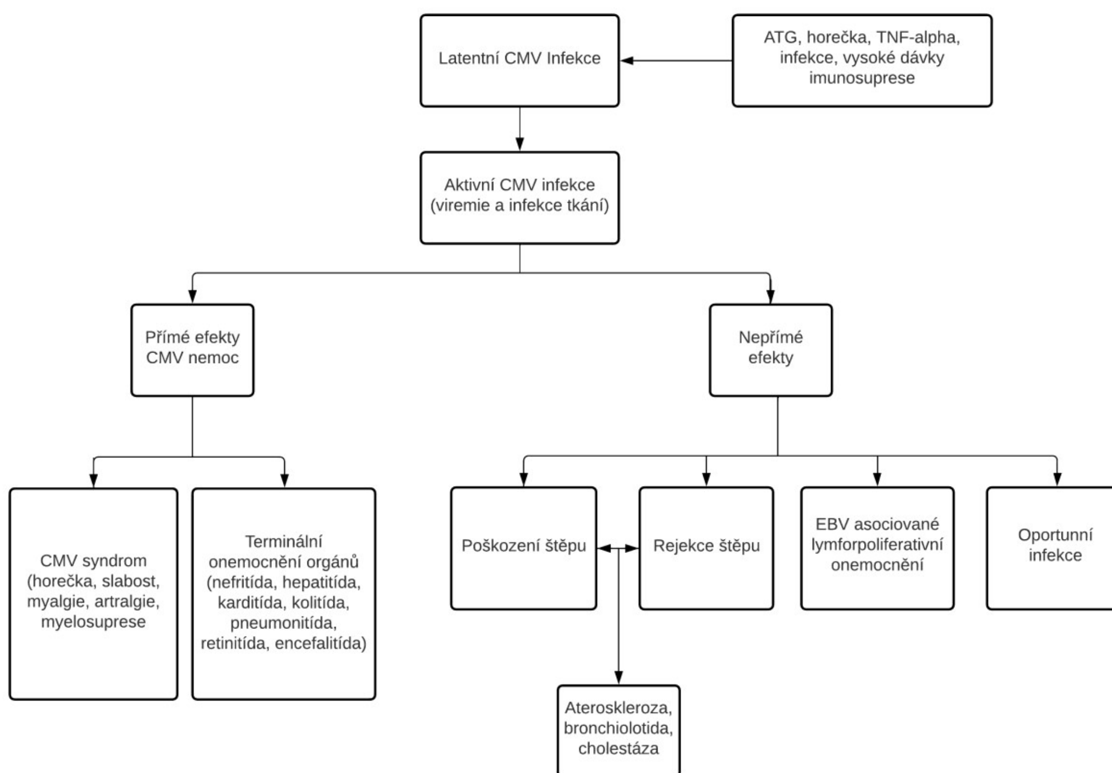
2.6.2.2 CMV infekce

CMV se přenáší slinami, sexuálním kontaktem, transplacentárně, kojením, krevní transfúzí, transplantací orgánů a kostní dřeně (83). Globální CMV seroprevalence, stanovena kvantifikací CMV IgG protilátek, byla v roce 2019 83% (95% UI: 78-88) (84).

Primární CMV infekce je standardně bezpříznaková a v těle pacienta pak celoživotně zůstane CMV v latentní fázi s občasnou reaktivací. Během latentní infekce CMV genom zůstává uložen v mononukleárních buňkách, včetně monocytů a makrofágů (85,86), lymfocytů (87), kmenových buněk (88), nezralých dendritických buněk (89) a endoteliálních buněk (90). Reaktivace CMV z latentního stavu je klíčový krok v patogenezi CMV infekce, tato reaktivace je často reakcí na imunosupresi, zánět, infekci, nebo stres (91). I když je u zdravých jedinců infekce CMV často bezpříznaková, u novorozených dětí, plodu v těle matky a pacientů s imunosupresivní léčbou, může způsobit řadu závažných klinických komplikací.

2.6.2.3 CMV u orgánových transplantací

U orgánových transplantací je CMV signifikantním zdrojem morbidity a mortality a je asociován s horším přežitím štěpu (92). Pacienti, kteří prodělali CMV primo-infekci a mají ve svém těle adaptivní imunitní paměť, která je tvořena CMV-specifickými $CD4^+$ a $CD8^+$ paměťovými T lymfocyty a imunoglobulinem G (IgG), by měli být chráněni před opakovanou virovou reaktivací (82). U orgánových transplantací může z důvodu užívání imunosupresivní léčby dojít k selhání adaptivní imunity. U CMV seropozitivních příjemců transplantovaného orgánu může vlivem snížení CMV specifické imunity dojít k rozvoji CMV infekce či CMV onemocnění. Nepřímým efektem virové reaktivace je výskyt oportunních infekcí, akutní nebo chronické rejekce, kardiovaskulárních onemocnění a diabetu (Obr. 7) (93).



Obr. 7: *Cytomegalovirová infekce, přeloženo z Fishman et al. (2007)* (94). *CMV může způsobit jak invazivní onemocnění („přímé efekty CMV") tak i doprovodné imunologické jevy („nepřímé efekty CMV"), včetně rejekce transplantovaného štěpu* (94).

Míra rizika CMV infekce po transplantaci orgánů je asociována s výskytem několika rizikových faktorů, mezi ně patří například CMV seropozitivita dárce nebo příjemce. Nejvíce rizikovou skupinou jsou dvojice, kdy je dárce CMV seropozitivní a příjemce CMV seronegativní (D+/R-), naopak transplantace kdy je jak dárce, tak i příjemce CMV seronegativní (D-/R-) je z hlediska reaktivace CMV nízkoriziková. Ve středním riziku jsou seropozitivní příjemci, kteří dostali transplantovaný orgán od seropozitivního dárce (D+/R+). Mezi další rizikové faktory pro vznik post-transplantační CMV infekce patří: deficit CMV specifické celulární imunity (CMV specifické $CD4^+$ a $CD8^+$ T lymfocyty), rejekce transplantátu, virová replikace a imunosupresivní léčba depletujícím králičím antithymocytovým globulinem (rATG).

2.6.2.4 Cytomegalovirus a rejekce

Přítomnost aktivní CMV infekce je u orgánových transplantací asociována s vyšším výskytem akutní a chronické rejekce (95,96). Latentní CMV infekce vyvolává silnou doživotní T buněčnou imunitu, která kontroluje CMV reaktivaci/reinfekci a funguje jako prevence CMV nemoci pomocí permanentní dynamické interakce mezi virem a CMV-reaktivními T buněčnými klony. Přestože primárním cílem paměťových CMV-specifických T lymfocytů je ochrana před další potenciální infekci, mohou u transplantovaného orgánu způsobit poškození tkáně.

Jsou 3 základní mechanismy, které mohou toto poškození vyvolat. Prvním z nich je přímý cytotoxický efekt na buňky štěpu, které jsou infikované CMV, druhým mechanismem je *bystander* aktivace, která vytvoří lokální prozánětlivé prostředí a třetím mechanismem je heterologní imunita - zkřížená reakce mezi CMV a alloantigeny (96).

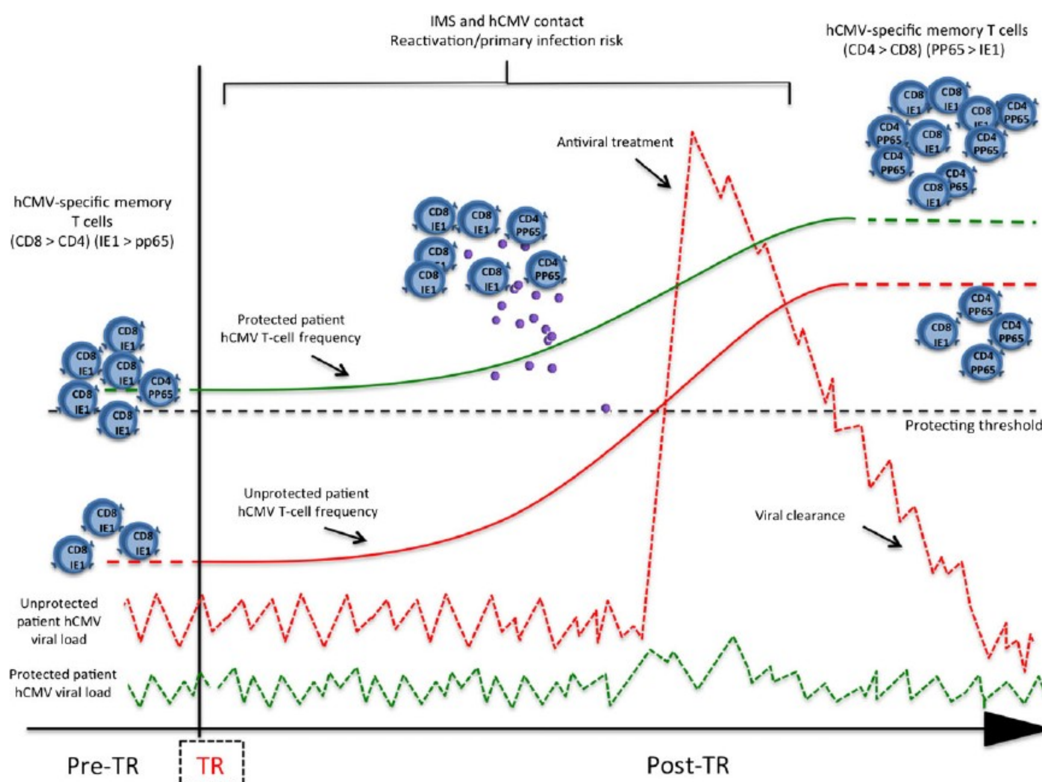
2.6.2.5 Monitorace CMV specifické adaptivní imunity

CMV infekce je jednou z hlavních komplikací po renální transplantaci, která má signifikantní vliv jak na život pacienta, tak i transplantovaného orgánu. Monitorace CMV specifické adaptivní imunity (humorální a T buněčné) v různých časových bodech po transplantaci se ukazuje jako zásadní v predikci rizika vzniku virové infekce. Predikce rozvoje CMV může napomoci k individualizaci terapie v klinické praxi. Ke zhodnocení humorální imunity slouží analýza anti-CMV protilátek v séru (IgM indikující akutní infekci a IgG předchozí infekci). Dynamika virové infekce může být sledována kvantifikací virové nálože CMV pomocí RT-PCR.

Celulární adaptivní imunita je pro kontrolu CMV infekce nezbytná, potenciální replikaci kontrolují jak $CD4^+$, tak $CD8^+$ T lymfocyty, obě subpopulace T buněk jsou v tomto procesu stejně důležité (Obr. 8) (97,98). Ukazuje se, že $CD8^+$ T buňky jsou zásadní při kontrole CMV replikace a $CD4^+$ T buňky se účastní dlouhodobé ochrany při udržování hladin virově specifických protilátek (65,99–101). Vysoce diverzifikovaná T- buněčná odpověď se vyvíjí mezi 4-6 týdnem po prvotní expozici antigenu. Paměťový kompartment je generován v závislosti na množství antigenu, míře virové replikace a typu infikované tkáně (102).

T lymfocyty rozeznávají antigeny pocházející z CMV specifických proteinů. Virové proteiny jsou exprimovány v různých stádiích virové replikace (časný, středně časný, pozdní) a plní odlišné funkce (kapsida, matrix/tegument, glykoprotein, regulační

protein atd.). Mezi nejvíce imunodominantní CMV antigeny patří IE-1 (immediately early -1) iniciační protein, a pp65 (phosphoprotein 65) matrix- tegumentový protein.



Obr 8: Schéma CMV specifické T- buněčné odpovědi u pacientů po orgánové transplantaci, přeloženo z Lucia et al. (2014) (103).

Pro monitoraci kinetiky a funkce CMV specifického T buněčného kompartmentu se používá několik různých esejí, mezi nejčastěji používané patří intracelulární barvení cytokinů (FACS), ELISPOT, tetramery (FACS) a quantiferon. Hlavní charakteristiky jednotlivých metod jsou shrnuty v tabulce 02.

	Intracelulární barvení cytokinů (FACS)	**ELISPOT (IFN-γ, IL2)**	**Tetramery (FACS)**	**Quantiferon (založen na ELISA)**
Materiál, množství	Periferní krev/ PBMC, 1ml/1x10^6	PBMC, 1x10^6	PBMC, 1x10^6	Periferní krev ¾ ml

Frekvence	% CMV-specifických CD8$^+$ nebo CD4$^+$/ IFN-γ buněk	CMV- specifických IFN-γ produkujících buněk	% CMV-specifických CD8$^+$ nebo CD4$^+$/ IFN-γ buněk	Detekce IFN-γ (IU)/ml
Čas	48h	36h	48h	24h
Protektivní práh (frekvence)	0.2-0.4%	8-11 IFN-γ spotů	nespecifický	0.1-0.2 IU/ml
Antigen pro stimulaci	15AA dlouhý peptidový pool obsahující antigen, nebo virový lyzát	15AA dlouhý peptidový pool obsahující antigen, nebo virový lyzát	15AA dlouhý peptidový pool obsahující antigen, nebo virový lyzát	Kolekce jednotlivých 15AA peptidů
Výhody	Vysoká sensitivita, bez HLA restrikce, nezávislá analýza CD4 a CD8	Vysoká sensitivita, bez HLA restrikce, reprodukovatelnost	Barvení jednotlivých epitop-specifických klonů	Vysoká sensitivita, Schváleno pro použití v klinické praxi v EU
Nevýhody	Potřebné vybavení (FACS), kvalifikovaný personál	Potřebné vybavení (ELISPOT), kvalifikovaný personál	HLA restrikce, potřebné vybavení (FACS)	HLA restrikce, vzácné HLA alely jsou vyloučeny z analýzy

Tabulka 02: Hlavní imunitní eseje pro stanovení lidské CMV specifické T buněčné odpovědi, přeloženo z Lucia et al. (2014) (103).

2.6.2.6 Terapeutická strategie u CMV

Vzhledem k riziku CMV nemoci u pacientů s vysokým rizikem se standardně podává protivirová profylaxe 6 měsíců (valgancyklovir), ostatním pacientům v riziku pak 3 měsíce. Samozřejmostí je pravidelná potransplantační kontrola virové replikace pomocí RT-PCR (103,104). Efekt indukční a udržovací imunosuprese na CMV primární infekci a nemoc je velice dobře popsán u CMV seronegativních příjemců (R-) transplantované ledviny. Depleční indukční terapie, např. rATG, je asociována s vyšším rizikem vzniku CMV infekce ve srovnání s nedepleční terapií (basiliximab) (105). rATG se používá jako prevence i léčba akutní rejekce u orgánových transplantací. rATG způsobuje lymfocytopenii a masivní T buněčnou depleci, která může vést ke zvýšenému výskytu infekcí, včetně cytomegalovirové. T buněčná repopulace, která může být v lymfocytopenických podmínkách podpořena thymopoezou, homeostatickou proliferací a antigenem indukovanou expanzi (106).

Předchozí studie zaměřené na analýzu dynamiky T buněk po léčbě rATG ukázaly perzistentní depleci $CD4^+$ T buněk a rychlou repopulaci $CD8^+$ T lymfocytů (107–109). U imunosuprimovaných pacientů často dochází k CMV reaktivaci a ke zvýšení frekvenci CMV-specifických $CD8^+$ T buněk oproti zdravým jedincům (110). Deficit paměťových buněk v lymfopenii v kombinaci s virovou reaktivací by mohl vést k CMV infekci, která by v časném potransplantačním období mohla mít fatální následky pro zdraví pacienta a funkčnost štěpu, proto je důležité u těchto pacientů použití profylaktické antivirové terapie.

3 Cíle práce

Cílem projektu bylo ověření role efektorových a paměťových T lymfocytů u pacientů před a po transplantaci ledviny se zaměřením na CMV-specifickou a dárcovsky-specifickou imunitní odpověď.

1. **Analyzovat vliv indukční terapie na CMV specifické efektorové/paměťové T lymfocyty po transplantaci ledviny od žijících dárců**

CMV reaktivní buňky byly stanoveny metodou ELISPOT, která kvantifikuje počet IFN-γ pozitivních efektorových /paměťových T lymfocytů po stimulaci CMV-specifickými antigeny (IE-1, pp65) před a 6M po transplantaci. Zajímal nás vliv imunosuprese (indukční i udržovací) na potransplantační vývoj CMV-specifické imunity u příjemců transplantované ledviny, kteří již v minulosti prodělali CMV primoinfekci a mají vyvinutou imunitní paměť proti CMV.

2. **Ověřit hypotézu heterologní imunity; předpokládali jsme, že CMV specifické paměťové T lymfocyty jsou aktivovatelné antigeny dárce, což by mohlo vést k přímému poškození transplantovaného orgánu**

Zkřížená reaktivita mezi CMV a alloantigeny byla ověřena pomocí NGS analýzy TCR-β identických klonů T lymfocytů, které reagovaly na přítomnost obou studovaných antigenů svou proliferací. Identické zkříženě reagující klony byly hodnoceny jak před samotnou transplantací v periferní krvi pacientů, tak i po transplantaci v renální biopsii.

3. **Studovat vliv délky dialyzační terapie na frekvenci periferních virově specifických efektorových/paměťových buněk a subpopulace DC, B a T lymfocytů u pacientů s chronickým selháním ledvin v terminálním stadiu (CKD5)**

CKD5 pacienti, kteří byli dlouhou dobu léčeni na dialýze, v uremickém prostředí, často vykazují obtížně definovatelné poškození imunitního systému. Naším cílem bylo zjistit, do jaké míry jsou ovlivněny paměťové/efektorové T lymfocyty virů způsobující hlavní oportunní infekce po transplantaci- CMV, BK, EBV. Dále nás

také zajímalo, zda dialýza ovlivňuje počty buněk jednotlivých subpopulací T a B lymfocytů a dendritických buněk.

4 Seznam vlastních publikací

4.1 Seznam použitých publikací

Stranavova L, Hruba P, Girmanova E, Tycova I, Slavcev A, Fronek J, Slatinska J, Reinke P, Volk HD, Viklicky O. The effect of induction therapy on established CMV specific T cell immunity in living donor kidney transplantation. Physiol Res. 2018 May 4;67(2):251-260. IF: 1. 670.

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5 Výsledky

5.1 Efekt indukční terapie na stanovení CMV specifické imunity v transplantaci ledvin od žijících dárců

Stranavova L, Hruba P, Girmanova E, Tycova I, Slavcev A, Fronek J, Slatinska J, Reinke P, Volk HD, Viklicky O.

Physiol Res. 2018 May 4;67(2):251-260

Infekce cytomegalovirem (CMV) ovlivňuje krátkodobé i dlouhodobé výsledky u imunosuprimovaných příjemců transplantovaných orgánů. Cílem této studie bylo zhodnotit účinek různých indukčních imunosupresivních režimů na počet CMV specifických T buněk u pacientů s již prokázanou CMV- specifickou imunitou. U 24 seropozitivních příjemců ledvin od žijících dárců byla stanovena frekvence CMV specifických T buněk metodou ELISPOT (Enzyme-linked ImmunoSpot) před a 6 měsíců po transplantaci. Příjemcovské periferní mononukleární buňky (PBMC) byly stimulovány CMV specifickými antigeny: časným IE1 (*Intermediate early*- IE1) a fosfoproteinem 65 (pp65), a následně byl stanoven počet buněk produkujících interferon gama (IFN-gama). Pacienti dostávali indukční léčbu buď depletujícím králičím antithymocytovým globulinem (rATG) nebo nedepletujícím basiliximabem a udržovací imunosupresi takrolimem / mykofenolát mofetilem / steroidy. Pacienti s indukcí rATG dostali také profylaxi valgancyklovirem. Zjistili jsme, že šest měsíců po transplantaci ledvin nebyly pozorovány žádné účinky indukční imunosuprese na počet CMV specifických buněk. Nebyly zjištěny žádné asociace mezi délkou dialýzy, předtransplantační CMV specifickou T buněčnou imunitou a pozdějším výskytem CMV DNAemií. Také nebyl prokázán žádný vliv CMV profylaxe na imunitu CMV specifických T buněk. Tato studie ukázala, že potransplantační imunosupresivní léčba nemá vliv na imunitu CMV specifických T buněk u CMV seropozitivních příjemců transplantovaných ledvin od žijících dárců, bez ohledu na depleci lymfocytů a CMV profylaxi.

Podíl na publikaci: kolekce materiálu od pacientů, experimentální část (izolace PBMC, ELISPOT), statistická analýza výsledků, příprava manuskriptu

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The Effect of Induction Therapy on Established CMV Specific T Cell Immunity in Living Donor Kidney Transplantation

L. STRANAVOVA[1], P. HRUBA[1], E. GIRMANOVA[1], I. TYCOVA[1], A. SLAVCEV[4], J. FRONEK[3], J. SLATINSKA[2], P. REINKE[5,7], H.-D. VOLK[6,7], O. VIKLICKY[1,2]

[1]Transplant Laboratory, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [2]Department of Nephrology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [3]Department of Transplant Surgery, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [4]Department of Immunogenetics, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [5]Department of Nephrology and Intensive Care Medicine, Charité University Medicine Berlin, Germany, [6]Institute of Medical Immunology, Charité University Medicine Berlin, Germany, [7]Berlin-Brandenburg Center for Regenerative Therapies, Charité University Medicine Berlin, Germany

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Summary

Cytomegalovirus (CMV) infection influences both short and long term outcomes in immunosuppressed organ transplant recipients. The aim of this study was to evaluate the effect of different induction immunosuppression regimens on CMV specific T cell response in patients with already established CMV immunity. In 24 seropositive living donor kidney recipients, the frequency of CMV specific T cells was determined by ELISPOT (Enzyme-Linked ImmunoSpot) assay prior and 6 months after transplantation. Recipients' peripheral blood mononuclear cells were stimulated with immediate-early (IE1) and phosphoprotein 65 (pp65) CMV-derived peptide pools and the number of cells producing interferon gamma (IFN-γ) was assessed. Patients received quadruple immunosuppression based either on depletive rabbit antithymocyte globulin (rATG) or non-depletive basiliximab induction and tacrolimus/mycophenolate mofetil/steroids. Patients with rATG induction received valgancyclovir prophylaxis. No effects of different induction agents on CMV specific T cell immunity were found at sixth month after kidney transplantation. There were no associations among dialysis vintage, pretransplant CMV specific T cell immunity, and later CMV DNAemia. Similarly, no effect of CMV prophylaxis on CMV specific T cell immunity was revealed. This study shows no effect of posttransplant immunosuppression on CMV specific T cell immunity in living donor kidney transplant recipients with CMV immunity already established, regardless of lymphocyte depletion and CMV prophylaxis.

Key words

Kidney transplantation • rATG • Thymoglobuline • Basiliximab • Cytomegalovirus • ELISPOT

Corresponding author

O. Viklicky, Department of Nephrology, Transplant Center, Institute for Clinical and Experimental Medicine, Videnska 1958/9, 14021 Prague, Czech Republic. E-mail: ondrej.viklicky@ikem.cz

Introduction

Cytomegalovirus (CMV) infection is the most frequent opportunistic infection occurring after organ transplantation. After CMV primo-infection, a robust adaptive immune response persists lifelong and CMV-specific CD4+, CD8+ T cells, and specific immunoglobulin G (IgG), have been implicated to inhibit virus reactivation (Crough *et al.* 2009). In organ transplantation, however, this protection may fail as a consequence of immunosuppressive therapy. In CMV seropositive graft recipients, the loss of CMV immune control after transplantation may be associated with direct effects (CMV infection and tissue invasive disease) and,

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more frequently, indirect effects which usually occur later on and include the opportunistic infections, acute or chronic rejection, cardiovascular diseases and diabetes (Couzi *et al.* 2015).

The effects of induction and maintenance immunosuppression on CMV primary infection and disease in CMV-seronegative graft recipients are well known. Lymphocyte-depleting induction agents as rATG may be associated with higher risk of CMV infection compared to non-depletive interleukin-2 (IL-2) receptor antagonists (Abou-Ayache *et al.* 2008, Huurman *et al.* 2006).

The impact of initial immunosuppression with or without antiviral prophylaxis on CMV specific immune surveillance in seropositive graft recipients remains less understood, with certain contradictory data published. In a recent study, the frequencies of pre-transplant CMV IE-1 specific T cells independently predicted the risk of post-transplant CMV infection regardless of CMV serostatus and immunosuppression (Bestard *et al.* 2013). Moreover, the negative CMV pp65-specific ELISPOT prior transplantation was associated with subsequent development of CMV infections after transplantation in CMV-seropositive kidney transplant recipients who did not receive CMV prophylaxis or preemptive therapy (Kim *et al.* 2015). Similarly, we observed the predictive value of a missing IE-1 T cell response for the development of CMV disease both in heart and lungs (Bunde *et al.* 2015) and in kidneys of renal transplant patients (Bunde *et al.* 2005, Nickel *et al.* 2009).

The superior outcome of kidney transplantation is usually reached in LD (living donor) recipients, mainly due to shorter dialysis time and younger recipient age. Recently, T cell repertoire has also been described to be age-dependent in patients under immunosuppressive therapy (Welzl *et al.* 2014). Similarly, the long term dialysis is associated with the premature aging of immune system (Betjes 2013). We hypothesized that in LD kidney transplant recipients the effect of depletive and non-depletive immunosuppressive agents on CMV-specific T cell immunity before and after transplantation may differ compared to previous reports on different populations.

Table 1. Patients demographics.

Variables	Total	rATG	Basiliximab	p
Patients (n)	24	12	12	
*Recipients age (years)**	43.6±10.5	42.4±13.2	44.7±6.9	0.8
*Donor age (years)**	48.7±9.89	51.0±8.5	46.5±10.7	0.2
Gender (male/female)	19/5	10/2	9/3	
Dialysis vintage (months)[#]	3.4 [0; 259.2]	1.4 [0; 259.2]	5.1 [0; 80.4]	0.7
HLA mismatch (n)	3.83 ±1.19	3.5 ± 1.21	4.0±1.1	0.5
PRA max (%)[#]	0 [0; 69]	11.5 [0; 69]	0 [0; 6]	0.03
Retransplantation (1st, 2nd, 3rd)	21, 1, 2	9, 1, 2	12, 0, 0	0.2
CMV prophylaxis (n)	12	12	0	0.001
Pretransplant CMV IgG serostatus				
*Donors (kAU/l)**	172.9±91.9	178.4±95.9	167.5±89.5	0.8
*Recipients (kAU/l)**	212.8±56.3	219.6±53.5	206.0±59.3	0.5
*Creatinine (µmol/l) 6 months**	135.5±16.2	138.8±19.9	130.5±8.6	0.5
*eGFR 6 months (ml/s)**	0.9±0.2	0.9±0.2	1.0±0.2	0.6
*eGFR 12 months (ml/s)**	0.9±0.2	0.9±0.3	1.0±0.2	0.7

[#] Median [min, max], * mean ± SD (range), kAU/l – King Armstrong unit per liter of serum, HLA – human leukocyte antigen, PRA – panel reactive antibodies measured every 3 months before transplantation. The highest number (PRA max) was considered for each patient.

Methods

Patient's characteristics

A total of 24 CMV seropositive patients who received kidney transplantation from LD between January 2014 and March 2015 were included in this single-center prospective study and were followed for 12 months. The study protocol was approved by the local ethics committee No. G14-08-38. The demographic data of patients are summarized in Table 1. Twelve patients

with panel reactive antibody (PRA)<20 % received induction with basiliximab (Simulect®, Novartis, Switzerland) and 12 patients with PRA>20 % received induction therapy based on rATG (Thymoglobuline®, Genzyme Corporation, Cambridge) with a mean cumulative dose 5 mg/kg within the first week after transplantation. All CMV seropositive patients treated with rATG received valgancyclovir prophylaxis (Valcyte®, Roche, Czech Republic) for 100 days.

Laboratory variables

To determine the allograft function, the estimated glomerular filtration rate (eGFR) using the chronic kidney disease epidemiology collaboration (CKD-EPI) equation was used (Ognibene *et al.* 2016). To evaluate CMV DNAemia, DNA was isolated from 200 µl plasma by NucleoSpin® Virus Kit (Macherey-Nagel, Germany). CMV polymerase chain reaction (PCR) was quantified by Artus CMV RGQ MDx Kit (Qiagen, Germany). Analytical sensitivity kit Artus, using the analyzer Rotor Gene 6000, was defined by the manufacturer as 69 CMV DNA copies/ml (p=0.05), therefore DNAemia was defined as >100 copies/ml.

PBMCs isolation and cryopreservation

Recipients´ PBMCs were isolated prior transplantation and 6 months after transplantation from the peripheral blood using standard Ficoll-Paque gradient centrifugation and then cryopreserved in liquid nitrogen for further processing as described previously (Gebauer *et al.* 2002).

ELISPOT assay

CMV specific T cells were evaluated by IFN-γ ELISPOT assay (Lucia *et al.* 2014, Nickel *et al.* 2009). Recipients' PBMCs were rested after thawing for 24 h in 5 % CO_2 atmosphere at 37 °C. After resting, the recipients' cells were washed and then seeded into the 96-well IFN-γ ELISPOT (AID, Germany) plate at 300,000 cells/100 µl per well. The cells were stimulated with whole protein-spanning overlapping CMV peptide pools (Miltenyi Biotec, Slovakia) (1 µg/ml of pp65 and IE-1) for 24 h in 5 % CO_2 atmosphere at 37 °C. As a positive control, cells were stimulated with 100 µl of pokeweed mitogen (AID, Germany) and as a negative control cells were incubated in medium (RPMI-1640 with glutamate, 10 % fetal bovine serum (FBS), 1 % Peniciline/Streptomycine) alone. After the incubation the numbers of spots (cells producing IFN-γ after stimulation) were measured and counted semi-automatically with ELISPOT reader (AID, Germany).

Statistical analysis

Data were analyzed by using Graphpad Instat 3 software (GraphPad Software, California, USA). Data normality was verified using the Kolmogorov-Smirnov test. Non-parametric data were analyzed using the Mann-Whitney U test. For data with normal distribution one-way analysis of variance (ANOVA) test was performed. The results were considered statistically significant when p<0.05.

Results

Pre- and post-transplant CMV specific cellular immunity

There was no clear association between the frequency of CMV specific T cells and dialysis vintage (Fig. 1). Eleven patients had undergone preemptive transplantation and thus they had no history of dialysis treatment.

There were no significant changes in CMV specific T-cell immunity 6 months after kidney transplantation either when compared to pre-transplant values. Numbers of IFN-γ producing cells stimulated with both pp65 and IE-1 antigens remained similar at both time points (Fig. 2). There were increases of CMV specific T cells after stimulation with pp65 peptide pool in 4 out of 24 patients only while in 3 out of 24 patients the frequency of those cells decreased (Fig. 2A). Similarly, after stimulation with IE-1 peptide pool, the IFN-γ producing cells increased in 4 out of 24 patients while decreasing in 5 out of 24 patients (Fig. 2B).

CMV specific cellular immunity and induction immunosuppression

There were no differences in CMV specific cellular response at transplantation when considering induction regimen (Figs 3A and 3B). The low-risk kidney transplant recipients with lower PRA initially treated with basiliximab, the anti CD25 monoclonal antibody, had similar level of CMV specific reactivity before transplantation as the patients with higher level of PRA, who received rATG, depleting T cells in the peripheral blood. Interestingly, initial T lymphocyte depletion caused by rATG had no effect of CMV specific cellular response at 6 months as the frequency of IFN-γ positive spots was similar.

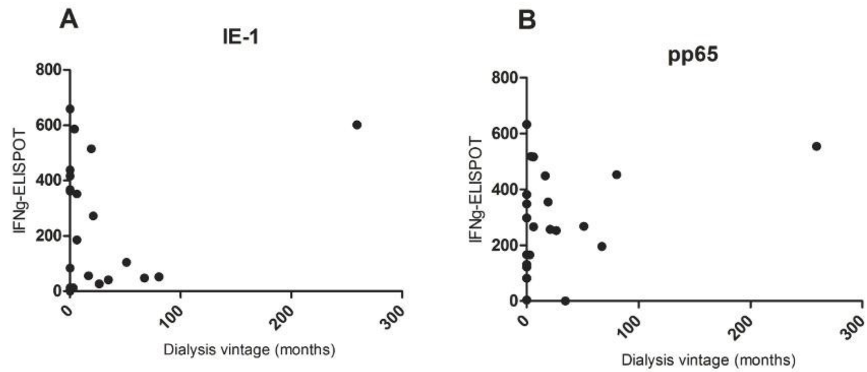

Fig. 1. Dialysis vintage and CMV specific immunity. Number of spots after stimulation with IE-1 (**A**) and pp65 (**B**) antigens.

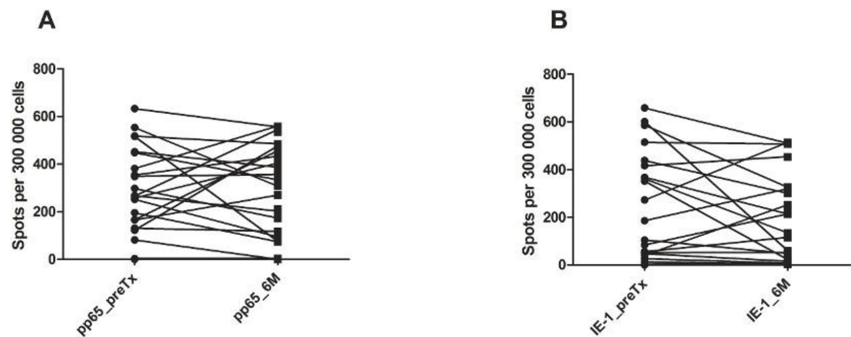

Fig. 2. Individual T cell reactivity development in seropositive recipients after stimulation with pp65 (**A**) and IE-1 (**B**) antigens prior and 6 months after transplantation.

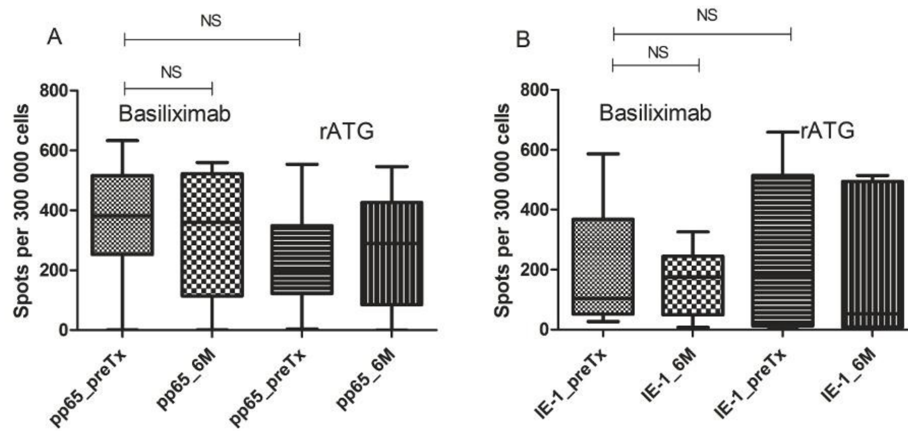

Fig. 3. Effect of induction treatment on T cell reactivity against CMV. Number of responder T cells producing IFN-γ after stimulation with pp65 antigen prior and 6 months after transplantation (**A**) and IE-1 antigen prior and 6 months after transplantation (**B**). All rATG treated patients received CMV prophylaxis while basiliximab treated patients did not.

Table 3. Demographic characteristics of patients with DNAemia.

Variables	Total	rATG	Basiliximab
Patients (n)	6	3	3
*Recipients age (years)**	45.7±10.1	50.8±11.6	40.5±5.3
*Donor age (years)**	49.2±11.4	54.0±12.4	44.3±8.6
Gender (male/female)	5/1	3/0	2/1
Dialysis vintage (months)#	0 [0; 6.2]	0 [0; 6.2]	0 [0; 6.2]
HLA mismatch (n)	3.8±1.2	3.6±0.5	4.3±1.3
PRA max (%)#	0 [0; 28]	0 [0; 28]	0 [0; 2]
CMV prophylaxis (n)	3	3	0
Retransplantation (1st, 2nd, 3rd)	6, 0, 0	3, 0, 0	3, 0, 0
Pretransplant CMV IgG serostatus			
Donors (kAU/l)	142.1±90.6	160.0±69.3	124.0±111.7
Recipients (kAU/l)	184.3±66.2	165.9±86.5	202.7±36.6
*Creatinine (umol/l) 6 months**	117.4±16.6	120.6±24.7	114.2±7.3

Median [min, max], * mean ± SD (range), HLA – human leukocyte antigen, PRA – panel reactive antibodies measured every 3 months before transplantation. The highest number (PRA max) was considered for each patient.

CMV specific cellular immunity and CMV DNAemia

The effect of CMV prophylaxis on CMV specific immunity was evaluated separately in patients with CMV-DNAemia (6 patients) and without post-transplant CMV-DNAemia (18 patients). Nine patients without DNAemia had received CMV prophylaxis with valgancyclovir and nine patients recieved no CMV prophylaxis. At 6 months, there were no statistically significant differences in IFN-γ producing cells stimulated either with pp65 or IE-1 antigens between CMV DNAemia negative patients with and without CMV prophylaxis. There was also no significant decrease of IFN-γ producing cells at 6 months compared to pre-transplant values in patients with CMV prophylaxis (data not shown). Borderline DNAemia (PCR>100 copies/ml) was observed in 5 out of 24 patients and high DNAemia (PCR>2,000 copies/ml) in a single patient (Table 2).

There were no differences in CMV specific T cell immunity at 6 months in patients with borderline DNAemia with and without CMV prophylaxis. In a single patient with high DNAemia who received CMV prophylaxis the number of IFN-γ producing cells was similar at both time points. Demographic characteristics of patients with DNAemia are shown in Table 3.

CMV specific cellular immunity and donor CMV serostatus

Among 24 CMV seropositive kidney transplant recipients, four patients received a kidney graft from CMV seronegative living donor (Table 4). In twenty recipients with seropositive donor (D+/R+) no significant changes were observed at 6 months in IFN-γ producing cells either after IE-1 or pp65 stimulation as compared to pre-transplant values. Four of these patients experienced positive DNAemia within first 100 days and in a single patient a DNAemia was observed at a later date. Interestingly, the increase in spots number after stimulation with both antigens pp65 and IE-1 in this patient was observed at 6 months. One patient with CMV seronegative living donor experienced borderline DNAemia (PCR>100 copies/ml) and in this patient the decrease in IFN-γ producing cells was observed. No other differences were observed in CMV specific immunity in recipients of CMV seronegative living donors.

Discussion

In this study, the changes in CMV specific cellular immunity in LD kidney transplant recipients with established humoral immunity were studied. The main observation of this study is the lack of impact of induction immunosuppression on CMV-specific T cell response, in both T cell depletive (rATG) and non-depletive (basiliximab) therapy in CMV-seropositive LD kidney transplant recipients. Moreover, there was no effect of short dialysis vintage in LD kidney transplant recipients on CMV specific T cell response.

Table 2. CMV specific immunity depending on PCR replication and CMV prophylaxis.

	CMV IFN-γ ELISPOT (spots) preTx pp65 *	CMV IFN-γ ELISPOT (spots) 6M pp65 *	p	CMV IFN-γ ELISPOT (spots) preTx IE-1*	CMV IFN-γ ELISPOT (spots) 6M IE-1*	p	CMV prophylaxis	CMV w/o prophylaxis	eGFR 6M	eGFR 12M	p
DNAemia neg. N=18	262.2 [1; 633]	320.5 [1; 559]	0.836	69.5 [0; 602]	87.5 [7; 514]	0.849	9 (50 %)	9 (50 %)	0.96±0.23	0.98±0.23	0.520
Middle DNAemia PCR>10^2, N=5	265.5 [81; 516]	173.5 [0; 545]	0.841	351.5 [12; 367]	134.5 [3; 319]	0.222	2 (40 %)	3 (60 %)	1.07±0.19	1.05±0.25	0.696
High DNAemia PCR>2.10^3, N=1	348	356		659	511		1 (100 %)	0	0.71	0.47	

* Data are presented as medians [min, max], # mean ± SD (range). Middle DNAemia ranged 728±939 copies.

Table 4. CMV specific immunity depending on donors´ serostatus.

CMV status	Onset of DNAemia	CMV prophylaxis	w/o CMV prophylaxis	CMV IFN-γ ELISPOT (spots) preTx pp65*	CMV IFN-γ ELISPOT (spots) 6M pp65*	p	CMV IFN-γ ELISPOT (spots) preTx IE-1*	CMV IFN-γ ELISPOT (spots) 6M IE-1*	p
D-/R+, n (%) N=4		2	2	99.5 [1; 517]	39 [1; 76]	0.771	44.25 [0; 351]	18.7 [8; 252]	0.685
	<100 d 1 (25 %)	0	1	516	76.5		351.5	22.5	
	>100 d 0								
D+/R+, n (%) N=20		9 (45)	11 (55)	282 [81; 633]	371 [0; 560]	0.421	228.7 [0; 659]	174 [3; 514]	0.413
	<100 d 4 (20 %)	1	2	232 [81; 348]	264 [0; 456]	0.685	364 [659; 12]	174 [3; 511]	0.485
	>100 d 1 (5 %)	1	0	265	545		185	319	

* Data are presented as medians [min, max].

Induction and maintenance immunosuppression therapy has a well-known effect on CMV primary infection and disease (Cavdar *et al.* 2008, Kim *et al.* 2012, Taherimahmoudi *et al.* 2009) when CMV seronegative recipient receives an organ from seropositive donor. In such a scenario all recipients have been receiving CMV prophylaxis with antiviral agents. Similarly, it is proposed that the seropositive recipients who received depletive induction regimen with rATG are at high risk for the CMV disease development and therefore they should receive prophylaxis as well. Interestingly, a general recommendation for CMV prophylaxis in CMV seropositive patients without depletive induction is yet to be clearly established. Some centers have been using a preemptive approach, rather than a CMV prophylaxis in those patients. Therefore, in our study we have evaluated the development of CMV cellular immunity in CMV seropositive recipients receiving depletive as well as non-depletive induction immunosuppression. While patients with T cell depletive immunosuppression received CMV antiviral prophylaxis, patients with T cell non-depletive immunosuppression did not. Interestingly, there were absolutely no differences between these two cohorts at 6 months after kidney transplantation regarding the CMV specific T cell immunity as measured by ELISPOT assay.

Does our observation mean that CMV prophylaxis with antiviral drugs is not necessary in CMV seropositive recipients regardless of the induction regimen? Probably not; all patients who had received rATG were treated with antiviral prophylaxis with valgancyclovir. However, we did not use such prophylaxis in patients receiving basiliximab. There were three cases of low and clinically not significant CMV viral replication after transplantation in that cohort despite well-established humoral immunity before and specific T cell immunity after transplantation. On contrary, the single case of significant post-transplant CMV replication and disease had received prophylaxis and had well established CMV humoral and cellular immunity despite T cell depletive regimen. Therefore, it is obvious that the presence of CMV specific effector memory T cells in the periphery is not sufficient to prevent CMV antigenemia (i.e. CMV reactivation). In combination with either preemptive or prophylactic antiviral therapy all patients were protected to CMV disease development.

CMV specific T cells play a crucial role in the control of viral replication (Egli *et al.* 2008, Mattes *et al.* 2008) and the effector memory T cells are to be recognized using the conventional ELISPOT assay as they produce IFN-γ after antigen stimulation (Calarota *et al.* 2013, Godard *et al.* 2004). Beside T cells, NK cells may produce IFN-γ after stimulation as well (Barabas *et al.* 2017, Han *et al.* 2016, Karlsson *et al.* 2003, Tischer *et al.* 2014). Our observation about no effect of post-transplant immunosuppression on the presence of effector memory T cells is in line with observation of others (Ayasoufi *et al.* 2013, Pearl *et al.* 2005). CMV specific effector memory T cells develop after primary infection and persist lifelong. Pearl *et al.* (2005) revealed that residual T cells after depletion therapy share a single phenotype corresponding with effector memory T cells (CD3+CD4+CD45RA-CD62L-CCR7-), the study suggested that effector memory T cells are selectively resistant to the therapeutic depletion therapy. In contrast to this, other parts of adaptive immune system (CD4+, CD8+, IgG) are thoroughly influenced by post-transplant immunosuppression (Carter *et al.* 2006, Gurkan *et al.* 2010, Zand *et al.* 2005) and therefore it is possible that homeostatic proliferation of T cells were not detected in our 2 time-points study.

In our study the CMV humoral immunity was already established prior to transplantation in all patients. Interestingly, a negative pre-transplant ELISPOT in both tested antigens was found in two patients. Abate *et al.* (2013) found the CMV specific memory effector T cells to be absent in 12 % of CMV seropositive adults when analyzed using ELISPOT and Quantiferon tests. Moreover, Sylwester *et al.* (2005) found some healthy individuals not to be able to correctly recognize pp65. Clearly, additional CMV antigens exist which were not used to stimulate recipient cells in our study (Elkington *et al.* 2003, Manley *et al.* 2004).

Another aim of our study was to examine CMV specific immunity in regards to CMV DNAemia. In our study there were no associations between pre-transplant CMV specific T cell immunity and later CMV DNAemia evaluated at 3 months or according to clinical situation. Contrary to our results, Bestard *et al.* (2013) showed the association between low frequencies of pre-transplant IE-1 specific T cells and the occurrence of CMV infection after transplantation. Moreover, a significant increase of IE-1 and pp65 specific T cells in patients who experienced CMV infection after transplantation was noticed (Tischer *et al.* 2014). However, our study was focused on CMV seropositive LD kidney recipients only while abovementioned study also evaluated deceased

donor kidney transplant recipients who were either seropositive or seronegative prior to transplantation. In living donor kidney transplant recipients, since both donor and recipients are generally younger, the patients have experienced mainly short-term dialysis, if any, and the ischemia reperfusion injury that might trigger viral replication is significantly shorter (Davis *et al.* 2005, Kayler *et al.* 2011, Mange *et al.* 2001).

In conclusion, our study shows no visible effects of post-transplant immunosuppression on CMV specific T cell immunity in peripheral blood of LD kidney transplant recipients with already established CMV immunity, regardless of lymphocyte depletion and CMV prophylaxis. This observation is significant, since most of previous studies included the deceased donor kidney transplantation only, while LD transplantations have been increasing in many countries worldwide. However, our data do not form a ground for changing guidelines or forming a recommendation, but rather warrants future larger prospective studies in this specific population.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

ANOVA, analysis of variance; CKD-EPI, chronic kidney disease epidemiology collaboration; CMV, cytomegalovirus; eGFR, glomerular filtration rate; ELISPOT, enzyme-linked immunospot; FBS, fetal bovine serum; IE-1, immediate-early; IFN-γ, interferon gamma; IgG, immunoglobulin G; IL-2, interleukin-2; LD, living donor; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; pp65, phosphoprotein 65; PRA, panel reactive antibody; rATG, rabbit antithymocyte globulin.

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5.2 Heterologní CMV- a Allo -reaktivita sdíleným repertoárem T-buněčných receptorů v transplantaci ledvin

Lucia Stranavova, Ondrej Pelak, Michael Svaton, Petra Hruba, Eva Fronkova, Antonij Slavcev, Klara Osickova, Jana Maluskova, Jiri Fronek, Petra Reinke, Hans-Dieter Volk, Tomas Kalina, Ondrej Viklicky

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Cytomegalovirová (CMV) infekce je asociována s rejekcí transplantovaného štěpu. Mechanismy vysvětlující tento fenomén jsou dosud špatně definovány. Ačkoliv zkřížená reaktivita T lymfocytů mezi alloantigeny a CMV byla předmětem mnoha hypotéz, přímý důkaz pořád chybí. V této monocentrické observační studii jsme testovali reakci pretransplantačních efektorových/paměťových T lymfocytů na CMV a alloantigeny u 78 příjemců ledviny od žijících dárců pomocí *Enzyme-Linked ImmunoSpot assay* (ELISPOT). Pro potvrzení hypotézy zkřížené reaktivity jsme analyzovali repertoár T receptorů ß (TCR- ß) u CMV a alloreaktivních T buněk z pretransplantační periferní krve pacientů pomocí NGS sekvenování u 11 CMV-seropozitivních a HLA I inkompatibilních pacientů. TCR- ß repertoár byl také analyzován v potransplantačních biopsiích transplantovaných štěpů. Byla nalezena signifikantní asociace mezi přítomností pretransplantačních efektorových/paměťových T buněk a akutní rejekcí a funkcí ledvinného štěpu ($p = 0.01$). Také jsme našli sdílené TCR-ß sekvence mezi CMV-IE1 a dárcovskými alloantigen-reaktivními T buňkami ve všech pretransplantačních vzorcích periferní krve od CMV-seropozitivních pacientů, kteří dostali štěp od HLA I inkompatibilních dárců. Identické TCR-ß sekvence byly rovněž nelezeny v potransplantační biopsií u pacientů se současnou CMV infekcí a rejekcí. Naše data ukazují na přítomnost funkčních, zkříženě reagujících T buněk a jejich klonotypů v periferní krvi a ve tkáni transplantované ledviny. Je proto pravděpodobné, že zkřížená reaktivita mezi dárcem a CMV, stejně jako zánět indukovaný CMV specifickými T lymfocyty, se účastní procesů ovlivňujících přežití a funkčnosti transplantované ledviny.

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Heterologous Cytomegalovirus and Allo-Reactivity by Shared T Cell Receptor Repertoire in Kidney Transplantation

Lucia Stranavova[1†], Ondrej Pelak[2†], Michael Svaton[2], Petra Hruba[1], Eva Fronkova[2], Antonij Slavcev[3], Klara Osickova[4], Jana Maluskova[5], Petr Hubacek[6], Jiri Fronek[7], Petra Reinke[8], Hans-Dieter Volk[8], Tomas Kalina[2‡] and Ondrej Viklicky[1,4*‡]

[1] Transplant Laboratory, Institute for Clinical and Experimental Medicine, Prague, Czechia, [2] CLIP – Childhood Leukaemia Investigation Prague, Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine, Charles University Prague and University Hospital Motol, Prague, Czechia, [3] Department of Immunogenetics, Institute for Clinical and Experimental Medicine, Prague, Czechia, [4] Department of Nephrology, Transplant Centre, Institute for Clinical and Experimental Medicine, Prague, Czechia, [5] Department of Transplant Pathology, Transplant Centre, Institute for Clinical and Experimental Medicine, Prague, Czechia, [6] Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine and Motol University Hospital, Charles University, Prague, Czechia, [7] Department of Transplant Surgery, Institute for Clinical and Experimental Medicine, Prague, Czechia, [8] BIH Centre for Regenerative Therapies, Berlin Centre for Advanced Therapies, Charité University Medicine Berlin, Berlin, Germany

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***Correspondence:**
Ondrej Viklicky
ondrej.viklicky@ikem.cz

†Shared first authorship

‡Shared senior authorship

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Cytomegalovirus (CMV) infection is associated with allograft rejection but the mechanisms behind are poorly defined yet. Although cross-reactivity of T cells to alloantigen and CMV has been hypothesized, direct evidence in patients is lacking. In this observational cohort study, we tested the pre-transplant effector/memory T cell response to CMV peptide pools and alloantigen in 78 living donor/recipient pairs using the interferon-gamma Enzyme-Linked ImmunoSpot (ELISPOT) assay. To prove the hypothesis of cross-reactivity, we analyzed by applying next-generation sequencing the T cell receptor ß (TCR- ß) repertoire of CMV- and alloantigen-reactive T cells enriched from peripheral pre-transplant blood of 11 CMV-seropositive and HLA class I mismatched patients. Moreover, the TCR-repertoire was also analyzed in the allograft biopsies of those patients. There was a significant association between the presence of pre-transplant CMV immediate-early protein 1 (IE-1)-specific effector/memory T cells and acute renal allograft rejection and function ($p = 0.01$). Most importantly, we revealed shared TCR-ß sequences between CMV-IE1 and donor alloantigen-reactive T cells in all pre-transplant peripheral blood samples analyzed in CMV-seropositive patients who received HLA class I mismatched grafts. Identical TCR sequences were also found in particular in post-transplant allograft biopsies of patients with concomitant CMV infection and rejection. Our data show the presence of functional, cross-reactive T cells and their clonotypes in peripheral blood and in kidney allograft tissue. It is therefore likely that CMV-donor cross-reactivity as well as CMV specific T cell elicited inflammation is involved in the processes that affect allograft outcomes.

Keywords: kidney transplantation, cytomegalovirus, ELISPOT, cross-reactivity, rejection, heterologous immunity, TCR repertoire

INTRODUCTION

Although the human cytomegalovirus (CMV) infection establishes a broad immunity which controls infection, rendering it asymptomatic in majority of immunocompetent hosts even if the CMV reactivates repeatedly following different stressors during life-time (1, 2). However, it might be associated with life-threatening complications in organ transplant recipients (3). Interestingly, it is widely acknowledged that, in addition to its direct pathogenic effects, CMV infection in organ transplant recipients is associated with more frequent acute and chronic rejection (4, 5). Although several possibilities have been proposed for those indirect negative effects, the mechanisms behind are poorly understood so far and direct proofs are missing. Persistent CMV infection elicits strong and lifelong T cell immunity that controls CMV reactivation/reinfection and prevents CMV disease by permanent dynamic interaction between the virus and the CMV-reactive T cell clones. However, CMV-reactive T cells can cause tissue damage by several mechanisms: (i) direct cytotoxic effect on CMV infected (allograft) cells, (ii) indirect bystander activation and proinflammatory milieu formation, and (iii) heterologous (cross-reactive) allorecognition (6).

The cross-reactivity of CMV-reactive effector T cells to HLA class I antigens has been discussed (7) and those cross-reactive cells were transiently found in the peripheral blood of kidney transplant recipients (8). T cell receptor (TCR) cross-reactivity has been suggested as primary means of increasing the effective size of T cell compartment, while cross-reactive memory cells have been shown to expand and activate more rapidly (9). Several mechanisms have been proposed for TCR cross-reactivity, including molecular mimicry and the ability of TCR to recognize different peptide-MHC complexes (10). Several other studies have shown the presence of cross-reactive virus-specific memory T cells and donor HLA molecules (7, 8, 11, 12). However, direct evidence of the role of heterologous TCR immunity in renal allograft rejection has not been shown so far.

Herein, we demonstrate that (i) the presence of CMV-reactive T cells pre-transplant predicts risk of acute allograft rejection, (ii) heterologous CMV- and donor-reactive cross-reactivity TCR-ß identical T cells pre-exists in patients prior to kidney transplantation, and (iii) identical cross-reactive T cell clones are detectable in renal allograft biopsies post-transplantation. Our data support the impact of heterologous immunity to CMV-IE1 and alloantigen among pre-transplant memory T cells on allograft outcome and indicate the need of adequate control not only by immunosuppression but also efficient antiviral strategies.

Abbreviations: TCR, T cell receptor; ELISPOT, Interferon-gamma Enzyme-Linked ImmunoSpot; IE-1, CMV immediate-early protein 1; pp65, Phosphoprotein 65; CI, Confidence interval; eGFR, Estimated glomerular filtration rate; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration equation; MLR, Mixed lymphocyte reaction; PBMCs, Peripheral blood mononuclear cells; NGS, Next-generation sequencing; VZV, Varicella-zoster virus; rATG, Rabbit polyclonal antithymocyte globulin; PRA, Panel-reactive antibody; CDR3, Complementarity-determining region 3; HR, Hazard ratio; FCS, Fetal calf serum; ROC, Receiver operating characteristic curve; AUC, Area under the curve.

MATERIALS AND METHODS

Patient Characteristics

In this observational cohort study, we evaluated the role of pre-transplant CMV-specific T cell immunity in acute rejection using an ELISPOT cohort consisting of 78 living donor kidney transplant recipients and their respective donors, all of whom underwent transplant surgery in the Institute for Clinical and Experimental Medicine in Prague between the years 2014 and 2017. The demographic data of the patients are summarized in **Table 1**. The peripheral blood of both donors and recipients was drawn pre-operatively to isolate PBMCs. All patients received tacrolimus, mycophenolate mofetil, and steroids as maintenance immunosuppression, initiated 48 h before the scheduled surgery. Patients at low immunological risk [panel-reactive antibody (PRA) <20%] received the anti-CD25 monoclonal antibody basiliximab (Simulect, Novartis), while patients at higher risk received rabbit polyclonal anti-thymocyte globulin (rATG, Thymoglobulin®, Genzyme Corporation) as induction immunosuppression. CMV prophylaxis with valganciclovir (Valcyte®, Roche) was given to CMV-seronegative recipients who had received grafts from seropositive donors or to CMV-seropositive recipients who had undergone rATG induction. Fourteen out of 78 patients experienced acute rejection episodes during the 1st year after transplantation and were treated as previously reported (13). For a detailed description of the histological findings, see **Table S1**.

For the analysis of the TCR repertoire of CMV- and donor alloantigen-reactive T cells (the "cross-reactive" cohort) 11 donor/recipient pairs were selected with primary low risk and pre-transplant CMV-seropositive living donor renal allograft recipients from the years 2014 to 2016. For a summary of the demographic data of these patients, see **Table 2**. All patients received tacrolimus, mycophenolate mofetil, and steroids as maintenance immunosuppression (initiated 48 h before the scheduled surgery) and basiliximab as induction immunosuppression. All patients underwent a 3-month protocol kidney graft biopsy according to the centre's standard practice, while case biopsies were performed to histologically verify clinically suspected acute rejection. In 5 out of 11 patients, histological proven acute rejection episodes occurred within 3 months after the operation. Histological findings are given in **Table S2**. In the case of this patient cohort and their respective living donors, peripheral blood was drawn prior to transplantation in order to isolate PBMCs and the allograft biopsy was performed, with 2–3 mm of the tissue samples stored in Ambion RNAlater® Stabilization Solution (Thermo Fisher Scientific) for future molecular evaluation.

All patients from the ELISPOT and cross-reactive cohorts as well as their respective donors gave their written informed consent to participate in the study. The local ethics committee approved the study protocol under No. G14-08-38.

IFN-γ ELISPOT Assay

In the "ELISPOT" cohort, allo- and CMV-specific T cells were assessed using the IFN-γ ELISPOT method according to recently described protocols (14, 15). Peripheral blood

TABLE 1 | Demographics of the "ELISPOT" patient cohort.

	Total	IE-1 positive	IE-1 negative	p
Patients (n)	78	31	47	
Recipients age (years)*	45.6 ± 13.2	49.0 ± 11.7	43.0 ± 13.7	0.032
Donor age (years)*	48.6 ± 10.9	50.0 ± 11.1	48.0 ± 10.47	0.372
Gender of recipients (M/F)	54/24	20/11	34/13	0.322
Dialysis vintage (months)#	1.7 [0; 259.2]	4.0 [0; 259.2]	0.4 [0; 85.0]	0.424
HLA mismatch*	3.5 ± 1.41	3.7 ± 1.4	3.4 ± 1.4	0.427
PRA max (%)#	0 [0; 69]	0 [0; 69]	0 [0; 36]	0.932
PRA max ≥ 20% n (%)	12 (15.4)	6 (19.4)	6 (12.8)	0.430
Retransplantation n (%)	7 (8.9)	4 (12.9)	3 (6.4)	0.324
CMV prophylaxis n (%)	37 (47.4)	14 (45.2)	23 (48.9)	0.744
Pretransplant CMV IgG serostatus				
D+/R+	52 (66.7)	26 (83.9)	26 (55.3)	0.009
D+/R–	8 (10.3)	0 (0)	8 (17.0)	0.015
D–/R–	6 (7.7)	0 (0)	6 (12.8)	0.038
D–/R+	12 (15.4)	5 (16.1)	7 (14.9)	0.882
CMV DNAemia				
PCR > 10^2 n (%)	9 (11.5)	6 (19.3)	3 (6.3)	0.079
Allo-positive ELISPOT n (%)	25 (32.1)	13 (41.9)	12 (25.5)	0.129
Induction Immunosuppression				
Basiliximab n (%)	49 (62.9)	19 (61.3)	30 (63.8)	0.151
Thymoglobulin n (%)	29 (37.1)	12 (38.7)	17 (36.2)	0.820
Rejection n (%)	14 (17.9)	11 (35.5)	3 (6.4)	0.001
eGFR 3M (mL/min)*	58.7 ± 12.6	53.2 ± 11.4	62.4 ± 12.2	0.003
eGFR 6M (mL/min)*	60.3 ± 13.7	55.0 ± 11.4	64.0 ± 13.9	0.006
eGFR 12M (mL/min)*	59.6 ± 13.5	55.5 ± 12.7	62.4 ± 13.5	0.119

#Median [min; max].
*Mean ± SD (range).

TABLE 2 | Demographics of the "cross-reactive" cohort.

Patients (n)	11
Recipients age (years)*	38.6 ± 13.7
Donor age (years)*	44.1 ± 13.4
Gender of recipients (M/F)	6/5
Dialysis vintage (months)#	3.9 [0; 25.9]
HLA mismatch*	3.7 ± 1.07
PRA max (%)#	0 [0; 13]
eGFR (mL/s)	1.25 ± 0.4
CMV prophylaxis n (%)	0 (0)
Pretransplant CMV IgG serostatus n (%)	
D+/R+	11 (100)
CMV DNAemia	
PCR > 10^2 n (%)	3 (27.2)
Induction immunosuppression	
Basiliximab n (%)	11 (100)
Rejection n (%)	5 (45.5)
eGFR 3M (mL/min)*	67.9 ± 11.2
eGFR 6M (mL/min)*	75.0 ± 25.9
eGFR 12M (mL/min)*	74.6 ± 12.2

#Median [min; max].
*Mean ± SD (range).

mononuclear cells (PBMCs) were isolated from heparinized peripheral blood samples of donors and recipients taken prior to transplantation (using standard density gradient centrifugation) and cryopreserved in liquid nitrogen as described previously (16). After thawing, PBMCs were re-suspended with complete media [RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin + streptomycin (50 U/mL) and 1.7 mM sodium glutamate] and left for 24 h at 37°C in a CO_2 incubator. Next, 3×10^5 recipient PBMCs were stimulated with CD3-depleted donor cells to detect allospecific T cells, and with CMV antigens [whole protein-spanning overlapping peptide pools of immediate-early protein 1 (IE-1) and phosphoprotein 65 (pp65), length of each is 15 amino acids with 11 amino acid overlap] (Miltenyi Biotec) to detect CMV-reactive T cells [using pokeweed mitogen from Autoimmune Diagnostika (AID), GmbH as a positive control]. CMV peptide pools were used in the concentration of 1 µg/mL of pp65 or IE-1. PBMCs were seeded in an ELISPOT plate and incubated for 24 h at 37°C in a CO_2 incubator. The ELISPOT kit used to detect IFN-γ-producing cells was obtained from AID. After 24 h incubation at 37°C in the CO_2 incubator, cells were removed and the ELISPOT plate processed according to the manufacturer's protocol. The resulting numbers of spots were measured semi-automatically using an ELISPOT reader (AID iSpot FluoroSpot Reader System ELR07 IFL).

Antigen Specific T Cells by Flow Cytometry and FACS Sorting

Antigen specific T cells (virus specific or donor reactive) were detected as proliferating CD8+ T cells by dye dilution technique. Eleven patients from the "cross-reactive" cohort were selected for flow cytometry sorting. Cryopreserved PBMCs from recipients and donors were thawed, suspended with complete media and left for 24 h at 37°C in a CO_2 incubator. After resting, donor PBMCs were inactivated with Mitomycin C (50 µg/1 mL) (Sigma-Aldrich) for 2 h at 37°C in a CO_2 incubator and washed twice with complete media (210 RCF/10 min). Afterwards, both recipient and donor PBMCs were labeled with the dilution dyes CellTrace™ Violet and Far Red Cell Proliferation kits (Thermo Fisher Scientific), respectively, according to the manufacturer's instructions. Labeled recipient PBMCs were aliquoted by 0.5 $\times 10^6$ in a 96-well plate (2 mL, V-bottom, Greiner Bio-One, GmbH) with a culture medium [RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin+ streptomycin (50 U/mL), 1.7 mM sodium glutamate, 0.00036% (v/v) β-mercaptoethanol, and 10 U/mL IL-2]. CellTrace™ Violet dilution dye labeled PBMC were stimulated with the following CMV antigens: 1 µg/mL of pp65, 1 µg/mL of IE-1 (Miltenyi Biotec), or whole CMV lysate (Vidia) for 6 days. To detect alloreactive and cross-reactive T cells, inactivated donor cells (ratio 1:1) were used as a stimulus (Far Red dye labeled). Additional controls consisting of unstimulated recipient cells and recipient cells with additional IL-2 (50 U/mL) (Sigma-Aldrich) were used to eliminate bystander cell proliferation (data not shown). After 6 days of stimulation, the cells were harvested in 5 ml tubes and washed once with PBS containing 2 mM EDTA. Antigen specific cells proliferate in response to antigen and loose their dilution dye (CellTrace

cells (**Figure 1A**). Interestingly, the pre-transplant presence of a CMV-reactive response, both to IE-1 and pp65 whole protein overlapping peptide pools, had a stronger predictive power of acute rejection [IE-1 and pp65: AUC = 0.70, cut-off = 122.5 at 69.8% sensitivity and 80% specificity, 95% confidence interval (CI): 0.54–0.87; $p = 0.014$ and AUC = 0.59, cut-off = 332 at 63.5% sensitivity; and 53.3% specificity, 95% CI: 0.44–0.74, $p = 0.27$, respectively] than the donor-alloreactive ELISPOT (AUC = 0.40, cut-off = 25 at 66.7% sensitivity and 25.0% specificity, 95% CI: 0.27–0.59, $p = 0.39$, **Figure 1B**). Moreover, a shorter rejection-free interval was observed in patients with a positive pre-transplant IE-1 ELISPOT (**Figure 1C**).

Univariate Cox regression analysis revealed as significant risk factors of acute rejection only pretransplant positive IE-1 ELISPOT [Hazard ratio (HR) = 6.8, 95% CI: 1.89–24.36, $p = 0.003$] and post transplant positive CMV viral load by PCR>10^2 (HR = 3.8, 95% CI: 1.2–12.2, $p = 0.024$) (**Table 3**). A multivariate Cox regression analysis adjusted for ATG induction treatment and CMV PCR > 10^2 revealed only IE-1 positive ELISPOT (HR = 6.2, 95% CI: 1.67–22.3, $p = 0.006$) to be independent risk factor of acute rejection.

Interestingly, significant correlations were also found between pre-transplant IE-1 ELISPOTs and kidney graft function (estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation) at 3, 6, and 12 months ($p < 0.001$, $p = 0.002$, and $p = 0.038$, respectively) (**Figures 1D–F**). The demographic characteristics of patients with positive and negative IE-1 ELISPOTs are summarized in **Table 1**.

Taken together, CMV-reactive cellular immunity predicts acute rejection and short-term outcome of renal allografts.

CMV- and Alloreactive T Cells Express Shared TCR Sequences

The strong association between the pre-transplant presence of CMV-reactive T cells and rejection prompted us to investigate the possible cross-reactivity of CMV-specific T cells to donor alloantigens by search for shared TCR sequences. First, we combined the donor alloantigen MLR with CMV-peptide pentamer staining to evaluate cross-reactivity at single cell level in pre-transplant peripheral blood mononuclear cells (PBMCs) (8). In contrast to previously published studies, we

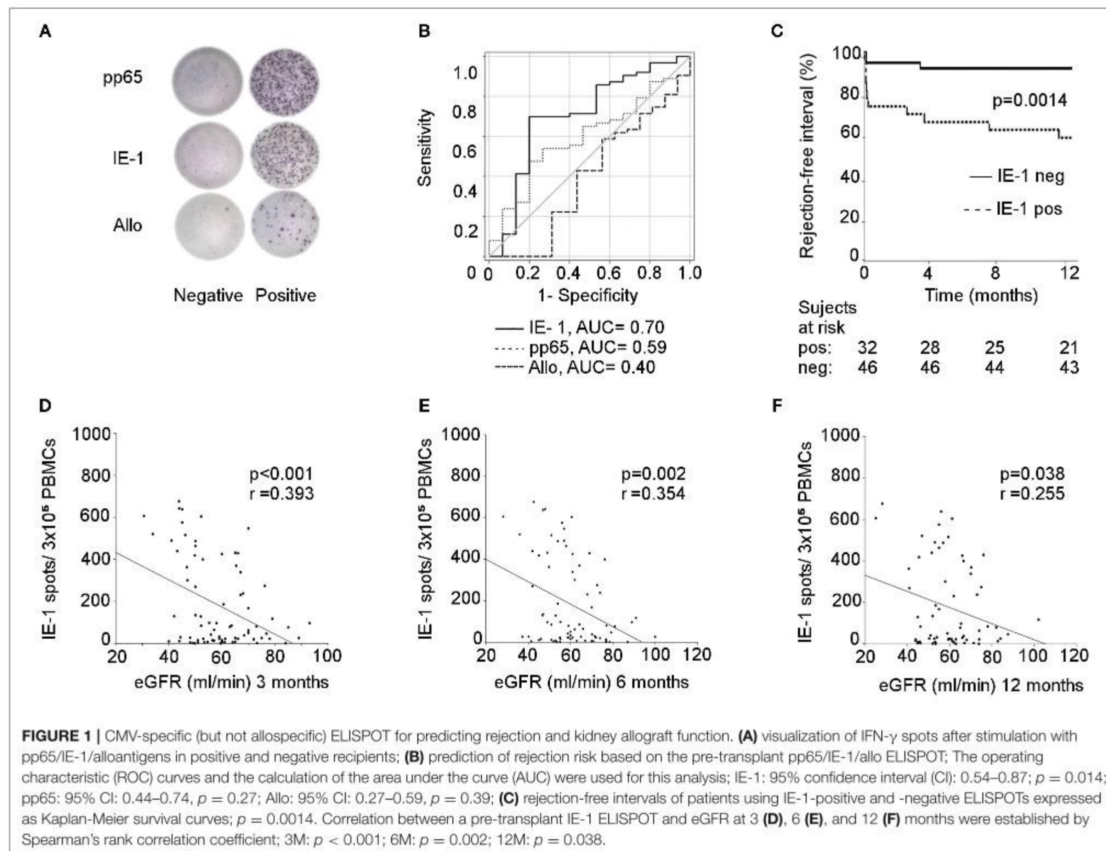

FIGURE 1 | CMV-specific (but not allospecific) ELISPOT for predicting rejection and kidney allograft function. **(A)** visualization of IFN-γ spots after stimulation with pp65/IE-1/alloantigens in positive and negative recipients; **(B)** prediction of rejection risk based on the pre-transplant pp65/IE-1/allo ELISPOT; The operating characteristic (ROC) curves and the calculation of the area under the curve (AUC) were used for this analysis; IE-1: 95% confidence interval (CI): 0.54–0.87; $p = 0.014$; pp65: 95% CI: 0.44–0.74, $p = 0.27$; Allo: 95% CI: 0.27–0.59, $p = 0.39$; **(C)** rejection-free intervals of patients using IE-1-positive and -negative ELISPOTs expressed as Kaplan-Meier survival curves; $p = 0.0014$. Correlation between a pre-transplant IE-1 ELISPOT and eGFR at 3 **(D)**, 6 **(E)**, and 12 **(F)** months were established by Spearman's rank correlation coefficient; 3M: $p < 0.001$; 6M: $p = 0.002$; 12M: $p = 0.038$.

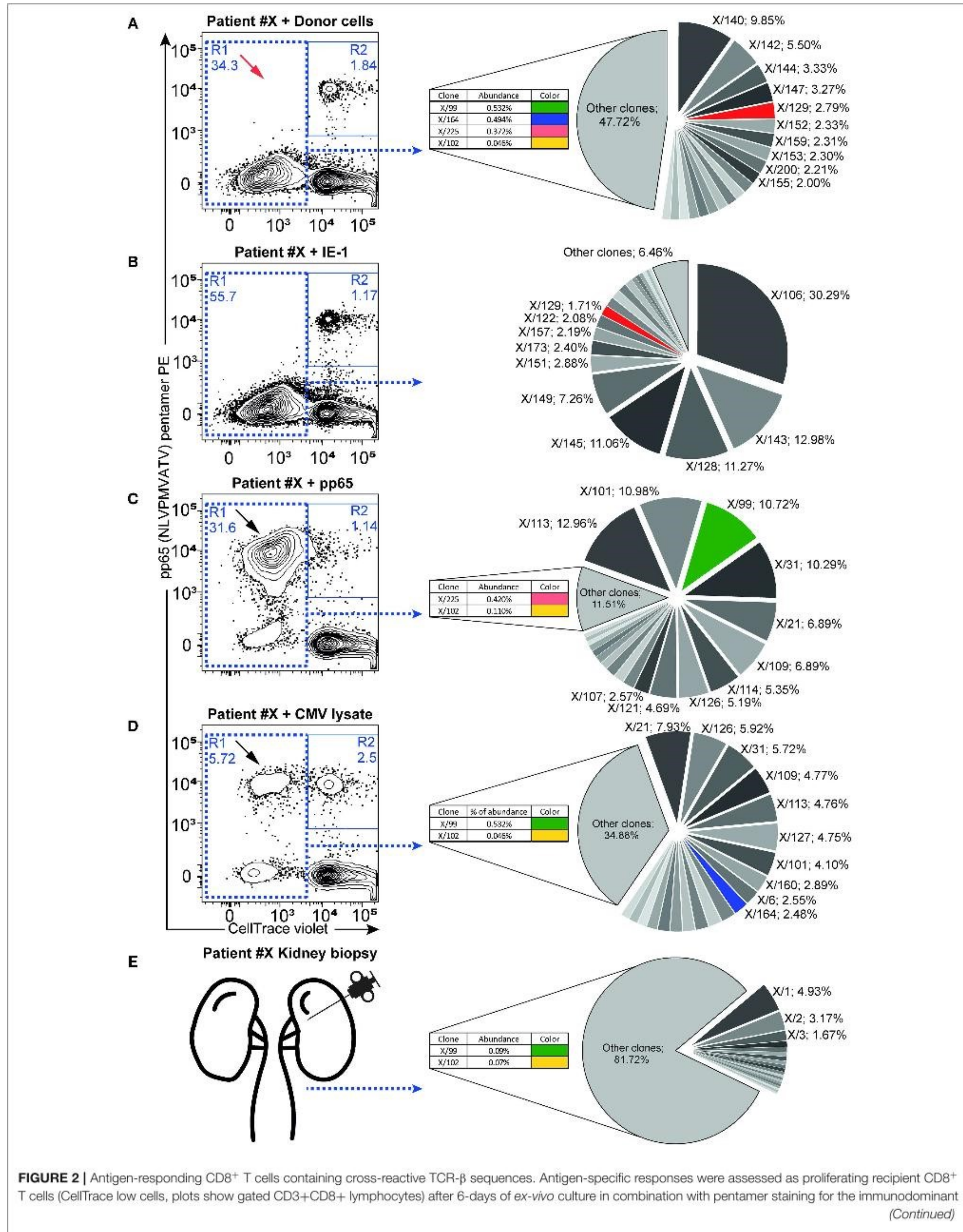

FIGURE 2 | Antigen-responding CD8⁺ T cells containing cross-reactive TCR-β sequences. Antigen-specific responses were assessed as proliferating recipient CD8⁺ T cells (CellTrace low cells, plots show gated CD3+CD8+ lymphocytes) after 6-days of *ex-vivo* culture in combination with pentamer staining for the immunodominant *(Continued)*

FIGURE 2 | (pp65: NLVPMVATV-specific) TCR receptor. Flow cytometry dot plots show the proliferation response of $CD8^{+}$ T cells to donor cells **(A)**, IE-1 **(B)**, pp65 **(C)**, and whole CMV lysate **(D)**. Proliferating cells in R1 were FACS-sorted and used for subsequent NGS TCR-β repertoire analysis. Twenty of the most abundant TCR-β sequences are represented in the pie chart graph (right panels), while additional minor cross-reactive clones are shown in inlets. Color codes highlight the same TCR sequence clones found in the respective antigen-responding cells or in the kidney **(E)**. Black arrows highlight antigen-specific proliferating T cells recognizing the immunodominant pp65 peptide, with red arrows indicating their absence from the donor cell-elicited response. Relative clone abundance is shown next to the clone name in 10 of the most abundant clones or in the inlets. One representative patient (#X) is shown, with a complete list of responding cell fractions. The amount of sorted cells and available NGS reads are given in **Table 4**, while the cross-reactive clones found are listed in **Table S5**.

detected no T cells cross-reactive to the immunodominant pp65 CMV peptide and donor cells, as detected by the co-staining for CMV-pp65 pentamer and cell tracking dilution following proliferation to alloantigen stimulation (**Figure 2A**). However, the response to dominant epitopes (pentamer staining) is lower compared to the proliferative response to the whole CMV peptide pool as demonstrated in 10 out of 11 CMV-seropositive patients (**Figures 2B–D** and summarized in **Figure S1**). In parallel, donor alloreactive T cells were present in all patient samples (including CMV pentamer-negative ones) prior to transplantation.

To investigate whether cross-reactivity would be present among the total pool of CMV-reactive T cells, we isolated antigen-reactive T cells (either reactive to CMV peptide pool or donor PBMCs) by FACS sorting (**Figures 2A–D**) and performed NGS of TCR-β sequences (**Table 4**). In 10 out of 11 patients, we acquired a sufficient amount of reads for analysis. We hypothesize that while pentamer staining only reveals single immunodominant CD8+ T cell clones, cross-reactivity may be caused by other less-dominant TCR clones. We were able to identify hundreds of distinct TCR-ß sequences from sorted antigen-reactive T cells from all patients (median 392 [241; 491], **Table S5**). Indeed, multiple clones sharing the same unique TCR-ß sequences were found in both CMV- and donor-reactive samples (**Figure 2**, right panels) from all patients, regardless of occurrence of rejection (**Table S6**).

Our results also provide evidence that both donor cells and CMV antigens can trigger identical T cell clones for proliferation showing functional responsiveness.

Shared Cross-Reactive TCR-ß Clonotypes Are Detectable in Renal Allograft Biopsies

Next, we sought to investigate whether cross-reactive TCR-ß clonotypes would be detectable in the allografts. Allograft biopsy samples were made available for 7 patients investigated for alloreactive and CMV-specific clonotypes (see "cross-reactive" cohort described above). In the kidney biopsy samples of 6 out of 7 patients, we were able to find identical TCR-β CDR3 sequences as in the alloreactive T lymphocytes pre-transplant. For the remaining patient, only 16 clones could be analyzed from the sequencing results of the MLR tube. Therefore, cross-reactive clones could have been missed due to the lower coverage of this sequencing library. In parallel, CMV-reactive TCR-ß clonotypes were found in the biopsy samples even at higher frequencies in the same patients, with a median of 3 clones per patient and a maximum of 11 (**Table 5**). The CMV-reactive clonotypes in the kidney covered 0.5–6.4% of all TCR-β sequences (**Table 5**) found in the kidney biopsies (see individual clones in **Table S6**). Finally, in 3 out of 7 biopsy samples, we detected CMV/donor alloantigen cross-reactive clonotypes identified pre-transplant in peripheral blood (**Table 5**).

Remarkably, in agreement with the acknowledged capacity of CMV-reactive T cell clones to expand upon CMV reactivation, cumulative abundance of CMV-reactive TCR-ß sequences was the highest (6.4 and 5.1%) in the two kidney tissue samples obtained from patients (No. VIII and X) suffering from significant CMV reactivation (viral load: 935 copies/ml and 1,020 copies/ml of plasma, respectively) concomitantly with biopsy-proven cellular rejection.

In summary, CMV-reactive and alloreactive clonotypes were found in all allograft biopsies patients analyzed, and in 3 out of 7 patients, we detected cross-reactive clonotypes as defined by pre-transplant analyses. Importantly, the highest number of these cross-reactive clones was observed in the two patients with CMV reactivation and concomitant cellular rejection.

DISCUSSION

The immunity in response to previous virus infections can modify the immune response to other antigens. Although heterologous immunity can be beneficial by boosting protective responses, it can also result in severe immunopathologies (6). Here, for the first time, we provide evidence that heterologous immunity can be detected in blood and biopsies of renal allograft recipients. Firstly, we found that high frequencies of CMV-reactive effector/memory (but not of alloreactive) T cells detected pre-transplant were associated with subsequent occurrence of T cell-mediated rejection. Secondly, multiple cross-reactive T cell clones (shared TCR-ß sequences) were found in both CMV- and donor-reactive T cells enriched from pre-transplant peripheral blood samples. Finally, TCR-ß sequences of alloreactive [CMV-reactive, and cross-reactive (CMV & MLR)] clonotypes were found in renal allografts; the latter particularly in association with CMV-associated T cell mediated acute rejection. Therefore, our data demonstrate that identical clonotypes of T cells can react in response to alloantigens as well as CMV antigens. This observation might explain how CMV reactivation, especially in the case of high viral load during uncontrolled replication, boosts directly not only the CMV but also the alloimmune T cell response.

Interestingly, in contrast to the association between high levels of pre-transplant CMV memory/effector response, in our study, we found no associations between pre-transplant donor-reactive memory/effector T cell response and acute rejection. Given the standardized, validated and robust ELISPOT method applied (previously used in a large European multicentre clinical trial; see www.biodrim.eu), it is unlikely that any

TABLE 4 | Percentages of proliferating CD8+ T cells (% CellTrace low), the number of sorted proliferating CD8+ T cells (sorted events), and the number of reads obtained after TCR-β next generation sequencing of sorted proliferating CD8+ T cells (No. of reads) in response to different stimulations.

Stimulation	Donor PBMCs cells			IE-1			pp65			CMV lysate			Kidney
Patient ID	% CellTrace low	Sorted events	No. of reads	% CellTrace low	Sorted events	No. of reads	% CellTrace low	Sorted events	No. of reads	% CellTrace low	Sorted events	No. of reads	No. of reads
I	35.5	11,142	33,778	49.6	13,014	28,014	31.2	5,258	13,246	12.3	1308	53188	16423
II	8.93	1,888	28,855	63.2	20,656	802	65.2	29,571	19,164	49.1	14738	3846	NA
III	36.1	10,516	10,804	36	3,081	56,507	16.9	933	71,615	15.6	1267	65789	12312
IV	33.6	12,458	35,175	52.6	7,945	51,790	41.5	8,673	25,332	7.5	2263	61281	NA
V	10.4	2,788	27,888	3.2	218	20,024	46.3	6,339	31,116	37.3	7534	16768	6915
VI	13.7	3,262	68,617	10.6	2,000	65,951	40.3	3,546	70,503	12.9	3194	104427	121
VII	24.3	3,428	46,562	57.4	70,911	1,702	10.1	2,901	51,597	8.3	5410	79025	20816
VIII	24.7	23,849	1,549	38.7	22,871	9,790	41.3	19,032	4,141	16.4	931	53528	20825
IX	36.5	3,731	37,273	0.1	8	0	7.75	302	33,931	2.8	200	56879	2799
X	34.3	18,084	26,346	55.7	20,473	1,898	31.6	11,140	32,570	5.7	2040	66330	19806
XI	7.7	2,289	70,817	67.9	18,835	140	NA	NA	NA	67.3	37150	143	NA

The kidney column only lists results from TCR-β next generation sequencing of isolated cells from fine needle biopsies where sorting of CD8+ T cells was not possible.

TABLE 5 | CMV-, Allo-, and Cross-reactive clones identified from blood pre-transplant are found in the kidney.

Patient ID	Number of shared clones between PBMCs and kidney from kidney		
	CMV specific (% of reads from all TCR-β sequences found in the biopsy)	Alloreactive (% of reads from all TCR-β sequences found in the biopsy)	Cross-reactive (% of reads from all TCR-β sequences found in the biopsy)
I	1 (0.5%)	1 (0.4%)	0%
III	1 (0.7%)	3 (2.4%)	2 (1.8%)
V	3 (1.8%)	6 (12.6%)	2 (3%)
VII	3 (0.5%)	2 (0.6%)	0%
VIII	11 (5.1%)	0%	0%
IX	0%	2 (9.2%)	0%
X	1 (0.1%)	7 (6.4%)	1 (0.1%)

The percentage of reads from clones that were identified in the functional assay as CMV-reactive, Allo-reactive, or in both tubes as Cross-reactive clones is shown as fraction of all the TCRβ sequences found in the kidney biopsies.

methodological bias occurred. An earlier study reported that higher pre-transplant T cell alloresponse was associated with acute allograft rejection in a study where patients received non-lymphocyte-depleting induction immunosuppression (23). Contrary, in our study some 40% of patients had received rATG T cell depletive induction immunosuppression. Similarly, pre-transplant allo-T cell responses have also been shown to correlate with lower post-transplant eGFR in patients with non-depleting induction (24). Apart from the association between CMV-specific memory/effector T cells and acute rejection, we found significant correlation with lower post-transplant eGFR at three different time-points during the first post-Tx year, rendering our observations more robust. Therefore, it is likely that the T cell-depletion strategy used in about half of our patients effectively reduced the available clonal size of alloreactive memory/effector T cells to a level that could be further controlled by maintenance immunosuppression.

Interestingly, there was weaker association of CMV-pp65- vs. CMV-IE-1-reactive T cells with acute rejection in our study. This phenomenon might be explained by higher CD8+ T cell response to IE-1 than to pp65 antigens (25).

In fact, subclinical CMV reactivation is frequently detected in over 30% of kidney transplant recipients despite CMV prophylaxis (26–28). We speculate that subtle localized CMV reactivations are even more frequent and, while undetected, provide antigen stimulation to CMV-specific T cells. This is in line with observations made by authors of a previous prospective randomized trial. They found that late-onset of CMV viremia, which developed in more than half of patients despite CMV prophylaxis, is associated with poorer outcomes (29).

Although it was reported that at least 151 of the 213 predicted CMV proteins, elicited T cell responses in at least one out of 33 donors (30), we and others could show that the T cell responses to IE-1 and pp65 CMV-proteins are the most dominant ones. Therefore, we concentrated in this study on the two immunodominant CMV proteins.

Applying the previously described method for detecting cross-reactive T cells based on MLR-reactivity combined with peptide-pentamer staining, was not effective in our scenario to detect cross-reactive T cells (7, 8, 31). One reason for this might be the use of unbiased PBMC samples with a scarcity of cross-reactive cells. In fact, as our access to patient material was limited by ethical reasons, we used only 5×10^5 T lymphocytes for functional stimulation, resulting after sort in limited yield of antigen-reactive T cells ranging from 3,846 to 104,427 and 1,888 to 23,849 CMV- and allospecific-proliferating T cells, respectively, for TCR repertoire analysis. Moreover, our recent data show that immunodominant epitopes for one particular HLA-type, as detected and enriched by peptide/dextramer staining, do not reflect the whole response to a particular CMV protein. Therefore, we developed recently the method of T cell stimulation by whole protein-spanning overlapping peptide pools covering almost all epitopes in a HLA-independent matter (32). Applying this method here, we could detect all three categories of CMV-, donor alloantigen-, and cross-reactive T cells in all patients with sufficient yield after sorting derived from pre-transplant blood samples despite limited amounts of reactive T cells (and resulting reads in NGS). These results show the potency of recipients' memory/effector T cell pool to react in case of CMV reactivation post-transplantation with both a protective CMV-specific and a putatively harmful CMV/allo-cross reactive response. In other words, CMV reactivation because of breakthrough through or after weaning of antiviral prophylaxis that might be amplified by TNF-release following ATG application can trigger putatively harmful alloresponse by crossreactivity (33). In line with this, we could detect cross-reactive TCR-ß clonotypes in the kidney biopsies of 3 out of 6 patients with sufficient yield for analysis. Whether the absence of detectable shared cross-reactive TCR-ß sequences in the remaining three biopsy samples is due to sensitivity problems or missing triggering by CMV is not clear, but the high abundancy in the samples just of the two patients suffering from enhanced CMV viral load and concomitant acute rejection supports their pathogenic role in CMV-associated graft injury.

In summary, our data show that within the large peripheral population of CMV-specific memory T cells there is a pool of cross-reactive T cell clonotypes that can produce effector T cells capable of migrating into kidney allografts. Moreover, these T cell clonotypes (when in the presence of chronic antigenic stimuli, such as CMV) may be susceptible to enhanced proliferation and allograft rejection. This phenomenon seems to be universal and corresponds with previous hypotheses about the cross-reactive virus-alloimmune response (12, 34). Specific allo-HLA cross-reactivity has been reported for EBV, CMV, varicella-zoster virus (VZV), and influenza A virus-specific T cells at clonal level, while cross-reactivity has been shown to be mediated by the same TCRs (35, 36). However, our data demonstrate for the first time their occurrence in the unbiased bulk T cell pool from peripheral blood and intragraft.

The limitations of this study must also be acknowledged. The analysis was confined by the limited number of patients and TCR-β chains; furthermore, TCR-α rearrangements were not examined. The configuration of TCR-β chains (including D segments) building in particular the CDR3 region ensures much greater variability of rearranged sequences than TCR-α. Therefore, TCR-β is considered more informative than TCR-α and has been widely used in similar studies. Aside of T cells several other cells (e.g., NK cells) may produce IFNγ after stimulation. Therefore, we phenotypized IFNγ-producing cells stimulated by CMV antigens by flow cytometry. However, the majority of IFNγ-producing cells were T lymphocytes (55%), while NK cells accounted for 5% of IFNγ-producing cells only. The "cross-reactive cohort" subjected TCR- β NGS comprised only by CMV seropositive donor-recipient pairs. Among CMV seropositive-donors cells the CMV-infected cells might be present (37). To minimize the risk of potential activation by CMV infected donor cells, we evaluated those donors for the presence of CMV in their peripheral blood and found none CMV genome.

Our data show that CMV-specific cellular response pre-transplant predicts rejection and document that surprisingly large proportion of patients harbors CMV and donor cross-reactive clones. CMV and donor cross-reactive T cells might thus directly damage the donor cells, being expanded by CMV antigenic stimulation during CMV reactivation. This effect might be supported by CMV specific response that builds inflammatory environment in the kidney. We recommend the approaches aimed at preventing CMV reactivation to be employed more aggressively; not only to prevent CMV disease but also to limit cross-reactivity-induced graft rejection.

In conclusion, we report that in our patient cohort the presence of cytomegalovirus IE-1-specific memory/effector IFN-gamma secreting T cells predict kidney transplant rejection and poorer 1 year graft function. Since we established the presence of functional, cross-reactive T cells and their clonotypes in peripheral blood, tracking the clonotypes directly in the kidney tissue, it is therefore likely that CMV-donor cross-reactivity as well as CMV specific T cell elicited inflammation is involved in the processes that affect allograft outcomes. Future studies should be carried out to determine whether more aggressive prevention and treatment of CMV reactivation might possibly limit alloimmune injury boosted by cross-reactive T cells.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The study protocol was approved by the Ethics Commintee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital under number: G14-08-38. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

OV and TK share senior authorship designed and supervised the research and wrote the manuscript. LS, OP, MS, PHr, AS, and EF performed the research, participated in the data analysis, and manuscript writing. JF, JM, PHu, and KO helped with carrying out the research. PR and H-DV helped in establishment of the ELISPOT technology and significantly contributed to writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02549/full#supplementary-material

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5.3 Dialyzační terapie je asociována s augmentací periferních B lymfocytů marginální zóny

Lucia Stranavova, Petra Hruba, Janka Slatinska, Birgit Sawitzki, Reinke, Hans-Dieter Volk, Ondrej Viklicky

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U pacientů s chronickým onemocněním ledvin v terminálním, 5., stádiu (CKD5), kteří jsou léčeni na dialýze a zůstávají dlouhou dobu v uremickém prostředí, se často vyskytuje několik špatně definovaných poruch imunitního systému. V této studii jsme hodnotili periferní virově-specifické efektorové / paměťové T buňky a jednotlivé subpopulace T, B lymfocytů a dendritických buněk (DC) pomocí metod ELISPOT a FACS u 74 nízko-rizikových pacientů, bez anti-HLA protilátek, čekajících na transplantaci ledviny. Pacienti byli rozděleni do dvou skupin: na dialyzovanou a preemptivní (bez předchozí zkušenosti s dialýzou). Zjistili jsme, že mezi těmito dvěma skupinami byl signifikantní rozdíl v počtu cirkulujících B lymfocytů marginální zóny (MZB) (IgD^{high} $CD27^{high}$) p=0.002). Pacienti léčeni na dialýze více jak 12 měsíců měli 4.2x vyšší riziko zvýšení absolutního počtu MZB (95%CI:1.6-11.2; P = 0.004). Rozdíly v počtu ostatních subpopulací T, B a DC nebyly pozorovány. Také počet efektorových/paměťových T buněk reaktivních na hlavní oportunistické virově-specifické antigeny (CMV, BKV, EBV) nebyl ovlivněn předchozí dialyzační léčbou. Nesensitizovaní CKD5 pacienti léčení dialýzou vykazují signifikantně víc cirkulujících MZB než ti, kteří nikdy nepodstoupili dialyzační terapii.

Podíl na publikaci: kolekce materiálu od pacientů, experimentální část (ELISPOT, FACS), statistická analýza výsledků, příprava manuskriptu

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Brief communication

Dialysis therapy is associated with peripheral marginal zone B-cell augmentation

Lucia Stranavova[a], Petra Hruba[a], Janka Slatinska[b], Birgit Sawitzki[c], Petra Reinke[c], Hans-Dieter Volk[c], Ondrej Viklicky[a,b,*]

[a] Transplant Laboratory, Institute for Clinical and Experimental Medicine (IKEM), Prague, Czech Republic
[b] Department of Nephrology, Institute for Clinical and Experimental Medicine (IKEM), Prague, Czech Republic
[c] BIH Centre for Regenerative Therapies, Berlin Centre for Advanced Therapies, Charité University Medicine Berlin, Berlin, Germany

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ABSTRACT

Chronic kidney disease stage 5 (CKD5) dialysis patients who stay long term in uremic environment often exhibit several, poorly defined, immune impairments. In this study, we assessed peripheral virus-specific effector/memory cells and subpopulations of T, B and DC cells using ELISPOT and FACS methods in 74 low-risk kidney transplant candidates without anti-HLA antibodies, prior to transplantation in pre-emptive (never experienced dialysis) and dialysis cohorts. There was difference in circulating marginal zone B cells (MZB) ($IgD^{high}CD27^{high}$) between dialysis patients and those receiving kidney grafts pre-emptively (P = .002). Patients treated on dialysis > 12 months had also 4.2-fold greater risk of increased absolute numbers of MZB (95%CI:1.6–11.2; P = .004). There were no other differences in B-, T- and DC-cell subsets. Numbers of effector/memory T cells reactive to major opportunistic virus-specific antigens (CMV, BKV and EBV) were not affected by dialysis. Non-sensitised dialysis-treated patients displayed significantly more circulating MZB compared to those CKD5 patients that had never undergone dialysis therapy.

1. Introduction

Chronic kidney disease stage 5 (CKD5) is associated with several, poorly defined, immune impairments [1]. Individuals receiving haemodialysis are at increased risk of bloodstream and other infections [2]. Interestingly, patients undergoing lengthy dialysis treatment have inferior kidney allograft outcomes to those transplanted pre-emptively ahead of initiation of dialysis [3–7]. Certain immune deviations related to dialysis therapy as well as cardiovascular diseases [8,9] are likely to play an important role. The deleterious effect of HLA and non-HLA antibodies on kidney allograft outcomes is widely acknowledged [10–14]. However, early acute rejection may occur even in pre-emptive transplantation and in patients without anti HLA antibodies and thus other memory-associated mechanisms may be involved. Among other alloimmune mechanisms, heterologous immunity – immune cross-reactivity towards bacterial or viral antigens and alloantigens – has been hypothesized as a potential factor [15,16]. Therefore, augmented cellular sensitisation towards bacterial and viral pathogens in patients on long-term dialysis may affect kidney transplantation outcomes.

In the recent study, CKD5 patients had fewer naïve T cells and a higher percentage of memory T cells. The long-term dialysis and especially systemic inflammation was associated with accelerated immunosenescence in T cell and monocyte compartments [17]. It can be reasonably assumed, that patients on long-term dialysis have an augmented cellular immune memory against various pathogens. The studies performed thus far on immune deviations in various dialysis cohorts are limited by their poorly defined control groups, with healthy controls unable to mimic uraemia-related disturbances [18–20]. Therefore, little is known about the effect of dialysis itself on these immune memory mechanisms.

In order to evaluate possible immune disturbances associated with dialysis treatment in CKD5 patients without anti-HLA antibodies prior

Abbreviations: CKD5, chronic kidney disease stage 5; ELISPOT, enzyme-linked immune absorbent spot; cPRA, calculated panel-reactive antibody; DSA, donor-specific antibody; HLA, human leukocyte antigen; CMV, cytomegalovirus; BKV, BK virus; EBV, Epstein-Barr virus; PBMCs, peripheral blood mononuclear cells; FCS, foetal calf serum; IE-1, immediate-early protein 1; Pp65, phosphoprotein 65; LT, large T antigen; VP1, viral capsid protein 1; EDTA, ethylenediaminetetraacetic acid; MZB, marginal zone B cells; SFU, spot-forming units; CRP, C-reactive protein; GN, glomerulonephritis

* Corresponding author at: Department of Nephrology, Transplant Centre, Institute for Clinical and Experimental Medicine, Videnska 1958/9, 14021 Prague, Czech Republic.

E-mail address: ondrej.viklicky@ikem.cz (O. Viklicky).

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Table 1
Demographic data of patients.

	Pre-emptive	Dialysis > 3 M	P value
Patients (n)	20	54	
Recipient age (years)[a]	53.4 [34.0; 66.6]	53.7 [18.9; 72.4]	0.74
Gender of recipient (M/F)	11/9	42/12	0.05
Dialysis duration (months)[a]	0	23.7 [3.6; 86.7]	< 0.01
Type of dialysis			
HD n (%)	0	33 (61.1)	< 0.01
PD n (%)	0	15 (27.8)	< 0.01
HD/PD n (%)	0	6 (11.1)	< 0.01
cPRA	0	0	
CRP[a]	2.7 [0.3; 2.7]	2.4 [0.2; 12.4]	0.84
Original disease			
Hereditary n (%)	3 (15.0)	8 (14.8)	0.98
GN n (%)	8 (40.0)	23 (42.6)	0.84
Diabetes, hypertension n (%)	3 (15.0)	15 (27.8)	0.25
Others n (%)	6 (30.0)	8 (14.8)	0.13

[a] Median [min; max], HD (haemodialysis), PD (peritoneal dialysis), cPRA (calculated panel-reactive antibody), CRP (C-reactive protein), GN (glomerulonephritis).

to kidney transplantation, we used an innovative, standardised immune monitoring panel based on the One Study and BIO-DrIM clinical trials. In contrast with CKD5 patients without dialysis, we found that dialysis treatment is associated with a time-dependent augmentation in circulating marginal zone B cells (MZB).

2. Materials and methods

2.1. Patient characteristics

This is prospective, exploratory, single-centre cohort study with 74 CKD5 patients in whom a panel of immune monitoring tests was evaluated ahead of scheduled transplant surgery in 2014–2018. The demographic data of patients are summarised in Table 1. Peripheral blood samples for Enzyme-linked immune absorbent spot (ELISPOT) and flow cytometry (FACS) analysis were collected immediately prior to kidney transplantation. Twenty of the 74 patients had never received dialysis treatment and, therefore, were assigned to the pre-emptive cohort. The dialysis cohort ($n = 54$) consisted of patients that had spent at least 3 months on dialysis. None of the patients had history of previous transplants; all patients were considered to be at low immunological risk without anti-HLA antibodies, tested by Luminex prior to transplantation (calculated panel-reactive antibody (cPRA) at 0%). All patients gave their written informed consent to participate in the study. The local ethics committee approved the study protocol under no. G14–08-38, 1619/16.

2.2. IFN-γ ELISPOT assay

Effector/memory T cells reactive to tested virus-specific antigens were assessed using the IFN-λ ELISPOT assay [21]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinised peripheral blood samples using standard Ficoll density gradient centrifugation. All cells were cryopreserved in liquid nitrogen for subsequent processing, as described previously [22]. After thawing, PBMCs were re-suspended with complete media (RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (FCS), penicillin + streptomycin (50 U/mL) and 1.7 mM sodium glutamate) and left for 24 h at 37 °C in a CO_2 incubator. Next, $3*10^5$ recipient PBMCs were stimulated with specific antigens to detect virus-reactive T cells: CMV antigens (whole protein-spanning overlapping peptide pools of immediate-early protein 1 (IE-1) and phosphoprotein 65 (pp65) – length of each is 15 amino acids with 11 amino acid overlap) (Miltenyi Biotec)) and BKV antigens (polyoma large T protein (LT), viral capsid protein 1 (VP1) and EBV peptide mix (all from Autoimmun Diagnostika, GmbH)); pokeweed mitogen (AID, GmbH) served as the positive control. All cells with antigens were seeded in an ELISPOT plate (AID) and incubated for 24 h at 37 °C in a CO_2 incubator. After incubation, cells were removed and the ELISPOT plate processed according to the manufacturer's protocol. The resulting numbers of spot-forming units (SFU) were counted semi-automatically using an ELISPOT reader (ELR07 IFL AID iSpot Fluoro-Spot Reader System). Positive ELISPOT results were pre-defined as at least 25 SFU per well after subtracting the negative control [21,23].

2.3. Flow cytometry staining protocol

Subpopulations of the analyzed leukocytes (DC, B and T cells) were assessed using the ONE Study flow cytometry panel, computing major surface marker antigens to monitor their main subsets [24]. Briefly, anti-coagulated peripheral blood was collected in a test tube with ethylenediaminetetraacetic acid (EDTA) immediately prior to transplantation (nearly end of dialysis) and stained within 4 h of collection with dry pre-formulated surface antibodies (DURAClone IM B cells, IM T cells and IM DC cells; Beckman Coulter) for 15 min at room temperature in the dark. For B-cell staining protocol, whole blood was additionally washed twice with PBS (10 mL PBS, 300G/10min) before the surface staining procedure. Thereafter, all staining protocols involved lysis and fixation with VersaLyse (Beckman Coulter) + 2.5% IOTest fixative solution (Beckman Coulter) for 15 min in the dark. Lysed cells were then washed twice with 3 mL PBS. The prepared pellet was re-suspended in PBS/fixation buffer (PBS + 0.1% NaN3 + 0.8% IOTest® 3 Fixative Solution (10×)) and measured using the Navios flow cytometer (Beckman Coulter). Kaluza software (Beckman Coulter) was used for flow cytometry data analysis.

2.4. Antibody panels

Fluorochrome-conjugated anti-human DURAClone panels were obtained from Beckman Coulter (Marseille, France). Panels of antibodies contained: B cells [IgD-FITC (clone IA6–2), CD21-PE (clone BL13), CD19-ECD (clone J3–119), CD27-PC7 (clone 1A4CD27), CD24-APC (clone ALB9), CD38-A750 (clone LS198–4-3), IgM-Pacific Blue (clone SA-DA4), CD45-Krome Orange (clone J33)]; T cells [CD45RA-FITC (clone 2H4), CD197/CCR7-PE (clone G043H7), CD28-ECD (clone CD28.2), CD297/PD1-PC5.5 (clone PD1.3.5), CD27-PC7 (clone 1A4.CD27), CD4-APC (clone 13B8.2), CD8-A700 (clone B9.11), CD3-A750 (clone UCHT-1), CD57-Pacific Blue (clone NC1), CD45-Krome Orange (clone J33); and DC cells [CD16-FITC (clone 3G8), CD14-PE (clone RMO52), CD1c-PC5.5 (clone L161), CD11c-PC7 (clone BU15), Clec 9A-APC (clone 8F9), CD123-A700 (clone SSDCLY107D2), HLA-DR-Pacific Blue (clone IMMU-357), CD45-Krome Orange (clone J33)]. To determine absolute numbers of sub-populations, the DURAClone IM Count Tube: CD45-FITC, 7-AAD (clone J33) and fluorescent beads were used.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad InStat 3 (GraphPad Software) and IBM SPSS 22 software. Normality of data distribution was tested using the Kolmogorov-Smirnov test.

As most variables were found not to correspond with standard normal distribution, only non-parametric statistical methods were used. The Mann-Whitney U and chi-square tests were used to compare the demographic characteristics of patient groups. The Mann-Whitney *U* test was also used as part of the FACS (T, B and DC) and ELISPOT (CMV, BKV and EBV) analysis. The Kruskal-Wallis test was used to compare the three groups of patients, analysing absolute numbers of circulating MZB in patients on different dialysis treatment spans (0 M, 3-12 M, > 12 M). To emphasise the relation of these cells to dialysis, receiver operating characteristic (ROC) curves and the area under the

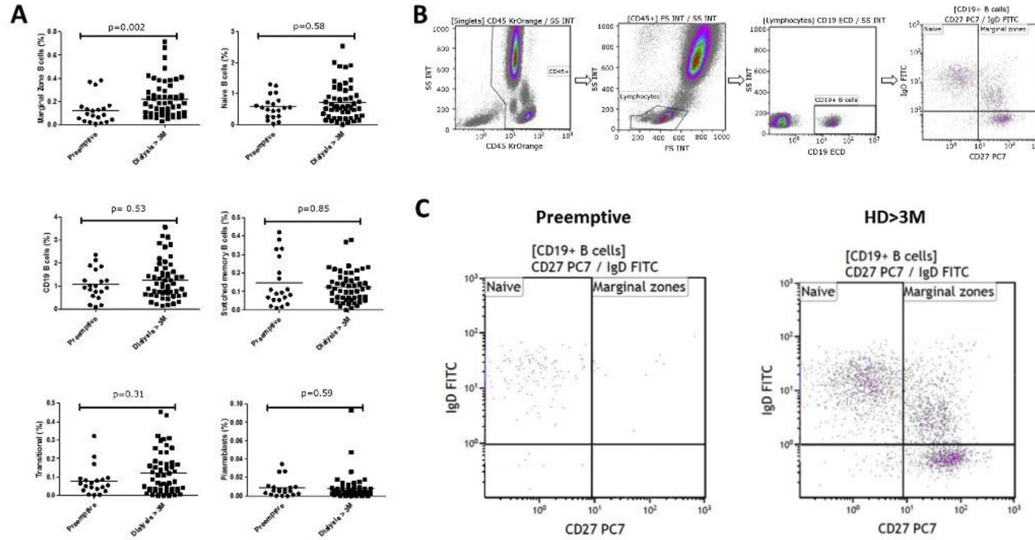

Fig. 1. (A) Circulating B-cell subpopulations in patients transplanted pre-emptively and in those on dialysis treatment. Differences are shown as percentages of each B-lymphocyte subset in nearly end of the dialysis period (immediately prior to transplantation) in peripheral blood. From upper left: marginal-zone IgD^+CD27^+ B cells, naïve $IgM^+IgD^+CD27^-$ B cells, $CD19^+$ B cells, switched-memory IgM^-CD27^+ B cells, transitional $IgMh^{igh}CD38^{high}CD24^{high}$ B cells, and IgM − $CD38^{high}CD27^{high}$ plasmablasts. The Mann-Whitney *U* test was used to compare groups, with individual *P* values displayed above the graphs; (B) gating strategy of circulating marginal-zone B cells; (C) visualisation of differences in the number of marginal zone B cells between pre-emptive and haemodialysis groups.

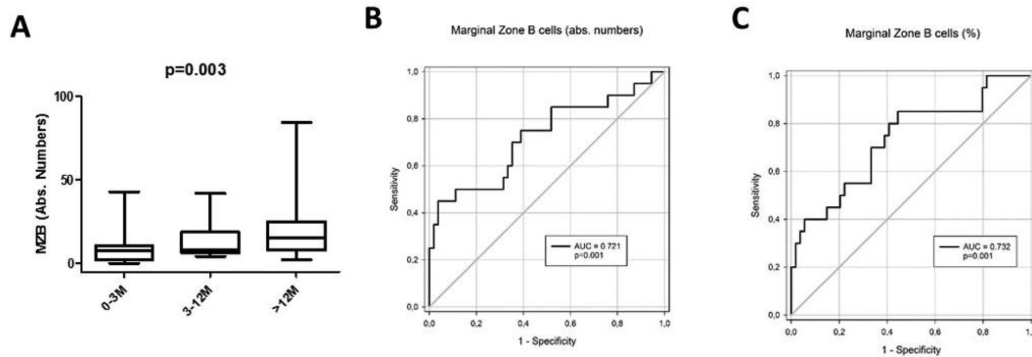

Fig. 2. (A) Changes in absolute numbers of marginal zone B cells in patients based on length of dialysis treatment (0 M, 3-12 M, > 12 M), Kruskal-Wallis test was used to compare the patients' groups, (B) receiver operating characteristic (ROC) curves based on absolute numbers and percentages (C) of marginal zone B cells and dialysis therapy.

curves (AUC) were also calculated. ROC analysis was also used for establishment the limit value for high/low numbers of MZB. Kaplan-Meier survival curves and the log-rank test were used to project rejection-free intervals for high/low numbers of MZB and to compare groups. Using binary logistic regression, a prediction model was calculated to estimate the risk of increased absolute MZB numbers in patients on dialysis > 12 M. All results with a *P* value of less than 0.05 were considered statistically significant.

3. Results

3.1. Dialysis treatment is associated with the augmentation of peripheral marginal zone B cells (MZB)

Dialysis-treated patients displayed significantly more circulating marginal zone B cells (MZB) (Fig. 1B) compared to those CKD5 patients who had never undergone dialysis therapy (P = .002, Fig. 1A, C). Interestingly, the numbers of circulating MZB were significantly higher in patients that remained on dialysis for long periods (P = .003, Fig. 2A). Binary logistic regression revealed that patients who remained on dialysis for > 12 months had a 4.2-fold greater risk of increased absolute

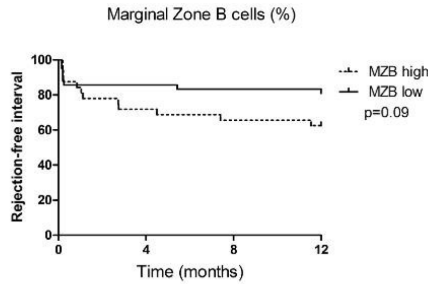

Fig. 3. Rejection-free intervals of patients using high/low numbers of Marginal Zone B cells (relative numbers) expressed as Kaplan-Meier survival curves; *P* value = .099. The limit value for high/low MZB was counted by ROC analysis: AUC = 0.64, cut off = 0.1083; sensitivity 60%: 95% confidence interval (CI): 36.05–80.88 and specificity 62.96%: CI: 48.74–75.71.

numbers of circulating MZB (95% CI: 1.6–11.2; *P* = .004) than patients of shorter stay on dialysis. Similarly, associations of dialysis duration with absolute and relative numbers of MZB are also suggested (Fig. 2A, B, C). The aim of our study was not designed to explore the effect of MZB cells on transplantation outcomes due to several confounding factors and limited patients numbers. However, two out of three pre-emptively transplanted patients exhibiting higher numbers of peripheral MZB experienced early acute rejection after transplantation. Therefore, we separately analyzed effects of low and high MZB on all types of rejections at one year. Interstingly, patients with higher MZB ahead of transplantation experienced non-significant trend towards higher rejection rate at one year (Fig. 3, Table 1). Clearly, such data needs to be considered preliminary and should be further validated in another studies.

Significantly, we observed no differences in the other B-cell subsets (CD19+, naïve, switched memory, transitional and plasmablasts) (Fig. 1A) or in T-cell subsets and dendritic cells between patient cohorts (Fig. 4).

3.2. Dialysis and virus-reactive effector/memory T cells

To investigate whether long-term dialysis would affect immunity against opportunistic viruses, an IFNγ-ELISPOT assay was performed to evaluate the presence of pre-existing virus-reactive (CMV, BKV, EBV) effector/memory T cells. Numbers of IFNγ-secreting cells after stimulation by pp65, IE-1, LT, VP1 and EBV peptide-mix antigens were similar between patients with and without dialysis history (Fig. 5 A-E). Immune deviations associated with dialysis treatment did not affect cellular immunity directed against major opportunistic viruses and the majority of patients remaining fully immuno-competent.

4. Discussion

Long-term dialysis therapy is associated with increased immune sensitisation against various pathogens. In this study, we evaluated

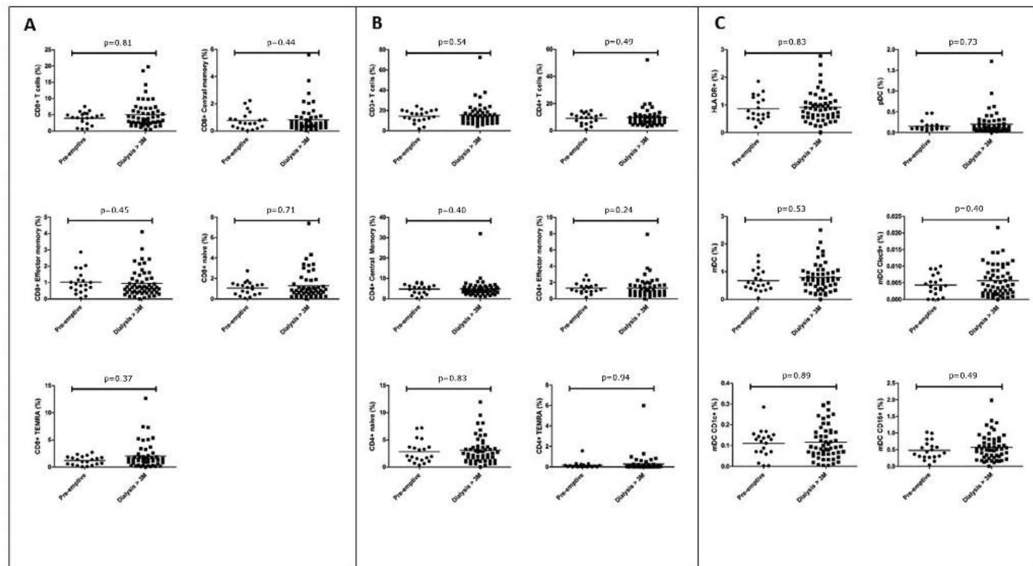

Fig. 4. Analyzed subpopulations of (A) CD8+ T cells, (B) CD4+ T cells and (C) DC cells in patients either transplanted pre-emptively or on dialysis. The Mann-Whitney *U* test was used to compare groups; *P* values are displayed above graphs.
Individual analyzed subpopulations:
A: CD8$^+$ T-lymphocyte subpopulations – CD8$^+$ T cells, CCR7$^+$CD45RA$^-$ central memory T cells, CCR7$^-$CD45RA$^-$ effector memory T cells, CCR7$^+$CD45RA$^+$ naïve T cells, CCR7$^-$CD45RA$^+$ TEMRA.
B: CD4+ T-lymphocyte subpopulations – CD4$^+$ T cells, CCR7$^+$CD45RA$^-$ Central memory T cells, CCR7$^-$CD45RA$^-$ Effector memory T cells, CCR7$^+$CD45RA$^+$ naïve T cells, CCR7$^-$CD45RA$^+$ TEMRA.
C: Dendritic cells (DC) – LIN$^-$ HLADR$^+$, LIN$^-$ CD11c$^-$ CD123$^+$ plasmacytoid dendritic cells (pDC), CD11c$^+$ myelod DC (mDC), Clec9$^+$ mDC, CD1c$^+$ CD16$^-$ mDC, Clec9$^-$ CD16$^+$ mDC.

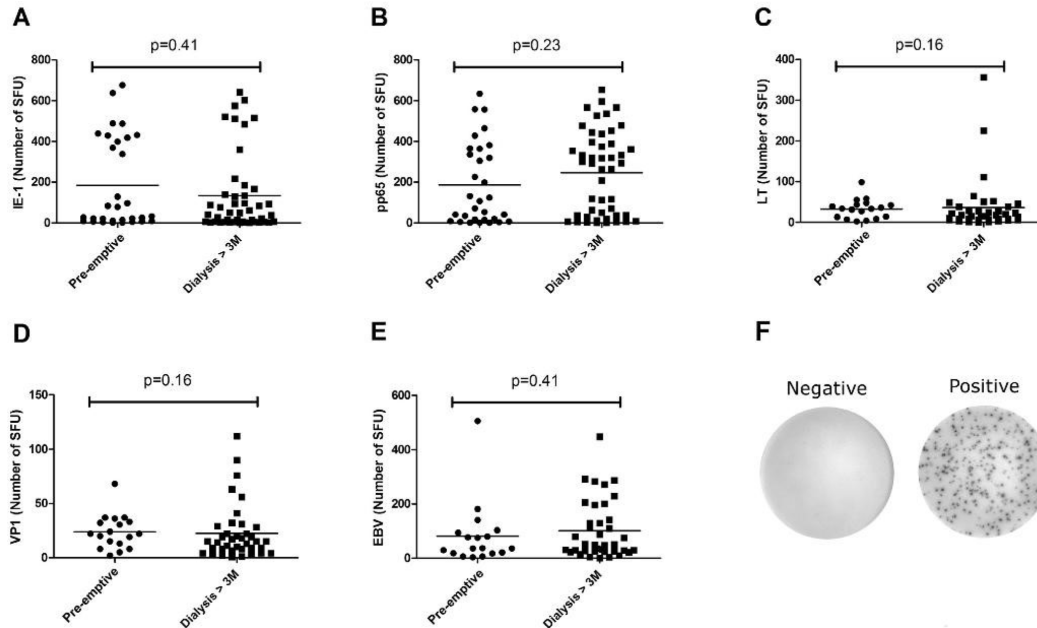

Fig. 5. Number of responder memory/effector T cells producing IFN-γ after stimulation with different virus-specific antigens: (A) CMV IE-1; (B) CMV pp65; (C) BKV LT; (D) BKV VP1; (E) EBV peptide mix; (F) example of positive and negative ELISPOT results, with ≥25 spot-forming units (SFU) measured by the ELISPOT reader (ELR07 IFL AID iSpot FluoroSpot Reader System) considered positive.

effector/memory T cells reactive to main opportunistic virus-specific antigens and peripheral lymphocyte phenotypic deviations using a panel of immune monitoring tests in patients with absent humoral allosensitisation. While we found that peripheral MZB cells were augmented in patients on long-term dialysis, we observed no differences in the other B-, T- and DC subsets or in opportunistic virus-specific effector/memory T cells.

Peripheral MZB cells are acknowledged as a "first-line defence" in the early antibody response against circulating blood antigens, especially microbials and polysaccharides [25,26]. They also maintain homeostasis through the clearance of apoptotic cells and cellular debris [27–29]. In dialysis patients, bloodstream infections are common, mostly due to contamination from dialysate, i.v. catheters and dialysis needles. MZB cells activate the primary immune response by rapidly expressing immunoglobulins in response to both T-cell-dependent and T-cell-independent antigens, thus contributing to humoral sensitisation [30]. Moreover, because MZB express a memory phenotype, [31–33] they can be easily activated once exposed to antigens. Long-term dialysis treatment accelerates immunosenescence, dramatically reduces naïve T subpopulations, and increases the percentage of memory T-cell phenotype compartments [17]. It can be therefore hypothesized exposure to a uraemic environment significantly reinforces immune memory in other parts of the adaptive immune system.

MZB are considered to be substantive producers of natural polyreactive antibodies with broad specificity and low affinity, which are predominantly IgM class with shorter H-CDR3 regions, and their impact on posttransplant graft condition is anticipated as well [34,35]. This study was not designed to evaluate posttransplant outcome mainly due to limited patient numbers and lower expected acute rejection incidence in low immunological risk population. Interestingly, in two out of three pre-emptively transplanted patients with higher peripheral MZB cells the acute T cell mediated rejection had occurred and there was a trend towards higher MZB cells in patients with rejection at one year.

In this study, we found no association between dialysis therapy and opportunistic virus-specific cellular immune responses. The level of immune competence – calculated as the number of antigen-reactive cells – was similar for pre-emptive and dialysis cohorts. This finding supports the widely held assumption that the established cellular immunity against viruses or absent opportunistic infection trigger in the long-term dialysis. Similarly, it is likely that dialysis therapy *per se* does not affect cellular immunity against opportunistic viruses.

This study has several strengths but also limitations. Among the firsts, our pre-emptive cohort represent an ideal control group for dialysis. Moreover, we used well-established and validated immune monitoring platforms to minimise technical bias.

Further, we included only patients without HLA antibodies, limiting the effect of humoral alloimmunity on MZB cells. Clearly, this study is cross-sectional in design: immune monitoring was performed at a single time point in advance of scheduled kidney transplantations. To provide a more in-depth understanding of the associations between MZB cells and post-transplant outcomes, a much larger prospective study is needed.

In conclusion, we report here for the first time an association between MZB and dialysis duration.

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Author contributions

LS, PH and OV performed the research, conducted the data analysis and wrote the manuscript. JS assisted with research and the management of patients. BS, PR and HDV helped with the preparation of ELISPOT and FACS assays and also contributed to the writing of the manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.trim.2020.101289.

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6 Diskuze

Paměťové T lymfocyty mají zásadní roli v transplantační imunologii. Ukazuje se, že přítomnost dárcovsky-specifických paměťových/efektorových T lymfocytů před transplantací je spojována s horší funkcí štěpu a s výskytem akutní rejekce (111,112).

V této práci jsme se zaměřili na studium CMV- a dárcovsky- specifických paměťových/efektorových T lymfocytů u transplantací ledvin.

V první části projektu jsme se zabývali změnami v potransplantační CMV specifické celulární imunitě u CMV seropozitivních příjemců ledviny od žijícího dárce. Hlavním pozorováním bylo, že indukční imunosuprese, nedepleční (basiliximab) ani depleční (rATG), nemá vliv na CMV-specifickou buněčnou odpověď u CMV seropozitivních příjemců transplantované ledviny v 6. měsíci po transplantaci

U pacientů, kteří se nacházejí ve vysokém riziku vzniku CMV infekce (CMV seronegativní příjemci, kteří dostali orgán od CMV seropozitivního dárce (R-/D+), a CMV seropozitivní příjemci (R+/D+, R+/D-), a kteří dostali depleční imunosupresi rATG) je dobře popsán vliv indukční a udržovací imunosuprese na vznik CMV nemoci a jsou známé obecné doporučení pro použití antivirové profylaxe valgancyklovirem (113–115). Naše pozorování, že potransplantační imunosuprese nemá vliv na přítomnost efektorových buněk je v souladu s pozorováním ostatních (116,117). Ve studii Pearl et al. (2005) zjistili, že reziduální T buňky po depleční terapii sdílejí stejný fenotyp, který koresponduje s paměťovými/efektorovými T buňkami. Studie naznačila, že paměťové/efektorové T buňky jsou selektivně rezistentní k terapeutické depleční terapii (117).

Paměťové/efektorové T lymfocyty byly v naší studii stanoveny metodou ELISPOT, kde jsou detekovány buňky produkující IFN-γ po stimulaci (61,118). T lymfocyty nejsou jediné, které produkují IFN-γ, například NK buňky exprimují tento cytokín (119–122). Autoři jiné studie potvrdili, že CMV specifické paměťové/efektorové T lymfocyty chybí u 12% CMV seropozitivních pacientů, výsledky byly měřeny testem ELISPOT a Quantiferon (123). Také Sylwester et al (2005) ukázali, že někteří dospělí nejsou schopni správně rozeznat pp65 antigen (124). V našem souboru jsme neprokázali CMV-reaktivní lymfocyty u 2 pacientů nehledě na pozitivní sérologii.

Dalším cílem naší studie bylo zhodnotit CMV specifickou celulární imunitu v kontextu CMV DNAemie, tj. potransplantační reaktivací CMV. Nenašli jsme žádnou

asociaci mezi pretransplantační CMV specifickou imunitou a pozdějším výskytem CMV DNAemie, která byla hodnocena 3 měsíce po transplantaci, nebo podle klinického stavu pacienta. K odlišným výsledkům dospěli Bestard et al. (2013), kde byla nalezena asociace mezi nízkým výskytem pretransplantačních IE-1 specifických T buněk a výskytem CMV infekce po transplantaci. Naše studie byla zaměřena na CMV seropozitivní příjemce ledviny od žijících dárců a výše zmíněná studie byla naopak provedena u příjemců, kteří dostali ledvinu od zemřelého dárce. Do studie byli zařazeni jak CMV seropozitivní, tak i CMV seronegativní příjemci.

V další části projektu jsme se zabývali vlivem CMV- a alloreaktivních paměťových/efektorových T lymfocytů na výskyt akutní rejekce. Zjistili jsme, že vyšší výskyt CMV- reaktivních paměťových/efektorových lymfocytů před transplantací byl asociován s vyšším výskytem rejekce zprostředkované T lymfocyty po transplantaci. Předchozí studie ukázaly, že vyšší předtransplantační dárcovsky-specifická T buněčná odpověď byla asociována s akutní rejekcí a nižším eGFR u pacientů, léčených nedepleční indukcí (111,112). V naši studii bylo 40% pacientů léčeno rATG, který efektivně zredukoval alloreaktivní paměťové/efektorové T lymfocyty na frekvenci, která může být kontrolována udržovací imunosupresí.

Nalezli jsme ale vztah mezi CMV-reaktivními paměťovými/efektorovými T lymfocyty a rejekcí a také signifikantní korelaci mezi těmito buňkami a nižším potransplantačním eGFR ve 3., 6. a 12. měsíci. Subklinická CMV reaktivace je opakovaně detekovatelná u 30% příjemců transplantované ledviny bez ohledu na CMV profylaxi (125–127). Domníváme se, že subtilní CMV reaktivace jsou mnohem častější, i když jsou nedetekované, a poskytují antigenní stimulaci pro CMV-specifické T buňky. To koresponduje s pozorováním ostatních autorů, kteří zjistili, že CMV virémie, která se vyskytuje u víc jak poloviny pacientů, bez ohledu na CMV profylaxi, je spojena s horší funkcí štěpu (128).

Další vysvětlení, které se nabízí, je přímý vliv heterologní imunity na funkci štěpu (129). Zjistili jsme, že zkříženě-reagující T buněčné klony (sdílející stejnou TCR-β sekvenci) byly nalezeny u obou CMV- i alloreaktivních T buněk v periferní krvi před transplantací a také v biopsii štěpu po transplantaci, kde mohou participovat v CMV-asociované T buňkami zprostředkované rejekci. Naše předchozí experimenty ukázaly, že imunodominantní epitopy pro konkrétní HLA alelu, obarvené komplexem peptid/dextramer nereflektovaly komplexní odpověď na CMV peptidy. Proto jsme použili metodu HLA-nezávislé T buněčné stimulace (130), kterou bylo možné

detekovat všechny 3 analyzované skupiny T buněk: i) CMV-specifické, ii) dárcovsky-specifické, iii) zkříženě reagující. Reaktivní buňky jsme vysortrovali a vyšetřili pomocí NGS.

Z výsledků vyplývá, že poměrně rozsáhlá kohorta periferních CMV-reaktivních buněk obsahuje subpopulaci zkříženě reagujících buněk, které jsou schopny migrovat do transplantovaného štěpu. Tyto zkříženě reagující klonotypy mohou v přítomnosti vhodných antigenních stimulů (CMV, allotransplantát) reagovat zvýšenou proliferací a následným poškozením štěpu. Tyto závěry korespondují s hypotézou heterologní imunity (131,132). Specifická allo-HLA zkřížená reaktivita již byla prokázána na klonální úrovni sdíleným repertoárem TCR u EBV, CMV, varicella-zoster (VZV) a chřipkového viru A (76,133). Naše data poprvé prokázaly výskyt zkříženě reagujících klonů nejen v periferní krvi, ale i v transplantované ledvině. Jde ale samozřejmě o předběžné výsledky, které musí být ověřeny na větším souboru.

Dále jsme se zabývali vlivem dialýzy na paměťové/efektorové T buňky, které reagují na oportunní viry (CMV, EBV, BK) a na periferní subpopulace T a B lymfocytů a dendritických buněk u pacientů bez humorální sensitizace. Zjistili jsme, že u pacientů s dlouhodobou dialyzační léčbou byly v periferní krvi zvýšené B lymfocyty marginální zóny (MZB). U ostatních analyzovaných subpopulací, včetně virově-reaktivních paměťových/efektorových buněk nebyly pozorovány signifikantní rozdíly.

Periferní MZB fungují jako „první linie obrany" proti cirkulujícím mikrobům a polysacharidům a pomáhají udržovat homeostázi odstraňováním apoptotických buněk a celulární debris (134–138). U pacientů na dialýze může docházet k bakteremii při eventuální kontaminaci dialyzátu, katétru a dialyzačních jehel. MZB exprimují paměťový fenotyp, a proto mohou být velice snadno aktivovány. Jakmile jsou vystaveny antigenům (T-dependentním i T-independentním) produkují polyreaktivní přirozené protilátky a tím přispívají ke zvyšování humorální sensitizace (139–141).

Dlouhodobá dialyzační léčba zvyšuje imunosenescenci, je spojena s redukcí naivních T lymfocytů a se zvýšenou frekvencí paměťových T buněk (142). Lze tedy předpokládat, že uremické prostředí výrazně alteruje imunitní paměť a jiné části adaptivního imunitního systému. U 2 pacientů s vysokým výskytem MZB před transplantací se akutní rejekce vyskytla do 1 roku. Jde ale samozřejmě o pilotní pozorování. Každopádně je možno usuzovat, že kromě humorální sensitizace spojené s krevními transfuzemi, předchozí transplantací nebo porody, jsou u nemocných s nezvratným selháním ledvin léčených dialýzou aktivovány další složky přirozené a

adaptivní imunity, které se mohou podílet na snadnějším vzniku rejekce po transplantaci.

7 Závěry

1. **Efekt indukční terapie na CMV specifické efektorové/paměťové T lymfocyty před a po transplantaci ledviny od žijících dárců**

Počet příjemcovských CMV-specifických buněk produkujících IFN-γ po stimulaci pp65 a IE-1 antigeny před a 6 měsíců po transplantaci ledviny je stejný bez ohledu na použitou indukční terapii.

2. **Zkříženě reagující T lymfocyty se nacházejí v periferní krvi pacienta před transplantací a jsou identifikovatelné také po transplantaci v ledvinném štěpu**

Pomocí NGS sekvenování jsme prokázali přítomnost heterologní imunity, resp. přítomnost T buněčných klonotypů se stejnými TCRβ CDR3 sekvencemi reagujícími jak na CMV, tak i na alloantigeny v krvi pacientů před a v ledvinném štěpu po transplantaci ledviny. Jejich přítomnost v biopsii štěpu ukazuje na přímé spojení mezi CMV specifickou imunitou a poškozením štěpu.

3. **Dialyzační léčba ovlivňuje vyšší výskyt B lymfocytů marginální zóny v periferii**

Delší pobyt na dialýze neovlivnil počet T buněk specifických pro nejčastější oportunní infekce u pacientů podstupujících transplantaci (CMV, BK, EBV). Tato terapie signifikantně ovlivnila počet cirkulujících B lymfocytů marginální zóny, ostatní analyzované subpopulace T, B a DC buněk zůstali nezměněné.

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