Jihočeská univerzita v Českých Budějovicích
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HABILITAČNÍ PRÁCE

Vliv klíštěcích slin na signalizaci v patogenem aktivovaných dendritických buňkách

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Anotace

Spirochéty *Borelia burgdorferi* a virus klíšťové encefalitidy (TBEV) jsou patogeny přenášené klíšťaty do hostitele spolu s klíštěcími slinami během sání. Modulací hostitelských obranných mechanizmů vytváří sliny v místě sání příznivé podmínky pro přenos patogenů, jejich přežití a množení v hostiteli. Tato asistence slin bývá označována jako slinami asistovaný přenos (saliva-assisted transmission, SAT). Cílem studií, které jsou součástí předložené práce, bylo rozpoznat mechanizmus působení klíštěcích slin a vybraných klíštěcích proteinů na dendritické buňky na úrovni buněčné signalizace a tím přispět k objasnění SAT efektu. Dendritické buňky tvoří první linii hostitelské obrany a jejích jedinečnost tkví v schopnosti spojovat nespecifickou a specifickou imunitu a tím ovlivňovat celkovou hostitelskou odpověď. Dendritické buňky rozeznávají patogeny pomocí specifických receptorů, což následně vede ke kaskádě signalizačních reakcí, výsledkem kterých je jejich aktivace. Vliv slin a tří klíštěcích proteinů, Sialostatinu L, Sialostatinu L2 a IRS-2 byl studován na dendritických buňkách infikovaných boreliemi, virem TBE, nebo aktivovaných relevantními ligandy.

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1. KLÍŠTĚ A KLÍŠŤATY PŘENÁŠENÉ PATOGENY

Klíšťata jsou vektory mnoha patogenů včetně spirochét rodu *Borrelia*, viru klíšťové encefalitidy (TBEV), *Francisella tularensis, Babesia microti*, *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis* a bakterií rodu *Rickettsia* (1, 2). Četnost a závažnost nemocí, které tyto patogeny způsobují, umocňuje význam studií věnovaných interakci vektor-patogen-hostitel.
Klíšťata jsou obligátní krevsající členovci, kteří parazitují na širokém okruhu hostitelů včetně savců, ptáků, plazů a obojživelníků. Řadí se do třídy Acari, řádu Ixodida, v němž se rozlišují dvě hlavní čeledi; čeleď *Ixodidae* (tzv. tvrdá klíšťata) a čeleď *Argasidae* (tzv. měkká klíšťata). Existuje přes 900 druhů klíšťat, z toho 700 z čeledi *Ixodidae* a 200 z čeledi *Argasidae*. Třetí je čeleď *Nuttalliellidae*, která má jenom jednoho zástupce. Tvrdá a měkká klíšťata se liší ve způsobu sání; měkká klíšťata sají rychle a opakovaně, zatímco tvrdá klíšťata sají jednou za několik dní v každém jejich vývojovém stadiu (3).
Z lékařského hlediska jsou významná hlavně tvrdá klíšťata, protože přenáší řadu významných patogenů. Na základě morfologických rozdílů se dělí na dvě skupiny: Prostriata a Metastriata. *Ixodes* je jediným rodem skupiny Prostriata, zatímco Metastriata reprezentuje 5 podčeledí: *Amblyomminae, Haemaphysalinae, Hyalomminae, Rhipicephalinae, Bothriocrotoninae* (4). V Evropě je nejdůležitějším klíštětem *Ixodes ricinus.*
Životní cyklus klíštěte trvá od 6 měsíců do 6 let, v závislosti na podmínkách. Sestává ze tří stádií, které zahrnují larvu, nymfu a dospělce. Klíště v každém vývojovém stádiu saje jednou, přičemž sameček (dospělec) klíštěte krev nesaje. Samička po oplodnění a plném nasátí klade vajíčka v počtu 400-20 000 (3). Klíšťata představují významný rezervoár klíšťaty přenášených patogenů. Jedním z nich jsou spirochéty *Borrelia burgdorferi* sensu lato.

B. BURGDORFERI SENSU LATO

Jedním z nejčastějších klíšťaty přenášených patogenů jsou spirochéty serokomplexu *B. burgdorferi* sensu lato způsobujících lymskou boreliózu. V Evropě a Asii jsou u člověka etiologickými agens lymské boreliózy hlavně tři druhy rodu *Borrelia: B. burgdorferi* sensu stricto (s.s.), *B. garinii* a *B. afzelii*, v USA je příčinou Lymské boreliózy *B. burgdorferi* s.s. Existuje početná skupina borelií, které jsou původcem tzv. návratné horečky (relapsing fever). Tyto borelie ovšem nejsou přenášeny tvrdými klíšťaty (1).
Lymská borelióza je multisystémové onemocnění, které se dělí do tří fází. Prvním symptomem je erythema migrans, červená skvrna šířící se v místě přisátí s postupným centrálním vyblednutím. Druhá fáze onemocnění se objevuje několik měsíců po první fázi, kdy se spirochéty šíří do sekundárních orgánů. Symptomy se liší v závislosti od druhu borelií, *B.burgdorferi* s.s. je asociovaná s artritidou a karditidou, *B.garinii* je neurotropní a vyvolává neuroboreliózu, zatímco infekce *B.afzelii* je spjata s kožními projevy (5). Třetí fáze nemoci má již chronický charakter a může způsobovat chronickou artritidu, karditidu, či těžší formu encefalomyelitidy. Symptomy prví fáze jsou jediným projevem onemocnění až v 20 % případů neléčených boreliových infekcí.

Borelie jsou gram-negativní bakterie spirálovitého tvaru, jejich vnější membrána obklopuje protoplasmatický cylindrický komplex skládající se z peptidoglykanu, vnitřní buněčné membrány a cytoplasmy. Borelie mají několik bičíků umístněných v periplasmatickém prostoru, které slouží k jejich charakteristickému šroubovitému pohybu a k udržení buněčného tvaru. Genom borelií sestává z jednoho lineárního chromozómu, 12 lineárních a 9 cirkulárních plazmidů (6, 7).
Borelie perzistují v přírodě v enzootickém cyklu mezi klíšťaty *Ixodes* spp. a širokým spektrem hostitelů včetně savců, plazů a ptáků. V Europě je primárním vektorem spirochet *B. burgdorferi* sensu lato klíště *I. ricinus*, v Asii *I. persulcatus*, v USA jsou to *I. scapularis* a *I. pacificus*. V rámci životního cyklu klíšťat se borelie přenáší transtadiálně z larvy na nymfu a z nymfy na dospělce. Transovariální přenos borelií zaznamenán nebyl, larvy klíšťat získávají spirochéty během sání na infikovaném hostiteli. Člověk je náhodný hostitel a obyčejně je nakažen nymfou nebo dospělcem. Borelie jsou na hostitele přenášeny slinami během sání klíštěte. V klíštěti jsou borelie zachycené ve střevě pomocí adhesinu OspA. Beheм sání klíštěte se borelie aktivují, dochází k down regulaci OspA a up regulaci OspC, což umožňuje uvolnění spirochét a jejich přenos do hostitele. Existuje několik studií, které popisuji, že přenos borelií je podporován klíštěcími slinami (8).

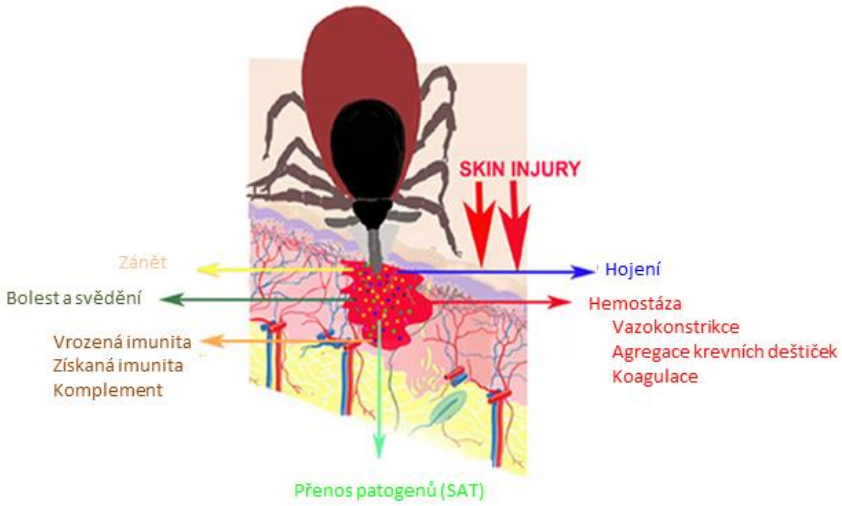
VIRUS KLÍŠŤOVÉ ENCEFALITIDY (TBEV)

Dalším patogenem, který je přenášen klíšťaty je virus klíšťové encefalitidy (TBEV). TBEV je neurotropní virus způsobující závažné onemocnění, encefalitidu. TBEV patří do rodu *Flavivirus*, čeledi *Flaviviridae* a spolu s virem Langat, virem Omské hemoragické horečky, virem horečky Kjasanurského lesa, virem Louping ill, či virem Powassan se řadí do komplexu flavivirů přenášených klíšťaty. Do komplexu flavivirů přenášených komáry, které jsou příbuzné s TBEV, patří virus Dengue, virus západonilské horečky (WNV), virus japonské encefalitidy nebo virus Zika. Tyto viry, s výjimkou viru Zika, podobně jako TBEV, způsobují encefalitidu (1).
Na základě serologických a sekvenčních analýz se TBEV rozděluje na 3 subtypy, evropský, sibiřský a dálněvýchodní, přičemž každý subtyp je asociovaný s různou závažností onemocnění. Evropský subtyp způsobuje mírnější onemocnění, naopak infekce dálněvýchodními kmeny může být fatální. Vektorem pro evropské kmeny je *I. ricinus*, pro sibiřské a dálněvýchodní kmeny je vektorem *I. persulcatus* (1).
Flaviviry jsou je obalené RNA viry, jejich genom tvoří jednovláknová pozitivně orientovaná nukleová kyselina. Genomová RNA je translatovaná do jednoho polypeptidového proteinu, který je štěpen virovými a buněčnými proteázami na několik nestrukturních (NS1, NS2A, NS2B, NS3, NS4A, NS4B a NS5) a strukturních proteinů (C, M a E) (9). Nestrukturní virové proteiny hrají důležitou roli jak v replikačním cyklu TBEV tak v inhibici hostitelské antivirové reakce. Strukturní proteiny se účastní vytvoření nukleokapsidu a infekční virové částice a zodpovídají za interakci s hostitelskou buňkou. Virus, po vazbě na specifický buněčný receptor, vstupuje do buňky klatrin-dependentní endocytózou. K uvolnění virové RNA z endosomálního váčku dochází po fúzi virové membrány s membránou endosomu následkem nižšího pH v pozdním endosomu. Následná translace virové RNA probíhá v cytoplazmě za vzniku polyproteinu, který je štěpen na jednotlivé virové proteiny. Virový genom je replikován v blízkosti membrány endoplazmatického retikula (ER) v tzv. replikačních vezikulech. Virová částice se formuje skládáním kapsidového proteinu a virového genomu, a následným pučením do lumenu ER. Takto vytvořená virová částice

dozrává v Golgiho aparátu, kde se po konformačních změnách prekurzorového M proteinu mění v plně infekční virus (10).
Přenos TBEV v rámci životního cyklu klíšťat je možný transovariálně, transstadiálně a horizontálně při sousání. K přenosu TBEV na člověka dochází během sání infikovaným klíštětem (jiný ovšem méně častý způsob nákazy je možný po požití nepasterizovaného mléka). Virus se přenáší s klíštěcími slinami do místa sání již krátce po přichycení klíštěte na hostitele. Nicméně, množství přeneseného viru narůstá 10 až 100 krát během sání (11). Rezidentní buňky kůže jsou místem, kde dochází k zmnožení viru ještě před detekovatelnou virémii. Keratinocyty, Langerhansové buňky, makrofágy a fibroblasty jsou vnímavé k infekci TBEV a jsou důležitým zdrojem viru v kůži. Langerhansovy buňky (typ dendritických buněk) mají schopnost migrovat do lymfatických uzlin, kde v důsledku dalšího zmnožení viru dochází k viremii a následné diseminaci viru do jiných orgánů jako jsou slezina, játra a kostní dřen. Konečným cílem viru TBE jsou buňky centrálního nervového systému (12).

KLÍŠTĚCÍ SLINY

Tvrdá klíšťata sají po dobu několika dní a je pro ně žádoucí zůstat hostitelem nezpozorovaný. Už po vniknutí klíšťecího hypostomu do kůže dochází k jejímu mechanickému poškození a narušení hemostázy, čímž se iniciuje koagulace, vasokonstrikce a agregace krevních destiček. Tyto procesy jsou potlačeny během sání klíštěte skrze účinky klíštěcích slin (13). Rovněž jsou inhibovány bolest a svědění. Dalším důležitým obranným hostitelským mechanismem je aktivace komplementu, zánětu a rekrutace leukocytů do místa sání. Aktivace nespecifické a adaptivní imunity jsou slinami ovlivněny ve prospěch sajícího klíštěte (14-17). Sliny mají prokazatelně pro klíště esenciální význam, protože mu umožňují zachovat hemostázu a potlačit hostitelskou imunitní reakci (Obr. 1).
Biologicky aktivní látky v klíštěcích slinách jsou proteinové, peptidové či ne-peptidové povahy. Většina proteinů a peptidů je syntetizována v slinných žlázách během sání. Transkriptomové studie ukázaly, že je sekretováno více než 500 různých proteinů a peptidů (18). Ty můžou být rozděleny do skupin na základě vzájemné příbuznosti. Nejvýznamnější skupiny tvoří lipokaliny, proteiny a peptidy obasahující Kunitzovu doménu, metaloproteázy, proteiny s bazickým koncem, cystatiny, ixostatiny a další (14).
Za anti-hemostatické účinky slin jsou zodpovědné prostaglandiny, apyrázy, metaloproteázy, disintegriny, inhibitory trombinu, FXa a FVIIa/TF komplexu, inhibitory kontaktní fáze a modulátory fibrinolýzy. Analgetický účinek slin se přiřazuje inhibitorům bradykininu a lipidovým mediátorům, endokanabionidům. Protizánětlivé účinky byly popsány pro histamin a serotonin vázající proteiny a inhibitory komplementu. Serpiny a cystatiny tvoří kategorii slinných komponentů, u kterých byl v rámci imunomodulačních účinku prokázán i protizánětlivý efekt. Významné imunomodulační účinky mají evaziny (proteiny vázající chemokiny) a imunoglobulin vázající proteiny (14, 19) Podrobnější popis imunomodulačních účinků slin a vybraných slinných komponentů bude v další kapitole.



Obr. 1. Interference klíštěcích slin s hostitelskou obranou během sání klíštěte. Sliny ovlivňují proces hojení, hemostázu, svědění, zánět, imunitní reakce a přenos patogenů. Převzato a upraveno z review (17).

SLINAMI ASISTOVANÝ PŘENOS (SAT)

Klíštěcí patogeny jsou přenášeny do hostitele během sání s klíštěcími slinami. Slinami ovlivněné prostředí v místě sání vytváří příznivé podmínky pro přenos patogenů z infikovaného klíštěte na hostitele i pro jeho zmnožení v hostiteli (8). Tato asistence slin při přenosu patogenů bývá označována jako slinami asistovaný přenos (saliva-assisted transmission, SAT). Sliny rovněž napomáhají přenosu patogenů z infikovaných klíštat na neinfikovaná klíšťata během sousání (cofeeding). K tomuto způsobu přenosu patogenů dochází nezávisle na virémii a je proto označován jako neviremický přenos. SAT byl poprvé popsán pro Thogoto virus (20), u kterého byla zvýšená transmise prokázána pod vlivem extraktu ze slinných žláz (salivary gland extract, SGE) z klíštěte *Rhipicephalus appendiculatus*. Později byl SAT prokázán použitím SGE a klíštěcích slin i pro jiné patogeny včetně TBEV (21), *B. burgdorferi* sensu lato (22-24), *Francisella tularensis* (25), *Rickettsia conorii* (26), Powassan virus (27) a African swine fever virus (28).
Mechanizmus pozitivního vlivu klíštěcích slin na přenos různých patogenů není zcela objasněn. Za SAT při přenosu bakteriálních patogenů mohou pravděpodobně imunomodulační účinky slin na hostitelské imunitní buňky (29, 30). Z důvodu unikátního postavení dendritických buněk (DC) v rámci hostitelské imunity byly právě DC intenzívně studovány s cílem podrobně popsat modulační účinky klíštěcích slin na DC a přispět tak k objasnění mechanizmu SAT. V případě virové infekce je role dendritických buněk dvojí, kromě modulace imunitní odpovědi jsou tyto buňky permisivní k virové infekci a jsou tedy i zdrojem virových částic.

2. DENDRITICKÉ BUŇKY

Dendritické buňky jsou buňkami vrozené imunity a jsou považovány za tzv. hlídače. Jejich unikátní úlohou je rozpoznat nebezpečí pomocí specifických receptorů a poslat varovné signály formou rozpustných mediátorů jiným imunitním buňkám, které hrozbu, např. v podobě mikrobiální infekce, potlačí.

Obecně mezi hlavní funkce dendritických buněk patří rozpoznání patogenního motivu, fagocytóza, zpracování antigenního peptidu a jeho prezentace na buněčném povrchu pomocí molekuly MHC II (hlavní histokompatibilní komplex třídy II). Prezentace antigenu naivním T lymfocytům, ke které dochází po migraci zralých DC do lymfatických uzlin, je tou vlastností, díky které jsou tyto buňky považovány za profesionální antigen prezentující buňky. Naivní T lymfocyty po interakci s DC diferencují na jednotlivé subtypy; rozlišují se Th1, Th2, Th9, Th17 a regulační T lymfocyty. K tomuto rozdělení dochází na základě cytokinů, které lymfocyty produkují. Převaha některého Th subtypů je označována jako polarizace imunitní odpovědi. Diferenciace T lymfocytů a tedy i směr polarizace závisí od složení cytokinů, které produkují DC při prezentaci. Je důležité zmínit, že během zpracování antigenu dochází k tzv. maturaci neboli zrání dendritických buněk, což je proces provázený zvýšenou expresí molekul MHC II a zvýšenou expresí kostimulačních molekul jako jsou např. CD80, CD86, CD83. Snížená exprese těchto molekul na povrchu DC negativně působí na vývoj specifické imunitní reakce. Funkční DC jsou tedy esenciální pro nastartování specifické adaptivní imunity (31).

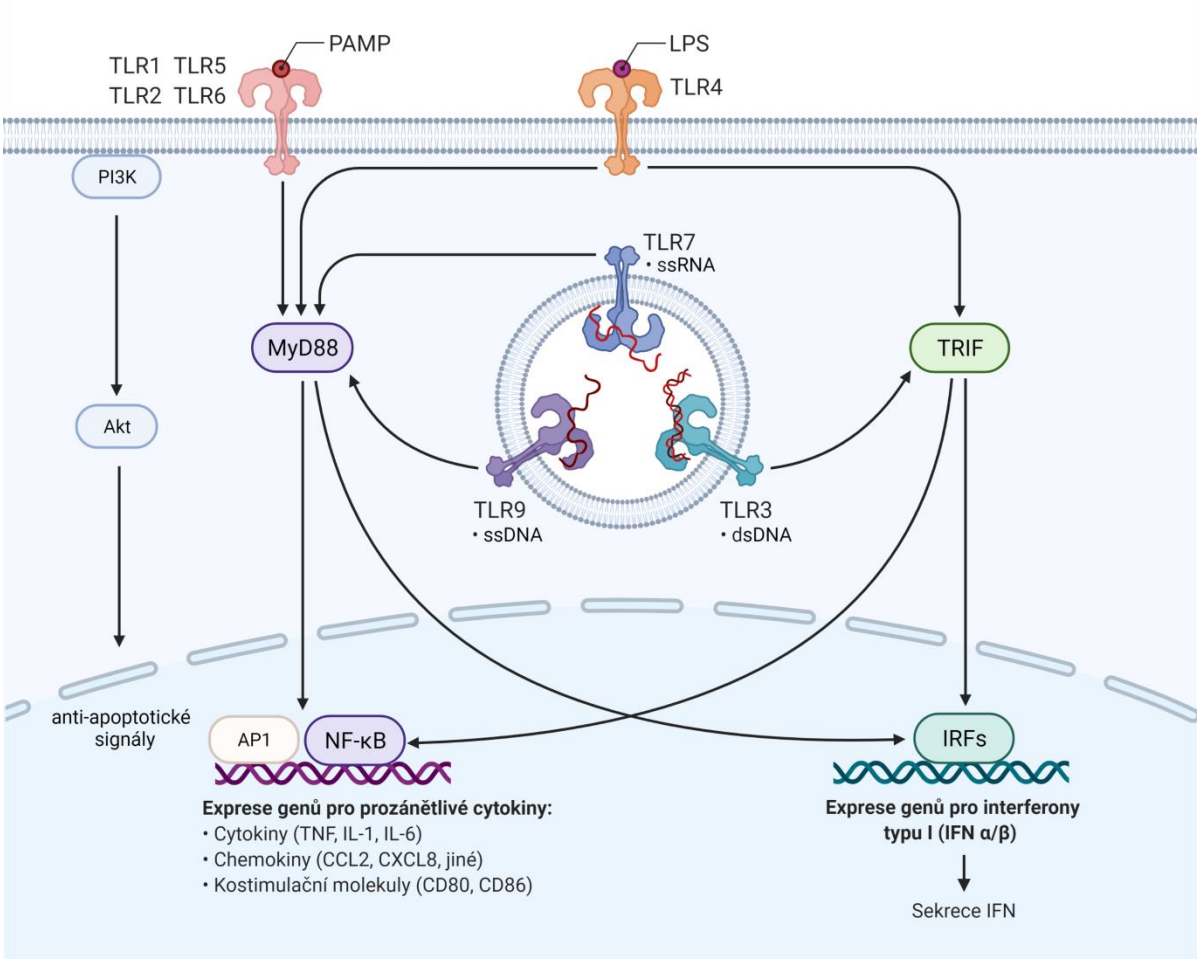
Existuje několik subtypů dendritických buněk, které se liší původem, lokalizací a funkcí (32). V *in vitro* studiích se používají dendritické buňky izolované ze sleziny (CD11c pozitivní buňky) nebo derivované z buněk kostní dřeně pomocí různých faktorů. Slezinné dendritické buňky jsou heterogenní populací buněk nesoucí znak $CD8^+$, $CD4^+$, nebo specifické znaky pro plasmacytoidní dendritické buňky (pDC) ($B220^+/CD11b^-/CD11c^+$). Derivací buněk kostní dřeně s růstovým faktorem GM-CSF vznikají myeloidní dendritické buňky (mDC) a z monocytů derivované makrofágy nesoucí kromě znaku CD11c i znak CD11b (33). Derivací buněk kostní dřeně s Flt-3L vznikají plazmacytoidní DC (pDC), mDC ($CD11b^+/CD4^+$) a lymfoidní DC ($CD103^+/CD8\alpha^+$) (34). V kůži se nachází rezidentní Langerhansovy buňky, dermální dendritické buňky a DC derivované z monocytů, tzv. monocyte-derived DC (tyto buňky vznikají z monocytů, které migrují do kůže v závislosti na podnětu). Liší se mezi sebou povrchovými znaky, transkripčními programy a funkcí (34).

DC rozpoznávají nebezpečí v podobě patogenních motívu PAMPs (pathogen associated molecular pattern) nebo DAMPs (danger associated molecular pattern) pomocí speciálních receptorů nazývaných pattern recognition recepotors (PRR). Tyto receptory se dělí na Toll-like receptory (TLR), Nod-like receptory (NLR), RIG-like receptory (RLR) a C-type lektin receptory (CLR). Aktivace těchto receptorů spouští kaskádu signalizačních reakcí vedoucích k aktivaci transkripčních faktorů zodpovědných za indukci interferonu a prozánětlivých genů (35).

TOLL-LIKE RECEPTORY A JEJICH SIGNALIZACE

Skupina Toll-like receptorů sestává z 10 členů u lidí a 13 členů u myší. Receptory se navzájem liší substrátovou specificitou a buněčnou lokalizací. TLR1, 2, 4, 5, 6 a 10 jsou lokalizované v cytoplazmatické membráně, zatímco TLR3, 7, 8, 9, 11, 12 a 13 jsou ukotveny v membráně endosomu. Obecně, povrchové TLR rozeznávají komponenty mikrobiálních

membrán, jako jsou lipopolysacharid (LPS), peptidoglykan, flagelin, lipoprotein a jiné. Endosomální TLR rozpoznávají nukleové kyseliny virů a nukleové kyseliny derivované z intracelulárních baktérií (35). Ligací TLR se iniciuje kaskáda signalizačních reakcí, které začínají rekrutací adaptorů obsahujících TIR (Toll/interleukin-1 receptor) doménu. I když existuje 5 adaptorů (MyD88, TRIF, TIRAP, TRAM a SARM), většina TLR signalizace je MyD88 nebo TRIF závislá. Následuje kaskáda fosforylačních reakcí končící aktivací nukleárního faktoru kappa B (NF-κB) a fosforylací serin-treoninových kináz rodiny mitogen-aktivovaných protein kináz (MAPK). Do rodiny MAPK kináz patří extracelulárně regulovaná kináza (Erk), p38 kináza a JNK kináza (taky označována SAPK-stresem aktivována kináza). MAPK nejsou transkripční faktory, ale regulují aktivitu mnoha transkripčních faktorů (AP1) a cytoplazmatických proteinu. TRIF závislá signalizace přes aktivaci interferon regulujících faktorů (IRF) iniciuje expresi genů pro interferon typu I. Výsledkem TLR signalizace je tedy indukce prozánětlivých a protizánětlivých cytokinů, interferonů, vývoj adaptivní imunity a kontrola apoptózy. Zjednodušené schéma na obr. 2 ilustruje signalizační dráhy aktivované jednotlivými TLR receptory.



Obr. 2. Zjednodušené schéma TLR signalizace. Receptory ukotvené v cytoplazmatické a endosomální membráně po rozpoznání specifických ligandů (PAMP, pathogen-associated molecular pattern) rekrutují adaptory MyD88 a TRIF. Následuje kaskáda reakcí, která vede k aktivaci transkripčních faktorů NF-κB, AP1 a IRFs. Výsledkem je exprese genů pro cytokiny, chemokiny, kostimulační molekuly a interferony typu I. Ligací některých receptorů dochází k aktivaci PI3K/Akt dráhy, která iniciuje expresi genů s anti-apoptotickými účinky. Lipopolysacharid (LPS) je PAMP, který se váže na receptor TLR4.

NOD-LIKE RECEPTORY A JEJICH SIGNALIZACE

Nod-like receptory jsou cytosolické receptory rozeznávající mikrobiální komponenty jako jsou peptidoglykany bakterií a endogenní alarminy. Alarminy (neboli DAMPs) jsou molekuly/proteiny asociované s nebezpečím a produkované v reakci na buněčný stres. Vazba ligandů na NLR receptory vede k aktivaci NF-κB signalizační dráhy a umocňuje prozánětlivou reakci buňky. Některé receptory ze skupiny NLR, která celkem čítá 22 členů u lidí a 34 u myší, oligomerizují a vytváří tzv. inflamazóm. V inflamazómu dochází, kromě aktivace NF-κB signalizační dráhy, i k aktivaci kapasázy-1 a kaspázy-11, a k následnému štěpení prekurzorů IL-1β a IL-18 do jejích aktivní formy, čímž se prozánětlivá reakce ještě víc zesílí. Signalizace přes NLR ovlivňuje kromě imunitní odpovědi i vícero buněčných procesů včetně buněčné smrti (36).

RIG-LIKE RECEPTORY A JEJICH SIGNALIZACE

Virová infekce je, kromě endosomálních TLR receptorů, detekována cytosolovými RIG-like receptory, které představují další skupinu PRR. RIG-like receptory vážou virovou RNA a využívají MAVS adaptor (mitochondrial antiviral signaling) k spuštění kaskády signálních drah vedoucích k aktivaci transkripčních faktorů NF-κB a IRF3 a IRF7. Následně se indukuje exprese prozánětlivých cytokinů a interferonu typu I. IFN aktivuje IFN receptor na téže buňce a na okolních buňkách a aktivuje signální dráhu JAK/STAT (Janus-activated kinase/signal transducer and activator of transcription), která vede k indukci interferon stimulujících genů (ISG). Produkty těchto genů zprostředkovávají protivirovou ochranu v infikovaných i neinfikovaných buňkách (35).

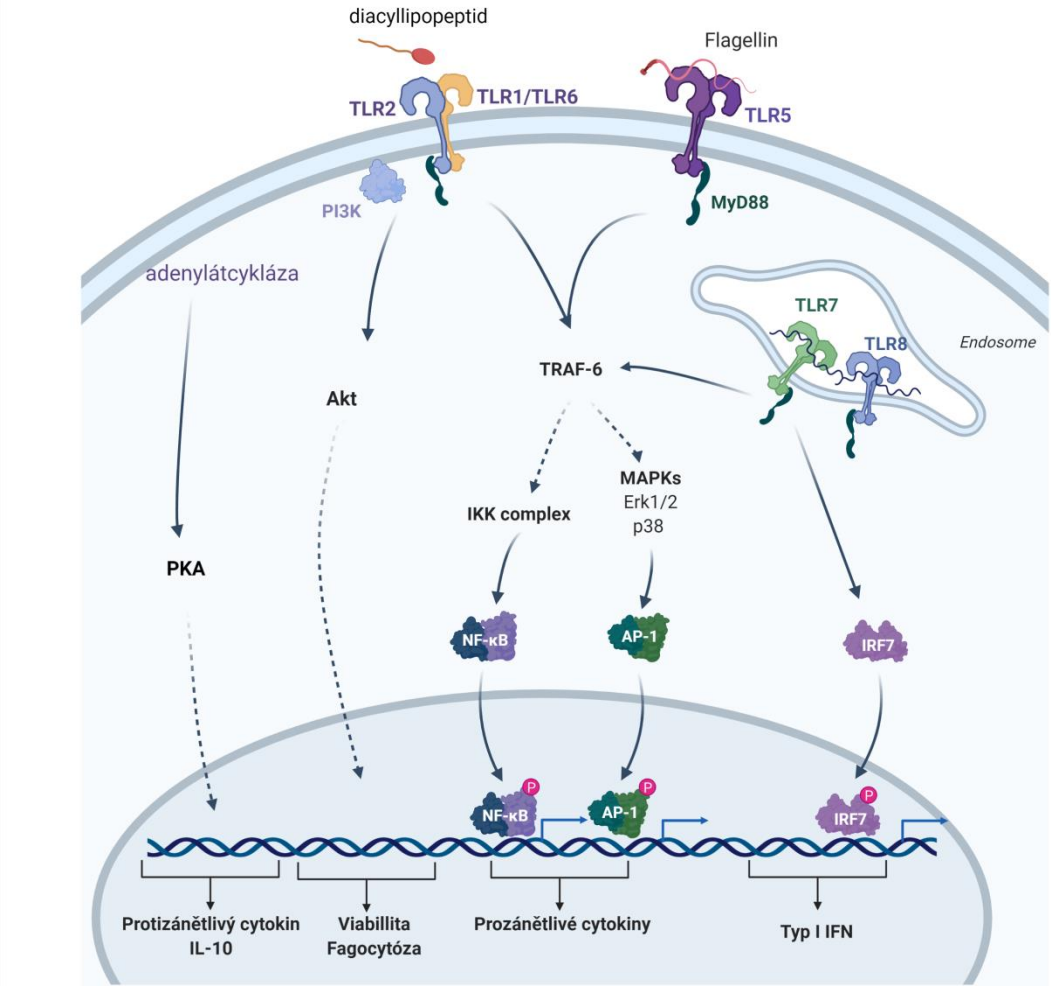
3. REAKCE HOSTITELE NA BORELIOVOU INFEKCI

Borelie, podobně jako jiné extracelulární bakterie, vyvolávají v hostiteli obranné reakce, kterých se účastní jak složky vrozené nespecifické imunity, tak složky získané imunity. Lipoproteiny borelií jsou rozpoznávány pomocí specifických receptorů na fagocytujících buňkách, jako jsou makrofágy, neutrofily a dendritické buňky, což vede k jejich aktivaci a následné produkci prozánětlivých cytokinů a chemokinů (37). Kromě receptorů na imunitních buňkách jsou borelie detekovány rozpustnými mediátory v podobě některých složek komplementu; vazba borelií na tyto složky vede k aktivaci komplementu alternativní cestou (38). Aktivace komplementu spolu s fagocytózou borelií buňkami vrozené imunity jsou klíčové mechanizmy, jakými hostitel eliminuje borelie. V rámci získané imunity se po boreliové infekci vyvíjí Th17 imunitní odpověď.

DENDRITICKÉ BUŇKY A MAKROFÁGY VERSUS BORELIOVÁ INFEKCE

Dendritické buňky a makrofágy jsou jedny z prvních buněk, které přicházejí do kontaktu s boreliemi. Boreliové lipoproteiny jsou rozeznávané receptory skupiny TLR, NLR a CLR (37). TLR receptory hrají v kontrole boreliové infekce klíčovou roli. Bylo zjištěno, že myši deficientní na společnou signální molekulu, adaptor MyD88, mají 250-krát vyšší počet spirochét než kontrolní ‚wild-type' myši (39, 40). Ze skupiny TLR, povrchové TLR2 a TLR5, a endosomální TLR7/8 a TLR9 jsou aktivovány boreliemi (Obr. 3). TLR2 je nejdůležitější pro indukci prozánětlivých cytokinů, zatím co endosomální receptory TLR7/8 a TLR9 zprostředkovávají indukci interferonu typu I (41-44). Produkce prozánětlivých i protizánětlivých cytokinů je závislá na fagosomální signalizaci, při které dochází ke kooperaci TLR2 a TLR8. Fagocytóza závislá na signalizační kaskádě PI3K/Akt (fosfatidylinositol-3 kinasa/Akt) je nezbytná pro plnou aktivaci fagocytujících buněk. Navíc infekce lidských monocytů boreliemi vede k indukci transkripce TLR2 a TLR8 (45). Zvýšená exprese TLR2 po stimulaci boreliemi byla pozorována i v dendritických buňkách (46). Ze skupiny NLR receptorů boreliové peptidoglykany rozeznává NOD2 receptor. Zdá se, že jeho úloha v kontrole boreliové infekce spočívá v indukci tolerance, protože při použití NOD2-deficientních myší byla artritida, indukovaná *B. burgdorferi* výraznější. V *in vitro* experimentech vedla absence NOD2 receptoru k nižší produkci prozánětlivých cytokinů (47).

Spirochéty *Borrelia* aktivují několik signálních drah ve fagocytujících buňkách. Jsou to dráhy, při kterých dochází k aktivaci NF-κB, kináz z rodiny MAPK jako jsou Erk1/2, p38 a JNK, PI3K a protein kinázy C (48-52). Pro produkci prozánětlivých cytokinů jsou důležité NF-κB a p38 MAPK, zatímco PI3K je zásadní pro fagocytózu (53). Je důležité zmínit, že borelie indukují protizánětlivý cytokin IL-10, který má supresivní efekt na indukci prozánětlivých cytokinů (54, 55). Indukce IL-10, která je zprostředkována i signalizační kaskádou cAMP/PKA, je jedním ze způsobů jakými borelie modulují imunitní systém (56, 57).

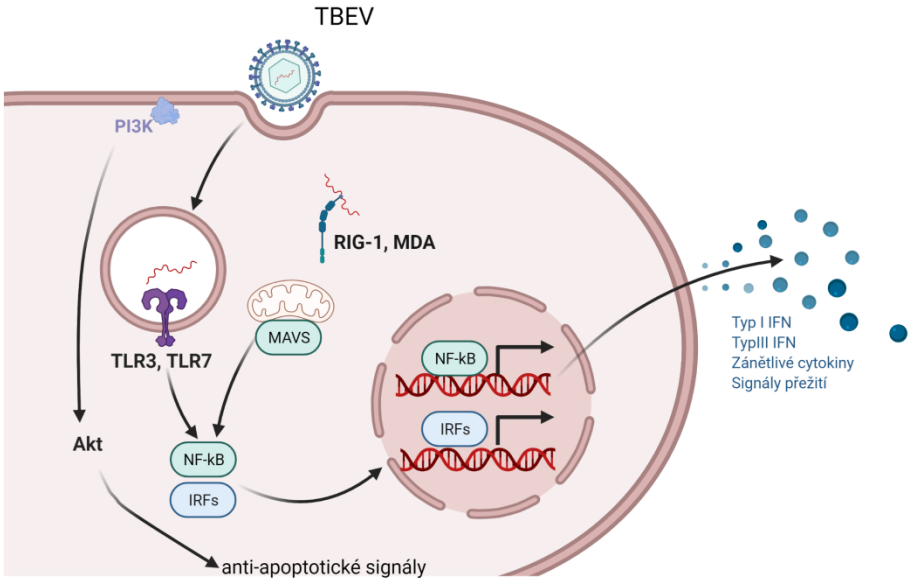


Obr. 3. Receptory skupiny TLR podílející se na rozpoznání boreliových antigenů. Receptory TLR, ukotvené v cytoplazmatické a endosomální membráně, využívají adaptor MyD88 a signalizují přes kinázu TRAF-6. Následně dochází k aktivaci transkripčních faktorů NF-κB a AP-1. Ligací endosomálních receptorů navíc dochází k aktivaci transkripčního faktoru IRF7. Boreliemi je aktivována i signalizační dráha PI3K/Akt a cAMP/PKA.

4. ROZPOZNÁNÍ FLAVIVIROVÉ INFEKCE DENDRITICKÝMI BUŇKAMI

Hostitelská buňka napadená flavivirem rozeznává virové struktury pomocí specifických receptorů. V rámci skupiny TLR jsou to endosomální TLR3 a TLR7, a cytosolické tzv. RIG-like receptory, konkrétně RIG-1 a MDA. Ligací zmíněných receptorů se aktivuje NR-κB, IRF a PI3K/Akt signalizační dráhy (Obr. 4). Exprese virus-specifických receptorů se liší mezi jednotlivými subtypy DC. pDC, z důvodu vyšší exprese endosomálních TLR7 a TLR9, reagují na virovou infekci robustní produkcí IFN typu I. I když tento typ buněk nemusí podporovat replikaci viru, virus buňky aktivuje a ty produkují IFN. V případě mDC, které vznikají derivací kostní dřeně s GM-CSF a jsou často modelovými DC, je virus TBE rozeznán pomocí cytosolických RIG-1 receptorů (58).

Existuje několik mechanizmů, jakými flaviviry unikají před hostitelským obranným aparátem. Interferon (IFN) je klíčovým faktorem v nastolení hostitelské antivirové obrany a je atakován virem na několika úrovních. Dvě nezávislé studie prokázaly, že produkce IFN je opožděná z důvodu maskování replikační RNA (59, 60). Důležitou roli v negaci interferonových účinků hraje nestrukturní protein NS5, který byl identifikován jako antagonista JAK/STAT signalizace (61). Kromě toho tento virový protein způsobuje snížení exprese receptorové podjednotky IFNAR1 na povrchu buněk (62). Jiný stupeň modulace byl odhalen na úrovni produkce IL-12, která je inhibována virem TBE v důsledku antagonistického účinku na IRF1 (63). Jedna z novějších studií odhalila, že virus TBE inhibuje buněčnou translaci čím interferuje s hostitelskou obranou (64).



Obr. 4. Rozpoznání flavivirové RNA receptory PRR. Virová RNA je po uvolnění z virové částice rozpoznána v endosomech receptory TLR3 a TLR7 nebo v cytosolu receptory RIG-1 a MDA. Následně dochází k aktivaci faktorů NF-κB a IRFs a spuštění odpovídajících transkripčních programů. Výsledkem je produkce interferonů a zánětlivých cytokinů. Iniciace anti-apoptotických signálů je zprostředkována aktivací PI3K/Akt signalizační kaskády.

5. VLIV KLÍŠTĚCÍCH SLIN NA HOSTITELSKÉ BUŇKY

Ve většině případů je pozitivní asistence slin přisuzována imunomodulační aktivitě látek obsažených ve slinách. Klíčové postavení DC v hostitelské imunitě je důvodem, proč byly právě tyto buňky intenzívně studovány i ve vztahu k infekci *B. burgdorferi* (29). Autoři používali k aktivaci buněk borelie, nebo relevantní TLR agonisty.

IMUNOMODULACE DENDRITICKÝCH BUNĚK KLÍŠTĚCÍMI SLINAMI

Funkce dendritických buněk jsou klíštěcími slinami ovlivněny téměř na všech úrovních. Cavassani *et al.* odhalili inhibiční efekt slin z *Rhipicephalus sanguineus* na diferenciaci, maturaci a funkci DC použitím agonisty TLR4 (65). Snížená produkce některých cytokinů byla pozorována v DC ovlivněných slinami z *I. scapularis*; tyto DC produkovali po stimulaci TLR2, TLR4 a TLR9 méně IL-12 a TNF a rovněž byla potlačena schopnost DC stimulovat antigen-specifickou $CD4^+$ proliferaci (66). Sliny z *R. sanguineus* negativně ovlivnily migraci DC k MIP-1α a MIP-1β a byla prokázána snížená exprese chemokinového receptoru CCR5 (67). Inhibiční vliv slin na migraci v důsledku snížené exprese chemokinových receptorů byl prokázán i u slin *A. cajennese* (CCR5 a CCR7) (68). Schopnost polarizovat Th odpověď k Th2 byla opakovaně prokázána v různých experimentálních modelech. Slezinné DC po interakci se slinami z klíšťete *I. ricinus* indukovaly Th2 diferenciaci z $CD4^+$ T lymfocytů *in vivo* i *in vitro* (69). Skalová a kol. studovali vliv slin z *I. ricinus* na schopnost slezinných DC aktivovaných agonisty TLR3, TLR7 a TLR9 aktivovat a polarizovat $CD4^+$ T buňky. Sliny výrazně snížily polarizaci Th1 a Th17. Snížená Th1 polarizace a silně indukovaný vývoj Th2 polarizované odpovědi byl potvrzen i *in vivo* experimentem, kde byl navíc prokázán výrazný inhibiční efekt na maturaci a migraci DC (70). Myši deficientní na Langerhansovy buňky indukovaly Th1 odpověď po infestaci *I. scapularis*, co potvrdilo důležitost Langerhansových buněk v inhibici klíšťaty- zprostředkované Th1 reakce (71).

V předložených pracích byly zjišťovány změny v signalizaci pomocí detekce aktivovaných forem jednotlivých signalizačních molekul. V případě použití patogenního organizmu se aktivuje několik receptorů najednou. A protože tyto receptory částečně sdílí signalizační dráhy, je obtížné odhalit, kde k interferenci slin se signalizací dochází. Teoreticky mohou sliny ovlivňovat rozpoznání patogenu příslušnými receptory nebo některé složky ‚upstream' signalizace. Použití specifických ligandů PRR umožňuje zjistit, která signalizační dráha je slinami ovlivněna.

Vliv klíštěcích slin na signalizační dráhy aktivované ligandem TLR2 a spirochétami *B. afzelii* v dendritických buňkách (1. předložená práce)

Lieskovská J., Kopecký J. (2012): Effect of tick saliva on signalling pathways activated by TLR-2 ligand and *Borrelia afzelii* in dendritic cells. Parasite Immunology 34, 421-429.

Prací popisujících vliv klíštěcích slin na funkci dendritických buněk infikovaných přímo boreliemi nebo TBEV není mnoho. Zkoumání interakce spirochét *B. afzelii* a myších dendritických buněk odhalilo negativní efekt slin *I.ricinus* na počet fagocytujících buněk, na produkci všech testovaných cytokínů a na schopnost indukovat proliferaci CD4 T lymfocytů (72). V předložené práci jsme navázali na tyto studie a analyzovali signální dráhy po stimulaci ligandem TLR2 - lipoteichoovou kyselinou (LTA) nebo spirochétami *B. afzelii* v myších slezinných DC (73). TLR2-závislá aktivace NF-κB, PI3K/Akt a Erk1/2 MAPK signálních drah byla negativně ovlivněna klíštěcími slinami a podobně byl inhibiční vliv pozorován i v buňkách stimulovaných boreliemi. Aktivace p38 MAPK indukovaná LTA nebo boreliemi zůstala beze změn. Použitím inhibitorů PI3K a Erk1/2 bylo prokázáno, že Erk1/2 kináza se podílí na indukci TNF, ale nemá vliv na produkci IL-10 v LTA stimulovaných buňkách. V buňkách, které byly aktivované boreliemi, inhibice Erk1/2, NF-κB a PI3K negativně ovlivnila jak produkci TNF, tak produkci IL-10. Z této analýzy vyplývá, že sliny ovlivňováním výše zmíněných signálních drah mohou přispívat ke snížené produkci jak prozánětlivých (TNF), tak protizánětlivých cytokinů (IL-10). Použitím specifického inhibitoru Protein kinázy A (PKA) H89 bylo zjištěno, že cAMP-PKA signalizační dráha je zodpovědná za slinami vyvolané zvýšení IL-10 v boreliemi aktivovaných DC. Data z této práce ukazují, že **klíštěcí sliny inhibují TLR2 závislou signalizaci a zvyšují produkci protizánětlivého cytokinů IL-10 interferencí s PKA signalizací, čímž modulují funkci DC na úrovni produkce cytokinů.**

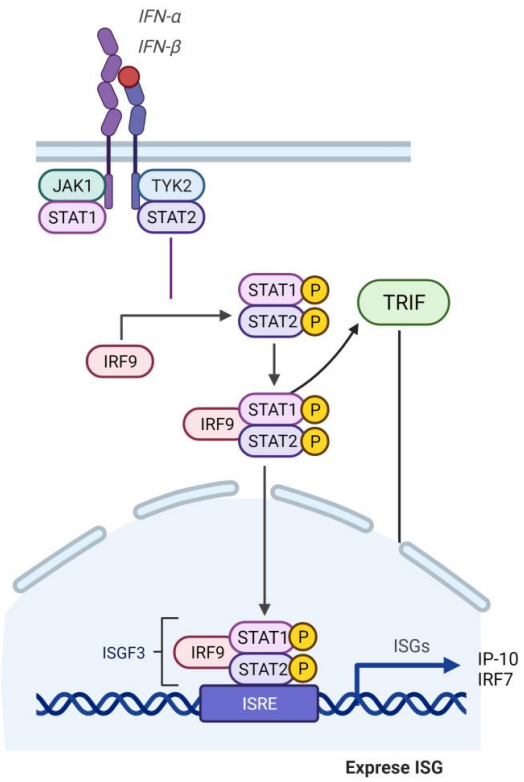
Modulace cAMP-PKA dráhy klíštěcími slinami z *R. sanguineus* byla prokázána Olivierou a kol. Autoři uvádí, že většina imunomodulačních efektů slin závisí na cAMP/PKA dráze, která je aktivována nukleosidem adenosinem a prostaglandinem (74). Autoři identifikovali v klíštěcích slinách adenosin v koncentraci přibližně 110 nmol/ml a prostaglandin E2 v koncentraci 35.2 ng/ml. Obě tyto nízkomolekulární složky inhibovaly produkci cytokinů prostřednictvím indukce cAMP-PKA dráhy. Přítomnost adenosinu a prostaglandinu byla zjišťována i v naší laboratoři ve slinách *I. ricinus;* adenosin nebyl nalezen a prostaglandin byl detekován v koncentraci 7 ng/ml (75). Koncentrace PGE2 naměřená ve slinách *I. ricinus* je nejnižší ze všech dosud testovaných klíštěcích slin. Co ve slinách *I. ricinus* aktivuje cAMP/PKA dráhu v DC aktivovaných boreliemi zůstává neobjasněno. Můžeme pouze spekulovat, že k aktivaci této dráhy dochází přes upregulaci neidentifikovaného povrchového receptoru.

Interference s Erk1/2 signalizací byla pozorována u klíštěcích slin z *R. sanguineus* v DC po stimulaci LPS (46) a ve fibroblastech stimulovaných PDGF v přítomnosti slin z *Dermacentor variabilis* (76). Na rozdíl od našich zjištění, sliny *R. sanguineus* působily inhibičně i na aktivaci p38 MAPK v LPS stimulovaných DC (46). Autoři naznačili, že klíštěcí sliny mohou indukovat regulační DC. Další signální molekula, která byla testována je IRAK (interleukin -1 asociovaná kináza). Bylo zjištěno, že sliny klíštěte *Dermacentor variabilis* upregulují IRAK po aktivaci zymozanem v makrofágové buněčné linii IC-21 (77).

Klíštěcí sliny potlačují interferonovou signalizaci v dendritických buňkách po infekci *B. afzelii* (2. předložená práce):

Lieskovská J., Kopecký J. (2012): Tick saliva suppress IFN signalling in dendritic cells upon *Borrelia afzelii* infection. Parasite Immunology 34, 32-39.

Interferon je znám především jako cytokin se silně antivirovým účinkem. Kromě toho je interferon významným regulátorem ovlivňujícím mnoho aspektů hostitelské imunitní a zánětlivé reakce. Interferonová signalizace a STAT1 aktivace jsou důležité např. pro diferenciaci a fenotypovou maturaci DC (78, 79),(80). Existují tři typy IFN, typ I je reprezentován IFNα a IFNβ, IFNγ je jediným zástupcem typu IFN II. Typem IFN III jsou IFNλ1, IFNλ2 a IFNλ3. Vazba IFN typu I (IFNβ) na IFN receptor iniciuje fosforylaci STAT1 a STAT2, které po vytvoření heterodiméru a asociaci s IRF-9 tvoří transkripční faktor ISGF3 (IFN-stimulovaný transkripční faktor). Ten nasedá na příslušný promotor (ISRE) a spouští transkripci interferonem stimulovaných genů (ISG) (Obr. 5) (81).
V rámci pochopení mechanizmu působení klíštěcích slin na funkci DC byl testován jejich účinek na interferonovou signalizaci ve slezinných dendritických buňkách měřením aktivace signální molekuly STAT1 (82). Bylo zjištěno, že STAT1 aktivace byla vlivem slin snížená po stimulaci rekombinantním IFNβ, i po stimulaci lipopolysacharidem (LPS) a boreliemi. Aktivace STAT1 je v boreliemi stimulovaných buňkách zprostředkována endogenním IFN, jehož produkci sliny neovlivnily. Pokles STAT1 fosforylace v boreliemi stimulovaných buňkách tudíž reflektuje negativní vliv slin na signalizaci aktivovanou IFN. IFN signalizace je esenciální i pro produkci IL-12; produkce IL-12 byla výrazně snížená v STAT1 (-/-) a IRFAR (-/-) myších (83). V souladu s těmito poznatky, klíštěcí sliny snižují produkci IL-12 v boreliemi a LPS stimulovaných DC. **Interference klíštěcích slin s IFN závislou signalizací může být jedním z mechanismů, které přispívají ke snížené maturaci DC a polarizaci imunitní odpovědi.**
Testováním extraktu ze slinných žláz (SGE) z klíštěte *Dermacentor reticulatus* na antivirový účinek IFN se zbývala práce Hajnické *et al* (84). Autoři zjistili, že antivirový účinek IFN na replikaci viru vezikulární stomatitidy (VSV) v myších fibroblastech byl výrazně potlačen SGE. Důležitost STAT-1 pro indukci ISG byla prokázána i v makrofázích stimulovaných *B. burgdorferi* (85).



Obr. 5. Interferonová signalizace. INFα a IFNβ vazbou na příslušný receptor iniciují tzv. JAK/STAT dráhu. STAT1 a STAT2 po fosforylaci dimerizují a vážou IRF9. Vytvořený komplex, označovaný jako ISGF3, spouští expresi interferonem stimulovaných genů (ISGs). Příkladem takových genů jsou IP-10 a IRF7.

Sliny z klíštěte *I. ricinus* zvyšují replikaci viru TBE v dendritických buňkách modulací Akt dráhy (3. předložená práce)

Lieskovská J., Páleníková J., Langhansová H., Chmelař J., Kopecký J. (2018): Saliva of *Ixodes ricinus* enhances TBE virus replication in dendritic cells by modulation of pro-survival Akt pathway. Virology 514: 98-105.

Tato studie byla zaměřena na testování vlivu klíštěcích slin na DC infikované virem klíšťové encefalitidy. Důraz byl kladen na analýzu signálních drah se stejným cílem jako v případě borelií, tj. poodhalit molekulární mechanismus, který je za účinkem slin a porozumět, jak sliny podporují přenos TBEV na hostitele. V případě bakteriální infekce je imunomodulace DC vnímána jako klíčový faktor zodpovědný za SAT. V případě virové infekce jsou infikované DC navíc zdrojem virových částic, protože podporují replikaci viru a migrací z kůže do lymfatických uzlin se podílí na přenosu viru v hostiteli.
V práci byly použity myší myeloidní DC derivované z kostní dřeně a kmen Hypr viru TBE. Bylo zjištěno, že klíštěcí sliny pozitivně ovlivňují množství viru v dendritických buňkách. Analýza vybraných signálních drah odhalila, že aktivace Akt a částečně i NF-κB a STAT1 je zvýšená v přítomnosti slin. Akt signalizace je známa pro své anti-apoptotické a proliferaci podporující účinky. Procento apoptotických buněk bylo dle očekávání po TBEV infekci slinami sníženo a životnost buněk byla slinami zvýšena. Použitím specifického inhibitoru kinázy PI3K, která fosforyluje Akt bylo demonstrováno, že PI3K/Akt signalizační dráha pozitivně ovlivňuje titr viru v DC a snižuje počet apoptotických buněk. Z výsledků vyplývá, že **zvýšením aktivity anti-apoptotické kinázy Akt sliny pozitivně ovlivňují replikaci viru** v mDC (86).
Vliv slin na aktivaci Akt ve virem infikovaných DC je jiný v porovnání s našimi předešlými výsledky, kdy byly dendritické buňky stimulovány TLR-2 ligandem nebo boreliemi a sliny snižovaly fosforylaci Akt (1. předložená práce). Důvodem může být rozdílná kinetika analýzy signálních drah ve virem infikovaných buňkách v porovnání s předchozími studiemi. Ve virem infikovaných buňkách byla aktivita signálních molekul analyzována po 3, 22 a 45 hodinách na rozdíl od TLR-2 stimulovaných DC, kdy trval nejdelší časový interval po stimulaci TLR-2 ligandem 1 hodinu. K aktivitě Akt v pozdějších intervalech můžou přispívat nově vznikající virionové částice, a tedy za zvýšenou aktivitu signálních drah v přítomnosti slin může i zvýšené množství replikujícího se viru. Skutečná příčina Akt upregulace není zatím objasněna, ale je pravděpodobné, že zvýšená aktivita této kinázy napomáhá k tomu, že buňky infikované virem jsou rezistentnější k apoptóze.
Touto prací jsme navázali na již dříve publikované studie, které vyšly z naší laboratoře (70, 87). Ty odhalily na modelu slezinných DC negativní vliv slin na jejich maturaci a na produkci TNF, IFNβ a IL-6. Přítomnost slin ve virem infikovaných slezinných DC neovlivnila množství produkovaného viru, na rozdíl od mDC, ale vedla k zvýšení procenta infikovaných buněk a snížení virem indukované apoptózy. Absenci vlivu slin na replikaci viru byla demonstrována i v práci zabývající se vlivem klíštěcích slin na dendritické buňky infikované virem krymzsko-kongské hemoragické horečky. Autoři zjistili, že klíštěcí sliny imunomodulují DC (kožní DC a Langerhansovy DC) a inhibují jejích migraci z místa sání (88).

Modulace hostitelské imunity klíštěcími slinami (4. předložená práce)

Kotál J., Langhansová H., Lieskovská J., Andersen J.F., Francischetti I.M., Chavakis T., Kopecký J., Pedra J.H., Kotsyfakis M., Chmelař J. (2015): Modulation of host immunity by tick saliva. J Proteomics. 2015 Jul 17;128:58-68.

V předloženém review jsou shrnuty účinky klíštěcích slin na funkce různých imunitních buněk, jak vrozené, tak i získané imunity. Prvotní imunitní reakce hostitele na sání klíštěte zahrnuje aktivaci imunitních buněk, které jsou součástí tzv. vrozené imunity. Jsou zastoupeny, kromě již zmiňovaných dendritických buněk, také makrofágy, monocyty, žírnými buňkami, granulocytárními buňkami jako jsou neutrofily, bazofily, eozinofily a NK buňkami. Makrofágy a neutrofily jsou profesionální fagocyty, které hrají důležitou roli v eliminaci patogenů a podílí se na rozvoji zánětu. Funkce těchto imunitních buněk je v mnoha směrech negativně ovlivněna klíštěcími slinami. Sliny z různých druhů klíšťat **inhibují produkci prozánětlivých cytokinů** IL-1α, IL-β, IL-6, IL-8, TNF, IFN-γ a **zvyšují produkci protizánětlivých Th2 cytokinů** IL-4 a IL-10 v aktivovaných makrofázích. Schopnost fagocytózy makrofágů i neutrofilů je slinami inhibována. U neutrofilů dochází ke snížené adhezi, rekrutaci, degranulaci a produkci reaktivních kyslíkových radikálů (ROS). Ovlivnění tzv. NETs (neutrophil extracellular traps, extrusion of neutrophil DNA) klíštěcími slinami pozorováno nebylo.
Buňky adaptivní imunity, B a T lymfocyty, jsou slinami rovněž ovlivňovány. Byla pozorována snížená proliferace B a T lymfocytů a snížená produkce cytokinů IL-2, IL-12 a IFN-γ. Naopak množství Th2 cytokinů (IL-4, IL-5, IL-6) je v přítomnosti slin zvýšeno. Opakovaně byla pozorována **polarizace imunitní odpovědi** k Th2 cytokinům vlivem slin z různých druhů klíšťat.

Příloha 1

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2. **Lieskovská J.**, Kopecký J. (2012): Tick saliva suppress IFN signalling in dendritic cells upon *Borrelia afzelii* infection. Parasite Immunology 34, 32-39.

3. **Lieskovská J.**, Páleníková J., Langhansová H., Chmelař J., Kopecký J. (2018): Saliva of *Ixodes ricinus* enhances TBE virus replication in dendritic cells by modulation of pro-survival Akt pathway. Virology 514: 98-105.

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Effect of tick saliva on signalling pathways activated by TLR-2 ligand and *Borrelia afzelii* in dendritic cells

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SUMMARY

Dendritic cells are a sentinel in defending against pathogens and tick saliva facilitates transmission of tick-borne pathogens by modulating the host immune response. The maturation of dendritic cells is inhibited by tick saliva. To elucidate the mechanism of this inhibition, we tested the impact of Ixodes ricinus *tick saliva on signalling pathways activated by Toll-like receptor (TLR-2) ligand and* Borrelia afzelii *in spleen dendritic cells. The activation of nuclear factor-κB (NF-κB) p65 and phosphatidylinositol-3 kinase (PI3K)/Akt pathways was decreased by tick saliva upon both TLR-2 and* Borrelia *stimulation. Among the mitogen-activated protein kinases (MAPK), the activation of extracellular matrix-regulated kinase (Erk1/2) was suppressed by tick saliva, but not p38. In response to spirochaetes, the amount of TNF-α decreased in the presence of tick saliva which was mediated by selective suppression of Erk1/2, NF-κB and Akt as tick saliva mimicked the effect of their specific inhibitors, UO126, IKK-IV and LY294002, respectively. Saliva-induced enhancement of IL-10 was not observed in the presence of specific inhibitor of Protein Kinase A (PKA), H-89, suggesting the involvement of PKA pathway in IL-10 production. Our cumulative data show that tick saliva interferes with several signalling pathways, thus modulating the immune functions of dendritic cells.*

Keywords Borrelia*, dendritic cells, tick saliva, Toll-like receptor signalling*

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INTRODUCTION

A number of immunomodulatory activities of tick saliva or salivary gland extract (SGE) have been reported, for example, the ability to inhibit the complement cascade (1), to prevent phagocytosis and production of superoxide by neutrophils (2), to inhibit the production of nitric oxide by macrophages (3), to impair NK cell function (4), and to reduce the antiviral effect of interferon (5). Polarization of the host immune response towards the Th2 cytokine profile has been repeatedly reported for saliva from various tick species (6). Finally, the principal role in this process was attributed to dendritic cells; the inhibitory effect of *Ixodes ricinus* tick saliva on their migration, maturation and antigen-presenting capacity was documented in addition to promoting the development of Th2-polarized immune response (7). Thus, tick saliva is able to enhance pathogen transmission by suppression of the host immune response (8).

At the frontline of the immune response, dendritic cells detect various pathogens by several conserved pattern recognition receptors including the Toll-like receptor (TLR) family. As a result of pathogen recognition, dendritic cells start producing immuno-regulatory cytokines affecting innate and adaptive immune responses and begin to mature, resulting in phenotypical and functional changes (9). Several types of TLR are recognized to date; TLR1, 2, 4, 5 are localized on the cell surface, while TLR7, 8, 9 are restricted to the endosomal membrane (10). To induce immunosuppression and Th2 prone immune response, parasites exploit TLR-2-mediated signalling resulting in release of IL-10 (11,12). Ligation of TLR-2 by lipoteichoic acid (LTA) leads to activation of several signalling pathways (13), including NF-κB, MAPKs (Erk1/2, p38), and PI3K/Akt-mediated cascades. These pathways positively or negatively influence cytokine production and maturation of dendritic cells. The NF-κB is crucial for the induction of pro-inflammatory cytokines, but also positively regulates the ability of dendritic cells to prime T cells to T helper type 2 (Th2) phenotype (14,15). Several functionally

independent, parallel MAP kinase signal transduction pathways are defined in mammalian cells. For example, p38 is critical for maturation of human derived dendritic cells in response to LPS (16) and induction of inflammatory cytokines (17). The activation of Erk1/2 MAP kinase was reported to be prone to Th2 biased response (18). Another pathway activated by various TLR ligands is mediated by the phosphatidylinositol 3-kinase (PI3K). PI3K cascade is involved in a variety of biological processes, including cell survival and proliferation (19). Activation of PI3K pathway poses a negative feedback mechanism regulating IL-12 production during DC activation and helps prevent the excessive Th1 polarization (20). In addition, the critical role of PI3K in TLR-dependent production of type I IFN in plasmacytoid dendritic cells has been shown (21). Overall, the pathways mentioned above play different roles in polarizing the phenotype of dendritic cells (15).

The molecular mechanism underlying how tick saliva affects immune cells is being investigated. The increased expression of TLR-2 receptor and inhibition of Erk 1/2 and p38 MAPK kinases activation were associated with impaired maturation of dendritic cells stimulated with LPS when saliva from the hard tick *Rhipicephalus sanguineus* was used (22). In addition, it was revealed that low molecular weight salivary constituents from *R. sanguineus* saliva (i.e. adenosine and prostaglandin-E_2) are responsible for most immunomodulatory effects in dendritic cells upon stimulation with Toll-like receptor agonists (23). We recently showed that in response to *Borrelia* spirochaetes and LPS, saliva from the hard tick, *I. ricinus,* inhibits STAT-1, the signalling molecule activated indirectly by interferon produced by dendritic cells. The attenuation of IFN signalling partly accounts for tick saliva immunomodulatory effects (24).

Tick saliva facilitates transmission of Lyme disease agent, *Borrelia burgdorferri* sensu lato to the host; the phenomenon termed 'saliva activated transmission' (25). *Borrelia* spirochaetes are recognized by several innate receptors, including TLR, Nod-like receptors and C-type lectin receptors (26). The binding of spirochaetal lipoproteins to TLR1/2 is critical for inducing pro-inflammatory cytokines, while activating endosomal Toll-like receptors (TLR7, 8, 9) is important for type I IFN induction (27,28). Induction of pro-inflammatory cytokines by *B. burgdorferi* is mediated by NF-κB and p38 MAP transduction pathways (29,30). Although tick saliva effects on dendritic cells stimulated with various TLR ligands or *Borrelia* spirochaetes have been described (7,31), little is known about the cell signalling pathways involved. This study was undertaken to elucidate the molecular mechanism which is behind the inhibitory effects of tick saliva.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (10 weeks of age) were obtained from Charles River Laboratories (Sulzfeld, Germany). Guinea pigs used for *I. ricinus* tick feeding were bred and maintained at the Institute of Parasitology, České Budějovice (Czech Republic). All experiments were performed with permission from local animal ethics committee.

Tick saliva collection

Pathogen-free adult *I. ricinus* ticks from the colony maintained at the Institute of Parasitology in České Budějovice (Czech Republic) were allowed to feed in groups of 20 mating pairs on guinea pigs. After 6 days, partially engorged female ticks were removed, immobilized, and a 10-μL glass capillary tube was fitted over their mouthparts. To induce salivation, 2 μL of 0·1 M pilocarpine solution in ethanol was applied on the dorsum of each tick. After 60 min, tick saliva was collected, pooled, and stored at −70°C. The concentration of pilocarpine in tick saliva was estimated at 4 mM by HPLC/mass spectrometry method. As a control, corresponding amounts of pilocarpine were added to samples not treated with tick saliva. All salivary samples were filtered through a 0·22-μm filter (Millipore, Billerica, MA, USA), and tick saliva protein concentration was determined using a Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) before use.

Bacteria

The CB-43 strain of *Borrelia afzelii* isolated from *I. ricinus* (32) was grown in Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma-Aldrich) supplemented with 6% rabbit serum at 34°C. The fourth passage was used in the experiments.

Dendritic cells isolation

Isolated mouse spleens were minced with scissors, digested in RPMI containing 1 mg/mL collagenase-D (Roche, Mannheim, Germany) at 37°C for 1 h, and passed through a 70-μm nylon cell strainer (BD Biosciences, Durham, NC, USA). Dendritic cells were isolated using magnetic beads conjugated with anti-CD11c (N418) Ab and MACS Column separation following the manufacturer's instructions (Miltenyi Biotec, Bergish Gladbach, Germany). Purified dendritic cells were cultured in RPMI supplemented with 10% heat-inactivated foetal calf serum (FCS), 50 μM 2-ME, 100 μg/mL penicillin, and 100 U/mL

streptomycin (all from Sigma-Aldrich). Purity of isolated dendritic cells (~90% CD11c+ cells) was determined by subsequent FACS analysis.

Cytokine measurements

Freshly isolated dendritic cells were cultured in 96-well plate at a concentration of 2×10^5 cells per well for 24 h. Following 2 h incubation with tick saliva, the cells were stimulated with 2·5 μg/mL of LTA (InvivoGen, San Diego, CA, USA) or *B. afzelii* in ratio 10 : 1 (10 spirochaetes to 1 cell), and cell-free culture supernatants were harvested after 24 h for TNF-α and 48 h for IL-10 determination. Both cytokines were determined using Ready-Set-Go! ELISA Set (eBioscience) following the manufacturer's instructions. The pilocarpine was added to the control sample at a concentration corresponding to that in tick saliva. In experiments when IKK-IV (Merck KGaA, Darmstadt, Germany), UO126 (Cell Signalling, Beverly, MA, USA), LY294002 (Sigma), and H-89 (Sigma-Aldrich) were used, cells were incubated with inhibitor for 30 min prior to the addition of *Borrelia* spirochaetes or LTA.

Immunoblotting

Freshly isolated dendritic cells were seeded at 1×10^6 cells per well in 24-well plate. On the following day, dendritic cells were incubated for 2 h with 20 μg of tick saliva per millilitre or corresponding amount of pilocarpine prior to the addition of LTA (2·5 mg/mL) or using live *B. afzelii* spirochaetes in ratio 10 spirochaetes per 1 cell. Following stimulation, the cells were lysed in a modified RIPA buffer [1% Nonidet P-40, 0·25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl (pH 7·5)] in the presence of protease inhibitors (10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin) and phosphatase inhibitors (25 mM sodium fluoride and 2 mM sodium orthovanadate). The cell lysates were centrifuged at 14 000 g for 10 min at 4°C, supernatants were mixed with 4× Laemmli sample buffer and then separated by SDS–PAGE using an 8% gel. The proteins were then transferred to Immobilon-P membranes, which were blocked for 1 h in Tris/saline buffer containing 0·1% Tween 20 and 5% nonfat milk. The blots were incubated overnight at 4°C with the anti-phospho-Erk1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-Akt (Ser473), and phospho-NF-κB p65 (Ser536) (all from Cell Signalling) or with anti-GAPDH (Santa Cruz Biotechnology), and anti-Erk1/2 antibody (Enzo Life Science) at dilution 1 : 1000. The proteins were visualized using enhanced chemiluminiscence (Pierce), and their abundance was analysed using the FUJI FILM Luminiscent Image Analyse LAS-3000. Densitometric analyses of the images were performed using Image J software, version 1.61 (http://rsb.info.nih.gov/nih-image/).

Statistical analysis

Student's *t* test was used to compare the differences between control and treated groups. A $P \leq 0{\cdot}05$ was considered statistically significant.

RESULTS

Tick saliva interferes with the activation of several signalling pathways in dendritic cells upon TLR-2 ligation

To determine how tick saliva interferes with signalling cascades triggered through TLR-2 receptor, we analysed pathways mediated by NF-κB, MAPK and PI3K/Akt – effectors implicated in controlling maturation of dendritic cells (15,33).

The effect of tick saliva on the MAPK activation was analysed by determining phosphorylation of the serine/threonine kinases, p38 and Erk1/2. Dendritic cells were cultured in the presence or absence of 20 μg tick saliva for 2 h followed by stimulation with LTA. This concentration of tick saliva (20 μg/mL) was chosen because it had the most pronounced effect on cellular activation (data not shown). As expected, the phosphorylation of both MAP kinases increased upon stimulation with LTA. The Erk1/2 activation was decreased in the presence of tick saliva compared with control samples at all time points monitored (at the time of maximum activation (30 min) the suppression by saliva reached 72·5%) (Figure 1a). Upon LTA stimulation, however, tick saliva did not affect the activation level of p38 (decrease at 30 min was only 17%) (Figure 1a).

The activation of NF-κB signalling pathway was examined by measuring phosphorylation of a member of NF-κB transcription factors family RelA (p65) containing transactivation domain in its C terminus. Dendritic cells were pre-incubated with tick saliva for 2 h prior to adding LTA. LTA-induced activation of Rel p65 at all time points tested and tick saliva attenuated activation of this signalling molecule by 80% at 30 min when NF-κB activation reached a peak (Figure 1b). The difference between NF-κB activation in the presence and absence of saliva was not detected after 60 min of adding LTA to the cells.

We then examined the PI3K/Akt signalling axis, a transduction pathway believed to inhibit signals that induce inflammatory cytokines (20). We assessed activation of PI3K/Akt pathway by determining the serine phosphorylation level of Akt, the PI3K downstream target. As seen in Figure 1(b), phosphorylation of Akt

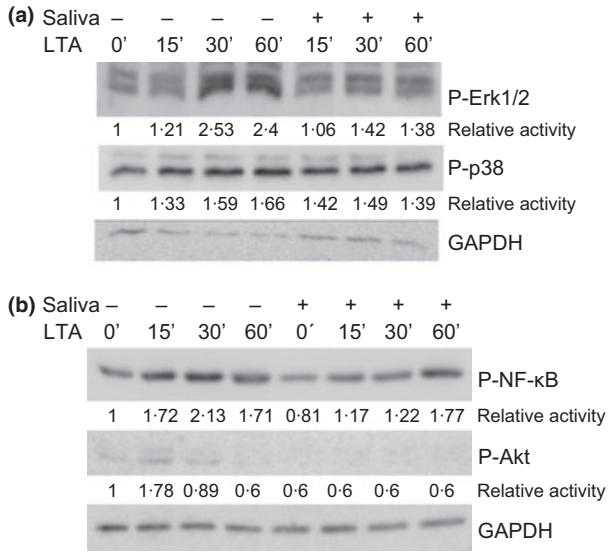

Figure 1 Effect of tick saliva on NF-κB, MAPK and Akt activation in dendritic cells upon TLR-2 ligation. After pre-incubation with tick saliva (20 μg/mL) or corresponding amount of pilocarpine for 2 h, dendritic cells were stimulated with 2·5 μg/mL lipoteichoid acid (LTA) for the time indicated. Cell lysates were analysed by Western blotting using specific antibody against phosphorylated Erk1/2, p38 MAPK (a), NF-κB and Akt proteins (b). Level of GAPDH protein is shown as an internal control. The phosphorylation of kinases was quantified using scanning densitometry and normalized by the GAPDH protein. Relative activities are corresponding to densitometric readings where values achieved in unstimulated control were set up to 1. Representative experiments and their densitometric readings are shown. Two independent experiments were performed.

(though only mildly induced) was suppressed by tick saliva upon ligation of TLR2.

Erk1/2 pathway (suppressed by tick saliva) is involved in the production of pro-inflammatory cytokine TNF-α upon TLR-2 ligation

Specific kinase inhibitors were utilized to determine whether identified kinases (that were suppressed by tick saliva) are directly involved in regulating pro-inflammatory or anti-inflammatory cytokines production. The production of pro-inflammatory cytokine (represented by TNF-α) by dendritic cells stimulated with LTA was assessed in the presence of tick saliva or in the presence of UO126 or LY294002, specific inhibitors of MEK (kinase upstream of Erk1/2) or PI3K, respectively (Figure 2a). LTA-induced TNF-α secretion had the tendency to be reduced by tick saliva ($P = 0{\cdot}1$) and was decreased by Erk1/2 inhibition ($P < 0{\cdot}05$). In contrast, the production of TNF-α was not influenced by PI3K inhibitor. The finding that the Erk1/2 inhibitor mimicked the effect of tick saliva suggested that saliva-attenuated TNF-α

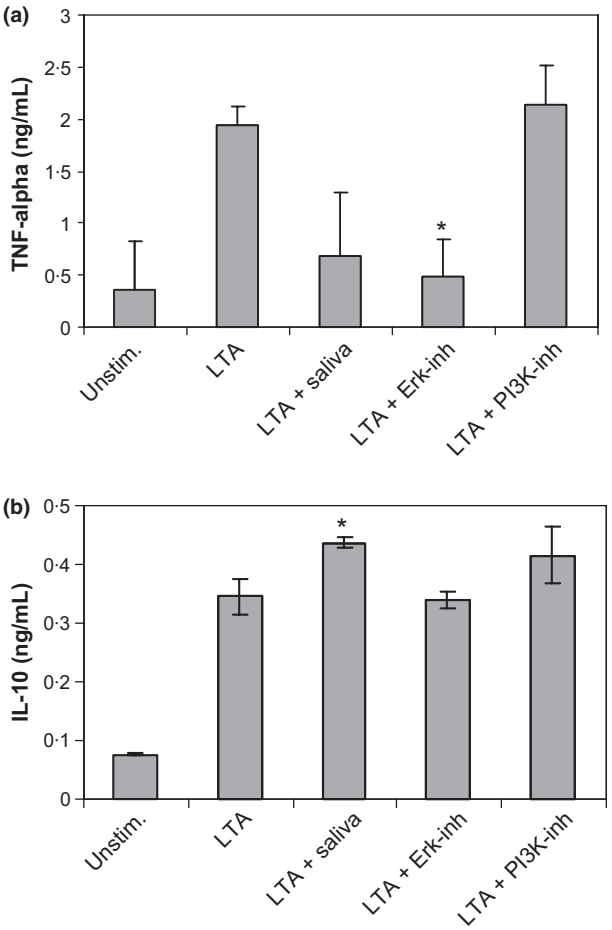

Figure 2 Effect of tick saliva and inhibitors of Erk1/2 and PI3K on the production of TNF-α and IL-10 by LTA-stimulated dendritic cells. Tick saliva was added to cultures 2 h prior to the stimulation at concentration 20 μg/mL. In some wells, dendritic cells were incubated with inhibitor for 30 min prior to the addition of LTA. Culture supernatants were collected 24 h or 48 h after ligand addition and analysed for presence of TNF-α (a) or IL-10 (b), respectively. Data are expressed as the mean cytokine concentration from triplicate wells ± SD. * indicates the effect of tick saliva and kinases inhibitors on LTA-stimulated cells significant at $P < 0{\cdot}05$.

production is mediated, at least in part, by inhibiting Erk1/2 signalling pathway.

Next, the IL-10 production (representing anti-inflammatory cytokine) was examined in the presence and absence of tick saliva (Figure 2b). Stimulation of dendritic cells with LTA led to an increase of IL-10 and the presence of tick saliva further enhanced the amount of IL-10 produced. When Erk1/2 inhibitor was applied, IL-10 production was not affected. For the PI3K inhibitor, we observed a slight increase in IL-10 production. We concluded that the production of IL-10 upon LTA stimulation seemed to

be independent of Erk1/2, but partly dependent on PI3K-mediated signalling pathways.

The activation of Erk1/2, NF-κB and Akt in response to *Borrelia* spirochaetes is attenuated by tick saliva

As we have shown that tick saliva suppresses some signalling pathways upon TLR-2 ligation, we anticipated whether saliva would have similar effects on signalling pathways activated by *B. afzelii* (bearing multiple TLR ligands). Dendritic cells were pre-incubated with tick saliva and live *Borrelia* spirochaetes were added as indicated. Activation of NF-κB, MAPK and PI3K/Akt signalling pathways was examined. Spirochaetes induced activation of all kinases tested (Figure 3a). The analysis of NF-κB pathway showed that phosphorylation of Rel p65 was decreased in the presence of tick saliva by 48·5% at 30 min ($P < 0·05$), and by 51·4% at 60 min ($P = 0·07$) (Figure 3b). Among the MAP kinases, tick saliva negatively affected activation of Erk1/2 (by 43·8% at 30 min, $P < 0·05$), but not p38. Activation of Akt kinase was also attenuated in the presence of tick saliva, though to a lesser extent (by 29·3% at 30 min, $P = 0·16$ and 25·8% at 60 min, $P = 0·07$) (Figure 3b). Overall, dendritic cells stimulated with *B. afzelii* shared similar signalling pathways affected by tick saliva, as those stimulated with LTA.

Erk1/2, NF-κB and Akt pathways, negatively influenced by tick saliva, are implicated in pro-inflammatory cytokine TNF-α production in response to Borrelia

To further investigate the relevance of the identified kinases with regard to the cytokine production by dendritic cells, we utilized NF-κB, Erk1/2 or PI3K inhibitors (IKK-IV, UO126 or LY294002 respectively). The amount of secreted TNF-α and IL-10 by dendritic cells upon infection with *B. afzelli* was measured in the presence or absence of tick saliva, IKK-4, UO126 or LY294002. As

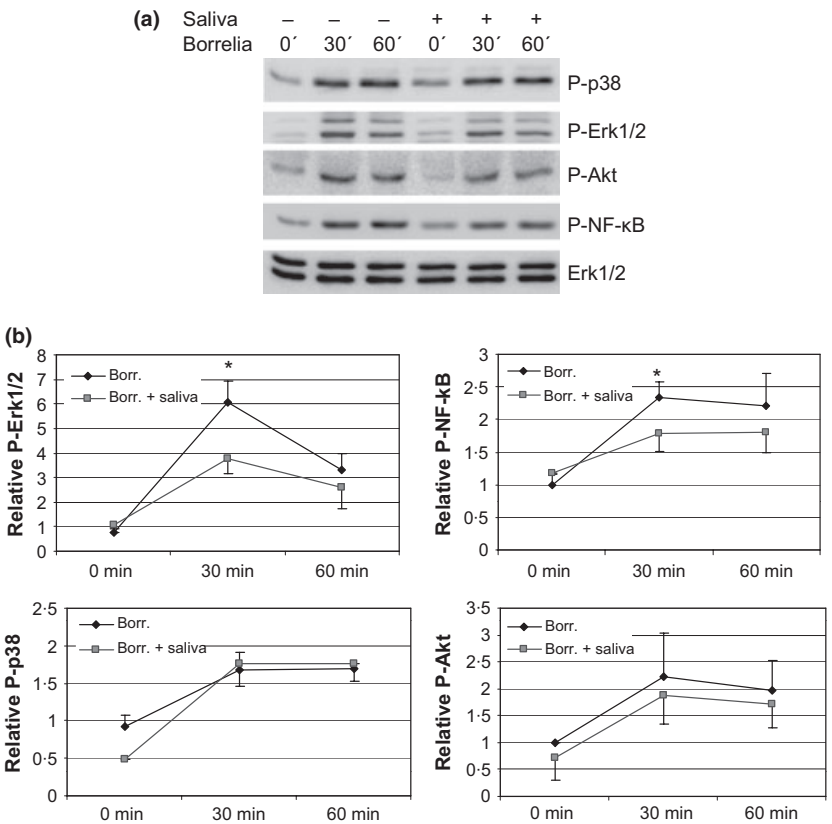

Figure 3 Tick saliva attenuates Erk1/2, NF-κB and Akt activation, but not p38 MAPK in dendritic cells in response to *Borrelia afzelii*. Dendritic cells were pre-incubated with tick saliva for 2 h (20 μg/mL) followed by the addition of live spirochaetes (10 spirochaetes per cell) and incubation for the time as indicated. Cell lysates were analysed by immunoblotting with antibody against phosphorylated Erk1/2, p38 MAPK, NF-κB and Akt (a). The total amount of protein was determined by reprobing the membrane with antibody against Erk1/2 protein. The phosphorylation of kinases was quantified using scanning densitometry and normalized by control protein (b). Each point represents the mean fold increase of three independent experiments. * indicates the effect of tick saliva significant at $P < 0·05$. Representative immunoblots are shown.

shown, the production of pro-inflammatory TNF-α was decreased by tick saliva and by all inhibitors (Figure 4a). The observation that tick saliva and all tested inhibitors had an inhibitory effect implicates that tick saliva may interfere with the production of cytokines and subsequently with the maturation of dendritic cells by attenuating NF-κB, Erk1/2 and PI3K/Akt signalling pathways.

In contrast, the production of IL-10 in response to spirochaetes was enhanced by tick saliva, but decreased in the presence of IKK-IV, UO126 or LY294002 inhibitors (Figure 4b). Based on the opposing effects of tick saliva and inhibitors on IL-10 production, we conclude that saliva-evoked enhancement of IL-10 is independent of pathways inhibited by the saliva.

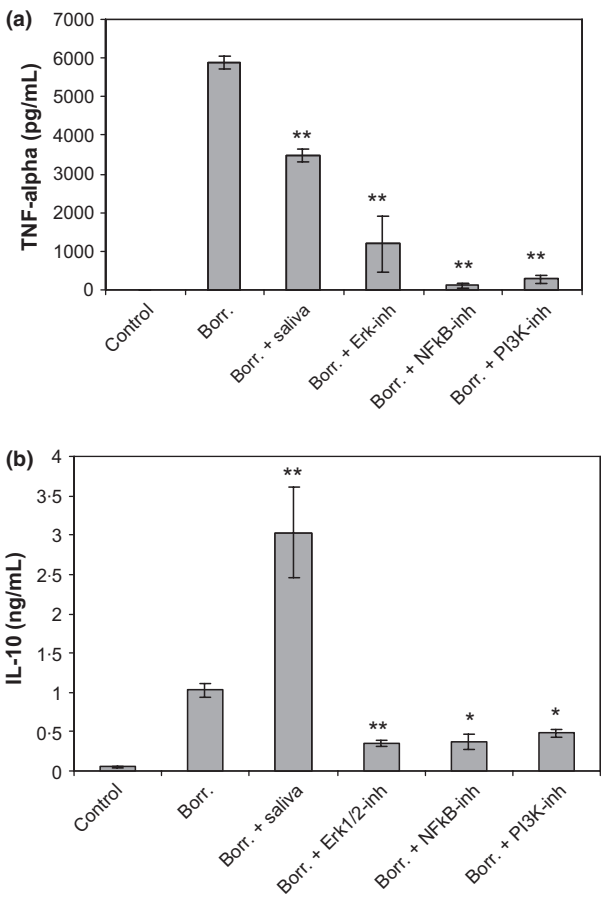

Figure 4 Effect of saliva and inhibitors of NF-κB, Erk1/2 and PI3K on the production of TNF-α and IL-10 cytokines in dendritic cells in response to *Borrelia afzelii.* Tick saliva was added to cultures for 2 h at concentration 20 μg/mL followed by addition of spirochaetes at ratio 10 : 1. In some wells, dendritic cells were incubated with inhibitor for 30 min prior to the addition of *Borrelia* spirochaetes. Culture supernatants were collected 24 h or 48 h after stimulation and analysed for presence of TNF-α (a) or IL-10 (b). Data are expressed as the mean cytokine concentration from triplicate wells ± SD. * indicates the effect of tick saliva or specific inhibitors significant at $P < 0{\cdot}05$, ** indicates the effect of tick saliva or specific inhibitors significant at $P < 0{\cdot}005$.

The saliva-induced enhancement of IL-10 production is mediated by Protein Kinase A (PKA) pathway

Most of the immunomodulatory effects of tick saliva from *R. sanguineus* in dendritic cells are mediated through activating the cAMP/PKA pathway (23). We set out to determine whether cAMP/PKA pathway is critical for IL-10 production. Therefore, the inhibitor of PKA, H89, was applied and the amount of secreted IL-10 in response to *Borrelia* was measured in the presence or absence of tick saliva. As seen in Figure 5, the amount of IL-10 was up-regulated by tick saliva. The presence of H-89 completely blocked saliva-induced enhancement of IL-10. This result suggests that the enhancement of IL-10 is dependent on PKA pathway.

DISCUSSION

Tick salivary constituents facilitate host transmission of tick-borne pathogens by modulating the host immune system. Dendritic cells play a critical role in recognizing pathogens and tick saliva negatively affects the maturation and differentiation of these cells (7). Still, which signalling pathways are influenced by tick saliva remains elusive. We recently reported that tick saliva from *I. ricinus* negatively interferes with IFN signalling (24). Herein, we expand our

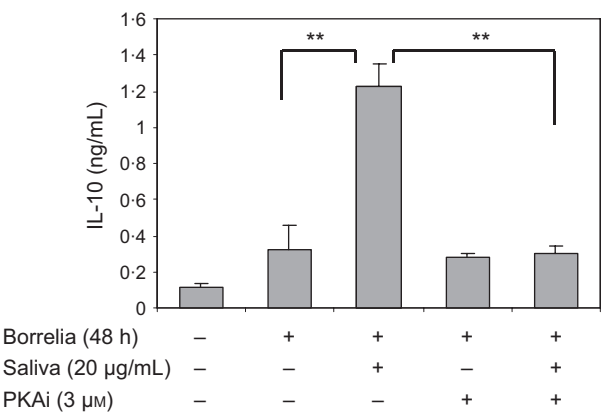

Figure 5 Saliva-induced enhancement of IL-10 production is dependent on PKA-mediated pathway. Pre-incubated with 20 μg/mL tick saliva for 2 h in the presence or absence of PKA inhibitor H89 (3 μM), dendritic cells were stimulated with *Borrelia afzelii* at ratio 10 : 1. Supernatants were collected 48 h after adding spirochaetes and analysed for IL-10 by ELISA. Data are expressed as the mean cytokine concentration from triplicate wells ± SD. ** indicates the effect of tick saliva or tick saliva and H89 significant at $P < 0{\cdot}005$.

study to describe the effects of tick saliva on activating signal transduction pathways in dendritic cells upon TLR-2 ligation and addition of *Borrelia* spirochaetes. Because of low numbers obtainable from skin dendritic cells, we used spleen dendritic cells as our model system. The implication of tick saliva effect on signalling cascades for induction of pro-inflammatory and anti-inflammatory cytokines is suggested.

Upon ligand binding to innate receptors (e.g. TLR), several signalling pathways are activated in dendritic cells. This activation leads to a production of cytokines whose composition determines the course of the immune response. We show here that tick saliva attenuated NF-κB pathway, negatively influenced Erk1/2 but not p38 MAPK pathway, and attenuated PI3K pathway in response to TLR-2 ligand and *Borrelia* spirochaetes. All inhibited pathways seemed to be involved in the induction of pro-inflammatory cytokine TNF-α. The saliva-induced enhancement of anti-inflammatory IL-10 production, however, appeared to be dependent on activation of cAMP/PKA pathway.

Balance between pro-inflammatory and anti-inflammatory cytokines determines the outcome of adaptive immune response; TNF-α is a pro-inflammatory cytokine and IL-10 is an anti-inflammatory cytokine. Pro-inflammatory cytokines production, including TNF-α, critically depend on NF-κB pathway (14). We showed that tick saliva severely decreased TLR-2 and *Borrelia*-dependent NF-κB activation. Moreover, the production of TNF-α was decreased in the presence of either tick saliva or the inhibitor of NF-κB in response to *Borrelia*. Our data confirm the involvement of NF-κB pathway in TNF-α induction and imply that by suppressing this pathway, tick saliva decreases TNF-α production. In addition, *B. burgdorferi* lipoproteins up-regulate chemokines and adhesion molecules in endothelial cells and fibroblasts that are also mediated by the activation of NF-κB (29). It is conceivable that saliva-induced suppression of NF-κB pathway may impact production of additional pro-inflammatory cytokines or chemokines as well.

Among MAP kinases, p38 has been associated with the production of pro-inflammatory cytokines such as IL-12, TNF-α and IL-1β (17,30). Oliveira *et al.* (22) have shown that the presence of tick saliva impaired p38 activation upon LPS (TLR4 ligand) stimulation in dendritic cells. In our experimental setting, however, the components of tick saliva did not affect activation of p38 upon TLR2 or *Borrelia* stimulation. As activation of p38 by LTA is weaker compared with LPS, it is possible that smaller differences in p38 activation could be missed.

Interestingly, another MAP kinase, Erk1/2 was clearly suppressed by tick saliva in response to both LTA and B*orrelia.* The impairment in Erk1/2 activation by tick saliva was reported in dendritic cells stimulated with LPS (22) and in fibroblasts when Erk1/2 activation was triggered with a growth factor affecting their migration (34). However, the signalling cascade mediated by Erk1/2 is important for Th2 biased response (18), and the ability of tick saliva to induce Th2 prone phenotype has been repeatedly reported (7). Our observation that tick saliva inhibits Erk1/2 phosphorylation suggests that there is a different mechanism of the immune response polarization than of that described by Dillon *et al.* (18). Indeed, an Erk-independent Th2 cell induction was also described in TLR-stimulated Langerhans dendritic cells (35).

What are the consequences of suppressing Erk1/2-mediated pathway in dendritic cells? We attempted to answer this question by comparing the effect of Erk1/2 inhibitor on pro-inflammatory cytokine TNF-α production with that of tick saliva. We showed that tick saliva mimicked the effect of Erk1/2 inhibitor upon both LTA and *Borrelia* stimulation. The suppression of Erk1/2 pathway may contribute to the decrease in pro-inflammatory cytokine production in addition to the effect on NF-κB-mediated pathway.

PI3K/Akt signalling pathway is considered to be inhibitory to TLR-2-mediated pro-inflammatory cytokines induction (20), and we show that tick saliva attenuated PI3K/Akt signalling in response to both TLR2 and *Borrelia*. Correspondingly, we found no positive association between saliva-induced dampening of PI3K/Akt pathway and TNF-α production. In response to *B. afzelii*, however, TNF-α production was sensitive to PI3K inhibition. As *Borrelia* activates several TLRs, signals mediated by TLRs other than TLR2 may be sensitive to PI3K and required for TNF-α induction in response to spirochaetes. Indeed, cooperative interaction between TLR2 and TLR8 in phagosomal signalling triggered by *B. burgdorferi* was demonstrated to be involved in pro- and anti-inflammatory cytokine production (36).

Recently, it has been shown that *I. ricinus* saliva components negatively influence the phagocytosis of tick transmitted pathogen *B. afzelii* (31). The inhibition of PI3K resulted in significantly decreased uptake of *B. burgdorferi* spirochaetes, pointing to the importance of PI3K in phagocytosis (37). We presume that the impaired phagocytosis of *B. afzelii* is a consequence of the saliva-induced attenuation of the PI3K/Akt signalling axis. In addition, the PI3K pathway is a key regulator of cellular adherence junctions (38). The impairment in PI3K transduction pathway may represent a link in the disruption of the actin cytoskeleton and altering cellular morphology induced by saliva from different tick species as described recently by Hajnicka *et al.* (39).

Development of Th2 prone immune response is characteristic for parasites, and IL-10 is a typical cytokine of the Th2 subset (11,12). The production of IL-10 seemed to be independent of pathways suppressed by tick saliva (NF-κB, Akt and Erk1/2). Interestingly, the inhibitory effect of tick saliva on the production of several cytokines was attributed to the activation of cAMP/PKA pathway (23). We did not directly test the activation of PKA by tick saliva, however, the PKA inhibitor completely reversed saliva-induced enhancement of IL-10. In agreement with the results by Oliveira *et al.*, we concluded that PKA-mediated pathway is critical for IL-10 up-regulation by tick saliva. Moreover, PKA pathway can contribute to saliva-induced decrease of other cytokines produced by dendritic cells, because PKA signalling suppresses cytokine production (40).

Overall, our data show that suppression of NF-κB-, Erk1/2- and Akt-mediated pathways, together with the activation of PKA signalling cascade, accounts for tick saliva modulation of dendritic cells in response to *B. afzelii* and TLR-2 ligand. This study thus contributes to a limited knowledge of how tick saliva acts at the signalling level.

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Tick saliva suppresses IFN signalling in dendritic cells upon *Borrelia afzelii* infection

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SUMMARY

Type I interferons (IFN-α and IFN-β) are crucial determinants of the host immune response and tick saliva modulates this response, thus facilitating the transmission of tickborne pathogens. The current study therefore examines the effect of Ixodes ricinus tick saliva on IFN-β signalling in murine dendritic cells using lipopolysaccharide (LPS) and Borrelia afzelii spirochaetes as inducers. Activated dendritic cells secret IFN that activates Signal Transducer and Activator of Transcription 1 (STAT-1). Our results show that Borrelia-induced activation of STAT-1 was suppressed by tick saliva. As the amount of secreted IFN-β was not influenced by tick saliva, the results indicated that saliva affected the interferon pathway at the IFN receptor or downstream of it. By using recombinant IFN-β, we show that tick saliva attenuates IFN-triggered STAT-1 activation. Tick saliva also inhibited LPS-induced IFN-β production suggesting that saliva interferes with the activation of the pathway that mediates IFN-β induction. Our data indicate that I. ricinus tick saliva may modulate the host immune response by attenuating the initial signal transduction pathway of type I IFN.

Keywords Borrelia*, dendritic cells, interferon signalling, tick saliva*

INTRODUCTION

Typical of blood-feeding arthropods, ticks have evolved mechanisms enabling them to overcome the host immune system for a successful blood meal. Several components of tick saliva influence the host immune response such as prostaglandins, proteases and protease inhibitors (1). Bioactive properties of saliva are dynamic and change in the course of blood feeding (2) – a change that correlates with differences in salivary gland transcriptome among various ixodid tick species (3). Reports on the immunomodulatory activities of tick saliva or salivary gland extract (SGE) include the ability to inhibit the complement cascade, prevent phagocytosis and production of superoxide by neutrophils, inhibit the production of nitric oxide by macrophages, impair NK cell function and reduce the antiviral effect of interferon (4–8). Particularly, the production of IFN-γ was negatively affected by SGE in host macrophages and lymphocytes (9,10). Polarization of the host immune response towards the Th2 cytokine profile has been repeatedly reported for saliva from various tick species (11). The migration, maturation and function of dendritic cells were inhibited by tick saliva, while promoting development of Th2 cytokine response (12).

Dendritic cells are considered a sentinel for immune response bridging naive and adaptive immunity. Several subsets of dendritic cells are identified based on their tissue distribution, cell surface markers and transcriptional programmes. Skin dendritic cells, comprising of dermal and Langerhans cells, encounter tick antigens at the tick feeding site and are surrounded by keratinocytes and fibroblasts that play a supplementary role in the cutaneous immune system (13). Several classes of pattern recognition receptors are involved in recognizing tickborne pathogens including Toll-like receptors (TLR), NOD-like receptors and C-type lectin receptors (14). Several types of TLRs are expressed either on the cellular surface membrane (TLR1,2,4,5) or are localized in endosomal membrane (TLR3,7,8,9). *Borrelia burgdorferi* sensu lato, the causative agent of Lyme disease, is one of many pathogens transmitted by ticks. *Borrelia* spirochaetes are sensed by immune cells (including dendritic cells) through TLR1, TLR2, TLR7, TLR8 and TLR9 (15–18), and the induction of IFN-α/β upon *Borrelia* infection is mediated by TLR7, TLR8 and TLR9 (17,18).

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Upon sensing the pathogen by TLR, dendritic cells transduce through several signalling pathways engaging two adaptor proteins: myeloid differentiation marker-88 (MyD88) and Toll-IL-1 receptor domain-containing adapter inducing IFNβ (TRIF) (19). Following TLR-4 ligation by lipopolysaccharide (LPS), both MyD88- and TRIF-dependent pathways lead to activating the latent transcription factor NF-κB (nuclear factor-κB) and mitogen-activated protein kinases (MAPK), while only TRIF-dependent pathway mediates interferon regulatory factor (IRF3/7) activation resulting in IFN-β induction (20). Upon endosomal TLR-7,9 activation, signal is transduced by MyD88 leading to activation of NF-κB and IRF3/7, while activating the latter results in IFN-α/β induction (19). Initial production of type I IFN is low and boosts in an autocrine manner to a more robust second wave of IFN, often called feedback amplification loop. Induction of proinflammatory cytokines depends on NF-κB pathway and MAPK pathway; however, type I IFNs also plays an important role in producing these cytokines – especially in the case of IL-12, which critically determines the ability of dendritic cells to prime T cells to Th1 phenotype (21,22).

There are few reports addressing the effect of tick saliva on TLR-mediated signalling pathways in dendritic cells. The inhibition of MAPK pathways (determined by Erk 1/2 and p38 activation) and increased expression of TLR-2 receptor was associated with impaired maturation of dendritic cells stimulated with LPS when saliva from the hard tick *Rhipicephalus sanguineus* was applied (23). Recently, Oliveira *et al.* (24) reported that the saliva-induced inhibition of proinflammatory cytokine production by dendritic cells is attributed to the induction of cAMP-PKA signalling by purine nucleoside adenosine and prostaglandin E2.

Type I IFN is represented by IFN-α and IFN-β, while IFN-γ is the only known type II IFN. Binding of type I and II IFNs to their receptors results in activating Janus-activated kinase (Jak)/STAT pathway. Tyrosine kinases Jak1/Tyk2 and Jak1/Jak2, for IFN-α/β and IFN-γ, respectively, become activated and phosphorylate the receptor chain enabling recruitment and phosphorylation of STATs. STAT-1/2 heterodimers associate with IFN regulatory factor (IRF-9) forming the IFN-stimulated transcription factor (ISGF3), or STAT-1 homodimers forming IFN gamma activation factor (GAF). These complexes translocate to the nucleus to induce IFN-stimulated genes from ISRE or GAS promoter elements, for type I or type II interferon responses, respectively (25). TLR-mediated induction of autocrine IFN-β and activation of the JAK-STAT pathway are important for dendritic cell maturation, expression of costimulatory molecules and antigen cross-presentation (26–29).

Our current study presents data on how tick saliva affects IFN signalling in dendritic cells stimulated with *Borrelia afzelii* and LPS. We demonstrate here that tick saliva suppresses STAT-1 activation but does not suppress the production of IFN-β induced by *B. afzelii*. The inhibition of STAT-1 phosphorylation was induced by tick saliva blocking JAK-STAT signalling, downstream of IFN receptor. When LPS was used for dendritic cells stimulation, tick saliva inhibited the induction of type I IFN. We propose that tick saliva-induced impairment of IFN signalling – in addition to inhibiting IFN-γ production (9) – is part of the mechanism on how saliva modulates the host immune response.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (10 weeks of age) were obtained from Charles River Laboratories. Guinea pigs used for *Ixodes ricinus* tick feeding were bred and maintained at the Institute of Parasitology, České Budějovice. All experiments were performed with permission from local animal ethics committee.

Tick saliva collection

Pathogen-free adult *I. ricinus* ticks from the colony maintained at the Institute of Parasitology in České Budějovice were allowed to feed in groups of 20 mating pairs on guinea pigs. After 6 days, partially engorged female ticks were removed and immobilized, and a 10-μL glass capillary tube (Sigma-Aldrich, St. Louis, MO, USA) was fitted over their mouthparts. To induce salivation, 2 μL of 0·1 M pilocarpine solution in ethanol was applied on the dorsum of each tick. After 60 min, the saliva was collected, pooled and stored at −70°C. The concentration of pilocarpine in saliva was estimated at 4 mM by HPLC/mass spectrometry method. Corresponding amount of pilocarpine was added to samples not treated with saliva as a control. All salivary samples were filtered through a 0·22-μm filter (Millipore, Billerica, MA, USA), and saliva protein concentration was determined using a Bradford reagent (Sigma-Aldrich) before use.

Bacteria

The CB-43 strain of *B. afzelii* isolated from *I. ricinus* (30) was grown in Barbour–Stoenner–Kelly-H (BSK-H) medium (Sigma) supplemented with 6% rabbit serum at 34°C. The fourth passage was used in the experiments.

Dendritic cells isolation

Isolated mouse spleens were minced with scissors, digested in RPMI containing 1 mg/mL collagenase-D (Roche, Mannheim, Germany) at 37°C for 1 h and passed through a 70-μm nylon cell strainer (BD Biosciences, Durham, NC, USA). Dendritic cells were isolated using magnetic beads conjugated with anti-CD11c (N418) Ab and MACS Column separation following the manufacturer's instructions (Miltenyi Biotec, Bergish Gladbach, Germany). Purified dendritic cells were cultured in RPMI supplemented with 10% heat-inactivated foetal calf serum (FCS), 50 μM 2-ME, 100 μg/mL penicillin and 100 U/mL streptomycin (all from Sigma-Aldrich). Purity of isolated dendritic cells (~90% CD11c+ cells) was determined by subsequent FACS analysis.

Cytokine measurements

Freshly isolated DCs were cultured in 96-well plate at a concentration of 2×10^5 cells per well for 24 h. Following 2 h incubation with tick saliva, the cells were stimulated with LPS (50 ng/mL) or *B. afzelii* in ratio 10 : 1 (10 spirochaetes to 1 cell) and cell-free culture supernatants were harvested after 4 and 22 h for IFN-β and after 72 h for IL-12 determination. IFN-β was determined using mouse IFN-β ELISA Kit (PBL Biomedical Laboratories, Piscataway, NJ, USA) and IL-12 using Ready-Set-Go! ELISA Set (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. The pilocarpine was added to the control sample at corresponding concentration.

Immunoblotting

Freshly isolated dendritic cells were seeded at 1×10^6 cells per well in 24-well plate. Next day, dendritic cells were incubated 2 h with indicated concentration of tick saliva or pilocarpine prior to the addition of LPS (50 ng/mL) or 5 U/mL of recombinant mouse IFN-β (PBL interferon source) or 5 U/mL of recombinant mouse IFN-γ (R&D Systems, Minneapolis, MN, USA). Following stimulation, the cells were lysed in a modified RIPA buffer [1% Nonidet P-40, 0·25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl and 50 mM Tris–HCl (pH 7·5)] in the presence of protease inhibitors (10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1 μg/mL pepstatin) and phosphatase inhibitors (25 mM sodium fluoride and 2 mM sodium orthovanadate). The cell lysates were centrifuged at 14 000×*g* for 10 min at 4°C, mixed with 4× Laemmli sample buffer and then separated by SDS-PAGE using a 6% gel. The proteins were then transferred to Immobilon-P membranes, which were blocked for 1 h in Tris/saline buffer containing 0·1% Tween 20 and 5% fat-free milk. The blots were incubated with antibody against phospho-STAT1 (Tyr701) (Cell Signaling, Beverly, MA, USA), STAT-1 protein or GAPDH (BD Transduction Laboratories, Lexington, KY, USA) at dilution 1 : 1000 overnight at 4°C. The proteins were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA), and their abundance was analysed using the GeneGenome CCD image system (Syngene, Ltd., Cambridge, UK). Densitometric analyses of the images were undertaken using NIH Image J version 1.61.

Statistical analysis

Student's *t*-test was used to compare the differences between control and treated groups. $P \leq 0{\cdot}05$ was considered statistically significant.

RESULTS

Tick saliva reduces amount of secreted IFN-β upon LPS but not *Borrelia afzelii* stimulation

First we determined whether IFN-β production is affected by tick saliva. Dendritic cells were stimulated with LPS or with live *Borrelia* spirochaetes following preincubation with 20 μg/mL of tick saliva. IFN-β was detected in the supernatant of LPS-stimulated cells, mainly 4 h after stimulation. The amount of secreted IFN-β was markedly decreased (by 85%, $P < 0{\cdot}05$) in the presence of saliva in comparison with stimulated cells preincubated with pilocarpine (Figure 1a). The IFN-β, secreted in response to *B. afzelii,* was detected in the supernatant at both intervals tested; however, it was not affected by tick saliva (Figure 1b).

Tick saliva suppresses STAT-1 activation upon addition of LPS and *Borrelia afzelii*

Next we analysed the activation of STAT-1 in dendritic cells after adding LPS in the presence or absence of tick saliva. STAT-1 phosphorylation, mediated by secreted IFN-β, was detected 2 h after LPS stimulation, and preincubation with tick saliva resulted in reduced levels (by average 60%, $P < 0{\cdot}05$) of STAT-1 activation (Figure 2a). The decrease in LPS-induced STAT-1 activation apparently reflected the decreased amount of IFN-β secreted by dendritic cells. Similarly, we analysed the effect tick saliva has on STAT-1 phosphorylation upon infection of dendritic cells with *B. afzelii* (Figure 2b). STAT-1 activation was observed as early as 2 h upon addition of spirochaetes. Interestingly, STAT-1 phosphorylation was markedly decreased in the presence of tick saliva at all time points analysed (by 100% at 2 h, by 100% at 4 h and by 84% at 6 h, $P < 0{\cdot}05$).

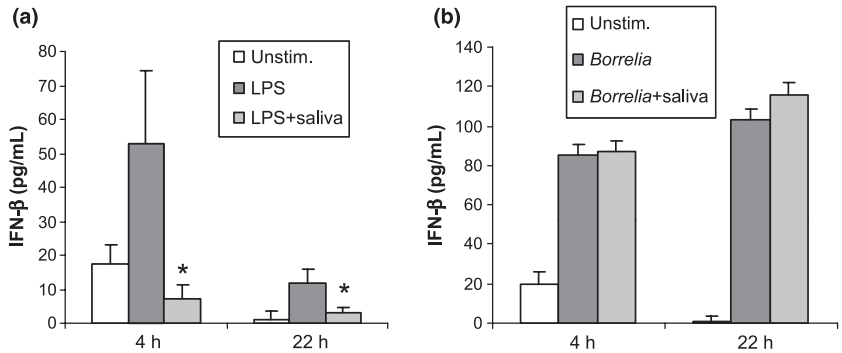

Figure 1 Tick saliva reduces production of IFN-β by dendritic cells stimulated with lipopolysaccharide (LPS) but not with *Borrelia afzelii*. Tick saliva at concentration 20 μg/mL was added to freshly isolated dendritic cells 2 h prior to the stimulation with 50 ng/mL of LPS (a) or live *Borrelia* spirochaetes (b) at the ratio of 10 spirochaetes per cell. Culture supernatants were collected 4 and 22 h after LPS/*Borrelia* addition and analysed for the presence of IFN-β. Results represent the mean from triplicate wells ± SD. *The effect of tick saliva significant at $P < 0{\cdot}05$. Data are representative of two independent experiments.

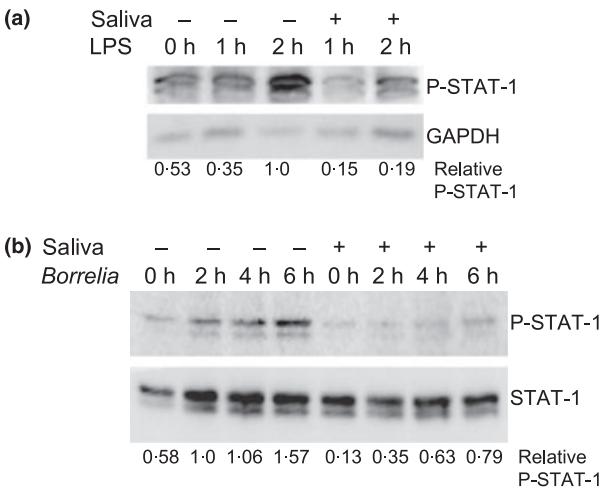

Figure 2 Tick saliva suppresses STAT-1 activation in dendritic cells upon lipopolysaccharide (LPS) and *Borrelia afzelii* stimulation. Dendritic cells were incubated for 2 h with tick saliva (20 μg/mL) and stimulated with 50 ng/mL of LPS (a) or live spirochaetes (10 spirochaetes per cell) (b) for the time as indicated. Cell lysates were analysed by immunoblotting for activation of STAT-1 protein. The membranes were re-probed with antibody against STAT-1 or GAPDH protein. The phosphorylation of STAT-1 was quantified using scanning densitometry and normalized by GAPDH (a) or STAT-1 (b) protein level. Relative activities are corresponding to densitometric readings where values achieved 2 h after IFN stimulation were set up to 1. Three independent experiments were performed with LPS and with *Borrelia* spirochaetes. Representative immunoblots are shown.

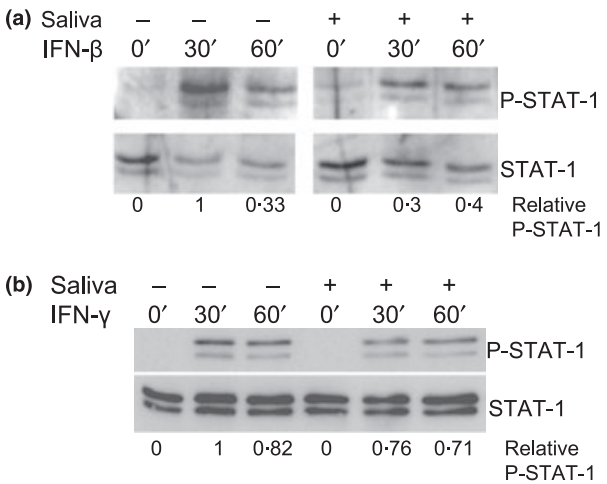

Figure 3 Tick saliva attenuates IFN-triggered STAT-1 activation. Dendritic cells were treated with five units of recombinant IFN-β (a) or IFN-γ (b) for indicated times in the presence or absence of tick saliva (20 μg/mL). Cell lysates were analysed by immunoblotting with anti-phospho-STAT-1 antibody. The membranes were re-probed with anti-STAT-1 antibody. The phosphorylation of STAT-1 was quantified using scanning densitometry and normalized by the STAT-1 protein level. Relative activities are corresponding to densitometric readings where values achieved 30 min after IFN stimulation were set up to 1. Three independent experiments were performed with IFN-β and with IFN-γ. Representative blots are shown.

Tick saliva attenuates IFN-triggered STAT-1 activation

As tick saliva suppressed STAT-1 phosphorylation upon *B. afzelii* infection in spite of normal amount of endogenously produced IFN-β, we tested whether tick saliva interferes directly with IFN-β-triggered JAK/STAT signalling pathway. The activation of STAT-1 was measured in the presence or absence of tick saliva in dendritic cells upon addition of recombinant IFN-β. As shown in Figure 3(a), IFN-β-induced STAT-1 phosphorylation was attenuated by tick saliva by 31·2% (±22·9%, $n = 3$) at the time of maximum activation (30 min). Degree of

STAT-1 attenuation varied between experiments (therefore not achieving a statistical significance); however, in each independent experiment, we observed inhibition of STAT-1 phosphorylation in the presence of tick saliva. To detect whether the effect of tick saliva on STAT-1 is restricted to type I interferon, recombinant IFN-γ was applied to induce STAT-1 activation. The experiment revealed that STAT-1 activation was also decreased by the saliva in response to IFN-γ (Figure 3b). Reduction reached 24% (±0·5%, $P < 0·005$) at 30 min after activation and 24·4% (±9·9%, $P < 0·05$) at 60 min after activation. Taken together, our data showed the inhibitory effect of saliva on STAT-1 activation in response to IFNs.

The effect of tick saliva on the production of IL-12p70 by dendritic cells upon stimulation with LPS and *Borrelia afzelii*

Type I interferon amplification loop is essential for the production of IL-12p70 by dendritic cells (31). For example, IL-12p70 was strongly reduced in bone-marrow-derived dendritic cells from STAT-1(−/−) and INFAR (−/−) mice (31). Furthermore, the dependence of differentiation and phenotypic maturation of dendritic cells on JAK/STAT signalling pathway was reported (26,32,33). Therefore, we measured the amount of IL-12p70 produced by dendritic cells stimulated with LPS and *B. afzelii*. Similar levels of IL-12p70 were secreted by cells in response to both, LPS and *Borrelia* spirochaetes. Preincubating dendritic cells with tick saliva resulted in 45% ($P < 0·05$) decrease in LPS-induced IL-12p70 production (Figure 4a), while *Borrelia*-induced IL-12p70 was inhibited by 94%, $P < 0.005$ (Figure 4b).

DISCUSSION

Dendritic cells interconnect innate and adaptive immunity as they are major antigen-presenting cells and crucial players in determining T cell polarization (34). As dendritic cells are strategically located at the site of arthropod-borne pathogen entry, the effect of tick saliva on these cells has been recently addressed. It has been documented that tick saliva inhibits dendritic cells maturation and function resulting in Th2 polarization of the immune response (12,35). In this study, we focused on how tick saliva effects type I IFN in dendritic cells stimulated with *B. afzelii* and LPS. Because of low numbers of obtainable skin dendritic cells, we used spleen dendritic cells as a model system.

Dendritic cells sense the extracellular environment by several pattern recognition receptors, including TLR, NOD-like receptors and C-type lectin receptors (14). Cells, after recognition of PAMPs (pathogen-associated molecular patterns), response via activating signal transduction pathways culminating in cytokine production and cell activation (36). Type I interferon is one of the earliest cytokines secreted upon bacterial or viral infection, and dendritic cells possess the ability to secrete and react to IFNs. Herein we report that tick saliva impaired activation of STAT-1, the signal transducer of the major signalling pathway activated by IFNs, in response to *B. afzelii.* Interestingly, STAT-1 phosphorylation in response to *B. afzelii* was inhibited in spite of normal level of IFN-β produced by autocrine signalling, suggesting that tick saliva interferes with IFN-triggered signalling. In the presence of tick saliva, STAT-1 activation induced by recombinant IFN-β was also attenuated, though, to a lesser extent. The impairment, however, in LPS-induced STAT-1 phosphorylation

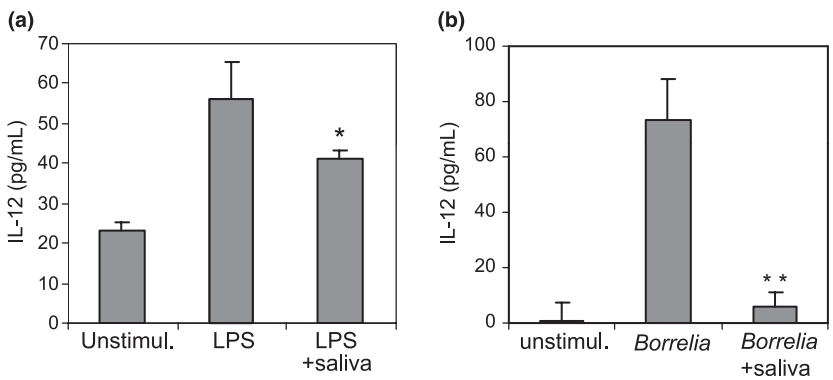

Figure 4 Tick saliva diminishes IL-12p70 secretion by dendritic cells exposed to lipopolysaccharide (LPS) or *Borrelia afzelii.* Tick saliva was added to dendritic cells at concentration 20 μg/mL 2 h prior to the stimulation with 50 ng/mL of LPS (a) or *B. afzelii* (b) at the ratio of 10 spirochaetes per cell. Supernatants were collected after 72 h and analysed by ELISA for the presence of IL-12p70. Results represent the mean from triplicate wells ± SD. *The effect of tick saliva significant at $P < 0·05$, **significant at $P < 0·005$. Data are representative of two independent experiments.

apparently resulted from the saliva-mediated decrease in IFN-β production.

As IFN-β induction mediated through TLR4 activation (with LPS) is TRIF-dependent and MYD88-independent (20), our results suggest that tick salivary constituents directly or indirectly target TRIF/IRF signalling pathway. The process that tick saliva interferes with IFN signalling, thus varies depending on the ligand used for inducing IFN (Figure 5). Reports do show that protein extract from tick salivary glands negatively effect IFN-α mRNA levels in human peripheral blood cells after LPS stimulation (37). Moreover, the inhibition of IFN-β induction upon LPS stimulation may be mediated by tick salivary prostaglandin E2 as the inhibitory effect of prostaglandin E2 on LPS-induced IFN-β production in macrophages at mRNA and protein levels was reported (38).

Contrary to LPS, induction of type I IFNs by *B. burgdorferi* spirochaetes is mediated by the TLR7, TLR8 and TLR9 pathways (17,18). Studies show that phagocytosis of spirochaetes is a prerequisite for IFN induction by *Borrelia* spirochaetes (16), and the uptake of *Borrelia* spirochaetes by macrophages is decreased under the influence of tick saliva (39). Our finding that tick saliva did not effect IFN-β production following *B. afzelii* stimulation was quite unexpected. As inhibiting phagocytosis of *Borrelia* spirochaetes by tick saliva is not complete, the number of engulfed spirochaetes was apparently sufficient for inducing IFN amount compared with that produced by cells untreated with saliva. In addition, we used *B. afzelii* for IFN induction contrary to other authors who used *B. burgdorferi* sensu stricto. Furthermore, as IFN-α was not the subject of this study, we do not exclude the concept that IFN-α, if secreted, is affected by tick saliva.

Binding of IFNs to their receptors activates JAK/STAT signalling pathway and leads to the induction of IFN-stimulated genes. Information about the signalling pathway leading to inducing IFN-stimulated genes by *Borrelia* spirochaetes is limited. The functional type I IFN receptor is required for inducting IFN-responsive genes by *Borrelia* in bone-marrow-derived macrophages (40). Petzke *et al.* (17) reported that in human immune cells, the induction of IFN-inducible genes, in response to *Borrelia*, is independent of type I IFN feedback signalling. Importantly, the critical role in *Borrelia*-induced IFN-stimulated genes was attributed to STAT-1 feedback amplification pathway (41). We report here that *Borrelia*-induced STAT-1 phosphorylation is suppressed by tick saliva and thus most likely affects the induction of IFN-stimulated genes.

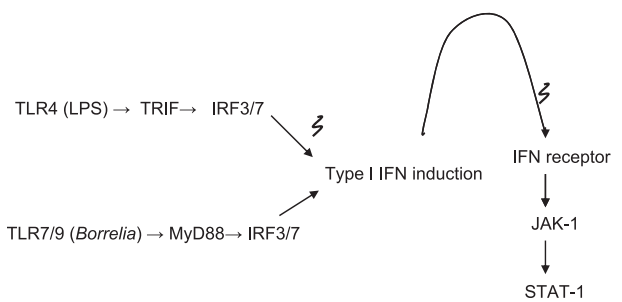

Figure 5 Schematic illustration of saliva interference with IFN induction and signalling upon lipopolysaccharide (LPS) and *Borrelia* stimulation. After LPS stimulation, saliva (≶) interferes with IFN-β induction. In response to *Borrelia,* saliva does not interfere with IFN-β induction but inhibits IFN signalling at IFN receptor level or downstream of it (as determined by STAT-1 phosphorylation).

As the level of produced IFN-β in response to *Borrelia* spirochaetes is not affected by tick saliva, we considered the possibility that saliva will interfere with IFN at the level of IFN-triggered signalling. Moreover, the inhibitory effect of SGE on antiviral action of interferon has been described (8). In our experiments, STAT-1 phosphorylation was attenuated by tick saliva upon addition of recombinant IFN-β. However, saliva-induced decrease in STAT-1 phosphorylation in response to recombinant IFN-β was not as pronounced as observed with *Borrelia* spirochaetes. Di Domizio (42) reported that phosphoinositide 3-kinase (PI3K) and p38 MAPK are implicated in the control of STAT-1 phosphorylation triggered by TLR7 ligand (and independent of type I IFN) in plasmacytoid dendritic cells. As *B. burgdorferi* is recognized by TLR7 (17), the decrease in STAT-1 activation upon *Borrelia* infection may be a partial consequence of tick saliva-induced suppression of p38 MAPK or PI3K pathway. Although the activation of p38 MAPK in dendritic cells upon *B. afzelii* infection was not affected by tick saliva, the activation of PI3K pathway was impaired, as judged by phosphorylation of its downstream target Akt (data not shown). Additional mechanisms are therefore conceivable that tick saliva affects STAT-1 activation by impairing PI3K/Akt pathway.

Phosphorylation of STAT proteins is controlled by proteolytic processing in addition to dephosphorylation by tyrosine phosphatases. STATs are targeted for ubiquitin ligation followed by degradation in proteasome or are cleaved by serine proteases resulting in forming novel STAT isoforms (43,44). The degradation of STAT-1 was not observed in dendritic cells by tick saliva. In contrast, tyrosine phosphorylation of STAT-1 in response to *Borrelia* spirochaetes and IFN-β was inhibited by tick saliva. Results thus suggest that neither cellular nor tick proteases are directly involved in the inhibition of STAT-1 activation by tick saliva.

Tyrosine phosphorylation of STAT-1 is the common component of both IFN-α/β and IFN-γ signalling

pathways. IFN receptor complexes responsible for phosphorylating STAT-1 are different in IFN-α/β- and IFN-γ-triggered signalling. The fact that tick saliva also inhibits IFN-γ-induced STAT-1 phosphorylation suggests that saliva exerts an effect either directly on STAT-1 or indirectly affecting a negative cellular regulator that acts on both pathways. The ability of tick saliva to impair IFN-γ signalling may also contribute to modulating the immune response by interfering with IFN-γ-mediated inactivation of the feedback inhibitory mechanism (mediated by IL-10) (45).

Type I interferon signalling and STAT-1 activation are important for many aspects of host immune and inflammatory reaction (46). The differentiation and phenotypic maturation of dendritic cells depend on JAK/STAT signalling pathway (26,32,33). Both TLR-ligand-induced phenotypic maturation and IL-12 production by dendritic cells are negatively affected by tick saliva (12,47). Our observation that tick saliva decrease *Borrelia*-induced IL-12 agrees with previous reports (47).

The ability of tick saliva to suppress *B. afzelii*-dependent STAT-1 activation in dendritic cells presented herein may apparently manifest in both innate and adaptive immune responses to an important tick-borne pathogen. To our knowledge, this is the first report addressing the mechanism and action of blood-feeding arthropod saliva on type I IFN signalling. We propose that tick saliva-induced impairment for STAT-1 activation may be a mechanism underlying impairment of dendritic cells activation following infection with *Borrelia* spirochaetes and partially contributing to Th2 polarization of the immune response.

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Saliva of *Ixodes ricinus* enhances TBE virus replication in dendritic cells by modulation of pro-survival Akt pathway

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ABSTRACT

It has been suggested that tick saliva facilitates transmission of tick-borne encephalitis virus (TBEV) to vertebrates. The mechanism of this facilitation has not been elucidated yet. Since dendritic cells (DCs) are among first cells attacked by the virus, we examined the amount of virus and changes induced by saliva in TBEV-infected DCs. We found that virus replication was significantly increased by saliva of *Ixodes ricinus* tick. Next, saliva-induced enhancement of Akt pathway activation was observed in TBEV-infected DCs. Akt mediated pathway is known for its anti-apoptotic and pro-survival effects. Accordingly, apoptosis of TBEV-infected DCs was declined and cellular viability increased in the presence of tick saliva. Saliva-induced enhancement of STAT1 and NF-κB was also observed in TBEV-infected DCs. In conclusion, we suggest that tick saliva provides pro-survival and anti-apoptotic signals to infected DCs via upregulation of Akt, which may have positive consequences for TBEV replication and transmission.

1. Introduction

Tick-borne encephalitis virus (TBEV) belongs to the family *Flaviviridae* of the genus *Flavivirus*. Flaviviruses, including mosquito-borne dengue virus (DEN), Japanese encephalitis virus (JEV), and West Nile virus (WNV), are enveloped viruses with single-stranded, positive sense RNA genome. The genome is transcribed as a single polyprotein and cleaved by viral and cellular proteases into three structural proteins (C, prM, and E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (reviewed in (Kaufmann and Rossmann, 2011)). After attachment, the virus is internalized into the host cell by clathrin-dependent endocytosis. Viral RNA replication takes place at the membranes of endoplasmic reticulum (ER) and Golgi-derived membranes called vesicle packets (VP).

Far Eastern subtype of TBEV can cause severe encephalitis with mortality rate up to 30% and neurological sequelae in 30–60% of survivors, European subtype infection is much milder (Mansfield et al., 2009). Transmission of TBEV to humans/vertebrates generally occurs following the bite of an infected tick. After the inoculation of virus via vector saliva, the initial round of viral replication is supported by skin dendritic cells (DCs). Infected DCs migrate to draining lymph nodes, and a second round of replication occurs in lymphoid tissues leading to viremia and systemic infection. In addition to DCs, migratory monocytes and macrophages support the replication of TBEV (Labuda et al., 1996).

Dendritic cells function as sentinels of host defense and as a professional antigen presenting cells bridge innate and adaptive immunity. Several types of dendritic cells are known including "classical" DCs, plasmacytoid DCs, monocyte-derived DCs, and Langerhans cells (Austyn, 2016). These multiple and heterogeneous subsets of DCs vary in localization, origin and cell surface marker expression. Classical DCs consist of both lymphoid ($CD8^+$) and myeloid ($CD8^-$, $CD11b^+$) type of cells. Lymphoid type of plasmacytoid dendritic cells (pDC) are characterised by robust production of type I IFN. In the skin, epidermal Langerhans cells and dermal dendritic cells are present. Myeloid dendritic cells that were used in this study can be generated from bone marrow cells by cultivating in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF).

Several reports suggested that saliva released during tick feeding facilitates transmission of tick-borne pathogens. This was documented for TBEV (Labuda et al., 1993), Powassan virus (Hermance and Thangamani, 2015), Thogoto virus (Jones et al., 1987), *Borrelia burgdorferi* sensu lato (Zeidner et al., 2002; Horka et al., 2009; Pechova et al., 2002), and *Francisella tularensis* (Krocova et al., 2003). Tick saliva

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contains numerous pharmacologically active molecules which modulate host defenses, including wound healing, hemostasis, inflammation, innate and adaptive immunity (Kotal et al., 2015). It was shown that salivary gland extract from *I. ricinus* inhibits the antiviral action of interferon in mouse fibroblasts (Hajnicka et al., 2000). The ability of tick saliva to inhibit IFN receptor signaling in mouse spleen dendritic cells was reported recently. The impairment was demonstrated by showing a decrease in phosphorylation of the signaling molecule STAT1 (Lieskovska and Kopecky, 2012). The effect of TBEV infection and tick saliva on phenotype and function of DC's was partially addressed. Tick saliva treatment increased the proportion of virus-infected cells, decreased virus-induced TNF-α and IL-6 and reduced virus-induced apoptosis (Fialova et al., 2010). DC migration, maturation and function were modulated by tick saliva in response to virus-specific TLR ligand (Skallova et al., 2008). The transduction pathways implicated in tick saliva-induced DCs modulation were not addressed.

Host cells recognize flaviviral nucleic acids via pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) in the endosomes or RIG-1-like receptors in the cytoplasm which ultimately leads to the activation of transcription factors, including interferon regulatory factor 3 (IRF3) and IRF7. In myeloid DCs, viral RNA is recognized by RIG-1 receptor (Robertson et al., 2009). This results in the production of interferon β (IFNβ) and consequent induction of interferon stimulated genes (ISGs) via the activation of JAK/STAT pathway. Such a canonical pathway of innate antiviral immunity results in the inhibition of viral replication. TBEV is able to circumvent the antiviral response by delaying the production of type I IFN (Overby et al., 2010; Miorin et al., 2012). In addition, the virus antagonizes IFN signaling by the protein NS5 which alters JAK-STAT pathway (Best et al., 2005), down-regulates the level of IFN- α and β receptor (IFNAR1) by binding to prolidase (Lubick et al., 2015), and antagonizes IRF1 (Lubick et al., 2015).

Phosphatidylinositol 3 kinase (PI3K)/Akt pathway is known as an anti-apoptotic and pro-survival signaling pathway playing a critical regulatory role in various cellular processes, including apoptosis, autophagy, RNA processing, endocytosis, and translation. Therefore it is targeted by many viruses (Diehl and Schaal, 2013). Diverse cellular Akt targets, including mTOR, GSK-3β and FOXO, confer the protection of cells through transcriptional and post-transcriptional regulation of pro-survival/pro-apoptotic proteins (Diehl and Schaal, 2013). Induction of apoptosis is considered a defense cellular mechanism after viral infection. Flaviviruses like DEN and JEV can evade this surveillance and block apoptosis via activating PI3K/Akt signaling (Lee et al., 2005). The activation of mTOR can provide essential substrates for pathogen replication, because it stimulates protein and lipid synthesis (Brunton et al., 2013). Simultaneously, Akt strengthens antiviral immune response mediated by interferon since it is essential for RIG-1/IRF3 signaling (Yeon et al., 2015). Altogether, Akt is the key regulator of apoptosis and survival having dual effect on replication of flaviviruses (Iranpour et al., 2016; Ghosh Roy et al., 2014).

In this study, we analyzed the levels of virus production, signal transduction and survival of TBEV-infected DCs in the presence or absence of tick saliva in order to reveal how tick saliva affects the efficiency of TBEV transmission from tick to the mammalian host.

2. Materials and methods

2.1. Animals

Female C57BL/6 J mice (10 weeks of age) were obtained from Charles River Laboratories. Guinea pigs, used for *Ixodes ricinus* tick feeding, were bred and maintained at the Institute of Parasitology, České Budějovice. All experiments were performed with permission from local animal ethics committee.

2.2. Tick saliva collection

Pathogen-free adult *I. ricinus* ticks from the colony maintained at the Institute of Parasitology in České Budějovice were allowed to feed in groups of 20 mating pairs on guinea pigs for 6–7 days. Afterwards, partially engorged female ticks were removed and immobilized, and a 10-μl glass capillary tube (Sigma-Aldrich) was fitted over their mouthparts. Salivation was induced by applying 2 μl of 0.1 M pilocarpine solution in ethanol on the dorsum of each tick. After 60 min, the saliva was collected, pooled and stored at − 70 °C. All saliva samples were filtered through a 0.22 μm filter (Millipore) and saliva protein concentration (ranged from 280 μg/ml to 900 μg/ml) was determined using a Bradford reagent (Sigma-Aldrich) before use.

2.3. Virus and infection

Hypr, a virulent strain of TBEV was used in the experiments. The virus was propagated in Vero E6 cells (ATCC No. CRL-1586) growing in RPMI 1640 medium (Biosera) supplemented with 3% fetal calf serum (FCS) (Biosera) and a mixture of penicillin and streptomycin (Sigma-Aldrich). Following virus infection, Vero cells were incubated for two days, and when signs of cytopathic effect were visible, infectious medium/supernatant was collected and the virus titer was determined by plaque assay. As a control to virus infected cells, the conditional medium from non-infected Vero cells was used.

2.4. Plaque assay

The porcine kidney stable cells (PS) (Kato and Inoue, 1962) were used to determine virus titer as described previously with minor modifications (De Madrid and Porterfield, 1969). PS were grown at 37 °C in L-15 medium supplemented with 3% newborn calf serum, penicillin and streptomycin (Sigma-Aldrich). Tenfold dilutions of the virus samples were placed in 24-well tissue culture plates and PS cells were added in suspension (10^5 PS cells per well). After adhesion (4 h later) the cells were overlaid with carboxymethylcellulose (1.5% in L-15 medium) and incubated for 5 days at 37 °C. Afterwards, the plates were washed with PBS and the cells were stained with naphthalene black (Sigma Aldrich). Virus titer was expressed as plaque forming units (PFU) per ml.

2.5. Generation of bone marrow-derived dendritic cells

Bone marrow-derived myeloid dendritic cells (BMDCs) were prepared as described before with minor modifications (Lutz et al., 1999; Brasel et al., 2000). Briefly, mice were sacrificed by cervical dislocation, intact femurs and tibias were removed, and bone marrow was harvested by repeated flushing with MEM. Bone marrow cells were seeded at concentration 2×10^5/ml in 10 cm-diameter Petri dishes in RPMI 1640 medium supplemented with 10% FCS, 50 mM HEPES, 2 mM glutamine, 50 μM mercaptoethanol, penicillin, streptomycin, and 30 ng/ml of recombinant mouse GM-CSF (Peprotech) and cultured for 8 days. Cells were fed on day 3 by adding 10 ml of fresh medium and on day 6 by replacing half of the volume with the fresh medium. On day 8, non-adherent cells were harvested and used as immature DCs.

2.6. Immunoblotting

DCs were seeded at 0.5×10^6 cells per well in 24-well plate. After 3 h, adhered DCs were incubated 2 h with tick saliva (10 μg/ml) prior to the infection with Hypr at multiplicity of infection (MOI) as indicated. After 1 h, 3 h, 22 h or 46 h cells were lysed in a RIPA buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.5)) in the presence of protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin) and phosphatase

inhibitors (25 mM sodium fluoride and 2 mM sodium orthovanadate). 20 μg of total proteins were separated by SDS-PAGE using an 8% gel and then electro-transferred to Immobilon-P membranes. Following blocking in 3% non-fat milk in TBS buffer the blots were incubated with the primary antibody overnight at 4 °C. Following antibodies were used: phospho-Akt (Ser^{473}), phospho-STAT1 (Tyr^{701}), phospho-NF-κB p65 (Ser^{536}), total Akt, STAT1, NF-κB p65, and β-actin (all from Cell Signaling). After washing, the membranes were incubated with secondary antibody conjugated with horse radish peroxidase (Cell Signaling). The proteins were visualized using enhanced chemiluminescence (Advansta), and their abundance was analyzed using CCD image system (Uvitec) and Image J software. Stripping of membranes was performed with Restore™ Western Blot Stripping Buffer (Thermo Scientific). Western blots images in Figures are supplemented with actual ratios of densitometric reading values of phospho-proteins to total proteins and /or to β-actin.

2.7. Flow cytometry

DCs were let to adhere on 96-well plate at the concentration of 1×10^6 cells per ml of complete culture medium without GM-CSF for 3 h. Tick saliva was added at concentration 10 μg/ml and incubated for 2 h. Cells were infected by Hypr at MOI 2. After 22 h and 46 h cells were collected, washed once in PBS with 1% FCS, fixed and stained with anti-caspase-3 antibody according the protocol (FITC active caspase-3 apoptotic kit, BD Biosciences) or anti-phospho Akt antibody conjungated with APC (BD Biosciences) followed by flow cytometry analysis. Flow cytometry was performed on FACS Canto II flow cytometer and data were analyzed using FACS Diva software, v. 5.0 (BD Biosciences). Percentage of active caspase-3 positive cells was determined by number of positive cells in FITC channel. The expression of phospho-Akt is shown as medium fluorescence intensity (MFI) in APC channel.

2.8. AlamarBlue assay

Viability of cells was measured by AlamarBlue® Cell Viability Reagent (Thermo Fisher Scientific), a redox indicator that yields a fluorescent signal in response to metabolic activity of the cells. DCs were seeded on 96-well plate at the concentration of 1×10^6 cells per ml of complete culture medium without GM-CSF, and 3 h later tick saliva was added at concentration 10 μg/ml. After 2 h incubation, cells were infected with Hypr at MOI 2. 22 h and 46 h upon infection, 1/10 vol of AlamarBlue® Cell Viability Reagent was added to cells for additional 4 h and fluorescence was measured in Synergy H1 microplate reader (BioTek) upon excitation and emission wavelengths 550 nm and 590 nm, respectively. The values from mock-infected cells were set to 100%.

2.9. Viral replication

DCs were preincubated or not with tick saliva (10 μg/ml) for 2 h or PI3K inhibitor LY294002 (10 μM, Sigma-Aldrich) for 30 min in 1.5 ml tubes followed by the infection with Hypr at MOI 2 at 37 °C. After one hour, cells were washed twice with RPMI medium to remove free virus and seeded on 96-well plate at the concentration of 1×10^6 cells per ml of complete culture medium without GM-CSF with or without tick saliva (10 μg/ml) or LY294002 (10 μM). Following 22 and 46 h incubation, plates with infected cells were transferred into at − 70 °C for at least one day and the virus titer was subsequently determined by plaque assay.

2.10. Statistical analysis

With the exception of Fig. 1C, two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test in GraphPad Prism, version 5.0 was used to compare the differences between control and treated groups. Flow cytometry data (MFI) were analyzed by 1-way ANOVA followed by Tukey test. $P \leq 0.05$ was considered as the level of statistical significance. * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$, **** - $P \leq 0.0001$.

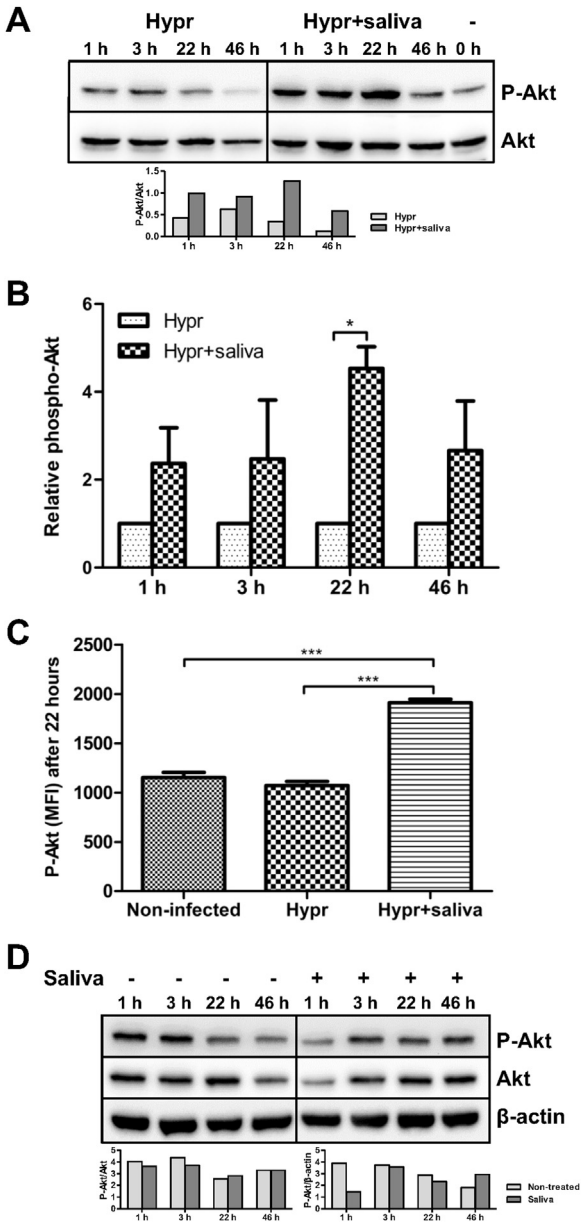

Fig. 1. The effect of tick saliva on the phosphorylation of Akt in TBEV-infected DCs. DCs were infected with Hypr (multiplicity of infection (MOI) 2) and cultured for indicated times in the presence or absence of tick saliva (10 μg/ml). (A) Cells were lysed and cellular lysates were further processed for analysis of Akt phosphorylation by western blotting using specific antibodies against phospho-Akt, Akt and β-actin. (B) Quantitative analysis of Akt phosphorylation from three independent experiments in TBEV-infected DCs treated by tick saliva. The Akt phosphorylation was normalized by total Akt level and values from Hypr-infected cells were set up to 1. (C) P-Akt was measured in fixed and permeabilized cells by staining with anti-P-Akt antibody followed by flow cytometry analysis. The expression of phospho-Akt is shown as medium fluorescence intensity (MFI). (D) DCs were incubated with tick saliva (10 μg/ml) for indicated time and phosphorylation of Akt was analyzed by western blotting. Data in column graphs are represented as means ± standard error of mean (SEM) from three independent experiment. * - $P \leq 0.05$, *** - $P \leq 0.001$.

3. Results

3.1. Tick saliva increases activation of Akt in Hypr-infected DCs

The activity of PI3K/Akt pathway is critical for survival of several viruses. We analyzed whether the phosphorylation of Akt, main downstream target of PI3K, is induced by TBEV in DCs and whether this is influenced by tick saliva in the time course of two days. We used Hypr, virulent strain of TBEV, to infect DCs at MOI 2. The infection of DCs by TBEV did not induce significant changes in the activation of Akt (data not shown). However, when DCs were infected with Hypr in the presence of saliva, the enhancement of Akt activation was observed at all time points tested (Fig. 1A, B). The same effect on Akt phosphorylation was seen in Hypr-infected cells when analyzed by flow cytometry (Fig. 1C). The statistically significant increase of Akt phosphorylation in the presence of saliva was detected by both methods at 22 h post infection (Fig. 1B and C). To see whether tick saliva alone is able to change Akt activation, the phosphorylation of this signaling molecule was evaluated. Phosphorylation of Akt was decreased shortly after saliva addition (1 h) and by time it had tendency to return to the level comparable to the one in untreated cells (Fig. 1D).

3.2. Tick saliva decreases the apoptosis and increases the viability of Hypr-infected DCs

The percentage of apoptotic cells infected by TBEV was determined. The apoptosis was evaluated by the measurement of executive protease caspase-3, which is involved in both extrinsic and intrinsic pathways of apoptotic activation. As seen in Fig. 2A, the percentage of active caspase-3 positive cells in Hypr-infected cells was relatively low (around 5% at 22 h p.i. and 10% at 46 h p.i.). However, the number of apoptotic cells decreased in saliva treated cells at both time points tested, reaching statistical significance at 46 h post infection (Fig. 2A).

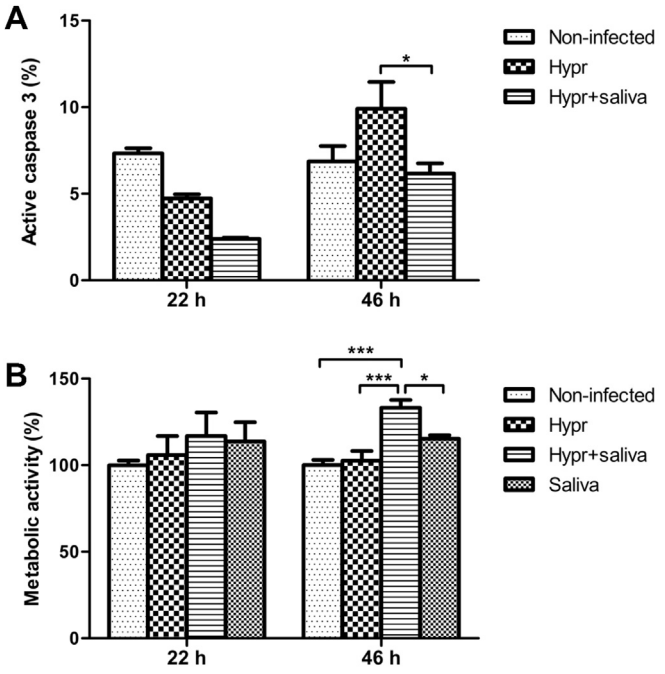

Fig. 2. The apoptosis and viability of TBEV-infected DCs in the presence of tick saliva. Dendritic cells were infected with TBEV strain Hypr at MOI 2 in the presence or absence of tick saliva (10 μg/ml). (A) The percentage of apoptotic cells was determined by flow cytometry; cells positive for caspase 3 were considered apoptotic. The percentage of apoptotic cells was measured 22 h and 46 h post infection. (B) Viability/proliferation of DCs was evaluated by AlamarBlue assay 22 h and 46 h after infection in the presence or absence of tick saliva. Three independent experiments were done to analyze apoptosis and two to analyze cellular viability. Data are represented as means ± SEM. * - $P \leq 0.05$, *** - $P \leq 0.001$.

Viability of TBEV-infected dendritic cells was evaluated by measuring the metabolic activity of cells using AlamarBlue assay. The infection by Hypr did not change the viability of cells, however, a significant increase was observed in the presence of tick saliva (Fig. 2B). Saliva itself did not influence DCs viability.

3.3. STAT1 and NF-κB phosphorylation are increased by tick saliva in Hypr-infected dendritic cells

Activation of RIG-1 receptor by viral RNA leads to induction of IFN and consequent activation of JAK/STAT signaling. To determine, whether this pathway is affected, we tested the phosphorylation of STAT1 in the presence or absence of tick saliva. Weak activation of STAT1 was detected in virus-infected cells 22 h and later after the infection and the increase in STAT1 phosphorylation was observed in the presence of saliva (Fig. 3A). The increase in P-STAT1 was partially caused by increased protein level of STAT1. Interestingly, STAT1 activation was also increased by tick saliva alone. As STAT1 phosphorylation reflects the level of IFN we have attempted to determine the level of IFN-β in saliva-treated and virus-infected cells. However, no detectable level of IFN-β was found in TBEV-infected DCs.

The transcription factor NF-κB is another RIG-1 downstream signaling molecule. When the activation of this molecule was tested no NF-κB activation was detected by virus alone. However, an increase of NF-κB phosphorylation was observed in the presence of tick saliva (Fig. 3B). It seems that both STAT1 and NF-κB signaling are enhanced by tick saliva.

3.4. The effect of the PI3K/Akt pathway inhibition on saliva-modulated apoptosis and signaling in Hypr infected DCs

The involvement of PI3K/Akt pathway in apoptosis and cell signaling was examined. To test whether PI3K inhibition can decrease the effect of tick saliva on the apoptosis, we measured the percentage of apoptotic cells in Hypr-infected cells in the presence of both PI3K inhibitor (LY294002) and tick saliva. The decrease in the number of apoptotic cells observed in the presence of tick saliva was only partially reversed by LY294002 (Fig. 4A) (without statistical significance). However the level of apoptosis seen in LY294002-treated cells was significantly decreased by tick saliva suggesting that saliva may overcome anti-apoptotic signal caused by PI3K inhibition, likely through PI3K-independent way.

Next we tested how presence of LY294002 influences saliva-provoked changes of STAT1 activation. The STAT1 phosphorylation was examined in Hypr-infected DCs in the presence of saliva and LY294002 (Fig. 4B). The virus-induced STAT1 phosphorylation was completely inhibited by LY294002 (lane 5). STAT1 phosphorylation, induced by the virus in the presence of saliva, was partially decreased by this inhibitor (lane 4 versus lane 3) suggesting that STAT1 induction may be both dependent and independent on PI3K. The efficiency of Akt inhibition by PI3K inhibitor was evaluated. While virus-induced Akt activation was fully dependent on PI3K activity, in the presence of saliva the Akt activation decreased only partially. Thus, it seems that both PI3K-dependent and independent ways of Akt activation exist in the presence of tick saliva.

3.5. Replication of TBEV in dendritic cells is increased by tick saliva and decreased by the inhibition of PI3K/Akt signaling

Finally, we set to determine whether the replication of TBEV in DCs is influenced by tick saliva. Dendritic cells were pre-incubated with tick saliva for 2 h, followed by the infection with TBEV at MOI 2. The viral titer was assessed 24 h and 48 h post infection (Fig. 5A) and in Hypr -infected cells it reached 10^5 PFU/ml. The significant increase in virus production was detected 24 and 48 h post infection in cells treated by

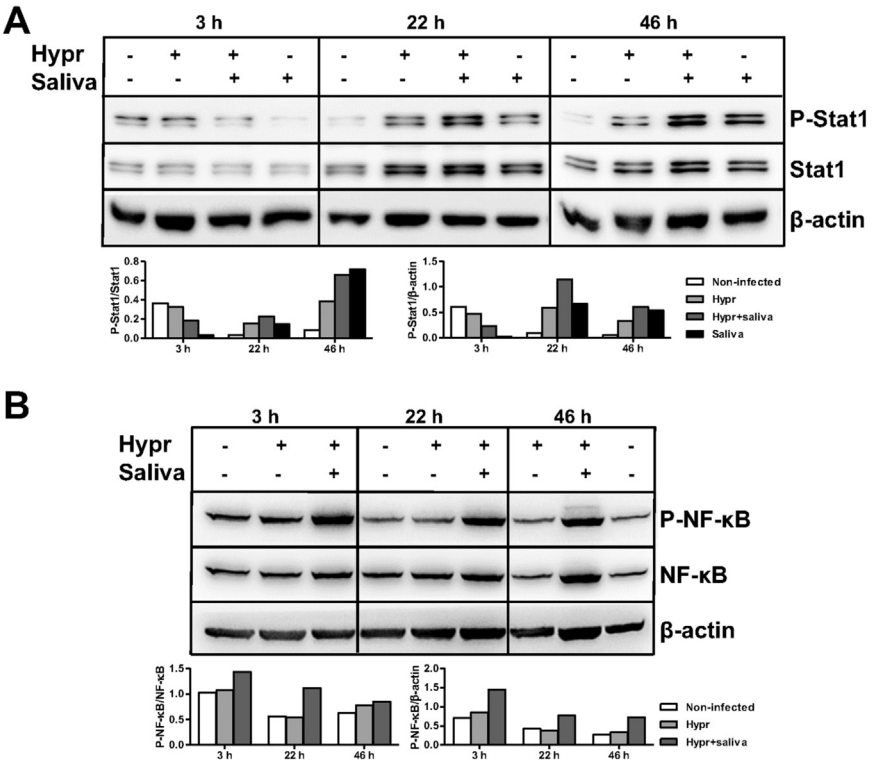

Fig. 3. The activation of STAT1 and NF-κB in TBEV-infected DCs. Dendritic cells were infected with TBEV strain Hypr at MOI 2 in the presence or absence of tick saliva (10 μg/ml). (A) Cells were lysed at indicated times and cell lysates were analyzed by immunoblotting with anti-phospho-STAT1 antibody. The membranes were, after stripping, re-probed with antibody against STAT1 protein. (B) Phosphorylation of NF-κB was examined by Western blotting using antibodies against phospho-NF-κB p65, total NF-κB p65 and β-actin. Three independent experiments were performed to examine STAT1 and NF-κB phosphorylation. Representative blots are shown.

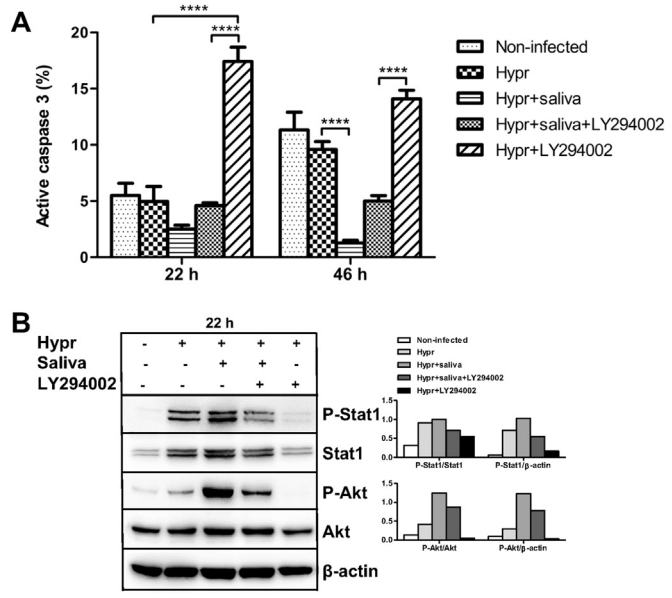

Fig. 4. A decrease of saliva effects on the apoptosis and signaling in Hypr infected DC by PI3K/Akt pathway inhibition. Dendritic cells were infected with TBEV strain Hypr at MOI 2 in the presence or absence of tick saliva (10 μg/ml) and LY294002 (10 μM) inhibitor. (A) The percentage of apoptotic cells was determined by flow cytometry; cells positive for caspase 3 were considered apoptotic. The percentage of apoptotic cells was measured 22 h and 46 h post infection. (B) Cells were lysed upon 22 h and cell lysates were analyzed by immunoblotting with anti-phospho-STAT1 and anti-phospho-Akt antibody. The membranes were, after stripping, re-probed with antibody against STAT1, Akt and β-actin protein. Two independent experiments were performed to test the effect of LY294002. Representative blots are shown.

saliva. Determination of viral E protein expression in infected DCs by flow cytometry showed its increase in the presence of saliva (data not shown). We conclude that tick saliva, in spite of enhanced activation of antiviral pathways, significantly increases the replication of TBEV in dendritic cells. To clarify the role of PI3K/Akt signaling in TBEV replication in dendritic cells we utilized the inhibitor of PI3K, LY294002. It was added to dendritic cells before virus adsorption. As seen in Fig. 5B, the inhibition of PI3K pathway resulted in significant decrease in virus production already 24 h post infection. The effect of LY294002 was more pronounced 48 h after infection, likely due to decreased viability of cells.

Effect of the combination of PI3K inhibitor and tick saliva on the replication of TBEV in dendritic cells was evaluated at 24 h post infection. A decrease of TBEV titer, observed in the presence of PI3K inhibitor, was reversed by the addition of tick saliva (Fig. 5C). It suggests that tick saliva potentiate virus replication independently of PI3K inhibition.

4. Discussion

The local skin site of tick feeding is an important focus of viral replication early after TBEV transmission by ticks. Infiltrating cells provide a vehicle for further dissemination of virus within the host as well as for the transmission of virus between infected and uninfected co-feeding ticks (Labuda et al., 1996). With regard to migratory ability of dendritic cells and permissiveness to TBEV replication *in vitro* we chose myeloid DCs to analyze tick saliva effects on TBEV-infected DCs to clarify the supportive effect of tick saliva on TBEV transmission.

The major finding in this study is that tick saliva significantly increased the replication of TBEV in DCs *ex vivo*. We assessed the degree of apoptosis and survival of TBEV-infected myeloid DCs and examined the changes at the level of signaling pathways activation in the presence of tick saliva.

Number of viruses positively affect survival of cells in order to complete their replication cycle (Ghosh Roy et al., 2014). Activation of PI3K/Akt signaling pathway is one of the strategies how viruses slow down apoptosis and thereby prolong viral replication in both acute and persistent infection (Diehl and Schaal, 2013). The analysis of signaling pathways activation revealed that the activation of anti-apoptotic PI3K/Akt pathway in DCs infected by TBEV is strongly enhanced by tick saliva. The major increase was detected one day after infection. Other flaviviruses, like DEN and JEV, activate PI3K/Akt signaling and

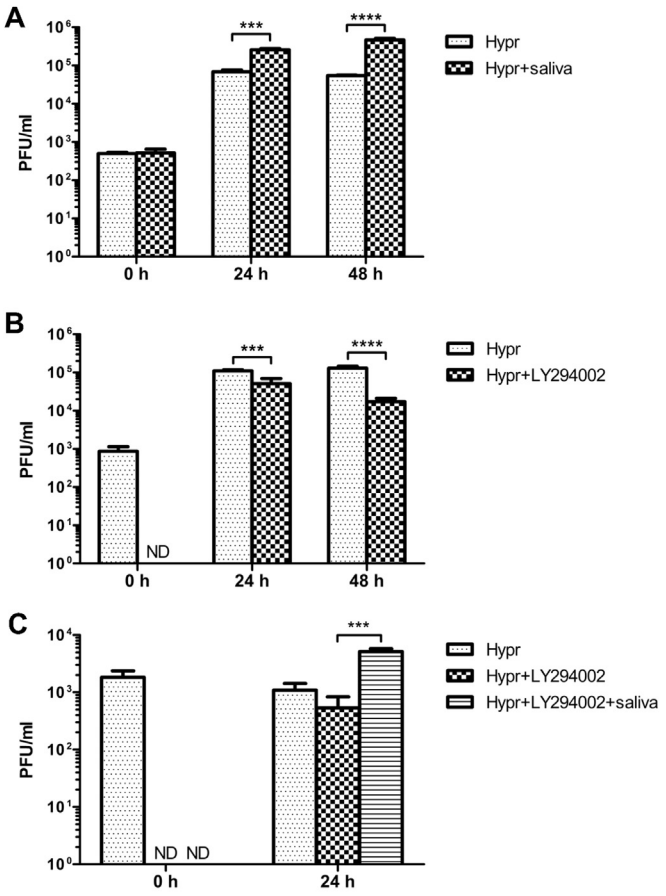

Fig. 5. Replication of TBEV in DCs. Dendritic cells were infected with TBEV strain Hypr at MOI 2 in the absence or presence of tick saliva (A) or PI3K inhibitor LY294002 (B) or both (C). Tick saliva (10 μg/ml) was added to cells 2 h prior infection, LY294002 (10 μM) was added to cells 30 min prior infection. Following 24 h and 48 h of incubation the virus titer was determined by a plaque assay as described in "Materials and Methods". Two experiments were done to examine the effect of tick saliva and representative one is shown. Data from two independent experiment were pooled together to show the effect of PI3K inhibitor on virus replication. *** and **** indicate the difference in virus production between control and saliva- or LY294002-treated cells significant at $P \leq 0.001$ and $P \leq 0.0001$, respectively.

blocking of PI3K signaling results in earlier induction of apoptosis (Lee et al., 2005). When the apoptosis was examined in TBEV-infected DCs, rather small percentage of cells were apoptotic (positive for active caspase-3) but still the significant decrease in number of apoptotic cells was found in saliva-treated cells. Similar effect of tick saliva was seen when spleen DCs were examined, though the percentage of apoptotic cells induced by TBEV was much higher (Fialova et al., 2010). The second parameter tested, relevant to activation of PI3K/Akt pathway, was the viability of cells. We found that the viability of TBEV-infected cells was significantly supported by tick saliva. The cellular viability was evaluated using redox-sensitive dye. The cellular redox environment is a balance between the production of reactive oxygen species (ROS), reactive nitrogen species (RNS), and their removal by antioxidant enzymes and small-molecular-weight antioxidants (Sarsour et al., 2009). The changes found between saliva-treated and untreated TBEV-infected cells likely reflect differences in the production of reactive species or antioxidant enzymes. Interestingly, decreased production of ROS was found in *Borrelia*-exposed neutrophils in the presence of *I. ricinus* saliva (Menten-Dedoyart et al., 2012). In any case, saliva-induced changes in the apoptosis and the viability of TBEV-infected DCs could likely influence the virus production. Of note, Akt can also positively affect the virus production though potentiation of virus entry and translation (Diehl and Schaal, 2013).

Akt and PI3K are essential for RIG-1 mediated antiviral signaling through crosstalk with IPS-1 (Yeon et al., 2015), thus higher activity of this pathway might enhance the activation of cellular antiviral defenses in saliva-treated cells. Indeed, two pathways involved in antiviral cellular defenses, JAK/STAT1 and NF-κB were strengthened by tick saliva in TBEV-infected DCs. It might seem that the potentiation of antiviral defenses is not beneficial for the virus and does not correlate with supportive effect of tick saliva. However, at least NF-κB activity is also critical for survival of cells. NF-κB is an inducible transcription factor that plays a role in the expression of over one hundred genes involved in immunity, inflammation, proliferation, and in defense against apoptosis. The expression of NF-κB target genes typically promotes cellular survival e.g. via increased expression of antioxidant proteins (Morgan and Liu, 2011). Thus increased viability of TBEV-infected cells by saliva may be mediated, in addition to Akt, via increased activation of NF-κB. If we consider the viability of infected cells as an important parameter of virus spreading *in vivo* then the potentiation of NF-κB pathway is desired.

In contrast to NF-κB, the STAT1 activation drives the expression of many pro-apoptotic genes (Kim and Lee, 2007). The enhancement of JAK/STAT1 pathway, observed by tick saliva, is not beneficial either for virus replication or cellular survival. However, the saliva-induced increase in STAT1 activation was observed only at later time points (22 h and later). Thus it is not expected that increased STAT1 activation, seen in the presence of tick saliva, could significantly influence the apoptosis or viability of cells within the first day or two. In addition, we previously showed the negative effect of tick saliva on IFN signaling when exogenous IFN was applied to activate STAT1 phosphorylation in spleen DCs (Lieskovska and Kopecky, 2012). The transient decline of STAT1 activation by saliva was also observed in TBEV-infected DCs at early time points (3-h interval is shown). The activation of STAT1 is likely mediated by interferon. However, significant induction of IFN-β was observed neither in infected nor saliva-treated cells (data not shown). We can consider that either another type of IFN mediates STAT1 activation or other receptors are responsible for STAT1 activation. For example, the activation of TAM receptors, which are involved in regulation of innate immune response and under certain circumstances cooperate with IFNR1A, triggers STAT1 phosphorylation. In fact, TAM receptors play critical role in determining the final outcome of IFN action, which can be either pro-inflammatory or immunosuppressive (Rothlin et al., 2007). In this context it is noteworthy that the saliva-induced increase of STAT1 phosphorylation is only partially dependent on PI3K phosphorylation, in contrast to STAT1 phosphorylation induced by virus alone. It suggests that PI3K-independent mechanism of Akt activation and consequently STAT1 phosphorylation does exist in saliva-treated TBEV-infected DCs. This is also supported by the experiment showing that the level of apoptosis, increased in the presence of PI3K inhibitor, is reversed by tick saliva in TBEV-infected DC. Altogether, tick saliva, through potentiation of Akt, NF-κB and STAT1 activation, has dual effect on the apoptosis and survival. Our data suggest that in TBEV-infected DCs, within first two days post infection, the anti-apoptotic and pro-survival effects of tick saliva prevail.

We can only speculate what causes the enhancement of Akt activation in TBEV-infected DCs. One possibility is that tick saliva facilitates the attachment of virus particles to cell surface. It has been suggested that Akt activation is triggered by binding of virus to host cell (Yeon et al., 2015) and the number of infected spleen dendritic cells was increased by tick saliva (Fialova et al., 2010). We assume that the percentage of TBEV-infected myeloid dendritic cells is increased by saliva too. Although we were not able to measure the percentage of positive or negative population, the expression of flaviviral E protein was increased in saliva-treated cells (data not shown). The surface molecules like integrins, TAM receptors or C-type lectin receptors are involved in attachment, entry, or binding of some flaviviruses (Perera-Lecoin et al., 2013). Saliva may induce conformational changes or

increase the expression of these receptors to facilitate binding of virus particles. Interestingly, tick saliva by itself influences the activation of Akt. The phosphorylation of Akt is transiently decreased at early time points (1–2 h) upon addition of tick saliva. We think the initial decrease of Akt phosphorylation can result from the weakened adherence observed already 1 h after the addition of saliva to cells (data not shown) and is likely mediated by perturbation of integrin signaling. The phenomenon of lost cellular adherence due to tick saliva effect was observed previously in polymorphonuclear leukocytes (PML) and endothelial cells (Francischetti et al., 2005; Montgomery et al., 2004). The downregulation of β2 integrins in PML and tick metalloprotease-mediated cleavage of α5β1 integrin in endothelial cells were implicated in saliva-induced cell detachment.

The most significant result of our study, in the context of virus transmission, is the observation of increased virus titer in DCs in the presence of tick saliva. The activation of Akt mediated pathway could apparently bring opposed effects on virus replication. Increased survival and decreased apoptosis, which is observed in tick saliva-treated cells, could have positive effect on virus replication. Facilitation of virus entry and stimulation of protein synthesis through regulation of mTOR complex 1 are among other processes which are positively regulated by Akt (Diehl and Schaal, 2013) and likely contribute to increased replication. The potentiation of antiviral cellular defenses, on the other hand, could impede virus replication. When the effect of PI3K inhibitor (leading to suppression of Akt) on TBE virus replication was analyzed in DCs, the significant decrease in the amount of produced virus was observed. It can be explained by a decrease of the endocytotic uptake, since endocytosis of several viruses is diminished after inhibition of PI3K/Akt pathway (Diehl and Schaal, 2013). To measure the uptake of virus particules by DCs in the presence of tick saliva is our future plan. Interestingly, it appears that saliva-induced effects accompanied by Akt phosphorylation are partially independent of its main upstream kinase PI3K since tick saliva could overcome the apoptotic effect of PI3K inhibition as well as the effect of PI3K inhibitor on the virus replication in TBEV-infected DCs. In agreement, the Akt phosphorylation in the presence of tick saliva was only partially reduced by PI3K inhibitor. Of note, blocking of PI3K activation did not affect JEV and DEN virus production, but accelerated the appearance of cytopathic effect (Lee et al., 2005).

Last important note which should be discussed in relation to TBEV transmission is pretreatment of cells by tick saliva before addition of the virus. Although TBE virus is inoculated by ticks together with saliva, some saliva inoculation into the skin precedes inoculation of the virus and it is probably transmitted by parts in accordance with repeated salivation and blood feeding. Considering that in general every 5–30 s there is an alteration between sucking and salivation (Kaufman, 1989) and the virus titer in salivary glands increases markedly (10–100 times) at least during the first three days since feeding commencement (Alekseev and Chunikhin, 1990), most of the virus inoculum comes into the skin already pretreated with tick saliva.

In conclusion, we report here that tick saliva significantly increased the replication of TBEV in DCs. It can be of particular significance for spreading of the virus from tick feeding site to draining lymph nodes. The effect of tick saliva on virus replication is complex. We suggest that the TBEV replication is at least partly affected by saliva due to modulation of Akt-mediated signaling resulting into the pro-survival and anti-apoptotic effect. However, it is not clear, whether the observed increase of Akt activation by tick saliva is the cause, the consequence, or both of increased virus replication in DCs.

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Review Article

Modulation of host immunity by tick saliva

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ABSTRACT

Next generation sequencing and proteomics have helped to comprehensively characterize gene expression in tick salivary glands at both the transcriptome and the proteome level. Functional data are, however, lacking. Given that tick salivary secretions are critical to the success of the tick transmission lifecycle and, as a consequence, for host colonization by the pathogens they spread, we thoroughly review here the literature on the known interactions between tick saliva (or tick salivary gland extracts) and the innate and adaptive vertebrate immune system. The information is intended to serve as a reference for functional characterization of the numerous genes and proteins expressed in tick salivary glands with an ultimate goal to develop novel vector and pathogen control strategies.

Significance: We overview all the known interactions of tick saliva with the vertebrate immune system. The provided information is important, given the recent developments in high-throughput transcriptomic and proteomic analysis of gene expression in tick salivary glands, since it may serve as a guideline for the functional characterization of the numerous newly-discovered genes expressed in tick salivary glands.

Contents

List of abbreviations: Akt, protein kinase B; BMDMs, bone marrow-derived macrophages; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CCL, chemokine (C–C motif) ligand; CCR, C–C motif receptor; CD, cluster of differentiation; ConA, concanavalin A; CTL, cytotoxic T lymphocytes; CXCL, chemokine (C–X–C motif) ligand; DC, dendritic cell; ERK, extracellular signal-regulated kinase; IDO, indoleamine 2,3 deoxygenase; IFN, interferon; Ig, immunoglobulin; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; LC, Langerhans cell; LFA-1, leukocyte function-associated antigen-1; LPS, lipopolysaccharide; MC, mast cell; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; NET, neutrophil extracellular trap; NF-κB, nuclear factor kappa light chain-enhancer of activated B cells; NK, natural killer; NO, nitric oxide; PBL, peripheral blood leukocytes; PGE_2, prostaglandin E_2; PI3k, phosphatidylinositol-3 kinase; PMNs, polymorphonuclear lymphocytes; RANTES, regulated upon activation, normal T cell expressed and secreted; ROS, reactive oxygen species; SGE, salivary gland extract; STAT, signal transducer and activator of transcription; sTNFRI, soluble TNF receptor I; TGF, transforming growth factor; Th, helper T cell; TLR, toll-like receptor; TNF, tumor necrosis factor; VLA-4, very late activation-4.

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1. Introduction

Ticks are obligatory blood-feeding arthropods that belong to the subclass *Acari*, order Ixodida, and three families: *Ixodidae* (hard ticks), *Argasidae* (soft ticks), and Nuttallielidae. Soft ticks feed repeatedly for minutes to hours, while hard ticks usually stay attached to their hosts and feed for several days or even weeks, but only once in each life stage [1,2]. The amount of blood ingested is species and life-stage specific, with females of some tick species increasing their volume up to 200 times by the end of blood feeding [3].

Ticks are important vectors that transmit a wide range of pathogens. The most common tick-borne pathogens are viruses and bacteria, but fungi, protozoa, and helminths can also be transmitted [4]. Clinically and epidemiologically, the most important tick-borne diseases are: tick-borne encephalitis (TBE), caused by the TBE virus; Lyme disease, caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex in Europe and *B. burgdorferi* sensu stricto in the USA; tick-borne spotted fever, caused by *Rickettsia* spp.; anaplasmosis, caused by *Anaplasma* spp.; and babesiosis, caused by *Babesia* spp. protozoa [5,6]. Pathogens have different life cycles, but the transmission usually begins with a tick biting an infected vertebrate host and pathogen uptake by the tick in the blood meal. Pathogens, e.g. *Borrelia* spp. spirochetes then stay in the midgut and wait until next feeding, which triggers their proliferation and migration through the midgut wall to hemocoel and, ultimately, to the salivary glands. Moreover, spirochetes interact with some midgut and salivary components that induce *Borrelia* proliferation or increase their infectious potential [7]. When the tick bites its next vertebrate host, pathogens are transmitted via tick saliva. In some tick species the pathogens are transmitted transovarially from the female to laid eggs, thus keeping the level of prevalence in the tick population [8]. Tick saliva has been shown to facilitate pathogen transfer to the vertebrate host by virtue of its pharmacological properties, including modulation of the vertebrate immune system [9–11]. Moreover, tick saliva contains toxins belonging to families also found in venomous animals, such as spiders or snakes, and that can induce paralysis and other toxicoses [12].

To secure uninterrupted blood uptake, ticks suppress and evade the complex physiological host immune and homeostatic responses that are raised against them. Hemostasis, which includes coagulation, vasoconstriction, and platelet aggregation, is the first innate host defense mechanism against the mechanical injury caused by intrusion of tick mouthparts into the host skin. This early vertebrate host response further includes complement activation and inflammation, with the host inflammatory response including, among other factors, rapid leukocyte infiltration after skin injury [13]. Keratinocytes, endothelial cells, and resident leukocytes such as mast cells, dendritic cells, and macrophages make immediate contact with tick saliva or the tick hypostome and are activated. Pro-inflammatory chemokines and cytokines including interleukin-8 (IL-8), tumor necrosis factor (TNF), and IL-1β are released to recruit neutrophils and other inflammatory cells to the area of tick infestation [14]. Following tick feeding, there is activation of both the cellular and humoral branches of vertebrate adaptive immunity [15]. Activated memory T and B cells (in the case of secondary infestation) amplify the host inflammatory response to ticks by releasing specific cytokines and producing antibodies that target tick salivary or mouthpart-derived antigens to activate complement or sensitize mast cells and basophils [9,14,15]. The strength and specificity of the host immune response and its effect on tick physiology depend on the host and tick species, the host's health, and its genotype [16]. The same is true for tick defense mechanisms, since both tick salivary components and host immune mechanisms have been co-evolving. As a result, the tick–host interaction can be considered an "arms race" between the new defense mechanisms developed by the host and the evasion strategies developed by ticks [17]. As an adaptation to blood feeding, ticks secrete a complex mixture of immunomodulatory substances in their saliva that suppress both innate and adaptive host immune responses that can cause pain, itch, blood flow disruption in the tick feeding cavity, or even direct damage to the tick, thereby subverting tick rejection and death [18–20]. Despite the specificity of tick salivary component targets, there is also redundancy at the molecular, cellular, and functional level [9,13]. The richness and diversity of tick salivary compounds have been established in several transcriptomic studies over the last 15 years and, more recently, by next generation sequencing (NGS) studies.

The rapid developments in NGS and proteomics are reflected in the recent progress made in tick research, in which several transcriptomic and proteomic studies have been published over the last few years. These studies represent a rich data source that provides the basis for functional studies and investigation of gene expression dynamics during tick feeding and different physiological states. For instance, significant differences in the salivary proteome of partially and fully engorged female *Rhipicephalus* (*Boophilus*) *microplus* ticks have been described [21]. More recently, a transcriptomic study described over 800 immuno-proteins in *Amblyomma americanum* saliva during 24–48 h of feeding [22]. A transcriptomic analysis of *Dermacentor andersoni* salivary glands resulted in over 500 singletons and 200 clusters in which a number of sequences with similarity to mammalian genes associated with immune response regulation, tumor suppression, and wound healing were identified [23]. By combining transcriptomic and proteomic approaches, nearly 700 proteins were identified in *D. andersoni* saliva after 2 and 5 days of feeding, from which 157 were postulated to be involved in immunomodulation and blood feeding [24]. Schwarz and colleagues performed a comprehensive study of *Ixodes ricinus* salivary and midgut transcriptomes and proteomes and found that the transcriptomic and proteomic dynamics did not 100% overlap in different tick tissues [25]. A recent study by Kotsyfakis and colleagues characterized transcriptional dynamics in the *I. ricinus* female and nymph salivary glands and midguts at various feeding time points [26], and established that some gene families show stage- and time-specific expression, possibly via epigenetic control. In addition, the genes encoding secreted proteins exhibited a high mutation rate, possibly representing a mechanism of antigenic variation, and analysis of the midgut transcriptome revealed several novel enzymes, transporters, and antimicrobial peptides [26]. A transcriptomic analysis of *Amblyomma maculatum* salivary glands revealed almost 3500 contigs with a secretory function [27]. Another sialome (salivary gland transcriptome) of *Amblyomma* ticks was published by Garcia and colleagues [28]: the authors analyzed samples from *Amblyomma triste* nymphs and females, *Amblyomma cajennese* females, and *Amblyomma parvum* females and focused on putative transcripts encoding anticoagulants, immunosuppressants, and anti-inflammatory molecules. A further study characterized *A. americanum* nymph and adult proteomes and compared the data with other *Amblyomma* species [29]. A *Rhipicephalus pulchellus* tick sialome study revealed differences between males and females [30], with the sequences identified used for a preliminary proteomic study to identify 460 male and over 2000 female proteins. A sialomic study was also performed in *Haemaphysalis flava* that revealed tens of thousands of genes, some of which were putative secreted salivary proteins thought to be involved with blood feeding and ingestion [31].

A *Rhipicephalus sanguineus* salivary proteome showed recycling of host proteins and their secretion back into the host [32]. Lewis and colleagues used a transcriptomic approach to characterize immunogenic *Ixodes scapularis* salivary proteins present after 24 h of feeding [33]; these appeared to be involved in tick feeding even before the majority of pathogens could be transmitted.

In addition to the analysis of secreted tick salivary proteins, tick-feeding lesions on the host have been analyzed by high-throughput and histological methods. Recently, the feeding lesion of *D. andersoni* was described in detail together with microarray analysis of host gene expression dynamics, thereby characterizing the inflammatory infiltrate at the feeding site and the changes occurring in the epidermal and dermal compartments near the tick [34,35]. The skin lesions examined from rats infested by *Ornithodoros brasiliensis* showed edema, muscle degeneration, and hemorrhage [36], with the rats themselves presenting with a bleeding tendency and signs of toxicosis. *O. brasiliensis* salivary gland homogenates delayed wound healing and had anti-proliferative or even cytotoxic activity on cultured epithelial cells [37]. An analysis of skin-draining lymph nodes in goats repeatedly infested with *A. cajennese* nymphs revealed an increased number of antigen presenting cells (APCs) such as B lymphocytes, macrophages, and dendritic cells [38]. A skin lesion from a human infested with female *Amblyomma testudinarium* was characterized by an inflammatory infiltrate and an eosinophilic cement in the center of the lesion [39]. Feeding lesions from rabbits injected with salivary gland extract (SGE) collected from *R. sanguineus* ticks after 2, 4, and 6 days of feeding showed signs of inflammation, especially at day four [40], suggesting that molecules present in *R. sanguineus* SGE have high immunogenicity and that immune reaction raised against SGE is stronger than the immunomodulatory action of *R. sanguineus* salivary effectors.

Such high-throughput studies in both ticks and hosts and complemented with histological information and detailed characterization of salivary components have made a valuable contribution to our knowledge of the dynamic processes occurring at the tick–host interface. However, experiments with saliva or SGE highlight the complexity of host modulation by the tick in vivo. Characterizing individual salivary components can help link specific pathophysiological events to particular molecules to provide a complete picture of tick–host interactions. In this review, we focus on the immunomodulatory actions of whole tick saliva or salivary gland extracts (SGE) rather than the effects of the individual salivary components, since these are reviewed elsewhere [13,41,42].

2. The role of tick saliva in modulating host hemostasis and complement

Ticks have developed various mechanisms to counteract the hemostatic responses of the host so that they can successfully feed on blood for many days [13,19]. Serine proteases are key players in host hemostasis and, therefore, are specifically targeted by the wide range of serine protease inhibitors present in tick saliva. The net result is that the physiological balance between host proteases and endogenous anti-proteases is impaired. Tick salivary secretions also contain vasodilators, platelet activation inhibitors, and coagulation modulators, as reviewed elsewhere [14,43,44].

Complement is a cascade of proteolytically-activated components that eventually leads to the creation of pores in the walls of microbes, leading to their destruction. There are three main complement activation pathways: classical, alternative, and lectin; the central reaction in all pathways is the conversion of complement component C3 to C3a and C3b [45,46]. The inhibition of the host alternative complement pathway is crucial for tick feeding and, indeed, the saliva of several *Ixodes* species inhibits this pathway [47,48]. In an in vitro study, the ability of tick saliva to counteract complement activity varied according to the animal species source of serum, with specificity shown towards the most common hosts for each *Ixodes* species [49]. Several anti-complement molecules have been identified to date; however, a detailed description is beyond the scope of this review. Further information about the role of complement in tick–host interactions can be found in the reviews by Schroeder and colleagues [50] or Wikel [14].

3. Innate immunity and tick saliva

Innate immune responses against tick feeding involve the activation of resident immune cells that initiate and promote the local inflammatory response as a reaction to skin damage. The resident leukocytes are macrophages, Langerhans cells (LCs), mast cells, or innate lymphoid cells, and pro-inflammatory mediators are also released by endothelial cells and keratinocytes [51]. These mediators and complement components are chemotactic for circulating inflammatory cells including neutrophils and monocytes.

4. Interaction of macrophages and monocytes with tick saliva

Macrophages are APCs as well as cytokine and chemokine producers [52]. They can be further divided into two different subpopulations: (i) bone marrow-derived hematopoietic macrophages, which circulate as monocytes and, after extravasation at the site of inflammation, differentiate into pro-inflammatory [53] or alternatively-activated macrophages [54] and (ii) tissue-resident macrophages of yolk sac origin that are found in many organs including the skin; the latter tend to be more immune-modulatory [55]. These macrophage subpopulations differ with respect to cytokine production, receptor expression, and their overall effect on any subsequent immune response [54,56,57].

Numerous interactions have been identified between macrophages, tick saliva or SGE, and pathogens, suggesting that they play a major role in host defenses against ticks and tick-borne infectious agents. The effects of saliva or SGE on macrophages are summarized in Fig. 1.

I. ricinus SGE inhibited superoxide and nitric oxide (NO) production by *Borrelia afzelii*-activated macrophages, which led to the inhibition of *Borrelia* killing in a murine host [58]. *I. ricinus* SGE also reduced phagocytosis of *B. afzelii* spirochetes by murine macrophages and inhibited IFN-γ- and *B. afzelii*-stimulated TNF production by macrophages [59]. It was recently shown that *I. ricinus* saliva could induce the production of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2) in splenocytes [60]. MCP-1 attracts

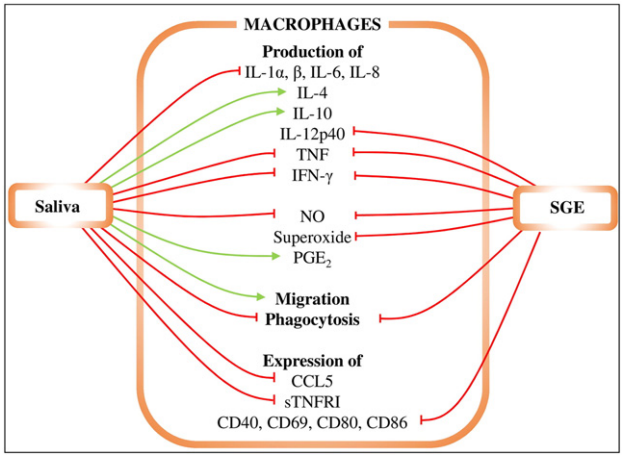

Fig. 1. The effects of saliva and SGE on macrophages. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits production of IL-1α, IL-1β, IL-6, IL-8, TNF, IFN-γ, NO, superoxide, and CCL5, as well as expression of sTNFRI and phagocytosis. Tick saliva increases production of IL-4, IL-10, and PGE_2 and macrophage migration. Tick SGE inhibits production of IL-12p40, TNF, IFN-γ, and NO, expression of CD40, CD69, CD80, and CD86, and phagocytosis.

monocytes, and MIP-2 is a chemokine secreted by monocytes and macrophages that is chemotactic for neutrophils.

Similarly, *I. scapularis* saliva inhibited TNF, IL-1β, IL-6, and IL-12p40 production by murine bone marrow-derived macrophages (BMDMs) after stimulation with lipopolysaccharide (LPS) or *Anaplasma phagocytophilum*. It was further reported to inhibit IL-8 secretion by human peripheral blood mononuclear cells (PBMCs) after TNF stimulation [61] and NO synthesis upon LPS stimulation [62].

Incubation with SGE isolated from *R. microplus*, a tick of veterinary importance, resulted in diminished expression of the co-stimulatory molecules CD80, CD86, CD40, and CD69 on the surface of bovine macrophages after 24 h of LPS stimulation, which was accompanied by a decrease in TNF, IFN-γ, and IL-12 production [63]. Conversely, CD86 expression was increased in the murine macrophage cell line RAW 264.7 in response to *R. microplus* SGE and LPS but not SGE alone. Furthermore, SGE had no effect on CD40 and CD80 expression [64]. However, both bovine primary macrophages and murine macrophage cell line displayed an increase of CD86 expression after 6 h incubation with LPS and SGE. [64]. These partially contradictory observations may be attributed to the host specific response. The difference may also originate from altered signaling in immortalized cell line, as CD86 upregulation was shown to be at least partially dependent on the ERK1/2 pathway and may, therefore, promote polarization of the immune response towards a less pro-inflammatory Th2 profile (see below) [64]. In another study, *R. sanguineus* saliva diminished NO production by IFN-γ-activated macrophages and thus impaired *Trypanosoma cruzi* killing. The authors suggested that decreased NO production was due to a saliva-induced cytokine imbalance, leading to decreased NO synthase activity [65]. Similar to the results with primary macrophages, SGE from *Rhipicephalus appendiculatus* affected cytokine production by the murine macrophage cell line JA-4. SGE from *R. appendiculatus* inhibited the transcription of IL-1α, IL-10, and TNF after macrophage stimulation with LPS. NO production was also lower, in accordance with the similar effect observed with *I. ricinus* saliva [58,66].

Dermatocentor variabilis (Table 1) saliva has been shown to impair phagocytosis and alter gene expression in the murine macrophage cell line IC-21, as well as increase basal and platelet-derived growth factor (PDGF)-stimulated macrophage migration and the expression of the Th2-specific cytokines IL-4 and IL-10 [67].

The tick salivary component prostaglandin E_2 (PGE_2) subverted macrophage secretion of pro-inflammatory mediators and was able to recruit fibroblasts to heal tick-bite wound [68]. In addition to PGE_2 from tick saliva, the saliva of *D. variabilis* upregulated PGE_2 secretion in IC-21 murine peritoneal macrophages and reduced secretion of the pro-inflammatory mediators CCL5, TNF, and soluble TNF receptor I (sTNFRI) via a PGE_2-dependent mechanism mediated by cAMP [68].

In summary, the tick saliva of various tick species inhibits the pro-inflammatory activities of macrophages, supporting a major role for macrophages in anti-tick defenses.

5. Dendritic cells and tick saliva

Dendritic cells (DCs) are APCs and are part of the innate immune system. After immature (unstimulated) DCs recognize and phagocytose pathogens, they mature and migrate to draining lymph nodes where they present antigens derived from the processed pathogen to CD4+ T cells, which subsequently launch an adaptive immune response. Thus, DCs initiate host adaptive immunity via presentation of pathogenic antigens. Two DC states exist: an immature form present in skin or mucosae and a mature form in lymphoid tissues. Langerhans cells (LCs) are a specialized resident cell type found in the vertebrate skin. Similar to macrophages, LCs have two origins and share many properties with macrophages [69]; therefore, they are sometimes considered to be a subtype of tissue macrophage [57]. Immature DCs primarily have an antigen uptake and presenting function, while mature DCs effectively stimulate T cells but have limited phagocytic activity. Several studies suggest that there are interactions between tick saliva and DCs [70–72]. For a review of the interactions between DCs, tick saliva, and *Borrelia*, see [73].

Oliveira and colleagues studied the effect of *R. saguineus* saliva on DC migration and function, and found that tick saliva reduced immature DC migration towards macrophage inflammatory proteins MIP-1α and MIP-1β but not MIP-3β [74]. Tick saliva also inhibited the chemokine RANTES by reducing expression of its surface receptor CCR5 [74]. DC maturation was impaired via toll-like receptor (TLR) signaling [75]. However, the inhibition of migration was limited to immature DCs. DC maturation and differentiation was inhibited in the presence of *A. cajennese* saliva [76]; in this study, the DCs showed reduced expression of CCR5 and CCR7 and, therefore, diminished migration towards the corresponding chemokines. Furthermore, tick saliva polarized cytokine production towards a Th2 phenotype. The authors suggested that most of the observed effects were due to the presence of PGE_2 in tick saliva [76]. *I. scapularis* saliva has displayed various effects on bone marrow-derived DCs: it inhibited TNF and IL-12 production upon stimulation of different TLRs, in particular TLR-2, TLR-4, or TLR-9 [77], and the DC's ability to stimulate antigen-specific CD4+ proliferation and IL-2 production was also suppressed [77]. LC-deficient mice induced Th1 responses after *I. scapularis* infestation, demonstrating the requirement for LCs in attenuating tick-mediated Th1 responses in regional lymph nodes [78].

CD40 or TLR3, 7, and 9 ligation impaired DC maturation, and *I. ricinus* saliva inhibited DC migration in vivo and antigen presentation [79]. *I. ricinus* saliva has also been shown to impair Th1 and Th17 polarization in DCs [79] and activation of specific CD4+ T lymphocyte subsets by *Borrelia*-exposed DCs [80]. In the latter study, *I. ricinus* saliva decreased DC phagocytosis of *B. afzelii*. Interestingly, *I. ricinus* saliva inhibited DC production of both Th1 cytokines (TNF and IL-6) and the Th2 cytokine IL-10 after 48 h (but not 24 h) of incubation with *B. afzelii* [80]. *I. ricinus* saliva also impaired DC maturation and production of TNF and IL-6 in response to infection with TBE virus [81]. Lieskovská and Kopecky studied the signaling pathways activated in DCs via TLR-2 ligand and *B. afzelii* in the presence of tick saliva [82]; upon both types of activation, the NF-κB and phosphatidylinositol-3-kinase (PI3K)/Akt pathways were inhibited by *I. ricinus* saliva. When activated by *Borrelia* spirochetes, TNF levels decreased in DCs due to selective suppression of ERK1/2, Akt, and NF-κB as a result of tick saliva mimicking the native inhibitors. Tick saliva also attenuated IFN-β production, and IFN-β triggered signal transducer and activator of transcription-1 (STAT-1) activation [83]. A summary of the known interactions between DCs and tick saliva is shown in Fig. 2.

6. Mast cells and tick saliva

Mast cells serve as sentinel cells and reside in many tissues. They are divided into two main types based on the presence of mast cell-specific proteases: connective tissue mast cells, which produce both tryptase and chymase (MC_{TC}), and mucosal mast cells, which produce only tryptase (MC_T) [84]; skin mast cells are of the first type. Upon exposure to pathogens or other stimuli, activated mast cells degranulate and release a variety of pre-stored mediators including vasoactive compounds, serine proteases, histamine, and cytokines. Activated mast cells also secrete newly synthesized mediators to recruit more inflammatory cells [85].

The immunological importance of mast cells in tick–host interactions remains unclear. Mast cell numbers increase after secondary or subsequent tick infestations, but remain unchanged during primary tick infestations [86–88]. The number of degranulated mast cells is also significantly higher after repeated tick infestations. Mast cell-deficient mice have been shown to develop some resistance to *D. variabilis* after repeated exposure, similar to wild type mice [89]. On the other hand, mast cell-deficient mice were not resistant to *Haemaphysalis longicornis*, with tick resistance re-established after mast cell injection [90,91]. Such

Table 1
The effects of tick saliva, SGE, or feeding on immune cell populations.

Tick	Saliva/SGE/Feeding	Effect	Reference
Macrophages			
Dermatocentor variabilis	Saliva	Impaired phagocytosis and altered gene expression, stimulation of migration	[67]
		Stimulation of PGE_2 production, inhibition of cytokine production	[68]
Ixodes ricinus	SGE	Inhibition of superoxide and NO production	[58]
		Inhibition of phagocytosis and TNF production	[59]
Ixodes scapularis	Saliva	Inhibition of cytokine production	[61]
		Inhibition of NO production	[62]
Rhipicephalus appendiculatus	SGE	Inhibition of cytokine and NO production	[66]
Rhipicephalus microplus	SGE	Altered surface molecule expression, inhibition of cytokine production	[63,64]
Rhipicephalus sanguineus	Saliva	Inhibition of NO production	[65]
Dendritic cells			
Amblyomma cajennese	Saliva	Inhibited maturation and differentiation; reduced migration due to decreased expression of receptors; polarization towards Th2 cytokines	[76]
I. ricinus	Saliva	Inhibited maturation, migration and antigen presentation; blocked Th1 and Th17 polarization	[79]
		Inhibited proliferation, phagocytosis and cytokine production	[80]
		Impaired maturation and cytokine production	[81]
		Inhibition of signaling pathways	[82,83]
I. scapularis	Saliva	Inhibition of proliferation and cytokine production	[77]
R. sanguineus	Saliva	Reduced migration, maturation and cytokine production	[74,75]
Basophils			
Amblyomma cajennense	Feeding	Increased amount of basophils in feeding cavity	[121]
Amblyomma dubitatu	Feeding	Increased amount of basophils in feeding cavity	[121]
Eosinophils			
Soft and hard ticks	Feeding	Increased amount of eosinophils in feeding cavity	[36,88,120–122]
Hard ticks	SGE	Inhibition of attraction to the feeding site	[123,124]
I. ricinus	Saliva	Basophil activation via MCP-1 released from splenocytes	[60]
Neutrophils			
Soft and hard ticks	SGE	Anti-IL-8 activity	[123,130]
Amblyomma americanum	SGE	Altered dynamics of chemokine activity	[125]
I. ricinus	Saliva	Decrease in ROS production	[132]
I. scapularis	Saliva	Inhibition of granule release, infiltration, phagocytosis	[133]
		Reduced adhesion of polymorphonuclear leukocytes	[134]
R. appendiculatus	SGE	Altered cytokines mRNA production by peripheral blood leukocytes	[170]
R. microplus	SGE	Inhibition of phagocytosis	[135]
Lymphocytes			
Soft and hard ticks	Saliva, SGE	Polarization of the immune response towards Th2 via cytokines	[66,71,139,159,161, 162,171,172]
Amblyomma variegatum	SGE	Inhibition of lymphocyte proliferation	[142]
Dermacentor andersoni	SGE	Reduced T cells proliferation	[149,150]
		Reduced Th1 cytokine production	[173,174]
	Saliva, SGE, feeding	Inhibition of integrin expression	[163]
	SGE, feeding	Increased IL-4 and IL-10 levels	[164]
Haemaphysalis bispinosa	Feeding	Reduction in T lymphocyte count and proliferation, increased CD4+/CD8+ ratio	[153]
Hyalomma anatolicum anatolicum	Feeding	Reduction in T lymphocyte count and proliferation, increased CD4+/CD8+ ratio, increase in circulating B lymphocyte count	[153]
I. ricinus	SGE	Inhibition of lymphocyte proliferation	[142]
		Suppression of B cell proliferation, inhibition of IL-10 production, reduction of markers on the surface of T and B cells	[143]
	Saliva	Inhibition of T cell proliferation	[144]
		Induction of Th2 differentiation of CD4+ T cells via dendritic cells	[71]
	Feeding	Increased CD4+/CD8+ ratio	[147]
		Inhibited proliferation and responsiveness	[145]
		Reduced amount of specific Ig against antigen, no change in total Ig amount	[148,157]
I. scapularis	Saliva	Inhibition of IL-2 production by T cells, inhibition of splenic T cell proliferation	[62,140,141]
	Feeding	Inhibition of Th17 immunity, priming of a mixed Th1/Th2 response during secondary infestation	[35]
	SGE, feeding	Increased IL-4 levels	[165]
R. appendiculatus	SGE	Inhibition of lymphocyte proliferation	[142]
R. microplus	Feeding	Decreased T and B lymphocyte percentage among PBLs	[151]
	Saliva	Decreased PBL responsiveness to phytohemagglutinin	[151]
		Inhibition of the blastogenic response of mononuclear cells	[175]
R. sanguineus	Feeding	Suppressed response to mitogens	[152]
	Saliva	Suppressed response to mitogens	[152]
	SGE	Suppressed Ig production by PBL	[156]
NK cells			
A. variegatum	SGE	Decreased NK cell activity	[168]
Dermatocentor reticulatus	SGE	Decreased NK cell activity	[167]
Haemaphysalis inermis	SGE	Decreased NK cell activity	[168]
I. ricinus	SGE	Suppression of NK cell cytotoxicity	[169]

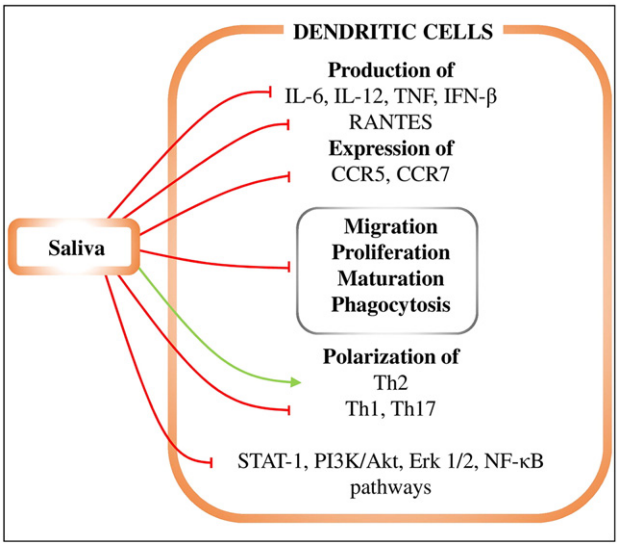

Fig. 2. The effects of saliva on dendritic cells. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits production of IL-6, IL-12, TNF, IFN-β, and RANTES cytokines. It also inhibits expression of CCR5 and CCR7, DC migration, proliferation, maturation, and phagocytosis, and STAT-1, PI3K/Akt, Erk1/2, and NF-κB signaling pathways. The saliva induces Th2 polarization while suppressing Th1 and Th17 differentiation.

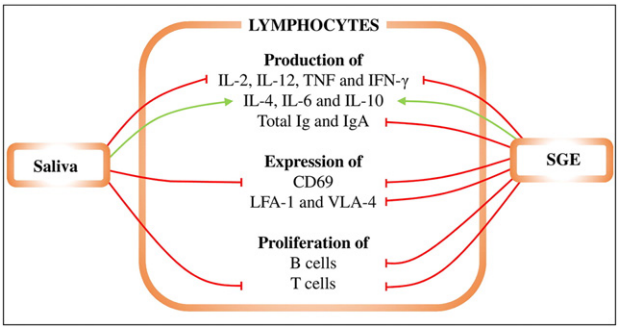

Fig. 4. The effects of saliva and SGE on B and T lymphocytes. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits T cell proliferation, CD69 expression, and production of IL-2, IL-12, TNF, and IFN-γ by lymphocytes. In contrast, it increases production of IL-4, IL-6, and IL-10. Tick SGE has the same effects as tick saliva and, furthermore, suppresses LFA-1 and VLA-4 expression, proliferation of B cells, and total Ig and IgA production.

differences might be due to species-specific host responses or other unknown factors. Highly tick-resistant zebuine cattle breeds have more dermal mast cells than taurine breeds [92]. F2 crossbreeds of these two cattle were resistant to *R. microplus* infestation, and infestation with *R. micrpoplus* larvae induced significant increases in dermal mast cell numbers. Mast cells are major producers of the inflammatory mediator histamine, and ticks can affect histamine actions by either binding histamine via histamine-binding lipocalins [93,94] or by promoting its secretion via histamine release factor [95], further evidence of the ambiguous role for mast cells in tick feeding responses. One explanation for histamine binding followed by its release can be explained by the need to suppress inflammatory responses at the early stage of feeding, followed by an increased need for vascular permeability during the rapid engorgement phase of tick feeding.

7. Granulocytes and tick saliva

Granulocytes are bone marrow-derived myeloid leukocytes that contain granules in their cytoplasm. The granulocyte group consists of three major cell types: basophils, eosinophils, and neutrophils [96].

8. Basophils and tick saliva

Basophils are IgE-activated granulocytes that, unlike tissue-resident mast cells, circulate in the blood. They play a critical role in the IgE-mediated development of chronic allergic reactions and inflammation [97,98], and they can also promote polarization towards Th2 responses by IgE-independent antigen presentation in mice [99,100]. Basophils are recruited to a tick-feeding site and accumulate in the host skin during second and consequent (but not primary) tick infestation, where they act as important tick rejection factors [101,102]. After migration to the site of injury, basophils degranulate and release mediators such as histamine to reject ticks in a host reaction known as cutaneous basophil hypersensitivity [103]. Similar to mast cells, histamine release from basophils can be mediated by tick histamine release factor binding [95]. Several studies have confirmed the role of basophils in acquired immunity against ticks in mice [102,104,105]. Basophils expressing the immunoglobulin Fc receptor were found to be responsible for antibody-mediated acquisition of *H. longicornis* resistance [102], with selective basophil ablation by diphtheria toxin leading to loss of resistance to *H. longicornis* feeding in subsequent tick infestations [102]. Basophils appear to play a non-redundant role in antibody-mediated acquired immunity against ticks [102].

As noted above, *I. ricinus* saliva increased MCP-1 production by stimulated splenocytes [60]. MCP-1 is a potent basophil activator that triggers their degranulation and histamine release [106].

Basophils can cause cutaneous basophilia, a mechanism of tick resistance [104,105]. The susceptibility or resistance of cattle to ticks (*R. microplus*) was associated with the number of basophils at the feeding site, with skin biopsies from tick-resistant breeds contain significantly more basophils than biopsies from susceptible breeds [107–109].

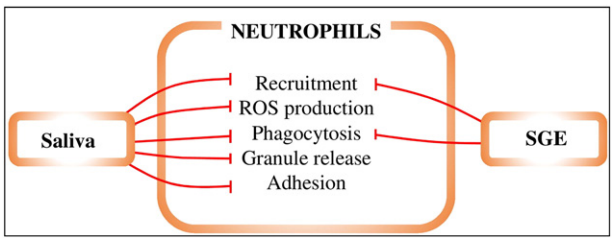

Fig. 3. The effects of saliva and SGE on neutrophils. Red lines represent inhibition, green lines enhancement. Tick saliva inhibits neutrophil recruitment, phagocytosis, adhesion, granule release, and production of ROS.

9. Eosinophils and tick saliva

Eosinophils are mainly present in mucosal areas in contact with the external environment such as the gut or lung mucosae. Their circulating levels are relatively low in healthy organisms, but increase during allergic reactions or parasitic infections [46]. Eosinophils produce cytokines, chemokines, and other mediators, some of which (e.g., indoleamine 2,3 deoxygenase; IDO) induce apoptosis and inhibit T cell proliferation [110,111]. Eosinophils are also rich in granules that contain cytotoxic effectors such as eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin, or major basic protein, which can cause mast cell (and probably also basophil) degranulation [112]. Finally, eosinophils are an important source of inflammatory and tissue repair-related molecules such as the transforming growth factors TGF-α and TGF-β1 and the extracellular matrix glycoprotein tenascin [113,114].

Repeated exposure to both soft and hard tick species raised eosinophil levels at the feeding site in many host species including cattle [115,116], dogs [117], guinea pigs [118,119], rabbits [86], mice [88],

woolless lambs [120], rats [36], capybaras [121], and even anteaters and armadillos [122]. The relationship between eosinophil number and tick resistance is not clear. Similar to mast cells, the susceptibility or resistance to ticks in cattle was associated with the number of eosinophils (and basophils) at the feeding site. Cattle breeds with more eosinophils (*Bos taurus indicus*, Nelores breed) appeared to be more resistant to *R. microplus* feeding than the *B. taurus taurus* Holstein breed with fewer eosinophils [107]. In contrast, the tick count on Nguni and Bonsmara cattle was positively correlated with the eosinophil count in skin biopsies from tick feeding sites, while the correlation was negative in the case of mast cells and basophils [109].

Ticks inhibit the chemokine-mediated attraction of eosinophils to tick feeding sites. SGE from many tick species blocked CCL3, CCL5, or CCL11 (eotaxin) eosinophil chemoattractant activity [123–126].

10. Neutrophils and tick saliva

Neutrophils are granulocytes with both phagocytosis and degranulation roles. They are highly motile cells and they have a relatively short lifespan. Neutrophils play an important role in the early stages of vertebrate immune homeostasis, such as during acute inflammation, but they also play a role in some chronic inflammatory diseases. Neutrophils are generally activated by pathogens and secrete effectors and mediators that promote inflammation by recruiting other leukocytes, and they also directly kill pathogens by releasing their granules [46,127]. They can also phagocytose and kill pathogens intracellularly [127].

Tick saliva modulates a local cutaneous immune response at the tick feeding site almost immediately after tick attachment, as shown by gene expression analysis of skin biopsies taken at several time points after the initiation of *I. scapularis* nymph feeding [128]. The expression of neutrophil-specific chemokines (CXCL1 and 5) was induced as early as 12 h after tick attachment to the host [128]. Neutrophil abundance in the skin was high during the first tick infestation compared to other cell types but decreased during subsequent tick infestations of the same host [120,129]. It is unknown whether the absence of neutrophils affects resistance of the host to ticks.

Saliva or SGE from soft and hard ticks have been shown to attenuate neutrophil functions such as recruitment by interfering with the neutrophil chemoattractants CXCL8 (IL-8) or CCL3 [123,124,126,130,131]. In one study, *I. ricinus* saliva significantly decreased neutrophil reactive oxygen species (ROS) production [132]. In contrast, the formation of neutrophil extracellular traps (NETs), which are formed by extrusion of neutrophil DNA and can retain and kill bacteria, was not affected by saliva [132]. *I. scapularis* (published as *Ixodes dammini*) saliva inhibited granule release and neutrophil infiltration and had an inhibitory effect on neutrophil phagocytosis of *B. burgdorferi* [133]. *I. scapularis* saliva also reduced polymorphonuclear leukocyte (PMN) adhesion by down-regulating β2-integrin expression and signaling, which decreased pro-inflammatory functions of PMNs [134]. Finally, SGE from *R. microplus* inhibited neutrophil phagocytic activity in cattle [135]. These data show that tick saliva inhibits several pro-inflammatory neutrophil properties that are deleterious to tick feeding but does not affect antibacterial NET formation, suggesting that tick salivary activity is specific. The effects of tick saliva and SGE on neutrophils are illustrated in Fig. 3.

11. T and B lymphocytes and tick saliva

Adaptive immunity relies on a wide range of antigen receptors (with varying antigen recognition specificities), which are clonally distributed in two types of lymphocytes: T cells and B cells. The induction of a specific immune response is only possible when a foreign antigen is recognized by the corresponding receptor. This first recognition signal is consolidated by the interaction of co-stimulatory molecules on T or B cells with those on APCs — such as dendritic cells or macrophages — that belong to the innate immune system. In this way, links are made between the cell populations that play dominant roles in the two branches of vertebrate immunity [136].

T cells are produced in the bone marrow from lymphoid progenitors and differentiate in the thymus. Mature T cells then migrate to the peripheral lymphoid tissues; they also circulate throughout the body [46]. Two major T cell subpopulations are recognized based on the co-receptor molecule expressed at the cell surface: CD4+ (T helper cells) and CD8+ T cells (which develop into cytotoxic T lymphocytes, CTLs). According to the secreted cytokine profile, CD4+ T helper cells can be further divided into several subpopulations that have different roles in immune responses [137], with Th1 and Th2 populations the most thoroughly studied in tick–host interactions thus far. Th1 populations are associated with host cellular and inflammatory responses, and Th2 populations with host humoral responses against ticks [138,139]. Fig. 4 illustrates how tick saliva and SGE influence T and B cell functions.

In 1985, *I. scapularis* (*dammini*) tick saliva was shown to inhibit IL-2 production by T lymphocytes, with PGE_2 proposed to be responsible for this effect [140]. Urioste and colleagues confirmed diminished IL-2 levels in the presence of *I. scapularis* saliva, and showed profoundly inhibited splenic T cell proliferation in response to stimulation with concanavalin A (ConA) or phytohemagglutinin in the presence of saliva [62]; however, they disproved the PGE_2 hypothesis, providing evidence that IL-2 is in fact inhibited by a proteinaceous salivary component. Later, in 2001, an unknown salivary component from *I. scapularis* was reported to bind IL-2 and inhibit T lymphocyte proliferation [141].

The inhibition of lymphocyte proliferation by SGE has also been reported in other tick species such as *I. ricinus*, *Amblyomma variegatum*, and *R. appendiculatus*, with species- and sex-specific differences shown for the effects of tick salivary gland antigens on human lymphocyte proliferation [142]. *I. ricinus* SGE suppressed isolated B cell proliferation and IL-10 production in response to LPS. CD69 activation marker expression on both activated T and B cells was also reduced [143]. *I. ricinus* saliva inhibited splenic T cell proliferation in response to ConA, and both SGE and saliva reduced the responsiveness of T cells draining to lymph nodes and sensitized splenic T cells [144]. The same observation was made with naïve splenic T cells [145]. T lymphocytes from mice infested 9 days previously with *I. ricinus* nymphs displayed suppressed responses to ConA stimulation compared to cells from naïve mice [145]. In contrast, the lymph node cell response to LPS was increased in infested mice compared to naïve mice [145]. The authors attributed the observed effect to increased B lymphocyte numbers or activity [145]. On the other hand, soluble salivary gland antigens derived from female *I. ricinus* ticks stimulated lymph node T cells from mice infested with *I. ricinus* larvae or nymphs, but not those infested with *Amblyomma hebraeum* nymphs [146]. A 65 kDa protein fraction (IrSG65) isolated from the salivary glands of partially fed *I. ricinus* females induced specific T cell proliferation in lymph node cells obtained from mice infested with *I. ricinus* nymphs [146]. Feeding of *I. ricinus* nymphs on BALB/c mice revealed that CD4+ T cells were more abundant than CD8+ cells [147], which changed from 2:1 upon primary tick infestation to 7:1 in tertiary tick infestation. The ratio of CD3+ and CD4+ T lymphocytes was identical in *I. ricinus* infested and control mice [148].

D. andersoni SGE reduced ConA-induced proliferation of T cells [149,150]. *R. microplus* feeding on cattle decreased the T lymphocyte percentage in peripheral blood lymphocytes (PBLs) [151], with the B lymphocyte percentage only lowering after repeated heavy infestations [151]. *R. microplus* saliva also suppressed PBL responsiveness to phytohemagglutinin [151]. *R. sanguineus* feeding on dogs suppressed ConA, phytohemagglutinin, and pokeweed mitogen-induced lymphocyte responses [152]. In the same study, SGE also suppressed all mitogen-stimulated blastogenic responses of lymphocytes from healthy dogs in vitro. Feeding of the *Haemaphysalis bispinosa* and *Hyalomma anatolicum anatolicum* ticks on sheep resulted in reduced circulating T lymphocyte counts as tick feeding progressed [153]. The authors showed that depletion of CD8+ populations and increased CD4+ T cell levels accounted for the observed effects [153]. Feeding of these two ticks also suppressed in vitro

proliferation of T cells isolated from the tick-infested animals [153]. The CD4+/CD8+ and B/T lymphocyte ratios were increased in all sheep during infestation with either *H. bispinosa* and *H. anatolicum anatolicum* [153]. Interestingly, reduced CD4/CD8 T cell ratios were observed in skin biopsies taken at primary and secondary infestation with *H. anatolicum anatolicum* ticks on sheep compared to healthy skin biopsies [154].

B cells also originate from lymphoid progenitors in the bone marrow [46]. Their further differentiation involves migration from the bloodstream to the spleen, where they develop into mature B cells. Mature B cells circulate between the spleen and lymph nodes. The role of B cells lies in the surface expression and secretion of immunoglobulins upon activation [155]. In immunity against ticks, B cells produce specific antibodies against tick salivary and gut antigens.

Both primary and secondary infestations of sheep with *H. anatolicum anatolicum* ticks caused a significant increase in circulating B lymphocytes over several days [153]. In dogs, *R. sanguineus* SGE was shown to suppress total immunoglobulin and IgA (but not IgM) production by PBLs in vitro upon activation with LPS or pokeweed mitogen [156]. It has also been observed that anti-BSA IgG and IgM levels decreased in mice immunized with BSA during *I. ricinus* feeding [148]. However, anti-BSA IgG and IgM production was not decreased when BSA was injected prior to tick infestation. Interestingly, this study did not demonstrate a shift towards the Th2-type immune response when anti-BSA IgG1 and IgG2a antibody levels were compared between mice groups [148]. It was later shown that total IgG and IgM antibody levels were not reduced in animal sera by tick infestation, anti-BSA antibody production was not delayed, and memory cell formation did not appear to be inhibited by tick saliva [157]. Tick saliva did not affect memory B cell production of either anti-BSA IgG or IgM [157].

Experiments with tick saliva or SGE have shown polarization of the immune response from Th1 to Th2 branches by suppression of Th1 and upregulation of Th2 cytokines in both mice and humans. This polarization leads to an attenuated inflammatory response, which is beneficial for tick survival and feeding [15,158]. Briefly, saliva or SGE inhibited secretion of IL-2, IL-12, TNF, and IFN-γ. In contrast, IL-4, IL-6, and IL-10 secretion was stimulated [66,139,159]. IL-10-specific neutralizing antibodies abrogated the suppressive effect of *I. ricinus* SGE on IFN-γ production [160]. IL-1α secretion was inhibited in JA-4 macrophage cells exposed to *R. appendiculatus* SGE [66]. In contrast, and in spite of their pro-inflammatory properties, IL-1α and IL-1β production was increased by Th1 lymphocytes and splenocytes after treatment with *I. ricinus* SGE [161,162]. This can be explained by the fact that IL-1 can also act as a co-stimulator for Th2 lymphocyte proliferation. One of the mechanisms described for the action of *I. ricinus* saliva involves a negative effect on DCs, which then prime naive CD4+ T cells to induce Th2 cell differentiation in vitro and in vivo [71].

Feeding of *D. andersoni* decreased expression of two integrins, leukocyte function-associated antigen-1 (LFA-1) and very late activation-4 (VLA-4), by lymphocytes [163]. The same effect was achieved by exposing tick-naïve mouse lymphocytes to both *D. andersoni* saliva and SGE [163]. Infestation with *D. andersoni* nymphs or intradermal administration of female or male tick SGE increased IL-4 and IL-10 transcript levels in the draining lymph nodes and skin of the host [164]. Intracellular IL-4 levels were significantly increased in CD4+ T cells [164], and increased IL-4 levels were also observed during *I. scapularis* nymph feeding or by intradermal application of SGE [165]. Primary *I. scapularis* infestation on mice was characterized by late induction of an innate immune response and by inhibition of pro-inflammatory Th17 immunity. During secondary tick infestation, a mixed Th1/Th2 response was elicited [35].

Ticks have evolved in various ways to circumvent adaptive immunity. Their saliva inhibits lymphocyte proliferation to reduce immune responses. Furthermore, ticks actively direct the immune response towards the Th2 arm that favors their feeding. The immunosuppressive properties of tick saliva also include inhibition of antibody production by B cells that could damage tick mouthparts and activate other cells or complement. The effects of tick saliva and SGE on lymphocytes are illustrated in Fig. 4.

12. Natural killer cells and tick saliva

Despite their lymphoid origin, natural killer (NK) cells are part of the innate immune system [46]. Their main function is microbial or tumor cell killing and the regulation of endothelial cell, dendritic cell, and macrophage interactions with T lymphocytes [166]. SGE from female *Dermatocentor reticulatus* ticks that fed for 3–6 days on mice decreased human NK cell activity, while SGE from unfed or 1 day-fed ticks had no effect. Weaker activity was reported for SGE from *A. variegatum* and *Haemaphysalis inermis* ticks [167,168], and NK cell cytotoxicity was suppressed after treatment with *I. ricinus* SGE [169].

13. Conclusions

Tick saliva clearly contains numerous different pharmacologically-active molecules that affect various immune cell populations and facilitate tick feeding. In this "systems biology" era, the effects of tick saliva described in this review can help in the design of experiments to discover specific salivary molecules that account for those effects. Although molecular biology and biochemical methods such as transcriptome and proteome analyses have provided excellent information about the genes expressed in the salivary glands of different tick species, the number of identified and functionally characterized salivary molecules remains limited. Ultimately, the goal is to fully uncover the complexity of how ticks modulate the host immune system so that this information can be used to pioneer the development of novel control strategies for ticks and tick-borne diseases and aid drug discovery.

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Conflicts of interest

The authors declare that no conflicts of interest exist.

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6. IMUNOMODULACE DENDRITICKÝCH BUNĚK SLOŽKAMI KLÍŠTĚCÍCH SLIN A JEJICH INTERFERENCE SE SIGNALIZACÍ

V posledních letech bylo identifikováno několik složek klíštěcích slin, které prokazatelně ovlivňovaly funkci DC. Prostaglandin E2 (PGE2), který je přítomen ve slinách *I. scapularis,* inhiboval DC v *in vitro* testech podobně jako klíštecí sliny, snižoval produkci IL-12 a TNF po stimulaci LPS (66). Autoři tvrdí, že PGE2 je zodpovědný za většinu efektů pozorovaných na DC. Podobně PGE2 z *Amblyomma sculptum* moduluje interakci DC s *Rickettsia rickettsii* (původce horečky Skalistých hor) *in vivo* a *in vitro* (89).
Multifunkční protein Salp15 (salivary gland protein 15) z *I. scapularis* ovlivňuje také funkci DC a to interakci s C-lektinovým receptorem II typu DC-SIGN (dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin). Výsledkem této interakce je snížená produkce prozánětlivých cytokinů a suprese aktivace T buněk po stimulaci lidských dendritických buněk LPS a *B. burgdorferi s.s.* Salp15 aktivuje novou Raf-1/MEK-závislou signalizační dráhu a DC aktivaci ovlivňuje na úrovni transkripce i post-transkripčných úprav. Výsledkem aktivace Raf/MEK dráhy je snížení stability transkriptů pro IL-6 a TNF a zhoršená remodelace nukleosómu v oblasti IL-12p35 promotoru (90).
Japanin, lipokalin z *Rhipicephalus appendiculatus,* výrazně mění expresi kostimulačních a koinhibičních receptorů, produkci prozánětlivých a protizánětlivých cytokinů po stimulaci *in vitro*, a navíc inhibuje differenciaci DC z lidských monocytů (91).

Funkce dendritických buněk je ovlivněná i **cystatiny**. Cystatiny jsou reverzibilní inhibitory papainu podobných cysteinových proteáz a legumainů. Rozdělují se do 4 podskupin; typ 1 (stefiny), typ 2, typ3 (kininogeny) a typ 4 (fetuiny) (92). Cystatiny popsané v klíšťatech jsou stefiny bez sekrečního signálu mající funkci ve vnitrobuněčném trávení hemoglobinu, nebo patří do skupiny 2 a fungují jako imunomodulátory sekretované do slin (93). Cystatiny regulují mnoho fyziologických procesů včetně prezentace antigenu, fagocytózy a produkce cytokinů. Substráty cystatinů jsou lyzosomální katepsiny, které se podílí na degradaci antigenů prezentovaných komplexem MHC II nebo na aktivaci kaspázy-1 (regulace inflamazómu, apoptózy a autofagie). Jedním z funkčně charakterizovaných klíštěcích cystatinů je Sialostatin L identifikován ve slinách *I. scapularis.* Sialostatin L snižuje v *in vitro* testech sekreci prozánětlivých cytokinů, expresi kostimulačních molekul a ovlivňuje rovněž T buňky po stimulaci LPS (94, 95). Mechanismus imunomodulace DC vlivem tohoto klíštěcího cystatinu spolu s příbuzným Sialostatinem L2 byl analyzován na úrovni signálních drah v dalších dvou předložených pracích (96, 97).

Další skupinou klíštěcích proteinů, které byly intenzívně studovány, jsou **serpiny**. Serpiny jsou inhibitory serinových proteáz, které regulují hemostázu a imunologickou odpověď. V klíšťatech jsou serpiny přítomné ve velkém počtu a modulace imunitní odpovědi je jednou z jejich důležitých funkcí. Do dnešního dne bylo dle sekvenční analýzy identifikováno přes 60 serpinů z tvrdých klíšťath, ovšem jenom několik z nich bylo charakterizováno z hlediska jejich funkce (např. IRIS, IRS-2 a RHS2) (98).
IRIS, první známý serpin z *I .ricinus* inhibuje leukocytární elastázu. Interferuje s koagulací, fibrinolýzou a narušuje adhezi krevních destiček. IRIS moduluje vrozenou i adaptivní

imunitní odpověď, indukuje Th2 odpověď a inhibuje prozánětlivé cytokiny. Je zajímavé, že schopnost inhibice prozánětlivých cytokinů je nezávislá na proteolytické aktivitě tohoto serpinu (10-13).
IRS-2 je serpin z klíštěte *I. ricinus.* Inhibuje proteázy produkované aktivovanými neutrofily (katepsin G) a žírnými buňkami (chymáza). Bylo prokázáno, že IRS-2 potlačuje zánět tím, že inhibuje migraci neutrofilů do postiženého místa (98). Poslední předložená studie se zabývá účinky tohoto serpinu na funkci DC.

Klíštěcí sialostatin L2 oslabuje interferonovou signalizaci v myších dendritických buňkách (5. předložená práce)

Lieskovská J., Páleníková J., Širmarová J., Elsterová J., Kotsyfakis M., Campos Chagas A., Calvo E., Růžek D., Kopecký J. (2015): Tick salivary cystatin sialostatin L2 suppresses IFN responses in mouse dendritic cells. Parasite Immunology. Feb;37(2):70-8.

V 5. předložené práci jsme se zabývali otázkou, jestli cystatiny Sialostatin L a Sialostatin L2 ovlivňují interferonovou signalizaci, podobně jako klíštěcí sliny. Vliv Sialostatinu L a příbuzného Sialostatinu L2 na IFN signalizaci byl analyzován na úrovni a) fosforylace STAT1 a STAT2, b) indukce ISG (IRF7 a IP-10) a c) účinku IFN na replikaci TBEV. Fosforylace STAT1 a STAT2 byla negativně ovlivněna oběma cystatiny v slezinných DC po aktivaci IFN-β. Důsledky této inhibice byly ověřovány testováním indukce vybraných ISG. Genová exprese IRF7 a IP-10 byla inhibována pouze Sialostatinem L2. Je pravděpodobné, že na inhibici ISG se podílí kromě oslabené IFN signalizace i jiná signální dráha, která je ovlivněná pouze Sialostatinem L2. **Interference Sialostatinu L2 s IFN signalizací** byla potvrzena experimentem, ve kterém Sialostatin L2 oslabil antivirový efekt IFN na replikaci TBEV.

Klíštěcí sialostatiny L a L2 rozdílně ovlivňují reakce dendritických buněk na spirochéty *Borrelia* (6. předložená práce)

Lieskovská J., Páleníková J., Langhansová H., Campos Chagas A., Calvo E., Kotsyfakis M., Kopecký J. (2015): Tick sialostatins L and L2 differentially influence dendritic cell responses to *Borrelia* spirochetes. Parasite&Vectors 15;8:275.

Předmětem této práce bylo testování účinků Sialostatinu L (Sialo L) a Sialostatinu L2 (Sialo L2) v dendritických buňkách po stimulaci ligandy receptorů TLR2, TLR7, TLR9 a *B. burgdorferi.* Bylo zjištěno, že tyto příbuzné cystatiny odlišným způsobem ovlivňují funkce DC. Oba cystatiny negativně ovlivňují produkci chemokinu MIPα, ale pouze Sialo L2 inhibuje produkci IP-10 v myeloidních DC (mDC) po stimulaci boreliemi. Analýza signálních drah v mDC odhalila, že pouze Sialo L2 snižuje aktivitu Erk1/2, NF-κB a Akt kinázy v TLR2 aktivovaných DC a působí inhibičně na Erk1/2 kinázu v boreliemi stimulovaných DC. Interference cystatinů s TLR7 a TLR9 závislou signalizací byla testována v plazmacytoidních DC na úrovni produkce IFNβ a exprese kostimulační molekuly CD86. Bylo zjištěno, že Sialo L snižuje jak produkci IFNβ, tak expresi CD86. Data tedy ukázala, že **Sialo L interferuje s TLR7 a TLR9 závislou signalizací a negativně ovlivňuje maturaci DC a následný rozvoj adaptivní imunitní odpovědi. Sialo L2 interferencí s TLR2 signalizací ovlivňuje produkci chemokinů, čímž potenciálně oslabuje iniciaci zánětlivé odpovědi.**

Analýze mechanizmů, jakými cystatiny Sialo L a SialoL2 působí na aktivaci buněk, se věnovalo několik studií. Jedna z nich, zaměřená na odhalení účinku Sialo L v žírných buňkách odhalila, že Sialo L interferuje s transkripčním faktorem IRF-4 a tím inhibuje produkci prozánětlivých cytokinů (IL-β) na úrovni RNA (99). V případě Sialo L2 se zdá, že tento cystatin neovlivňuje jenom TLR signalizaci, ale interferuje i s aktivací inflamazomu v makrofázích aktivovaných *Anaplasma phagocytophilum.* Pozorovaný efekt je specifický pro inflamazom aktivovaný *A. phagocytophilum,* protože aktivace inflamazomu kanonickými aktivátory NLRP3 a NLRC4 nebyla ovlivněna cystatinem Sialo L2 (100). Sialo L2 je jedním z mála proteinů klíštěcích slin, který lze považovat za SAT faktor pro borelie; *in vivo* experimentem bylo prokázáno, že intradermální injekce borelií se Sialo L2 vede ke zvýšenému množství spirochét v kůži (101).

Kromě výše zmíněných cystatinů byly testovány i cystatiny pocházejících i z jiných druhů klíšťat. Cystatin OmC2 ze slin měkkého klíštěte *Ornithodoros moubata* byl studován na buněčné linii MUTZ-3 (modelových nezralých dendritických buňkách). Internalizace OmC2 vedla ke snížení exprese MHC II a CD86 (102).

Cystatin *z Rhipicephalus haemaphysaloides,* pojmenovaný RHcyst-1 na rozdíl od výše popsaných cystatinů nemá vliv na maturaci DC, ale inhibuje diferenciaci BMDCs. Ko-inkubace T buněk s DC vede v přítomnosti RHcyst-1 ke snížené produkci TNFα, IFNγ and IL-2 v porovnání s kontrolní skupinou (103).

Zcela nový mechanismus účinku byl popsán pro DsCystatin z klíštěte *Dermacentor silvarum* vyskytujícího se v Číně. Tento cystatin snížil expresi kostimulačných molekul v LPS-stimulovaných DC a významně inhiboval indukci prozánětlivých cytokinů (IL-1β, IFNγ, TNFα, and IL6) v makrofázích derivovaných z kostní dřeně. Autoři odhalili, že DsCystatin-

interferuje s TLR4 signalizací přes aktivaci autofagie. Výsledkem byla degradace signální molekuly TRAF6 a následná inhibice NF-κB, přičemž inhibice NF-κB byla pozorována po stimulaci LPS i *B. burgdorferi*. Imunosupresivní účinky tohoto cystatinu byly potvrzeny na myším modelu, kdy byly symptomy artritidy indukované *B. burgdorferi* či Freundovým adjuvans slabší v přítomnosti DsCystatinu (104).
Iristatin je zatím jediný cystatin z klíštěte *I. ricinus*, který byl funkčně a strukturně charakterizován (105). Široké spektrum imunosupresívních účinků tohoto cystatinu bylo prokázáno v buňkách vrozené i adaptivní imunity. Produkce cytokínů IL-2, IL-4, IL-9 a IFNγ T-lymfocyty, produkce IL-6 a IL-9 žírnými buňkami a produkce oxidu dusnatého makrofágy byly vlivem Iristatinu snížené.

Serpin IRS-2 klíštěte *I.ricinus* ovlivňuje diferenciaci Th17 prostřednictvím inhibice signální dráhy IL-6/STAT3 (7. předložená práce)

Páleníková J, Lieskovská J, Langhansová H, Kotsyfakis M, Chmelař J, Kopecký J. (2015): Ixodes ricinus salivary serpin IRS-2 affects Th17 differentiation via inhibition of the interleukin-6/STAT-3 signaling pathway. Infection&Immun. May;83(5):1949-56.

Testování účinku IRS-2 na boreliemi stimulované dendritické buňky jsme se věnovali v sedmé předložené práci (106). Bylo zjištěno, že IRS-2 inhibuje produkci IL-6, což následně ovlivňuje vývoj Th17 lymfocytů. Th17 lymfocyty hrají důležitou roli v imunitní odpovědi na extracelulární patogeny, mezi které patří i borelie. Interleukin 6, produkovaný dendritickými buňkami po stimulaci boreliemi, je klíčový pro vývoj Th17 lymfocytů a při jeho absenci nedochází k aktivaci STAT3 signální dráhy. IRS-2 inhibuje produkci IL-6, aktivaci STAT3 signální dráhy a snižuje procento Th17 lymfocytů po boreliové infekci. Data prokázala, že klíštěcí serpin **IRS-2 moduluje adaptivní imunitní reakci během boreliové infekce pomocí inhibice produkce IL-6**.
Z řad klíštěcích serpinů byl nedávno prokázán imunomodulační efekt serpinu RHS2 z *R. haemaphysaloides* na dendritické buňky. RHS2 inhibuje diferenciaci a maturaci DC, snižuje produkci prozánětlivých cytokinů a zvyšuje IL-4, inhibuje aktivaci CD4 a CD8 T buněk po stimulaci LPS. Autoři zjistili, že hladina mRNA a fosforylace signálních molekul p38, ERK a STAT byly během diferenciace DC z buněk kostní dřeně působením RHcyst-1 sníženy (107).

Příloha 2

5. **Lieskovská J.**, Páleníková J., Širmarová J., Elsterová J., Kotsyfakis M., Campos Chagas A., Calvo E., Růžek D., Kopecký J. (2015): Tick salivary cystatin sialostatin L2 suppresses IFN responses in mouse dendritic cells. Parasite Immunology. Feb;37(2):70-8.

6. **Lieskovská J**., Páleníková J., Langhansová H., Campos Chagas A., Calvo E., Kotsyfakis M., Kopecký J. (2015): Tick sialostatins L and L2 differentially influence dendritic cell responses to *Borrelia* spirochetes. Parasite&Vectors 15;8:275.

7. Páleníková J., **Lieskovská J.**, Langhansová H., Kotsyfakis M., Chmelař J., Kopecký J. (2015): *Ixodes ricinus* salivary serpin IRS-2 affects Th17 differentiation via inhibition of the interleukin-6/STAT-3 signaling pathway. Infection&Immun. May;83(5):1949-56.

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Tick salivary cystatin sialostatin L2 suppresses IFN responses in mouse dendritic cells

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SUMMARY

Type I interferon (IFN), mainly produced by dendritic cells (DCs), is critical in the host defence against tick-transmitted pathogens. Here, we report that salivary cysteine protease inhibitor from the hard tick Ixodes scapularis, sialostatin L2, affects IFN-β mediated immune reactions in mouse dendritic cells. Following IFN receptor ligation, the Janus activated kinases/signal transducer and activator of transcription (JAK/STAT) pathway is activated. We show that sialostatin L2 attenuates phosphorylation of STATs in spleen dendritic cells upon addition of recombinant IFN-β. LPS-stimulated dendritic cells release IFN-β which in turn leads to the induction of IFN-stimulated genes (ISG) through JAK/STAT pathway activation. The induction of two ISG, interferon regulatory factor 7 (IRF-7) and IP-10, was suppressed by sialostatin L2 in LPS-stimulated dendritic cells. Finally, the interference of sialostatin L2 with IFN action led to the enhanced replication of tick-borne encephalitis virus in DC. In summary, we present here that tick salivary cystatin negatively affects IFN-β responses which may consequently increase the pathogen load after transmission via tick saliva.

Keywords *tick, dendritic cell, interferon, cystatin*

INTRODUCTION

Ticks are blood-feeding parasites that transmit many pathogens of medical importance (e.g. Lyme disease agent *Borrelia burgdorferi*, tick-borne encephalitis virus, etc.). Hard ticks feed on their host for several days and successful feeding depends on a cocktail of salivary proteins which are injected into the host (1). These proteins have antihaemostatic, anti-inflammatory and immunomodulatory properties and support transmission of pathogens (2).

Sialostatin L2 (Sialo L2) and sialostatin L (Sialo L) are inhibitors of cysteine peptidases, which have been characterized in the hard tick *Ixodes scapularis.* Both sialostatins are strong inhibitors of cathepsin L, while sialostatin L strongly inhibits also cathepsin S, a protease which plays an important role in the processing of antigens (3, 4). Sialostatin L2 is necessary for tick feeding success (5) and is one of the salivary molecules identified as saliva activated transmission factors; particularly, sialostatin L2 supports *Borrelia burgdorferi* transmission *in vivo* (6). The precise mechanism behind this effect is however not clear. In addition, it has been reported that sialostatin L2 inhibits caspase-1-mediated inflammation during *Anaplasma phagocytophilum* infection (7).

Numbers of immunosuppressive effects of sialostatin L have been demonstrated. Sialostatin L inhibited the inflammatory reaction induced by carrageenan and caused decline of the T-cell line CTLL-2 proliferation (4). Further, the asthma symptoms were severally suppressed by sialostatin L in the model of experimental asthma. The inhibition of host hypersensitivity was due to suppression of the asthma promoting cytokine IL-9 (8). With regard to dendritic cell function, sialostatin L can inhibit LPS-induced dendritic cell maturation and proliferation of Ag-specific CD4+ T lymphocytes (9).

Dendritic cells**,** known as professional antigen-presenting cells, play a critical role in initiating and modulating immune response elicited upon recognition of pathogens. Upon sensing pathogens by pattern recognition receptors, including Toll-like receptors (TLR), DCs produce several cytokines and chemokines which in turn regulate, in auto-

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crine and paracrine manner, the establishment of an innate immune response (10). DCs are producers but also key responders to IFN.

Type I IFNs, represented by IFN-α and IFN-β, play an important role in direct antiviral defence as well as linking the innate and adaptive immune responses. Type I IFNs bind to their heterodimeric IFNα/β receptor containing IFNAR-1 and IFNAR-2, both members of cytokine receptor superfamily (11). The binding of IFN to its receptor triggers the internalization of IFNAR-1 and IFNAR-2 and results in activation of associated tyrosine kinases Tyk2 and JAK1, which in turn phosphorylate signal transducer and activator of transcription (STAT)-1 and STAT-2. Upon activation, STATs bind to IFN-stimulated response elements in the promoter of IFN-stimulated genes (ISG) (11), for example IFN regulatory factor (IRF)-1 and IRF-7. Products of ISG confer to these cells an antiviral status. Type I interferons also regulate the production of CX chemokine ligand 10 (CXCL10/IP-10), as well as interleukin-12 (12).

In the previous report, we demonstrated that tick saliva suppresses IFN signalling in dendritic cells upon LPS and *Borrelia* infection (13). In this study, we analysed the possible effect of tick salivary cystatins on the host immune response through interfering with type I IFN action.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (10 weeks of age) were obtained from Charles River Laboratories. All experiments were performed with permission from local animal ethics committee.

Preparation of recombinant cystatins

Recombinant cystatins were expressed in *Escherichia coli* followed by purification of active protein, as previously described (3, 6). LPS contamination was removed by Arvys Proteins using the detergent extraction method. After this procedure, the presence of endotoxin was estimated with a sensitive fluorescent-based endotoxin assay (PyroGene recombinant factor C endotoxin detection system; Lonza Biologics); estimated presence of endotoxin was 4×10^{-5} endotoxin U/μg protein (approximately 3×10^{-14} endotoxin g/μg protein) for both cystatins. When testing the recombinants, the endotoxin level was <2 pg per ml.

Spleen dendritic cells isolation

Mouse spleens were minced with scissors, digested in RPMI containing Liberase DL (0·67 U/mL) and DNase I (0·2 mg/mL) (both from Roche) for 30 min at 37°C and passed through a 70-μm nylon cell strainer (BD Falcon). Spleen dendritic cells (sDCs) were isolated using magnetic beads conjugated with anti-CD11c (N418) Ab and MACS Column separation following the manufacturer's instructions (Miltenyi Biotec). Purified sDCs were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Sigma-Aldrich). Purity of isolated sDC (approximately 90% CD11c+ cells) was determined by subsequent FACS analysis.

Derivation of dendritic cells from bone marrow

Bone marrow-derived dendritic cells (bmDCs) were prepared as described before by Inaba *et al.* (14), with minor modifications. Briefly, mice were sacrificed by cervical dislocation, intact femurs and tibias were removed, and bone marrow was harvested by repeated flushing with MEM. Bone marrow cells (10^6/mL) were cultured for 7 days in 6-well plate in RPMI 1640 medium supplemented with 10% FCS, 50 mM HEPES, 2 mM glutamine, 50 μM 2-mercaptoethanol, penicillin, streptomycin, amphotericin B and 30 ng/mL of recombinant mouse GM-CSF (R&D). On day 3 and 5, nonadherent cells were depleted and 70% of medium was replaced with the fresh medium. On day 7, nonadherent cells were harvested, washed in fresh medium and used as immature DCs.

IP-10 and IFN-β measurement

Freshly isolated sDCs were cultured in 96-well plate at a concentration of 2×10^5 cells per well for 20 h. Following 2 h incubation with tick cystatins (3 μM), the cells were stimulated with LPS (100 ng/mL) (from *Escherichia coli* K-235, Sigma-Aldrich). Cell-free culture supernatants were harvested 6 h after stimulation and used for IFN-β determination by LEGEND MAX™ mouse IFN-β ELISA Kit (BioLegend) (detection limit for IFN-β was 1·8 pg/mL) or after 24 h for IP-10 determination using Murine IP-10 ELISA Development Kit (PeproTech), following the manufacturer's instructions.

Quantitative PCR – measurement of IRF-7 gene expression

Freshly isolated sDCs were cultured in 24-well plate at a concentration of 2×10^6 cells per well for 18 h. Following 2 h incubation with tick cystatins (3 μM), the cells were

stimulated with LPS (100 ng/mL) for 6 h. Cells were lysed and total RNA was isolated using Nucleospin RNA II kit (Macherey-Nagel, GmbH & Co. KG, Duren, Germany) according to the manufacturer's instructions. 1 μg of total RNA was used for transcription to cDNA by High capacity RNA-to-cDNA kit (Applied Biosystems). 1 of 10 of cDNA was added to qPCR reaction using a cycler Rotor-Gene 3000 and Rotor-Gene 6.0.19 software (Corbett Research). IRF-7-specific primers, forward 5′-CACTGAG TTCTGAACCTA-3′, reverse 5′-GTTGGTAACAGGTAG GAA-3′, and FAM-labelled probe 5′-ACCACAAGTTCTC AAACCTCATCTG-3′ with BHQ1 quencher (Sigma) were used. The relative expression of IRF-7 was determined by comparative CT method (15), where mouse-β-actin was used as a housekeeping gene (Applied Biosystems). All reactions were performed in triplicates.

Flow cytometry – surface expression of IFNAR-1

sDCs were cultured in 96-well plate at a concentration of 2×10^5 cells per well for 20 h. Following 2 h incubation with sialostatins (3 μM), the cells were stimulated with 10 U of IFN-β for 30 and 60 min. Cells were collected (using cold 5 mM EDTA in PBS) and stained with anti-mouse-IFNAR-1 antibody (clone MAR1-5A3) or with corresponding isotype control, both conjugated with phycoerythrin (BioLegend). CD11c positive cells (detected by anti-CD11c antibody conjugated with APC, eBioscience) were analysed for surface expression of IFNAR-1. Dead cells were excluded from analysis using propidium iodide. Flow cytometry was performed on FACS Canto II cytometer using FACS DIVA software, v. 5.0 (BD Biosciences) and FLOWING software 2.

Immunoblotting

Freshly isolated dendritic cells were seeded at 1×10^6 cells per well in 24-well plate. Next day, DCs were incubated 2 h with indicated concentration of tick cystatins prior to the addition of 5 U/mL of recombinant mouse IFN-β (PBL interferon source). Following stimulation, the cells were lysed in a modified RIPA buffer (1% Nonidet P-40, 0·25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl and 50 mM Tris-HCl (pH 7·5)) in the presence of protease and phosphatase inhibitors (10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM PMSF, 1 μg/mL pepstatin, 25 mM NaF and 2 mM $NaVO_3$). The protein extracts, mixed with Laemmli sample buffer, were separated by SDS-PAGE and transferred to Immobilon-P membranes. Following blocking in TBS-containing 5% fat-free milk, the blots were incubated overnight with the antibodies against phospho-STAT-1 (Tyr^{701}), phospho-STAT-2 (Tyr^{689}), STAT-1 protein, STAT-2 protein, (all from cell signalling except phospho-STAT-2 antibody, which was from Millipore) and GAPDH (Santa Cruz Biotechnology). The proteins were visualized using enhanced chemiluminescence (Pierce), and their abundance was analysed using CCD image system (Chemi-Doc™ MP Imaging System) and IMAGE LAB software, v. 4.1 (Bio-Rad).

Virus and infection

Low-passage TBE virus strain Neudoerfl (a generous gift from Prof. F. X. Heinz, Medical University of Vienna), a prototype strain of the European subtype, was used in the experiments. Bone marrow-derived DCs were seeded to 96-well plate at concentration 2×10^5 per well. 24 h later, the cells were infected by the virus at multiplicity of infection (MOI) of 10 (5 μL of virus suspension in RPMI 1640 medium with 10% FCS was added into each well). After virus adsorption for 1 h at 37°C and 5% CO_2, the cells were washed with PBS, and complete medium without or with sialostatin L2 (3 μM) was applied. After another two hours post-infection, recombinant mouse IFN-β (PBL interferon source) was added to the final concentration of 100 U per ml. At 48 h post-infection, supernatant medium from the wells was collected and frozen at −70°C. Virus titres were determined by plaque assay.

Plaque assay

The porcine kidney stable cells (PS) were used to determine virus titre according to protocol described previously with minor modifications (16). PS were grown at 37°C in L-15 medium supplemented with 3% newborn calf serum and mixture of penicillin and streptomycin (Sigma-Aldrich). Tenfold dilutions of the virus samples were placed in 24-well tissue culture plates, and PS cells were added in suspension (10^5 of PS cells per well). After incubation for 4 h, the suspension was overlayed with carboxymethylcellulose (1·5% in L-15 medium). After incubation for 5 days at 37°C, the plates were washed with PBS and the cells were stained with naphthalene black (Sigma-Aldrich). Virus titre was expressed as PFU/mL.

Statistical analysis

Student's *t*-test in Medcalc 11.2.0.0 program or one-way analysis of variance (ANOVA) followed by Bonferroni test in GRAPHPAD PRISM, version 5.0, was used to compare the differences between control and treated groups. $P \leq 0{\cdot}05$ was considered statistically significant.

RESULTS

Tick salivary cystatins attenuate IFN-β-triggered JAK/STAT signalling pathway

Here, we tested tick salivary cystatins for their potential to interfere with IFN signalling. Major signalling pathway activated by IFN is JAK/STAT pathway (11). Both, STAT-1 (consisting of isoforms α and β) and STAT-2 proteins are phosphorylated in response to IFN. Therefore, the phosphorylation of these STATs was measured in spleen dendritic cells activated by recombinant IFN-β (5 U/mL) in the presence or absence of sialostatins for indicated times. Three independent experiments were performed with sialostatin L and two with sialostatin L2. Both cystatins attenuated the phosphorylation of STAT-1 induced by IFN-β (Fig. 1a). While the most prominent decrease by sialostatin L was seen at the time point 30 min and 60 min upon IFN-β addition (76·32% decrease at 30 min (±9·74%, $n = 3$, $P = 0{\cdot}01$) and 83·44% decrease at 60 min (±18·56%, $n = 3$, $P = 0{\cdot}03$), respectively), the effect of sialostatin L2 at the same time points was 46·68% decrease ±19·24% ($n = 2$) and 54·28% decrease ±32·68% ($n = 2$), respectively.

The phosphorylation of STAT-2 was induced with similar kinetic as STAT-1, and the inhibitory effect of both sialostatin L and sialostatin L2 was observed (Fig. 1b). Sialostatin L decreased the phosphorylation of STAT-2 by 63·44% at 30 min (±12·45%, $n = 3$, $P = 0{\cdot}05$), by 59·64% at 60 min (±0·36%, $n = 3$, $P = 0{\cdot}02$) and by 42·77% at 120 min (±3·38, $n = 3$, $P = 0{\cdot}1$). The phosphorylation of STAT-2 was decreased by sialostatin L2 at 60 min by 19·9% (±4·51%, $n = 2$) and at 120 min by 21·2% (±25·67%, $n = 2$). The effect of sialostatins on IFN signalling was also tested in peritoneal macrophages; however, no differences in STAT-1 activation were observed (data not shown). The inhibitory effects of sialostatins on IFN signalling thus seem to be restricted to dendritic cells.

Tick cystatins do not influence internalization rate of IFN receptor

To understand how sialostatins attenuate STATs phosphorylation, we first tested at which step sialostatins interfere with IFN signal transduction pathway. IFN receptor is the most upstream molecule, which could be targeted by sialostatins. IFN receptor consists of two chains, IFNAR-1 and IFNAR-2. Upon ligation, IFNAR-1 is internalized and rate of its internalization influences downstream signalling [reviewed in references (17, 18)]. We determined the kinetics of receptor internalization by measuring the amount of IFNAR-1 on the cellular surface by flow cytometry. Dendritic cells were preincubated with sialostatin L or sialostatin L2 for 2 h and stimulated with recombinant IFN-β for 30 min. The amount

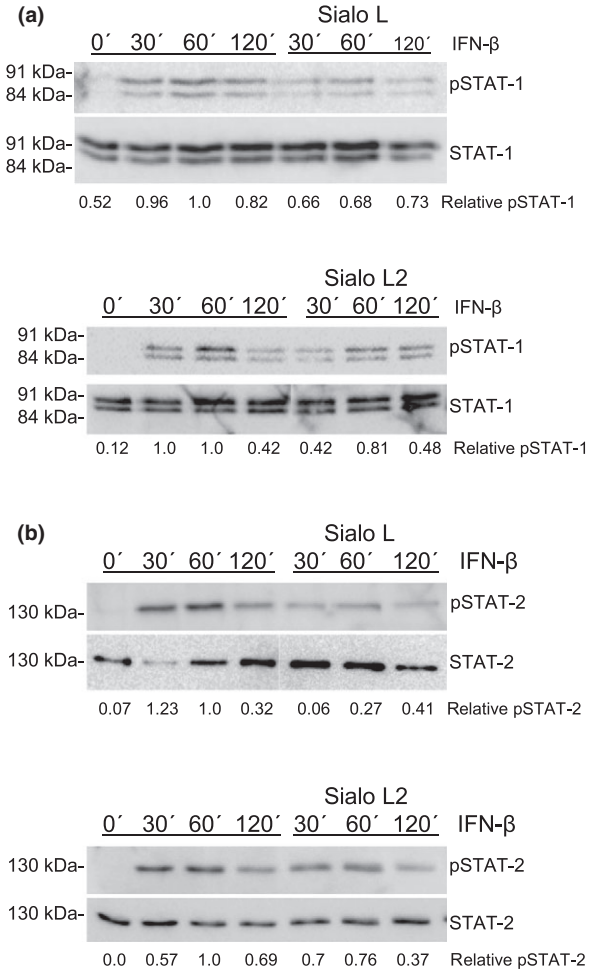


Figure 1 Tick cystatins attenuate IFN-β signalling at the level of JAK/STAT activation in dendritic cells. Spleen dendritic cells were stimulated with five units of IFN-β for indicated times in the presence or absence of 3 μM sialostatin L and sialostatin L2. Cell lysates were analysed by immunoblotting with anti-phospho-STAT-1 antibody (a) or anti-phospho-STAT-2 antibody (b). The membranes were after stripping reprobed with antibody against STAT-1 protein (a) or STAT-2 protein (b), what served as a control. Bands corresponding to the phosphorylated forms of STAT-1 (pSTAT-1) or STAT-2 (pSTAT-2) and nonphosphorylated forms of STATs were quantified using scanning densitometry. Relative phosphorylations/activities of STATs were normalized by the STAT-1 or STAT-2 protein levels, respectively (relative activity = pSTAT/STAT; densitometric measurements of both STAT-1 isoforms were pooled together). Relative activities of STAT-1 and STAT-2 achieved 60 min after IFN stimulation were set up to 1 for better illustration. Three independent experiments were performed with sialostatin L and two with sialostatin L2. Representative blots are shown.

of IFNAR-1 decreased upon IFN-β addition as expected. In the presence of either cystatins, the clearance of IFNAR-1 from cellular surface was however comparable to the control (Fig. 2a). The preincubation of cells with sialostatins for even longer time did not affect the basal surface level of IFNAR-1 (Fig. 2b, data shown for sialostatin L). This result suggests that sialostatins do not interfere with IFN signalling at IFNAR-1 level but rather influence transduction downstream of IFN receptor.

Induction of interferon-stimulated gene IRF-7 and production of IP-10 is suppressed by sialostatin L2 in LPS-activated dendritic cells

Stimulation of cells with TLR-4 agonist causes the release of IFN-β which in turn activates IFN-receptor-mediated signalling cascade and results in the induction of IFN-stimulated genes, such as the transcription factor IRF-7 and the chemokine IP-10 (19–21). We wondered whether the impairment of IFN signalling, observed in the presence of cystatins, would result in the failure to induce IRF-7 and IP-10 in LPS-stimulated spleen DC. We determined the level of IRF-7 gene expression in dendritic cells incubated with LPS for 6 h in the presence or absence of sialostatin L and sialosatin L2 (Fig. 3a). LPS induced 1·89-fold increase of IRF-7 expression as compared to nonstimulated control. While sialostatin L did not affect gene expression of IRF-7, the induction of IRF-7 was completely inhibited by sialostatin L2.

The amount of chemokine IP-10 produced by LPS-stimulated dendritic cells was measured by ELISA (Fig. 3b). LPS stimulation induced comparable level of IP-10 as 10 U of IFN-β, and the significant decrease was observed in the presence of sialostatin L2 but not sialostatin L. IP-10 thus represents another ISG, together with IRF-7, which is affected by sialostatin L2.

To exclude the possibility that observed impairment in ISG by sialostatin L2 is due to the decline of IFN-ß production, we measured the amount of IFN-β after LPS stimulation. Dendritic cells were stimulated by LPS for 6 h, and the secreted IFN-β was measured in culture

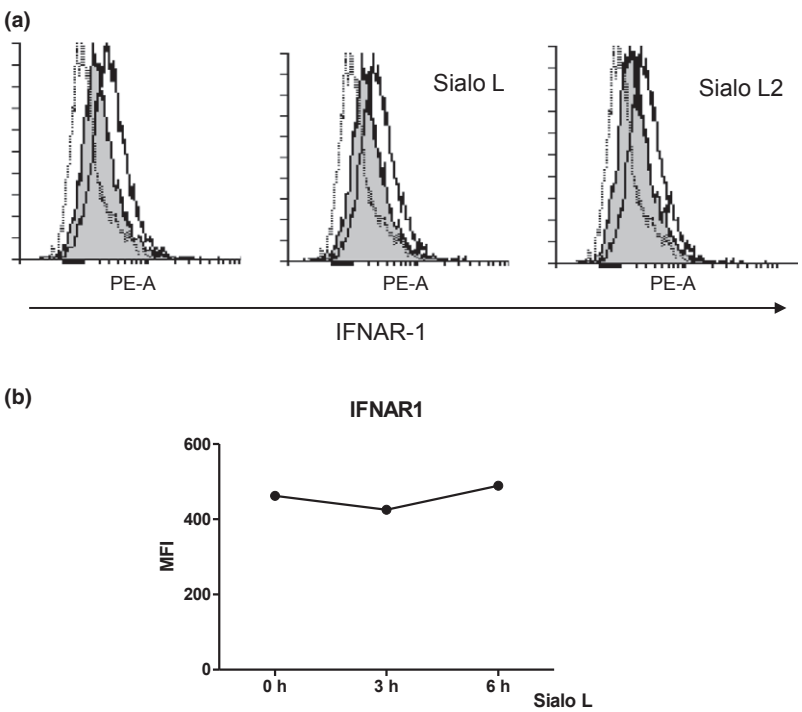
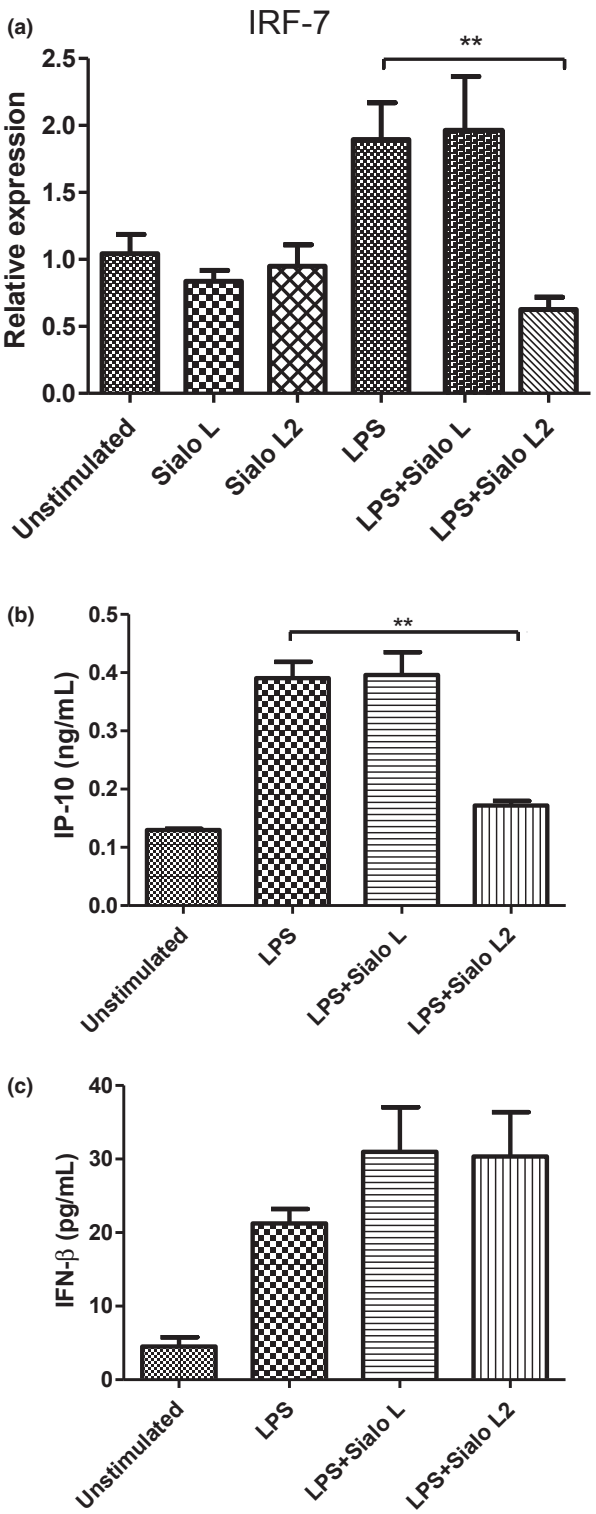


Figure 2 Sialostatins do not impact the internalization rate of the IFN receptor (IFNAR-1). (a) Dendritic cells were preincubated with 3 μM sialostatins L or L2 for 2 h and stimulated with IFN-β (10 U/mL) for 30 min. Cells were collected, stained with anti-IFNAR-1 antibody and analysed by flow cytometry. The surface expression of IFNAR-1 was determined in CD11c positive cell population. Representative histograms are shown, empty histograms with solid line represent unstimulated cells, grey-filled histograms represent IFN-β-stimulated cells, and dotted line histograms are isotype control. (b) Dendritic cells were incubated with 3 μM sialostatin L for the times as indicated. Cells were collected, stained and analysed as in A. Mean fluorescence intensity (MFI) values of IFNAR-1 are shown.



supernatant. As shown in Fig. 3c, none of tested sialostatins influenced IFN-β production in LPS-activated spleen DC.

Figure 3 Sialostatin L2 impairs induction of interferon-stimulated genes, IRF-7 and IP-10 in LPS-stimulated DC and does not affect the level of secreted IFN-β. (a) Dendritic cells were activated by 100 ng/mL of LPS for 6 h in the presence or absence of sialostatins L and L2 (both 3 μM). Transcript level of IRF-7 was determined by qPCR using specific primers for IRF-7. Expression of IRF-7 was normalized to β-actin transcript. Data from three independent experiments were pooled together and relative expression of IRF-7 is shown. (b) Dendritic cells were activated by 100 ng/mL of LPS in the presence or absence of sialostatin L and sialostatin L2 (both 3 μM). Culture supernatants were collected 24 h after ligand addition and analysed for the presence of IP-10. (c) Dendritic cells were treated as in (a). Culture supernatants were then collected and analysed by ELISA for IFN-β. Data are expressed as the mean cytokine concentration from triplicate wells ± SD. Two independent experiments were performed (for b and c), and data are shown from the representative one. **Indicates the effect of cystatin on IRF-7 induction/IP-10 production significant at $P < 0{\cdot}01$.

Sialostatin L2 promotes replication of TBE virus in dendritic cells and compromises the antiviral action of IFN-β

The above-described findings prompted us to investigate whether sialostatin L2 would impact the replication of tick-borne encephalitis virus in dendritic cells. We used Neudoerfl strain to infect dendritic cells derived from bone marrow by GM-CSF. Spleen cells were substituted by bone marrow DC because they produce less IFN-β than spleen dendritic cells and therefore are more appropriate for showing the effect of exogenously added IFN-β. Following virus adsorption, sialostatin L2 was added to cell cultures and virus titre was measured 48 h later as described in 'Materials and Methods'. As shown in Fig. 4, the replication of TBE virus was significantly enhanced by sialostatin L2. The effect of IFN-β on virus replication was determined by adding 100 U/mL of recombinant IFN-β 2 h after virus adsorption in the presence or absence of sialostatin L2. The antiviral effect of IFN-β was significantly compromised by sialostatin L2.

DISCUSSION

The effective inhibition of the host immune response is essential for feeding success of ticks and as a side effect it supports transmission and dissemination of tick-transmitted pathogens (2). Intact type I IFN signalling pathway is required for protection against viral infection. We report in this paper that tick salivary cystatin, sialostatin L2, attenuates IFN signalling. More specifically, it (i) interferes with IFN-triggered signal transduction by decreasing phosphorylation of STAT-1/2 proteins, (ii) negatively

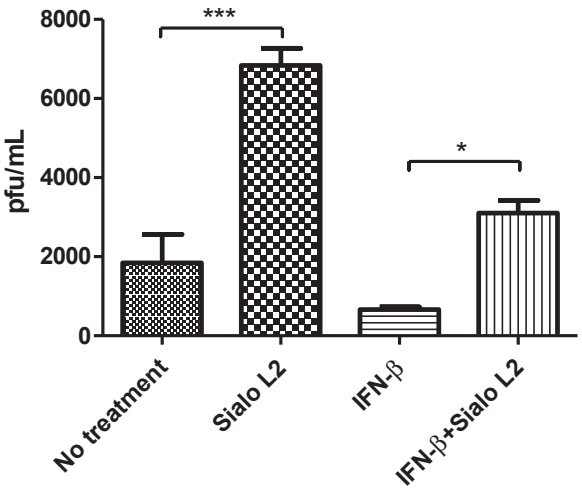


Figure 4 Sialostatin L2 promotes replication of TBE virus in dendritic cells and compromises the antiviral action of IFN-β. Bone marrow-derived dendritic cells were infected with Neudoerfl strain of TBE virus at MOI 10. After adsorption of the virus, recombinant IFN-β (100 U/mL) and/or sialostatin L2 (3 μM) were added to the cells. Following 48 h lasted incubation, the virus titre was determined as described under 'Materials and Methods'. ***Indicates the difference in virus production between control and sialostatin L2-treated cells significant at $P < 0{\cdot}001$; indicates the difference in virus production between IFN-β-treated and IFN-β+ Sialo L2-treated cells significant at $P < 0{\cdot}05$.

affects IFN-β-mediated induction of IFN-sensitive genes in LPS-stimulated cells and (iii) promotes TBE virus replication in dendritic cells *in vitro*.

Type I interferons, represented by IFN-α and IFN-β, are ones of the earliest cytokines secreted upon viral or bacterial infection, and dendritic cells are able to secret IFNs and react to them (22, 23). Dendritic cells play a key role in the recognition of pathogens, and as professional antigen-presenting cells, they determine the development of adaptive immunity. By releasing soluble mediators, including type I IFN, they influence the innate type of immunity as well.

Binding of IFN to its cognate receptor activates JAK/STAT signalling pathway and leads to the induction of IFN-stimulated genes (11). Interference of tick cystatins with IFN-receptor-triggered signal transduction was detected at the level of STATs phosphorylation. We found that STAT-1 and STAT-2 phosphorylation was decreased by both sialostatin L and sialostatin L2. Several ways how cystatins can affect the phosphorylation of STATs can be considered. The phosphorylation of STAT proteins is controlled by proteolytic processing in addition to dephosphorylation (17, 24). The degradation of STAT proteins was not observed by sialostatin L or sialostatin L2, so it is apparently degree of phosphorylation/dephosphorylation which is influenced by these polypeptides. Whether cystatins interfere directly with STATs phosphorylation or affect some upstream molecule is not clear. At least, the possibility that cystatins affect IFN signalling at the receptor level can be excluded as the internalization rate of interferon receptor (IFNAR-1) was unaffected by either cystatin. Type I IFN, when secreted from DC, not only establishes an antiviral state in themselves and other cells, but negatively effects their phenotypic and functional activation (25). In agreement, LPS-induced maturation of bone marrow DC was impaired by sialostatin L, so defective IFN signalling, observed by us could contribute to this effect (9).

Immunomodulatory effects of several cystatins from various parasites were reported pointing to the importance of these inhibitors in parasite–host interaction [reviewed by (26)]. The interference of parasitic cystatin with the IFN action is a novel finding. In fact, there is only one reported case showing the inhibition of IFN signalling by a cystatin, particulary cellular cystatin B (27, 28). Cystatin B is co-expressed with STAT-1 in human macrophages, inhibits IFN-β response by preventing phosphorylation of STAT-1 and is associated with increased HIV-1 replication in human macrophages. Interestingly, these effects are likely mediated by cystatin interaction with proteins lacking any proteolytic function (28). Thus, cystatins, although primarily recognized as inhibitors of cysteine proteases, can function as regulatory proteins.

In DC, the stimulation with TLR-4 agonist leads to the production of IFN-β. Released IFN-β binds to IFN receptor and induces the expression of IFN-stimulated genes, such as the transcription factor IRF-7 and the chemokine IP-10 (20, 21, 29). We show that the induction of both, IRF-7 and IP-10, was impaired by sialostatin L2 in LPS-stimulated dendritic cells. IRF-7 is an important transcription factor involved in positive regulation of TLR signalling and is also required for robust IFN induction (30). Furthermore, it has been shown that IRF-7 is critical for the induction of antiviral IFN-α response during West Nile virus (another member of Flaviviridae family) infection (31). We can speculate that sialostatin L2 could similarly interfere with antiviral host response through downregulation of IRF-7.

The consequences of impaired IFN signalling for pathogen transmission/growth can be expected. And indeed, the replication of TBE virus in bone marrow dendritic cells was enhanced in the presence of sialostatin L2, possibly as a consequence of impaired IFN signalling. Moreover, the antiviral action of exogenously added recombinant IFN-β was also compromised by Si-

alo L2. To show this effect, we had to substitute spleen DC by bone marrow DC. It was due to the lack of antiviral effect of exogenous IFN-β on TBE virus replication in spleen DC (presumably because of high endogenous level of IFN-β). Thus, for mechanistic part of the study, we utilized spleen DC and the biological effect of sialostatin L2 was shown in bone marrow DC. Of note, the promotion of *Borrelia burgdorferi* 'growth' by sialostatin L2 was observed *in vivo* when intradermally injected into mice (6).

In summary, we present here a novel finding that tick salivary cystatin sialostatin L2 attenuates the interferon responses in dendritic cells. The suppression of IFN-stimulated genes induced in LPS-stimulated dendritic cells and the enhancement of the TBE virus replication in DC by sialostatin L2 have been demonstrated. The inhibitory effect of tick cystatin on interferon responses in host dendritic cells is a newly described mechanism elucidating the role of tick saliva in the transmission of tick-borne pathogens.

ACKNOWLEDGEMENT

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Parasites & Vectors

RESEARCH Open Access

Tick sialostatins L and L2 differentially influence dendritic cell responses to *Borrelia* spirochetes

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Abstract

Background: Transmission of pathogens by ticks is greatly supported by tick saliva released during feeding. Dendritic cells (DC) act as immunological sentinels and interconnect the innate and adaptive immune system. They control polarization of the immune response towards Th1 or Th2 phenotype. We investigated whether salivary cystatins from the hard tick *Ixodes scapularis*, sialostatin L (Sialo L) and sialostatin L2 (Sialo L2), influence mouse dendritic cells exposed to *Borrelia burgdorferi* and relevant Toll-like receptor ligands.

Methods: DCs derived from bone-marrow by GM-CSF or Flt-3 ligand, were activated with *Borrelia* spirochetes or TLR ligands in the presence of 3 μM Sialo L and 3 μM Sialo L2. Produced chemokines and IFN-β were measured by ELISA test. The activation of signalling pathways was tested by western blotting using specific antibodies. The maturation of DC was determined by measuring the surface expression of CD86 by flow cytometry.

Results: We determined the effect of cystatins on the production of chemokines in *Borrelia*-infected bone-marrow derived DC. The production of MIP-1α was severely suppressed by both cystatins, while IP-10 was selectively inhibited only by Sialo L2. As TLR-2 is a major receptor activated by *Borrelia* spirochetes, we tested whether cystatins influence signalling pathways activated by TLR-2 ligand, lipoteichoic acid (LTA). Sialo L2 and weakly Sialo L attenuated the extracellular matrix-regulated kinase (Erk1/2) pathway. The activation of phosphatidylinositol-3 kinase (PI3K)/Akt pathway and nuclear factor-κB (NF-κB) was decreased only by Sialo L2. In response to *Borrelia burgdorferi*, the activation of Erk1/2 was impaired by Sialo L2. Production of IFN-β was analysed in plasmacytoid DC exposed to *Borrelia*, TLR-7, and TLR-9 ligands. Sialo L, in contrast to Sialo L2, decreased the production of IFN-β in pDC and also impaired the maturation of these cells.

Conclusions: This study shows that DC responses to *Borrelia* spirochetes are affected by tick cystatins. Sialo L influences the maturation of DC thus having impact on adaptive immune response. Sialo L2 affects the production of chemokines potentially engaged in the development of inflammatory response. The impact of cystatins on *Borrelia* growth *in vivo* is discussed.

Keywords: Dendritic cells, *Borrelia burgdorferi*, Tick cystatin, Signalling

Background

Borrelia burgdorferi, the causative agent of Lyme disease, is transmitted to mammals through the bite of infected *Ixodes* ticks. In the skin, dendritic cells (DC) are among the first immune cells to come into contact with *B. burgdorferi* [1]. *B. burgdorferi* elicits a potent cytokine/chemokine response through activation of multiple pattern recognition receptors on innate immune cells, including Toll-like receptor (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) [2]. TLRs have an essential role in the control of *B. burgdorferi* burden, because mice deficient in the common TLR signaling molecule myeloid differentiation primary response 88 (MyD88), have up to 250-fold more spirochetes than the wild-type controls [3, 4]. Among Toll-like receptors (TLRs), TLR-2 has been found to be the most important receptor for induction of pro-

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inflammatory mediators, whereas endosomal receptors TLR-7 and TLR-9 mediate type I interferon production [5–9]. All these TLRs utilize MyD88 as adaptor molecule, however, TLR-2 dependent inflammatory responses to *B. burgdorferi* can also be mediated by Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) [10]. *Borrelia* spirochetes activate multiple signalling pathways through these adaptors, including nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPK) (extracellular matrix-regulated kinase (Erk) 1/2, p38, Janus N-terminal kinase (JNK)) [11–13], phosphatidylinositol-3 kinase (PI3K) [14], and Protein kinase C (PKC) pathways [15]. The p38 MAPK and NF-κB are critically involved in the expression of pro-inflammatory cytokines [12, 16], whereas PI3K pathway is fundamental for optimal phagocytosis [14]. *Borrelia* also strongly induces anti-inflammatory cytokine IL-10, which has overall suppressive effect on induction of pro-inflammatory mediators [17, 18].

Dendritic cells, as a part of innate immune system, produce several cytokines and chemokines which in autocrine and paracrine manner regulate the establishment of an innate immune response, including the recruitment of monocytes, macrophages, and neutrophils [19]. In addition, DC upon sensing pathogens undergo the maturation process, characterized by increased expression of co-stimulatory molecules, which is necessary for proper presentation of antigen to naïve T-cells. *In vitro*, dendritic cells can be obtained by culturing of bone- marrow cells in the presence of two cytokines, granulocyte-macrophage colony-stimulated factor (GM-CSF) or Fms-like tyrosine kinase 3 ligand (Flt-3L), respectively. By GM-CSF, the myeloid subset of dendritic cells can be generated, while with the Flt-3 ligand, lymphoid- type of plasmacytoid dendritic cells (pDC) can be obtained [20, 21]. The pDC are characterised by robust production of type I IFN [22]. These subsets of DC differ in the cytokine profiles they induce in T cells *in vivo* [23].

Dendritic cells are key players in host defense against tick-transmitted borreliae [1]. However, many functions of DC are negatively influenced by tick saliva [24–26]. In addition to prostaglandin E2 [27], purine nucleoside adenosine [28] and Salp15 [29], tick cystatins are also involved in the effect of tick saliva on dendritic cells [30].

Sialostatins L and L2 are cysteine protease inhibitors of the hard tick *Ixodes scapularis.* Both are strong inhibitors of cathepsin L [31, 32], but sialostatin L also inhibits cathepsin S. Immunosuppressive effects of Sialo L have been demonstrated in T cell line CTLL-2 [32] and lipopolysaccharide-activated DC [33]. Expression of Sialo L2 is greatly enhanced by feeding and is necessary for tick feeding success [34]. In addition to being able to enhance the growth of *Borrelia burgdorferi in vivo* [35], this sialostatin has been shown to inhibit the inflammasome formation during infection with *Anaplasma phagocytophilum* in macrophages through targeting caspase-1 activity [36].

In order to understand how Sialo L2, a tick salivary molecule, can support *Borrelia* establishment in the host, we studied the effect of tick cystatins on DC maturation and function. The effect on the production of chemokines, IFN-β and signalling pathways activated in dendritic cells by *Borrelia* spirochetes and relevant TLR ligands was analysed.

Methods

Animals

Female C57BL/6 mice (10 weeks of age) were obtained from Charles River Laboratories. All experiments were performed with permission from Local animal ethics committee of the Institute of Parasitology, Biology Centre ASCR České Budějovice, PID 167/2011.

Bacteria

The strain of *Borrelia burgdorferi* sensu stricto obtained from ATCC collection was grown in Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma) supplemented with 6 % rabbit serum at 34 °C. The fourth passage was used in the experiments.

Preparation of recombinant cystatins

Recombinant cystatins Sialo L and Sialo L2 were expressed in *Escherichia coli* followed by purification of active protein, as previously described [31, 35]. LPS contamination was removed by Arvys Proteins using the detergent-based extraction method. The presence of endotoxin was estimated with a sensitive fluorescent-based endotoxin assay (Lonza Biologics) and was $<3 \times 10^{-14}$ endotoxin g/μg protein for both cystatins. The endotoxin level did not exceed 2 pg/ml during testing the effect of cystatins on DC.

Generation of bone-marrow-derived dendritic cells

Bone-marrow derived conventional dendritic cells (DC) and plasmacytoid (pDC) dendritic cells were prepared as described before [20, 21], respectively, with minor modifications. Briefly, mice were sacrificed by cervical dislocation, intact femurs and tibias were removed, and bone marrow was harvested by repeated flushing with MEM. To derive conventional DC, bone marrow cells (10^6/ml) were cultured for 7 days in 6-well plate in RPMI 1640 medium supplemented with 10 % FCS, 50 mM HEPES, 2 mM glutamine, 50 μM mercaptoethanol, penicillin, streptomycin, amphotericin B, and 30 ng/ml of recombinant mouse GM-CSF (Sigma-Aldrich). Half of the medium was replaced with the fresh medium on day 3 and 5. On day 7, non-adherent cells were harvested and used as immature DC.

To analyse the effects of cystatins on DC differentiation, 10^5 bone-marrow cells were seeded in 96-well plate in the

same medium as described above (including GM-CSF) and the Sialo L or Sialo L2 were added to the culture on day 3 to final concentration 3 μM. Cells were fed on day 5 and 7, and harvested on day 9. Surface expression of MHC class II was determined by flow cytometry within CD11c-positive population.

To derive plasmacytoid cells, bone marrow cells (1.5×10^6 / ml) were cultured for 8 days in 6-well plate in RPMI 1640 medium supplemented with 10 % FCS, sodium pyruvate, glutamine, penicillin, streptomycin, amphotericin B (PAA) and 100 ng/ml of recombinant human Flt-3L (R&D Systems). Half of the medium was replaced once after 4 days of culture. On day 8, non-adherent cells were harvested, washed in fresh medium and used in subsequent experiments.

IFN-β measurement

Freshly derived pDC were seeded in 96-well plate at a concentration of 2×10^5 cells per well. Following 2 h incubation with Sialo L or Sialo L2 (each 3 μM) the cells were stimulated with spirochetes at MOI = 10 (10 spirochetes per 1 cell), imiquimod (R837, 2 μg/ml) (InvivoGen), or CpG (ODN1668, 50 nM) (Enzo Life Sciences). MOI = 10 was sufficient to activate DC as shown previously [37]. IFN-β was determined in cell-free culture supernatants harvested 5 and 16 h after stimulation using LEGEND MAX™ mouse IFN-β ELISA Kit (BioLegend) following the manufacturer's instructions.

Chemokine measurements

BMDC were seeded at concentration 0.5×10^6 or 2×10^5 cells per well in 24-well plate or 96-well plate, respectively. Next day DCs were incubated 2 h with Sialo L or Sialo L2 (both 3 μM) and then *B. burgdorferi* was added at MOI = 10. After 24 h, cell-free supernatants were collected and analysed in Proteome Profiler™ antibody array according the manufacturer's instructions (R&D). The chemokines were visualized by enhanced chemiluminescence and the abundance of signal was measured using CCD image system (ChemiDoc™ MP Imaging System) and Image Lab software, v. 4.1 (BIO-RAD). Alternatively, the amount of secreted chemokines (IP-10, MPC-1, MIP-1α, MIP-1β, and MIP-2) was determined in cell-free culture supernatants using ELISA kits (PeproTech) following the manufacturer's instructions.

Flow cytometry

Bone marrow-derived pDC were seeded on 96-well plate at the concentration of 1×10^6 cells per ml of complete culture medium with Flt-3L and pretreated with either Sialo L or Sialo L2 (both 3 μM). After 2 h, cells were activated either with imiquimod (2 μg/ml), CpG (ODN1668, 50 nM) or *B. burgdorferi* spirochetes (MOI = 10). After 24 h incubation, cells were washed once in PBS with 1 % FCS and stained for flow cytometry analysis with anti-CD11c-PE mAb, anti-MHCII-AlexaFluor700 mAb, anti-CD86-APC mAb (all from eBioscience), anti-CD11b-FITC mAb, and anti-B220-PE-Vio770 mAb (both from Miltenyi Biotech). Dead cells were excluded from analysis using propidium iodide. Flow cytometry was performed on FACS Canto II flow cytometer and data were analysed using FACS Diva software, v. 5.0 (BD Biosciences). Plasmacytoid DCs were gated from living single cells as CD11c+, CD11b- and B220+. Levels of expression of CD86 were measured as MFI of APC.

Immunoblotting

BMDC were seeded at 0.5×10^6 cells per well in 24-well plate. Next day DCs were incubated 2 h with tick cystatins (each 3 μM) prior to the addition of LTA (2 μg/ml) for 15, 30, and 60 min or *Borrelia* spirochetes (MOI = 10) for 15, 30, 60, and 120 min. Afterwards, cells were lysed in a RIPA buffer (1 % Nonidet P-40, 0.25 % sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.5)) in the presence of protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin) and phosphatase inhibitors (25 mM sodium fluoride and 2 mM sodium orthovanadate). 20 μg of total proteins were separated by SDS-PAGE using an 8 % gel and then electrotransferred to Immobilon-P membranes. The blots were incubated overnight at 4 °C with the antibody recognizing phospho-NF-κB p65 (Ser^{536}), phospho-p44/42 MAPK (Erk1/2) (Thr^{202}/Tyr^{204}), phospho-p38 MAPK (Thr^{180}/Tyr^{182}), phospho-Akt (Ser^{473}), total NF-κB p65, p44/42 MAPK (Erk1/2), p38 MAPK, Akt, and β-actin (all from Cell Signalling) followed by incubation with secondary antibody conjungated with horse radish peroxidase. The proteins were visualized using enhanced chemiluminescence (Pierce), and their abundance was analysed using CCD image system (ChemiDoc™ MP Imaging System) and Image Lab software, v. 4.1 (BIO-RAD).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Bonferroni test in GraphPad Prism, version 5.0 was used to compare the differences between control and treated groups. $P \leq 0.05$ was considered statistically significant and is marked by one star, $P \leq 0.01$ is marked by two stars.

Results

Sialostatin L2 decreases the MIP-α and IP-10 production by dendritic cells in response to *Borrelia burgdorferi*

Numbers of chemokines known to recruit leukocytes to the infection site are upregulated in DC during *Borrelia* infection [18, 38]. We aimed to determine the effect of cystatins on chemokine production by bone-marrow derived dendritic cells upon *Borrelia* stimulation. We utilised

proteome chemokine array to screen which chemokines are induced by *Borrelia* spirochetes, and which might be affected by sialostatins. Addition of borreliae resulted in a 3.5-fold increase of neutrophil-recruiting chemokine CXCL1 (KC), 4.5-fold rise of CXCL10 (IP-10) and 18.6-fold increase of monocyte/macrophage recruiting chemokine CCL3/CCL4 (MIP-1α/β). Two-fold rise and less was observed in case of CXCL2 (MIP-2), CCL5 (RANTES), CCL2 (MCP-1/JE), CXCL5 (LIX), and CXCL16 (Fig. 1a). The production of all tested chemokines was reduced by Sialo L2, except for KC and MIP-1γ which remained unchanged (MIP-1α/β by 23 %, MIP-2 by 18 %, RANTES by 15 %, JE by 29 %, LIX by 25 %, CXCL16 by 32 % and IP-10 by 44 %). Sialo L, in contrast to Sialo L2, did not influence either of these chemokines in the array. The effect of sialostatins on selected chemokines (MIP-1α, MIP-1β, IP-10, MIP-2, and JE) was further analysed by ELISA. The inhibitory effect of sialostatin L2 was confirmed for two chemokines; the production of MIP-1α and IP-10 was significantly decreased (Fig. 1b, c). However, we did not observe any effect of sialostatin L2 on other tested chemokines (data not shown). Interestingly, MIP-1α was inhibited also by sialostatin L. This was not seen in the proteome array likely because of using pan antibody not able to distinguish between MIP-1α and MIP-1β.

The effect of sialostatin L2 on the signalling pathways activated by LTA and *Borrelia burgdorferi* in dendritic cells

Induction of proinflammatory mediators by *B. burgdorferi* is mediated by multiple signalling pathways through ligation of several TLRs. Because TLR-2 is known to be strongly activated by *Borrelia* lipoproteins, we first tested the activation of signalling pathways upon addition of lipoteichoic acid (LTA), a ligand for TLR-2, in the presence or absence of both cystatins. The pathways important for induction of pro- inflammatory cytokines and chemokines were analysed: Erk1/2 and p38 MAP kinases, NF-κB, and PI3K/Akt pathways (Fig. 2a). Sialo L2 attenuated phosphorylation of Erk1/2 (decrease by 72 % at 60 min), while Sialo L decreased this signalling molecule by 37 % at 60 min (Fig. 2b). The activation of p38 MAP kinase remained unchanged in the presence of both cystatins (Additional file 1a). Interestingly, the activation of PI3K pathway, measured by the phosphorylation of its downstream target Akt, was decreased by 76 % in the presence of Sialo L2. No such effect was

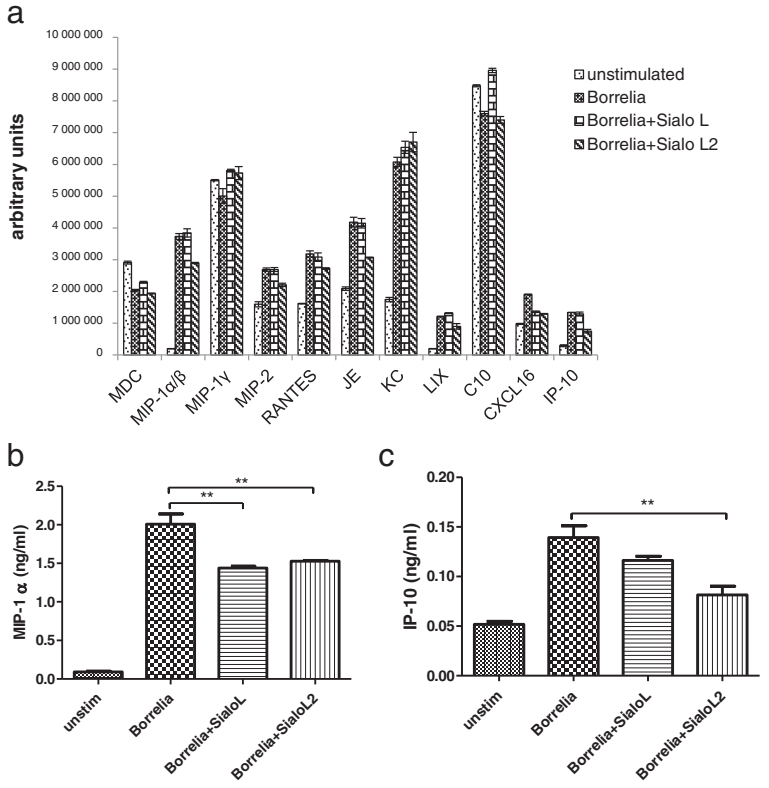

Fig. 1 Sialostatin L2 inhibits the MIP-α and IP-10 production by dendritic cells in response to *Borrelia burgdorferi*. Dendritic cells were exposed to *B. burgdorferi* at MOI = 10 in the presence or absence of sialostatin L (3 μM) or sialostatin L2 (3 μM). After 24 h, supernatants were collected and used in proteome chemokine array. The chemokines were visualized by enhanced chemiluminescence and their amount is expressed as arbitrary units (**a**). Similarly, supernatants from cells treated as described above were analysed in ELISA assay for the presence of MIP-1α (**b**) and IP-10 (**c**). Graphs (in **b**, **c**) show results of one representative experiment out of at least three independent experiments performed

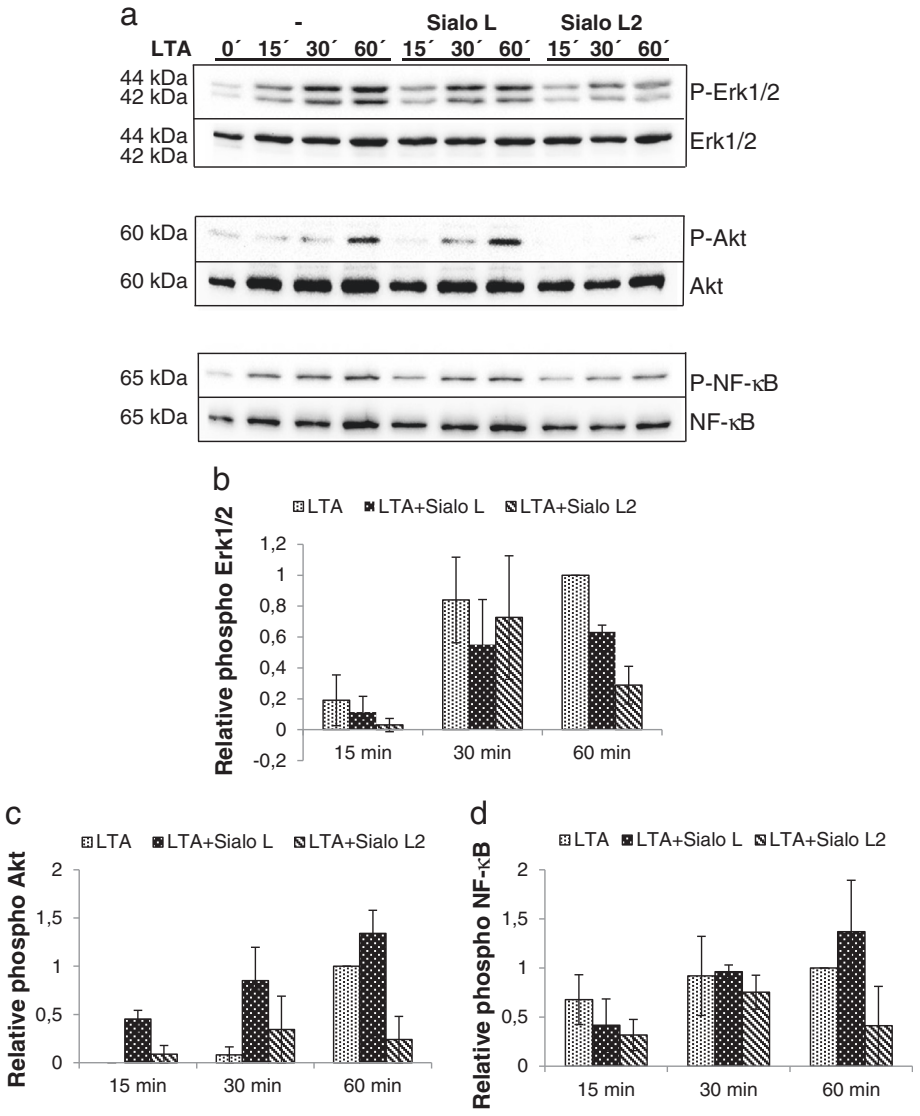

Fig. 2 Effect of sialostatins on the signalling pathways activated by LTA in dendritic cells. Dendritic cells were seeded in 24-well plate. Next day DCs were incubated 2 h with tick cystatins (both 3 μM) prior to the addition of LTA (2 μg/ml) and further incubated for indicated times. Afterwards, cells were lysed and obtained protein extract was further analysed by immunoblotting using antibodies recognizing phosphorylated form of tested kinases. Membranes were stripped and reprobed with antibodies against total kinase protein, which served as a control (**a**). Proteins were visualized by enhanced chemiluminescence. Bands were quantified using scanning densitometry and phosphorylation/activities of Erk1/2 (**b**), Akt (**c**), and NF-κB (**d**) kinases were normalized by total kinase protein level (relative activity = phospho kinase/total kinase). Three independent experiments were performed and representative blots are shown. Graphs represent the average ± SD from 2-3 experiments, the relative phosphorylation of kinase achieved at 60 min upon LTA stimulation was set up to 1 to allow pooling of data

observed in case of Sialo L (Fig. 2c). The phosphorylation of NF-κB was decreased by sialostatin L2 by 59 % at 60 min (Fig. 2d).

Similarly, dendritic cells were exposed to *B. burgdorferi* and the effect of Sialo L2 and Sialo L on signalling pathways was analysed. All tested pathways were activated with different kinetics compared to LTA. The phosphorylation of Erk1/2 kinase was impaired by Sialo L2 (decrease by 45 % at 30 min and by 22 % at 60 min) and by Sialo L by 29 % at 60 min (Fig. 3a, b). The phosphorylation of p38 MAPK, NF- κB and Akt remained unaffected in the presence of both Sialo L2 and Sialo L (Additional file 1b).

Sialostatin L decreases production of IFN-β in plasmacytoid dendritic cells activated by *Borrelia burgdorferi* and TLR-7 ligand

It has been shown that Borreliae are able to induce type I IFNs (IFN-α and IFN-β) in macrophages and dendritic cells and that this induction is mediated by endosomal TLR7/8 and TLR9 receptors [7–9]. Plasmacytoid (pDC)

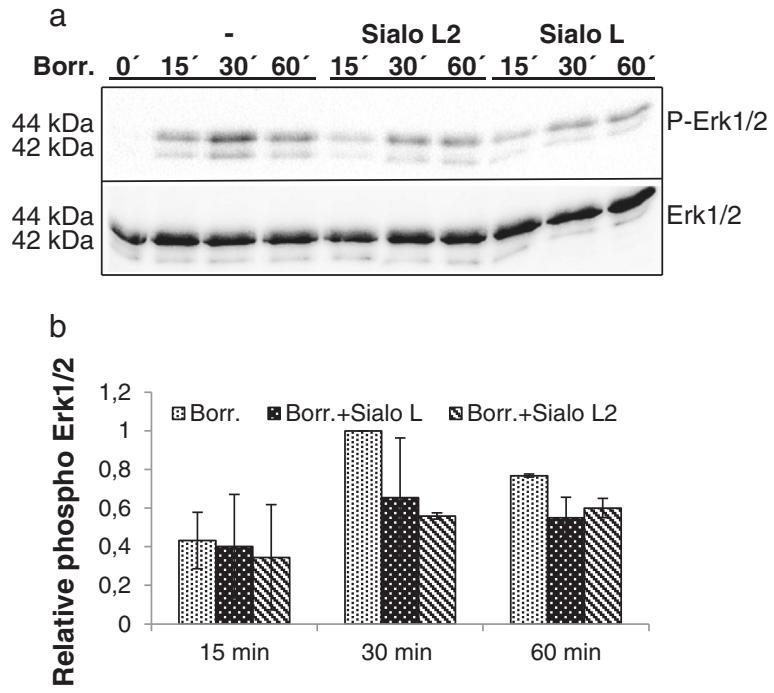

Fig. 3 Effect of sialostatin L2 and sialostatin L on the Erk1/2 signalling pathway activated by *Borrelia burgdorferi* in dendritic cells. Dendritic cells were seeded in 24-well plate. Next day DCs were incubated 2 h with sialostatin L2 and sialostatin L (both 3 μM) prior to the addition of Borreliae (MOI = 10) and further incubated for indicated times. Afterwards, cells were lysed and obtained protein extract was analysed by western blotting using antibodies against phosphorylated form of Erk1/2 and total Erk1/2 (**a**). Proteins were visualized by enhanced chemiluminescence. Relative phosphorylation/activity of Erk1/2 (**b**) kinase is shown; signal corresponding to phosphorylated kinase was adjusted to total kinase level. Three independent experiments were performed with sialostatin L2 and two with sialostatin L and representative blots are shown. Graph represent the average ± SD from 2 experiments, the phosphorylation of kinase achieved at 30 min upon *Borrelia* addition was set up to 1 to allow pooling of data

dendritic cells were chosen to examine the effect of tick cystatins on *Borrelia,* TLR-7, and TLR-9 - induced production of IFN-β. This subset of DC is known for great production of type I IFN and higher expression of TLR-7 and TLR-9 [39]. pDC were activated with *Borrelia* spirochetes, imiquimod (TLR-7 agonist), and CpG (TLR-9 agonist) in the presence or absence of cystatins and subsequently the amount of IFN-β was determined at indicated time points (chosen according to Petzke *et al.* [9] (Fig. 4). Upon addition of *Borrelia* spirochetes to cells we observed the induction of IFN-β mainly at later time point and this induction was significantly decreased by sialostatin L (Fig. 4a). Similarly, the amount of secreted IFN-β was significantly decreased by sialostatin L upon TLR-7 ligation at both tested time points (Fig. 4b). The presence of sialostatin L2 did not influence the amount of produced IFN-β. When pDC were stimulated with CpG, the production of IFN-β was more robust and increased with time. Sialostatin L decreased the IFN-β production by almost 50 % (without statistical significance), and sialostatin L2 remained without effect (Fig. 4c).

Sialostatin L negatively affects TLR-7 and TLR-9 mediated maturation of DCs but does not influence *Borrelia burgdorferi* induced maturation

Dendritic cells, upon sensing pathogen, undergo process of maturation, which is accompanied by an increase of expression of some co-stimulatory molecules, like CD86, CD40, and CD80. We wondered whether cystatins Sialo L and Sialo L2 can influence the maturation of plasmacytoid dendritic cells stimulated by *B. burgdorferi*, TLR-7 and TLR-9 ligands. The expression of co-stimulatory molecule CD86 was analysed by flow cytometry. The phenotype of pDC is shown in Fig. 5d, pDC were gated as CD11c+, CD11b-, and B220+ cells. As expected, addition of *Borrelia* spirochetes led to the increase of CD86 expression (Fig. 5a). However, the expression of CD86 increased to the comparable levels also in the presence of tested cystatins. Thus *Borrelia-* induced maturation was not affected by cystatins. On the contrary, in imiquimod-stimulated pDC, was observed small but significant decrease in CD86 surface expression in the presence of sialostatin L compared to control (Fig. 5b). Similarly, the increase of CD86 expression on DC, induced by ligation of TLR-9, was

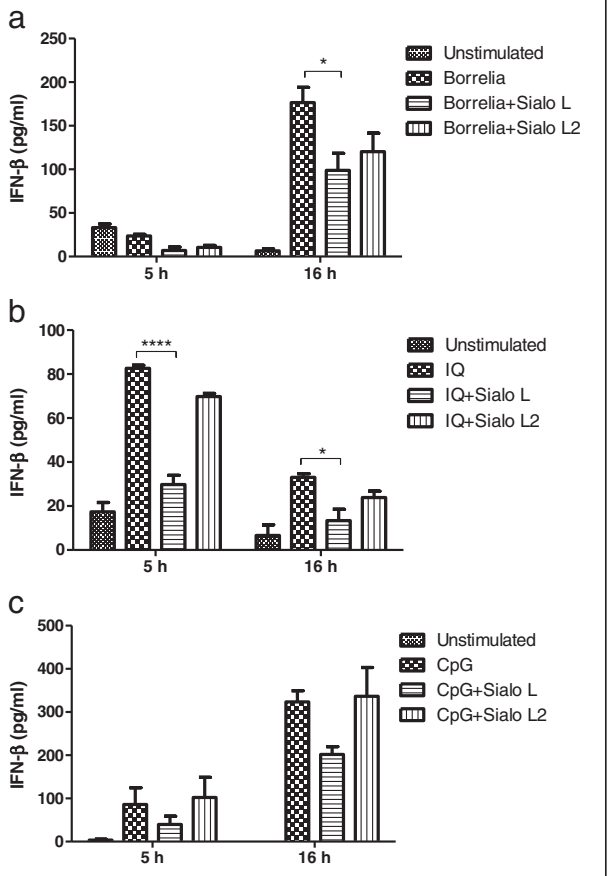

Fig. 4 The effect of tick sialostatins on the production of IFN-β in plasmacytoid dendritic cells activated by *Borrelia*, TLR-7, and TLR-9 ligands. Plasmacytoid dendritic cells were activated with *B. burgdorferi* at MOI = 10 (**a**), imiquimod (2 μg/ml) (**b**) or CpG (50 nM) (**c**) in the presence or absence of sialostatins (both 3μM). Supernatants were collected 6 and 16 h after stimulation and analysed by ELISA assay

inhibited by sialostatin L, but not sialostatin L2 (Fig. 5c). Thus sialostatin L negatively affects TLR-7, and TLR-9 mediated maturation of DC but does not significantly affect *Borrelia*-induced maturation.

Sialostatin L reduces differentiation of bone-marrow DC

As salivary molecules have an opportunity to enter bone marrow through the bloodstream, we decided to examine the influence of cystatins on the differentiation of dendritic cells from bone-marrow cells. The experiment was performed according to Sun *et al.* [40]. Bone-marrow cells were cultured (differentiated) in the presence of GM-CSF and on day 3 sialostatin L or sialostatin L2 were added to the cultures. After 8 days, cells were harvested and the expression of MHC class II molecules was determined. As shown in Fig. 6, among the CD11c positive cells, the number of MHCII positive cells reached 65.85 %. In the presence of sialostatin L the number of MHCII - positive cells decreased significantly to 43.91 %. Sialo L2 did not affect significantly the percentage of MHC class II positive cells.

Discussion

Sialo L2 and Sialo L are tick salivary cystatins, which are together with other salivary compounds released by the hard tick *I. scapularis* into the wound during tick feeding. During this process *B. burgdorferi* could be transmitted to the host. In response to *Borrelia* spirochetes, dendritic cells and other skin-resident immunocompetent cells become activated which leads to the production of proinflammatory mediators attracting further immune cells to the site of infection and activating them. These events can lead to clearing of most bacteria. It has been shown that Sialo L2, when injected intradermally into the mice, increased the burden of *Borrelia* spirochetes in the skin [35]. We hypothesized that observed effect could result from Sialo L2 evoked changes in dendritic cells function. Therefore we analysed the effect of Sialo L2 and related cystatin Sialo L on the immuno-modulatory function and signal transduction of mouse bone-marrow derived dendritic cells (DC) activated by *Borrelia* and relevant TLR ligands. We found that these two tick cystatins differentially modulate the function of DC. While Sialo L2 inhibited the production of chemokines MIP-1α and IP-10 in response to *Borrelia* spirochetes and attenuated the activation of Erk1/2, PI3K/Akt, and NF-κB pathways in response to TLR-2 ligation (the major receptor activated by spirochetal lipoproteins), the related cystatin Sialo L suppressed the production of IFN-β and attenuated the maturation and differentiation of DC.

In our *ex vivo* experiments, *Borrelia*-stimulated bone-marrow dendritic cells secreted several chemokines, including neutrophil-, monocyte/macrophage-, and T cell-recruiting chemokines, similarly as was reported by other studies [18, 38]. Sialo L2 suppressed significantly production of two chemokines, MIP-1α and IP-10. MIP-1α is a chemotactic factor for mononuclear cells, T cells, and mast cells and plays a role in differentiation of type 1 Th lymphocytes. IP-10 is a CXC chemokine and attracts, in addition to monocytes and Th1 cells, also NK cells [41]. We predict that the recruitment of these cells could be impaired by Sialo L2 *in vivo*.

Dendritic cells are among the first immune cells to come into contact with *Borrelia* in the skin [1]. Phagocytosis of *Borrelia* spirochetes leads to production of various proinflammatory cytokines [42] including chemokines. Inhibitory effect of sialostatin L2 on the production of chemokines attracting inflammatory cells into tick feeding site can lead to reduced inflammation due to tick saliva effect [43]. Reduced influx of inflammatory cells could facilitate establishment and proliferation of spirochetes in the skin [44].

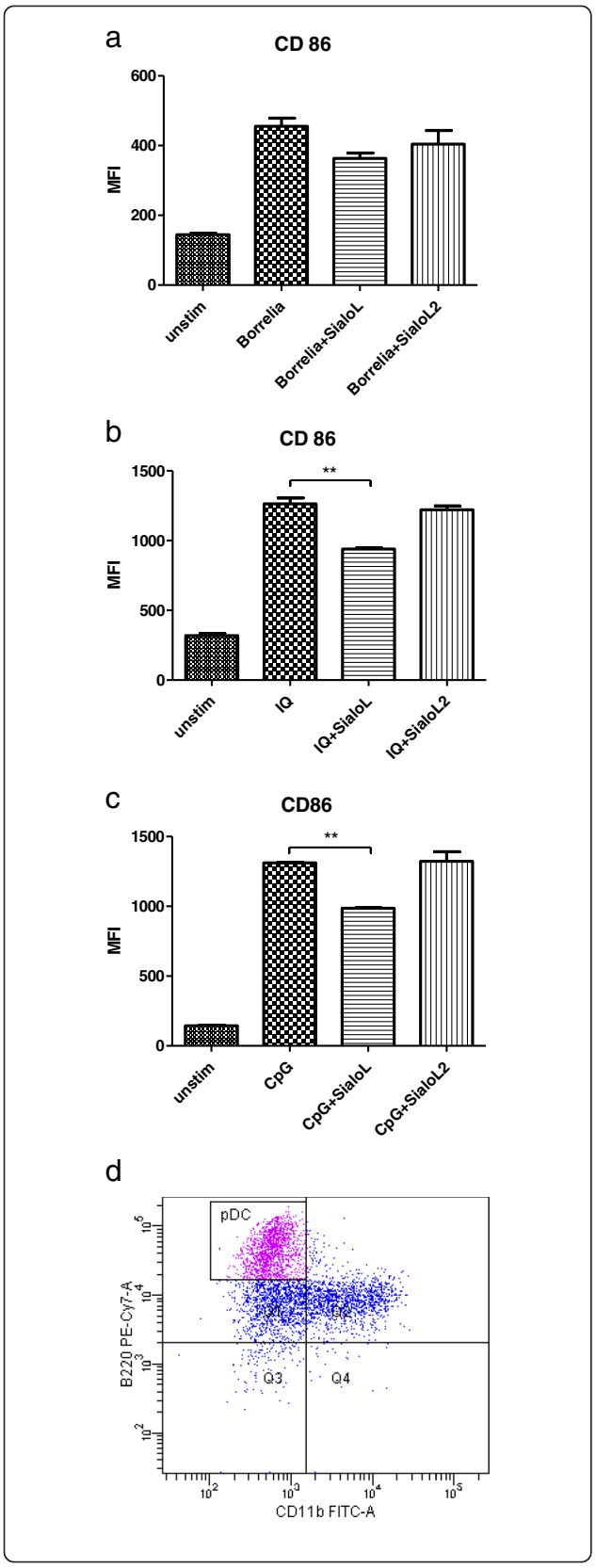

Fig. 5 Maturation of plasmacytoid dendritic cells induced by *Borrelia*, TLR-7, and TLR-9 ligation in the presence of sialostatins. Plasmacytoid dendritic cells were activated with *Borrelia* at MOI = 10 (**a**), imiquimod (2 μg/ml) (**b**), or CpG (50 nM) (**c**) in the presence or absence of sialostatins (both 3 μM). The expression of costimulatory molecule CD86 was analysed by flow cytometry among CD11c+, CD11b-, and B220+ cell population. Medium fluorescence intensity (MFI) is shown. The phenotype of plasmacytoid DC from CD11c population is shown (**d**)

Dendritic cells are equipped with several pattern recognition receptors (PRR), which sense Borreliae, including TLR, NLR, and LTR [2]. To reveal the mechanism of Sialo L2 effect on chemokine production by *Borrelia*-activated DC, we analysed the activation of chosen signalling molecules first upon TLR-2 ligation. TLR-2 is robustly activated by *Borrelia* lipoproteins [5] and critically involved in production of pro-inflammatory mediators, including chemokines. Moreover, TLR have an essential role in the control of *B. burgdorferi* burden [2, 4], which is enhanced by Sialo L2 *in vivo* [35]. The most pronounced effect of Sialo L2 on activation of tested signalling molecules in response to LTA was observed on phosphorylation of Akt, the downstream target of PI3K pathway. Interestingly, even the basal level of this kinase was decreased by Sialo L2. Consequences of PI3K pathway inhibition can be predicted. The inhibition of PI3K significantly impaired induction of chemokine and cytokine genes via TLR-2 in DC, including IP-10 [45]. Of note, PI3K pathway plays an important role in phagocytosis of *Borrelia* spirochetes by macrophages [14]. The inhibition of Akt phosphorylation was not observed by Sialo L2 in *Borrelia*-activated DC, possibly due to weak activation of this kinase.

The other pathway attenuated by Sialo L2 (in LTA and *Borrelia* activated DC) was Erk1/2 mediated cascade. Both, Erk1/2 and PI3K kinases are indispensable for induction of MIP-1α and MCP-1 in LTA stimulated murine macrophages [46]. IP-10 induction is mediated by IFNs (often produced in response to microbial products) and its upregulation is associated with the activation of JAK1, JAK2/STAT1 and MAPK pathways [47–49]. The decline of MIP-1α and IP-10 production in *Borrelia*-activated DCs by Sialo L2 could be thus mediated via inhibition of the Erk1/2 and PI3K signalling pathways. Recently, we have found that Sialo L2 attenuates IFN signalling triggered by IFN-β or LPS which leads to the suppression of interferon stimulated genes like IRF-7 and IP-10 [50]. The decrease of IP-10 production by Sialo L2 in response to *Borrelia* spirochetes could be in part also a consequence of impaired IFN/JAK/STAT signalling.

The third pathway influenced by sialostatin L2 upon LTA stimulation was NF-κB pathway. The involvement of NF-κB pathway in the induction of proinflammatory

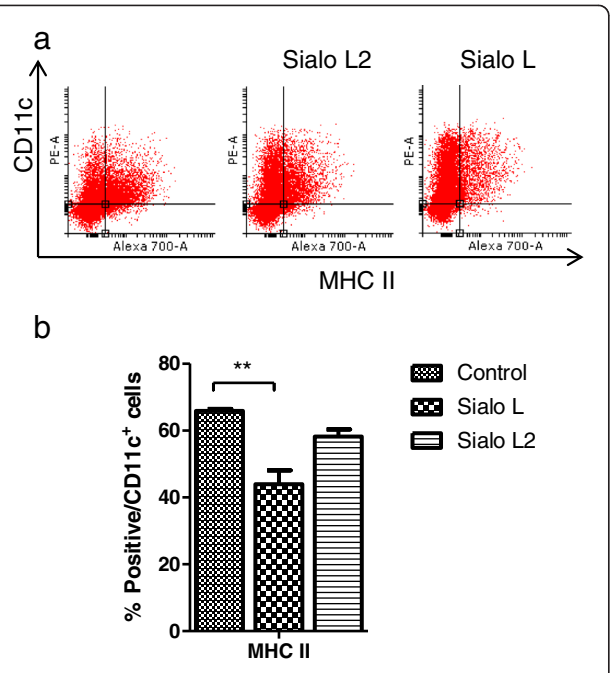

Fig. 6 Differentiation of dendritic cells from bone-marrow cells is inhibited by sialostatin L. Bone-marrow cells were cultured in the presence of GM-CSF and on day 3 the Sialo L or Sialo L2 (3 μM) were added to cultures. After 8 days, cells were harvested and analysed by flow cytometry for surface expression of MHC class II and CD11c (**a**). Differentiated cells (MHC II and CD11c positive) are shown as a percentage of total CD11c positive cells (**b**). The representative of three independent experiments is shown

mediators was documented; e.g. TLR-2/NF-κB/MAPK signalling plays a key role in IL-8 induction in macrophage cell line THP-1 exposed to *B. burgdorferi* [51]. We however did not detect any defect in the activation of this pathway in response to borreliae. Since dendritic cells sense borreliae by several PRR [2], the moderate effect of Sialo L2 on signals triggered through TLR-2 could be masked by signals triggered through other receptors.

In addition to chemokines, type I interferons are important cytokines modulating immune response to pathogens. *B. burgdorferi* is able to induce type I IFN and this induction is mediated through endosomal receptors TLR-7 and TLR-9 [6–9]. Plasmacytoid DC are major producers of type I IFN [52]. We found out that in plasmacytoid dendritic cells, the amount of produced IFN-β in response to *Borrelia* spirochetes and TLR-7 activation was decreased by sialostatin L and only weakly or not at all by sialostatin L2. IFN is pleotropic cytokine which recruits NK cells, has a direct antiviral effect on cells, and links the innate and adaptive immunity.

The down-regulation of IFN-β production by Sialo L in *Borrelia*/TLR-7/TLR-9 stimulated cells may have further consequences for the development of adaptive immune responses. In general, type I interferon directly influences the fate of CD4+ and CD8+ T cells during the initial phases of antigen recognition contributing to Th1 commitment and negatively regulating Th2 and Th17 differentiation [53]. Down-regulation of interferon can bring about an opposite effect. Moreover as sialostatin L inhibits production of IL-12 and TNF-α by DC as well as their differentiation [30], it probably leads to Th2 polarization of the immune response which is advantageous for *Borrelia* establishment in the skin [54]. In addition to modulation of the Th differentiation, type I IFNs also positively influence DC maturation [55, 56].

Indeed, we show that the maturation of plasmacytoid DC induced by TLR-7 or TLR-9 ligands was also decreased by Sialo L (judged by expression of co-stimulatory molecule CD86). When the maturation of DC was initiated by borreliae, only statistically not significant decline in CD86 expression was observed in the presence of Sialo L, presumably due to the fact that *Borrelia* spirochetes are weaker inducers of maturation then TLR ligands. In agreement, it was previously published that Sialo L inhibits the maturation of DC induced by LPS; it negatively affects the expression of the costimulatory molecules CD80 and CD86 [30]. Thus, Sialo L influenced function of dendritic cells in a different way in comparison to Sialo L2.

We did not investigate the mechanism which is behind the declined IFN-β production due to sialostatin L effect. However, since cathepsin L has been implicated in processing of TLR-9 [57], and sialostatins L and L2 are strong inhibitors of this protease [35], we could speculate that the decline of IFN-β is the result of impaired TLR-9 processing. Moreover, the amount of endogenously produced IFN-β was not affected by sialostatins in splenic DCs stimulated with TLR-4 agonist, where no processing had occurred [50].

Finally we examined the effect of tick cystatins on the differentiation/derivation of dendritic cells from bone marrow and found that Sialo L negatively affects the number of differentiated dendritic cells (MHC class II and CD11c positive cells). MHC class II molecule is necessary for the presentation of antigen to naive T-cells. As cathepsin S is implicated in the processing of the invariant chain within MHC class II antigens and sialostatin L strongly inhibits this protease [30], it seems likely that the decrease in MHC class II expression is mediated through inhibition of cathepsin S [58]. The inhibitory effect on differentiation of BMDC (measured by expression of MHC class II molecules) was also reported for cystatin rHp-CPI from murine nematode parasite *Heligmosomoides polygyrus* [40].

Conclusions

We show here that two related tick sialostatins affect different functions of dendritic cells. While sialostatin L influences the maturation of DC in part through the inhibition of IFN-β having thus an impact on adaptive immune response, sialostatin L2 affects, through attenuation of several signalling pathways, the production of chemokines engaged in the development of inflammation.

Additional file

Additional file 1: Effect of sialostatins on the signalling pathways activated by LTA and *Borrelia burgdorferi* in dendritic cells. Dendritic cells were seeded in 24-well plate. Next day DCs were incubated 2 h with tick cystatins (both 3 μM) prior to the addition of LTA (2 μg/ml) or Borreliae (MOI = 10) and further incubated for indicated times. Afterwards, cells were lysed and obtained protein extract was further analysed by immunoblotting using antibodies recognizing phosphorylated form of tested kinases. Afterwards, membranes were reprobed with antibodies against total kinase protein (a) or β-actin (b) which served as a control. Proteins were visualized by enhanced chemiluminescence.

Competing interests
We declare no financial competing interests. There are no non-financial competing interests.

Authors' contributions
JL carried out the signalling pathways experiments, participated in the design of the study and drafted the manuscript, JP performed the immunoassays, HL carried out flow cytometry experiments, AC, EC and MK contributed by design and preparation of recombinant cystatins and JK participated in the design of the study and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Ixodes ricinus Salivary Serpin IRS-2 Affects Th17 Differentiation via Inhibition of the Interleukin-6/STAT-3 Signaling Pathway

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Th17 cells constitute a subset of CD4$^+$ T lymphocytes that play a crucial role in protection against extracellular bacteria and fungi. They are also associated with tissue injury in autoimmune and inflammatory diseases. Here, we report that serpin from the tick *Ixodes ricinus*, IRS-2, inhibits Th17 differentiation by impairment of the interleukin-6 (IL-6)/STAT-3 signaling pathway. Following activation, mature dendritic cells produce an array of cytokines, including the pleiotropic cytokine IL-6, which triggers the IL-6 signaling pathway. The major transcription factor activated by IL-6 is STAT-3. We show that IRS-2 selectively inhibits production of IL-6 in dendritic cells stimulated with *Borrelia* spirochetes, which leads to attenuated STAT-3 phosphorylation and finally to impaired Th17 differentiation. The results presented extend the knowledge about the effect of tick salivary serpins on innate immunity cells and their function in driving adaptive immune responses.

Ticks are bloodsucking arthropods, major vectors of human pathogens like *Borrelia burgdorferi* and tick-borne encephalitis virus. Ticks from the family Ixodidae (hard ticks) require several days to fully engorge. During feeding, ixodid ticks remain tightly attached to their host (1, 2). To avoid attack from the host immune system during the feeding period, tick saliva contains two groups of molecules, the first with antihemostatic and the second with immunomodulatory properties. These groups include both proteinaceous and nonprotein molecules (3). One group of immunomodulatory proteins is represented by serine proteinase inhibitors (serpins), a large superfamily of structurally related, but functionally diverse, proteins that control essential proteolytic pathways (4, 5). Recently, three serine protease inhibitors, namely, purified human urinary trypsin inhibitor (UTI) and two synthetic serpins, gabextate mesilate (FOY) and nafamostat mesilate (FUT), which are widely used in treatment of acute inflammatory disorders, such as disseminated intravascular coagulation (DIC), have been shown to attenuate allergic airway inflammation and remodeling in a murine model of chronic asthma. These effects were associated with inhibition of Th2 cytokines (interleukin-4 [IL-4], IL-5, IL-6, and IL-13) and Th17 cell functions. These serpins also inhibited NF-κB activation in lung tissues (6).

Until now, more than 60 serpins have been identified at the sequence level in ixodid ticks, but only two serpins from *Ixodes ricinus* have been further functionally characterized (7–9). The first known *I. ricinus* serpin, Iris (*I. ricinus* immunosuppressor), is known to preferentially target leukocyte elastase. It also interferes with the contact phase coagulation pathway, fibrinolysis, and disrupts platelet adhesion. Moreover, Iris has the ability to modulate both innate and adaptive immunity. It affects T lymphocyte and macrophage responsiveness, and it induces a Th2-type response and inhibits the production of proinflammatory cytokines. Interestingly, it was shown that the anti-inflammatory properties of the protein are independent of its proteolytic activity and are mediated through its exosite domain (10–13).

IRS-2, the second described serpin from *I. ricinus*, targets cathepsin G and chymase. Both enzymes are part of the acute inflammatory response and are produced by activated neutrophils (cathepsin G) and mast cells (chymase). Moreover, IRS-2 is able to inhibit swelling and the migration of neutrophils into the inflamed tissue (14). The effects of IRS-2 on other cells of innate and acquired immunity have not been described so far.

Dendritic cells (DCs) are known as antigen-presenting cells and play a critical role in initiating and modulating the immune response. With their ability to recognize, process, and present antigens on their surfaces and thus activate T lymphocytes, DCs form a unique link between innate and acquired immunity (15, 16). Depending upon the recognized pathogens and other stimuli produced by activated DCs, such as cytokines and chemokines, T lymphocytes differentiate into cytotoxic CD8$^+$ or helper CD4$^+$ cells, which can further differentiate into various subsets (17). The IL-6/STAT-3 signaling pathway leads to differentiation of CD4$^+$ T lymphocytes into the Th17 subset. IL-6, a pleiotropic cytokine produced by dendritic cells in response to invading pathogens, binds to IL-6 receptors on T cells and activates the signaling pathway, leading to phosphorylation of the transcription factor STAT-3, an essential molecule for Th17 differentiation (18, 19). Th17 cells participate in host defense against extracellular bacteria and fungi by mediating the recruitment of neutrophils and macrophages into infected tissues. It is also known that regulation of Th17 cells plays a significant role in the pathogenesis of various inflammatory and autoimmune disorders (20–22). Moreover, it was shown that Th17 cells are involved in

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the development of severe destructive arthritis caused by the Lyme disease spirochete *B. burgdorferi* (23).

The objective of this study was to analyze the effect of a tick salivary serpin on dendritic cells and its consequences for the development of proinflammatory cells, like Th17 lymphocytes.

MATERIALS AND METHODS

Experimental animals. Specific-pathogen-free C57BL/6 mice (6- to 10-week-old females) were purchased from Charles River Laboratories. The animals were maintained under standard conditions in the animal house facility of the Institute of Parasitology, Biology Centre AS CR, České Budějovice. All experiments were performed with permission of the Czech animal ethics committee.

Recombinant IRS-2. Recombinant serpin from *I. ricinus*, IRS-2, was overexpressed in *Escherichia coli* BL21(DE3) pLysS cells. The expressed protein accumulated in inclusion bodies, which were separated. Refolded and concentrated IRS-2 was purified using a standard chromatographic method (fast protein liquid chromatography [FPLC]) (14, 24). Lipopolysaccharide (LPS) contamination was removed by Arvys Proteins Company using the detergent-based method.

Bacteria. *B. burgdorferi sensu stricto* ATCC 35211 isolated from *I. ricinus* was grown in Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma-Aldrich) supplemented with 6% rabbit serum at 34°C. The number of spirochetes was calculated by dark-field microscopy according to the method of Magnuson et al. (25). The fourth to sixth passages were used in the experiments.

Splenic DC isolation. Isolated mouse spleens were minced with scissors, digested in RPMI containing 0.25 mg/ml Liberase DL (Roche) and 0.2 mg/ml DNase I (Roche) at 37°C for 30 min, and passed through a 70-μm nylon cell strainer (BD Falcon). The dendritic cells were isolated using magnetic beads conjugated with anti-CD11c antibody (Ab) and magnetically activated cell sorting (MACS) column separation following the manufacturer's instructions (Miltenyi Biotec). The purified dendritic cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 μg/ml penicillin, and 100 U/ml streptomycin (all from Sigma-Aldrich). The purity of the isolated dendritic cells (~90% $CD11c^+$ cells) was determined by subsequent fluorescence-activated cell sorter (FACS) analysis.

$CD4^+$ T cell isolation. The fourth day after subcutaneous infection of mice with 1×10^5 *Borrelia* spirochetes, isolated mouse spleens were passed through a 70-μm nylon cell strainer (BD Falcon), and $CD4^+$ T cells were isolated using magnetic beads conjugated with anti-CD4 Ab and MACS column separation following the manufacturer's instructions (Miltenyi Biotec). Purified $CD4^+$ T cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 μg/ml penicillin, and 100 U/ml streptomycin (all from Sigma-Aldrich). The purity of the isolated $CD4^+$ T cells (≥90% $CD4^+$ $CD62L^{high}$) was determined by FACS analysis.

Specific activation of $CD4^+$ T lymphocytes. Purified splenic DCs were seeded at 5×10^4 cells per well in 96-well plates and stimulated with *Borrelia* spirochetes (5×10^5 per well) and IRS-2 (6 μM). After 24 h incubation, the medium was removed, and 3×10^5 freshly isolated *Borrelia*-primed $CD4^+$ T lymphocytes in 200 μl of culture medium were added to each well. The T cells were incubated with DCs for 3 days before restimulation with phorbol myristate acetate (PMA) (20 ng/ml) and ionomycin (1 μM) (both Sigma-Aldrich). Cell-free culture supernatants for IL-17 and IL-9 assessment were harvested at 2, 6, 12, 24, and 48 h after restimulation.

To determine the number of IL-17-producing Th cells, *Borrelia*-exposed DCs and *Borrelia*-primed $CD4^+$ T cells were cocultured as described above. On day 5 of coculture, the cells were restimulated with PMA and ionomycin and, after an additional 2 h, treated with monensin (2 μM; eBiosciences). The cells were then incubated for 4 h before staining was performed with anti-IL-17 antibody conjugated with phycoerythrin (PE) (eBioscience).

Cytokine measurement. Freshly isolated dendritic cells were seeded at 2×10^5 cells per well on 96-well plates. The next day, the DCs were stimulated with *B. burgdorferi* spirochetes at a multiplicity of infection (MOI) of 10 (2×10^6 per well) in the presence or absence of IRS-2 (6 μM). Cell-free culture supernatants were harvested 2, 9, 12, 24, or 48 h after stimulation and used for detection of IL-1β, IL-6, IL-10, and tumor necrosis factor alpha (TNF-α) with Ready-Set-Go! enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) following the manufacturer's instructions. Cell-free culture supernatants for IL-17 and IL-9 assessment were prepared as described in "Specific activation of $CD4^+$ T lymphocytes" above, and the amount of cytokines was measured with a Ready-Set-Go! ELISA kit (eBioscience) following the manufacturer's instructions. All reactions were performed in triplicate.

RNA extraction, quantitative real-time PCR, and mRNA half-life determination. To assess relative mRNA expression, DCs were seeded at 2×10^6 cells per well in 24-well plates. The next day, the DCs were stimulated with *Borrelia* spirochetes at an MOI of 10 (2×10^7 per well) in the presence or absence of IRS-2 (6 μM) and incubated for 6 or 12 h. RNA was then isolated with the Nucleospin RNA II kit (Macherey-Nagel) following the manufacturer's instructions. The quality and concentration of the isolated RNA were assessed by measurement on a Nanophotometer P-330 (Implen). cDNA was synthesized with the High-Capacity RNA-to-cDNA kit (Applied Biosystems). Real-time PCR analysis was performed with a TaqMan gene expression set (Applied Biosystems) containing primers and probe specific for IL-6 and β-actin using a Rotor Gene 3000 and Rotor-Gene 6.0.19 software (Corbett Research). The relative expression of IL-6 mRNA was determined by the comparative threshold cycle (C_T) method (26), where the mouse β-actin gene was used as a housekeeping gene (Applied Biosystems). All reactions were performed in triplicate.

Immunoblotting. Freshly isolated dendritic cells were seeded at 1×10^6 cells per well in 24-well plates. The next day, the DCs were stimulated with *Borrelia* spirochetes at an MOI of 10 (1×10^7 per well) in the presence or absence of IRS-2 (6 μM). Following stimulation (15, 30, and 60 min for C/EBP, phosphorylated NF-κB [p-NF-κB], p-CREB, p-p-38, and p-ERK1/2 and 6 and 16 h for p-STAT-3), the cells were lysed in a modified RIPA buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5) in the presence of protease and phosphatase inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml pepstatin, 25 mM NaF, and 2 mM $NaVO_3$). The protein extracts, mixed with Laemmli sample buffer, were separated by SDS-PAGE and transferred to Immobilon-P membranes. Following blocking in Tris-buffered saline (TBS)-containing 5% fat-free milk, the blots were incubated overnight with the antibodies against C/EBP, phospho-STAT-3 (Tyr^{705}), phospho-NF-κB (Ser^{536}), phospho-CREB (Ser^{133}), phospho-p38 (Thr^{180}), and phospho-ERK1/2 (Thr^{202}) (all from Cell Signaling) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β-actin (Santa Cruz Biotechnology). The proteins were visualized using enhanced chemiluminescence (Pierce), and their abundances were analyzed using a charge-coupled-device (CCD) imaging system (ChemiDoc MP Imaging System) and Image Lab software v. 4.1 (Bio-Rad).

To assess the level of phosphorylated STAT-3 in T lymphocytes, freshly isolated dendritic cells were seeded at 1. 5×10^5 per well in a 96-well plate. After 6 h, the DCs were stimulated with *Borrelia* spirochetes (1×10^7 per well) in the presence or absence of IRS-2 (6 μM). The next day, cell-free culture supernatants were harvested and added to freshly isolated *Borrelia*-primed $CD4^+$ T lymphocytes (9×10^5 per well). Following stimulation (15 and 30 min), the cells were lysed, and Western blotting was performed as described above with phospho-STAT-3 (Tyr^{705}) and β-actin antibodies.

Flow cytometry. $CD4^+$ cells were prepared and stimulated as described in "Specific activation of $CD4^+$ T lymphocytes" above. After 4 h of restimulation with PMA, ionomycin, and monensin, the cells were harvested (using cold 5 mM EDTA in PBS) and stained with anti-CD4 antibody (conjugated with allophycocyanin [APC]; eBioscience). After wash-

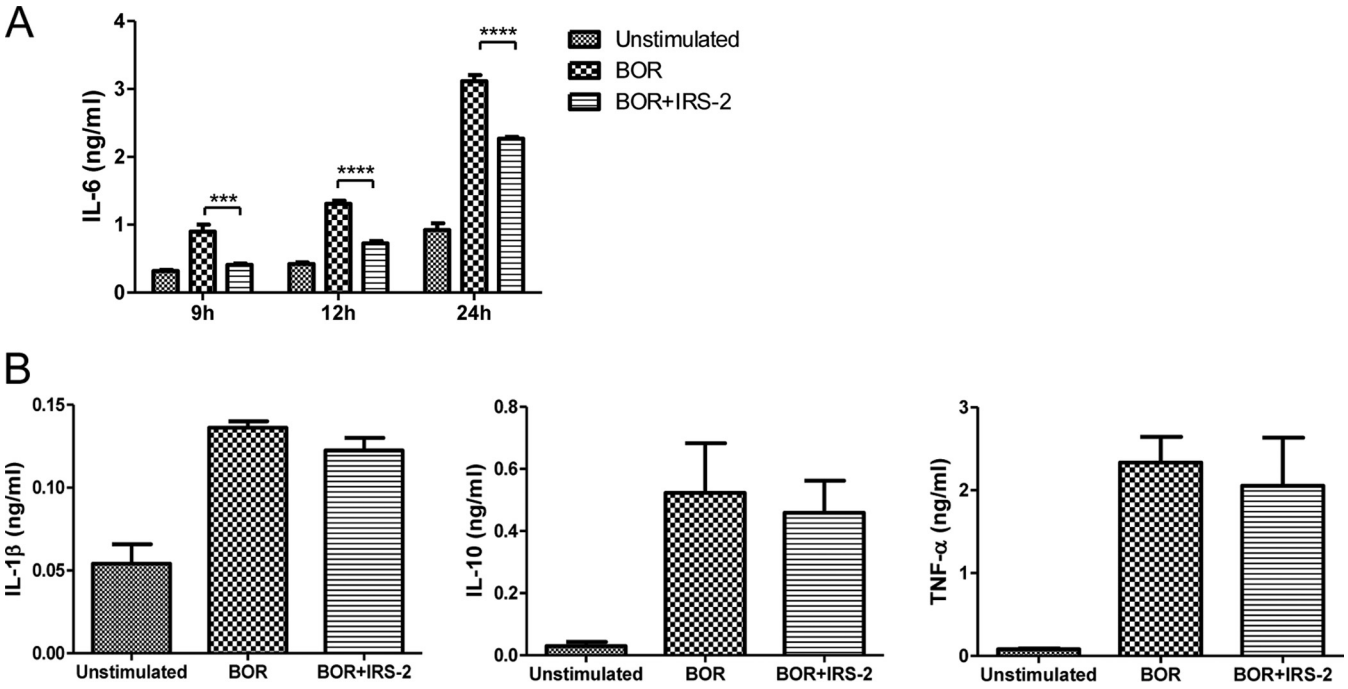

FIG 1 IRS-2 selectively inhibits IL-6 production by DCs. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (BOR) (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). (A) Culture supernatants were harvested 9, 12, and 24 h after stimulation, and the amount of IL-6 was determined by ELISA. Two independent experiments were performed, and data from a representative experiment are shown. The data are expressed as the mean cytokine concentrations from three wells plus standard errors of the mean (SEM). *** and ****, effects of IRS-2 on IL-6 production were significant at P values of <0.001 and <0.0001, respectively. (B) Culture supernatants were harvested 24 h (TNF-α) or 48 h (IL-1β and IL-10) after stimulation, and the presence of cytokines was detected by ELISA. Three independent experiments were performed, and the data were pooled. The data are expressed as the mean cytokine concentrations from nine wells plus SEM.

ing, the cells were fixed and permeabilized with a Foxp3/transcription factor staining buffer set (eBioscience) and labeled with anti-IL-17A antibody (conjugated with PE; eBioscience). The prepared cells were resuspended in cold PBS with 1% FCS. Flow cytometry was performed on a FACSCanto II cytometer using FACS Diva software v. 5.0 (BD Biosciences).

Statistical analysis. One-way analysis of variance (ANOVA) followed by a Bonferroni test in GraphPad Prism, version 5.0, was used to compare the differences between control and treated groups. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

IRS-2 selectively inhibits IL-6 production by DCs upon stimulation with *Borrelia* spirochetes. Cytokines produced by activated DCs play a key role in shifting the immune response toward particular Th subsets. To investigate the effects of IRS-2 on the production of different pro- and anti-inflammatory cytokines by DCs, immature DCs were stimulated with *Borrelia* spirochetes in the presence or absence of IRS-2 for 9, 12, 24, or 48 h, and the production of IL-1β, IL-6, IL-10, and TNF-α was measured.

Serpin significantly inhibited the production of IL-6 in DCs (Fig. 1A), whereas the production of other cytokines remained unaltered (Fig. 1B). The same inhibitory effect of IRS-2 on the production of IL-6 was also observed in the PMJ2-R cell line (macrophages) and primary neutrophils (data not shown).

IRS-2 inhibits IL-6 production at the level of mRNA. Gene expression can be regulated by many mechanisms at many stages, including chromatin accessibility, transcription activation, mRNA nuclear export, mRNA decay, and translation. To understand the mechanism of IL-6 decline caused by IRS-2, the expression of the IL-6 gene was measured in DCs activated with *Borrelia* spirochetes in the presence or absence of IRS-2. mRNA specific for IL-6 was determined by quantitative RT-PCR. As shown in Fig. 2, the IL-6 transcript level was slightly increased after as little as 6 h (no significant effect of IRS-2 was observed). However, the mRNA of IL-6 was severely suppressed by IRS-2 at a later time point (12 h). We concluded that a decline in IL-6 production is the result of impaired

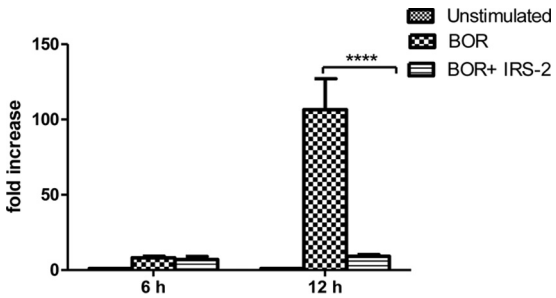

FIG 2 IRS-2 inhibits IL-6 production at the level of mRNA expression. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). The transcript level of IL-6 was determined by quantitative PCR (qPCR) using specific primers for IL-6. The gene expression of IL-6 was normalized to the β-actin transcript. Two independent experiments were performed, and the data were pooled. The data are expressed as the average fold IL-6 mRNA increase (plus SEM) from six wells compared with the control. ****, the effect of IRS-2 on the relative expression of IL-6 mRNA was significant at a P value of <0.0001.

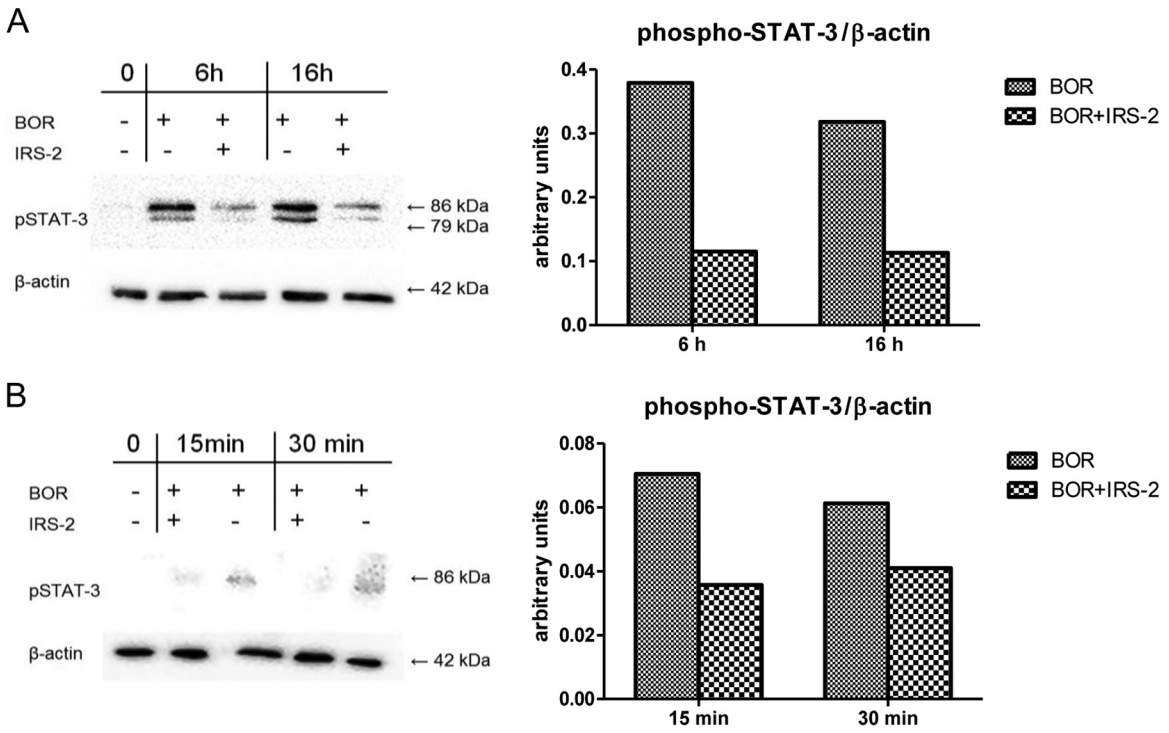

FIG 3 IRS-2 inhibits STAT-3 molecule phosphorylation. (A) Splenic dendritic cells (sDCs) were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). Six and 16 h after stimulation, cell lysates were prepared and analyzed by immunoblotting with anti-phospho-STAT-3. The membranes were reprobed with antibody against β-actin. The phosphorylation of STAT-3 was quantified using chemiluminescence and normalized to the β-actin protein level. The two bands represent different isoforms (α and β) of pSTAT-3 that are present in DCs. (B) Freshly isolated *Borrelia*-primed $CD4^+$ T cells were stimulated with 24-h supernatants from sDCs stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). Fifteen and 30 min after stimulation, cell lysates were prepared and analyzed by immunoblotting with anti-phospho-STAT-3. The membranes were reprobed with antibody against β-actin. The phosphorylation of STAT-3 was quantified using chemiluminescence and normalized to the β-actin protein level.

gene expression in IRS-2-exposed cells after activation with *Borrelia* spirochetes (Fig. 2).

Decreased stability of IL-6 mRNA is often responsible for a decline in IL-6 production. Moreover, inhibition of IL-6 production due to increased IL-6 mRNA decay was observed with another tick salivary protein (27). Therefore, we investigated whether the same mechanism could be responsible for the IRS-2-induced effect. Splenic DCs were stimulated with *Borrelia* spirochetes in the presence or absence of IRS-2. After 9 h, actinomycin D was added to block mRNA synthesis, cells were harvested (after 1 and 2 h), and mRNA decay was determined. The IL-6 mRNA half-life observed in the presence of IRS-2 was comparable to that of control cells stimulated only with *Borrelia* spirochetes (data not shown). This result suggests that the impaired gene expression of IL-6 is not due to impaired stability of IL-6 mRNA.

In our effort to reveal the mechanism of the IRS-2 effect on IL-6, we further tested whether signaling pathways leading to induction of IL-6 are affected by IRS-2.

Gene expression of IL-6 is controlled by several transcription factors and signaling molecules, including NF-κB, C/EBP, CREB, and kinases p38 and ERK1/2 (28–32); therefore, the phosphorylation of these molecules was tested. DCs were stimulated with *Borrelia* in the presence or absence of IRS-2 for 15, 30, and 60 min. After stimulation, cell lysates were prepared and analyzed by immunoblotting. The phosphorylation of none of these signaling molecules was inhibited by IRS-2, so we concluded that induction of the IL-6 gene is intact and does not seem to be responsible for decreased IL-6 transcript expression (data not shown).

IRS-2 impairs Th17 differentiation via inhibition of the IL-6/STAT-3 signaling pathway. It is well known that the major transcription factor activated by IL-6 is STAT-3. STAT-3 phosphorylation is mediated through the association of IL-6 with the IL-6 receptor (IL-6R) and the signal transducer glycoprotein 130 (gp130), followed by subsequent activation of Janus kinases (19). Since the production of IL-6 in DCs was strongly inhibited by IRS-2, we expected that the phosphorylation of the STAT-3 signaling molecule would be decreased. DCs were activated with *Borrelia* spirochetes in the presence or absence of IRS-2, and the level of phosphorylated STAT-3 molecules was determined 6 and 16 h after activation. Indeed, a marked decrease of phospho-STAT-3 was observed (Fig. 3A). *Borrelia*-primed T lymphocytes were activated with supernatants from DCs (stimulated for 24 h with *Borrelia* spirochetes in the presence or absence of IRS-2), and the phosphorylation of STAT-3 in T lymphocytes was also decreased, likely due to diminished production of IL-6 by DCs (Fig. 3B).

The IL-6/STAT-3 signaling pathway is known to be crucial for development of the Th17 subset (18, 20). The main effector cytokines produced by Th17 cells are IL-17 (IL-17A), which is a hallmark of the subpopulation; IL-21; IL-22; and IL-9 (22, 33, 34). We predicted that the inhibition of IL-6/STAT-3 signaling by IRS-2 could lead to impaired Th17 differentiation, and therefore, the number of Th17-producing cells and the amounts of IL-17 and

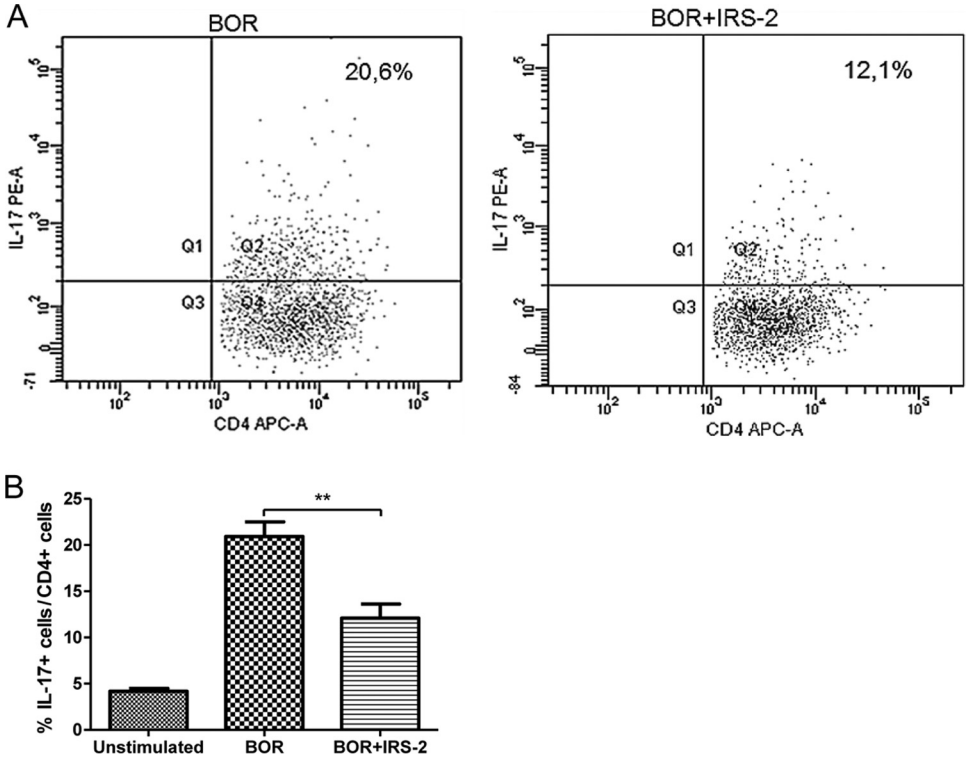

FIG 4 IRS-2 reduces the number of IL-17-producing CD4$^+$ T cells. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). After 24 h, freshly isolated *Borrelia*-primed CD4$^+$ T cells were added to the cultured DCs, and the cells were cocultured for 5 days. Then, T lymphocytes were restimulated with PMA and ionomycin, treated with monensin, and stained for IL-17. (A) Flow cytometry dot plots of T lymphocytes treated with DCs stimulated with *Borrelia* in the presence or absence of IRS-2. Quadrant 2 (Q2) shows CD4$^+$ IL-17$^+$ cells. (B) The percentage of IL-17-producing cells was determined in live CD4$^+$ cells. The data are expressed as the mean percentages of CD4$^+$ IL-17$^+$ cells from triplicate wells plus SEM. **, the effect of IRS-2 on the presence of IL-17-producing cells was significant at a *P* value of <0.01.

IL-9 were determined. Splenic DCs were stimulated with *Borrelia* spirochetes in the presence or absence of IRS-2. After 24 h, freshly isolated *Borrelia*-primed CD4$^+$ T cells were added to the cultured DCs, and the cells were cocultured for the next 5 days. Afterward, the cells were restimulated with PMA and ionomycin. To detect the Th17 subset, intracellular staining for IL-17A was performed, and the cells were analyzed by flow cytometry. To block cellular transport, monensin was added to the restimulated cells. As seen in Fig. 4A and B, the number of IL-17-producing CD4$^+$ T cells was significantly decreased by IRS-2.

To measure the production of IL-17 and IL-9 cytokines, the coculture of DCs and *Borrelia*-primed CD4$^+$ T cells lasted 3 days before restimulation with PMA and ionomycin. The supernatants were then collected at various time points and analyzed. The levels of both IL-17 and IL-9 were significantly decreased in the presence of IRS-2 (Fig. 5A and B). The reduced levels of the measured cytokines, together with the decreased number of IL-17-producing CD4$^+$ T cells, in the presence of IRS-2 clearly indicate that IRS-2 inhibits Th17 differentiation.

DISCUSSION

During coevolution with their hosts, ticks evolved various mechanisms enabling them to avoid the hosts' hemostatic and immune systems and successfully finish their blood meals.

In recent years, attention has been focused on identification and functional characterization of particular tick salivary proteins that are responsible for antihemostatic and immunomodulatory effects (3).

Thanks to this intensive research, many tick salivary substances that have immunomodulatory effects on various immune cell populations have been identified. Among these substances, molecules that can affect DC functions seem to play important roles, since DCs are among the first cells present at the site of inflammation and can further modulate or shift the immune response by driving T cell differentiation.

Here, we describe specific and extensive inhibitory effects of the tick salivary serpin IRS-2 from the hard tick *I. ricinus* on Th17 differentiation mediated by impairment of the IL-6/STAT-3 signaling pathway.

Proteins from the serpin superfamily are involved in fundamental biological processes, such as blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation, and tumor suppression (35, 36). From this enumeration, it is apparent that tick serpins can be expected to play a role in tick feeding, suppressing both the antihemostatic and immune responses of the host. To date, only two *I. ricinus* serpins have been functionally characterized (12, 14).

We showed that IRS-2 decreased IL-6 at the protein and mRNA levels in spleen dendritic cells activated by *B. burgdorferi*. A decrease by Sapl15, the best-studied tick salivary protein, in the IL-6 level in response to *B. burgdorferi* was also observed. Salp15 binds to DCs via the DC-SIGN receptor, which results in activa-

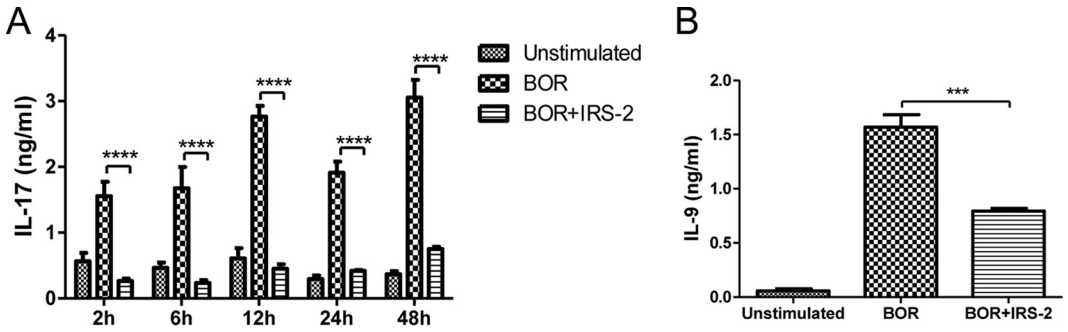

FIG 5 IRS-2 reduces levels of Th17 cytokines. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). After 24 h, freshly isolated *Borrelia*-primed $CD4^+$ T cells were added to the cultured DCs, and the cells were cocultured for the next 3 days. Afterward, the cells were restimulated with PMA and ionomycin, and the production of cytokines was analyzed at various time points. (A) Cell supernatants for IL-17 assessment were harvested 2, 6, 12, 24, and 48 h after restimulation and analyzed by ELISA. The data are expressed as the mean cytokine concentrations from triplicate wells plus SEM. Two independent experiments were performed, and data from a representative experiment are shown. ****, the effect of IRS-2 on the IL-17 level was significant at a *P* value of <0.0001. (B) IL-9 production was assessed by ELISA 24 h after restimulation. The data are expressed as the mean cytokine concentrations from triplicate wells plus SEM. ***, the effect of IRS-2 on IL-9 production was significant at a *P* value of <0.001.

tion of the serine/threonine kinase Raf-1/mitogen-activated protein kinase (MEK)-dependent signaling pathway and subsequently in a decrease of IL-6 and TNF-α mRNA stability and impaired nucleosome remodeling at the IL-12p35 promoter in human DCs activated with *B. burgdorferi* (27). However, the authors point to the fact that the addition of rabbit polyclonal anti-Salp15 antibodies abrogates the capacity of *I. ricinus* saliva to inhibit IL-12, but not IL-6 and TNF-α, which might be due to the presence of other molecules in tick saliva that are able to block IL-6 and TNF-α. In our study, we proved that one of these molecules, which can be responsible for IL-6 inhibition, is the salivary serpin IRS-2. To reveal the possible mechanism of IRS-2 effects, mRNA for IL-6 was assessed. However, it turned out that, in contrast to Salp15, IRS-2 does not act through impaired stability of IL-6 mRNA. In addition, monitoring of signaling pathways important for induction of IL-6 did not show any defect that led us to the conclusion that gene induction is not impaired. There is a positive-feedback loop in IL-6/STAT-3 signaling (IL-6 binds to IL-6R on a cell and activates phosphorylation of the STAT-3 molecule, which in turn boosts the production of autocrine IL-6), so direct inhibition of STAT-3 phosphorylation by IRS-2 could explain the observed decrease in IL-6 mRNA expression and, subsequently, IL-6 production (37). However, this option was also excluded (data not shown). Thus, we did not reveal the precise mechanism of the IRS-2 effect.

It has been shown that tick saliva and tick salivary proteins, like Salp15, Japanin, and sialostatin L, can modulate the T cell response by modulating DC accessory functions or directly by interaction with $CD4^+$ T cells. It was well demonstrated that tick saliva or salivary gland extract (SGE) diminishes the production of Th1-related cytokines and increases the production of Th2-related cytokines. Salp15 specifically binds to CD4 molecules on the surfaces of $CD4^+$ T (helper) cells, which results in inhibition of T cell receptor-mediated signaling, leading to reduced IL-2 production and impaired T cell proliferation (38). Japanin, a lipocalin from *Rhipicephalus appendiculatus*, specifically reprograms the response of DCs to a wide variety of stimuli *in vitro*, altering their expression of costimulatory and coinhibitory transmembrane molecules and secretion of proinflammatory, anti-inflammatory, and T cell-polarizing cytokines (it blocks LPS-induced secretion of Th17- and Th1-promoting cytokines); it also inhibits the differentiation of DCs from monocytes (39). Recently, Horka et al. showed that cystatin from *I. scapularis*, sialostatin L, which also inhibits several dendritic cell functions, can inhibit IL-9 production by Th9 cells, thus preventing the development of experimental asthma (40). Another cystatin, OmC2 from the soft tick *Ornithodoros moubata*, can also suppress the host adaptive immune response by reducing TNF-α and IL-12 production and the proliferation of antigen-specific $CD4^+$ T cells (41).

In line with these reports, our data show that the serpin IRS-2 is another tick salivary protein that is able to modulate T cell differentiation. We demonstrated that inhibition of *Borrelia*-induced IL-6 production in the presence of IRS-2 in DCs was accompanied by decreased phosphorylation of the STAT-3 signaling molecule, which is essential for the development of Th17 cells. Indeed, the impairment by IRS-2 of Th17 development was observed and was demonstrated by a decreased amount of IL-17 produced and by flow cytometry assessment of intracellular IL-17 in $CD4^+$ T lymphocytes cocultured with activated DCs. Similar results, showing that tick saliva inhibits the Th17 subset, were reported by Skallova and colleagues, who showed that saliva-exposed DCs failed to induce efficient Th1 and Th17 polarization and promoted development of Th2 responses (42). Interestingly, treatment with Salp15, which also inhibits IL-6 production in dendritic cells, was shown to increase the differentiation of Th17 cells *in vivo*, as evidenced by higher IL-17 production from PLP139-151-specific $CD4^+$ T cells isolated from the central nervous system and the periphery (43).

Th17 cells, a quite recently described subpopulation of $CD4^+$ T lymphocytes, can be characterized by production of the hallmark cytokine IL-17. Overproliferation of Th17 cells is connected with many severe autoimmune diseases, like human psoriasis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, asthma, and some bacterial and fungal infections. However, it is well established that Th17 cells not only play an important role in autoimmunity, but also function in the clearance of specific types of pathogens that require a massive inflammatory response and are not adequately dealt with by Th1 or Th2 immunity. Thus, the Th17 response can be triggered by many bacteria, including *Borrelia* spirochetes (22). Infante-Duarte showed that *B. burgdorferi*

lysate is able to induce massive amounts of IL-17 in T cell cultures and that microbe-induced IL-17 production can mediate infection-induced immunopathology in Lyme disease (44). Involvement of the Th17 subset in the development of severe destructive arthritis in patients with Lyme disease was also demonstrated by Burchill et al. (23). A causative protein, neutrophil-activating protein A (NapA) from *B. burgdorferi*, which is able to stimulate IL-17 production in synovial-fluid-derived T cells and could thus be crucial for the induction and maintenance of Lyme arthritis, was identified (45). Moreover, it is well described that synthetic or human-derived serpins, which are commonly used in the treatment of many autoimmune diseases, are able to decrease Th17 differentiation (6).

All these findings highlight the importance and potential of the *I. ricinus* serpin IRS-2 described here as a prospective molecule in many pharmaceutical applications.

In conclusion, here, we present a newly described ability of the *I. ricinus* salivary serpin IRS-2 to inhibit Th17 differentiation upon *B. burgdorferi* exposure via inhibition of the IL-6/STAT-3 signaling pathway, thus extending the knowledge about the effect of tick salivary serpins on innate immunity cells and their function in driving the adaptive immune response. This paper contributes to the understanding of tick saliva-mediated modulation of the host immune system.

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7. VÝZNAM, ÚSKALÍ A PERSPEKTIVY VÝZKUMU KLÍŠŤĚCÍCH SLIN

KLÍŠŤĚCÍ SLINY JAKO PODPORA PRO DISEMINACI PATOGENŮ

Existuje několik potenciálních mechanizmů, kterými lze vysvětlit pozitivní efekt klíštěcích slin na přenos a diseminaci patogenů z klíštěte do hostitele. Imunomodulace hostitelské odpovědi v podobě inhibice vrozené a adaptivní imunitní reakce je pravděpodobně nejdůležitější (15, 19, 30). Obecně se jedná o ovlivňování různých funkcí imunitních buněk s cílem utlumit svědění, bolest, inhibovat zánět, či navodit toleranci k sajícímu klíštěti. Udržení homeostázy je klíčové pro klíště a patogen této situace využívá. Podrobnější popis imunomodulace byl již zmíněn v předešlých kapitolách.

Kromě vlivu na funkci imunitních buněk mohou být za SAT efekt zodpovědné klíštěcí proteiny, které přímo interagují s patogenem a tím pozitivně ovlivňují jeho přežití. Na spirochéty *Borrelia burgdorferi* se např. váže klíštěcí protein Salp15, čímž ochraňuje spirochéty před lýzou závislou na protilátkách (108, 109). Komplementový systém je důležitý obranný mechanismus hostitele, který významně eliminuje boreliovou infekci. V klíštěcích slinách byly identifikovány složky, které inhibují aktivaci alternativní i lektinové dráhy komplementu; např: ISAC, Sapl20 a TSLPI (tick salivary lectin pathway inhibitor) (110-112). Přímá vazba klíštěcího faktoru na TBE virus dosud nebyla popsána. Protože počet TBEV pozitivních dendritických buněk je v přítomnosti klíštěcích slin zvýšený (87), existenci podobného faktoru jako je Salp15 nelze vyloučit. Vazba klíštěcího proteinu na patogen nemusí být vždy pro patogen výhodou. V případě viru Dengue bylo zjištěno, že glykoprotein přítomný v slinách komára *Aedes aegypti* (patřící do D7 proteinové rodiny) se váže na Dengue virus a inhibuje virovou infekci (113).

V případě virové infekce můžeme uvažovat o dalších způsobech, jak dochází k SAT efektu. Retence rezidentních antigen prezentujících Langerhansovych buněk v kůži v důsledku jejich snížené migrace do spádových lymfatických uzlin, která je pozorována v přítomnosti klíštěcích slin (70), může přispívat k lokálnímu navýšení počtu infekčních virových částic v kůži a podpořit diseminaci viru při tzv. sousání. Z hlediska diseminace viru v rámci hostitele, se pak snížená migrace DC jeví spíše antagonisticky. Další z teoretických možností, jak by sliny mohly podpořit přenos nebo diseminaci patogenu v místě sání, je zvýšená rekrutace monocytárních fagocytů, které jsou vnímavé k virové infekci. Tento mechanizmus SAT efektu se uplatňuje v případě arbovirů přenášených komáry (114). U klíšťat se obecně potlačení lokálního zánětu v místě sání považuje za faktor podporující diseminaci patogenu/viru (115). Recentní studie zaměřené na analýzu časných stadií infekce virem TBE ovšem naznačují, že infikované klíšťata během prvních tří hodin sání vytváří zánětlivé mikroprostředí; byly pozorovány změny v expresi cytokinů a chemokinů na transkripční úrovni (116). Je tedy představitelné, že zvýšená rekrutace monocytů může mít podíl na produkci viru v kůži (21). Navíc, v *in vitro* testech bylo prokázáno, že migrace makrofágové linie IC-20 je zvýšená vlivem slin z klíštěte *Dermacentor variabilis* (77). V případě virové infekce jsou kromě imunitních buněk zdrojem viru i jiné rezidentní buňky kůže, jako jsou keratinocyty a fibroblasty (117). Zvýšení replikace viru v permisívních buňkách může také přispět k SAT efektu. V *in vitro* testech byl titr viru pozitivně ovlivněn klíštěcími slinami kromě myeloidních dendritických buněk (86) i v myších primárních

keratinocytech (118). Keratinocyty tvoří kožní bariéru a mají schopnost produkovat řadu cytokinů, včetně interferonů se silně antivirovým účinkem, či prezentovat antigen (119). Zvýšená replikace Dengue viru v lidských keratinocytech byla např. prokázána pod vlivem slin komára *Aedes aegypti* (120). Tento účinek byl zprostředkován potlačením genové exprese interferonů (IFNα, IFNβ, IFNγ). Podobně byl podpůrný vliv slin komárů rodu *Aedes* a *Culex* na replikaci viru západonilské horečky prokázán díky inhibici antivirového účinku interferonu (119). Stejný mechanismus účinku byl prokázán i s extraktem slinných žláz klíštěte *Dermacentor reticulatus* analýzou replikace viru vezikulární stomatitidy (84). Oslabení IFN signalizace (na úrovni snížení fosforylace signální molekuly STAT1) klíštěcími slinami *I. ricinus* jsme pozorovali i v naší laboratoři (82). Efekt klíštěcích slin na replikaci TBEV závisí na typu použitých buněk. V makrofágové linii PMJ2-R klíštěcí sliny replikaci TBEV naopak inhibují (121). Je pravděpodobné, že sliny ovlivňují více buněčných parametrů, které působí proti sobě. Parametr, který převáží, určuje konečný efekt slin na replikaci viru.

Jedním z takových parametrů mohou být hostitelské exosomy. Exosomy jsou mikrovezikuly o velikosti 30-150 nm, které hrají důležitou roli v mezibuněčné komunikaci (122). Mnoho flavivirů, včetně Dengue, Zika, WNV and Langat viru, využívá exosomální dráhu ve svůj prospěch (54, 123-125) a poslední studie naznačují, že tyto nanočástice se podílejí i na diseminaci viru, kolonizaci hostitele a patogeneze (126, 127). Na modelu lidské keratinocytové linie bylo např. prokázáno, že exosomy derivované z keratinocytů se podílí na přenosu viru Langat (124). Zda sliny ovlivňují biogenezu, sekreci nebo obsah hostitelských exosomů není známo. Nutno zmínit, že i exosomy derivované z klíštěcích a komářích buněk, které jsou obsaženy ve slinách, hrají významnou roli v imunomodulaci hostitelské odpovědi (127).

Pozitivní účinek slin na přenos patogenů byl popsán i u krátce sajících členovců jako jsou např. komáři *Aedes aegypti*, kteří přenášejí mnoho medicínsky významných virů, včetně viru Zika, Dengue, Chikungunya a viru žluté zimnice. Je nutno uvést, že sání komárů je nejenom kratší, ale odlišné také způsobem sání; komáři sají z malých krevních kapilár, na rozdíl od klíšťat, které jsou tzv. ‚pool-feeders' a sají z vytvořené krvácející ranky. Princip SAT efektu komářích slin spočívá v modulaci hostitelské imunitní reakce a zvýšené rekrutace virus-permisivních buněk do místa sání (114, 128). Sliny komára *A.aegypti* negativně ovlivňují rekrutaci leukocytů a mění antivirovou signalizaci v místě inokulace virem západonilské horečky (129). V případě viru Chikungunya bylo zjištěno, že sliny *A.aegypti* zvyšují replikaci viru ve fibroblastech interferencí s interferonovou signalizací (130). Lokální zánětlivá reakce hostitele vyvolaná slinami zvyšuje závažnost arbovirové infekce u Zika a Dengue (114). Tato skutečnost se vysvětluje tím, že komáry přenášené arboviry využívají ‚zánětlivé niky' tvořené virus-permisivními myeloidními buňkami na podporu jejich replikace a diseminace *in vivo*. Je zajímavé, že efekt slin na antivirovou hostitelskou reakci vůči viru Zika a DENV nebyl pozorován.

SAT FAKTORY Z KLÍŠTĚCÍCH A KOMÁŘÍCH SLIN

V současné době bylo identifikováno několik slinných komponentů, které se mohou označit jako SAT faktory. Jednotlivé SAT faktory se liší podle druhu patogenů a jejich přenašečů (30). Klíštěcí SAT faktory pro borelie zahrnují již zmiňovaný protein 15 (Salp15) ze slin *Ixodes scapularis*, který se přímo váže na borelie a chrání je před poškozením protilátkami (108). Kromě toho se tento protein váže na CD4 koreceptor a inhibuje CD4

závislou T-buněčnou aktivaci (131). Ve slinách klíštěte *I. ricinus* byl objeven homolog glutation peroxidázy (Salp25D), který má silné anti-oxidační účinky a snižuje nasátí klíšťat u myší imunizovaných tímto proteinem (132). Inhibitor komplementu TSLP (tick salivary lectin pathway) chrání před lýzou komplementem (133). Histamin release faktor (tHRF) je dalším z proteinů, které podporují přenos borelií na hostitele. Imunizace tímto faktorem interferuje se sáním krve a přenosem borelií (134). SAT faktor pro TBEV v klíštěti *I. ricinus* nebyl dosud identifikován.

I v komářích slinách už bylo nalezeno několik komponent pozitivně ovlivňujících přenos virových patogenů. Ve slinách *A.aegypti* byla identifikována proteáza CLIPA3, která cílí na proteiny mezibuněčné hmoty (extracelulární matrix) a tím zvyšuje replikaci viru Dengue (135). Protein *Aedes aegypti* venom allergen-1 (AaVA-1) je faktor, který podporuje přenos virů Dengue a Zika přes aktivaci autofagie (136). Další slinný faktor LTRIN zvyšuje přenos viru Zika prostřednictvím vazby na lymfotoxinový β receptor, čímž inhibuje NF-κB závislou signalizaci a produkci prozánětlivých cytokinů. Ve slinách *A. aegypti* byly identifikovány i proteiny, které mají antivirový účinek; příkladem je D7 protein, který se přímo váže na virion a inhibuje infekci virem Dengue *in vitro* a *in vivo* (113). Porušení endoteliální bariery, která separuje kůži od krevní cirkulace a slouží jako fyzikální obrana proti invazi virů, je dalším ze způsobů, jak sliny komárů podporují diseminaci virů (128).

Kritickým faktorem při výzkumu klíštěcích slin a SAT efektu je složení použitých slin, které se mění v průběhu sání a liší se mezi jednotlivými druhy klíšťat (19, 30). V současné době se většinou používají sliny, které se získávají z klíšťat nasátých cca 6-7 dnů a jejich salivace se iniciuje pilokarpinem nakapaným na klíště. Použití slin z klíšťat nasátých kratší dobu (např. 3 dny) je bohužel technicky těžce proveditelné. Složení slin se liší i mezi klíšťaty, která jsou prosta patogenů nebo infikovaná (137). Změny na úrovni genové exprese v slinných žlázách byly pozorované v klíšťatech infikovaných *B. burgdorferi* či TBEV (138, 139). Je pravděpodobné, že složení slin se mění v důsledku aktivace obranných mechanizmů klíštěte a patogeny těchto změn pak využívají ve svůj prospěch při přenosu do hostitele (137). Dávka a pravděpodobně i kmen/druh testovaného patogenu rovněž ovlivňuje prokazatelnost SAT efektu. Příkladem může být infekce virem Powasan, kdy bylo zjištěno, že SGE z *I. scapularis* výrazně ovlivňuje průběh nemoci při nižší dávce viru 10^3 pfu, naopak efekt SGE není patrný při dávce 10^6 pfu (27).

VAKCINAČNÍ A TERAPEUTICKÝ POTENCIÁL SLOŽEK SLIN Z KREV SAJÍCÍCH ČLENOVCŮ

Výzkumu klíštěcích molekul a jejich funkční charakteristice se věnuje řada laboratoří po celém světě. Primárním cílem studia SAT efektu a klíštěcích SAT faktorů je identifikovat potenciální kandidáty pro přípravu vakcín. Většina ‚klasických' vakcín je zaměřená proti antigenům patogenů. Alternativně je možné připravit vakcíny, které jsou zaměřené proti klíštěcím SAT faktorům, tzv. přenos blokující vakcíny (115). Jako potenciální kandidáti na vakcínu proti boreliím jsou v současnosti v preklinickém stádiu testování nejméně 4 SAT faktory pro borelie; Salp15, Salp25D, TSLP a tHRP (140). Cementový protein z *Rhipicephalus appendiculatus* (64TRP) je testován jako potenciální vakcína proti TBEV (115). V stadiu preklinických studií je momentálně testování vakcinačního potenciálu slinných proteinů HqCRT, HICRT a rBmCRT (z klíštěte *Haemaphysalis*) vůči babesióze a theilerióze, a sialostatinu L2 (z *I. scapularis*) vůči anaplazmóze (140). Jiným přístupem k odhalení klíštěcích faktorů ovlivňujících sání a přenos patogenů je analýza hostitelské odpovědi po opakovaném sání, které vyvolává v hostiteli rezistenci na klíšťata. Zajímavé je,

že se tato reakce liší mezi myšmi a morčaty (141). U morčat se získaná anti-klíštěcí imunita projevuje v podobě předčasného uvolnění klíštěte z hostitele a zhoršeného sání. Rozdíly v hostitelské odpovědi myší a morčat byly pozorované na úrovni FcεRI signalizace, aktivace komplementu a koagulačních drah.

Kromě vakcinačního potenciálu se neméně důležitým jeví využití farmaceutického potenciálu proteinů klíštěcích slin. V *in vivo* a *in vitro* testech byly identifikovány proteiny s anti-koagulačními, imuno-modulačními, proti-zánětlivými a proti-nádorovými účinky, které mohou být perspektivně využity v humánní medicíně (142). Za zmínku stojí lipokaliny, histamin vázající proteiny, evasiny, Salp15, Sialostatin L, Ixolaris, Amblyomin-X, rEV576 nebo IAFGP. Lipokalin OmCI z měkkého klíštěte *Ornithodoros moubata* inhibuje klasickou i alternativní dráhu komplemetu vazbou na C5 složku. Na zvířecích modelech autoimunitních onemocnění jako jsou myastemia gravis nebo antifosfolipidový syndrom (APS) dokázal významně snížit projevy nemoci (143, 144). Tento klíštěcí inhibitor komplementu je v současnosti v preklinické fázi testovaní (např. při hemoglobulinurii). Histamin vázající proteiny, jako např. Ra-HBP2, jsou perspektivní v léčbě alergického astmatu (145). Evazíny jsou glykoproteiny vážící (hostitelské) chemokiny, čímž inhibují migraci leukocytů do místa sání. Infiltrace/rekrutace imunitních buněk, kterou zprostředkovávají chemokiny provází mnoho imunopatologických onemocnění. Různé evazíny byly identifikovány napříč různými druhy klíšťat (146) a jejích využití v humánní medicíně je perspektivní. Např. evasin-1 vážící chemokíny CCL3, CCL4 a CCL18 blokuje rekrutaci neutrofilů a má výrazný terapeutický efekt při plicní fibróze (147). Již několikrát zmiňovaný protein Salp15 by mohl být užitečný při léčbě alergického astmatu (148). Vliv Sialostatinu L byl testován na zvířecím modelu roztroušené sklerózy; jeho aplikace vedla k zmírnění symptomů nemoci a oddálení rozvoje experimentální autoimunitní encefalomyelitidy (EAE) (95). Potenciál Sialostatinu L v léčení alergického astmatu byl demonstrován na myším modelu a za hlavní mechanismus jeho účinku je považována schopnost inhibice interleukinu-9 (149). Některé z klíštěcích proteinů vykazují protinádorový efekt a jsou již ve stadiu preklinického testování. Příkladem jsou Ixolaris nebo Amblyomin X (150). Ixolaris je protein ze slin klíštěte *Ixodes scapularis*, který ovlivňuje koagulační kaskádu hostitele inhibicí komplexu FVIIa/TF, čímž zabraňuje aktivaci faktoru X (151). Amblyomin-X je inhibitor serinových proteáz Kunitzova typu, který byl izolován ze slin klíštěte *Amblyomma cajennense.* Tento protein má schopnost inhibovat enzym FXa a indukovat apoptotickou buněčnou smrt (150). Schopnost ovlivňovat apoptózu a autofagii je mechanizmem jejich protinádorového účinku. Využití klíštěcích proteinů jako terapeutik je perspektivní, ale zároveň vyžaduje modifikaci proteinů za účelem inhibice jejích imunogenicity, která není žádoucí (152). Běžným způsobem takové modifikace je např. post-translační úprava terapeutických proteinů, tzv. PEG-ylace (153).

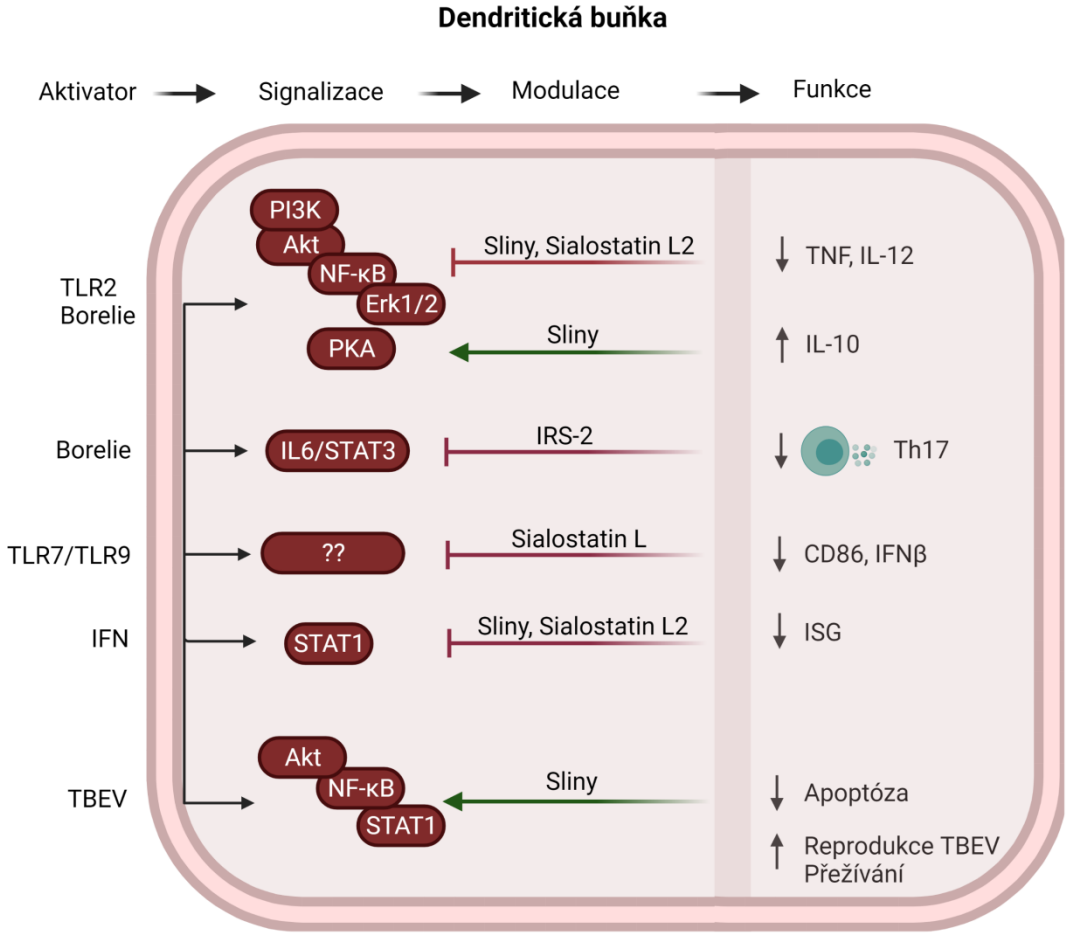
I v komářích slinách již bylo identifikováno několik složek pozitivně ovlivňujících přenos virových patogenů a počet faktorů, které jsou potenciální kandidáti na vakcínu blokující přenos, se postupně zvyšuje. Několik proteinů ovlivňujících replikaci virů Dengue a Zika je již v preklinické fázi testování (140). Příkladem jsou již dříve zmíněné proteiny; proteáza CLIPA3, protein *Aedes aegypti* venom allergen-1 (AaVA-1), LTRIN či protein aegyptin.

8. SHRNUTÍ

Předložené studie přinesly cenné informace o účincích klíštěcích slin a tří klíštěcích inhibitorů Sialostatinu L, Sialostatinu L2 a serpinu IRS-2 na funkci dendritických buněk. Bylo zjištěno, k jakým změnám dochází ve stimulovaných dendritických buňkách na úrovni signalizačních drah (Obr. 6).

- Sliny inhibují TLR2-závislou signalizaci a IFN signalizaci, ale pozitivně působí na dráhu Protein kinazy A (PKA) v boreliemi stimulovaných buňkách.
- Sliny pozitivně ovlivňují Akt, NF-κB a STAT1 signalizaci v buňkách infikovaných virem TBE.
- Sialostatin L2 inhibuje kromě IFN signalizace i TLR2-závislou signalizaci, konkrétně NF-κB, Akt a Erk1/2 dráhy.
- Sialostatin L interferuje s TLR7- a TLR9-závislou signalizací.
- IRS-2 inhibuje IL6/STAT3 dráhu.

Analýza změn v signalizaci umožňuje identifikaci buněčných procesů, které jsou ovlivněny slinami a ve funkci imunitních buněk hrají důležitou roli. Výsledkem působení slin a klíštěcích molekul byly změny na úrovni produkce cytokinů, exprese interferon stimulujících genů (ISG), maturace, apoptózy, produkce viru a polarizace adaptivní odpovědi. Imunomodulace buněk vlivem slin byla potvrzena na několika úrovních včetně polarizace DC směrem k Th2 fenotypu (zvýšení IL-10) a inhibice jejich maturace (snížení CD86). Zvýšená produkce viru v infikovaných dendritických buňkách rovněž koreluje s pozitivním vlivem slin na přenos viru TBE. Souhrn výsledků z předložených publikací je znázorněn na obrázku 6.
Pochopení mechanizmů, kterými klíště čelí hostitelské obraně, aby úspěšně dokončilo všechny vývojové stádia svého života, a identifikace SAT faktorů jsou přínosné nejen pro obecné poznání, ale mohou přispět i k úspěšnému vývoji vakcín, které v případě borelií, navzdory letitému úsilí několika vědeckých týmů, dosud neexistují. Navíc, porozumění způsobu přenosu a patogeneze infekčních nemocí vyvolaných klíšťaty přenášenými patogeny může být přínosné i pro studium patogenů, které jsou přenášené jinými krev sajícími členovci. Zvlášť významný je terapeutický potenciál některých složek klíštěcích slin, který představuje zajímavou alternativu v léčení různých imunopatologických a onkologických onemocnění. Funkční charakteristika klíštěcích proteinů a odhalení mechanizmů jejich vlivu je pro potenciální využití v medicíně nezbytné a je předmětem našeho současného bádání.



Obr. 6. Vliv klíštěcích slin, Sialostatinu L, Sialostatinu L2 a serpinu IRS-2 na signalizaci a funkci dendritických buněk stimulovaných boreliemi, virem TBE, TLR ligandy a interferonem (IFN). Sliny inhibují TLR2-závislou signalizaci a IFN signalizaci, ale pozitivně působí na dráhu Protein kinazy A (PKA) v odpovědi na TLR2 ligand a na Akt, NF-κB a STAT1 signalizaci ve virem infikovaných buňách. Sialostatin L2 inhibuje kromě IFN signalizace i TLR2-závislou signalizaci, konkrétně NF-κB, Akt a Erk1/2 dráhy. Sialostatin L interferuje s TLR7- a TLR9-závislou signalizací. IRS-2 inhibuje IL6/STAT3 dráhu. Výsledkem působení slin a klíštěcích molekul je ovlivnění DC na úrovni produkce cytokinů, exprese interferon stimulujících genů (ISG), maturace, apoptózy, produkce viru a polarizace adaptivní odpovědi. Souhrn z přiložených publikací.

9. SEZNAM ZKRATEK

AP1	transkripční faktor, z angl. *activator protein*
Akt	Proteinkináza B
ATG	z angl. *autophagy related genes*
ATP	adenosintrifosfát
CD	diferenciační skupina, z angl. *cluster of differentiation*
cAMP	cyklický adenosinmonofosfát
cDC	konvenční dendritické buňky
CLR	z angl. *C-typ lectin receptor*
DAMPs	z angl. *damage (danger)-associated molecular patterns*
DC	dendritické buňky
DENV	dengue virus
DNA	deoxyribonukleová kyselina
ER	endoplazmatické retikulum
Erk1/2	extracelulárně regulovaná kináza
Flt-3L	z angl. *FMS-like tyrosine kinase 3 ligand*
GM-CSF	z angl. *granulocyte macrophage colony stimulating factor*
hpi	hodiny po infekci
IFN	interferon
IL	interleukin
IRF	interferon regulační faktor
IRAK	interleukin-1 asociovaná kináza
ISGF	interferonem stimulovaný genový faktor
ISGs	interferonem stimulované geny
JAK	z angl. *Janus-activated kinase*
JEV	virus japonské encefalitidy
JNK/SAPK	z angl. *Jun N-terminal kinase/stress-activated protein kinase*
LGTV	Langat virus
LPS	lipopolysacharid
LTA	lipoteichoová kyselina
MAPK	mitogenem aktivovaná protein kináza
MAVS	adaptor, z angl. *mitochondrial antiviral signaling*
MDA5	receptor, z angl. *melanoma differentiation- associated gene 5*

mDC	myeloidní dendritické buňky
MHC	hlavní histokompatibilní komplex (z angl. *major histocompatibility complex*)
mTOR	serin/threoninová kináza (z angl. *mammalian target of rapamycin*)
MyD88	adaptor, z angl. *myeloid differentiation factor 88*
NF-κB	nukleární faktor κB
NLR	z angl. *NOD- like receptor*
NOD	z angl. *nucleotide-binding oligomerization domain*
NS	nestrukturní protein
PAMPs	z angl. *pathogen-associated molecular patterns*
pDC	plazmacytoidní dendritické buňky
PI3K	fosfatidylinositol-3-kináza
PKA	protein kináza A
PRR	receptor rozpoznávající patogen (z angl. p*athogen recognition receptors*)
RIG-I	typ cytoplazmatického receptoru (z angl. *retinoic acid-inducible gene I*)
RLR	z angl. *RIG-like receptor*
RNA	ribonukleová kyselina
ROS	reaktivní formy kyslíku
SARM	adaptor, z. angl. *sterile alpha and armadillo-motif containing protein*
SAT	saliva-assisted transmission
SGE	salivary gland extract
STAT	z angl. *signal transducer and activator of transcription*
TBEV	virus klíšťové encefalitidy (z angl. *Tick-borne encephalitis virus*)
TGF	z angl. *transforming growth factor*
Th	pomocné T lymfocyty
TIR	Toll/interleukin-1 receptor
TIRAP	z angl. *TIR domain-containing adaptor*
TLR	Toll-like receptor
TNF	z ang. t*umor necrosis factor*
TRAM	z angl. *TIR domain-containing adaptor inducing interferon-beta related adaptor molecule*
TRAF	z angl. *TNF receptor-associated factor*
TRIF	z angl. *TIR domain-containing adaptor inducing interferon-beta*
WNV	virus západonilské horečky

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