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Charakteristika spontánní regrese melanomu u MeLiM prasat

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doktorská disertační práce

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Prohlašuji, že svou disertační práci "Charakteristika spontánní regrese melanomu u MeLiM prasat" jsem vypracovala samostatně a s použitím odborné literatury a dalších informačních zdrojů, které jsou citovány v práci a uvedeny v seznamu literatury.

V Praze dne 6. 8. 2020 _____

Obsah

1 Úvod

Zvyšující se počet onkologických pacientů vede k přetrvávajícímu zájmu o výzkum přesných mechanismů vedoucích ke vzniku jednotlivých nádorových onemocnění. Protože je proces tumorigeneze ve většině případů polyfaktoriální, nelze jeden faktor povýšit nad ostatní, a proto je důležité, aby se studie zabývaly celou problematikou komplexně, protože jedině tak lze nalézt pro tuto skupinu nemocných účinnou léčbu. K tomuto poznání slouží v základní části výzkumu *in vitro* metody na buněčných liniích, které pomáhají lépe pochopit jednotlivé kroky v metabolismu nádorových buněk a zároveň umožňují ověřit účinky nových léků na zdravé i postižené buňky. Další náhled na problematiku onkologických onemocnění umožňují pokusy *in vivo* na zvířecích modelech, u kterých lze průběh nádorového onemocnění i potencionální léčbu studovat v maximální komplexnosti.

Jedním ze zvířecích modelů je i prasečí linie Melanoma-bearing Libechov Minipig, která je využívána pro výzkum spontánní regrese maligního melanomu. Fenomén spontánní regrese je zajímavý tím, že k němu dochází zcela bez umělého zásahu do organismu, a tak se možnost jeho využití v lékařské praxi jeví jako velice přitažlivá. Bohužel z důvodu nedostatku pacientů, u kterých se tento proces vyskytl, máme zatím pouze strohé informace o jeho etiologii.

Nádory kůže zahrnují několik různých typů, kdy maligní melanom má nižší incidenci v populaci než bazaliom nebo spinaliom, ale je s ním spojena nejvyšší úmrtnost. Jeho vznik je nejčastěji spojován s délkou expozice kůže UV záření, avšak jeho patofyziologie je závislá i na dalších faktorech včetně dědičné predispozice, které bylo využito při šlechtění všech pokusných linií prasat s tímto onemocněním.

2 Literární přehled

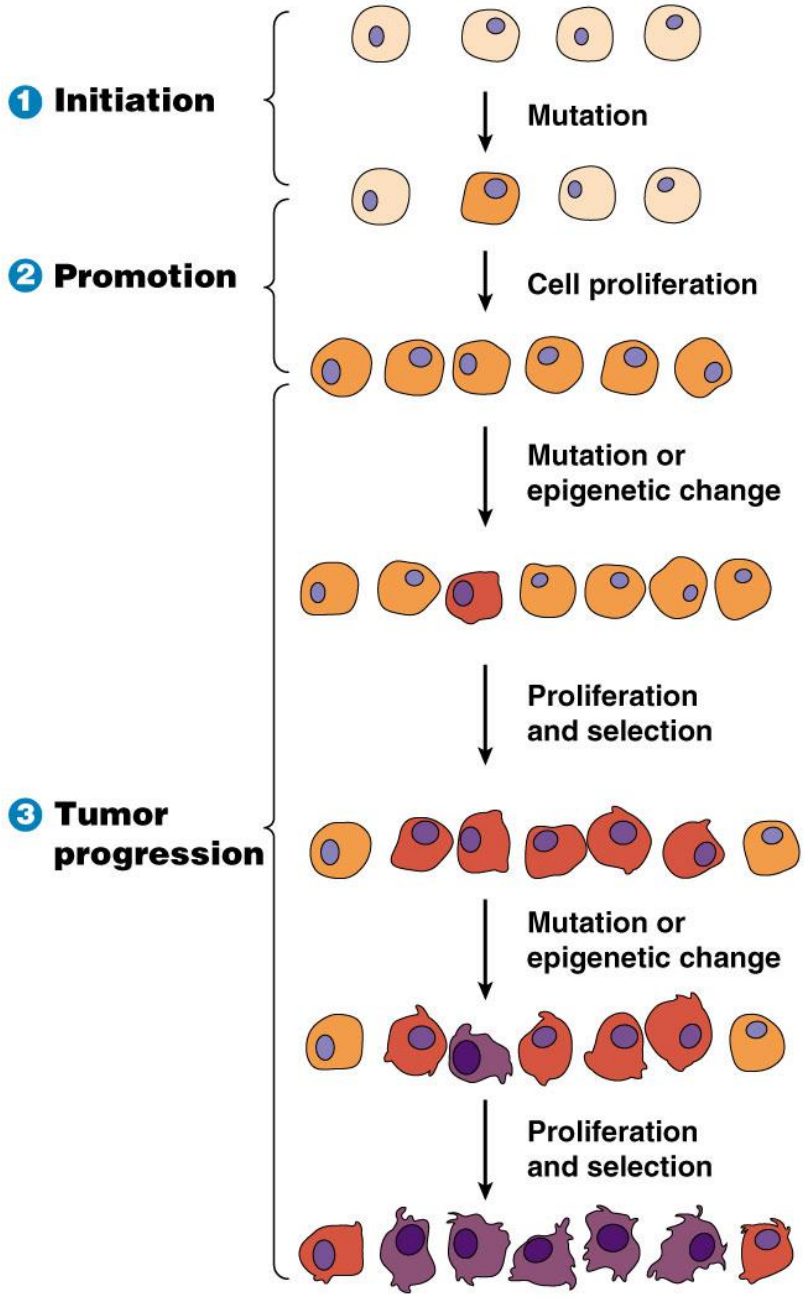
2.1 Nádorová onemocnění a příčiny jejich vzniku

Základní, nikoliv jedinou podmínkou vzniku nádorového bujení jsou mutace v genech kritických pro kontrolu buněčného cyklu. Během života se v DNA akumulují změny, které aktivují protoonkogeny a inaktivují tumor supresorové geny (Hoejimakers 2001). Maligní transformace může postihnout jakoukoliv buňku a z toho důvodu se nádorová onemocnění mezi sebou značně odlišují, což ztěžuje jejich terapii. Primárně se nádory dělí na benigní, které jsou lokalizované na místě svého vzniku, a maligní, které jsou naopak invazivní nejen pro okolní zdravou tkáň, ale prostřednictvím oběhového a lymfatického systému, pro celé tělo. Benigní a maligní nádory jsou klasifikovány podle typu buňky, ze které vznikly. Většina nádorových onemocnění spadá do jedné ze tří hlavních skupin: karcinomy, sarkomy a leukemie nebo lymfomy. Nádory se dále klasifikují podle tkáně a typu buněk (Adam et al. 2011; Závadová 2015).

2.1.1 Vznik nádorů

Vzniku nádoru předchází několikastupňový proces, než se ze zdravé buňky stane kolonie nádorových buněk. Hlavními iniciátory maligního procesu jsou environmentální faktory (UV záření, ionizující záření, genotoxické látky), buněčné metabolity (superoxidové anionty, hydroxylové radikály, peroxid vodíku), autoimunitní poruchy, patogeny (viry, bakterie, paraziti) a genetické predispozice (Hoejimakers 2001; Perrino et al. 2019).

Vývoj nádorové buňky se dělí na několik fází (viz Obrázek 1). Nejdříve dochází k tzv. iniciaci, kdy dojde k mutaci, která však sama o sobě většinou nevede k vývoji nádoru. Výsledkem je vznik preneoplastické buňky. Následována je tzv. promocí, kdy je iniciovaná buňka vystavena promotoru, který umožní vlastní nádorovou transformaci a dochází k preferovanému klonálnímu růstu preneoplastických nebo neoplastických buněk. Poslední fází je progrese, která nastává v pozdější fázi vývoje nádoru. Dochází k akumulaci dalších mutací, což je spojeno s rychlejší proliferací, vyšší invazivitou a vznikem metastáz. Preneoplastická populace se mění v čistě neoplastickou a z benigního nádoru se stává nádor maligní (Slaga et al. 1982; Boyd & Reade 1988; Pitot & Dragan 1991; Vincent & Gatenby 2008).



Obrázek 1: Hlavní stupně vývoje nádoru (Převzato z: www.mun.ca)

2.1.2 Infekce a zánět

Celosvětově se počet nádorových případů spojených s infekcí odhaduje zhruba na 15 %, kdy se toto číslo pohybuje v rozmezí od 7% v rozvojových zemích do 22% ve vyspělých zemích. (Pisani et al. 1997). Je však nutné podotknout, že před rozvojem nádorového onemocnění infekci samotnou většinou doprovází další faktory. Mechanismy, které vedou k rozvoji nádorů lze shrnout do tří skupin (Kuper et al. 2000; Krejsek a Kopecký 2004). Prvním je vyvolání chronického zánětu. Ten

způsobuje zvýšenou proliferaci buněk, u kterých může dojít k DNA poškození a následné maligní transformaci (Cohen et al. 1991; O´Byrne & Dalgleish 2001), též je doprovázen vznikem reaktivních forem kyslíku a dusíku (ROS a RNOS), které poškozují DNA, proteiny, buněčné membrány a modulují enzymatickou aktivitu a genovou expresi. Neopomenutelná je tvorba cytokinů, jež můžou zvyšovat proliferační aktivitu nádorových buněk a tím ovlivnit schopnost tvorby metastáz. Některé faktory zánětového mikroprostředí jsou pak odpovědné za procesy neoangiogeneze. Ta umožňuje zvětšování objemu nádorové masy (Ohshima & Bartsch 1994; Krejsek a Kopecký 2004). Dalším mechanismem je schopnost některých patogenů přímo transformovat hostitelský genom vložením aktivních onkogenů, případně inhibovat nádorové supresory nebo stimulovat mitózu. Tak tomu je např. u viru Estein-Barrové, viru žloutenky typu B a C, Kaposi sarcoma-associated herpesviru, lidského papillomaviru a bakteire *Helicobacter pylori*. Posledním mechanismem je schopnost vyvolání imunosuprese s následnou imunodeficiencí (např. virus HIV), kdy u takto postižených osob mívá průběh onemocnění agresivní charakter (Beral & Newton 1998; Bouvard et al. 2009).

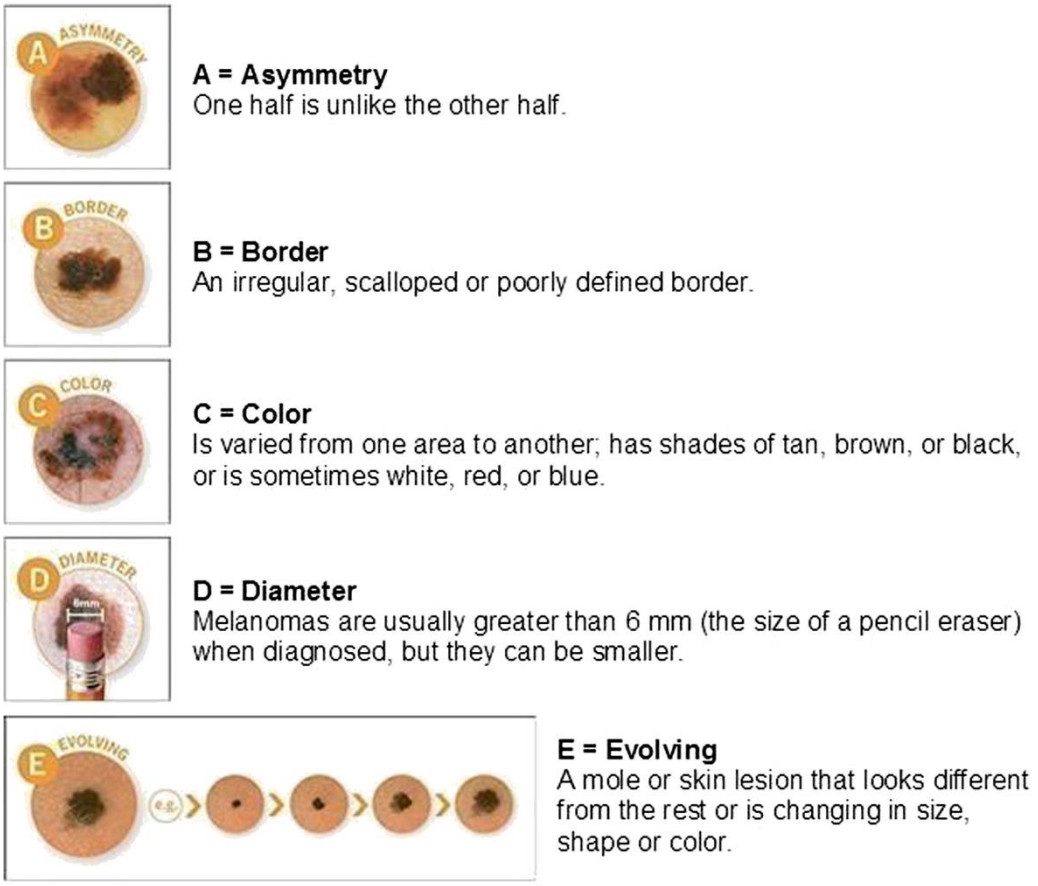
2.2 Melanom

Maligní melanom (MM) vzniká maligní transformací melanocytů (buňky produkující pigment melanin), které se nacházejí primárně v kůži. Mimo ní se melanocyty vyskytují v očích, epitelu sliznic a mozkových plenách (Lo & Fisher 2014), a proto mohou být melanomy rozděleny podle místa původu na nejběžnější kožní melanom, uveální melanom a slizniční melanom (Ali et al. 2013). Mezi hlavní faktory ovlivňující vývoj MM patří: intenzita pigmentace kůže, délka expozice kůže ultrafialovým (UV) zářením (Gilchrest et al. 1999; Lea et al. 2007) a dědičná predispozice (přibližně 10% melanomů má dědičný původ) (Rivers 1996). Zajímavé je, že se MM zřídka vyskytuje i mimo europoidní rassu, kdy je jeho výskyt 10-20 x nižší než u bílé populace, ale pokud už je přítomen, tak se obvykle jedná o agresivnější formu nodulárního nebo lentiginózního melanomu (Bataille & de Vries 2008).

MM je nejvážnější formou ze všech nádorů, které postihují kůži (Camilio et al. 2014). To je zapříčiněno zejména jeho nenápadností, poměrně rychlou

proliferací a častým sklonem k tvorbě metastáz. Pro zajištění příznivé prognózy je důležité, aby byl odhalen včas (ve stupni I-III), podle Clarkových kritérií, které dělí progresi melanomu do stupně I-IV (Clark et al. 1969).

MM se dělí do čtyř různých podtypů: superficiálně se šířící melanom, lentigo maligna melanom, nodulární melanom a akrální lentiginózní melanom. Časná diagnostika melanomu se provádí na základě vyhodnocení ABCDE kritérií: A-asymetrie, B-okraj, C-variabilita barvy, D-průměr, E-vývoj (viz Obrázek 2) (Friedman et al. 1985; Abbasi et al. 2004).



Obrázek 2: Schéma ABCDE (Převzato z: Tsao et al. 2015)

MM je jeden z deseti nejčastějších typů nádorů s neustále rostoucím výskytem nových případů. Výskyt se pohybuje od 6-10 případů/100 000 obyvatel v jižní Evropě do 50-60 případů/100 000 obyvatel v Austrálii (Agarwala 2008; Cornish et al. 2009; Maire et al. 2013). Ve Spojených státech patří melanom k pěti nejčastějším nádorům u mužů (Guy et al. 2015).

2.2.1 Melanomové mikroprostředí

Mikroprostředí MM je tvořeno nejenom maligními buňkami, ale také komplexními interakcemi s jinými buňkami, jako jsou keratinocyty, fibroblasty, imunitní buňky a endoteliální buňky krevních cév a lymfatických uzlin, jakož i jejich extracelulární produkty, které se podílejí na vzniku a růstu nádorů (Lacina et al. 2015; Dvorankova et al. 2017).

Ve zdravé kůži po UV ozáření začnou keratinocyty produkovat hormon melanotropin, který stimuluje melanocyty, aby začaly tvořit melanin. Vezikuly obsahující melanin (melanozomy) se pak přesunou do keratinocytu, kde tento pigment chrání jádro před UV-poškozením DNA (D´Orazio et al. 2013; Lo & Fisher 2014). Fibroblasty jsou nejčetnější buňky v pojivové tkáni a jsou hlavními producenty komponentů extracelulární matrix. Ve zdravé kůži produkují fibroblasty faktory regulující proliferaci, diferenciaci a přežívání melanocytů (např. faktor kmenových buněk (SCF), základní růstový faktor fibroblastů (bFGF), hepatocytový růstový faktor (HGF), transformující růstový faktor (TGF)) nebo faktory stimulující melanogenezi (keratinocytový růstový faktor (KGF), neuregulin-1 (NRG-1) (Wang et al. 2017). Fibroblasty také sekretují cytokiny IL6, IL8 a chemokin GROα. Sekrece IL6 a IL8 se významně zvyšuje v přítomnosti keratinocytů nebo nádorových buněk (Kolar et al. 2012). Fibroblasty spojené s nádory (CAF) jsou buňky, které se vyskytují zejména v stromatu nádoru. In vitro kokultivační experimenty ukazují, že CAF podporují migraci a invazivitu melanomových buněk a tato migrace je závislá na míře sekrece interleukinu 6 (IL6) a interleukinu 8 (IL8). Aplikace protilátek blokujících aktivitu IL6 a IL8 plně inhibují migraci buněk melanomu in vitro (Jobe et al. 2016). CAF z melanomu také ovlivňují keratinocyty a mimo jiné u nich indukují expresi keratinu typu 14 a vimentinu (Kucera et al. 2015).

Extracelulární produkty jsou základní součástí nádorového mikroprostředí. Nejen buněčné interakce s extracelulární matrix, ale také enzymy (například proteázy pro remodelaci matrix), sekreční faktory (včetně cytokinů, chemokinů, růstových faktorů, angiogenních faktorů atd.), extracelulární vezikuly, jako jsou exosomy (Weidle et al. 2017; O´Loghlen et al. 2018), dostupnost živin a kyslíku (Ratnikov et al. 2017) a další faktory se podílejí na kontrole progrese nádoru.

Léčebná manipulace s mikroprostředím nádorů se jeví jako velmi slibný přístup v léčbě MM (Lacina et al. 2018).

2.2.2 Cytokiny v melanomu

Cytokiny jsou proteiny, které se účastní buněčné signalizace a vnitřní i mezibuněčné komunikace. Jsou produkovány širokým spektrem buněk, ale v onkologickém výzkumu se věnuje největší pozornost cytokinům produkovaným imunitními buňkami. Cytokiny ovlivňují různé funkce od regulace zánětlivé reakce přes regulaci buněčného růstu, diferenciaci, chemotaxi, angiogenezi a mnoho dalších. Z analytického hlediska jsou cytokiny převážně malé proteiny (peptidy), kdy jejich molekulová hmotnost může být v rozmezí 6-70 kDa (Stenken & Poschenrieder 2015).

V nádorech představují cytokiny klíčové regulátory, které podporují migraci, invazi a metastázování buněk. Exprese a aktivita cytokinů je deregulována u mnoha typů nádorů (Yao et al. 2016). Transformované buňky produkují prozánětlivé cytokiny, chemokiny a růstové faktory, které podporují přežití buněk a proliferaci, podporují zánět a angiogenezi. Výsledkem je zvýšení počtu imunitních a stromálních buněk v nádoru. Mediátory sekretované rostoucím nádorem, včetně cytokinů, dále přispívají k proliferaci buněk, angiogenezi a zánětu, ale také k remodelování matrix, změně exprese adhezivních molekul a zvýšení vaskulární permeability. To vše zvyšuje tvorbu metastatického mikroprostředí (Atretkhany et al. 2016; Yao et al. 2016; Herraiz et al. 2017).

IL8 je popisován jako chemotaktický faktor pro neutrofily, nicméně má další funkce v angiogenezi a aktivaci matrix-metaloproteinasy. Angiogeneze a tvorba metastáz MM mohou být provázeny sekrecí IL8 z stromatu nádoru společně s jeho signalizací prostřednictvím receptoru CXCR2 (Liu et al. 2016; Herraiz et al. 2017). Sérové hladiny IL8 korelují s pokročilostí nádoru (Sanmamed et al. 2014) a IL8 byl navržen jako cirkulující biomarker melanomu (Alegre et al. 2015). Podobně jako IL8, tvorba HGF stromálními buňkami a aktivace Met receptoru HGF ovlivňuje invazivitu MM. Zvýšené hladiny HGF v krvi, stejně jako přítomnost exosomů obsahujících Met jsou spojeny s melanomovými metastázami a s rezistencí na léčbu (Filitis et al. 2015; Matsumoto et al. 2017). Chemokiny CCL17 a CCL22, které jsou

produkované makrogágy infiltrujícími nádor, pomáhají k infiltraci Treg do nádoru, což udržuje imunosupresivní mikroprostředí v MM (Fujimura et al. 2016).

2.2.3 Léčba melanomu

Standardní léčba MM zahrnuje chirurgické odstranění, chemoterapii, imunoterapii, radiační terapii a biochemoterapii (Payette et al. 2009; Maire et al. 2013). Primárním přístupem je lokální excize nádoru. Biopsie sentinelové uzliny je prvním krokem, pokud je nález pozitivní, pak se pokračuje exstirpací regionálních lymfatických uzlin (Khan et al. 2011). Standardní léčbou metastazujícího MM je také chemoterapie. Nejčastěji je používáno chemoterapeutikum dacarbazin, ale bohužel je míra odpovědi pouze mezi 10 a 20 % a doba přežití se pohybuje okolo 8 měsíců. Další používaná chemoterapeutika jsou temozolomid, carmustin, lomustin, cisplatin atd (Garbe et al. 2011; Finn et al. 2012).

Radioterapie je k léčbě využívána od roku 1970, zvláště v případě neproveditelnosti chirurgické excize. Radioterapie se též používá v radiochirurgii u mozkových metastází MM, brachyterapii uveálního melanomu a v terapii MM lokalizovaného na hlavě a krku (Khan et al. 2011).

T-lymfocyty mají ústřední úlohu v imunitním boji proti nádorům a jsou tak hlavním cílem melanomových imunoterapií. Stupeň infiltrace T-buněk a T-buněčný fenotyp v nádoru jsou důležitými prediktory odezvy pacientů na imunoterapii (Gasser et al. 2017). Výzkum se zaměřuje na vyhledávání melanomových antigenů specifických cytotoxických T-lymfocytů, které by mohly být použity v terapii (Floe et al. 2017). Adoptivní buněčná terapie s nádor infiltrujícími T-lymfocyty izolovanými z nádoru pacienta, expandovanými *in vitro* a aplikovanými pomocí infúze má pozitivní výsledky v léčbě metastazujícího MM (Zikich et al. 2016). Na druhou stranu infiltrace nádoru imunosupresivními buňkami, jako jsou regulační T-lymfocyty (Tregs) nebo makrofágy M2, sekreční protizánětlivé cytokiny, jako je TGF a IL10 a pro-angiogenní faktory nebo exprese PD-ligandu se vztahují k nepříznivé prognóze. Tyto imunosupresivní buňky představují cíle potenciálních imunoterapií (Fujimura 2016; Ouyang et al. 2016; Mignogna et al. 2017). Další imunitní buňky přítomné v stromatu nádoru jsou dosud zkoumány méně. Jedná se o přirozené zabíječe (NK buňky, natural killer cells) (Tarazona et al. 2015),

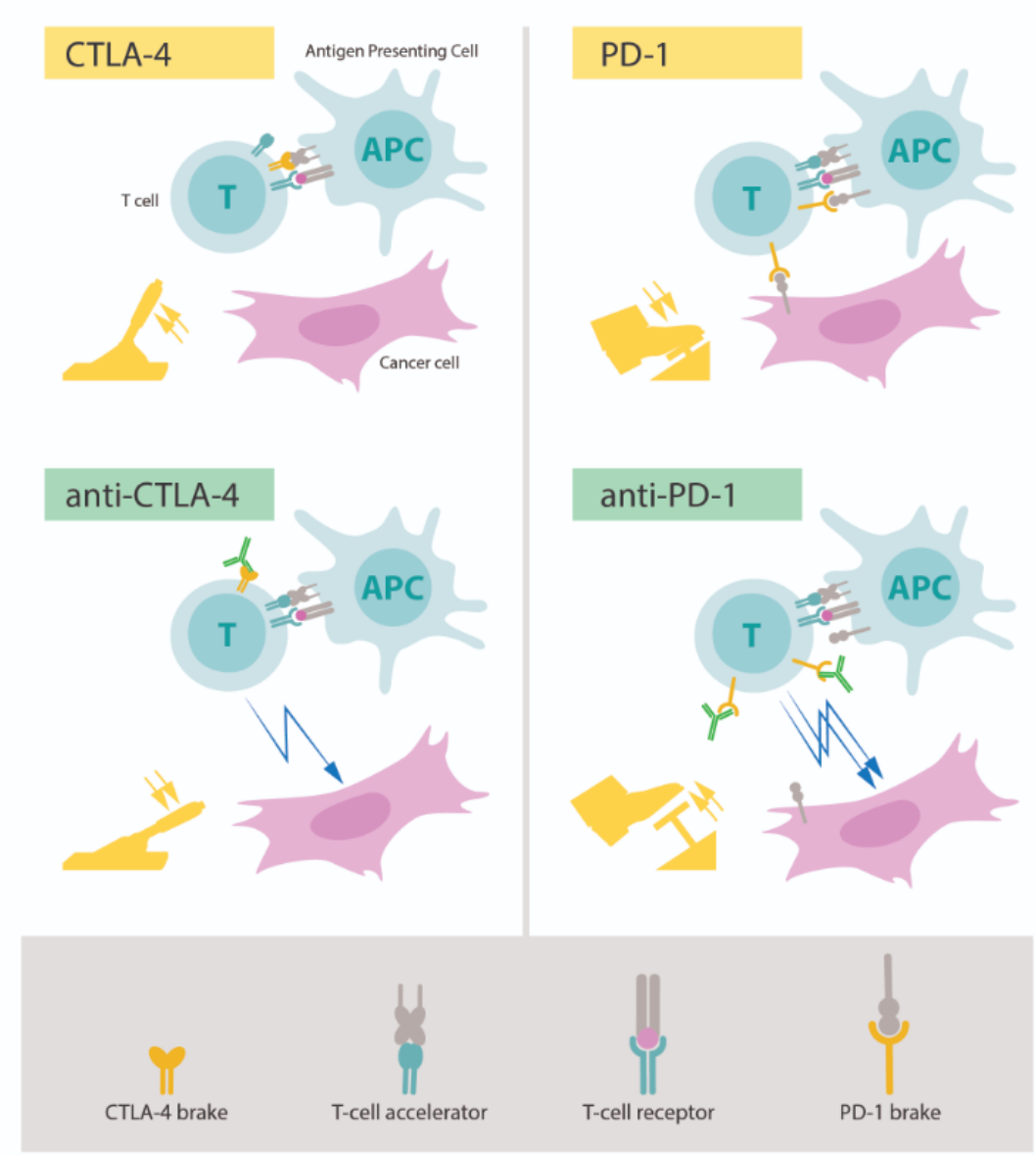
plazmacytoidní dendritické buňky (Saadeh et al. 2016), B-lymfocyty (Chiaruttini et al. 2017) atd.

Nejčastěji používané imunoterapeutické postupy pro léčbu MM jsou založeny na aplikaci IL2. Až 20% nádorů vykazuje odpověď na tuto cytokinovou terapii. Nicméně léčba je často doprovázena nežádoucími účinky. Jedná se nejen o méně závažný chřipkový syndrom, gastrointestinálními příznaky a zvýšení hmotnosti, ale i o závažnější příznaky jako je multiorgánová toxicita (srdce, plíce, játra, ledviny a centrální nervová soustava) (Creagan et al. 1984; Robinson et al. 1986; Rosenberg et al. 1987; Dutcher et al. 1989). V současné době se dosahuje účinnější terapeutické odpovědi s nižším výskytem vedlejších nežádoucích účinků při kombinaci cytokinové léčby s jiným léčivem, např. monoklonálními protilátkami (Maker et al. 2002; Aris et al. 2017), inhibitory kinázy (Ryu et al. 2017), T lymfocyty infiltrujícími nádory (Dudley et al. 2002), chemoterapeutickými látkami (Atkins et al. 2008), stereotaktickou tělovou radioterapií (Seung et al. 2012) nebo peptidovou vakcínou (Sosman et al. 2008, Schwartzentruber et al. 2011). Bohužel dlouhodobé odpovědi je dosaženo pouze u 6-10 % pacientů a léčba je navíc komplikována vysokou toxicitou léčiva (Finn et al. 2012). Lepší odezva nastává při použití interferonu-α 2b (Garbe et al. 2011). Ale i zde přetrvává limitace z důvodu toxicity léku. Příznaky toxicity jsou podobné chřipce a jsou důvodem předčasného ukončení léčby až u 25 % pacientů (Kim et al. 2002).

Protein CTLA-4 (cytotoxic T-lymphocyte associated protein 4) a protein PD-1 (programmed cell death protein 1) exprimované na povrchu T lymfocytů patří mezi kontrolní body imunitního systému. Jejich funkce je ochrana před vznikem autoimunitních reakcí, jelikož oba působí jako brzda T lymfocytů, i když každý na principu jiného mechanismu (viz Obrázek 3). Použitím monoklonálních protilátek inhibujících receptory CTLA-4 (ipilimumab a tremelimumab) a PD-1 (pembrolizumab, nivolumab a cemiplimab) došlo k aktivaci protinádorové imunity (Urwyler et al. 2020). Klinické studie prokázaly jejich účinnost v léčbě metastazujícího melanomu, kdy kombinace anti-PD-1 a anti-CTLA-4 prokázala významně vyšší míru odezvy ve srovnání se samotným ipilimumabem (57,6 % vs, 19 %). Nejčastějšími nežádoucími účinky této kombinované léčby byly průjem, únava a svědění (Larkin et al. 2015; Larkin et al. 2017). Dalším

imunoterapeutickým přístupem je inhibice mutace BRAF pomocí léku Sorafenib. Ten je účinný v kombinaci s chemoterapeutiky (carboplatina, paclitaxel) a míra odpovědi je 31% ve fázi testování I/II (Agarwala 2008).

Biochemoterapie je definována jako kombinace chemoterapie (např. dacarbazin) a imunoterapie (např. IL2). Biochemoterapeutická léčba se volí u pacientů s metastazujícím MM (Verma et al. 2008). Několik klinických studií ve fázi II objektivně ukazuje reakci na léčbu u 50-60 % pacientů, úplná remise je u 10-20 % pacientů a medián přežití pacientů je mezi 11- 12 měsíci (O´Day et al. 2002). Navzdory pokroku v léčbě melanomu zůstává účinnost imunoterapií stále nepředvídatelná a u mnoha pacientů se nakonec vyvine rezistence na léčbu (Luke et al. 2017).



Obrázek 3: Mechanismus působení protilátek anti-CTLA4 a anti PD-1. Kontrolní body imunitní reakce, které se nacházejí zejména na T lymfocytech, zásadním způsobem kontrolují imunitní odpověď na antigeny. Jedná se buď o aktivační, nebo právě inhibiční receptory. CTLA-4 a PD-1 jsou molekuly navzájem strukturně podobné, ale mají jiné biologické vlastnosti a také jiné ligandy. Protilátky (zelené) proti CTLA-4 blokují inhibiční signál, který je přítomen na antigen prezentujících buňkách, což vede k aktivaci T lymfocytů a útoku na nádorové buňky. Protilátky proti PD-1 inhibují vazbu mezi ligandy PD-L1 a PD-L2, které jsou přítomny mimo jiné i na nádorových buňkách, a tak umožňují aktivaci T lymfocytu (Převzato z: www.nobelprize.org).

2.3 Melanomové antigeny

Mutace v genomu nádorové buňky mohou způsobit, že se začnou exprimovat mutantní proteiny, které jsou specifické pro určitý typ nádorového onemocnění a neexprimují se na zdravých buňkách (tzv. neoantigeny). Tyto neoantigeny jsou atraktivním imunitním cílem, protože jejich selektivní exprese na nádorových buňkách může minimalizovat imunitní toleranci a riziko autoimunity (Yarchoan et al. 2017).

2.3.1 MART-1 (Melan-A)

MART -1 se vyskytuje ve většině čerstvých vzorků MM a 60 % buněčných linií melanomu. Jedná se o nejčastěji rozpoznávaný melanomový antigen cytotoxickými T lymfocyty (Tc) a tumor infiltrujícími lymfocyty u HLA-A2 pozitivních pacientů s metastazujícím MM (Rosenberg et al. 1998; Saleh et al. 2001). Signifikantně snížená exprese MART-1 byla u pacientů s různými MM a u pacientů s metastazujícím MM, u kterých byla pozorována úplná regrese MM. Byl prokázán vztah mezi přítomností MART-1 specifickými Tc lymfocyty a vymizením MART-1 antigenu (Saleh et al. 2001; Martin et al. 2014).

Účinnost adoptivního přenosu melanom specifických lymfocytů byla prokázána v různých studiích. Bylo zjištěno, že TIL jsou oligoklonální (obohacené o $CD8^+$ T reaktivní buňky), klonální nebo polyklonální Melan-A specifické Tc lymfocyty (Vignard et al. 2005). Vakcína obsahující antigeny Melan-A a TYRP, která byla použita u pacientů s metastazujícím MM vedla k částečné regresi nádoru a k zvýšené depigmentaci kůže (Jager et al. 2000). Významné odpovědi bylo dosaženo v 50 % případů (43 % částečná a úplná odpověď, 7 % stabilní onemocnění) ve studii fáze II, která se týkala adoptivního přenosu MART-1 specifických $CD8^+$ T buněčných klonů (Khammari et al. 2009).

2.3.2 gp100

gp100 je melanocytický diferenciační antigen specificky rozeznávaný HLA-A T lymfocyty. Předpokládá se, že hraje roli v regresi nádoru. Je vysoce imunogenní, a proto je vhodným cílem pro imunitní systém (Zhou et al. 1999). U pacientů s regresí MM i vitiligem, kteří byli vakcinováni gp100, byly nalezeny specifické T lymfocyty (Speeckaert et al. 2011). Toto zjištění může vést k indukci imunitního systému v reakci proti antigenu a může být pozorována i depigmentace.

Místní aktivace nádorově reaktivních T lymfocytů je kritickým krokem pro zahájení regrese nádoru (Kaufman et al. 2005).

2.3.3 Tyrosináza a TYRP 1 a 2

Tyrosináza je antigen asociovaný s melanomem rozpoznávaný $CD8^+$ T lymfocyty. Jedná se o slibného kandidáta na imunoterapii MM. $CD8^+$ T lymfocytární reakce je zaměřena proti jednomu z epitopů tyrozinázy (Wolchok et al. 2007). TYRP 1 a 2 patří k rodině Cu^{2+}/Zn^{2+} metaloenzymů, se kterými sdílí několik sekvenčních homologií (Ghanem & Fabrice 2011). Bylo prokázáno, že protilátky reagují s antigeny tyrozinázou i TYRP 1 a 2, které jsou přítomny na melanocytech i melanomových buňkách, a mohou indukovat mechanismy vedoucí k vitiligu podobné depigmentaci MM (Christou et al. 2010).

2.3.4 HERV-K

Jedná se o rodinu lidských endogenních retrovirů K, která obsahuje 30-50 provirů. Existují dvě různé rodiny HERV-K I a II. Exprese HERV-K v plné délce mRNA je detekována ve většině lidských tkání, chybějící část env a rec mRNA pouze u MM a teratokacinomu. Sekvence vztahující se k HERV-K-env genového proviru s krátkým otevíracím rámcem je základem pro HERV-K-MEL (Buscher et al. 2005).

Tento s melanomem související antigen je tvořen nona- a dekapeptidy a exprimován u většiny MM (kůže, oči) a dysplatických névů, nikoliv však v nepostižené kůži. Tento melanomový znak je omezený HLA-A2 při prezentaci $CD8^+$ cytotoxických T lymfocytů (Buscher et al. 2005; Krone et al. 2005; Tran et al. 2013). Bylo zjištěno, že sdílí homologní sekvenci s více než 70 lidskými patogeny (*Streptococcus pyogenes*, *Serratia marcescens*, *Mycobacterium bovis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria* sp., *Staphylococcus aureus* atd.). Předchozí expozice zmíněným patogenním peptidům identických s HERV-K-MEL může vést ke zkřížené reaktivitě proti MM Tc buňkami, což může vést k regresi MM (Vignard et al. 2005; Tran et al. 2013).

2.4 Spontánní regrese melanomu

Spontánní regrese (SR) je definována jako částečné nebo úplné vymizení nádoru bez jakéhokoliv terapeutického zásahu. Jsou popsány různé klinické příznaky SR: ztráta pigmentu léze nebo jejího okolí, redukce velikosti, telangiectasie a jizvení (Maio 2012; Bramhall et al. 2014). K SR melanomu dochází 6x častěji než u jiných malignit. Tento výsledek však může být způsoben jeho lokalizací na kůži a pigmentací, což velice usnadňuje detekovatelnost SR v porovnání s malignitami vnitřních orgánů (Maio 2012; Martin et al. 2014).

Až 50 % primárních MM prochází SR. Nicméně u metastazujícího MM se jedná o vzácný jev. SR metastazujícího MM byla dobře zdokumentována pouze u 0,23% případů (Maio 2012; Tran et al. 2013). Tak velké variace v hlášené regresní prevalenci mohou být způsobeny různými hodnotícími kritérii používanými ve studiích (Aung et al. 2017). Nicméně, regrese se vyskytuje častěji v počátečních stádiích nádorů (dle Breslowových kritérií pod 1,5 mm) (Blessing & McLaren 1992). U pacientů s mnohočetnými asynchronními melanomy byla incidence SR vyšší u následných melanomů ve srovnání s primárním nádorem, což naznačuje zvýšenou protinádorovou imunitu vyvolanou primární lézí (Martin et al. 2014).

SR metastazujícího MM se vyskytuje ve stejné míře u obou pohlaví nezávisle na věku, naopak SR primárního MM je v poměru muži/ženy 2:1. SR u metastazujícího MM je nejčastěji pozorována na kůži a v podkoží, následně u metastáz v lymfatických uzlinách, plicích, játrech, mozku a střev (Bramhall et al. 2014).

Prognostický význam SR primárního MM je stále kontroverzní. Hlavní problém spočívá v přesném vymezení a vyhodnocení SR (Payette et al. 2009; Ribero et al. 2013). Podle některých studií je SR považována za negativní prognostický faktor, jiné naopak uvádějí, že u pacientů s výskytem SR došlo k zvýšení 5 letého přežití (Payette et al. 2009; Maire et al. 2013; Martin et al. 2014; Ribero et al. 2013).

2.4.1 Mechanismy spontánní regrese

Nejčastější faktory vedoucí k SR MM jsou operační trauma, infekce a vliv imunitního systému. Dalšími zdokumentovanými faktory spojenými s SR metastazujícího MM jsou transfuze krve, aplikace Bacillus Calmete-Guérin (BCG),

vakcíny proti vzteklině a endokrinní faktory (těhotenství, alternativní terapie xeroderma pigmentosum, diabetes, nefrolitiasa, hypertrofie prostaty a žaludeční vředy) (Bramhall et al. 2014; Martin et al. 2014). Regrese je důsledkem interakce mezi maligní nádorovou buňkou a hostitelským imunitním systémem, což vede k nahrazení nádorové tkáně fibrózou, dále k degeneraci melanomových buněk, k infiltraci nádoru melanofágy, proliferujícími lymfocyty a telangiectasii. V oblastech kompletní regrese vymizí melanomové buňky ve vrstvě dermis i epidermis (Payette et al. 2009).

2.4.2 Faktory související s imunitním systémem

Z publikovaných prací je zřejmé, že SR je výsledkem účinné imunitní odpovědi proti buňkám MM. V regredujícím MM jsou pozorovány různé složky imunitního systému, jako jsou nádor infiltrující $CD4^+$ a $CD8^+$ T lymfocyty (Maio 2012, Mukherji 2013; Ribero et al. 2013; Tran et al. 2013; Martin et al. 2014). Regrese MM je spojena s autoimunitním vitiligem a depigmentací pokožky. Vitiligo bylo pozorováno u pacientů s MM a několik studií toto pozorování dalo do souvislosti s protinádorovou odezvou. Histologická analýza biopsie vitiliga ukázala početnou infiltraci $CD8^+$ T lymfocytů. Tyto T lymfocyty mají klonální nebo oligoklonální T buněčný receptorový profil. U pacientů, kterým byl diagnostikován MM, a zároveň u nich bylo pozorované vitiligo, byl zaznamenán vyšší počet specifických $CD8^+$ T lymfocytů ve srovnání s pacienty s MM bez vitiliga (Maio 2012). Vitiligo je z těchto důvodů považováno za příznivý prognostický faktor u pacientů s MM, což je zapříčiněno zejména vztahem mezi vitiligem a imunitní odpovědí. Bylo prokázáno, že lymfocyty infiltrující nádory (TIL) jsou převážně αβ T lymfocyty a regredující MM vykazuje klonální amplifikaci těchto buněk. Tyto buňky vykazují cytolytickou aktivitu proti autologním MM. (Mukherji 2013)

U MM se vyskytují různé typy antigenů, které jsou rozpoznávány cytotoxickými T lymfocyty. Nejčastěji se jedná o melanocytární diferenciační antigeny, které jsou také nejvíce imunogenní. Jedná se o Melan-A/MART-1, gp 100, tyrozinázu, TYRP 1 a 2 atd (Maio 2012; Martin et al. 2014).

Jelikož jsou nádorové buňky nízce imunogenní, tak mohou uniknout detekci imunitním systémem (Zhang et al. 2011). T regulační lymfocyty (Treg) hrají klíčovou roli v obraně proti nádorovému bujení. Základním mechanismem je

specifické rozpoznávání a destrukce nádorových buněk a vznik specifické imunitní paměti proti nádorovým mechanismům. Tyto buňky jsou definovány jako podskupina $CD4^+$ T lymfocytů exprimující CD25 a FoxP3 (Adeegbe & Nishikawa 2013).

V laboratorní i klinické praxi jsou rozdělovány na přirozené (n) a indukované (i) Treg. Přirozené Treg ($CD4^+FoxP3^+$, nTreg) jsou tvořeny v thymu a jsou lépe prozkoumány než iTreg, které jsou na thymu nezávislé (jsou produkovány v periferních lymfatických orgánech, mohou také exprimovat FoxP3). nTreg i iTreg bez ohledu na jejich původ mají společnou schopnost potlačit efektorové T lymfocyty. Protože nTreg jsou tvořeny v thymu (pod vlivem thymického stromálního lymfopoetinu aktivovaného CD11c pozitivními dendritickými buňkami a kostimulačními molekulami CD28, PD1, CD40L a IL2), lze očekávat, že hrají významnou roli při regulaci protinádorové odpovědi. Proto lze předpokládat, že nízké hladiny Treg v krvi nebo nádorové tkáni mohou hrát významnou roli při regresi nádoru (Adeegbe & Nishikawa 2013; Fessler et al. 2013). Zvýšené hladiny Treg byly detekovány u pacientů s MM a u karcinomu ledviny. Snížené hladiny Treg buněk byly pozorovány u pacientů, kteří reagovali na léčbu vysokými dávkami IL2 (Ascierto et al. 2010).

Vakcinace autologními dendritickými buňkami (DC) byla použita u pacientů s různými typy nádorů. Nejlepší výsledky byly získány u pacientů s MM vyskytujícím se v kůži nebo v lymfatických uzlinách (Edele et al. 2014). Imunologická odpověď byla pozorována v klinických studiích u pacientů s MM vakcinovaných nezralými, peptidem nebo nádorovým lyzátem plněnými DC (Berger & Schultz 2003). Úspěšná regrese byla pozorována u 6 z 11 (55,0%) pacientů s pokročilým metastazujícím MM (Godelaine et al. 2003).

Cytotoxický efekt NK buněk byl dobře zdokumentován v mnoha studiích. Vyšší pozitivita CD56 byla pozorována u regredujícího MM, naopak nižší pozitivita byla u neregredujícího MM (McKay 2011). Makrofágy asociované s nádorem (TAM) jsou detekovány v MM, ale jejich role v MM progresi / regresi je kontroverzní. Aktivované TAM mohou ovlivnit některé růstové vlastnosti MM - angiogenezi, tvorbu stromatu nebo růst buněk MM (regrese/progrese). Účinek TAM na regresi MM může být způsobený přímým cytotoxickým účinkem nebo indukcí buněčné lýzy (Hussein 2006).

2.4.3 Faktory související s mikroorganismy

Pojem mikrobiom je souhrnné označení pro všechny mikroorganismy, které žijí v hostitelském těle nebo na něm (Roy & Trincheri 2017). V případě kůže je mikrobiální rozmanitost ovlivněna různými faktory, jako je životní prostředí, pohlaví a genetika, kdy bylo prokázáno, že nadmořská výška ovlivňuje kožní mikrobiom u lidí i prasat (Avena et al. 2016; Zeng et al. 2017). Kožní mikrobiom hraje důležitou roli nejen v lidském zdraví, ale má významný vliv i pro další savce, ryby a obojživelníky (Grice et al. 2010; Nakatsuji et al. 2013; Rodrigues Hoffmann et al. 2014; Smeekens et al. 2014; Walke et al. 2014; Larsen et al. 2015, Ross et al. 2018), jelikož jeho základní vlastností je, že zajišťuje bariéru proti vniknutí patogenních bakterií (Sanford & Gallo 2013). Též byl prokázán jeho vliv na SR MM, jelikož stimulace hostitelského imunitního systému během probíhající infekce zlepšuje přirozenou imunitní obranu proti nádoru. Přínos infekce v případech spojených se spontánní regresí metastazujícího MM je vysoký – 28 % (Bramhall et al. 2014).

V experimentech s Coley toxinem bylo popsáno, že došlo k regresi nádoru několik hodin po injekci toxinu. Přerušení léčby na jeden den vedlo k znovuobjevení nádoru z jeho zbytkové tkáně. Protinádorová imunita byla zprostředkována vrozenou a nespecifickou imunitní reakcí spíše než pomalejší adaptivní imunitou. Z tohoto důvodu byla doporučena každodenní aplikace Coley toxinu (Yamadaet et al. 2002; Hoption et al. 2006).

Některé patogeny exprimují antigeny, které zkříženě reagují s nádory asociovanými antigeny. Thomsen-Friedenreich a Tn parazitární antigeny byly detekovány u více než 80% pacientů s nádorovým onemocněním a zdá se, že jsou potenciálními markery pro klinické použití. V séru získaném od pacientů s parazitární infekcí (*Echinococcus*), je zkřížená reaktivita často pozorována se séry získanými od pacientů s karcinomem. Je zajímavé, že tato séra jsou často přítomna u pacientů s méně rozsáhlými nádory. Protilátky proti těmto sdíleným antigenům můžou potenciálně zacílit nádorové buňky, které mají být zničeny, nebo zvýšit prezenci antigenů T lymfocytům, a tak indukovat protinádorovou odpověď (Oikonomopoulou et al. 2013).

T lymfocyty jsou odpovědné za buněčnou imunitu a B buňky za humorální imunitu. Úloha B buněk při destrukci nádorů zahrnuje lýzu zprostředkovanou

aktivací komplementu a usnadnění na protilátkách závislou buněčnou cytotoxicitu. CTL a NK buňky jsou důležité při aktivaci lýzy nádoru. CTL buňky rozpoznávají antigeny prezentované hlavním histokompatibilním komplexem a NK buňky vyhledávají a zabíjejí nádorové buňky, což hraje důležitou roli v prevenci metastáz (Cann et al. 2002). Lymfocyty a buňky prezentující antigen, jako jsou makrofágy a dendritické buňky (DC), infiltrují nádor v případě přítomnosti infekce. Po navázání PAMP (pathogen-associated molecular patterns) na toll-like receptory antigen prezentujících buněk dochází k jejich aktivaci a prezentaci antigenu. Aktivace vede k produkci důležitých kostimulačních molekul, jako jsou B7 a IL12, což vede k aktivaci imunitního systému (Pardoll & Topalian1998). DC mohou být primárně stimulovány lipopolysacharidy gramnegativních bakterií nebo jinými bakteriálními případně virovými produkty. Velké množství bakteriálních lipopolysacharidů indukuje produkci cytokinů, tymocytů a cytotoxických T lymfocytů (CTL). I*n vitro* bylo prokázáno zvýšení teploty. Nádorové buňky jsou na teplo citlivější než normální buňky, takže infekce způsobující hemoragickou nekrózu může z důvodu horečky vyvolat kolaps vaskulatury nádoru (Hobohm 2001; Mager 2006). Horečnatá onemocnění mohou hrát roli v remisi nádorů, protože horečka může vést k uvolnění kaskády prozánětlivých faktorů schopných stimulace DC a vedoucích k aktivaci T lymfocytů (Mager 2006).

Infekce *Toxoplasma gondii* u melanomových myší měla významný vliv na regresi MM bez souběžné aktivace imunitního systému, produkce oxidu dusného makrofágy a zvýšení IL12 a TNF. Základním mechanismem tohoto pozorování byla syntéza rozpustných faktorů s antiangiogenním účinkem. Doprovodným syndromem byla hypoxie následována nekrózou buněk (Yamada et al. 2002; Patyar et al. 2010).

SR MM je často spojována s výskytem specifických mikroorganismů. Coley toxin (tepelně usmrcený *Str. Pyogenes* a *S. marcescens*) a vakcína BCG (*M. bovis*) byly použity pro terapii MM. Bylo též zdokumentováno, že aplikace bacillus Calmette-Guérin vakcíny (BCG) nebo těžká infekce v dětství může být ochranou proti MM. Mechanismy působení Coley toxinu proti MM zahrnují čtyři kritické kroky, aby bylo navozeno protinádorového účinku - rozvoj infekce s horečkou, postupné zvyšování dávek toxinu, přímé injekce toxinu do tumoru (pokud je to možné) a aplikace toxinu minimálně po dobu 6 měsíců (prevence recidivy nádoru)

(Mager 2006). Terapeutický účinek toxinu je založen na aktivaci vrozené a adaptivní imunitní odpovědi. Tento proces je spuštěn prostřednictvím aktivace receptorů typu Toll (TLR) a /nebo jinými receptory rozpoznávající patogen. Lipopolysacharidy a nemetylované CpG motivy bakterií působí jako agonisté TLR. Vazba mezi agonistou a TLR iniciuje aktivaci imunitního systému, uvolnění prozánětlivých cytokinů a indukci horečky (Maletzki et al. 2012; Singh & Overwijk, 2015). Terapeutický účinek toxinu je propojen právě s rozvojem horečky, kdy po vyvolání horečky 38–40° C byla často pozorována SR nádorů (Jessy 2011). Navíc je vaskulatura tumoru křehčí než v nepostižené tkáni. Infekce vedoucí k hemoragické nekróze může vyvolat kolaps nádorové vaskulatury (Mager 2006). Též produkce cytokinů je odpovědná za terapeutický účinek toxinu. Zvýšená exprese TNFα, INF, ILlb, IL6 a IL12 byla pozorována u pacientů po aplikaci toxinu (Hoption Cann et al. 2003; Karpinski & Szkaradkiewicz 2013; Koller et al. 2016).

Protinádorový účinek BCG vakcíny je založen na zesílení imunitní odpovědi proti nádorovým buňkám. Klíčový efekt je přičítán aktivaci NK buněk (Chakrabarty 2003; Felgner et al. 2016). Terapeutický účinek BCG vakcíny je propojen s aktivací TLR2, TLR4, TLR9 a MyD88 cestou. Též byla zdokumentována indukce lokální imunitní odpovědi - infiltrace granulocyty, makrofágy a T lymfocyty (především stimulace Th1 odpovědi), exprese IL2, TNFα a INFγ cytokinů. Dále byly v moči pacientů detekovány cytokiny IL1, IL6, IL8, IL10, IL12, IL18 a MCSF (Hoption Cann et al. 2003; Sengupta et al. 2010; Gandhi et al. 2013; Iqbal & Hussain 2014; Ni et al. 2016).

Též bylo popsáno, že aplikace vakcíny proti tetanu vedla k vyvolání mechanismů vedoucích k SR MM. Již popsaná horečka byla často spojena s následnou SR MM, což bylo prokázáno i u *L. monocytogenes*, *P. aeruginosa*, *Salmonella* spp., *E. coli*, *Clostridium* spp., *Lactobacillus* spp. a *Bidobacterium* spp (Sengupta et al. 2010; Baird et al. 2013; Karpinski & Szkaradkiewicz 2013; Tran et al. 2013; Yamamoto et al. 2016).

SR MM lze vyvolat i produkcí streptokinázy (bakteriální enzym *S. pyogenes*). Tento enzym katalyzuje tvorbu plazminu z plazminogenu a fibrinu z fibrinogenu, což způsobí spuštění kaskády proteáz, které degradují plazmatické proteiny a proteiny extracelulární matrix. Tyto mechanismy jsou pro nádorové buňky fatální,

jelikož naruší jejich extracelulární matrix, a tak pozastaví jejich následný růst a sníží riziko metastáz (Mager 2006).

2.5 Zvířecí modely MM

Pro studium patogeneze melanomu a usnadnění vývoje cílených terapií, bylo vytvořeno několik zvířecích modelů tohoto onemocnění, včetně myší, prasat, koní, psů, dánií, kuřecích embryí (studium migrace buněk nervové lišty) atd. (Swerdlow et al. 1986; Markovic et al. 2007; Bataille & DeVries 2008; Cornish et al. 2009). Spontánně se vyskytující a UV indukovaný MM byl popsán u ryb *Xiphophorus* a vačice *Monodelphis domestica*. Iniciace MM u morčat a křečka syrského je indukována chemickými karcinogeny (např. DMBA). U dalších zvířat existují různé principy, na kterých jsou zmíněné modely založeny (indukované, dědičně založené) (Ha et al. 2005; Beaumont et al. 2014). U většiny zvířecích modelů MM progreduje, SR MM není příliš frekventovaná. Typickými modely SR MM jsou prasata, u kterých je tento fenomén často pozorován.

2.5.1 Prasečí modely SR

Prasata jsou nejlepším modelem pro sledování SR MM. V současnosti jsou tři linie prasat s dědičným melanomem: Sinclair linie, Mnichovská miniaturní linie a Melanoma-bearing Libechov Minipig linie. U všech těchto linií se melanomy vyvíjí spontánně v prenatálním i postnatálním období. Příznaky SR jsou doprovázené halo efektem kolem melanomů nebo lokálním případně systémovým vitiligem. Běžně se tyto změny vyskytují u pigmentovaných zvířat (černá, hnědá, zrzavá) nikoliv u bílých zvířat (Millikan et al. 1974; Wanke et al. 1998; Horak et al. 1999).

2.5.2 Sinclair linie

Tato prasata byla vyšlechtěna na Hormelově institutu v Minnesotě a později se stádo přestěhovalo na univerzitu v Missouri (Millikan et al. 1974). První prasata s kožním melanomem se objevila v roce 1967, kdy počáteční výskyt pigmentovaných kožních lézí byl 21 % (Strafuss et al. 1968). V následujících

generacích byl výskyt lézí vysoce ovlivněn selektivním chovem, díky kterému se podařilo jejich výskyt zvýšit až na 60 % (Millikan et al. 1974; Hook et al. 1979).

Na černých prasatech se vyskytují rozmanité primární kožní léze s proměnlivou velikostí a vzhledem, které jsou často přítomny již po narození (vrozené) nebo se vyvinou postnatálně. Naopak u rezavých selat se žádné léze nevyskytují. Kožní pigmentované léze mají různé histopatologické formy a vykazují podobnost s lidskými lézemi. Jsou klasifikovány jako benigní névy, superficiálně se šířící melanom nebo nodulární melanom metastazující do lymfatických uzlin a viscerálních orgánů (plíce, játra). Kožní nádory během postnatálního života spontánně regredují, což je často doprovázeno lokální nebo generalizovanou depigmentací kůže a štětin. Též je pozorována úplná regrese melanomu včetně metastáz v regionálních lymfatických uzlinách (Manning et al. 1974; Millikan et al. 1974; Oxenhandler et al. 1979; Hook et al., 1982; Misfeldt & Grimm 1994) Podíl zvířat s regresí melanomu se pohybuje mezi 85 % a 100 %. Podrobné histologické hodnocení regredujících melanomů ukázalo bifázický imunologický proces, kdy první fáze proběhne především během druhého měsíce po narození a je charakterizována masivní infiltrací makrofágů. V druhé fázi (počínaje začátkem čtvrtého měsíce věku) dochází k infiltraci lymfocytů a úplné regresi melanomů (Greene et al. 1994).

2.5.3 Mnichovské miniaturní prase

Tato prasata byla vyšlechtěna na univerzitě v Mnichově v roce 1986. Zakladatelem stáda byl jeden kanec s melanomem a dvě zdravé prasnice. Selektivní chov zvířat postižených melanomem zvýšil výskyt maligních nádorů až na 70 % a též se u tmavě pigmentovaných (černých a červených) zvířat vyskytovaly benigní melanocytové léze. Kožní léze byly přítomny již po narození nebo se většinou vyvinuly během prvních dvou měsíců života. Jednotlivé melanomové léze se liší od plochých přes mírně vyvýšené melanocytární névy až po nodulární invazivní melanomy. Poměrně často jsou pozorovány metastázy do regionálních lymfatických uzlin, méně často pak do jiných orgánů. Invazivní melanomy mají výraznou tendenci k SR nádoru s lokální a generalizovanou depigmentací (Muller et al. 1995; Wanke et al. 1998; Muller et al. 2001).

2.5.4 Melanoma-bearing Libechov Minipig

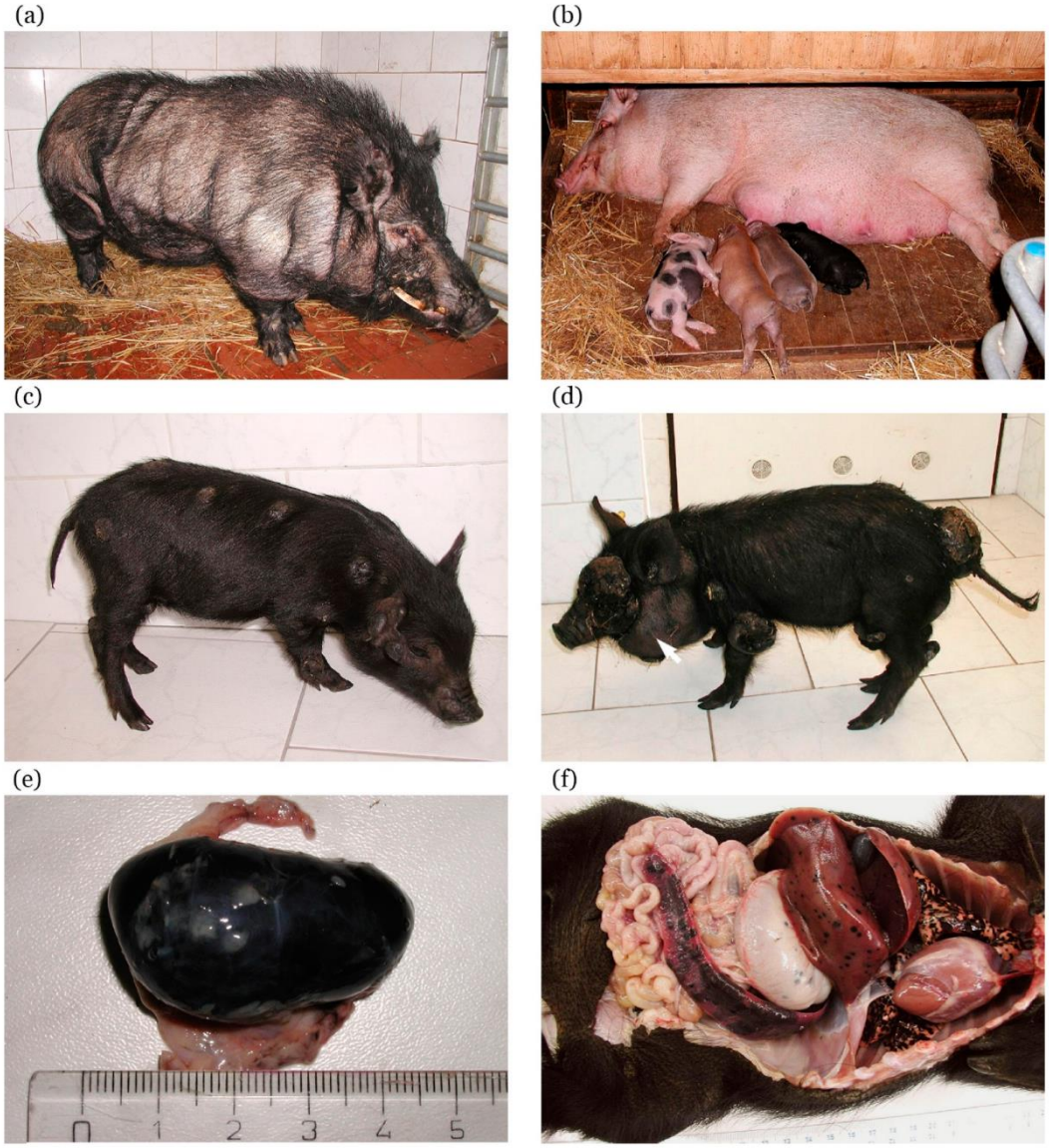
Linie Melanoma-bearing Libechov Minipig (MeLiM) byla vyšlechtěna v Ústavu živočišné fyziologie a genetiky AV ČR. Prvních několik černých selat s kožními melanomy se objevilo v roce 1989. Selektivním šlechtěním zvířat s melanomem se potvrdila genetická predispozice k melanomu (incidence kolem 50 %) (Horak et al., 1999; Hruban et al., 2004). Dlouhodobé sledování MeLiM prasat ukázalo, že se hodnoty incidence melanomu lišily v závislosti na míře postižení rodičů. Z tohoto důvodu byla do šlechtitelského programu zahrnuta nejvíce postižená prasata, čímž se zvýšila incidence melanomu na zhruba 80 %. V současné době je chováno osm prasnic a čtyři kanci linie MeLiM pro produkci selat používaných v experimentech (Horak et al. 2019).

Prasata MeLiM mají různé barevné rázy, ale převažuje černá. Zřídka se vyskytují zvířata rezavá, hnědá nebo bílá (s černými skvrnami). U barevných zvířat se mohou občas objevit drobné bílé znaky. Černá prasata jsou nejvíce postižena melanomem. Kožní nádory jsou obvykle vícenásobné, černé z důvodu vysokého obsahu melaninu v melanosomech, nodulárního typu (s lokální nekrózou u větších nádorů) a jsou distribuovány na všech částech těla. U rezavých a hnědých zvířat se vykytuje pouze jeden nebo několik málo kožních melanomů a bílá prasata s černými skvrnami jsou bez kožních lézí. U postižených prasat se také objevují névy a povrchově se šířící melanomy. Podobně jako u Sinclair prasat se kožní léze vyskytují již po narození nebo se objevují krátce poté během prvních dvou měsíců postnatálního života. Rostou exofyticky a dosahují velikostí asi 15–70 mm, výjimečně až 150 mm. Maligní charakteristika melanomu v linii MeLiM je potvrzena přítomností četných metastáz. Obvykle se vyskytují v lymfatických uzlinách, plicích a slezině. Silně postižená zvířata také vykazují metastázy v jiných viscerálních orgánech, jako je žaludek, játra, tenké a tlusté střevo, slinivka břišní, ledviny, srdce a thymus (Fortyn et al. 1994a; Fortyn et al. 1994b; Fortyn et al. 1998; Horak et al. 1999).

U menší části postižených selat (asi 5–30 %) melanomy progredují. Progrese melanomu je typická především pro černá selata, zatímco u rezavých a hnědých je velmi vzácná. Kožní melanomy s věkem zvířete rostou a dosahují značné velikosti. Tato silně postižená selata zpočátku zaostávají v přírůstcích tělesné hmotnosti za jejich méně postiženými sourozenci s projevy spontánní regrese. V pozdější fázi

zhubnou a vyvine se u nich silná kachexie. Též jsou pozorovány rozsáhlé metastázy v plicích, mízních uzlinách a slezině (viz Obrázek 4). Metastázy v lymfatických uzlinách, zejména v cervikálních a inguinálních, jsou také makroskopicky viditelné u některých zvířat vzhledem k jejich rostoucí velikosti. Kromě toho jsou metastázy přítomny v játrech, různých částech gastrointestinálního systému, thymu, srdci a mozku (Fortyn et al. 1998; Horak et al. 1999). Zvířata s progredujícím melanomem obvykle umírají během prvních tří měsíců věku (Horak et al. 2019).

Charakteristickým rysem melanomů u MeLiM prasat je SR, pozorovaná zhruba u 75 % postižených zvířat (Horak et al. 1999), ale v současné době pozorujeme její výskyt až u 90-95 % zvířat. Po počátečním období růstu se nádory začnou zplošťovat, zmenšovat se a měnit barvu z černé na šedou. Tělesná hmotnost prasete dosahuje normální nebo téměř normální hodnoty. Melanomová regrese je obvykle spojena s depigmentací kůže a štětin. SR začíná řídce rozptýlenými bílými štětinami na těle. Je také pozorován halo efekt kolem některých melanomů. Depigmentace se pak postupně rozšiřuje do okolních částí těla. Tato depigmentace se někdy šíří téměř po celém těle, což vede k tomu, že původně černé prase je téměř bílé (Horak et al. 1999; Vincent Naulleau et al. 2004). Tyto změny barvy kůže naznačují aktivaci imunitních buněk proti antigenu, který je společný melanomovým buňkám a normálním melanocytům. SR je velmi dynamický proces, během kterého se melanomové buňky postupně ničí a nádorová tkáň je nahrazena fibrózní tkání. Zdá se, že věk 10 týdnů je zlomem v přechodu mezi počáteční fází růstu melanomu a následnou fází spontánní regrese u MeLiM selat (Planska et al. 2015; Planska et al. 2018). Spontánní regrese nenastává synchronně u všech melanomů. Její trvání závisí na počtu a velikosti melanomů. Celý proces spontánní regrese je obvykle ukončen ve věku 6-12 měsíců (Horak et al. 2019).



Obrázek 4: a) černý MeLiM kanec po spontánní regresi melanomu bez jakýchkoli změn pigmentace (věk tři roky); b) původně černá MeLiM prasnice (věk čtyři roky) po spontánní regresi melanomu (s téměř úplnou depigmentací) společně se selaty ve věku tří týdnů; c) MeLiM sele s kožními melanomy (stáří šest týdnů); d) MeLiM sele s velkými nodulárními melanomy s lokální nekrózou a začínající kachexií (stáří sedm týdnů). Zvětšená cervikální lymfatická uzlina je označena šipkou; e) velmi zvětšená inguinální lymfatická uzlina zcela infiltrovaná metastazujícími melanomovými buňkami; f) pitva MeLiM selete (věk čtyři týdny). Četné melanomové metastázy ve viscerálních orgánech (plíce, játra, žaludek a slezina) jasně dokumentují maligní charakteristiku melanomu u MeLiM prasat. (Převzato z: Horak et al. 2019)

3 Hypotézy

H1: Specifická subpopulace T lymfocytů se liší v různých fázích spontánní regrese melanomu u MeLiM prasat

H2: Jednotlivé hematologické parametry se liší v různých fázích spontánní regrese a progrese melanomu u MeLiM prasat

H3: Melanom a zdravá kůže se liší svým mikrobiomem u MeLiM prasat

4 Cíle práce

C1: Ověřit, že se specifická subpopulace $CD4^+CD8^+$ T lymfocytů liší mezi progredujícími a regredujícícmi MeLiM prasaty a zároveň, že se neliší mezi jednotlivými nádory na jednom zvířeti

C2: Změřit hematologické parametry u progredujících a regredujících MeLiM prasat a stanovit, které parametry se v závislosti na skupině zvířat od sebe liší

C3: Stanovit mikrobiální profil melanomu a zdravé kůže u MeLiM prasat

5 Výsledky a diskuze

5.1.1 Identifikace specifické subpopulace $CD4^+CD8^+$ T lymfocytů a jejích rozdílů mezi progredujícími a regredujícícmi MeLiM prasaty

Cílem práce bylo identifikovat specifické imunitní buňky v periferní krvi a melanomech, které se liší mezi progredujícími a regredujícími MeLiM prasaty.

U MeLiM prasat byl prokázán v periferní krvi (Melanoma-Associated T lymphocytes, MATL) i melanomech (Tumor Infiltrating Lymphocytes, TIL) výskyt specifických $CD4^+CD8^{high}$ T lymfocytů (DP). Počet MATL se v prvních dvou měsících života neliší mezi MeLiM a kontrolními bílými prasaty, avšak jejich počet se významně zvýší, jakmile dojde k regresi melanomů. Od věku 6 měsíců tvoří MATL vždy významnou populaci, která se rovná populaci buněk $CD8^-$. Při bližší charakterizaci těchto buněk bylo zjištěno, že mají významně ($P<0,05$) nižší expresi CD3 ve srovnání s $CD8^-CD4^+$ buňkami. Dále jsou tyto buňky negativní pro CD45RA, CD45RC, MHC-II (SLA-DQ) a pozitivní pro CD5. Tento imunofenotyp a časový výskyt DP je podobný i pro TIL, jedinou výjimkou je SLA-DQ, kdy v nádoru byly přítomny $SLA\text{-}DQ^+$ TIL DP. Spektratypingová analýza DP TIL CD4 ukázala prominentní 72 bp dlouhý pás v superrodině Vβ IV-VI, což ukazuje na přítomnost specifického klonu, který je rozšířen v důsledku regrese melanomu. Dále bylo v průběhu analýz objeveno, že část DP TIL izolovaná pouze z regredujících melanomů je pozitivní po značení protilátkou FQ4C6 (neznámá izoforma CD45 antigenu). Z tohoto důvodu byly $FQ4C6^{hi}$ DP buňky také podrobeny CDR3 spektratypingu, kdy bylo potvrzeno, že tyto buňky obsahovaly taktéž pouze prominentní 72 bp pás. Tento výsledek byl následně potvrzen klonováním a sekvenováním výsledného PCR produktu.

Výskyt periferních dvojitě pozitivních αβ T buněk je důsledkem zvláštnosti imunitního systému prasat, který vyplývá z permanentní exprese molekul CD8α po jejich aktivaci (Saalmuller et al. 2002). Periferní dvojitě pozitivní αβ T buňky mají tedy svůj původ v pomocných T lymfocytech, které se podílejí na imunitní paměti (Saalmuller et al. 1989; Zuckermann & Husmann 1996; Sinkora et al. 1998). Z tohoto pohledu je potřeba zmínit velmi důležitou úlohu T pomocných lymfocytů při regresi melanomu. Toto je v přímém vztahu s lidskými studiemi, které ukazují, že ačkoli jsou cytotoxické αβ T buňky pravděpodobně hlavními efektorovými T

buňkami během rejekce nádoru, kooperace s T pomocnými buňkami je nezbytná pro účinnou protinádorovou odezvu (Li et al. 2017). Expresní profil dvojitě pozitivních buněk dále podporuje tento závěr, neboť jsou CD45RA negativní, což je typické pro paměťové buňky a nikdy nemají vysokou expresi CD25, která je typická pro regulační T lymfocyty (Kaeser et al. 2008). Proto DP buňky považujeme za efektorové. Navíc exprese $CD8^{hi}$ na buňkách DP není tak vysoká jako u TIL, což dále podporuje závěr, že CD8β (který je specifický pro TIL) není na těchto buňkách exprimován (Saalmuller et al. 2002; Sinkora & Butler 2009). Výskyt DP buněk u MeLiM prasat naznačuje, že úloha DP při regresi melanomu není druhově specifická. Je důležité, že TIL izolované z různých prasat a odlišných melanomů z jednoho zvířete jsou téměř identické. Analýza $FQ4C6^{hi}$ TIL z regredujících prasat navíc odhalila mono-specifický TCR, kdy stejný TCR byl sdílen mezi jednotlivými prasaty. Tyto výsledky ukazují, že za regresi nádoru je odpovědných pouze několik (možná jeden) klon T lymfocytů.

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The role of αβ T-cells in spontaneous regression of melanoma tumors in swine

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Tumor regression

ABSTRACT

Using a porcine model, we describe Melanoma-Associated $CD4^+CD8^{hi}$ T-lymphocytes (MATL) in peripheral blood that increase during melanoma regression. These MATL possess the $CD4^+CD8^{hi}$ phenotype and they have their direct counterparts in Tumor Infiltrating Lymphocytes (TIL) isolated from melanoma loci. Both MATL and $CD4^+CD8^{hi}$ TIL have a similar expression of selected markers indicating that they represent effector/memory αβ T-cell subset. Moreover, although TIL also contain $CD4^-CD8^+$ T-cells, only $CD4^+CD8^{hi}$ TIL expand during melanoma regression. Importantly, TIL isolated from different pigs and different melanoma loci among the same pig have similar composition of CD4/CD8 subsets, indicating that the composition of the MATL and TIL compartment is identical. Analysis of sorted cells from regressing pigs revealed a unique MATL subpopulation with mono-specific T-cell receptor that was further analyzed by sequencing. These results indicate that pigs regressing melanomas possess a characteristic population of recirculating T-cells playing a role in tumor control and regression.

1. Introduction

Spontaneous regression of tumor is defined as a partial or complete disappearance in the absence of any treatment. This phenomenon has been detected more frequently in malignant melanomas in comparison with other solid tumors (Maio, 2012). Up to 50% of human primary melanomas undergo spontaneous regression but the regression of metastatic melanomas is rare as only 0.23% cases have been well documented since 1866 (Kalialis et al., 2009).

There are several animal models of spontaneously regressing tumors, one of which is Melanoma-bearing Libechov Minipig strain (MeLiM) (Fortyn et al., 1994). MeLiM is a unique model of hereditary malignant melanoma. There are two courses of disease. More prevalent include progression and metastasis period followed by spontaneous regression. In comparison to other spontaneously regressing models, some MeLiM continue with progression of disease (Fortyn et al., 1994; Horak et al., 1999). This unique feature enables the comparison of regressive and progressive courses.

Other features including histopathological, immunohistochemical, biochemical and molecular biological diagnoses show parallels with human melanomas (Horak et al., 1999; Vincent-Naulleau et al., 2004; Geffrotin et al., 2004). Tumors are usually multiple and nodular with exophytic growth, i.e. the most aggressive form of melanoma. They are localized randomly on all parts of body. Diameters range from 5 to 120 millimeters (Horak et al., 1999). The depth of penetration corresponds to level IV and V according to the Clark's classification for human melanomas (Hruban et al., 2004; Planska et al., 2015). Some melanomas have cracked surface and they can also bleed. Tumor cells are

Abbreviations: CDR3, Complementary Determining Region 3; DP, Double Positive; mAbs, monoclonal Antibodies; MATL, Melanoma-Associated $CD4^+CD8^{hi}$ T-Lymphocytes; MeLiM, Melanoma-bearing Libechov Minipig; PBL, Peripheral Blood Leukocytes; SP, Single Positive; TCR, T-Cell Receptor; TIL, Tumor Infiltrating Lymphocytes; WSB, Washing and Staining Buffer

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Table 1
Mouse anti-pig mAbs used in this study.

mAb	clone	isotype	origin
anti-CD3	PPT3	IgG1	H. Yang, Institute of Animal Health, Pirbright, UK
anti-CD3-FITC	BB23-8E6-8C8	IgG2a	BD Pharmingen, cat. no. 559582
anti-CD4	10.2H2	IgG2b	J. K. Lunney, Animal Parasitology Institute, Beltsville, MD
anti-CD4-PE	74-12-4	IgG2b	SouthernBiotech, cat. no. 4515-09
anti-CD5	b53b7	IgG1	M. D. Pescovitz, Indiana University, Indianapolis, IN, USA
anti-CD8α	76-2-11	IgG2a	M. D. Pescovitz, Indiana University, Indianapolis, IN, USA
anti-CD8α-APC	76-2-11	IgG2a	SouthernBiotech, cat. no. 4520-11
anti-CD25	K231-3B2	IgG1	C. R. Stokes & K. Haverson, University of Bristol, UK
anti-CD45 isoform	FQ4C6	IgG1	L. Dixon & C. Netherton, The Pirbright Institute, Pirbright, UK
anti-CD45RA	FG2F9	IgG1	H. Yang, Institute of Animal Health, Pirbright, UK
anti-CD45RC	MIL-5	IgG1	C. R. Stokes & K. Haverson, University of Bristol, UK
anti-CD90	5E10	IgG1	BD Pharmingen, cat. no. 555595
anti-SLA-DQ[a]	K274.3G8	IgG1	C. R. Stokes & K. Haverson, University of Bristol, UK

[a] SLA–DQ = Swine Leucocyte Antigen type DQ, MHC class II antigen.

often disseminated to the inguinal and cervical lymph nodes which are enlarged and black in the cross section. Numerous distant metastases are frequently established. These can be found predominantly in lungs, spleen and liver, and they are macroscopically visible. However, spontaneously regressing melanomas rebuilt tumor tissue into fibrous tissue during several months after the birth. Regression of melanomas is also associated with autoimmune vitiligo (skin depigmentation). Peak point for switch from growth to regression is the 10th week of age (Planska et al., 2015).

Regression is a consequence of the interaction between malignant tumor cells and the host immune system resulting in the replacement of tumor tissue by fibrosis, degenerated melanoma cells, melanophages and proliferating lymphocytes, and telangiectasia (Payette et al., 2009). It is believed that the spontaneous regression of melanomas is a result of an effective immune response of tumor infiltrating $CD4^+$ helper and $CD8^+$ cytotoxic αβ T-cells (Weide et al., 2012). Usage of T-cell activation substances was shown to prolong survival of melanoma patients, and adoptive T-cell therapies cause rejection of established melanomas (Li et al., 2017). Although $CD8^+$ cytotoxic T-cells were found to be the main effector T-cells, co-transfer with $CD4^+$ is essential for effective antitumor response (Li et al., 2017). However, underlying mechanisms are not well understood and complex information about the phenotype of lymphocytes that are important for regression of melanomas is missing. Here we report on the detailed phenotypic profile of αβ T-cells isolated from regressing MeLiM pigs, and analyze their T-cell receptor (TCR) repertoire to show that a subpopulation of $CD4^+CD8^+$ double-positive (DP) T-cells with mono-specific TCRβ is overrepresented during and early after regression. Mature peripheral DP T-cells represent a substantial lymphocyte subset in swine (Saalmuller et al., 1989). Their frequency in peripheral blood, lymphoid organs and tissues is associated with immune system stimulation and adaptive immune memory development after birth (Sinkora et al., 1998). Our results are in agreement with these findings and might be a useful clue for explaining the mechanism of melanoma regression in humans and help in diagnosis and treatment of this disease.

2. Materials and methods

2.1. Experimental animals

Animals used in the study were MeLiM breed pigs described earlier (Fortyn et al., 1994; Horak et al., 1999) and white control Minnesota/Gottingen miniature crossbred pigs bred in Libechov (Baxa et al., 2013). Thirty-nine MeLiM pigs with one or more skin melanoma and nine control white pigs were included. Ages of animals were 1 month–2.5 year, both males and females at different stages of melanoma progression or regression. This experimental study was performed in accordance with the Project of Experiments approved by the Central Animal Science Committee of the Czech Academy of Sciences, following the rules of the European Convention for the Care and Use of Laboratory Animals.

2.2. Sample collection and recovering of cells

Peripheral blood was collected into EDTA/K3 tubes (BD Vacutainer) and erythrocytes were removed using EasyLyse (Dako) according to manufacturer instructions. Acquired peripheral blood leukocytes (PBL) were washed twice in the Washing and Staining Buffer (WSB, 0.2% gelatin form cold water fish skin and 0.1 sodium azide in calcium-free isotonic phosphate buffered solution, pH 7.2; all chemicals from Sigma-Aldrich). Skin tumors were excised under systemic i.m. anesthesia using a combination of Zoletil 100 (tiletamine 2 mg/kg and zolazepam 2 mg/kg, Virbac), Narketan (ketamine 2 mg/kg, Vetoquinol) and Xylazine (2 mg/kg, Riemser Arzneimittel AG). Local anesthesia during excision of the tumor was done s.c. by 1% Mesocain (Zentiva). Skin wounds closure was done in two layers and disinfection cover was applied (Solutio Novikov, Fargon). Post-surgery i.m. analgesic included Vetalgin (30 mg/kg; Intervet International BV) in combination with antibiotic coverage and Betamox (15 mg/kg; Norbrook). Cells from tumors were isolated either by enzymatic tissue digestion (100 units/ml of type IV collagenase from Clostridium histoliticum; Sigma-Aldrich), or by mechanical dissociation using a fine stainless metal mesh after cutting the tumor into small pieces (about 0.5 cm). Comparison of enzymatic and mechanical isolation has shown that the latter isolation technique provided higher numbers of lymphocytes in tumor cell suspension. Therefore, mechanical isolation was selected as the superior approach and all data shown use this method. Dissociated tumor samples containing all debris were washed twice in WSB, resuspended in 1 ml of WSB and used for surface staining without further lymphocyte isolation to minimize redundant manipulation.

2.3. Immunophenotyping

Direct or indirect surface staining with pre-titrated mouse anti-pig primary monoclonal antibodies (mAbs) was used for immunophenotyping (Table 1). Briefly, cocktails of neat (indirect staining) or fluorochrome-conjugated mAbs were incubated with the cell suspension for 20 min at room temperature or on wet ice depending on whether or not sodium azide, which can interfere with cell integrity at room temperature, was present in cell suspensions. After two washes in WSB, directly stained samples were used for flow cytometry. Indirect staining continued by adding 100 µl of WSB containing 10% heat inactivated non-immune goat serum (Sigma-Aldrich) to block Fc-receptors. After 10 min, 100 µl of optimally titrated fluorochrome-conjugated secondary antibodies (anti-IgG1-FITC, anti-IgG2b-RPE, anti-IgG2a-APC) were added in appropriate combinations and the mix was

incubated for 10 min, washed twice in WSB, resuspended in 300 µl of WSB and kept on ice until further analysis. Indirect triple color immunophenotyping in pigs relies on human serum adsorbed anti-mouse isotype-specific goat polyclonal antibodies (Southern Biotech) that exert no inter-isotype reactivity as described elsewhere (Sinkora and Sinkorova, 2014).

2.4. Flow cytometry and sorting

Samples were acquired, analyzed and sorted on a standard FACSAria II cell sorter (Becton Dickinson) operated by FACSDiva™ 6.1.3 acquisition/analysis software. Cytometer Setup and Tracking module was used for sorter setup. Experiment standardization and compensation matrix stabilization was done according to manufacturer's instruction. Unstained pig PBL and fully stained multicolor samples were used for instrument settings optimization and single stained lymphocytes for spectral overlap of FITC, PE and APC elimination. Propidium iodide (Sigma-Aldrich) at the concentration of 0.1 µl/ml was used for live/dead cell discrimination by its infrared (730–810 nm) fluorescence. Samples isolated from tumor tissue were analyzed without any other purification after surface staining. We found attempts to remove melanin and debris from the samples difficult, time consuming and inefficient. Instead, only 1 g sedimentation of a tumor sample ready for flow cytometry was applied for 10 min. Supplemental Figs. 1A–B shows the difference between freshly suspended and 1 g sedimented sample. Under such conditions, reasonably low clog incidence was encountered and the samples could be analyzed relatively smoothly. Some problems with clogging occurred in the sample line as melanin particles stick to the tubing material. However, short (5 s) flushing back of the sample line has always allowed us to continue measuring the sample. The “Append data” option in the FACSDiva software allowed collection of a sufficient number of events for reasonable analysis even when sample line had to be flushed several times per sample. In any case, the presence of residual melanin in analyzed suspension interfered with light scattering and made standard lymphocyte identification in the forward scatter (FSC) versus side scatter (SSC) diagram quite difficult. We have thus decided to use SSC Height parameter instead of the generally recommended Peak Area, which has proven quite useful and made identification of lymphoid population easier (Supplemental Fig. 1, C-F).

2.5. Cell sorting

Candidate populations were sorted from PBL preparations based on scatter characteristics and surface marker selection. After doublet discrimination using both scatter detectors and gating for cell with lymphoid characteristics in the FSC-A/SSC-A diagram, 4-way sorting of four T-cell subsets with selected immunophenotype was performed and collected in eppendorf tubes prefilled with PBS. Immediately after sorting, which never lasted more than 10 min, the cells were spun at 400 g and pellets of 30–100 thousand sorted cells were dissolved in 5 µl of TRI Reagent (Sigma-Aldrich, St. Louis, MO) per thousand of cells. Cell homogenates were frozen for subsequent RNA isolation.

2.6. RNA isolation, PCR amplification and detection of transcripts

In a particular experiment, only the same amount of sorted cells dissolved in TRI Reagent was used for isolation of total RNA according to a protocol recommended by the manufacturer. Total cDNA was prepared using random hexamer primers (Invitrogene, Carlsbad, CA). Previous studies showed that porcine TCRβ uses 19 groups of Vβ genes in seven families (VβI – VβVII) (Butler et al., 2005). According to similarities in the Vβ leader sequences, these seven Vβ families were grouped into three Vβ superfamilies, i.e. (1) VβI - VβIII, (2) VβIV - VβVI and (3) VβVII. Recent studies and complete genomic sequencing confirmed the existence of ten Vβ genes, seven of which are expressed (Eguchi-Ogawa et al., 2009), corresponding to earlier identified Vβ families. Analyses of the expressed repertoire revealed that usage of Vβ superfamilies in αβ T-cells corresponds to ~20% for VβI - VβIII families, ~50% for VβIV - VβVI families and ~30% for VβVII family (Butler et al., 2005). For these reasons, each cDNA preparation was amplified in three concurrent analyses for one of three Vβ superfamilies (VβI - VβIII families, VβIV - VβVI families and VβVII family) (Butler et al., 2005, 2011; Butler and Sinkora, 2013). The 1st round PCR targeted the original cDNA preparation while the 2nd round PCR targeted the 1st round PCR products. All primers and PCR conditions used for amplifications were published earlier (Sinkora et al., 2014). The 3rd round PCR is discussed in section 2.7.

2.7. Complementary determining region 3 (CDR3) spectratyping

The diversity in the αβ T-cell repertoires is overwhelmingly determined by the diversity in the CDR3 region of TCR (TCRBV-CDR3) (Sinkora et al., 2007, 2017; Stepanova and Sinkora, 2012). Thus, length analyses of CDR3 regions using polyacrylamide sequencing gels provides a clonotypic analysis of porcine αβ T-cells showing their level of clonality (Holtmeier et al., 2004; Sinkora et al., 2013). Technically, the second round of PCR products for each Vβ superfamily was subjected to the 3rd round of PCR that involved incorporation of radioactive adenosin 5′-ATP γ-^{32}P triphosphate nucleotide labeled Cβ primer (Sinkora and Sinkorova, 2014). The products were separated on sequencing gels that were subsequently dried and their radioactive images were obtained by Storage Phosphor Screens BAS-IP MS scanned in fluorescent Image Analyser FLA-7000 (Fujifilm corporation, Tokyo, Japan).

2.8. Cloning and sequencing

Cloning was performed as described previously (Sinkora et al., 2000). Briefly, PCR amplified and purified Vβ products were re-amplified using Pfu-polymerase and isolated fragments were cloned into EcoRV digested pBluescript II SK phagemids by T4 DNA ligase blunt-end ligation (all chemicals New England Biolabs, Ipswich, MA). The ligation mixture was used to transform DH5α competent cells (Invitrogene, Carlsbad, CA). Clones containing DNA fragments of right size after XhoI/XbaI restriction digest were sequenced by commercial Sanger sequencing (SEQme, Dobris, Czech Republic).

2.9. Statistical analyses

Differences among the experimental values were analyzed by one way analysis of variance (ANOVA), Tukey's multiple comparisons test using GraphPad Prism 6 software (GraphPad Software, San Diego, CA).

3. Results

3.1. Identification of melanoma-associated double-positive T-lymphocytes (MATL) in peripheral blood

In agreement with previous findings (Saalmuller et al., 1989; Sinkora et al., 1998), PBL of melanoma-free individuals from the MeLiM breed showed increasing frequency of mature peripheral DP T-cells with age. While less than 14% of CD4$^+$ T-cells co-express the CD8 antigen on their surface in the 6-week old piglet (Fig. 1A), 30% of CD4$^+$ T-cells are DP at the age of 8 months (Fig. 1B). In the first two months of life, CD4 and CD8 expression profile does not significantly differ in MeLiM from that in their age-matched healthy control animals (see below). However, long-term screening of PBL has revealed different CD4/CD8 expression profile during the phase of massive melanoma regression and later on, when anti-melanoma/melanocyte immune response develops and skin vitiligo progresses. Fig. 1C and D demonstrate the difference between DP T-cells from age-matched melanoma-free control pigs and tumor-regressing pigs respectively, and show a clearly

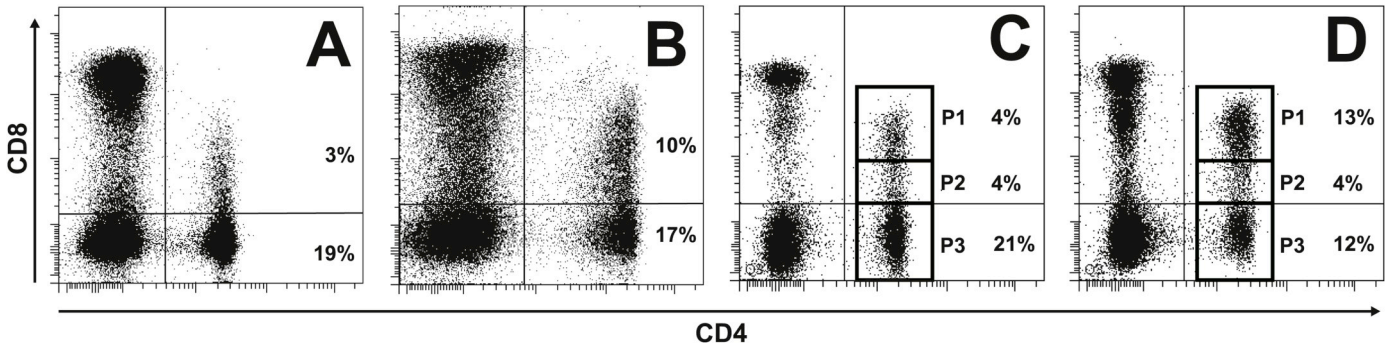

Fig. 1. Representative analyses of CD4 versus CD8 expression profile in different pigs. Lymphocytes identified by scatter characteristics were analyzed in a melanoma free MeLiM pig at the age of 6 weeks (**A**) and 8 months (**B**). The proportion of DP T-cells within the $CD4^+$ compartment was calculated using quadrant statistics. Within the lymphoid compartment, the percentage of DP T-cells (upper right quadrant) was related to the percentage of all $CD4^+$ T-cells (the sum of upper right and lower right quadrant). Therefore, 13.6% (100% x 3/22) and 30% (100% x 12/40) of DP T-cells existed among $CD4^+$ T-cells at the age of 6 weeks and 8 months, respectively. The same approach was used for analyses of age-matched (8 months) control healthy animal (**C**) and melanoma bearing littermates in the later stages of tumor regression (**D**). Gating strategy is shown and distinguishes $CD8^{hi}$ DP T-cells (P1), $CD8^{lo}$ DP T-cells (P2) and $CD8^-$ T-cells (P3) among $CD4^+$ lymphocytes.

distinguishable population of DP T-cells (P1 in Fig. 1D). Such MATL bear higher amounts of CD8 on the surface. Quantification of MATL (P1) revealed that their ratio to other $CD4^+$ αβ T-cells (P2 + P3) was about 1 (Fig. 1D) while in melanoma-free control pigs stayed below 0.2.

3.2. Characteristics of peripheral MATL

In addition to their unique DP phenotype, MATL exert higher FSC values than other CD4/CD8 αβ T-cell subsets (Fig. 2B). In fact, MATL are the largest lymphocyte subset according to FSC values ($P < 0.05$ for P1 vs. P2, and $P < 0.01$ for P1 vs. P3). To further characterize peripheral MATL we have used triple color immunophenotyping using anti-CD4 and anti-CD8 mAb in combination with a panel of mAbs directed against other available lymphocyte surface markers (Fig. 2C–I). Only $CD4^+$ αβ T-cells were gated and further analyzed, and only pigs in the late stages of melanoma regression were included. In all ten pigs, MATL exert significantly ($P < 0.05$) lower CD3 expression than their $CD4^+$ single positive (SP) counterparts (Fig. 2C). Moreover, MATL were negative for both CD45 isoforms studied (Fig. 2D and E), CD5 expression was slightly higher than on $CD4^+$ SP cells (Fig. 2F) and MHC class II antigens type SLA-DQ were basically missing, although a tiny population of SLA-DQ^+ was always found on DP T-cells (Fig. 2G). However, due to MATL predominance in the DP compartment, infrequent SLA-DQ^+ cells probably represent different population. Interestingly, MATL appear to have bi-variant CD25 expression with about half of cells always being $CD25^{lo}$ (Fig. 2H). We detected high CD25 expression typical for Treg only within the DP population with lower CD8 expression and also within the $CD4^+$ SP population (Fig. 2H). We have also included Thy-1 antigen (CD90) into analysis, which showed some positivity in DP compartment. However, due to relatively small number of positive events it remains to be determined whether $CD90^+$ population belongs to MATL or represents a distinct DP T-cell population.

3.3. Frequencies of peripheral MATL

To bring more evidence that MATL may play a significant role in melanoma control and regression we have studied the proportion of circulating MATL and other $CD4^+$ αβ T-cell subpopulations to total $CD4^+$ cells at different age and spontaneous tumor regression stage (Fig. 3). Until the age of approximately 4 months, $CD8^{hi}$ MATL population is small (Fig. 3A) and $CD8^-$ population strongly prevails among $CD4^+$ T-cells in most of individuals: about 80% in 2-month old piglets and almost 70% at 4 months (Fig. 3C). Importantly, this period is characterized by a different onset of spontaneous regression, which is demonstrated by analysis of 3 months old piglets with earlier onset of spontaneous regression having about 40% of MATL in the periphery (Fig. 3A; white boxed). From the age of 6 months, when massive spontaneous melanoma regression occurs in all MeLiM pigs, MATL are always a considerable population and they equaled or even out-numbered the $CD8^-$ population.

3.4. Characterization of tumor infiltrating lymphocytes (TIL)

Knowing the phenotype of MATL in peripheral blood we asked a question whether or not a similar subset can be found among TIL. As described in Materials and Methods section, raw samples containing a high quantity of melanin were directly analyzed using cumulative gating on live cells, singlets and lymphocytes (Fig. 4A–C) using FSC-H/SSC-H parameters (Supplemental Fig. 1). The resulting CD4/CD8 analysis showed that TIL are mostly composed of DP and $CD8^+$ SP subsets (Fig. 4D). In some preparations, $CD4^+$ SP populations were basically missing. As we can never exclude the presence of contaminating non-lymphoid cells and debris, contribution of mentioned subsets ($CD8^+$ SP + DP + $CD4^+$ SP; Fig. 4D) was normalized to 100%.

The described approach of TIL identification was used for characterization of melanoma-positive piglets and three aged pigs (originally labeled M5, M6 and P118) that had regressing but still existing melanomas at different time points (Fig. 4E). In all samples, $CD8^+$ SP were always dominating over DP cells. However, chronological analysis revealed initial influx of $CD8^+$ SP after 4 months (compare piglets at 2 and 4 months with pigs M5 and M6 at 6 months) followed by expansion of DP cells. Thus, there is a clear trend of decreasing proportion of $CD8^+$ SP and increasing proportion of DP and $CD4^+$ SP cells with advanced age (Fig. 4E).

Analysis of addition cell surface markers expressed on $CD4^+$ TIL (Fig. 4F) showed quite similar immunophenotype compared to MATL (Fig. 2D–I). The vast majority of TIL DP cells are also CD45RA and CD45RC negative and CD5 positive. Similarly to MATL, about 50% of TIL DP cells are CD25 low and a minority of expresses CD90. The only difference between MATL and TIL DP cells was found in MHC class II antigen expression. While few if any MATL bear SLA-DQ (Fig. 2G), SLA-DQ^+ TIL DP cells can be found within the tumor tissue (Fig. 4F).

3.5. TIL isolated from different sources are similar

Similarity between MATL and TIL DP cells initiated a series of experiments analyzing TIL isolated from several MeLiM individuals with comparable melanoma regression. In these analyses, we acquired as many events as possible from each melanoma preparation to unambiguously show the differences. As shown in representative analysis for three pigs (Fig. 5A–C), the proportions of $CD8^+$ SP, DP and $CD4^+$ SP cells were very similar at the late stage of melanoma regression. These

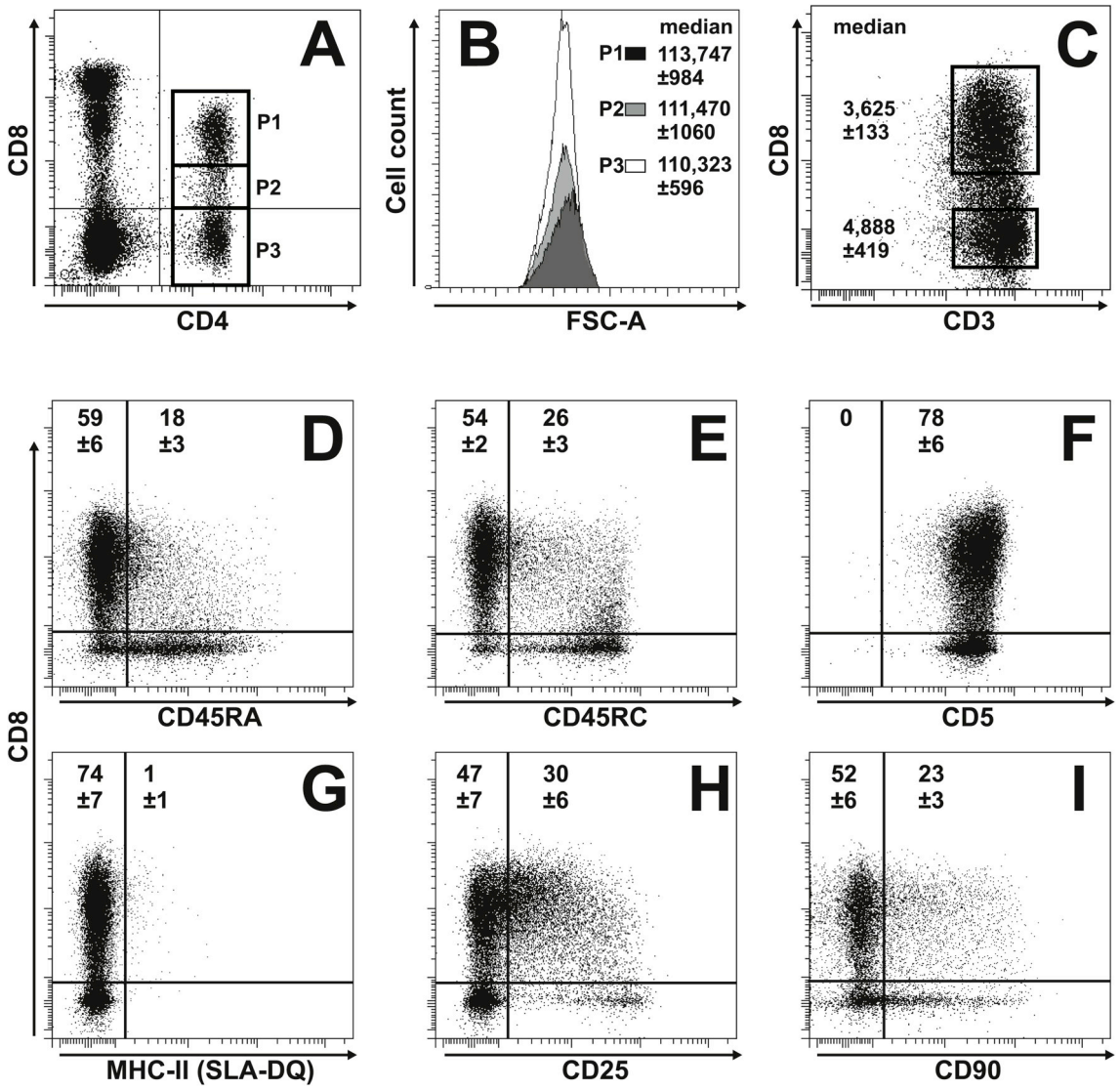

Fig. 2. Characteristics of peripheral MATL. Ten pigs in the late stages of melanoma regression were stained for CD4 and CD8 expression (**A**), and $CD4^+$ lymphocytes were gated into three subsets: $CD8^{hi}$ DP (population P1), $CD8^{lo}$ DP (population P2) and $CD8^-$ (population P3). Further analysis of their size (FSC-A) showed that $CD8^{hi}$ DP cells possess the largest diameter when compared to $CD8^{lo}$ DP and the smallest $CD8^-$ T-cells (**B**). Median FSC-A Peak Area is also indicated inside the dotplot with errors that represent the standard deviation. Note that median for P1 is significantly different ($P = 0.05$ to 0.01) from P2 and P3, which are on the same level of significance. Analysis of $CD4^+$ T-lymhocytes (P1 + P2 + P3) also showed that cells with the highest CD8 surface density (containing the MATL population) have slightly lower but significantly different ($P < 0.05$) CD3 expression than $CD8^-$ subsets (**C**). The same analysis of only $CD4^+$ T-lymhocytes (P1 + P2 + P3) were done also for other lymphocyte markers that included CD45RA (**D**), CD45RC (**E**), CD5 (**F**), CD25 (**G**), MHC-II (**H**) and CD90 (**I**) in 8 months old pigs with melanoma. Note that MATL represent the population with the highest CD8 expression so that these cells can reliably be identified in individual expression profiles. Statistic for 3–4 pigs is shown in each plot and errors represent the standard deviation.

findings instigated another question of whether or not TIL composition differs among individual melanoma loci in the same pig of the late stage of spontaneous melanoma regression (Fig. 5D). Again, the proportions of $CD8^+$ SP, DP and $CD4^+$ SP cells were almost identical.

3.6. MATL with mono-specific TCRβ are overrepresented early after regression of melanoma in MeLiM pigs

The knowledge of Vβ genomic sequences (see section 2.6.) allows a screening for expressed TCR repertoire by CDR3 spectratyping (Sinkora et al., 2014). This approach was used in sorted subpopulations of PBL-derived αβ T-cells from MeLiM pigs with regressing melanomas (Fig. 6). Spectratypic CDR3 length analysis of $CD8^+$ SP (P1) and $CD8^-/CD8^{lo}$ subsets of $CD4^+$ lymphocytes (P2-P3) showed polyclonal distribution of TCRβ repertoire in all three Vβ superfamilies (Fig. 6J-L). This indicates no expansion of certain αβ T-cell clones. However, when $CD4^+CD8^{hi}$ TIL were examined (P4), a prominent 72 bp long band in Vβ IV-VI superfamily was discovered (Fig. 6K). This indicates the presence of specific clone that is expanded as a result of melanoma regression. Note that no such band was noticed in control animals or any animal before melanoma regression (Fig. 6K, P4 with asterisk). During a course of this study, we have accidentally discovered that a part of DP TIL isolated from pigs regressing melanomas stains in high density by FQ4C6 antibody, which possibly recognize unknown isoform of CD45 antigen (Fig. 6F). Such high expression was not observed before regression (Fig. 6I). For this reason, $FQ4C6^{lo}$ and $FQ4C6^{hi}$ DP cells were also sorted (Fig. 6G and H respectively) and subjected to CDR3 spectratyping (Fig. 6J-L). Interestingly, while $FQ4C6^{lo}$ DP cells (P5) displayed polyclonal distribution of TCRβ repertoire for Vβ IV-VI family (Fig. 6K) in which the previously detected prominent 72 bp band disappeared, $FQ4C6^{hi}$ DP cells (P6) contained only the prominent 72 bp band that was originally detected in the $CD4^+CD8^{hi}$ population (Fig. 6K).

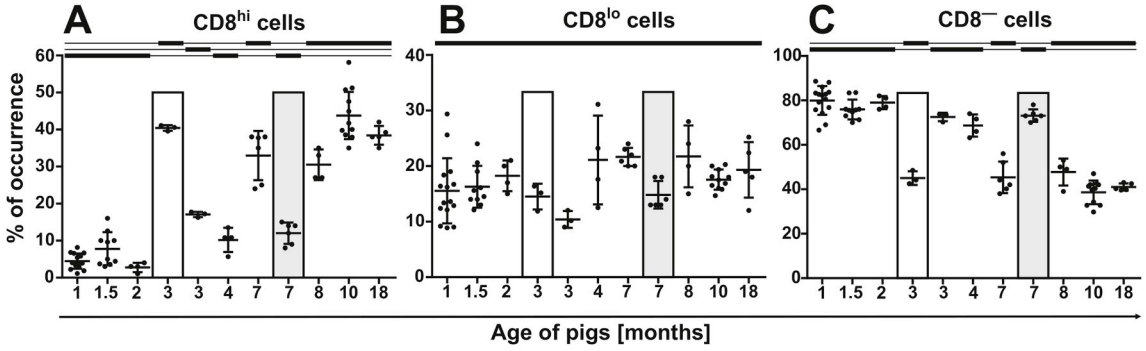

Fig. 3. The proportions of $CD8^{hi}$ (**A**), $CD8^{lo}$ (**B**) and $CD8^{-}$ (**C**) αβ T-cell subsets in the peripheral blood $CD4^{+}$ T-cell compartment during melanoma regression. The data have been normalized to 100% of $CD4^{+}$ T-cells. Each dot in the graphs represents an individual animal at given time (x-axis) and the mean for all animals in the same group is indicated by a long horizontal line. Error bars represent the standard deviation. Bold horizontal lines above graphs indicate groups of animals that show the same level of statistical significance (not statistically significant). A group of three MeLiM pigs with earlier onset of spontaneous melanoma regression at 3 months are white boxed. White pig control group of piglets at 7 months is gray boxed. After 6 months, melanoma associated increase of $CD8^{hi}$ DP T-cell numbers were studied in 3 MeLiM individuals designated M5, M6 and P118, and similar readouts from these piglets were grouped: 7 months = 6.5–7.5 months; 8 months = 8–8.5 months; 10 months = 10–12.5 months; 18 months = 18–20.5 months.

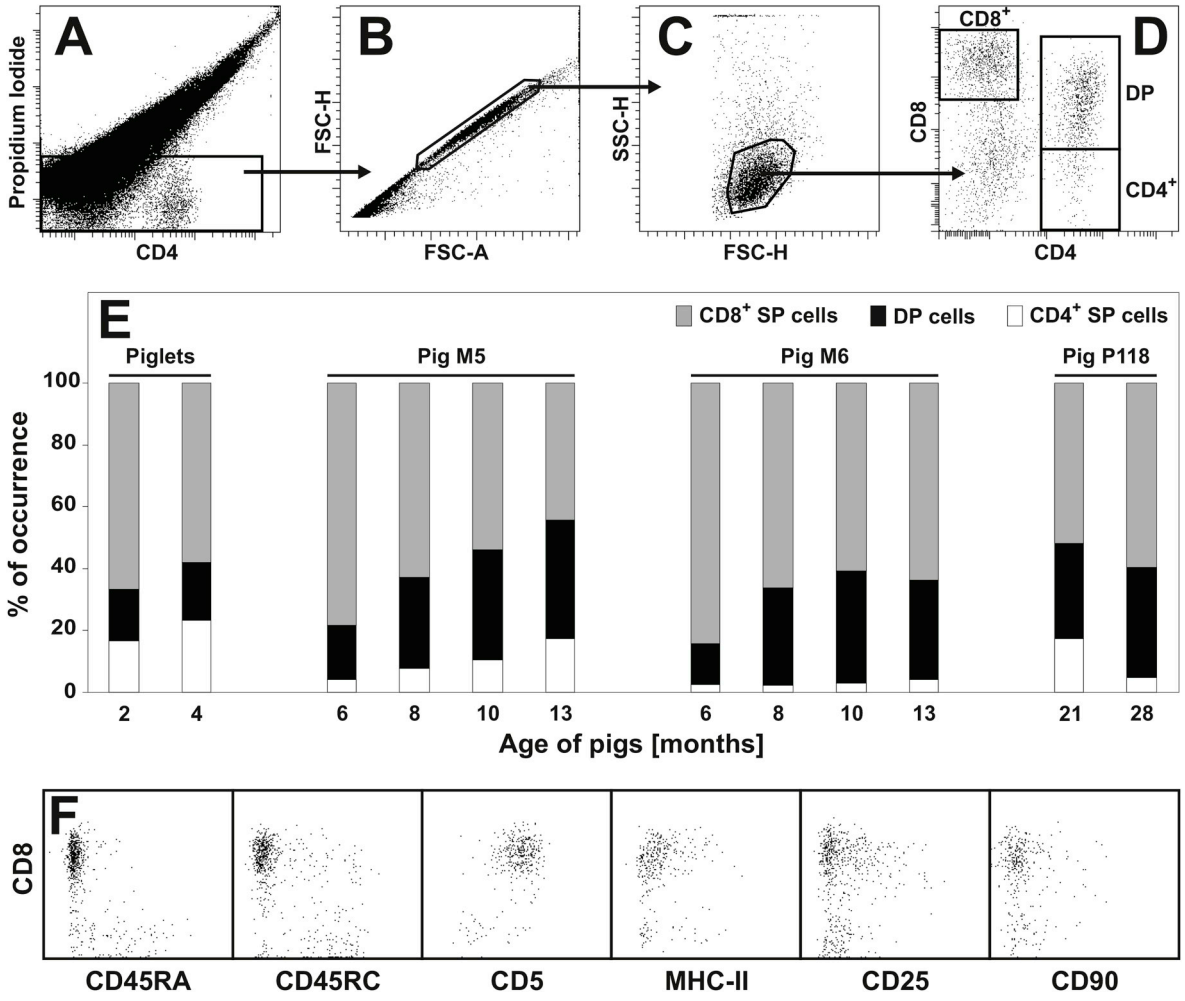

Fig. 4. Analysis of TIL during the course of melanoma regression. After mechanical tissue disintegration, washing and 1 g sedimentation, TIL were identified and analyzed by cumulative gating for live cells using propidium iodide exclusion (**A**), singlets excluding also melanin and debris (**B**) and lymphocytes (**C**). Resulting CD4/CD8 profile was used to analyze individual TIL subsets (**D**). Analyzed groups in different time points (**E**) included a group of young melanoma-positive piglets and three pigs (M5, M6 and P118) that had regressing but still existing melanomas. Note that data were normalized to 100% of $CD8^{+}$ SP + DP + $CD4^{+}$ SP cells to exclude differential contribution of other cell types and debris. Representative analysis of additional $CD4^{+}$ gated TIL phenotype using the same panel of markers as for MATL is also shown (**F**). This profile was comparable in all 6 pigs.

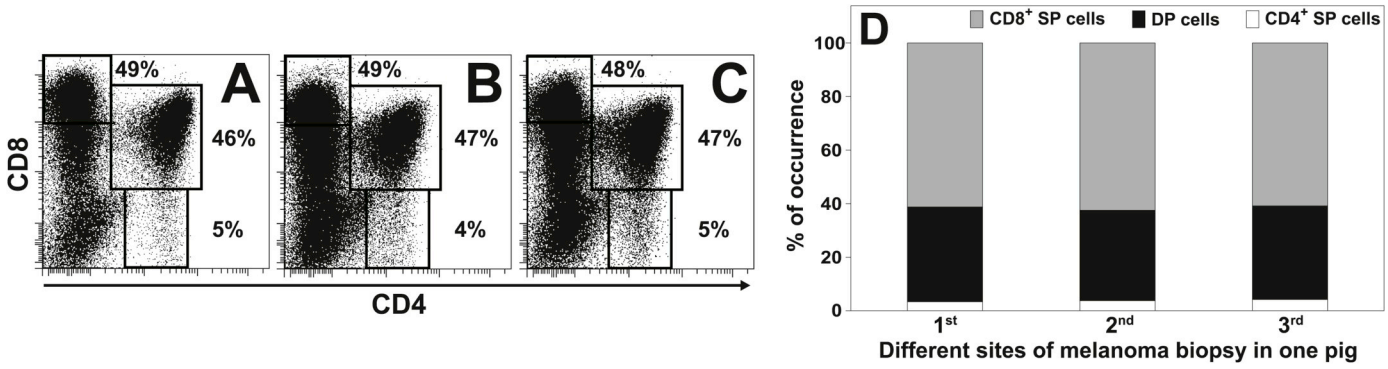

Fig. 5. TIL isolated from different sources at the late stage of tumor regression are similar. Flow cytometry analysis of TIL isolated from three different MeLiM pigs (**A-C**; 28 months old) in the late stage of melanoma regression. The proportions of three gated populations were normalized to 100% to exclude differential contribution of other cell types and debris. The same analysis was done for 3 different melanomas collected from one pig at the same time of late stage tumor regression (**D**). The data were also normalized to 100%.

3.7. Mono-specific FQ4C6^{hi} DP cells represent only one T-cell clone

The existence of only one prominent band in sorted FQ4C6^{hi} DP cells from regressing melanomas (Fig. 6K, population P6) may indicate that these MATL carry mono-specific TCRβ receptor. For this reason, we have cloned and sequenced the resulting PCR product. In all cases of recovered PCR clones, only one identical sequence has been retrieved (Fig. 7). The length of CDR3 region including J-segment (from 3′ end of V-region to 5′ end of C-region) was 72 bp, which is in agreement with previously detected prominent band (Fig. 6E). BLAST analysis showed 98% homology of V-region to the whole genome shotgun (GenBank: NC_010460.4). However, further screening showed 100% homology with previously identified clone DP-105 (GenBank: AY691127.1; Butler et al., 2005). We therefore conclude that the V-region does not contain

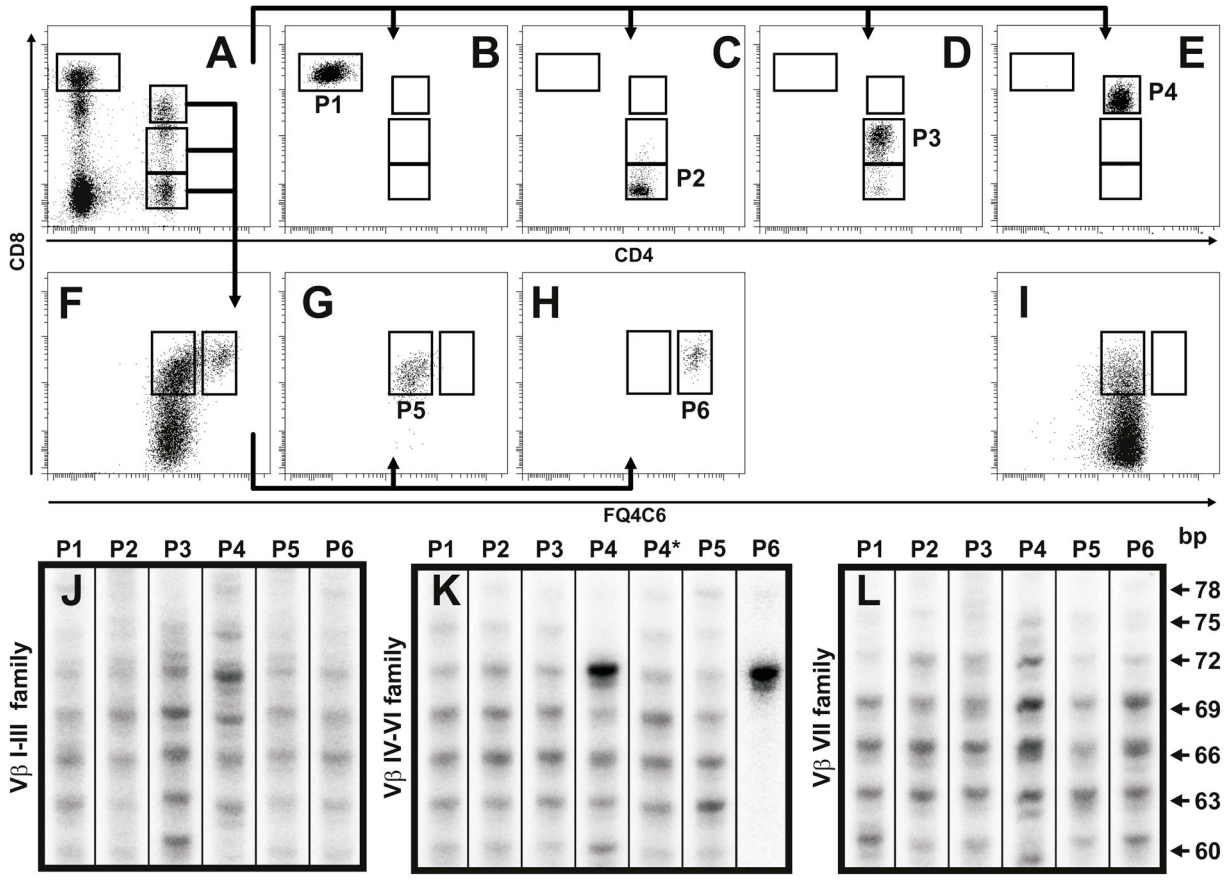

Fig. 6. Flow cytometry sorting and CDR3 spectratype of MATL. PBL from piglets with regressing melanomas was analyzed for CD4 and CD8 expression (**A**) and four populations (P1-P4) were sorted into individual tubes (reanalysis of sorted cells is shown in **B-E**). Gated CD4$^+$ cells were further divided according to staining with FQ4C6 antibody (**F**) and sorted for CD4$^+$CD8^{hi} FQ4C6^{lo} (**G**) and CD4$^+$CD8^{hi}FQ4C6^{hi} (**H**) populations (P5 and P6 respectively). The second sorting was included because of finding that population P6 is missing before melanoma regression (**I**). Each sorted population was thereafter examined by CDR3 length analysis (spectratype) of rearranged TCRβ in three concurrent analyses that include primers for VβI - VβIII families (**J**), VβIV - VβVI families (**K**) and VβVII family (**L**). Note that population P4 sorted from control animal with no signs of melanoma is indicated by asterisk. The results are representative of two independent experiments on two animals in which only the same amount of sorted cells was analyzed and the same number of amplification PCR cycles was used. Lengths of CDR3 including J-segment are indicated on extreme right and include number of base pairs (bp).

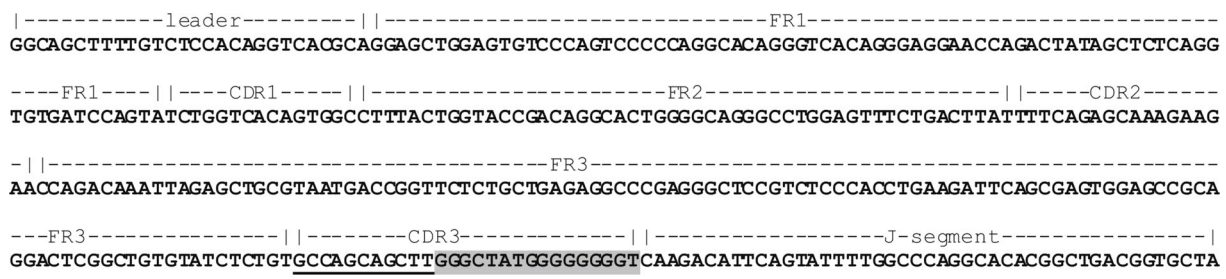

Fig. 7. The sequence of mono-specific TCRβ retrieved from sorted $CD4^+CD8^{hi}CD45R^-$ MATL. Individual frameworks (FR) and CDR portions are indicated above sequence. According to original nomenclature (Butler et al., 2005), the V-region belongs to the TRVB7 gene. Putative N-nucleotides (16 bp) are gray highlighted and D-segment (11 bp) is underlined. CDR3 has a length of 9 amino acids (27 bp) and J-segment (45 bp) is TRBJ3-3 gene (Eguchi-Ogawa et al., 2009).

any somatic mutation. An interesting feature is a longer CDR3 region containing 16 N-nucleotides with high G/C content (75%). As a result, translated CDR3 region is rich in Glycine with "GYGGG" motif.

4. Discussion

Melanoma regression in the MeLiM model is accompanied by increased occurrence of specific MATL that have their direct counterparts in $CD4^+CD8^{hi}$ TIL isolated from melanoma loci. The occurrence of peripheral DP αβ T-cells in swine is a result of porcine immune system peculiarity because the expression of CD8α molecules is permanent after activation (Saalmuller et al., 2002). Therefore, peripheral DP αβ T-cells are progenies of T-helper cells that form the effector/memory pool (Saalmuller et al., 1989; Zuckermann and Husmann, 1996; Sinkora et al., 1998). From this point of view, the current report shows highly relevant role of T-helper cells in melanoma regression. This is in direct correlation with human studies showing that although cytotoxic αβ T cells are probably the main effector T-cells in tumor rejection, co-transfer with T-helper cells is essential for effective antitumor response (Li et al., 2017). The expression profile of MATL and TIL DP cells further supports this conclusion as they are CD45RA negative, which is typical of memory cells, and they never carry CD25 high expression, which is typical for Treg cells (Kaser et al., 2008). Therefore, MATL are effector and non-regulatory T-cells. Moreover, the expression of $CD8^{hi}$ on MATL and TIL DP cells is not as high as for cytotoxic T cells (that can be classified as bright), which further supports the conclusion that CD8β (which is specific for cytotoxic T cells) is not expressed on these cells (Saalmuller et al., 2002; Sinkora and Butler, 2009). On the other hand, our unpublished results in human solid tumors reveal a similar population of DP T-cells. Further investigation showed that they may exert cytotoxic activity as they contain perforin and granzyme B. This would agree with the findings of Bagot et al. (1998) that show a clone of DP cells with $CD4^+/CD8^{dim}$ phenotype isolated from cutaneous infiltrate of patient with T-cell lymphoma. These cells were MHC I restricted and cytolytic against autologous tumor cells *in vitro*. Additionally, our unpublished results from human solid tumors showed that DP T-cells were uniformly negative for CD27, CD28, CD25 or CD127, which reflects their highly differentiated status and excludes them from the Treg compartment. In contrast, surface expression of CD56 and CD57 may suggest that they belong to the NKT cell population. Anti-CD45RO reactivity also indicates that this clinically underestimated subset may represent a differentiated population playing an important role in tumor control and regression. In any case, anti-tumor actions of DP αβ T-cells were also described in other tumor types (Overgaard et al., 2015) and also in cutaneous lesions caused by mycosis fungoides (De Marchi et al., 2014). Their presence was associated with a slightly slower progression of disease. Specifically for human malignant melanomas and their metastases, an increased numbers of DP cells were observed in about 60% of melanomas compared to peripheral blood (Desfrancois et al., 2010). High proportion of these cells produced TNF-α in response to autologous melanoma cells. They were also characterized by higher secretion of IL-13, IL-4 and IL-5 compared to SP cells (Desfrancois et al., 2010). All these findings prompt a further characterization of DP cells in tumor rejection. Our work strongly indicates that their role in melanoma regression is not species-specific.

An important finding of our studies is that TIL isolated from different pigs and different melanoma loci among the same pig are almost identical. This strongly suggests idea that the composition of the MATL and TIL compartment is extremely similar within the body during active anti-tumor immune response. Moreover, an analysis of sorted $FQ4C6^{hi}$ αβ T-lymphocytes from regressing pigs revealed a mono-specific T-cell receptor repertoire, and the same TCR was shared between individual pigs. Such results indicate that only a few (possibly one) T-cell clones are responsible for tumor regression. If this is the case, adoptive cell transfer therapy could be a very effective way of systemic cancer treatment. This is indeed demonstrated by several studies and probably explains why adoptive cell transfer is the most common therapy for melanomas (Melanoma Research Alliance website). However, none of trials targeted only αβ T-helper lymphocytes as a candidate and decisive cell population. In this way, TCR transducted T-helper cells would seem to be the best approach.

5. Conclusions

This report shows that peripheral melanoma-associated as well as tumor-infiltrating $CD4^+CD8^{hi}$ αβ T-lymphocytes are considerably involved in spontaneous regression of melanoma tumors in porcine model. Both populations share the similar expression profile between different pigs and different melanoma loci among the same pig, and possess mono-specific T-cell receptor. Such findings indicate that regressing melanomas possess a characteristic population of recirculating effector/memory αβ T-cells playing a role in tumor control and regression.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2018.10.001.

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5.1.2 Porovnání hematologických parametrů mezi progredujícími a regredujícími MeLiM prasaty

Cílem práce bylo změřit hematologické parametry u progredujících a regredujících skupin MeLiM prasat a stanovit, které parametry se v závislosti na skupině zvířat od sebe liší.

Skupina MeLiM prasat s progresí melanomu byla charakterizována černou barvou kůže i štětin bez vybělování, s exofyticky rostoucími melanomy, u nichž se někdy objevovala ulcerace, lokální nekróza a krvácení. Tato zvířata vykazovala nejnižší růstovou křivku, a dále nejnižší hodnoty v parametrech: počet červených krvinek (RBC), hematokrit (HCT), hemoglobin (HGB), a naopak se vyznačovala nejvyšším počtem krevních destiček (PLT). Další dvě skupiny MeLiM prasat se od sebe lišily přítomností vybělování v průběhu spontánní regrese melanomů, kdy zvířata s vybělováním měla mírně nižší růstovou křivku a v parametrech RBC, HCT a HGB se na začátku experimentu více blížily hodnotám progredujících prasat. Kontrolní skupina bílých prasat měla nejnižší hodnotu PLT, naopak skupina progredujících prasat měla hodnoty nejvyšší a od 6. do 11. týdne věku byl tento rozdíl statisticky významný ($P<0,05$). Zajímavým zjištěním bylo, že se skupiny významně nelišily v počtu bílých krvinek (WBC), což lze vysvětlit tím, že nebyly stanoveny diferenciální počty různých populací WBC. Koncentrace železa v plazmě byla u progredujících zvířat a u zvířat s depigmentací statisticky významně nižší ($P<0,05$) než u kontrolních zvířat, a však i u MeLiM prasat bez depigmentace byla výrazně snížena.

Celkový počet krevních destiček je hematologickým parametrem, který je ovlivněn u onkologických pacientů, kdy zvýšený počet krevních destiček je spojen s pokročilejší fází onemocnění a horší prognózou. Cytokiny trombocytů hrají významnou roli v progresi nádoru, tvorbě metastáz, angiogenezi a imunosupresi (Leblanc & Peyruchaud 2016; Plantureux et al. 2018). Zdravá selata vykazovala nejnižší hodnotu PLT v průběhu celého experimentu, přičemž nejvyšší hodnoty byly pozorovány u progredující skupiny P v ranně postnatálním období (ve věku od pěti do osmi týdnů), což odpovídá progresi melanomu. Počet PLT by tedy mohl být použit pro rozlišení selat MeLiM s progresí melanomu a spontánní regresí v časně postnatálním období života, kdy ještě nebyly pozorovány jiné rozdíly (např. nízká versus normální tělesná hmotnost, nízké versus vysoké RBC a HTC).

U nádorových onemocnění je dále často pozorován zvýšený počet WBC u pacientů. Tato leukocytóza související s nádorem je spojena se špatnou prognózou, včetně zvýšené mortality, morbidity, pooperačních komplikací, vyšší mírou selhání léčby, a kratší dobou přežití bez progrese u pacientů s karcinomem děložního čípku (Mabuchi 2011; So 2014), kolorektálním karcinomem (Moghadamyeghaneh 2015) a nemalobuněčným karcinomem plic (Holgersson 2012). U našich pokusných zvířat vykazovaly regredující skupiny nižší hodnoty WBC než u normálních bílých kontrolních prasat do 10. týdne věku. Progredující skupina překvapivě odhalila podobně vysokou hladinu WBC jako kontrolní skupina zdravých selat. Tento nález lze vysvětlit nedávným pozorováním u několika selat MeLiM s progresí melanomu pomocí pokročilého laserového systému Advia 120 Hematology System (Siemens Healthcare Diagnostics Inc.), který umožňuje stanovit diferenciální počty WBC. Zjistili jsme značně vysoký počet neutrofilů, který byl zodpovědný za vysoký počet WBC u těchto zvířat.

Anémie vzniklá nedostatečným množstvím železa v organismu je závažnou komplikací v současném chovu prasat, protože zásoby železa u novorozených selat jsou velmi omezené. Příjem železa mlékem není schopný dostatečně pokrýt požadavky selat, což znamená, že se u nich vyvíjí anémie během prvních dvou týdnů po narození. Doplnění železa pomocí intramuskulární injekce selatům krátce po porodu je běžně používanou technikou k překonání tohoto problému (Svoboda et al. 2017). V souladu s touto praxí byl všem selatům v experimentech aplikován dextran železa třetí den po narození. Tento postup byl účinný v kontrolní skupině a během sledovaného období zajišťoval stabilní koncentraci železa v krevní plazmě. Naopak všechny tři skupiny MeLiM vykazovaly snížené hladiny železa od začátku pozorování a tyto hladiny během experimentu dále klesaly. Tyto snížené koncentrace železa u prasat s melanomy odpovídaly nižším hodnotám HGB, HCT, MCV, MCH a RBC.

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Relationship between haematological profile and progression or spontaneous regression of melanoma in the Melanoma-bearing Libechov Minipigs

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ABSTRACT

Haematological parameters, plasma iron concentration, and bodyweight were monitored in Melanoma-bearing Libechov Minipigs (MeLiM) from 5 to 18 weeks old. Animals with melanoma progression (P group) and spontaneous regression (SR group) were compared.

The P group showed the lowest median values of red blood cell counts (RBC), haematocrit (HCT), haemoglobin concentration (HGB), and bodyweight, whereas the control white (tumour-free) pigs (C group) revealed the highest mean values of these parameters. The mean values of pigs with SR fell between the P and C groups. In addition, a stable concentration of plasma iron was found in the C group, while iron deficiency that increases with age was observed in the MeLiM groups. These results indicate that MeLiM are affected by cancer-related microcytic hypochromic anaemia. The lowest values of HGB, RBC, and HCT, together with the highest number of platelets (PLT) in the P group correspond to melanoma progression. Higher values of these parameters and lower PLT in the MeLiM pigs with SR reflected health improvement due to the destruction of melanoma cells during spontaneous regression. Monitoring of these haematological parameters can help distinguish MeLiM piglets with progression and spontaneous regression of melanoma in the early stages of postnatal development. The findings of this study correspond to findings in human patients in which cancer-related anaemia, thrombocytosis, and iron deficiency are often diagnosed.

Introduction

Melanocytes are naturally occurring cells in the epidermis that are responsible for the production of melanin, an endogenous pigment protecting the skin from harmful ultraviolet radiation (Dunki-Jacobs et al., 2013). Outside the skin, melanocytes also occur in other pigment-containing tissues. Melanomas that arise from malignant transformation of melanocytes can be divided into several subtypes in humans, according to their origin; the most common are cutaneous melanoma, mucosal melanoma, and uveal melanoma (Ali et al., 2013). Factors that influence the development of human melanoma include the degree of skin pigmentation, exposure of the skin to UV radiation (Gilchrest et al., 1999; Lea et al., 2007), and hereditary predisposition, given that about 10% of melanomas are hereditary (Rivers, 1996). The first choice for treating melanoma is surgical excision, followed by adjuvant chemotherapy and/or immunotherapy since melanoma is considered an immunogenic tumour (Haanen, 2013).

Spontaneous regression of various types of human tumours has been described in the literature, including melanoma. This phenomenon is characterised by the disappearance of melanoma cells without any therapeutic intervention. The specific mechanisms involved in spontaneous regression are not yet known in detail, but the influence of various factors, such as endocrine status, nutrition, genetic predisposition, the effect of infections, psychological interventions, and particularly the immune system, is assumed (Papac, 1996; Ricci and Cerchiari, 2010; Jessy, 2011; Cervinkova et al., 2017). Iron can also play an important role in this

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respect because its overload negatively influences both innate and adaptive immunity (Walker and Walker, 2000; Porto and De Sousa, 2007). Patients with hereditary haemochromatosis show extensive intestinal absorption of iron from food. This results in a pathological iron overload that decreases phagocytosis of monocytes and macrophages, decreases proliferative capacity, number and activity of T-helper cells and impairs generation of cytotoxic T lymphocytes compared to healthy controls. However, a high level of iron is essential for rapidly proliferating cancer cells, and iron depletion can selectively induce apoptosis in tumour cells (Richardson et al., 2009; Bystrom and Rivella, 2015).

Animal cancer models allow for detailed study and understanding of the biological processes associated with malignant transformation and tumour development. The Melanoma-bearing Libechov Minipig (MeLiM) is a useful melanoma model that was established at the Institute of Animal Physiology, Genetics (IAPG) of the Czech Academy of Sciences. MeLiM have a genetic predisposition to melanoma (Geffrotin et al., 2004; Hruban et al., 2004). Skin tumours (usually of the nodular type) occur in the early postnatal period in about half of all piglets (Horak et al., 1999). The tumours are very dark, almost black due to the very high content of melanin in melanosomes. An important feature of the MeLiM model is that the porcine tumours reveal the expression of enzymes (Borovansky et al., 2003), and proteins (Rambow et al., 2008) typical of human melanoma. In MeLiM with cutaneous melanomas, organ metastases are regularly observed, mainly in the spleen, lymph nodes, lungs, and about one-third of the affected pigs die due to melanoma progression (Fortyn et al., 1994; Horak et al., 1999)

A characteristic property of the MeLiM melanoma is its tendency to spontaneous regression, which is observed in about two-thirds of all tumour-bearing animals (Horak et al., 1999). Distinct macroscopic changes are observed related to spontaneous regression of MeLiM melanoma. The original dark exophytic skin tumours stop their growth and become flatter and greyish. Moreover, tumour regression is followed usually by skin and bristle depigmentation. These colour changes start around the tumours and then spread to surrounding parts of the body (Horak et al., 1999; Vincent-Naulleau et al., 2004). Therefore, the MeLiM model is unique, allowing the study of animals with both cancer progression and spontaneous regression. MeLiM piglets with melanoma progression or spontaneous regression need to be identified in the early postnatal period, because macroscopic changes do not occur until about 3 months of age, when the piglets with melanoma progression are dying.

The aim of this work was to monitor haematological parameters and iron concentration in MeLiM pigs from 5 to 18 weeks of postnatal life, and to look for differences in these parameters that could distinguish those with melanoma progression and spontaneous regression early in postnatal life.

Materials and methods

Animals

MeLiM piglets are usually entirely black at birth, but completely rusty individuals and those that are rusty with black or white spots occasionally appear. This variability in coat colour is caused by the crossing of differently coloured breeds and strains of pigs, when establishing the MeLiM model (Horak et al., 1999). In this study, only entirely black piglets with multiple skin melanomas were used. The study was approved by the Central Animal Science Committee of the Czech Academy of Sciences (Approval No. 173/2012; Approval date, 25 June 2012), following the rules of the European Convention for the Care and Use of Laboratory Animals.

The MeLiM piglets were housed in a farrowing pen together with the sow from birth until 12 weeks of age, and then restrained in separate pens. They were given unlimited access to water and fed with commercial mixtures, corresponding to their age. In accordance with veterinary practice in pig breeding, iron supplementation of all piglets was done via an intramuscular injection of 100 mg of iron dextran per animal (Ferribion; Bioveta, Czech Republic) on the third day of age. A total of 26 MeLiM piglets with multiple skin melanoma were included in this experiment. The piglets were weighed and the colour of their skin and bristles, as well as the colour and shape of their skin tumours, were monitored from 5 to 12 weeks old at weekly intervals, and finally at 18 weeks. All piglets were retrospectively categorised into three groups on the basis of ascertained changes in live weight, coat colour, and the appearance of skin tumours: P group (melanoma progression; $n = 7$), SR-d group (spontaneous regression of melanoma without depigmentation; $n = 9$) and SR + d group (spontaneous regression of melanoma with depigmentation; $n = 10$). Because there were no completely healthy (skin lesion-free) piglets in the MeLiM strain litters that were studied, white miniature pigs of the same age were used as controls (C group; $n = 8$). Control piglets were fed and iron was supplemented, in the same way as for the MeLiM piglets. This white strain of miniature pigs is derived from the same parental herd as the MeLiM strain (Hruban et al., 2004) and is kept at the IAPG Libechov for other experimental purposes.

Haematological parameters and plasma iron concentration

Samples of peripheral blood were collected from the external jugular vein into K3-EDTA tubes (Vacuette) and subsequently analysed by an automatic haematological veterinary analyser (ABC Vet, ABX Hematologie) to determine the following haematological parameters: the white blood cell count (WBC), red blood cell count (RBC), the concentration of haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and the number of platelets (PLT).

Plasma iron concentration was measured by differential pulse voltammetry via a three-electrode arrangement using a 797 VA Computrace (Metrohm) and an 889 IC Sample Center (Metrohm). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was used as the working electrode. The Ag/AgCl/KCl electrode was chosen as the reference electrode, and the Pt electrode served as the auxiliary electrode. The analysed samples were deoxygenated prior to being measured with argon (99.999%) for 120 s. After de-aeration, the iron ions were accumulated onto the surface of HMDE with the following settings: scan at −0.6 V to −1.3 V, deposition potential 0 V, accumulation time 0 s, pulse amplitude 0.05 V, pulse time 0.06 s, step potential 5.951 mV, time of step potential 0.1 s. The volume of the injected sample was 20 μL, and the volume of electrolyte in the electrochemical cell was 2400 μl. Iron ion concentration was detected in the presence of 0.1 M $KBrO_3$ containing 0.3 M NaOH and 0.01 M triethanolamine. The characteristic peak of iron ions was observed at the potential of −1.08 V.

Statistical analysis

Differences in haematological values and bodyweights between individual animal groups were analysed with Kruskal-Wallis tests using Statistica software (version 12, StatSoft). Post-hoc comparisons of mean ranks of all pairs of groups by multiple comparisons of mean ranks for all groups was computed as described by Siegel and Norman (1988). Differences were considered statistically significant at $P < 0.05$.

Results

MeLiM pigs with melanoma progression

Long-term monitoring of bodyweight, coat colour, and the appearance of skin tumours enabled the retrospective categorisation

of the experimental MeLiM piglets into three groups. The P group, featuring melanoma progression, was characterised by black, exophytically growing skin melanomas, which sometimes showed ulceration, local necrosis, and bleeding. No changes in bristles and skin colour were observed during the monitoring period. The weight of this group was the lowest out of all three MeLiM groups (Fig. 1). The animals in this group revealed growth retardation and emaciation that deepened with age. One piglet of the P group died due to melanoma progression and metastases at 10 weeks, and all other piglets had to be euthanased for ethical reasons at 12 weeks.

MeLiM pigs with spontaneous melanoma regression

Animals with SR were sorted into SR-d and SR + d groups, according to the extent of the preservation of bristle and skin pigmentation and/or their depigmentation, respectively. Initially, the skin melanomas of both the SR-d and SR + d groups were visually the same as in the P group. The first signs of spontaneous regression were observed around 10–12 weeks old, when the skin tumours began to flatten and become greyish. The skin and bristles were black without any depigmentation during the entire experiment in the SR-d group, while variable degrees of coat depigmentation were found in the SR + d group. This process accompanying the spontaneous regression in animals of this group was usually first observed in the vicinity of tumours, and the depigmentation then expanded to the surrounding areas of the body. The bodyweight of the SR + d group was slightly lower than for the SR-d group. The fastest growth and the highest weight gains and bodyweight were seen in the white pigs of the C group (Fig. 1).

Red blood cell parameters

Significant differences were observed between the control pigs (C group) and the MeLiM pigs with melanoma progression (P group). The C group revealed the highest number of RBC (Fig. 2) and the highest values of other parameters related to erythrocytes (HCT, HGB, MCV, MCH; Figs. 3–6). The P group showed the lowest values of most of these parameters. The values of the haematological parameters of both groups with spontaneous regression (SR-d, SR + d) ranged between the C and P groups, with the values of the SR + d group being closer to the P group. The differences among the groups for RBC, HCT, and HGB appeared at the beginning of the study (at 5 weeks old) but were not statistically significant. Despite the fluctuation in the medians of these parameters, it was possible to record their gradual increase during the monitored period in the C group. A pronounced elevation was also observed for RBC in both the SR-d and SR + d groups (reaching a similar level to the C group by the end of the experiment). The same tendency was observed in the P group up to 9 weeks old, but then RBC gradually decreased (until 12 weeks to almost the initial low value). This anaemia correlated with rapid cancer progression in these melanoma-bearing pigs. Unlike RBC, the other RBC-related parameters (HCT, HGB, MCV, and MCH) in all three MeLiM groups showed a significant decline until 7–9 weeks, after which they gradually increased, reaching almost normal levels in the SR-d and SR + d groups at 18 weeks. In the P group, a similar tendency was observed (until 12 weeks), but HCT and HGB showed a clear reduction between 11 and 12 weeks, correlating with marked anaemia at this age.

Platelets

In the C group, the lowest counts of PLT) were generally observed with a gradual slight decline from 5 to 18 weeks (Fig. 7). Initial PLT values of the SR-d and SR + d groups were similar to the C group; a moderate increase in the SR-d group until 7 weeks old was followed by a slow decrease until 18 weeks. The SR + d group exhibited generally higher levels of PLT than the SR-d group; the initial increase of PLT was very pronounced, and persisted until 9 weeks. This parameter then dropped, so the PLT count was only slightly higher at the end of the experiment than in the SR-d group. The P group revealed the most obvious thrombocytosis and the highest PLT from 5 to 8 weeks; thereafter (until 12 weeks), the PLT count was similar to that of the SR + d group.

Leukocytes

The number of WBC in the C group increased until 8 weeks, and then decreased slightly below the initial level (Fig. 8). These changes can be considered a normal postnatal developmental dynamic. Surprisingly, a similar trend was observed in the P group until 10 weeks, but unlike in the C group, the high level of WBC persisted. Both the SR-d and SR + d groups showed the lowest WBC at 5 weeks; then it increased, reaching roughly the normal level at the end of experiment.

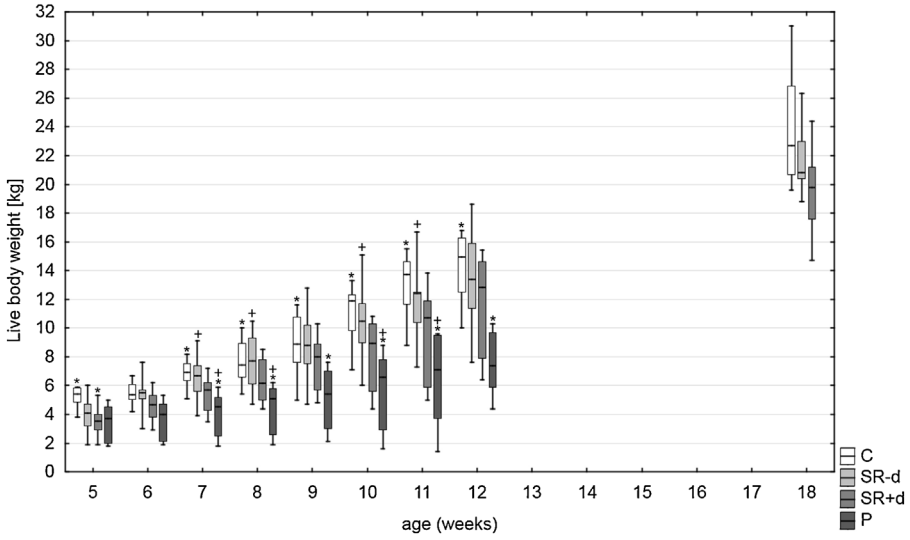

Fig. 1. Box plot for bodyweight. Bold lines indicate medians, box plots indicate 25th to 75th percentiles, and bars indicate minimum and maximum for all data. The asterisks and plus signs indicate statistical significance ($P < 0.05$).

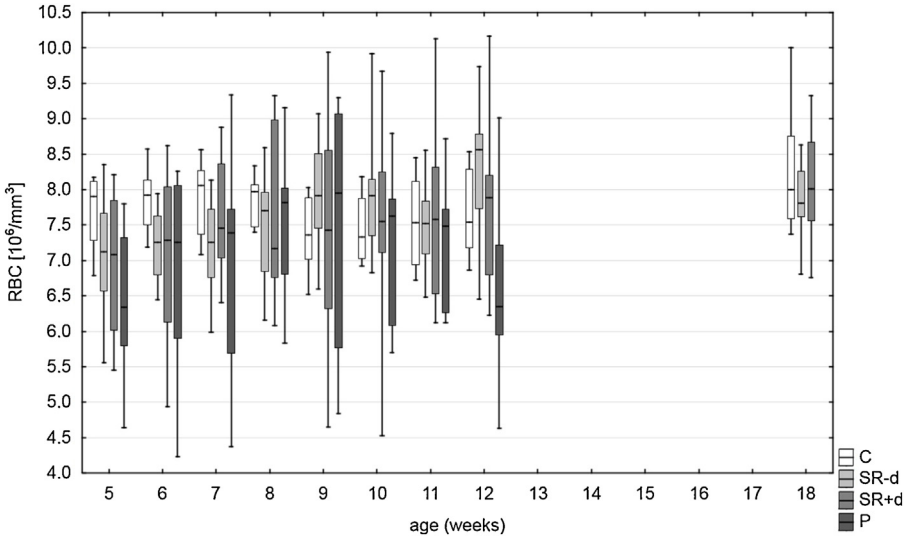

Fig. 2. Box plot for RBC. Bold lines indicate medians, box plots indicate 25th to 75th percentiles, and bars indicate minimum and maximum for all data. No statistically significant differences were observed for this parameter.

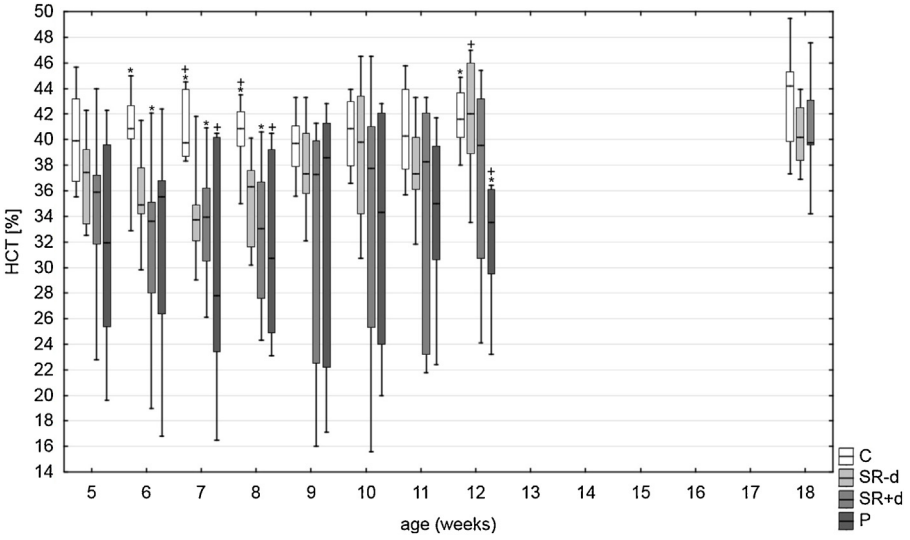

Fig. 3. Box plot for HCT. Bold lines indicate medians, box plots indicate 25th to 75th percentiles, and bars indicate minimum and maximum for all data. The asterisks and plus signs indicate statistical significance ($P < 0.05$).

Concentration of plasma iron

All three MeLiM groups had a similarly low concentration of plasma iron, which gradually declined during the monitored postnatal period to a very low level. However, the C group showed a stable and significantly higher iron level, in comparison to the MeLiM pigs (Fig. 9).

Discussion

Different haematological reference intervals are given in the literature for pigs that reflect the various breeds, ages, and bodyweights of the animals used for analysis, along with their health status, housing, and diet (Elbers et al., 1992; Klem et al., 2010; Cooper et al., 2014; Perri et al., 2017; Ventrella et al., 2017). Miniature pigs are clearly distinct from commercial pigs, mainly in their lower bodyweight and earlier sexual maturity. Moreover, the MeLiM strain is unique due to its cancer, which can modify the animals' haematological parameters, so we can expect reference intervals for the haematological parameters that differ from those of commercial pigs. For these reasons, we included the healthy miniature pigs (C group) in our experiment. The animals in this control group were of the same age and were housed and fed identically to the MeLiM groups. Changes in haematological parameters observed in the C group served as reference values, reflecting normal postnatal development. The values of all three MeLiM groups were compared with controls to reveal the effect of melanoma progression or spontaneous regression.

The monitoring of haematological parameters is an important part of the diagnosis and treatment of cancer diseases. A leukocytosis is often observed in cancer patients, and is associated with poor prognosis, including increased mortality, morbidity, postoperative complications, higher treatment failure rates, and shorter progression-free survival in patients with uterine cervical cancer (Mabuchi et al., 2011; So et al., 2014), colorectal cancer (Moghadamyeghaneh et al., 2015), and non-small cell lung cancer (Holgersson et al., 2012). In our experimental animals, the SR-d and

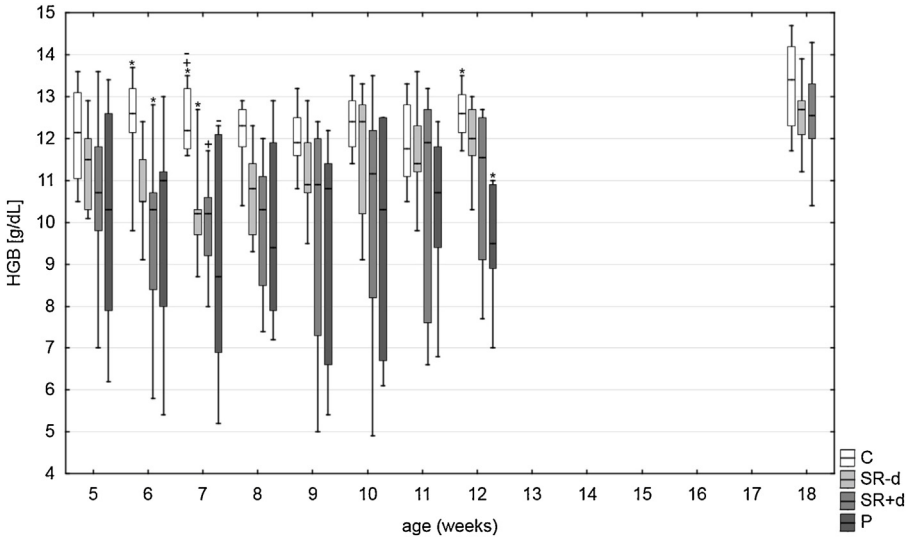

Fig. 4. Box plot for HGB. Bold lines indicate medians, box plots indicate 25th to 75th percentiles, and bars indicate minimum and maximum for all data. The asterisks and plus and minus signs indicate statistical significance ($P < 0.05$).

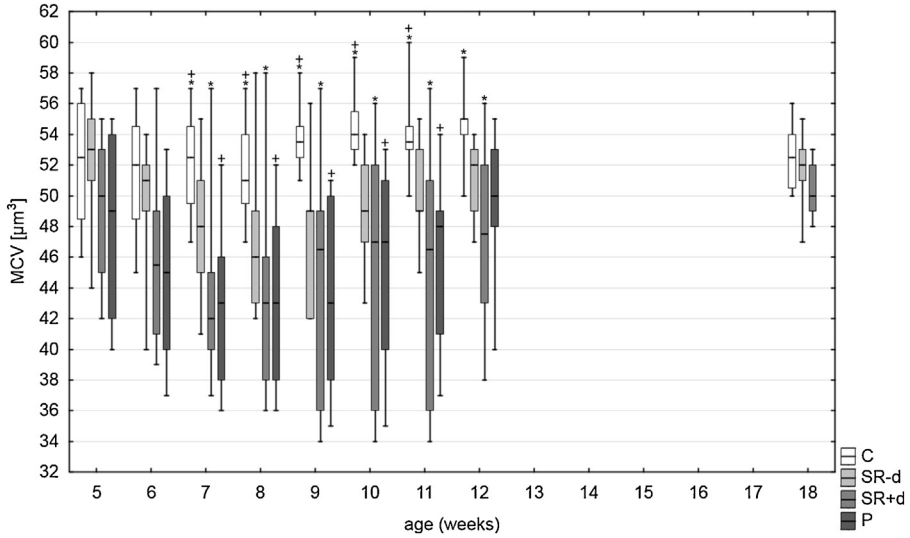

Fig. 5. Box plot for MCV. Bold lines indicate medians, box plots indicate 25th to 75th percentiles, and bars indicate minimum and maximum for all data. The asterisks and plus signs indicate statistical significance ($P < 0.05$).

SR + d groups showed only slightly lower (statistically non-significant) values of WBC than normal (melanoma-free) control pigs (the C group) until 10 weeks. Surprisingly, the P group (with the greatest cancer burden) revealed a similarly high level of WBC as the C group. This finding could be explained by our recent observation of several new MeLiM piglets with melanoma progression using the advanced laser-based Advia 120 Hematology System (Siemens Healthcare Diagnostics Inc.), which allows differential WBC counts to be determined. We ascertained a considerably high count of neutrophils that was responsible for the high number of WBC in these animals. This result is in agreement with studies of solid tumour patients, in which increased numbers of neutrophils have been observed in and linked to tumour progression and poor clinical outcomes, due to their tumour-promoting and immune-suppression effects (Dumitru et al., 2013; Ocana et al., 2017).

Platelet count is another haematological parameter that is affected in cancer patients. Thrombocytosis has been observed in patients with pancreatic cancer (Suzuki et al., 2004), ovarian cancer (Allensworth et al., 2013), colorectal cancer (Long et al., 2016), and gastric cancer (Pietrzyk et al., 2016). Elevated platelet counts are associated with a more advanced stage of the disease and a worse prognosis. Platelet cytokines play a significant role in tumour progression, metastasis, angiogenesis, and immunosuppression (Leblanc and Peyruchaud, 2016; Plantureux et al., 2018). Our results are in agreement with those observed in cancer patients. The C group (healthy piglets) showed the lowest PLT values throughout the experiment, while all MeLiM pigs revealed thrombocytosis. The highest numbers of PLT were observed in the P group in the early postnatal period (at 5–8 weeks of age), corresponding to melanoma progression. Also, both groups with SR exhibited increased PLT counts in comparison with the C group, but the values of PLT in the SR + d and SR-d group were lower than those in the P group. Thus, PLT count could be used to distinguish MeLiM piglets with melanoma progression and spontaneous regression early in postnatal life, when other differences (e.g. low versus normal bodyweight, low versus high RBC and HTC) are not yet observed. However, the automated PLT count may not

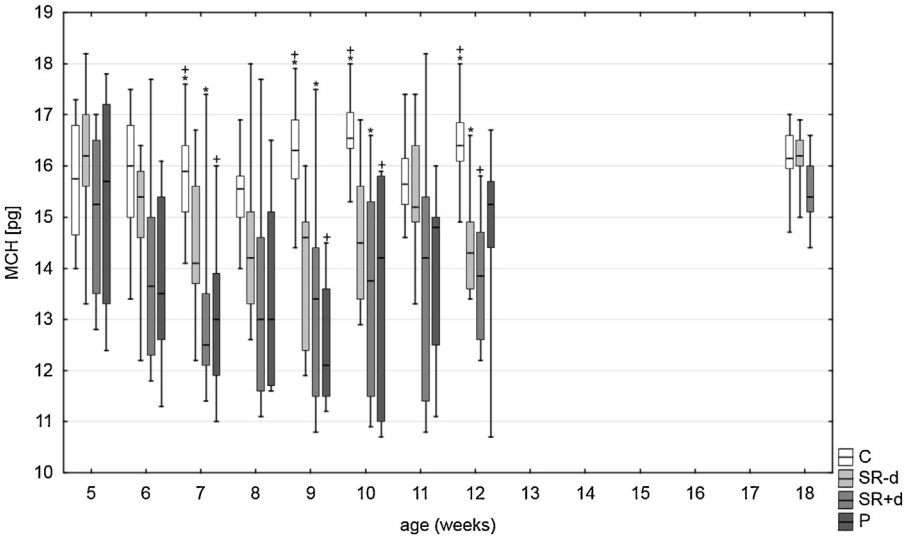

Fig. 6. Box plot for MCH. Bold lines indicate medians, box plots indicate 25th to 75th percentiles, and bars indicate minimum and maximum for all data. The asterisks and plus signs indicate statistical significance ($P < 0.05$).

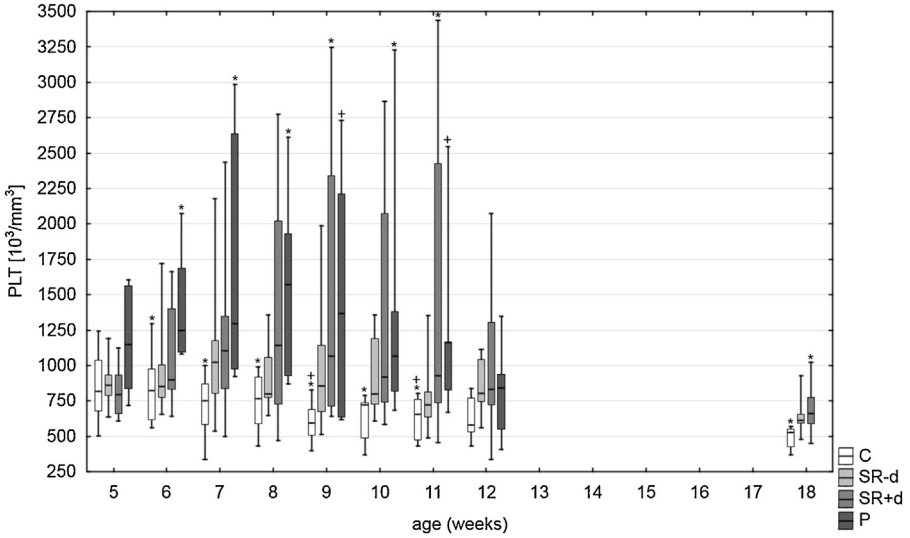

Fig. 7. Box plot for PLT. Bold lines indicate medians, box plots indicate 25th to 75th percentiles, and bars indicate minimum and maximum for all data. The asterisks and plus signs indicate statistical significance ($P < 0.05$).

express the exact PLT count due to EDTA-dependent pseudothrombocytopenia reported in humans (Lippi and Plebani, 2012) as well as in miniature pigs (Erkens et al., 2017). Because we did not evaluate blood smears in parallel with automatic haematological analyses, we cannot exclude PLT clumping artificially lowering automated platelet counts. Thus, the real PLT count could be higher than shown in this article. The presented PLT results should be verified in another study of the MeLiM model that would investigate this phenomenon, including microscopic evaluation of blood smears.

Iron is an essential element for normal body development. Iron-deficiency anaemia is a serious complication in contemporary indoor pig breeding because the iron reserve of newborn piglets is very limited, and their milk intake is insufficient to cover their requirements for iron, meaning that piglets develop anaemia during the first 2 weeks after birth. Thus, iron supplementation by intramuscular injection of iron dextran to suckling piglets shortly after birth is a commonly applied technique to overcome this problem (Svoboda et al., 2017). In accordance with this practice, all piglets in our experiment were injected with iron dextran on the third day after birth. This procedure was effective in the healthy C group and ensured stable iron concentrations in blood plasma throughout the monitored period. On the contrary, all three MeLiM groups showed decreased iron levels at the beginning of the observation, and these levels further declined during the experiment. These reduced iron concentrations in melanoma-bearing pigs corresponded to lower HGB, HCT, MCV, MCH, and RBC values in comparison with the C group. Because the melanoma-bearing pigs showed these parameters below the normal level (of the C group), we can conclude that the MeLiM piglets were affected by microcytic hypochromic anaemia that is likely cancer-related.

Paraneoplastic anaemia and iron deficiency are often found in cancer patients with various types of malignancies. Its severity correlates with the stage of cancer. Cancer-related anaemia is a multifactorial process associated with the increased inflammatory cytokines produced by various immune cells as a reaction of the immune system to tumour cells. Specifically, interleukin 6 (IL-6) induces secretion of the iron-regulatory hormone hepcidin, the

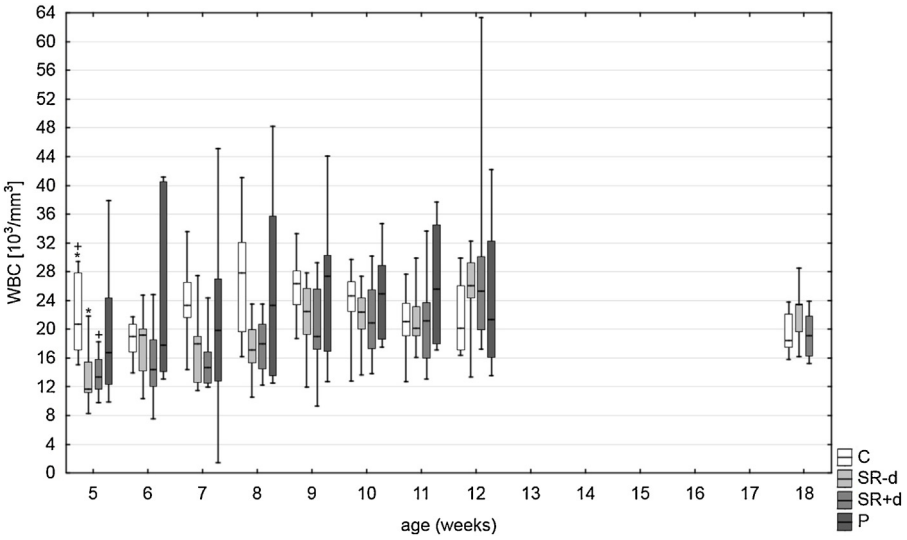

Fig. 8. Box plot for WBC. Bold lines indicate medians, box plots indicate 25th to 75th percentiles, and bars indicate minimum and maximum for all data. The asterisks and plus signs indicate statistical significance ($P < 0.05$).

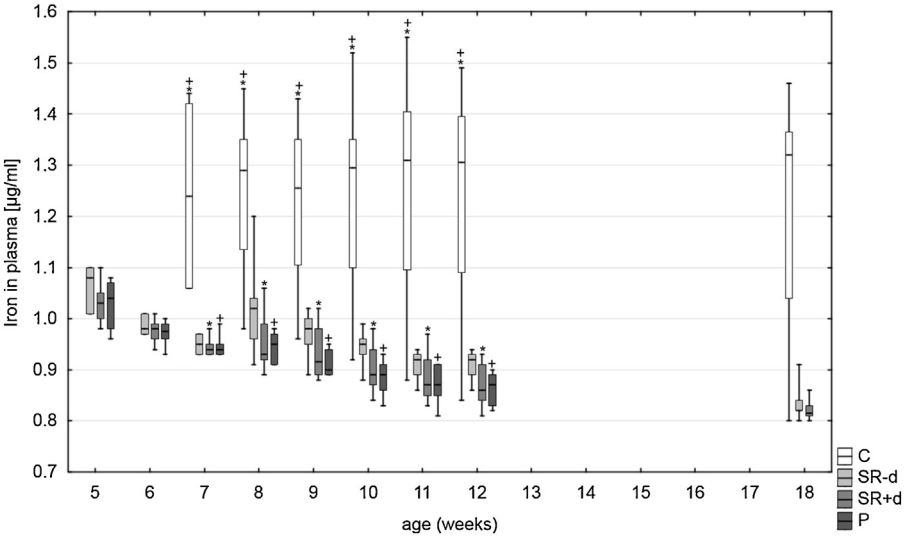

Fig. 9. Box plot for plasma iron. Bold lines indicate medians, box plots indicate 25th to 75th percentiles, and bars indicate minimum and maximum for all data. Asterisks and plus signs indicate statistical significance ($P < 0.05$).

key regulator of the iron homeostasis. Binding of hepcidin to its receptor, the cellular iron exporter ferroportin 1, blocks a release of iron from iron-storage cells such as tissue macrophages and jejunal enterocytes into the circulation, and the absorption of iron from the gut. These steps reduce the serum iron concentration and transferrin saturation, resulting in iron deficiency, iron-restricted erythropoiesis, and anaemia. Other cytokines produced by immune cells (such as tumour necrosis factor-α, interferon-γ, IL-10) also influence iron homeostasis and participate in the development of iron deficiency and anaemia in cancer patients (Weiss, 2005; Adamson, 2008; Ganz and Nemeth, 2009; Ludwig et al., 2013; Naoum, 2016; Pfeifhofer-Obermair et al., 2018). The iron deficiency (ID) related to inflammation and iron sequestration is referred to as functional ID, unlike absolute ID when body iron stores are depleted. Various methods (such as measurement of serum iron, cytokines, regulators of iron metabolism, and saturation of serum transferrin) can be used to diagnose ID (Brugnara, 2003; Polin et al., 2013). In our study, we detected only the concentration of plasma iron that showed decrease in all three MeLiM groups compared to the control group. Because we did not apply any other method, we cannot decide if ID is absolute or functional. Regardless, we can speculate that the observed ID may be associated with thrombocytosis, as demonstrated in patients with inflammatory bowel disease (Kulnigg-Dabsch et al., 2013; Voudoukis et al., 2013) and iron deficiency anaemia (Park et al., 2013).

In this study, the P group revealed the lowest values and the C group the highest values of the analysed haematological parameters at most monitored ages, with the exception of the PLT count, for which the opposite relationship between both groups was observed. The SR + d group showed values similar to, or slightly higher than, the P group, while the values of the SR-d group fell between the SR + d and C groups, or near to the C group. These differences between the groups (together with similar differences in bodyweight) were often statistically non-significant. The lowest values of RBC, HCT, and HGB in the P group correlated with melanoma progression and the greatest tumour burden in this group of animals. Higher values were observed in both MeLiM

groups with SR with lower tumour burden due to the destruction of melanoma cells during the process of spontaneous regression. We cannot explain the observed differences between the SR-d and SR + d groups in relation to the cancer. Since we were not able to monitor the number and size of metastases in internal organs, the extension of the cancer was assessed on the basis of skin melanomas and their macroscopic appearance alone. In this regard, we did not find any significant difference between the two groups. Therefore, we can only speculate that the SR + d pigs could be more affected by organ metastases than the SR-d pigs, and that the bristles and skin depigmentation, used as a criterion to distinguish between the animals of both groups, is a side effect of an anti-melanoma immune reaction arising in the affected pigs only at a higher cancer burden. Because this study did not show statistically significant differences in the analysed haematological parameters between individual groups of experimental animals, these results need to be verified in further studies with this model.

Conclusions

Basic haematological parameters, plasma iron, and bodyweight of MeLiM pigs with hereditary melanoma were influenced by cancer burden, similar to human patients. Tumour-related iron deficiency was observed in all MeLiM piglets. Pigs with melanoma progression had microcytic hypochromic anaemia indicated by decreased RBC, HCT, HGB, MCV, and MCH, and low bodyweight, together with increased PLT counts. Higher values for these haematological parameters and lower PLT were observed in pigs with spontaneous regression of melanoma, suggesting health improvements due to the destruction of melanoma cells during spontaneous regression. The observed differences between pigs with melanoma progression and those with spontaneous regression of melanoma, together with an evaluation of overall health status, bodyweight, and staging, could serve to distinguish them in the early stages of postnatal development. These data could potentially be used for the development of new melanoma therapies or detailed analyses of spontaneous regression.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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5.1.3 Stanovení mikrobiálního profilu melanomu a zdravé kůže u MeLiM prasat

Cílem práce bylo zjistit, jak se liší mikrobiom mezi melanomem a zdravou kůží u MeLiM prasat.

Při analýze mikrobiomu u MeLiM prasat byly nejčastěji detekovány bakterie z kmene *Firmicutes, Fusobacteria, Actinobacteria* a rodu *Lactobacillus, Staphylococcus, Streptococcus, Fusobacterium* a *Trueperella,* kdy jejich distribuce byla rozdílná mezi zdravou kůží a melanomem. Povrchový mikrobiom melanomu totiž vykazoval výrazně vyšší mikrobiální rozmanitost než zdravá kůže, což mohlo být částečně způsobeno ulcerací melanomu. Počet kopií 16S rRNA genu *Fusobacteria spp.* byl ve vzorcích melanomu vyšší ve srovnání se zdravou kůží. Též byl vyšší v melanomu progredujících zvířat. Počet *Fusobacterium necrophorum* se navíc zvyšoval s věkem u selat s progredujícím melanomem až do 10. týdne věku, měření ve 12. týdnu již nebylo relevantní, jelikož z pěti progredujících zvířat zůstala na živu pouze dvě. V melanomu byl dále zjištěn zvýšený počet *Staphylococcus hyicus*, *Staphylococcus chromogenes*, *Enterococcus faecalis* ve srovnání se zdravou kůží. V té naopak převažoval *Staphylococcus cohnii*. Rozdíly v zastoupení mikrobiomu mezi zdravou kůží a melanomem byly statisticky významné ($P<0,05$) a byly potvrzeny na základě několika analýz.

Výsledky obou studií ukázaly významný rozdíl v kožním mikrobiomu mezi různými místy stěru, a to mezi melanomem a zdravou kůží v jeho blízkosti. Hlavní kmen přítomný v mikrobiomu kůže u různých zvířat byl *Firmicutes,* kde *Staphylococcus, Streptococcus* a *Lactobacillus* byly dominantní rody. Mnoho studií naznačuje antiproliferativní účinky *Lactobacillus* v různých typech nádorů: karcinom prsu, nádory krku, kolorektální karcinom a nádory ústní dutiny (Motevaseli et al. 2016; Asoudeh-Fard et al. 2017; Cheng a kol. 2017; Jacouton et al. 2017; Marschalek et al. 2017; Motevaseli et al. 2017; Rasouli et al. 2017; Motevaseli et al. 2018; Yang et al. 2018). Dalším příkladem je *Staphylococcus epidermidis*, který produkuje protein 6-HAP. Ten přispívá k selektivnímu antiproliferativnímu účinku u transformovaných nádorových buněk, čímž brání hostitele proti růstu kožní neoplazie (Nakatsuji et al. 2018). Ve vzorcích melanomu byly přítomny kromě *Firmicutes* i *Fusobacteria* a *Actinobacteria*. Množství *F. nucleatum* v melanomu se zvyšovalo s věkem u progredujících zvířat a naopak se

snižovalo u regredujících zvířat, což naznačuje, že *Fusobacteria* podporují proliferaci nádoru. *Fusobacteria* jsou často spojována s kolorektálním karcinomem, karcinomem pankreatu a nádory dutiny ústní (Castellarin et al. 2012; Kostic et al. 2012; Flanagan et al. 2014; Gallimidi et al. 2015; Mitsuhashi et al. 2015; Holt & Cochrane 2017). Dále bylo ukázáno, že *F. nucleatum* je schopno podporovat proliferaci nádorů, protože nádory vázané na *F. nucleatum* jsou chráněny před útokem imunitních buněk a inhibují cytotoxické NK buňky prostřednictvím interakce Fusobakteriálního proteinu Fap2 s receptorem TIGIT. *F. nucleatum* je spojeno s různými nádory včetně melanomu (Gur et al. 2015). *Trueperella pyogenes* je oportunní patogen způsobující pyogenní infekci u prasat a jiných zvířat. Ve společné přítomnosti s některými rizikovými faktory vyvolává hnisavé léze a je schopna způsobit infekci u lidí (Zhao et al. 2013; Jarosz et al. 2014). *Streptokoky dysgalactiae subspecies equismilis* (SDSE) způsobují rozmanité povrchové a imunologicky zprostředkované onemocnění u lidí a též byly spojeny s těžkou hnisavou meningoencefalomyelitidou u prasete v Yorkshiru (Brandt et al. 2009; Kasuya et al. 2014). *Staphylococcus hyicus* je agens odpovědné za exsudační epidermitis způsobující mírné až těžké kožní léze u prasat (Andresen 2005; Park et al. 2013) kvůli své produkci exfoliativních toxinů některými kmeny. *S. hyicus* může způsobit sepse i u lidí (Casanova et al. 2011). Skutečnost, že *S. hyicus* dokáže způsobit kožní onemocnění, vyvolává myšlenku, že se může účastnit i progrese melanomu, ačkoli by bylo potřeba dalších experimentů pro ověření této hypotézy.

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ORIGINAL ARTICLE

Melanoma-related changes in skin microbiome

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Abstract

Melanoma is the least common form of skin tumor, but it is potentially the most dangerous and responsible for the majority of skin cancer deaths. We suggest that the skin microbiome might be changed during the progression of melanoma. The aim of this study is to compare the composition of the skin microbiota between different locations (skin and melanoma) of a MeLiM (Melanoma-bearing Libechov Minipig) pig model (exophytic melanoma). Ninety samples were used for PCR-DGGE analysis with primers specifically targeting the V3 region of the 16S rRNA gene. The profiles were used for cluster analysis by UPGMA and principal coordinate analysis PCoA and also to calculate the diversity index (Simpson index of diversity). By comparing the obtained results, we found that both bacterial composition and diversity were significantly different between the skin and melanoma microbiomes. The abundances of *Fusobacterium* and *Trueperella* genera were significantly increased in melanoma samples, suggesting a strong relationship between melanoma development and skin microbiome changes.

Introduction

The term microbiome refers to the community of microorganisms, including bacteria, archea, viruses, fungi, and eukaryotes living in or on a host body (Roy and Trinchieri 2017). Most microorganisms which cover the skin are harmless commensals. Many factors influence skin microbial diversity such as the environment, host gender, species, and genetics. It was demonstrated that altitude affects the skin microbiome of pigs and humans, and indicated that the skin microbiome adapts to extreme environmental conditions (Zeng et al. 2017). The composition and the complexity of the microbial community vary between different skin sites and depend to the physiological characteristics: local skin anatomy, lipid content, pH, sweat, and sebum secretion, which differentiate the skin into three categories: sebaceous (oily), moist, or dry (Grice et al. 2009; Byrd et al. 2018).

Various studies have shown that the skin microbiome plays an important role in human health and the health of other host species such as mice, dogs, fish, and amphibians (Grice et al. 2010; Nakatsuji et al. 2013; Smeekens et al. 2014; Rodrigues Hoffmann et al. 2014; Walke et al. 2014; Larsen et al. 2015). Indeed, it is responsible for providing a strong barrier effect which protects the skin against pathogenic bacteria (Sanford and Gallo 2013). In addition, it has been shown that germ-free mice are more vulnerable to skin infection (Naik et al. 2012). Several reports suggest a relationship between the composition of microbial communities in different human body habitats and disease (Dethlefsen et al. 2007).

The association between microbiota and cancer is a recent hypothesis. The microbiome is related to both cancer causation and the consequences of cancer treatment. The microbiota, particularly the gut microbiota, is able to modulate the response to cancer therapy such as chemotherapy, radiotherapy, and improve anticancer efficacy (Kaczor and Fabno 2017; Roy and Trinchieri 2017). However, only a few studies have considered the changes in the skin microbiome in skin cancer. One study focused on the characterization of the cutaneous microbiome of melanoma and melanocytic nevi, suggesting that the skin microbiome may not be a useful diagnostic tool

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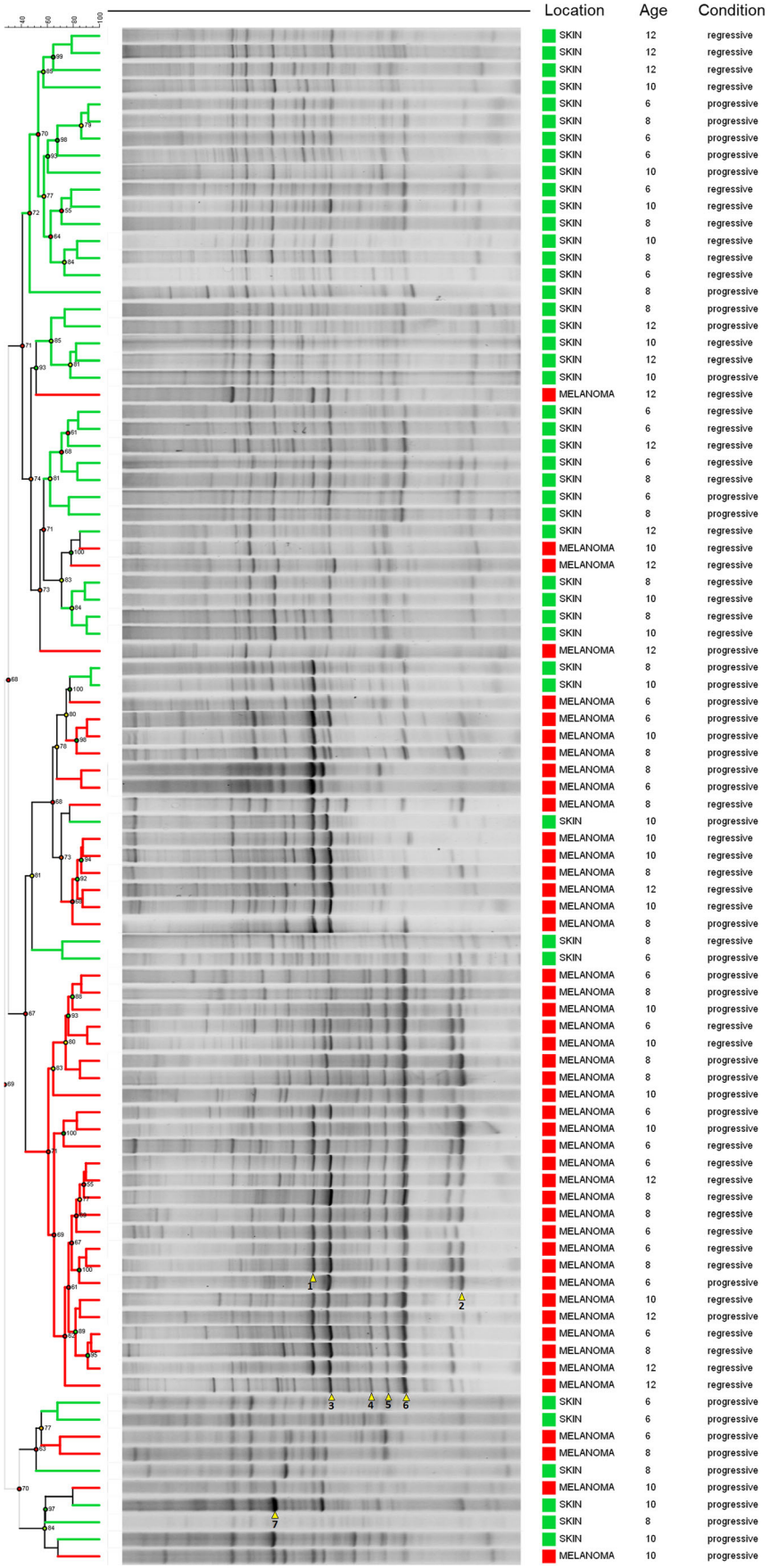
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for melanoma and melanocytic nevi (Salava et al. 2016). Another study showed that the skin microbiota composition was different in the cancer cachexia patient group from the healthy control group (Li et al. 2014).

Melanoma is a malignant melanocytic neoplasm with a high mortality and increasing incidence rates over the past few years. Four subtypes of melanoma are classically recognized using clinical and pathological criteria: superficial spreading, lentigo maligna, acral lentiginous, and nodular (McGovern et al. 1973; Balch et al. 2009). Polyploidy melanoma is considered to be an exophytic variant of the nodular form in which the bulk of the tumor is located above the surface of the skin, and it may be pedunculated or sessile (Cutler et al. 2000; Hikawa et al. 2014).

Pigs are considered to be the best model species for cutaneous studies due to the structural similarities of their skin to human skin. Little is known about the porcine skin microbiome. McIntyre et al. (2016) found that the pig skin bacteriome is similar to that of humans at the phylum level, and it largely consists of Firmicutes (55.6%), Bacteriodetes (20.8%), Actinobacteria (13.3%), and Proteobacteria (5.1%); however, there are differences between anatomical locations at the species level. The aim of this study was to estimate the relationship between the skin microbiome and melanoma development in the pig model. The proposed hypothesis is the existence of significant variability in the skin microbiome at the different locations (skin and melanoma) of the pig model MeLiM (Melanoma-bearing Libechov Minipig) with regressive and progressive disease development.

Materials and methods

Animals and tissue samples

Thirteen piglets were housed in the farrowing pen together with the sow from birth until the age of 12 weeks. They had an unlimited access to water and commercial feed mixtures according to their age. The melanoma and cutaneous scrapings ("melanoma" and "skin") were sampled using a sterile blade from the defined area (4 cm^2) of the back. The cutaneous scrapings were always obtained 5 cm from the surface of the selected melanoma. The samples were immediately placed into a test tube with an enzymatic lysis buffer and subsequently frozen at − 80 °C. Samples were collected four times from the same locations at 2-week intervals (weeks 6, 8, 10, and 12). Out of the 13 animals, eight developed the regressive form of the disease and five animals developed the progressive form of the disease and died before the end of the experiment.

◀ **Fig. 1** Dendrogram analysis of PCR-DGGE-derived microbial profiles. Numbers indicate bands identified by sequence analysis. 1: *Staphylococcus hyicus* (100% similarity), 2: *Trueperella pyogenes* (98.9%), 3: *Streptococcus dysgalactiae* (99.3%), 4, 5 and 6: *Fusobacterium necrophorum* (98.3%), 7: *Lactobacillus crispatus* (99%)

DNA isolation

Total DNA was isolated from tissue samples with a DNeasy PowerBiofilm Kit (Qiagen) according to the manufacturer's protocols. The disintegration step was performed with a FastPrep (MP Biologicals) device for 60 s at a maximum speed of 6.5 m/s. The elution was done with 50 μL of elution buffer.

PCR-DGGE analysis

Isolated DNA was used to prepare PCR amplicons of the V3 region of 16S rRNA to analyze the skin microbiome of two groups of pigs with regressive or progressive melanoma. One Taq PCR ready mix was used with primers and PCR conditions according to Muyzer et al. (1993). The PCR products were loaded onto 9.5% acrylamide gel with a denaturating gradient (35–60% urea), and the electrophoresis was run at 60 V for 18 h in a DCode Universal Mutation Detection System (Bio-Rad). The gels were stained in SYBR Green and visualized with a Gel Doc XL+ (Bio-Rad). The bands of interests were also cut from the gel; the DNA was eluted into 100 μL of dH_2O. The eluted DNA was reamplified, purified with a QIAquick PCR purification kit (Qiagen), and sequenced by a commercial service (SEQMe). The sequences were identified using the BLASTn tool (Madden et al. 1996).

Quantitative PCR

Specific primers were used to quantify the amount of Fusobacteria in each sample: FUSO1 (forward primer, 5′-GAG AGA GCT TTG CGT CC-3′) and FUSO2 (reverse primer, 5′-TGG GCG CTG AGG TTC GAC-3′) (adapted from Nagano et al. 2016) to target conserved regions of the 16S rRNA gene for *Fusobacterium spp.* In *F. nucleatum* (GenBank Accession Number AJ133496), the binding position of the FUSO1 primer is 212–228 and the FUSO2 primer binds to positions 821–804, flanking a fragment of 610 bp. The standard curve was generated by using the PCR product of the 16S gene of Fusobacteria using primers and PCR conditions according to Nagano et al. The PCR product was purified with a commercial kit (QIAquick PCR purification kit, QIAGEN), the concentration was measured with a NanoDrop spectrophotometer (Thermo Scientific), and the number of copies was calculated. Serial dilutions were prepared (from 10 to 10^6 copies of the gene).

The qPCR was performed in a 20-μL final volume consisting of 10 μL of EliZyme qPCR Mix (Elisabeth Pharmacon), 0.8 μL of 10 μmol/L forward and reverse primers, 7.4 μL of distilled water, and 1 μL of DNA template.

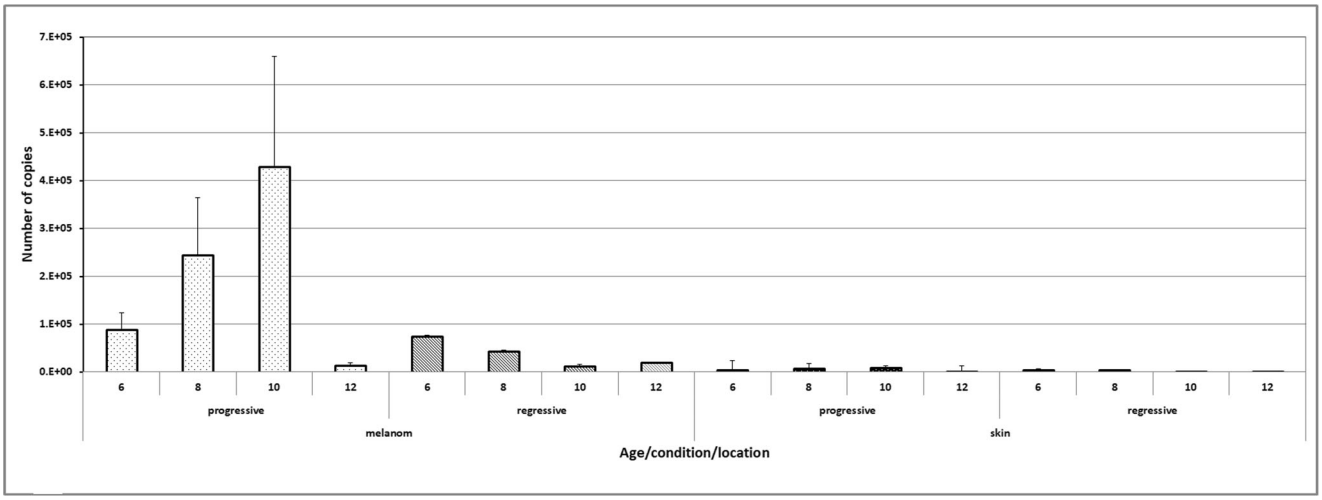

Fig. 2 Comparison of 16S rRNA gene copy numbers of *Fusobacterium necrophorum* according to age, condition, and location of different samples

Amplifications were carried out in a 96-well optical plate by mx3005P real-time PCR (Stratagene).

Data analysis

The DGGE electropherograms were analyzed by BioNumerics version 9.5 (Applied Maths NV). The profiles were used for cluster analysis by UPGMA, to visualize differences in microbiome diversity by PCoA analysis and also to calculate the diversity index (Simpson index of diversity).

The statistical significance of differences in skin bacterial diversity (Simpson index of diversity) between location categories (skin and melanoma) was determined using GraphPad Prism 6 (GraphPad Software, Inc.). Two-way ANOVA analysis was used to determine differences in skin microbiome diversity between different locations in the skin of different animals with regressive and progressive tumor development.

Results

DGGE profile analysis of microbiome of different locations

Each lane of the PCR-DGGE gel (Fig. 1) represents one sample taken from different locations (skin and melanoma) of different groups of animals (regressive or progressive disease). The DGGE profiles of the two animal groups (regressive and progressive) show the variable and distinct composition of microbiota in different locations (skin and melanoma).

Table 1 Bacterial diversity of skin and melanoma samples

Location	Index mean ± Std. dev
Skin	0.061 ± 0.015
Melanoma	0.070 ± 0.016

The examination by sequencing of the abundant bands extracted from different positions (labeled in the Fig. 1) in DGGE profiles reveal that the dominant bacterial phyla found in this study were *Firmicutes*, *Fusobacteria*, and *Actinobacteria*. The most prevailing genera present in several samples were *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Fusobacterium*, and *Trueperella*. The predominant and distribution of bacterial genera were different between skin and melanoma samples.

Quantification of Fusobacteria

The number of copies of the 16S rRNA gene of Fusobacteria was higher in melanoma samples than in healthy skin, and in progressive animals more than the regressive group (Fig. 2). We observed a proportional relationship between the age and number of copies of the 16S rRNA gene of Fusobacteria in melanoma samples of the progressive group of animals except for the age of 12 weeks, because just two animals remained alive until 12 weeks, and the others died earlier. There is an inverse proportional relationship between age and the number of copies of the 16S rRNA gene of Fusobacteria in melanoma samples of the regressive group of animals. The highest amount of Fusobacteria was found in melanoma samples of progressive animals at 10 weeks of age.

Microbiome diversity analysis in melanoma and healthy skin samples

The Simpson index of diversity was calculated to estimate the bacterial diversity in the different locations (Table 1). The diversity was slightly higher in the melanoma samples than that in skin samples (Table 1). The 95% confidence intervals (CI) were calculated using Tukey's multiple comparisons test for determining a significant difference between the skin and melanoma. A significant difference was observed between skin and

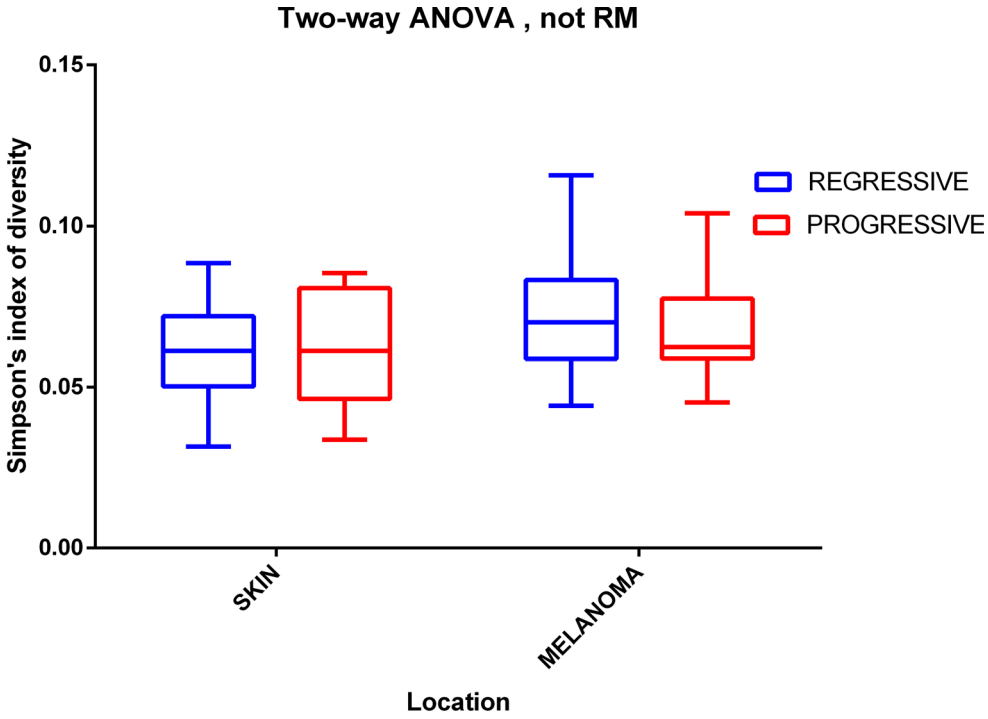

Fig. 3 Variation of microbiome diversity in different locations of progressive and regressive animals. $p = 0.39837$

melanoma microbiome diversity, where the 95% confidence interval of difference was from −0.01696 to −0.0008623. Two-way ANOVA analysis indicated that the skin microbiome diversity in the two groups of animals, regressive and progressive, were no different in skin samples, but there were significantly differences in the melanoma samples (Fig. 3).

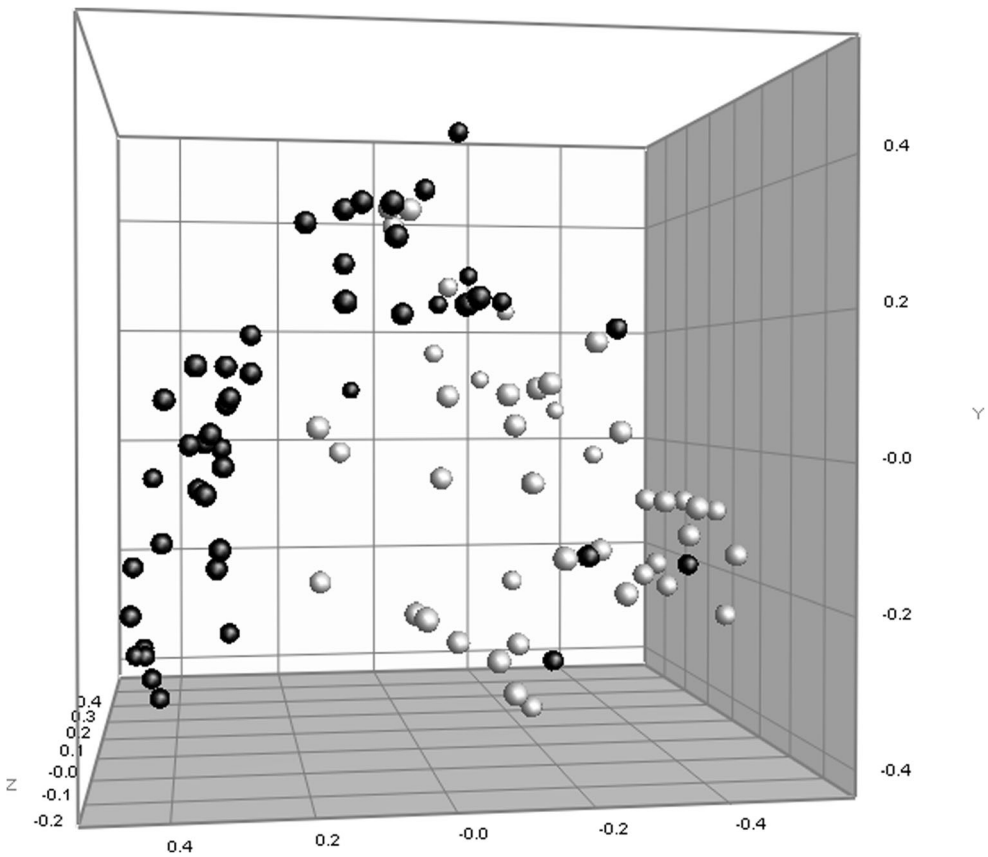

Fig. 4 Bacterial community diversity in different locations visualized based on Pearson's correlation of principal coordinate analysis (PCoA). Black: melanoma, white: skin

Principal coordinate analysis (PCoA) shows two apparent clustering arrangements were identified: "melanoma cluster" in black and "skin cluster" in white (Fig. 4). Similarly to the UPGMA clustering analysis (Fig. 1), a clear difference and separation between samples based on location (skin or melanoma) were observed.

Discussion

The results revealed a notable difference in skin microbiome between the different locations: skin and melanoma. All the results of bacterial diversity, UPGMA clustering analysis, 95% confidence intervals (CI) for determining a significant difference using the Simpson index of diversity for statistical analysis, and principal coordinate analysis (PCoA) showed that the bacterial diversity was significantly different between the skin and melanoma surface, which confirmed our proposed hypothesis. The microbiome diversity in the two groups of animals also exhibited different profiles.

The skin microbiome plays an important role in host defense and provides protection against cancer and infectious pathogens. However, there might be bacteria that help the tumor avoid immune cell attack.

The microbiome variability of the different locations was different in both bacterial composition and abundance. The major phylum present in the microbiome of the skin of the different animals was Firmicutes, where *Staphylococcus*, *Streptococcus*, and *Lactobacillus* were the dominant genera.

Many studies imply the antiproliferative effects of *Lactobacillus* in different cancer types: breast cancer, cervical cancer, colorectal cancer, and oral cancer (Asoudeh-Fard et al. 2017; Cheng et al. 2017; Jacouton et al. 2017; Marschalek et al. 2017; Rasouli et al. 2017; Yang et al. 2018; Motevaseli et al. 2016, 2017, 2018).

Another example, some bacterial species such as *Staphylococcus epidermidis* enhance the system's immune function. The *S. epidermidis* strain produces the protein 6-HAP, which contributes to the selective antiproliferative function against transformed tumor cells, thus defending the host against the tumor growth of skin neoplasia (Nakatsuji et al. 2018).

In the melanoma samples, in addition to Firmicutes, other phyla were found such as Fusobacteria and Actinobacteria, which make the microbiome more rich and complex. However, we only found the presence of *Fusobacterium* and *Trueperella* genera in the microbiome of melanoma samples, which have a higher abundance of *Staphylococcus* and *Streptococcus* than skin microbiome samples. In addition, the amount of *F. nucleatum* increases with time (age) in melanoma samples of animals with the progressive disease and decreases in regressive animals (Fig. 3), indicating that Fusobacteria favors the tumor's progress.

Fusobacteria are often associated with colorectal, pancreatic, and oral cancer (Castellarin et al. 2012; Kostic et al. 2012; Flanagan et al. 2014; Gallimidi et al. 2015; Mitsuhashi et al. 2015; Holt and Cochrane 2017). Further, it was shown that *F. nucleatum* is able to promote tumor proliferation and enhance tumor progression. A tumor-based immune evasion mechanism was described, where *F. nucleatum*-bound tumors are protected against immune cell attack and inhibit natural killer (NK) cell cytotoxicity via the interaction of the Fusobacterial protein Fap2 with the immune cells inhibitory receptor TIGIT. *F. nucleatum* can bind to many different tumor types, including melanoma (Gur et al. 2015). *Trueperella pyogenes* is an opportunistic pathogen causing pyogenic infection in pigs and other animals. In the co-presence of some risk factors, it gives rise to purulent lesions. Additionally, *Trueperella pyogenes* is able to cause infection in humans (Zhao et al. 2013; Jarosz et al. 2014). The *Streptococcus dysgalactiae* subspecies *equismilis* (SDSE) causes a variety of superficial and immunologically mediated diseases in humans, and it was associated with a severe disseminated suppurative meningoencephalomyelitis in a Yorkshire pig (Hughes et al. 2009; Kasuya et al. 2014). *Staphylococcus hyicus* is the agent responsible for exudative epidermitis (a disease commonly known as "greasy pig" disease) which causes mild to severe skin lesions in swine (Park et al. 2013). *S. hyicus* can cause sepsis in humans, since it was identified in the clinical specimen of a farmer who had been in close contact with his piglet (Casanova et al. 2011).

Conclusions

In this study, we have evaluated the skin microbiome of pigs. The high similarity of pig skin to human skin makes pigs a potentially useful animal model for skin microbiome and skin disease studies. In this study, the pig skin microbiome was characterized by a significant difference in the diversity of the microbiome between healthy skin and melanoma tissue. These results are encouraging for the further characterization of the association of microbial community alterations and skin disease, especially melanoma and skin cancer, to understand the functional roles of the skin microbiome during melanoma development and to comprehend the correlations and interactions between the skin microbiome and disease. These studies may involve the prediction or diagnosis of disease by identifying a specific skin microbe (biomarker) related to the progress of melanoma.

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ANALYSIS OF CUTANEOUS MICROBIOTA OF PIGLETS WITH HEREDITARY MELANOMA*

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Malignant melanoma may be a life-threatening disease caused by various conditions. Cutaneous bacteria could play a role in melanoma development or regression. The present work aimed to analyze the bacterial species present on the epidermis of piglets with hereditary melanoma. Bacteria isolated by swabs directly from melanomas and healthy epidermis were analysed using MALDI-TOF mass spectrometry. From the total of 290 isolates, 92 were identified, while the extraction by ethanol turned out to be more efficient compared to identification by direct transfer. *Staphylococcus sciuri, Staphylococcus cohnii,* and *Lactococcus lactis* were significantly more frequent on healthy skin, whereas *Staphylococcus chromogenes*, *Staphylococcus hyicus,* and *Enterococcus faecalis* have thrived significantly better on melanoma. Overall, the results indicate that the microbiota of melanoma is different from that of healthy epidermis, so piglet skin bacteria inspections are recommended.

bacteria, skin, cancer, pig, MeLiM

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INTRODUCTION

Malignant melanoma is the most aggressive form of skin cancer. Due to its metastatic potential it causes up to 90% of deaths from skin cancer (Cervinkova et al., 2017)spontaneous regression of the metastatic form of tumour is a rare phenomenon observed in only 0.23% of cases. The most frequently mentioned factors leading to spontaneous regression of MM are operative trauma, infection, vaccination (BCG and rabies vaccines. Malignant melanoma is classified into 4 different clinical subtypes: superficial spreading melanoma, lentigo maligna melanoma, nodular melanoma, and acral lentiginous melanoma (McCourt et al., 2014). The incidence of melanoma is generally more common in people from higher socioeconomic class, thus the largest incidence occurs in Australia and USA, followed by western European countries. Most affected has been the Caucasian race, however melanoma occurs also in other races (Little, Eide, 2012). Not only skin phototype, but also duration of sunlight exposure without sun protection use as well as genetic factors are considered as main predisposing factors (Bataille, de Vries, 2008).

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The microbiota seems to play a crucial role in the development of tumour in mice and rat models where germ-free or antibiotics-treated rats or mice have usually fewer tumours, although the evidence has been found mainly for colorectal and liver cancer (Schwabe, Jobin, 2013). Grivennikov et al. (2012) also found that metabolism of certain bacterial species can also enhance tumour growth. The association between skin cancer and wound-caused inflammation is also known. Hoste et al. (2015) showed that tumour incidence correlates with wound size, bacteria and chronic inflammation, and that exposure to bacterial flagellin, the protein present in flagellated bacteria, promotes tumour formation in mice. Tran et al. (2013) discovered that tetanus–diphtheria–pertussis vaccination enhanced a spontaneous regression of metastatic melanoma.

The aim of our work was to evaluate if there is a correlation between the composition of bacterial species and incidence of melanoma on skin of pigs.

MATERIAL AND METHODS

The bacterial samples were collected by MTS-002 Swabs with Amies medium with activated charcoal (Med-Lab trade, Czech Republic) from four eight-week-old MeLiM (melanoblastoma-bearing Liběchov minipigs) piglets with hereditary melanoma (Horak et al., 1999). The animals were housed in the farrowing pen together with the sow and they had an unlimited approach to water and commercial feed mixtures according to their age. Melanoma and cutaneous smears were harvested from the defined area on the back or proximal part of the limbs. The cutaneous smears were sampled from the site 5 cm far from the edge of selected melanoma. By this procedure we obtained eight samples, four melanoma and four controls. The samples were immediately inoculated using spread-plate technique on 9 mm Petri dishes containing the following media (all Oxoid, UK): CM0003 Nutrient agar for isolation of total aerobic bacteria, CM0619 Wilkins-Chalgren anaerobe agar for isolation of total anaerobic bacteria, and CM0145 Staphylococcus medium No. 110 for isolation of staphylococci.

The plates were subsequently cultivated at 37°C for 24 h for total aerobes and staphylococci and at 37°C for 24 h in anaerobic atmosphere created by an AnaeroGen Atmosphere Generation System (Oxoid). After the cultivation, 56 colonies from Nutrient agar, 24 colonies from Wilkins-Chalgren anaerobe agar, and 24 colonies from Staphylococcus medium were isolated and propagated at 37°C for ٢٤ h in vials containing CM0067 Nutrient broth No. 2 (Oxoid). In addition, 88 colonies from Nutrient agar, 80 colonies from Wilkins-Chalgren anaerobe agar, and 24 colonies from Staphylococcus medium were directly used for MALDI TOF analysis.

After cultivation, the obtained well grown isolates underwent the identification by MALDI TOF mass spectrometry on an AutoFlex Speed mass spectrometer (Bruker Daltonik, Germany) using ethanol-formic acid extraction (Schulthess et al., 2013)the direct transfer-formic acid method with on-target formic acid treatment, and ethanol-formic acid extraction. At first, 1 ml of each sample was centrifuged at 14 500 g for 3 min, the supernatant was discarded, and the surface of pellet was washed by 70% ethanol in order to remove the residual growth medium. After another centrifugation, the ethanolic supernatant was discarded and the pellet was resuspended in 15 µl of 70% formic acid (Fisher Scientific, USA) and 15 µl of acetonitrile (LC-MS Chromasolv®), centrifuged, and supernatant was used for MALDI-TOF analysis. The 1 µl of supernatant was applied on MTP 384 polished steel target plate (Bruker Daltonik) and allowed to air dry. Then, all samples were overlaid by 1 µl of α-cyano-4-hydroxycinnamic acid dissolved in acetonitrile, water, and trifluoroacetic acid (50 : 47.5 : 2.5) as a MALDI matrix (Bruker Daltonik).

Direct transfer method was carried out by the application of a selected colony directly from the agar plate to MALDI target and, overlaying by 1 µl of formic acid and 1 µl of acetonitrile. After drying out, the sample was overlaid by MALDI matrix as in the previous procedure.

After drying out at room temperature, the plate containing samples obtained by both described procedures was inserted into an AutoFlex Speed mass spectrometer (Bruker Daltonik) and measured in positive linear mode in the detection mass range 2–20 kDa. To optimise the experimental conditions, a solution of Bruker Bacterial Test Standard (BTS; Bruker Daltonik) containing *Escherichia coli* DH5 alpha extract was used to calibrate the system, according to manufacturer's instructions. The obtained data were evaluated by

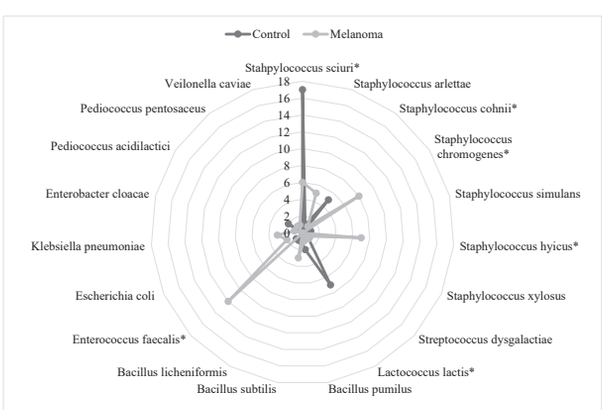

Fig. 1. Occurrence of species successfully identified by MALDI-TOF mass spectrometry
species with asterisk showed a significant difference ($P < 0.05$) between proportions of control and melanoma groups by Chi-squared test

MALDI Biotyper Real Time Classification software (Bruker Daltonik). For the statistical evaluation the samples that were not identified properly and samples reaching less than 1.800 MALDI Biotyper score value were excluded. The samples with more than 1.800 score value, but comprising other species identified by the score differing less than 0.200 were also excluded. This approach has been used based on manufacturer's instructions where the score between 1.700 and 1.999 indicates probable genus identification, score between 2.000 and 2.299 indicates secure genus and probable species identification, and score over 2.300 indicates secure species identification.

Statistical evaluation was performed by Statgraphics Centurion XV (StatPoint) using Chi-squared test at a significance level of $P = 0.05$. Differences in variability of bacterial species between melanoma and control group were evaluated for each growth media separately and altogether.

RESULTS

Bacterial colonies were formed on all three tested media. We managed to identify 92 isolates out of 290 in total where the analysis by ethanol-formic acid extraction has been much more effective as we successfully identified 56 out of 97 isolates giving 57.73% success rate compared to direct transfer method where we successfully identified only 36 out of 193 giving 18.65% success rate. Identified species are shown in Fig. 1.

In terms of groups we successfully identified to the species level 39 isolates from healthy epidermis (control group) and 53 isolates from melanoma.

The most abundant bacterium was *Staphylococcus sciuri* identified 23 times in total (17 times in control group and 6 times in melanoma group). This species falls with the proportion 0.26 beyond the limit ($P < 0.05$ confidence level). Other identified bacterium worth mentioning was *Lactococcus lactis* isolated in 7 cases exclusively from control group by Wilkins-Chalgren anaerobe agar. The 0.00 proportion of *L. lactis* is also beyond the limit ($P < 0.05$). On the contrary, the melanoma group showed exclusive abundance of *Staphylococcus chromogenes*, which was identified 8 times in this group and not even once in control group which means that the proportion 0.00 of this strain is also beyond the limit ($P < 0.05$). The same case displayed *Staphylococcus hyicus*, which was identified 7 times in melanoma group and not even once in control group and also lays beyond the limit (proportion 1.00, $P < 0.05$). *Enterococcus faecalis* was detected 12 times in melanoma group and only once in control group and with the proportion 0.92 falls beyond the limit ($P < 0.05$). Last significant proportion was displayed by *Staphylococcus cohnii* identified 5 times in control group and once in melanoma group which makes a proportion of 0.17 and falls beyond limit ($P < 0.05$).

Comparison using Chi-square test showed a clear significant difference between the proportions of bacteria in control and melanoma group ($P < 0.0001$). A significant difference was observed even when comparing identified bacteria from Nutrient agar ($P = 0.006$) and Wilkins-Chalgren agar ($P = 0.01$) alone. Comparison of proportions in Staphylococcus medium did not find significant differences ($P = 0.17$) due to small amount of isolates.

DISCUSSION

Schulthess et al. (2013)the direct transfer-formic acid method with on-target formic acid treatment, and ethanol-formic acid extraction reported that the direct transfer preparation method with the use of formic acid and without the use of ethanol for MALDI-TOF identification was equally successful for species identification as the ethanol-formic acid extraction. Our experiment does not confirm this claim as 53.85% of successfully identified species is much more than 18.75% obtained by the direct transfer method. The authors cited identified Gram-positive cocci from their strain collection which can be the cause of our different findings whereas we identified more heterogeneous samples containing not only Gram-positive cocci, but also bacilli and Gram-negative bacteria from *Enterobacteriaceae* family. Wilson et al. (2017) also performed multiple extraction methods for MALDI-TOF identification and they achieved much a more successful identification (89.9%) by the direct transfer than in our work. However, they identified bacteria from collection as did Schulthess et al. (2013) in their study and not samples originating directly from the environment. With regard to our findings, we recommend to perform the ethanol-formic acid extraction to identify unknown bacteria from the piglet skin or a similar environment.

McIntyre et al. (2016)normal cutaneous flora likely has been selected for because it potentiates or, at minimum, does not impede wound healing. While pigs are the gold standard model for wound healing studies, the porcine skin microbiome has not been studied in detail. Herein, we performed 16S rDNA sequencing to characterize the pig skin bacteriome at several anatomical locations. Additionally, we used bacterial conditioned-media with in vitro techniques to examine the paracrine effects of bacterial-derived proteins on human keratinocytes (NHEK reported that more than 50% of bacteria identified on pig skin belong to the *Firmicutes* phyla. We observed that all bacteria identified from healthy skin belong to *Firmicutes* phyla which could be probably due to different method of identification considering that McIntyre et al. (2016) used 16s rDNA sequencing which is a method able to identify non-cultivable strains of bacteria.

Regarding the isolated bacteria, the *Staphylococcus sciuri* that has been found significantly more frequently on healthy skin is commensal bacterium frequently isolated from humans and animals, but its presence has also been reported in human and animal infections. Some members of the species have been shown to be pathogenic and could carry multiple virulence and resistance genes (Nemeghaire et al., 2014). Chen et al. (2007) isolated *S. sciuri* even from pericardial fluid from piglets diseased with exudative epidermitis. In our case we found *S. sciuri* mainly on healthy skin so we assume that this species has no involvement in melanoma proliferation or it has protective effect against melanoma formation.

Lactococcus lactis is another bacterium present significantly more often on healthy skin of examined piglets. Its role in melanoma regression is up to date unknown, but *L. lactis* is a generally known probiotic bacterium and it produces lactic acid that has been investigated as an inhibitor on melanin synthesis in melanoma cells (Usuki et al., 2003). On the contrary, Brand et al. (2016) found out that lactic acid suppressed T and NK cells and thus promoted tumor growth in their study, although it is not known how does lactic acid contribute to activation or deactivation of other immune cells. Generally, lactic acid suppresses the growth of pathogenic bacteria (Coates et al., 2014; Hor, Liong, 2014), so more investigation is needed to reveal the role of lactococci in skin cancer.

Staphylococcus cohnii has also occurred in a significantly higher rate in healthy skin. The occurrence of *S. cohnii* has mostly been reported in animals (Kloos et al., 1976), but it has been shown that it is able to produce a biofilm similarly as *S. sciuri* (Garza-Gonzalez et al., 2011). Just like in the case of *S. sciuri*, there are no data that *S. cohnii* would play a role in melanoma incidence in pigs or in humans.

Exudative epidermitis associated with the abundance of *S. sciuri* has been a result of *Staphylococcus chromogenes* strain in the study carried out by Andresen et al. (2005)which is recognised as the causative agent of exudative epidermitis (EE. Other authors do not consider *S. chromogenes* as a an etiological agent of disease, especially in pigs (Sato et al., 2004), although it can develop mastitis in cattle (Devriese et al., 2002; Enger et al., 2015)and (2. *S. chromogenes* has been isolated exclusively from melanomas in our study. However, due to the lack of data regarding its toxicity in pigs we assume that its presence on melanoma could be a consequence, not a cause.

When we consider the role of *Staphylococcus hyicus* that has been also isolated exclusively from melanomas, we get a different finding. Multiple authors blame this bacterium as a causative agent of exudative epidermitis in pigs (Andresen, 2005; Park et al., 2013) due to its production of exfoliative toxins by some strains. The fact that *S. hyicus* is able to trigger the skin disease induces the idea that it can participate on the melanoma progression although a lot more investigation needs to be carried out to prove this claim.

Enterococcus faecalis has been significantly more abundant on melanoma. This species is a member of commensal flora of animals, but it can also cause infections, e.g. in wounds (Hwang et al., 2011). Its role in melanoma is, as well as in bacteria mentioned above, unknown.

Staphylococci are normal inhabitants in healthy animals (Skalka, 1991)in pigs and in domestic fowl, using the method of selective isolation of strains. A total of 6066 samples was examined; 4567 strains were revealed and they included all known species, except Staphylococcus caseolyticus, S. saccharolyticus, S. schleiferi and S. lugdunensis. 3.8% of strains failed to be identified with any species. The test samples were taken from slaughtered animals, only in calves intravital smears of tonsils were examined. The species most frequently isolated from the tonsil tissue in adult cattle were as follows: S. aureus (19.9%, but apparently some strains can switch to detrimental mode if favorable conditions occur. The question needed to be answered is if they can be cause or consequence of melanoma. This article demonstrates that several staphylococcal species appear on melanoma more than on healthy skin and other species *vice versa*. The same goes for *Enterococcus faecalis* and *Lactococcus lactis*, which are both lactic acid bacteria but meanwhile *E. faecalis* occurred mostly on melanoma, *L. lactis* occurred only on healthy skin. *E. faecalis* is able to survive and thrive in various niches (Nallapareddy, Murray, 2008)growth in 40% serum, a biological cue with potential clinical relevance, elicited adherence of all 46 E. faecalis strains tested to fibronectin and fibrinogen, but not to elastin; the adherence levels were independent of strain source and was eliminated by treating cells with trypsin. As previously reported, serum also elicited collagen adherence. While prolonged exposure to serum during growth was needed for enhancement of adherence to fibrinogen, brief exposure (<5 min and can cause diseases, so its role on skin could be detrimental.

CONCLUSION

In this work, we managed to isolate and describe various bacteria from melanoma and healthy skin of piglets. Our results indicate that ethanol-formic acid extraction should be used to identify bacteria from piglet skin by MALDI-TOF.

More research needs to be carried out to verify the significance of bacteria in influencing melanoma regression or promotion. Nevertheless, we managed to find the differences in the microbial composition of healthy piglet skin and melanoma.

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Spontaneous regression of malignant melanoma - is it based on the interplay between host immune system and melanoma antigens?

Monika Cervinkova[a,b], Petra Kucerova[a] and Jana Cizkova[a,c]

Malignant melanoma (MM) is the most aggressive and uneasily treatable form of skin cancer. Up to 90% of deaths because of skin tumours are estimated to be caused by this malignancy. Spontaneous regression is described as a partial or complete disappearance of cancer. It can be defined if the clinical and histological diagnosis of malignancy is verified and any therapeutic intervention potentially inducing mechanisms leading to regression has not been applied. Regression occurs more frequently in melanoma than in other types of tumours; it is reported to be six times higher than in other malignancies. Up to 50% of primary MM is reported to undergo spontaneous regression. However, spontaneous regression of the metastatic form of tumour is a rare phenomenon observed in only 0.23% of cases. The most frequently mentioned factors leading to spontaneous regression of MM are operative trauma, infection, vaccination (BCG and rabies vaccines) and immunological factors. Other well-documented circumstances associated with regression of metastatic MM include blood transfusion and various endocrine factors. *Anti-Cancer Drugs* **00:000–000 Copyright © 2017 Wolters Kluwer Health, Inc. All rights reserved.**

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Introduction

Malignant melanoma (MM) develops because of neoplastic transformation of pigment-producing cells localized primarily in the skin, and further in the ear, gastrointestinal tract, eye, oral cavity, genital mucosa and leptomeninges. Accumulation of various mutations occurs in cells affecting the genes responsible for cell proliferation, differentiation and apoptosis (Ras–Raf–Mek–Erk and PI3–Kinase–Akt pathways and the *CDKNA2* gene). V599E *BRAF* ($\leq$75% patients with MM and 70% human cell lines) and *NRAS* mutations are observed most frequently [1–5].

In epidemiological data, sunlight is mentioned as the major predisposing factor for the development of MM. There is an increased risk of MM in individuals with a history of repeated sunburn in childhood. Pigmentation of skin, and colour of the hair and eyes are very important as well [1,3,4].

MM is the most aggressive and uneasily treatable form of skin cancer [2]. Up to 90% of deaths because of skin tumours are estimated to be caused by this malignancy. Prognosis depends on the stage of disease disclosure according to Clark's criteria (I, II, III and IV stages). In many publications, V stage is also mentioned. Standard therapy comprises surgery, chemotherapy, immunotherapy, radiation therapy and biochemotherapy [6,7].

Nondisseminating melanomas are usually well treatable; however, in patients with the metastatic form of MM, long-term survival is extremely low [2]. Stage of the disease is the main criterion for long-term survival. Whether malignancy is diagnosed in early stages I–III and it is limited to skin and/or regional lymph nodes, curability is 40–90%. In stage IV, where metastases are already established, the 5-year survival rate is generally less than 15% [8].

MM is classified into four different subtypes – superficial spreading melanoma, lentigo maligna, nodular melanoma and acral lentiginous melanoma. Assignment into individual categories is usually performed according to the ABCDE scheme (A – asymmetry, B – borders irregularity, C – colour variability, D – diameter, E – evolving). Superficial spreading melanoma is usually associated with acute intermittent exposure to sunlight. Lentigo maligna is more common in patients with skin chronically exposed to the sun [3]. Repeated casual exposure to sunlight appears to be a protective factor against the development of MM in the White population [9].

The incidence of MM has increased globally (>4% for women and 5%/year for men) in the White population in the last few decades, especially in individuals intensively exposed to sunlight. More than 160 000 of new cases of

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MM are diagnosed every year. The incidence varies from 6–10/100 000 in Southern Europe to 50–60 cases/100 000 inhabitants in Australia [7,8,10].

MM is the most common form of cutaneous malignancy in the White population. Pale skin, blue eyes and blond or red hair are major predisposing factors for the development of MM [11]. MM is rarely also found in the 'non-White' population. The incidence is described to be 10–20× lower than that in the White population, but if the affection occurs, more aggressive lentiginous and nodular MM are often observed [9].

Present nevi are mentioned to be a significant predictor of the potential risk for the development of MM [9]. A link between MM occurrence and pre-existing nevus is mentioned in 25% of cases. Fortunately, only a small percentage of dysplastic nevi may develop into MM [11,12]. Dysplastic nevus syndrome is an inherited 'disease' that predisposes during the whole lifetime to an increased risk of development of MM and requires regular dermatological controls [11] (*http://www.cancer.org/cancer/skincancer-melanoma/detailedguide/melanoma-skin-cancer-risk-factors*).

The incidence of MM related to sex is nearly equivalent. MM usually affects relatively young individuals, especially the middle-aged population. MM is one of the most frequent malignancies affecting individuals younger than 30 years of age, especially women. Superficial melanoma affects primarily young individuals, and nodular MM and lentigo maligna affects mainly older populations [9,10] (*http://www.cancer.org/cancer/skincancer-melanoma/detailedguide/melanoma-skin-cancer-risk-factors*). Some studies show a possible significant effect of oestrogen, progesterone, hormonal contraceptives, a woman's age at first delivery, number of children and age of onset of menopause on the subsequent risk of development of MM [12].

In individuals with weakened immune systems (organ transplantation, HIV/AIDS syndrome, cancer, or other form of immunosuppression), there is a 3–4 times higher risk of development of MM. These individuals have to use a sunscreen with a factor higher than 50, in risk hours (11 a.m. to 3 p.m.) they should avoid direct sunlight and the most exposed areas of the human body should be covered with long, light-coloured clothes [12,13] (*http://www.cancer.org/cancer/skincancer-melanoma/detailedguide/melanoma-skin-cancer-risk-factors*).

Treatment

The standard treatment of MM includes surgery (MM diagnosed in an early stage), chemotherapy, immunotherapy, radiation therapy and biochemotherapy.

The primary approach in MM treatment is local excision of the tumour. Sentinel lymph node biopsy with lymph node dissection is reserved for advanced infiltrating lesions with a high risk of spreading to regional lymph nodes [14].

The standard treatment for patients with metastatic MM is chemotherapy. Dacarbazine is the most frequently used drug. Unfortunately, the response rate is rather low, ranging between 10 and 20%, and the overall survival rate is 8 months. Other chemotherapeutic drugs used are temozolomide, carmustine, lomustine, cisplatin, etc. [15,16].

Radiotherapy has been used frequently for the treatment of MM since 1970, especially if the surgical excision is limited because of cosmetic reasons or in patients in whom surgical procedure cannot be performed (comorbidity, risk of total anaesthesia). Radiotherapy also finds its application in radiosurgery in brain metastases of MM, plaque brachytherapy of uveal melanoma and in therapy of MM localized on the head and neck [14].

The most frequently used immunotherapeutic approaches for the treatment of MM are based on application of high doses of interleukin-2 (IL-2). A long-term, durable response is achieved only in a small percentage of patients (6–10%) and the therapy is often complicated by the high toxicity of medicines [16]. Interferon-α 2b was reported to be the first 'drug' in the treatment of MM that significantly improved long-term survival of patients [15]. Toxicity of interferon-α is rather high, represented by moderate to severe flu-like symptoms that could be the reason for termination of the therapy in up to 25% of patients [17].

Sorafenib targets and inhibits the BRAF mutation. It was tested in several clinical trials (phases II and III). Sorafenib is effective in combination with chemotherapeutics (dacarbazine, carboplatin, paclitaxel) in MM as a single drug is ineffective against this malignancy. The combination of sorafenib, carboplatin and paclitaxel showed a response rate of 31% (stable disease rate 54%) in clinical trials in phase I/II and a median time to progression of malignancy of 8.8 months [8]. PLX4032 (vemurafenib, RG7204) is successfully applied in the treatment of cutaneous MM. The response rate is about 70% [14,15]. GSK2118436 and RAF265 are other BRAF inhibitors tested in clinical trials [15].

Ipilimumab and tremelimumab (human anti-CTLA-4 monoclonal antibodies) have been tested in phase I and II clinical trials. These antibodies were applied alone or in combination with another form of therapy (peptide vaccines, chemotherapy). Response rates ranged between 15 and 20% [8]. Responses to CTLA-4 antibodies are stable (>70%), but therapy requires long-term administration (12 weeks or more) to achieve a late-onset response [15].

Biochemotherapy is defined as a combination of chemotherapy (e.g. dacarbazine) and immunotherapy procedures (e.g. IL-2). Biochemotherapy is the treatment of choice for patients with metastatic MM [18]. Several II phase clinical trials objectively show responsiveness to treatment in 50–60% of patients, complete remission

between 10 and 20% and a median patient survival between 11 and 12 months [19].

Spontaneous regression of melanoma

Definition of spontaneous regression

Spontaneous regression is described as a partial or complete disappearance of cancer. It can be defined if the clinical and histological diagnosis of malignancy has been verified and any therapeutic intervention potentially inducing mechanisms leading to regression has not been applied. Significant period of regular follow-up after running this process must be achieved and the histological evaluation of regressing tissue must be provided.

Various clinical signs of the spontaneous tumour regression are described – loss of pigment of lesion or surrounding resulting in hypopigmentation (halo phenomenon), size reduction, telangiectasia and scars [20,21].

Regression occurs more frequently in MM than in other types of tumours; it is six times higher than in other malignancies. It should be noted that the increase reported can be only the consequence of MM characteristics wherein they are pigmented and primarily occurring on the skin. Therefore, they are more easily detectable and evaluable than malignancies of the internal organs – for example, lung or breast tumours, etc. [21,22].

Up to 50% of primary MM undergo spontaneous regression. However, spontaneous regression in metastatic MM is a rare phenomenon. Spontaneous regression of metastatic MM was detected only in 0.23% of cases. There are only 76 well-documented cases in the literature from 1866 [21,23] .'Thin melanomas' show focal regression in 7–61% of lesions. Spontaneous regression of MM with metastases occurs equally in both sexes in patients of different ages. This is in contrast with complete spontaneous regression of primary MM, where the male : female ratio is 2 : 1. Spontaneous regression of metastatic MM is observed most commonly in cutaneous and subcutaneous deposits, followed by lymph nodes, pulmonary, hepatic, cerebral and intestinal metastasis [20].

Prognostic significance of regression of primary malignant melanoma

Prognostic significance of the regression of the primary MM has been discussed for several years and it still remains highly controversial. The main problem remains the exact definition and evaluation of regression (identical differentiation between complete and partial regression) [6,24].

In some studies, it is considered to be a negative prognostic factor. Significant signs of regression of primary MM in association with other factors (age <40, ulceration, Clark level IV, etc.) increase the risk of dissemination from the primary MM into the sentinel lymph nodes [24].

It was found to be an unfavourable prognostic factor particularly in 'thin melanomas'. One study has shown that 36 thin (0.76 mm) regressing MM metastasized in 45.5% compared with 12% of MM with no clinical and histological signs of regression [6]. The hypothesis that dissemination of MM to regional lymph nodes can stimulate an immune response resulting in a regression of the primary lesion has also been reported [24].

However, other studies reported that regression does not increase the risk of metastasis [24].

Of 41 patients with regressing cutaneous MM, only one patient was diagnosed with metastatic MM [6].

Follow-up of patients with regression of primary malignant melanoma

Subsequent data were obtained during the follow-up of patients with regression of primary MM.

In a study evaluating 486 patients with cutaneous MM (stage I), it was reported that MM regression decreased the 10-year survival rate. This rate was significantly lower in patients with regressing MM (79%) compared with a group of patients with nonregressing MM (95%). Severe regression of MM significantly decreases the disease-free period. Of the group of 214 MM patients without metastases, severe regression was observed in 35 patients. A disease-free interval of 5 years was observed in 72% of these patients with severe regression compared with 94% of patients without regression or moderate regression [6].

Progression of disease was observed only in 33/349 (9.5%) patients with regressing MM compared with 314/1344 (23.4%) patients with nonregressing MM. Regression of primary MM is beneficial for patients with MM stages I–II and it is not the occasion to realize sentinel lymph node biopsy [24].

Martin and colleagues hypothesized that patients who have suffered from MM could have an enhanced immune reaction against MM-associated antigens. In the study, patients with multiple MM were included. Successive MM regressed significantly more (42.1%) compared with the first MM (21.05%). It can be proposed that first MM could act as immunostimulant and could increase antitumour immunity against MM. Moreover, metastatic MM was diagnosed only in patients with no regression of MM [22].

Maire and colleagues published the results of 103 patients (IV stage of MM). Immunotherapy was not applied in most cases. The occurrence of autoimmune markers (antithyroid and antinuclear autoantibodies) and no awareness of primary MM lesion are associated with longer survival of MM patients (stages III and IV of MM) [7].

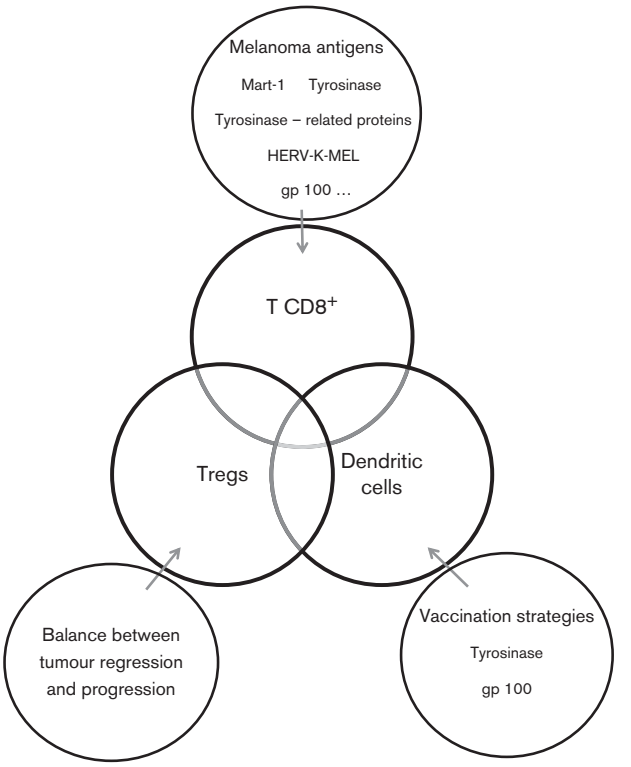
Possible mechanisms involved in the spontaneous regression of malignant melanoma

The most frequently mentioned factors leading to spontaneous regression of MM are operative trauma, infection and immunological factors. Other well-documented conditions associated with regression of metastatic MM include blood transfusion, application of Bacillus Calmette-Guérin (BCG) and rabies vaccines and endocrine factors (pregnancy, alternative therapies xeroderma pigmentosum, diabetes, nephrolithiasis, prostatic hypertrophy and gastric ulcers). Some of these factors can contribute towards initiation of regression and others can contribute towards maintenance of clinical conditions, enabling its contituation. Surgical trauma is often associated with the initiation of cancer regression [20,22].

Factors related to the host immune system

Regression is a consequence of interaction between malignant tumour cells and the host immune system, resulting in the replacement of tumour tissue with fibrosis, degenerated melanoma cells, melanophages, proliferating lymphocytes, proliferating lymphocytes and telangiectasia. In areas of complete regression, melanoma cells are absent in both the dermis and the adjacent epidermis [6]. Components of the immune system likely involved in spontaneous regression of MM are shown in Fig. 1.

Fig. 1



Factors related to immune system.

Immune cells

CD4$^+$ and CD8$^+$ T lymphocytes

There is evidence that spontaneous regression is the result of an effective immune response against MM cells. Various components of the immune system such as tumour-infiltrating CD4$^+$ and CD8$^+$ T cells are observed in regressing MM. As MM progresses, the immune system may alter the phenotype of the tumour by a process known as immunosculpting, resulting in the development of MM cells capable of avoiding the destruction mechanism of the immune system [21,25].

Regression of MM is associated with autoimmune vitiligo and skin depigmentation. Vitiligo was observed in patients with MM and several studies have reported that it may be associated with antitumour response. Histological analysis of the vitiligo biopsy showed infiltrating CD8$^+$ T cells. Moreover, T cells infiltrating the vitiligo have a clonal or an oligoclonal T-cell receptor profile. A high number of melanoma-associated specific T CD8$^+$ was observed in the peripheral blood of MM patients with vitiligo compared with patients without this phenomenon [21]. Vitiligo is reported to be a good prognostic factor for patients with MM. The mutual relationship between vitiligo and good prognosis for patients with MM means unequivocal determination of immune response. The halo effect and depigmentation are clinical symptoms of this process, although these features do not prove spontaneous regression itself. Laboratory results verify the diagnosis of regression.

It has been shown that tumour-infiltrating lymphocytes (TILs) are mostly α/β T cells and regressing MM show clonal amplification of T cells. These cells isolated from regressing MM show cytolytic activity against autologous MM [25].

Enhanced immune response to specific antigens expressed on tumour-associated melanocytes has been suggested in patients with MM. Different types of antigens are expressed in MM and recognized by cytotoxic T (T_c) lymphocytes. Melanocyte differentiation antigens are the most frequently detected and they are also the most immunogenic. These include Melan-A/MART-1, gp 100, tyrosinase, tyrosine-related proteins 1, 2, etc. [21,22].

Melanoma antigens

MART-1 (Melan-A)

MART-1 is expressed in most fresh MM samples and 60% of melanoma cell lines. It is the most frequently recognized melanoma tumour antigen by T_c cells and TILs in HLA-A2-positive patients with metastatic MM [26,27]. Significantly reduced expression of MART-1 was found in patients with various MM and in patients with metastatic MM in whom complete regression of MM was observed. A relationship between the presence of MART-1-specific T_c lymphocytes in blood and loss of the MART-1 antigen was found [22,26]. The efficiency

of adoptive transfer of melanoma-specific lymphocyte has been proven in different studies. TILs were found to be oligoclonal (enriched in $CD8^+$ T reactive cells), clonal or polyclonal Melan-A-specific T_c cells [28]. Vaccines containing Melan-A/MART-1/tyrosinase antigens were used for immunization of patients with metastatic MM. Partial regression of MM and increased depigmentation of skin were observed following an injection of this antigen [29]. A significant response was achieved in 50% (43% partial and complete response; 7% stable disease) MM in the phase II study on adoptive transfer of Melan-A-specific $CD8^+$ T-cell clones [30].

gp100

gp100 is a melanocyte differentiation antigen specifically recognized by HLA-A-restricted T cells. It is suggested to play a role in tumour regression. It is highly immunogenic and thus it is proposed to be a specific target for the immune system [31]. Specific T cells in both regressing MM and vitiligo-like lesions were found in patients with MM vaccinated using gp100 [32]. This phenomenon can lead to the induction of an immune response against MM antigens and depigmentation can be observed. Kaufman *et al.* [33] emphasize that local activation of tumour-reactive T cells is a critical step in the initiation of tumour regression.

Tyrosinase and tyrosinase-related proteins

Tyrosinase is a melanoma-associated antigen recognized by $CD8^+$ T cells from MM patients. It is a promising candidate for immunotherapy of MM. $CD8^+$ T-cell response is directed against individual epitopes of tyrosinase [34]. Tyrosinase-related proteins belong to a family of Cu^{2+}/Zn^{2+} metalloenzymes sharing several sequence homologies [35]. Christou *et al.* [36] reported that antibodies cross reacting with tyrosinase, tyrosine-related protein 1, 2 antigens present on melanocytes and melanoma cells can induce mechanisms leading to vitiligo-like depigmentation and regression of MM.

HERV-K-MEL

The human endogenous retrovirus K family (HERV-K) contains 30–50 proviruses. Two different families are known – HERV-K types I and II. The expression of HERV-K full length mRNA is detected in most human tissues, and deleted *env* and *rec* mRNA only in MM and teratocarcinomas. The sequence related to the *HERV-K-env* gene provirus with a short opening frame is the base for HERV-K-MEL [37]. This melanoma-associated antigen consisting of nona or decapeptides is present in most of the MM (cutaneous, ocular) and dysplastic nevi, but not in normal skin. The melanoma marker is HLA-restricted (HLA-A2 namely) in presentation to $CD8^+$ T_c cells [23,37,38]. It was found that it shares sequence homologies with more than 70 human pathogens (*Streptococcus pyogenes*, *Serratia marcescens*, *Mycobacterium bovis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria* sp., *Staphylococcus aureus*, etc.) [23,38]. Previous exposition to the mentioned pathogens with peptides identical to HERV-K-MEL can induce cross-reactivity against MM by T_c cells and can lead to regression of MM [23,38].

An overview of other melanoma antigens and more detailed information are presented in Table 1.

T regulatory cells

T_c lymphocytes play a key role in the immune system defense against the cancer development. Particularly, specific recognition and destruction of tumour cells and production of specific immune 'memory' against cancer are the underlying mechanisms. The problem is that tumour cells are mostly poorly immunogenic and thus they may escape detection by the immune system [55]. T-regulatory cells (T_{reg}) contribute significantly toward these undesirable mechanisms. T_{reg} cells are defined as a subset of $CD4^+$ T cells expressing high levels of CD25 and transcription factor forkhead box P3 (Foxp3) as well [56]. In laboratory and clinical practice, they are commonly divided into natural (n) and induced (i) T_{reg}s. nTregCD4$^+$ Foxp3$^+$ produced in thymus are better explored cells than iT_{reg} thymus-independent cells (produced in peripheral lymphoid organs), which can also express Foxp3. nT_{reg} and iT_{reg}, irrespective of their genesis, share the common ability to suppress effector T cells. As nT_{reg} are generated in the thymus (under the effect of thymic stromal lymphopoietin-activated CD11c-positive dendritic cells, co-stimulatory molecules CD28, PD-1, CD40L, IL-2 as well), they can be expected to play a significant role in the regulation of antitumour response. Therefore, it can be proposed that low or lower levels of T_{reg}s in blood or tumour tissue can play a significant role in tumour regression [56,57].

Elevated T_{reg} levels were detected in patients with MM and renal cell carcinoma. Decreased T_{reg} levels were observed in patients who responded to treatment with high doses of IL-2 [58].

Dendritic cells

Vaccination with dendritic cells (DC) was administered in patients with different types of cancer. The best results were obtained in patients with MM occurring on skin or lymph nodes [59]. Immunological responses were observed in a clinical study with MM patients vaccinated with immature, peptide-loaded or tumour lysate-loaded DCs [60]. Banchereau *et al.* [61] reported that CD34-derived DCs have also been used in MM vaccination trials where intensive immune response and regression of tumour were detected.

Godelaine *et al.* [62] reported that regression of MM was observed in 6/11 (55.0%) of patients with advanced metastatic MM vaccinated with monocyte-derived DC

Table 1 Melanoma antigens associated with spontaneous regression of malignant melanoma

Melanoma antigens	Antigen group	Physiological function	Mechanism of action	Therapeutic usage	References
Melan-A	Differentiation antigen	Melanosomal membrane protein function unknown	Recognized by T_c cells and TIL – HLA A2 patients with MM	Vaccine trials Phase I: recombinant adenoviruses encoding MART-1 or gp100 vaccine Phase II study: adoptive transfer of Melan-A-specific $CD8^+$ T-cell clones	[26,27,30,39,40]
Tyrosinase	Differentiation antigen	Essential protein in melanin biosynthesis	Recognized by T_c cells	Vaccine trials Phase I: tyrosinase DNA vaccine	[34,39,41,42]
Tyrosinase-related proteins 1,2	Differentiation antigen	TYRP1: important role in melanocytes – activation of pigmentation, cell proliferation and differentiation TYRP2: important role in the melanin biosynthesis pathway	Cross reacting antibodies – vitiligo and regression of MM	Vaccine trials Phase I: TYRP1 cDNA – patients in stage III/IV of MM	[35,36,39,43]
gp100	Melanocyte differentiation antigen	Crucial role in melanosome formation	Recognized by HLA-A restricted T cells, highly immunogenic	Specific vaccines – vitiligo, regression of MM	[31,32,39,44]
HERV-K-MEL	Melanoma-associated antigen	Sequence homologies with more than 70 human pathogens, sequence homology in OREBP (oxygen-responsive element binding proteins) – increased oxidative stress in cell	Recognized by HLA-A2 restricted T_c cell, cross-reactive T_c cells, regression of MM	Previous exposition to pathogens – induction of the mechanism leading to MM regression	[23,38,45]
MAGE-A1	Tumour (cancer testis) antigen	Surface protein of melanoma cells, function unknown	Recognized by T_c cells	Vaccine trials Phase I/II: limited number of patients with MM regression	[39,46–48]
MAGE-A3	Tumour (cancer testis) antigen	Mediation of fibronectin-controlled progression and metastasis	Recognized by T_c cells	Vaccine trials Phase II/III: patients in IV phase of MM	[49–51]
NY-ESO-1	Tumour (cancer testis) antigen	Function unknown	NY-ESO-1 expression – MM progression, increased tumour thickness and reduced number of TILs	Vaccine trials NY-ESO-1 peptides, full-length NY-ESO-1 protein, NY-ESO-1 DNA – phase II clinical trials	[52–54]

TILs, tumour-infiltrating lymphocytes; TYRP, tyrosinase-related proteins.

and pulsed with the MAGE-3.A1 peptide and *Clostridium tetani* toxoid or tuberculin.

Natural killer cells

The cytotoxic effect of natural killer cells in MM has been reported in many studies. McKay and colleagues evaluated the occurrence of natural killer cells in regressing, nonregressing MM and regressing nevi. The highest CD56 positivity was detected in regressing MM; however, nonregressing MM showed the lowest positivity [63].

Tumour-associated macrophages

Tumour-associated macrophages (TAM) are detected in MM, but their role in MM progression/regression is controversial. Activated TAMs can affect some characteristics of MM growth – angiogenesis, formation of stroma or growth of MM cells (regression/progression). The effect of TAMs on MM regression is supposed to be caused by a direct cytotoxic effect or induction of cell lysis [64].

Receptors on immune cells

Activation or inhibition of receptors on immune cells by specific ligands can play a significant role in MM regression/progression as well. In Table 2, the major pattern recognition receptors involved in these processes are reported.

Factors related to microorganisms

It was suggested that stimulation of the host immune system during an on-going infectious process enhances the natural immune defence against the tumour. The contribution of the infection in cases associated with spontaneous regression of metastatic MM is high – about 28% [20].

Spontaneous regression of MM is often associated with the occurrence of specific microorganisms. Maurer and Kolmel reported 68 cases of advanced MM regression where a febrile episode preceded this process in 21 patients. Specifically, erysipelas (*Str. pyogenes*) was diagnosed in nine cases [80]. Coley's toxin (heat-killed *Str. pyogenes* and *S. marcescens*) and BCG (*M. bovis*) or vaccinia vaccines were used for the therapy of MM. It was also proposed that application of BCG or vaccinia vaccine in childhood or a severe infectious process can be protective against MM. Application of the tetanus–diphthteria–pertussis vaccine to induce mechanisms leading to spontaneous regression of MM has also been described. Many authors have reported that pyrexia was the most often condition linked to subsequent spontaneous regression of MM. *L. monocytogenes*, *P. aeruginosa*, *Salmonella* spp., *E. coli*, *Clostridium* spp., *Lactobacillus* spp., *Bidobacterium* spp., etc. have also been reported [23,81–83].

Other factors

Infection with *Toxoplasma gondii* in melanoma bearing mice had a significant effect on MM regression without concurrent activation of the immune system. Synthesis of soluble factors with an antiangiogenic effect is the proposed underlying mechanism. Hypoxia arose as an accompanying symptom; subsequently, necrosis and cell death were observed [84,85].

The production of streptokinase (enzyme of *Str. pyogenes*) was assumed to be one of the possible ways to induce the spontaneous regression of MM. This enzyme converts plasminogen into plasmin, further fibrinogen to fibrin, and degradation of plasma and extracellular matrix proteins is observed. The reduction of size and spreading of metastases of MM are observed [86].

Animal models of malignant melanoma

Animal models are used as an effective tool for the study of MM. Spontaneously occurring and UV-induced MM have been described in a *Xiphophorus* fish model and opossum *Monodelphis domestica* model. Development of MM in guinea-pig and the Syrian hamster models is induced by chemical carcinogens [e.g. 7,12-dimethylbenz [a]anthracene (DMBA)]. Mouse, swine and horse models are other commonly used mammalian models of MM. There are various principles on which the mentioned models are based (induced, hereditary based, etc.) [87,88].

The majority of animal models of MM are progressing models and spontaneous regression of MM is not frequently described. An overview of progressing mouse models is presented in Table 3.

This review deals with mechanisms leading to spontaneous regression of MM. We thus focused on porcine models of MM because the spontaneous regression of MM is frequently observed in these models.

Porcine models of spontaneous regression

Pigs appeared to be the best animal model for the spontaneous regression of MM. Currently, there are three lines of pigs with hereditary melanoma: Sinclair swine, Munich miniature swine troll and Melanoma bearing Libechov minipigs (MeLiM). In all of these breeds, tumours develop spontaneously both prenatally and postnatally, and symptoms associated with spontaneous regression, the halo effect around melanomas and local or systemic vitiligo commonly occur in black-coloured or red-coloured animals, not in white-coloured animals [93–95].

Sinclair swine

Sinclair swine (previously Hormel swine) was developed at the Hormel Institute in Minnesota and was later part of the herd moved to the University of Missouri Sinclair Comparative Medicine Research Farm as a model organism for studies of chronic disease and ageing [95].

Table 2 Pattern recognition receptors versus spontaneous regression of malignant melanoma

Pattern recognition receptors	Ligands	Role in MM regression/progression	References
TLR			
TLR2	Various microbial components: G^+ bacteria – peptidoglycan, lipoteichoic acid Mycobacteria – lipoarabinomannan *Neisseria* spp. – porins Yeast – zymosan	TLR2 activation: ↑ invasivity and immunosuppressive factor production in B16–F10 MM cells TLR2 inhibition (specific antibody) and TLR9 activation (CpG ODN): ↑ antitumour response, ↓ immunosuppressive environment of MM, pulmonary metastases development, mortality of tested mice TLR1/2 agonist (bacterial lipoproteins): ↓ FoxP3 expression in T_{regs}, ↑ T_c cells function – new therapeutic strategies development	[55,65–67]
TLR4	G^- bacteria: lipopolysaccharide Fungus: mannan Viruses: envelope proteins Host: heat-shock proteins 60, 70, fibrinogen	TLR4 activation (LPS): ↑ migration of $TLR4^+$ MM cells, TLR4 inhibition (TLR4 and MyD88 knockdowns) – ↓ migration of $TLR4^+$ MM cells vs. TLR4 activation inside tumour (intratumoural LPS application) ↑ antitumour response, DC cells activation, antigen presentation, INF-γ production by $CD4^+$ T cell TLR4 activation: elucidation role and mechanisms - novel antitumour vaccine adjuvant	[68–72]
TLR9	Bacteria, mycobacteria: CpG-DNA Virus: DNA	TLR9 activation (TLR9 agonist): intradermal or perilesional application – therapeutic effect in phase I testing in MM patients, ↑ IL-6, IL-12p40, IFN-γ induced protein (IP)-10, and TNF-α levels, T_c cells	[68,69,73,74]
FPR			
FPR	Bacteria-*N*-formyl peptides	FPR activation (FPR agonist): ↓ growth of B16–F10 MM, ↑ NK cells in MM tissue, ↑ migration of NK (activation of ERK) FPR inhibition (FPR antagonist): ↑ growth of B16–F10 MM in mice	[75,76]
Scavenger receptors			
MARCO	Nucleic acids, bacteria: lipopolysaccharides, oxidized lipoproteins, numerous endogenous proteins	MARCO: important role in TLR-induced DC activation MARCO inhibition: subcutaneous application of $MARCO^{-/-}$ TP-DC – ↑ migration DC to lymph nodes, INF-γ production by $CD4^+$ T cell, MM regression, survival in B16 MM mice MARCO inhibition (specific antibody): ↑ migration DC to lymph nodes, INF-γ production by $CD4^+$ T cell, therapeutic effect in B16 MM mice	[77–79]

DC, dendritic cells; IL-6, interleukin-6; INF-γ, interferon-γ; LPS, lipopolysaccharide; MM, malignant melanoma; NK, natural killer cells; TNF-α, tumour necrosis factor-α.

Initially, the incidence of melanoma in animals was about 11% [96], but with successive breeding, the incidence increased to more than 70% [97]. In the swine, the following types of lesions were observed: flat lesions, elevated lesions, raised blue tumours, ulcerative tumours, systemic pigmented tumours and congenital tumours [95]. During the growth phase of the melanoma, metastatic spread into the lymph node and visceral organs usually occurred [95,98]. Spontaneous regression of melanoma is accompanied by depigmentation. Depigmentation of the skin and bristles starts on the legs, around the genitalia, around the eyes and naturally around the tumours. Some of the animals have completely gray-white skin at the end of the depigmentation [95].

Munich miniature swine troll

Munich miniature swine Troll has been established at the University of Munich. In this breed, the incidence of melanoma is almost 50%. Melanoma lesions vary from flat or slightly elevated melanocytic nevi to raised nodular invasive melanoma. However, metastases into regional lymph nodes and less frequently into other organs are observed quite often. Invasive melanomas have a pronounced tendency for completely spontaneous tumour regression with local and generalized depigmentation [94].

Melanoma bearing libechov minipigs

MeLiM has been established at the Institute of Animal Physiology and Genetics of the CAS. These pigs have a genetic predisposition for melanoma and therefore this type of tumour has a high occurrence at an early postnatal period. At first glance, the tumours are very dark, almost black, because of the high content of melanin in melanosomes, which contributes towards their strong mechanical resistance [93,99]. An important feature of the MeLiM model is that tumours express typical human melanoma enzymes [99] and melanoma antigens [100]. In animals with cutaneous melanoma organ metastases are also regularly observed, mainly in the spleen, lymph nodes and lungs, and about 5% of the animals die because of the melanoma progression [93,101]. The advantage of this model is the opportunity to monitor continuously the progression of the disease from totally benign to fully malignant lesions, but identification of the different phases of malignancy is still difficult using currently available methods. Furthermore, it is possible to observe different types of lesions in one animal [102].

A characteristic feature of MeLiM melanomas is spontaneous regression, observed in about 75% of afflicted animals [93,103], but currently, there has been an increase in the percentage of regression in animals of up to 90–95%.

Table 3 **Mouse models of malignant melanoma**

Mouse models of MM	Characteristics of models	Advantages	Disadvantages	References
Chemically induced models	Induced by chemical carcinogens – **12dimethylbenz(a)anthracene (DMBA)** and **12Otetradecanoylphobol13acetate (TPA**): used in combination with UV radiation, xenotransplantation or genetic engineering	Fully functional immune system of model (useful for immunotherapeutic studies – vaccines), cytokines, antibodies, used for the development of syngeneic transplantation models	Lack of clinical relevance to human MM	[89]
Syngeneic transplantation models	Harding-Passey melanoma in BALB/c vs. DBA/2F1 mice, Cloudman S91 melanoma in DBA/2 mice, **B16 melanoma in C57BL/6 mice:** derived from chemically induced MM in C57BL/6J mouse, expresses low levels of MHC I molecules, difficult recognition by T_c cells – poorly immunogenic, expresses MM-associated antigens – TRP-2 and/or gp100 – target for autologous T cells, used for testing of immunotherapeutic interventions – cytokines, immune-modulating antibodies, vaccines or their combination	Immunocompetent mice, used for the study and modulation of immune response to MM and for development of anticancer immunotherapy	Lack of knowledge with respect to tumour formation and progression, immunotherapy => tumour protection in numerous animal models vs. sporadic regression of tumours in clinical trial	[89–91]
Xenograft transplantation models	Transplantation of human MM cells into immunodeficient mice (**Nude (nu/nu), SCID, SCID-Beige, NOD, NOD/SCID** and **NSG mic**e): metastatic MM studies	Easy implementation, rapid results	Necessity of immunodeficient mice, limited information on interaction between tumour and host stromal cells	[89,92]
Genetically engineered models	Transgenic and/or knockout mice: activation of oncogenes relevant to human MM (e.g. ***BRAF***, ***NRAS***) and/or inactivation of suppressor genes (e.g. ***CDKN2A*** or ***PTEN***) ***Tyr-SV 40* model** (repression of *p53* and *pRb* genes): first GEM, spontaneous or UV-induced development of MM ***Cdkn2a*$^{-/-}$*, Tyr-HRAS* model:** targeting of more specific MM genes, spontaneous development of MM, no metastases, occurrence of another types of tumours (sarcomas, lymphomas) ***BRaf*^{V600E} model:** study of MM metastases, study of drugs – prevention of development of MM metastases ***HGF/SF*-transgene *Ink4a*$^{ARF-/-}$, *Tyr-N-Ras*^{Q61K} *INK4A*$^{-/-}$, *BRaf*^{V600E} *Pten*$^{-/-}$ models:** metastatic forms of MM (local or distant)	Study of the role of altered genes/pathways – reliable and repeatable models, functional immune system, study of the role of immune system in MM biology and resistance to treatment	Cost and time consuming	[88]

GEM, genetically engineered models; MM, malignant melanoma; UV, ultraviolet.

Conclusion

MM is one of the most uneasily curable form of skin cancer. The incidence of this cancer is still increasing worldwide. The success of MM treatment depends on the stage of the disease at diagnosis. Primary MMs are usually well treatable, but therapy of disseminated MM is often unsuccessful and long-term survival is extremely low.

Spontaneous regression of MM is frequently observed in primary MM, significantly less in metastatic MM. Study and evaluation of the possible mechanisms of spontaneous regression of MM in clinical cases should be carried out in more detail. We hope that application of these 'body's own' mechanisms in MM treatment could reduce the side effects of standard MM therapy (chemotherapy, radiotherapy, etc.)

The phenomenon of spontaneous regression of MM is very important for our Laboratory of Tumour Biology. At the Institute of Animal Physiology and Genetics, Czech Academy of sciences, v.v.i., Liběchov was established as the porcine MeLiM model of spontaneously regressing melanoma. Clarification of the possible mechanisms leading to spontaneous regression of melanoma in these pigs could be potentially useful in the treatment of MM in humans.

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Conflicts of interest

There are no conflicts of interest.

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Review

Advances in Proteomic Techniques for Cytokine Analysis: Focus on Melanoma Research

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Abstract: Melanoma is a skin cancer with permanently increasing incidence and resistance to therapies in advanced stages. Reports of spontaneous regression and tumour infiltration with T-lymphocytes makes melanoma candidate for immunotherapies. Cytokines are key factors regulating immune response and intercellular communication in tumour microenvironment. Cytokines may be used in therapy of melanoma to modulate immune response. Cytokines also possess diagnostic and prognostic potential and cytokine production may reflect effects of immunotherapies. The purpose of this review is to give an overview of recent advances in proteomic techniques for the detection and quantification of cytokines in melanoma research. Approaches covered span from mass spectrometry to immunoassays for single molecule detection (ELISA, western blot), multiplex assays (chemiluminescent, bead-based (Luminex) and planar antibody arrays), ultrasensitive techniques (Singulex, Simoa, immuno-PCR, proximity ligation/extension assay, immunomagnetic reduction assay), to analyses of single cells producing cytokines (ELISpot, flow cytometry, mass cytometry and emerging techniques for single cell secretomics). Although this review is focused mainly on cancer and particularly melanoma, the discussed techniques are in general applicable to broad research field of biology and medicine, including stem cells, development, aging, immunology and intercellular communication.

Keywords: cytokine; cancer; melanoma; secretome; proteomics; T-cell; biomarker; ultrasensitive; immunoassay; mass spectrometry

1. Introduction to Melanoma and Cytokines

1.1. Melanoma

Melanoma is one of the ten most common types of cancer with a steadily increasing incidence. In Europe, 100,300 of the new cases and 22,200 of deaths from melanoma arose in 2012 [1]. In United States melanoma belongs to the 5 most prevalent cancers in males [2].

Melanoma results from malignant transformation of melanocytes, which are naturally occurring pigmented cells in the epidermis. Melanocytes are responsible for the production of an endogenous pigment melanin, protecting the skin from harmful ultraviolet radiation [3]. Melanocytes originate from neural crest. Outside the skin, melanocytes occur in eye, mucosal epithelia and meninges [4] and therefore melanomas can be divided according to the place of origin to the most common cutaneous melanoma, uveal melanoma and mucosal melanoma [5]. Factors influencing the development of

melanoma include: the degree of skin pigmentation, the length of skin exposure to Ultraviolet (UV) radiation [6,7] and hereditary predisposition, as about 10% of melanomas have familial occurrence [8].

To study melanoma pathogenesis and facilitate development of targeted therapies, several animal models have been developed, including mouse, pig, horse, dog, zebrafish, chick embryo (to study neural crest cell migration) and others (reviewed in [9–12]).

1.2. Spontaneous Regression

Melanoma belongs to the tumours with the highest rate of documented spontaneous regression [13]. Spontaneous regression is the partial or complete disappearance of a malignant tumour in the absence of anticancer treatment. Melanoma regression is mostly partial, with rare reports of complete regression (38 documented cases in 2005 [14]). Melanoma regression occurs probably in 3–5% of tumours [15,16], although regression rates up to 50% have been documented [17]. Such a large variation in reported regression prevalence may be caused by various evaluation criteria used among studies [18]. Nonetheless, regression occurs more frequently in early stages of tumours (Breslow's thickness bellow 1.5 mm) [15] and is very rare (0.23%) in melanoma metastases [17].

Spontaneous regression is frequently accompanied by tumour infiltration by CD8+ and also CD4+ T-cells [18] and vitiligo, a hypopigmented skin lesion, which may be associated with an antitumour response to melanocytes [19]. Interestingly, in patients with multiple asynchronous melanomas, the incidence of spontaneous regression was higher in successive melanomas compared to primary tumour, suggesting an increased anti-cancer immunity evoked by the primary lesion [20].

As the immune system plays an active role in melanoma regression, a strong evidence is provided that melanoma would be amenable by immunotherapies [19].

1.3. Melanoma Treatment and Immunotherapies

While early stages of melanoma can be treated by surgical excision, tumours in later stages with vertical growth and metastasis are refractory to therapies. The tumour excision can be supplemented with chemotherapy and/or immunotherapy, as melanoma is typically an immunogenic tumour [16]. First cytokine therapies of melanoma were realized in the 1980s with interleukin 2 (IL-2) and interferon α (IFNα) and up to 20% of tumours showed response to such therapies. However, the therapy was often accompanied by toxic effects, including flu-syndrome, gastrointestinal symptoms and weigh gain but also more serious symptoms like multi organ (heart, lung, liver, kidney and central nervous system) toxicity [21–24].

Nowadays, better therapeutic responses with a lower incidence of side effects are achieved by combination of cytokine application with other approaches, e.g., monoclonal antibodies [25,26], kinase inhibitors [27], tumour-infiltrating T-cell [28], chemotherapeutic agents [29], stereotactic body radiotherapy [30] or peptide vaccines [31,32]. The overall survival of advanced-stage melanoma patients has improved dramatically in the last 5 years with development of immunotherapy by monoclonal antibodies blocking cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell-death protein 1 (PD-1) and with application of B-Raf or MEK kinase inhibitors in BRAF gene mutant tumours [33]. Despite the progress in melanoma treatment, the efficacy of immunotherapies is still unpredictable and many patients eventually develop resistance to treatment [33].

1.4. Tumour Microenvironment

Cells in tissues are living in precisely defined microenvironments, called also the niche or the stroma. Similar to adult tissue stem cells, where the microenvironment is essential for stemness maintenance, the environment in malignant tumours is indispensable for control of the cancer stem cell division and tumour growth [34,35]. Melanoma microenvironment is formed not only by intrinsic malignant cells but also by complex interactions with other cells, such as keratinocytes, cancer-associated fibroblasts (CAFs), immune cells, blood/lymphatic endothelial cells as well as their extracellular products, that all participate in tumour formation and growth [34,36]. To characterize

the tumour microenvironment, Paulitschke et al. analysed proteins secreted by individual cell types using shotgun proteomics [37]. Secretomes of normal skin human fibroblasts, CAFs from mouse melanoma, primary human melanocytes, M24met melanoma cells, human umbilical vein endothelial cells (HUVECs), monocyte-derived dendritic cells and cancer-associated fibroblasts isolated from bone marrow of multiple myeloma patients have been compared. Several extracellular matrix proteins and matrix remodelling enzymes have been commonly identified across samples. Macrophage migration inhibitory factor (MIF) was identified in all samples except of the human fibroblast secretome. Moreover, secretion of Interleukin-20 (IL-20), a structural protein of melanosomes Melanocyte protein Pmel 17, Melanoma antigen gp75 and Melan-A protein were typical for primary melanocytes [37].

Crosstalk between keratinocytes and melanocytes exists already in a healthy skin. After UV irradiation, keratinocytes produce the α-melanocyte stimulating hormone (αMSH) that stimulates melanocytes to produce melanin. Melanin-containing vesicles (melanosomes) are then transferred to keratinocytes, where the pigment protects keratinocyte nuclei from UV-caused DNA damage [4,38]. In melanoma, epidermis surrounding the tumour exhibits increased thickness. Transcriptomic study identified Basic fibroblast growth factor (bFGF), GRO1 oncogene (GROα, CXCL1), IL-8 and Vascular endothelial growth factor A (VEGF-A) to participate in such an effect of melanoma cells on keratinocytes [39].

Fibroblasts are major cells in the connective tissue and are main producers of extracellular matrix components. In healthy skin, fibroblasts also produce factors regulating proliferation, differentiation and survival of melanocytes (e.g., Stem cell factor (SCF), bFGF, Hepatocyte growth factor (HGF), Transforming growth factor-β (TGFβ)) or stimulating melanogenesis (Keratinocyte growth factor (KGF), Neuregulin-1 (NRG-1)) (reviewed in [40]). Normal fibroblasts are also capable of secretion of IL-6, IL-8 and GROα and the interleukin 6 and 8 release significantly increases in presence of either keratinocytes or cancer cells (FaDu hypopharyngeal carcinoma epithelial cells) [41]. Cancer-associated fibroblasts are frequent cells in tumour stroma. *In vitro* co-culture experiments show that CAFs promote migration and invasiveness of melanoma cells and such migration is dependent on IL-6 and IL-8 secretion. Application of antibodies blocking the IL-6 and IL-8 activity fully inhibits the melanoma cell migration *in vitro* [42]. Increased IL-6 and IL-8 expression have previously been well documented to correlate with tumour progression (reviewed in [43,44]). CAFs from melanoma influence also keratinocytes and, among others, induce expression of keratin type 14 (marker of proliferating basal layer keratinocytes) and vimentin (marker of epithelial-to-mesenchymal transition) in keratinocytes [45].

Among the immune cells infiltrating tumour, the T-lymphocytes play a central role in anti-cancer immunity and are thus in main focus of melanoma immunotherapies. The degree of T-cell infiltration and T-cell phenotype in the tumour are important predictors of response of patients to cancer immunotherapy [46]. An effort is applied to the search for melanoma antigen-specific cytotoxic T-cells that could be used in therapy [47]. Adoptive cell therapy with tumour infiltrating T-lymphocytes, isolated from patient's tumour, *in vitro* expanded and applied via infusion, is already showing positive outcomes as an effective treatment for metastatic melanoma [48]. On the other hand, tumour infiltration by immunosuppressive cells, such as regulatory T-cells (Tregs) or immunosuppressive tumour-associated macrophages (M2 TAMs), secreting anti-inflammatory cytokines, such as TGFβ and IL-10 and pro-angiogenic factors, or expressing a PD-ligand, relates to unfavourable prognosis. Such immunosuppressive cells represent targets of potential immunotherapies [49–51]. Other immune cells present in tumour stroma, such as natural killer (NK) cells [52], plasmacytoid dendritic cells [53], B-lymphocytes [54] or others, are less investigated. Nonetheless, immune cell components of malignant melanoma could highlight new predictive biomarkers for response to immunotherapy and indicate new immunotherapeutic approaches [51].

Extracellular products are fundamental parts forming the tumour microenvironment. Not only cellular interactions with extracellular matrix but also enzymes (e.g., matrix remodelling proteases), secreted factors (including cytokines, chemokines, growth factors, angiogenic factors, etc.), extracellular vesicles (EVs), such as exosomes [55,56], EV transferred miRNAs [57], nutrient and oxygen availability [58] and other factors participate in control of tumour progression.

Therapeutic manipulation of tumour microenvironment seems to be a highly promising approach in cancer therapy [35].

1.5. Cytokines

Cytokines are proteins that participate in cell signalling, intercellular communication and in many cellular and immunological functions. Cytokines are produced by a broad range of cells but in oncological research the most attention is paid on cytokines produced by immune cells. Cytokines exert various functions from regulation of inflammatory response, through regulation of cell growth, differentiation, chemotaxis, angiogenesis and many others. From analytical point of view, cytokines represent mostly small proteins (peptides), however, the molecular mass can cover ranges from approximately 6 to 70 kDa [59].

In cancer, cytokines represent key regulators that promote migration, invasion and metastasis of cells. The expression and activity of cytokines are deregulated in many cancer types [60]. Transformed cells produce pro-inflammatory cytokines, chemokines and growth factors that support cell survival and proliferation and promote inflammation and angiogenesis. This results in recruitment of immune and stromal cells into the tumour. Mediators secreted by the growing tumour, including cytokines, further contribute to the cell proliferation, angiogenesis and inflammation but also to a matrix remodelling, adhesive molecule expression changes and increased vascular permeability, leading to a formation of metastatic microenvironment [60–62].

Diagnostic potential and prognostic significance of cytokines in cancer have already been documented. Interleukin 8 is recognized as a chemotactic factor for neutrophils, however, it possesses additional functions in angiogenesis and matrix-metalloproteinase activation. Angiogenesis and metastases of melanoma may be accompanied by secretion of IL-8 from tumour stroma together with its signalling through CXCR2 receptor [62,63]. Serum levels of IL-8 correlate with tumour stage [64] and IL-8 has been suggested as a circulating biomarker of melanoma [65]. Similar to IL-8, production of HGF by stromal cells and activation of Met receptor by HGF, influences melanoma invasiveness. Elevated HGF levels in blood as well as presence of Met-containing exosomes are connected to melanoma metastases and resistance to therapy [66,67]. Chemokines CCL17 (Thymus and activation regulated chemokine (TARC)) and CCL22 (C-C motif chemokine 22) produced by tumour infiltrating macrophages may help to recruit Tregs to tumour and maintain an immunosuppressive tumour microenvironment in melanoma [50]. Another study analysed cytokine profile of cerebrospinal fluid in brain metastasis of melanoma. Elevated levels of IL-8, Macrophage inflammatory protein-1β (MIP-1β), Interferon gamma-induced protein 10 (IP-10) and TARC, have been reported and such molecules represent potential metastasis predicting biomarkers [68].

In a study of therapeutic effects of ipilimumab (monoclonal antibody targeting CTLA-4), an increased secretion of IL-1β, IL-2, IL-4, IL-5, IL-7, IL-8, IL-10, IL-13, IL-17, Granulocyte-colony stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), HGF, IFNγ, Monocyte chemoattractant protein 1 (MCP-1, CCL2) and VEGF from peripheral blood mononuclear cells (PBMCs) showed a trend towards better recurrence free survival in melanoma [69]. Interestingly, these cytokines belong to various functional groups with pro-inflammatory and anti-inflammatory effects [69]. Possibilities of therapeutic administration of cytokines to induce anti-tumour responses in melanoma have been reviewed by Xu et al. [70]. Cytokine employment to melanoma treatment is currently mostly limited to IL-2 [71] and IFNα [72], although attempts of application of GM-CSF encoded by oncolytic virus have been done [73]. Detecting the number of individual cytokines is

useful for planning of targeted therapeutic approaches for melanoma or it can serve as an important element in evaluating of the effectiveness of an ongoing treatment.

The purpose of this review is to give an overview of recent advances in techniques for the detection and quantification of cytokines at the protein level, applicable to melanoma research. Techniques covered span from single molecule to multiplex assays, including emerging ultrasensitive techniques and analysis of individual cytokine producing cells. Developments in technologies of detection from colour enzymatic reaction through chemiluminescence, fluorescence to ultrasensitive antibody detection coupled to PCR amplification, surface plasmon resonance and other electrochemical, optical or mechanical immunosensing led to sensitive detection of picogram to even femtogram per millilitre values. In this review, the term cytokine is used in a broader meaning and covers also chemokines, growth factors, angiogenic factors and other factors, as levels of such molecules reach similarly low concentrations and such molecules often possess a pleiotropic effect that may participate in regulation of similar cellular processes. Although this review is focused mainly on cancer and particularly melanoma research, the discussed techniques are in general applicable to broad research field of biology/medicine, including stem cells, development, degeneration, immunology and inter-cellular communication and signalling.

2. Cytokine Detection Techniques

Cytokines are key players in inter-cellular communication and immune system regulation and an extensive effort is applied on cytokine research in cancer. Significant development of proteomic techniques achieved in the last 10 years led to establishment of powerful tools for cytokine detection and quantification. However, accurate and reproducible measurement of cytokines faces several challenges. These include low cytokine concentrations (reaching picograms per millilitre), dynamics in cytokine secretion in time (transient secretion), cytokine binding to other proteins in the sample or interference with other proteins/antibodies in the assay [74]. Moreover, the biological effect may result from a large number of cytokines that act in complicated regulatory networks [74,75], which places demands not only on simultaneous detection of multiple cytokines but also may lead to convoluted interpretation of proteomic results. Current proteomic techniques for cytokine analysis are based on mass spectrometry and immunoassays.

2.1. Mass Spectrometry

Mass spectrometry (MS) is a fundamental technique in proteomics, applicable for protein identification and quantification. In a classical bottom-up proteomic workflow, proteins are isolated (enrichment or prefractionation step can be added) and digested by specific protease (e.g., trypsin) to smaller peptides. Peptides are then concentrated and desalted, separated by high-performance liquid chromatography (HPLC), ionised and analysed by MS. Tandem mass spectrometer (MS/MS) measures exact mass-to-charge (m/z) ratio of peptides (precursor ions) in MS experiment and their fragment ions in MS/MS experiment. This approach allows very specific identification and quantification of peptides/proteins.

Despite being a powerful proteomic technique, application of MS to cytokine analysis is challenging. Several limitations of MS should be considered: (1) MS is able to detect only charged (ionised) peptides but not all peptides are ionised with the same efficiency. Moreover, proteins with low molecular weight (including most cytokine molecules) provide less peptides and taken together with low abundance, these peptides will not be detected without enrichment or prefractionation [76]. (2) Sensitivity of shotgun MS is usually insufficient for detection of low abundant proteins (such as cytokines) in complex biological samples (blood serum or plasma) without employment of sample depletion or prefractionation. For example, the concentrations of serum albumin and IL-6 in human plasma differ by 10 orders of magnitude [77]. (3) MS-based approach is highly reproducible but quality controls (e.g., internal standard proteins/peptides) are essential for control of protein digestion efficacy, HPLC peptide separation and MS performance. Shotgun proteomics (data dependent acquisition

(DDA), see below) is partially stochastic, because precursor ions are selected for fragmentation by computer-controlled algorithm according to ion signal intensities, thus slightly different lists of identified proteins may yield from repeated analysis of the same complex sample, especially in case of low abundant proteins. On the other hand, targeted methods (selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), see below) do not suffer this issue and offer robust and reproducible analysis [76]. (4) Protein identification depends on protein databases and may suffer from insufficient coverage for non-human organisms (e.g., animal models used in biomedical research). As an example from our experience, the Swiss-Prot database (UniProt Release 2017_10) contains 1421 reviewed proteins for pig (*Sus scrofa*), compared to 20,239 reviewed proteins for human (*Homo sapiens*). Although there is a possibility to use reference proteome based-on genome assembly to get deeper coverage, data has to be evaluated carefully, as protein duplications or errors in protein sequence occur.

Taken together, MS approaches provide relatively easy multiplexing capability and higher specificity but lower sensitivity than immunoassays.

2.1.1. Data Dependent Acquisition (DDA)—Shotgun Proteomics

Shotgun proteomics aims to identify all (detectable) proteins that are present in a sample without prior knowledge or pre-selection of analytes. Shotgun proteomics requires use of tandem mass spectrometers and the method works in cycles. Firstly, a scan for precursor ions is done (MS experiment). Then a predefined number of precursor ions with the highest signal intensity is automatically selected for fragmentation. The resulting fragment ions are then analysed in a MS/MS experiments. When all precursor ions meeting selection criteria are fragmented and analysed, new cycle starts. Depending on the sample, tens to thousands of proteins can be identified or quantified by such an approach. However, due to partial stochasticity of ion selection for fragmentation, it is necessary to verify quantification results of DDA measurement by an independent method (e.g., western blot).

Although shotgun proteomics is capable of detecting analytes in the femtomole or attomole range, its sensitivity is mostly insufficient for detection of cytokines in blood plasma [77]. However, cytokines might still be detectable by MS in culture media conditioned by cells *in vitro*, as the culture medium (particularly a serum-deprived formula) represents less complex sample and as the cytokine production *in vitro* may be higher (due to more or less uniform cell populations cultured in a limited volume) and more synchronized (e.g., by cell activation). Nonetheless, the cytokine production by cultured cells may not fully resemble the *in vivo* physiological state.

Shotgun analysis of secretomes of melanoma cell lines from three different metastases of the same patient was performed by Rocco et al. [78]. Cytokine Growth/differentiation factor 15 (GDF15) was identified in all three secretomes. Moreover, Melanoma-derived growth regulatory protein (MIA) and Osteopontin (SPP1) were identified in secretome of one particular metastatic cell line [78]. Several cytokines, including GDF15, IL-6, IL-8, IL-18 and MIA, have been identified by shotgun MS in a study of secretomes of lung metastases derived from melanoma and breast cancer [79]. Interestingly, the GDF15, also called Macrophage Inhibitory Cytokine-1 (MIC-1), has been previously recognized as a promising biomarker of metastatic melanoma [80].

2.1.2. Data Independent Acquisition

Data independent acquisition (DIA), in contrast to DDA, provides information about quantity of all detected proteins, without specific selection of certain proteins (peptides) for analysis. DIA, for example Sequential Windowed Acquisition of all Theoretical Mass Spectra (SWATH-MS) method, is used for a discovery-based approach. All precursor ions in a specific window of m/z values in MS experiment are fragmented together and these fragments are detected in MS/MS experiment. This approach allows for identification and quantification of peptides, which would not be selected for fragmentation and thus remained undetected in DDA analysis. Assay library is essential for identification of peptides from such complex spectra from MS/MS experiments. The library is generated by shotgun measurements of the same samples, or for deeper coverage of proteome,

assay library can be prepared by measurements of many samples of different cell types, tissues or sample fractionations. Human proteome assay library containing more than 10,000 proteins is publicly available [81]. SWATH-MS approach was shown to be reproducible with high selectivity, accuracy and sensitivity [82]. Capability of SWATH-MS to serve as a tool in search for biomarkers, even in a clinical research, was reviewed recently [83].

The DIA method enabled identification of several growth factors and cytokines in medium conditioned by malignant cells. Lin et al. analysed glycoproteins in secretomes of colon adenocarcinoma cell line and its derived metastatic cells [84]. They identified 568 proteins in total, with 421 proteins suitable for quantification, including cytokines such as GDF15, Insulin-like growth factor II (IGF-II), Macrophage colony-stimulating factor 1 (M-CSF) and TGFβ-1. Study of secretomes from malignant mesothelioma cell lines and mesothelial cells revealed 421 secreted proteins including M-CSF, MIF and TGFβ-1 [85].

2.1.3. Selected Reaction Monitoring and Multiple Reaction Monitoring

SRM and MRM approaches have been developed for targeted analysis and quantification of selected protein(s) in a complex sample. Such approaches rely on a specific tandem MS instrument—triple quadrupole (QQQ). First quadrupole (Q1) selects precursor ions of particular m/z, which are then passed to the second quadrupole (Q2) that serves as a collision cell, where precursor ions are fragmented by collisions with molecules of inert gas (e.g., helium). The third quadrupole (Q3) then selects specific type of fragment ions of particular m/z (Figure 1). The first and third quadrupole thus works as m/z filters analysing predefined pair of precursor and fragment ions, so-called transition. This approach cannot be used for discovery analyses, because this method enables measurement of predefined transitions only. Specificity of the measurement is assured not only by Q1 and Q3 but also by elution of peptides from HPLC system (retention time). Addona et al. proved that MRM approach with standardized protocol is robust and reproducible between different laboratories and MS platforms [86].

MRM method is capable to absolutely quantify 142 proteins (312 peptides) from non-depleted and non-enriched human plasma in concentration ranges 31 mg/mL to 44 ng/mL in one 43 min analysis [87]. Interestingly, purchase of a MS instrument for MRM measurements represents approximately same expense as a development of five new ELISA assays [88].

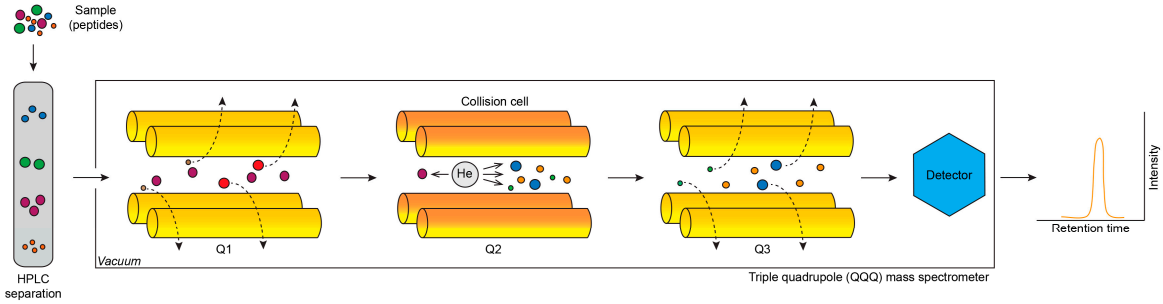

Figure 1. Principle of SRM assay for targeted protein quantification by mass spectrometry. Peptides from sample are separated by reversed-phase HPLC and eluted with specific retention times. Ionised peptides are analysed by triple quadrupole (QQQ) MS instrument. In quadrupole Q1, particular peptide with preset m/z value is selected (precursor ion, purple colour) and passed to Q2, whereas other ions are filtered out (dashed line arrows). In Q2, fragmentation of the selected precursor ion occurs, due to collisions with molecules of inert gas (helium—grey colour). Originating fragment ions are filtered by Q3 (dashed line arrows) and only fragment ion with preset m/z value (yellow colour) is passed to detector, where fragment ion intensity is recorded. As a preset pair of precursor and fragment ion with particular m/z (transition) is measured, the SRM method is very specific.

In focus on low abundant proteins, Bredehöft et al. developed MRM method for quantification on human IGF-1 after its immunoaffinity isolation from plasma samples with detection limit 20–50 ng/mL [89]. Anderson et al. used Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) approach to purify peptides of IL-6 and tumour necrosis factor-α (TNFα) from human plasma samples and employed SRM for their precise quantification [90]. The SISCAPA method was later used for quantification of other cytokines (e.g., G-CSF and Interleukin-1 receptor antagonist (IL-1ra)) with detection limits approximately 1 ng/mL in human plasma [91].

2.1.4. Immunoassays Coupled with MS Analyses

Sherma et al. described Mass Spectrometric Immunoassay (MSIA) approach for detection and quantification of MIF in human serum samples [92]. MIF was firstly enriched by MIF antibodies and then measured by MALDI-TOF (Matrix-assisted laser desorption ionisation-Time-of-flight) MS instrument. This method allows for quantification of MIF in concentration 1.56–50 ng/mL with a good correlation to ELISA. Moreover, different proteoforms of MIF (posttranslationaly modified, e.g., by cysteinylation) could be identified and quantified [92].

Direct immunoaffinity desorption/ionization (DIADI) MS is a recently developed method involving affinity capture of analytes by specific antibodies on protein chip and detection/quantification by MS instrument [93]. Protein chips (e.g., antibodies, lectins etc.) are prepared by ambient ion landing on MALDI plate. Samples are then added on the MALDI protein chip, covered with matrix and analysed by MALDI MS instrument. For example, detection limit for leptin in serum is 160 ng/mL. Performance of this approach was proved by detection of haptoglobin in human serum from 116 patients [94]. This method allows to distinguish two isoforms of haptoglobin with affinity to the same antibody.

2.2. Immunoassays for Quantification of Secreted Cytokines

Proteomic techniques for cytokine detection/quantification rely mainly on immunoassays. Immunoassays take advantage of high sensitivity of antibodies to detect proteins of interest. In the detection of cytokines secreted to body fluids or cell culture media, common immunoassays mostly reach sensitivity in 1–100 pg/mL protein concentrations that is 10^9 times lower than concentration of the most abundant blood plasma proteins. Sandwich immunoassays in various formats are mostly used for secreted cytokine quantification (Figure 2).

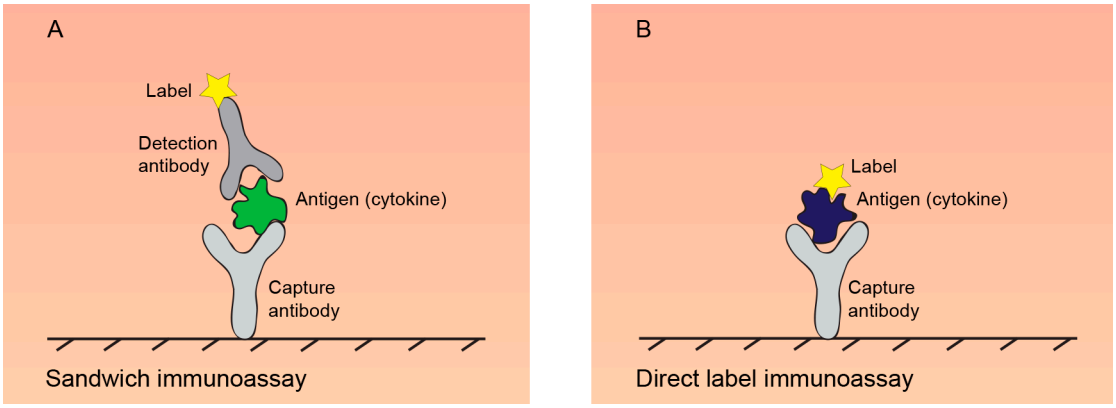

Figure 2. Principles of common immunoassays. (**A**) Sandwich immunoassays use matched pair of capture and detection antibody. Capture antibody is immobilized on a solid support, such as in 96-well plate, on beads, glass or a membrane array. Detection antibody conjugated to an enzyme, fluorescent tag or DNA tag, enables the quantification of cytokine; (**B**) in direct label assays, the proteins in analysed sample are labelled by fluorescent tags and incubated with an array of antibodies.

Quantification of cytokine levels by immunoassays usually depends on calibration curves, constructed from signal intensities of known standard concentrations. The availability of epitopes and specific/nonspecific binding of antibodies to antigens are strongly influenced by the sample composition (e.g., presence of other proteins, ions, salts, pH, or sample viscosity). Minimizing of such matrix effects, e.g., by resembling the sample matrix composition (sample background) also in calibration standards, or by sample dilution, is a prerequisite for low assay background and proper quantification. Moreover, suitable controls, e.g., negative, positive, in-house quality controls (sample that has been previously measured) or even spike-in controls should be included to validate assay performance. Following sections list immunoassays in the order based on the level of multiplexing, i.e., the number of simultaneously measurable analytes.

2.2.1. ELISA

ELISA (enzyme-linked immunosorbent assay), developed in 1971 [95,96], is a crucial technique for single analyte quantification in basic research and also in clinical settings. The sandwich ELISA, due to combination of two specific antibodies, enable analysis of complex samples with high specificity for analyte detection without the need of sample pre-fractionation [97]. The sample is incubated with a capture antibody immobilized in 96-well plate. Then, the sample is washed out and the detection antibody is added. Detection antibody can be directly conjugated to an enzyme, or additional secondary detection antibody-enzyme conjugate is used. After addition of substrate, the enzyme produces a coloured product that is detected in the microplate reader. The capture and detection antibodies must be validated to work together (to bind to different epitopes on the antigen). In cytokine analysis, the commercial ELISA ready-to-use kits with optimized antibody pairs are available for broad spectrum of targets and species.

ELISA immunoassays are quite popular because of their high specificity, sensitivity, rapid turnaround time, convenience, the ease of performance and a relatively low cost [98]. However, in the need of multiple analyte measurement, running of multiple ELISAs places substantial demands on time, costs and sample amounts. The multiplex techniques may overcome such issues.

2.2.2. Western Blot

Despite being developed almost 40 years ago [99], western blotting still represents a key and powerful biochemical technique for protein detection and relative quantification. Unlike ELISA, which is suitable for cytokine detection in solution, western blotting is preferably suited for analysis of intracellular cytokines and cytokines in complex biological samples and tissues. Western blotting is based on separation of denatured proteins in polyacrylamide gels followed by protein transfer to nitrocellulose or polyvinylidene difluoride membranes and detection by specific antibodies. Although the western blotting may not be as sensitive as ELISA in cytokine detection, it represents irreplaceable technique that provides additional information of protein molecular weight. Thus, western blotting can be applied to distinguish splice variants or detect cytokine molecule degradation and can also distinguish inactive precursors from the active forms. For example, IL-1β, a pro-inflammatory and pleiotropic cytokine abundant in tumours, is produced as 31 kDa precursor that is further processed by caspase-1 to a mature active 17 kDa form [100]. Western blotting enables to distinguish processed and unprocessed caspase-1 and/or IL-1β molecules and indirectly provides information about protein activation [101,102].

Western blotting can be also applied to study protein phosphorylation using phospho-site-specific antibodies. For instance, such approach was applied to study VEGF receptor phosphorylation in uveal melanoma cell lines to monitor effects of VEGF-A and its inhibitor (bevacizumab) on cell proliferation, migration and invasion and production of other cytokines [103]. Protein phosphorylation and intracellular signalling pathways can be effectively studied by multiplex techniques and various kits are offered on market.

Techniques for subcellular fractionation prior cytokine detection can distinguish nuclear and cytoplasmic localization [104,105]. Most of the cytokines are localized in the cytoplasm of the producing cell before secretion. However, for some cytokines, such as IL-1 family members IL-1α [106], IL-33 and IL-37 [107–109], nuclear localization has been detected. Nuclear localisation of IL-1α is mostly observed in resting cells, where IL-1α is probably acting as a transcription factor regulating cell growth and differentiation [100,109]. Similarly, nuclear localisation of a potent pro-inflammatory cytokine TNFα was detected. The TNFα release from stimulated cells could be blocked by leptomycin B, a specific inhibitor of nuclear protein export [110].

2.2.3. Electrochemiluminescence Immunoassays

Electrochemiluminescence (ECL) is an electrochemical reaction converting electrical energy to emission of light. ECL can be used for sensitive detection of biomolecules (reviewed in [111–113]). The first ECL immunoassay has been developed in nineties [114], utilizing ruthenium complexes for electrochemiluminescence reaction [115]. Currently, ECL is widely used in routine and custom immunoassays in clinical, preclinical and research testing. The research ECL immunoassay platforms are commercially available from Meso Scale Diagnostics (MSD) Company (Rockville, MD, USA). Their Multi-Array technology allows simultaneous quantification of one to ten analytes using a convenient format of 96 or 384-well plates. In the bottom of each well, carbon electrodes are placed, each pre-coated with one or several anti-cytokine capture antibodies. After incubation with sample, the detection antibodies, conjugated to an electrochemiluminescent label (sulfo-tag), are added. The sulfo-tags emit light when electricity is applied on the plate electrodes and light intensity is measured to quantify analytes in the sample. The Multi-Arrays ensure low background, as only labels near the electrode surface are detected. The arrays provide high sensitivity, broad dynamic range of quantification and similar results of absolute quantification, comparable to bead-based cytometric assays [116–118]. Kits for detection of hundreds of analytes are available with additional possibility to build custom assays.

ECL immunoassays, using both clinical or research platforms, find broad application in cancer research to detect tumour antigens (e.g., CEA, CA-125, PSA) and have also been applied to study cytokines associated with the disease [119–122].

2.2.4. Antibody Arrays

In the past 10 years, popularity and availability of multiplex immunoassays, that enable quantification of up to tens (theoretically of hundreds) of analytes in a single measurement, have grown rapidly. The reasons arise mainly from the fact that single cytokine is often insufficient as a true disease biomarker. As molecules and cells act in complicated networks and regulation of multiple entities leads to the observed effect, a more global view requiring multiplex platforms is needed to understand true disease biology [123]. Simultaneous measurement of multiple analytes saves time, costs and, importantly, sample requirements. Antibody arrays are mostly used in form of bead-based arrays (such as Luminex assays, Austin, TX, USA) or planar arrays (antibody arrays and antibody microarrays) [123,124].

Bead-Based Arrays

Bead based multiplex assays belong to the most used formats of multiplex cytokine assays, due to their high accuracy, sensitivity, reproducibility, broad dynamic range of quantification, high throughput and capability to quantify multiple analytes using as low as 25 to 50 μL of the sample. Such arrays have been built on the principle of sandwich ELISA, with the capture antibodies immobilized on microbeads. The microbeads possess internal fluorescence label that is unique for each measured analyte. The detection antibodies are mostly biotinylated and a streptavidin-phycoerytrhin conjugate is used for cytokine detection and quantification. Cytokine measurement is performed in a flow cytometer or dedicated Luminex instrument based on 2 measured signals. In the typical Luminex

settings, the red laser (classification laser) excites the bead internal dye to identify each microsphere particle (analyte). At the same time, the cytokine amount is quantified from fluorescence intensity of phycoerythrin, which has been excited by a green laser (detection laser).

Two types of microbeads may be used in the assays, polystyren or magnetic beads. Polystyren beads were historically the first developed for Luminex assays. However, their incubation and washing using filter 96-well plates may be accompanied by leaking and clogging problems, leading to reduced accuracy and precision [125]. Such problems have been overcome by development of magnetic beads, with fast and highly effective washing performed by hand-held magnet or automated magnetic plate washer.

Using Luminex assays, theoretically up to 100 analytes can be measured simultaneously. However, to avoid cross reactivity of antibodies and incorrect results, practical level of multiplexing reaches around 30 analytes in one panel. Combination of panels leads to hundreds to thousand detectable analytes. Currently, kits for human, mouse, rat, canine, monkey and porcine samples are available to cover various model organisms, with permanently growing number of covered analytes and species. Moreover, custom assays can be built by antibody coupling to magnetic beads and Luminex Corp. provides support for their customers in custom assay development.

Detection range of bead-based arrays spans over 3–4 orders of magnitude with the sensitivity reaching low picogram/mL concentrations. As in other immunoassays, good pipetting technique is critical to ensure high precision and accurate results. Using precise reversed pipetting and optimized dilution of standards, the lower limit of detection may be expanded below 1 pg/mL for some cytokines (Figure 3) [126]. Correct quantification at the lower end of the calibration curve is often crucial for setting a baseline of the control samples, e.g., in analysis of healthy control human samples or *in vitro* cultured unstimulated cells and non-immune cells, where the cytokine production is mostly low. In such cases, concentration of conditioned cell culture medium prior analysis may seem to be advantageous to increase cytokine concentrations. However, the sample concentration (e.g., by 3 kDa MWCO centrifugation filters) is not bringing the expected benefits. In our experiments, concentration of culture medium samples to 10% of its original volume led to only 1–5 time increase of the cytokines compared to original unconcentrated sample. Cytokine molecule degradation, adhesion to the filter, protein aggregation/precipitation or other factors may participate in the protein loss. Opposite situation occurs in plasma or serum samples, where increasing sample concentration is practically impossible due to already high protein content. Interestingly, from our experience, 2-fold diluted serum or plasma provide better cytokine concentration readings (e.g., lower variation of sample replicates) than use of undiluted samples. Sample dilution may lead to reduction of sample viscosity ensuring better availability of epitopes to antibodies. Reduction of matrix effect in diluted samples was observed also by Rosenberg-Hasson et al. [127].

Bead-based arrays find broad applications in cancer research, including melanoma research. Immunotherapeutic predictive or prognostic values of serum cytokines were tested in melanoma patients treated by adjuvant interferon [128] and ipilimumab [69]. Blood plasma cytokines have been analysed in patients with unresectable in-transit extremity melanoma, treated by isolated limb infusion with melphalan [129]. In uveal melanoma, bead-based arrays have been used to monitor response of PBMCs cells to stimulation by differentiation and tumour antigens [130] and to detect cytokines secreted into vitreous humour [131]. At the cellular level, Luminex assay was used to map cross-talk between fibroblasts and epithelial cells [41].

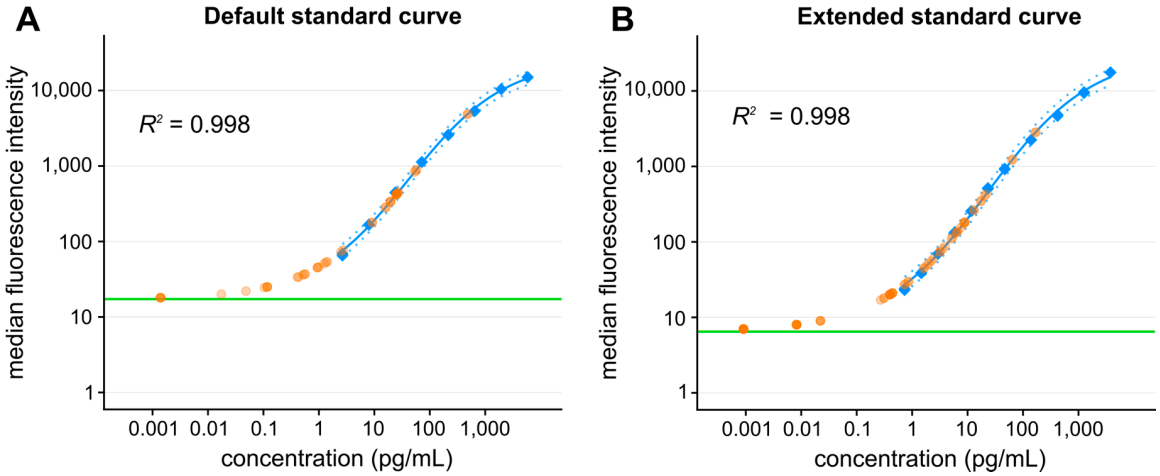

Figure 3. Optimization of standard curve to detect low amounts of IFNα by Luminex array. (**A**) Typical calibration curve covers 3–4 LOGs of cytokine concentration; (**B**) for some cytokines, additional data points at the lower end of the curve enable quantification of low cytokine concentration in samples and expansion of quantification over 4 orders of magnitude in cytokine concentration. Standard curves are calculated by drLumi package [132] in R statistical environment, full line shows standard curve fit to calibration standards (blue diamonds), dotted lines show prediction interval. Data points of real samples of experimental pig model body fluids are shown as orange circles. Three circles with the lowest IFNα concentration represent healthy control untreated animals.

Planar Antibody Arrays

Antibody arrays are widely recognized as a reliable and robust methodology for mining multiplex data from complex proteomes with high sensitivity, high specificity and high throughput [123]. The first antibody arrays have been developed by Chang in 1983 by spotting antibodies in 10 × 10 and 20 × 20 grids on 1 cm^2 area of glass cover slips [133]. Current arrays are mostly based on sandwich or direct label layout (Figure 2). Sandwich arrays use antibody pairs that have to be validated and checked for cross-reactivity to every other antigen/antibody in the array. From that reasons, practical sizes of the arrays mostly cover between 10 and 80 analytes. By combination of several arrays, detection and quantification of up to 1000 secreted human proteins, such as cytokines, chemokines, adipokines, growth factors, proteases, soluble receptors and other proteins, in human samples can be achieved [134]. In research on animals, up to 200 mouse, 67 rat, 50 porcine, 30 bovine, canine and rabbit, 20 ovine and 10 equine, feline, Rhesus Monkey, chicken and dolphin cytokines can be currently quantitatively measured by combination of antibody arrays (data based on on-line catalogues of major producers Raybiotech, Abcam and RnD).

Antibodies are printed mostly on nitrocellulose membrane or glass slide as a solid support (Figure 4). Membrane arrays are usually semi-quantitative (enable fold-change readings) with chemiluminescent or fluorescent detection and are easily processed similarly as western blot membranes without the need of specialized laboratory equipment. Glass slide arrays accommodate denser antibody prints and are mostly quantitative with fluorescent detection. Scanning of fluorescent arrays requires array scanner but is commonly offered as a payed service. For easy handling, glass slide arrays mostly consist of 2 × 8 subarrays, arranged in the format of 2 columns of the 96-well plate, enabling analysis of up to 16 samples or calibration standards on one slide. Four slides can be adopted into a frame resembling 96-well plate, enabling automation. Antibodies are spotted in several replicates for variability evaluation and proper quantification.

Bead-based and planar antibody arrays have been utilized or are potentially applicable to a wide range of cancer studies, including search for diagnostic and prognostic biomarkers, evaluation of antitumour treatment efficacy, understanding of the biology underlining tumourigenesis and tumour progression [123,135,136], including mapping the role of tumour microenvironment and inter-cellular cross-talk [137].

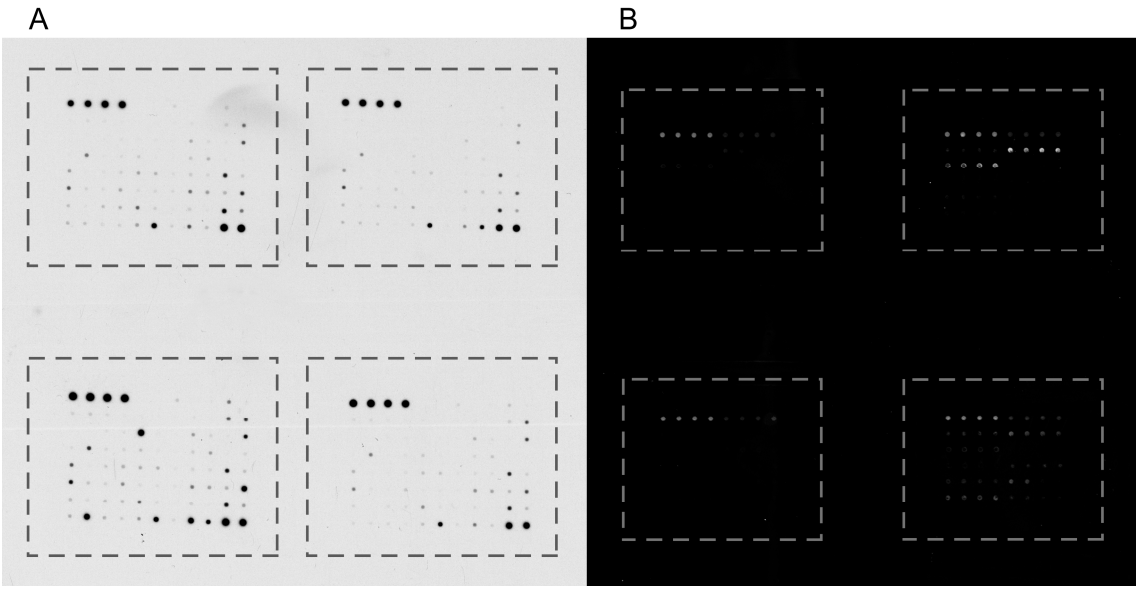

Figure 4. Planar antibody arrays for detection of secreted cytokines. (**A**) Membrane array with chemiluminescent detection; (**B**) glass array with fluorescent detection. Positions of individual subarrays (4 individual samples) are highlighted by dashed line.

2.3. Emerging Techniques for Ultrasensitive Detection of Secreted Cytokines

Current technological improvements led to development of ultrasensitive techniques that significantly enhance the detection of analytes at low concentration in pg/mL to fg/mL range [138–140]. Beside cytokine analysis, the ultrasensitive techniques are designed for detection of biomarkers present in the blood plasma/serum at very low levels to facilitate diagnostics of early stages of diseases, such as in neurodegenerative [140], cardiovascular diseases [141], inflammation or cancer [142]. Ultrasensitive methods are proposed to improve sensitivity, selectivity, simplicity and minimize the sample matrix effects together with minimizing the sample volumes. In addition to traditional colorimetric, chemiluminescent and fluorescent detection, the emerging technologies rely on electrochemical, optical [143], mechanical or surface plasmon resonance [144] biosensing, reviewed in [74,142,145–147]. Developments in nanotechnologies enable construction of microfluidic devices and biochips [74]. Moreover, concentration of an analyte to minimal volumes using femtoliter-sized wells, optical fibres, microfluidics compartments, or nanodroplets enables readout at the level of single events [148,149]. Combination of microfluidics and nanotechnologies with a variety of ultrasensitive reporters has a potential to reach unprecedented sensitivities approaching 1000 protein molecules per microliter, i.e., the aM–fM range [142,150]. However, most of the emerging techniques utilize purified proteins and simple sample matrices. The robustness of such techniques in the context of complex biological samples still remains to be demonstrated before their potential application in biological research and clinical practice [142].

Selected more or less established ultrasensitive technologies are mentioned in the following paragraphs. Due to the large variety of emerging technologies for cytokine biosensing, it is out of the scope of this review to provide global information on all of them. Several reviews have been published in the last 3 years, where the more detailed information on biosensing can be found [59,74,143–146].

2.3.1. Single Molecule Counting (Singulex)

Single Molecule Counting (SMC) technology, originally developed by Singulex Inc. (Alameda, CA, USA), combines bead-based immunoassay with Single Molecule Counting detection [151,152]. Classical 96-well plate format bead-based immunoassay is used to form sandwich complexes (magnetic bead coated by capture antibody—antigen—fluorescently labelled detection antibody). Then, the sandwich complex is disrupted and eluted fluorescently-labelled detection antibodies are analysed using proprietary digital SMC technology. SMC counts single molecule fluorescent signals ("flashes") using laser confocal microscope digital counter. SMC assay kits offer detection of cytokines in human samples with sensitivity in sub-pg/mL levels. Custom assays for various analytes can be built. Adaptation of the SMC technology employing 3 different lasers enabled multiplex (3-plex) detection of IL-4, IL-6 and IL-10 levels in blood plasma of healthy donors with single pg/mL sensitivity [153].

2.3.2. Single Molecule Array (Simoa)

Single Molecule Array (Simoa), developed by Quanterix Corp. (Lexington, MA, USA), is a digital ELISA that reaches astonishing sensitivity up to sub fg/mL concentration [138]. Simoa is based on classical sandwich immunoassay on magnetic beads followed by digital detection. Antigen is captured by an antibody immobilized on magnetic microbeads and by addition of biotinylated detection antibody and streptavidin-labelled enzyme, an immunocomplex is formed. Beads are then loaded into a microwell array consisting of 50,000 wells (46 fL wells, each can accommodate only one bead) and sealed in the presence of fluorogenic substrate. Due to extremely small reaction volume, the concentration of fluorescent product easily reaches a detectable range even if only one enzyme molecule is present in the well [154]. Very low analyte concentrations, i.e., zero or one antigen on each bead, can be directly determined from number of fluorescent wells and total number of beads in the array by digital readout (0 or 1 signal). In higher analyte concentrations, direct quantification from fluorescence intensity (analogue readout) is applied. Simoa is open for multiplexing by using beads labelled by distinct fluorescent dyes [155] and currently up to 10 analytes can be simultaneously measured. Multiplex Simoa for simultaneous detection of 6 cytokines showed 0.01–0.03 pg/mL limit of detection [156]. In another study, Simoa assays for 10 cytokines in human serum samples have shown limit of detection between 0.001–0.217 pg/mL [154]. Kits for various analytes are available from Quanterix Corp.

2.3.3. Immuno-PCR

ImmunoPCR, originally described in 1992 [157], takes advantage of high specificity of immunoassays and high sensitivity of polymerase chain reaction (PCR). Polymerase enzyme allows for an exponential signal amplification compared to the linear relation between substrate and product in conventional ELISA and provides up to 1000× higher sensitivity than common ELISA [158–160].

ImmunoPCR in sandwich format, identically to sandwich ELISA, is performed in 96-well plate coated by the capture antibody. Unlike to enzyme-antibody conjugates used for detection in ELISA assay, the immunoPCR utilizes detection antibody covalently conjugated to DNA and detected by quantitative PCR. Commercial ready-to-use covalent conjugates (Imperacer®, Chimera Biotec, Dortmund, Germany) or kits for their production are now available [159]. Detailed protocol for immunoPCR assay development for IL-6 in plasma was described by [161]. Applications of immuno-PCR for the detection of early stage cancer were currently reviewed in [162,163].

Although the merits of immuno-PCR are obvious (e.g., ultra-sensitivity, good reproducibility and universality), its main limitation is a high background and the need of extensive washing steps [160]. Proximity ligation assay (PLA) and Proximity extension assay (PEA) have been developed recently to reduce nonspecific binding and shorten processing time of immunoPCR.

2.3.4. Proximity Ligation Assay

Proximity ligation assay (PLA) was originally described by Fredriksson et al. [164], who used a DNA aptamer with affinity to platelet-derived growth factor (PDGF) protein to quantify PGDF. Homodimer PDGF-BB can accommodate two aptamer molecules, each of them having extensions for primer binding and additional extension to be joined by ligation upon hybridization to a common connector oligonucleotide. Real-time detection of PCR products could detect as low as 10^{-20} molar protein concentrations [164].

In current proximity ligation assays, antibodies binding pairwise to adjacent epitopes of target proteins are used. In case of direct PLA, such antibodies are biotinylated and combined with oligonucleotides covalently attached to streptavidin at either their 5′- or 3′-ends. In indirect PLA, unmodified primary antibodies risen in 2 different species are used, which are detected with secondary antibodies conjugated to the DNA strand [165].

Kits and reagents are now commercialized, including Duolink® (Merck, Darmstadt, Germany), TaqMan® Protein Assay (Thermo Fisher Scientific, Waltham, MA USA), ProQuantum High-Sensitivity Immunoassays (Thermo Fisher Scientific) or Proximity Ligation Assay (Abnova, Taipei, Taiwan). The applications of PLA enable also studies of protein interactions, phosphorylations or *in situ* subcellular localisation.

2.3.5. Proximity Extension Assay

Proximity Extension Assay (PEA) is an alternative to PLA. The main difference between the two techniques is that the ligation event of PLA is replaced by a DNA polymerisation step in PEA, which minimizes the background noise and improves assay sensitivity [165]. PEA assays use matched antibody pairs linked to either 3′ or 5′ ends of unique single-stranded DNA sequences. The other (unoccupied) ends of ssDNA are complementary, allowing for pair-wise annealing with the other oligonucleotide and extension by a DNA polymerase. Each oligonucleotide contains a primer-binding site. Thus, only in case of specific binding of both antibodies to one protein and complementary ssDNA sequence hybridization, the PCR product is formed and quantified by qPCR. Such PEA has been commercialized by Olink (Uppsala, Sweden). Unlike conventional immunoassays, where multiplexing degree is limited by unspecific binding (cross reactivity) of antibodies, in PEA only matched DNAs are amplified, which enables high specificity and high degree of multiplexing. The Proseek Multiplex panels (Olink) enable analysis of 92 protein biomarkers across 96 samples simultaneously, using 1 µL of sample.

2.3.6. ImmunoMagnetic Reduction Assay

Magnetic susceptibility reduction as a bioanalytical technique was described in 2006 [166], where biotinylated magnetic nanoparticles were used to detect avidin. Current immuno magnetic reduction (IMR) assays are using capture antibodies immobilized on magnetic nanoparticles. Such nanoparticles are homogeneously dispersed in a solution and oscillate by application of external multiple alternating current (AC) magnetic fields (i.e., are susceptible to the magnetic field). Upon addition of the analysed sample, nanoparticles become heavier due to the capture of analyte molecules by antibodies. This results in reduced response of nanoparticles to the magnetic field. Degree of reduction of AC magnetic susceptibility corresponds to the amount of the target molecules present in the sample. Highly sensitive magnetometer detectors enable to detect proteins at pg/mL concentrations [167].

The main advantage of the IMR assay is that it is simple technique that does not need washing steps to remove unbound reagents. Moreover, the assay is highly sensitive and quantitative. For example, concentration ranges 1–50,000 pg/mL and 0.3 fg/mL–300 pg/mL of amyloid-β1-42 and α-synuclein, respectively, could be covered [147,167]. Unlike sandwich immunoassays, the IMR assay uses just one antibody (“capture” antibody). The assay specificity is ensured by oscillatory

movements of nanoparticles, where weak non-specific antigen-antibody interactions are disrupted due to centrifugal forces evoked on nanoparticles by application of magnetic fields.

The IMR assay is currently aimed at identification of early stages of diseases, such as neurodegenerative diseases [147,167,168], cancer [169–171] and viral infections [172]. In cancer research, assay kits for VEGF [169] and carcinoembryonic antigen [170] are available.

Despite sensitive and elegant cytokine quantification by immunotechniques, the absolute cytokine concentrations should be interpreted with caution. An extensive inter-assay comparison of 9 emerging or well-established platforms for cytokine quantification was performed by Yeung et al. to evaluate 4 selected cytokine (IL-6, TNFα, IL-17a, IL-2) levels [173]. Despite using the same reference set of human serum samples, different immunoassays yielded different quantitative results. With a primary focus on assay sensitivity and accuracy, Simoa (Quanterix) and Erenna (Singulex) platforms showed the best performance, followed by V-plex (MSD) and Ella (ProteinSimple; an automated ELISA using microfluidic cartridge) assays [173]. Similar comparison of 14 immunoassays (ELISA, bead-based arrays and MSD) with absorbance, chemiluminescence, electrochemiluminescence and fluorescence detection of IL-1β and IL-6 showed significant inter-laboratory and inter-assay variations [174].

2.4. Single-Cell Analyses

Immunoassays are frequently aimed at measuring cytokine production by large numbers of cells, such as cytokines released into the tumour tissue, to body fluids (e.g., blood) or cytokines released by cells cultured *in vitro*. Thus, the cytokine producing cell populations are mostly heterogeneous and only the average response of cells is measured. Analysis of rare subsets of cells (like antigen-specific T-cells or B-cells) by common methods is challenging, if not impossible, as such cells contribute only a minor component of the total measurement [175]. Thus, techniques to detect cytokine production by single cells have been developed.

2.4.1. ELISpot

Enzyme-linked ImmunoSpot (ELISpot) assay has been originally developed for detection of antibody producing cells [176]. Later, ELISpot was adapted to enumerating of activated T-cells secreting IFNγ among human peripheral blood lymphocytes [177]. Since then, ELISpot became popular as an easy and highly quantitative assay for detection of single cell producing cytokine and monitoring cellular response to various stimuli [178].

In this technique, cells are incubated in wells of 96-well plate coated with a capture antibody that captures the secreted cytokine. After the removal of cells, enzyme-conjugated detection antibody is added and cytokine production is visualized by a colour reaction. Position of each cytokine-producing cell is visualized as a spot. Number and size of the spots is detected by ELISpot reader and Spot forming cells (SFC) are counted [179,180].

ELISpot is adaptable not only to the evaluation of a variety of T-cell functions but also to B-cells and innate immune cells. In cancer research, ELISpot is often employed for evaluation of T-cell response to cancer therapy in phase I and II trials. ELISpot might be a useful biomarker assay to predict cancer vaccine efficacy [178,181].

2.4.2. Flow Cytometry

The first device, which had all the fundamental elements of a flow cytometer, was built by Louis Kamentsky in 1965 [182]. Interestingly, the first cell sorter was introduced in the same year [183]. Flow cytometry is based on single-cell analysis, which allows to show the frequency of cytokine-expressing cells together with characterization of producing cell population using staining of intracellular cytokines together with cell markers [184,185] (Figure 5). In the intracellular cytokine staining, cells are activated to produce cytokines and at the same time treated by substances that prevent cytokine secretion (such as Brefeldin A or monensin [186]). Subsequently, the cells are fixed and permeabilized to allow binding of the specific anti-cytokine antibodies [187–189]. Flow cytometry allows multiparameter

analysis and the number of analysed parameters depends on the cell population, cytokines of interest and the sophistication of the cytometer. However, not all cytokines can be detected using a single protocol and demanding optimization of staining may be required to detect dim, low-frequency, or newly characterized cytokines [184]. Moreover, the presence of a cytokine within cells does not necessarily mean that it would be released from cells or exhibit biological effects *in vivo* [190].

The most frequently studied cytokine producing cells are T-lymphocytes [190] but also other cell populations, including basophils [191], neutrophils [192,193], NK cells [194], monocytes [192] and mast cells [195] are investigated. In melanoma, flow cytometry was applied to study mechanisms of effects of immunotherapies, such as a PD-1 protein blockade by monoclonal antibodies pembrolizumab or nivolumab [196–198], CTLA-4 blockade with ipilimumab [198–201] or high-dose IL-2 therapy [202,203]. Intracellular cytokine staining can be used to monitor tumour antigen-specific T-cell functions [204–206] in the search for anti-tumour competent cytotoxic T-cells.

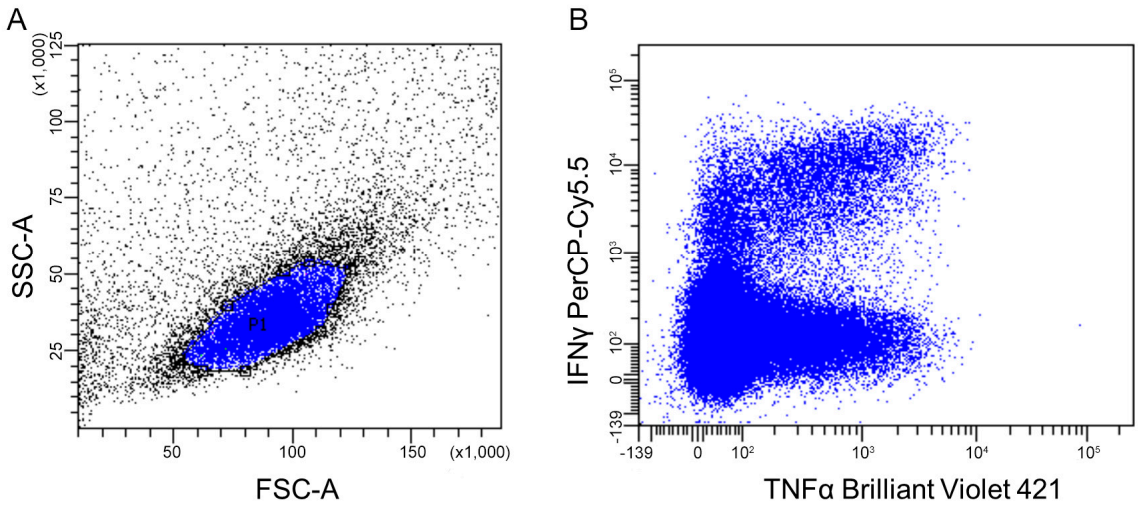

Figure 5. Application of flow cytometry to detect intracellular cytokines in porcine lymphocytes. (**A**) Activated cells with lymphoid scatter characteristics (highlighted in blue) from Melanoma–bearing Libechov Minipigs; (**B**) expression of IFNγ and TNFα in these cells.

2.4.3. Mass Cytometry

In 2009, Bandura et al. developed new approach combining flow cytometry and mass spectrometry—mass cytometry (also called CyTOF) [207]. This approach allows real time quantitative single cell analysis of theoretically up to 3000 cells per second and simultaneous measurement of 60 distinct markers (proteins or other biomolecules) in one cell. As in classical flow cytometry, cells are stained by specific antibodies but these antibodies are conjugated with element tags (stable isotopes of metals) instead of fluorescent dyes. Analysis of cells is performed by inductively coupled plasma time-of-flight mass spectrometer (ICPTOF-MS). The main advantages of this technique are high resolution and sensitivity together with high number of markers for simultaneous measurement with no need for compensation of emission spectral overlaps in comparison to fluorescence-based flow cytometry [207]. However, this technique has also several drawbacks—a lack of forward and side light scatter information, impossibility of cell sorting implementation (because cells are vaporized during analysis) and challenging quality control and statistical analyses of results [207,208]. Study of a human hematopoietic continuum stimulated by cytokines proved that results from mass cytometry are comparable and provide similar information value as fluorescence flow cytometry [209].

Although mass cytometry is a relatively new technique, it is well-established and broadly accepted by scientific community and protocols for intracellular cytokine staining for mass cytometry analysis are similar to protocols for classical flow cytometry analyses [208,210]. However, only several studies characterized cytokine production in immune cells using mass cytometry. Fisher et al. noticed dysregulated production of cytokines in myelofibrosis, when they monitored 12 selected cytokines, e.g., IFNγ, IL-6, MIP1β and TNFα [211]. O'Gorman et al. analysed expression of 16 cytokines (IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-8, IL-12p40, IL-17A, Perforin, GM-CSF, IFNα, IFNγ, MCP1, MIP1β and TNFα) in monocytes in patients with systemic lupus erythematosus [210]. Developments in multiplex mass cytometry for assessment of T-cell antigen specificity, applicable in search for targets of anti-cancer therapies, are discussed in [212].

2.4.4. Single Cell Arrays

Although the ELISPOT assay and flow cytometry are widely accepted in the preclinical testing, they rely on analysis of cells cultured in bulks, where the cells are under influence of paracrine factors produced by other cells. Response of individual cells (e.g., T-cell stimulation by cancer antigen) can be monitored by currently developed microtools for single secretome analysis. Accommodation of single cells into separated compartments can be achieved in valved microfluidic chambers (similar to microfluidic chips used to single-cell RNA sequencing) or in an array of subnanoliter wells [175].

Initial single cell array for secreted cytokines employed 81,400 microwells (~0.1 nL each) moulded on a poly(dimethylsiloxane) slab. The array was loaded with PBMCs cells (~1 cell per well) and then inverted onto a glass slide coated with a specific capture anti-IL-6 or anti-IFNγ antibody. After incubation, appropriate detection antibodies conjugated to fluorescent dyes were added and the fluorescence was recorded by microarray scanner [213]. In the following study, anti-IFNγ capture antibodies were incorporated into a hydrogel that was used for coating of a glass slide. Microwells of 20 μm diameter were formed by photolithography on top of the antibody-containing hydrogel layer and filled with cells. Such array was used to determine IFN-γ secretion by individual stimulated T-lymphocytes [214].

Simultaneous detection of multiple cytokines was achieved by further improvement of the single cell arrays. Han et al. used an array of 125 picolitre volume wells to accommodate single cells. Such an array was covered by a cover-slip, coated by mixture of 3 capture antibodies. Mixture of detection antibodies conjugated to 3 distinct fluorescent labels and a microarray scanner have been used to simultaneously determine levels of IFN-γ, IL-2 and TNFα in a secretome of stimulated T-cells [215]. In another study, high-density antibody barcode array chip together with subnanolitre microchamber array were used to determine secretion of 14 cytokines. The assay performance was documented for both cell lines (human A549 and U937 cell lines) as well as primary cultures isolated from human tumours (glioblastoma, meningioma) [216]. Further combination of spatial and spectral encoding led to co-detection of 42 immune effector proteins in single cells [217].

In focus on melanoma, Ma et al. developed a microfluidic chip for quantification of dozen effector molecules secreted from tumour antigen–specific cytotoxic T-lymphocytes that were actively responding to melanoma and compared with healthy donor controls [218]. Two types of tumour antigen-specific cytotoxic T-cell were evaluated: (i) T-cells transgenic for T-cell receptor specific for melanoma-associated antigen recognized by T-cells 1 (MART-1) protein and (ii) *ex vivo*–expanded tyrosinase-specific T-cells. A microfluidic chip with 3 nanolitre volume microcompartments was used to detect secretion of 10^4 individual cells. The cytokine production was detected by dense bar code antibody array—set of 12 capture antibodies immobilized in form of bars across each chip microcompartment. After incubation with cells, chip chambers have been flushed with biotinylated detection antibody and streptavidin-phycoerythrin was used for target quantification. Although the individual T-cells of each type were phenotypically similar, functional heterogeneity in active tumour antigen–specific CTLs was observed among single cells [218]. The heterogeneity among phenotypically similar cytokine producing cells was documented not only for T-lymphocytes but also

for macrophages [217,219] and NK-cells [220]. The single-cell studies demonstrate that only very low percentage of immune cells gets activated upon stimulation.

2.5. Other Techniques for Cytokine Detection

Immunoassays are powerful techniques to quantify cytokines but they may provide only limited information on the cytokine activity. As the immunoassays may suffer from detection of biologically inactive cytokines (e.g., IL-6 bound to its soluble receptor [221]) or cytokine fragments, or may be influenced by matrix effects, antibody unspecific binding or cross-reactivity, bioassays can be applied to detect biological activity of cytokines. Bioassays are using cells (and, mostly historically, also experimental animals) to monitor cytokine effects. The monitored target cell response may include induction of proliferation, differentiation or growth inhibition, chemotaxis, phagocytosis, cytotoxicity/apoptosis, induction of antiviral or antimicrobial activity, or up-regulation of expression of intracellular, surface membrane or secreted proteins [98,222]. Despite their unique power to measure biological activity of cytokines, bioassays, like all biological systems, are inherently variable and also laborious. Thus, bioassays are increasingly replaced by immunoassays, even at the cost of loss of information on the cytokine activity [222].

Many other techniques are applied beyond proteomics to detect cytokines. Analysis of cytokine gene expression is robust, fast and easy technique applicable even to very limited sample amounts. However, the relation between mRNA level and activity of the secreted protein is difficult to estimate, mainly due to regulations on posttranscriptional [223] as well as posttranslational level (secretory pathway, storage prior release, activating proteolytic cleavage, binding of secreted cytokine to neutralizing molecules, etc.). Nevertheless, transcriptomic analysis in combination with other techniques contributes to comprehensive view on cytokine regulation in biologic systems.

Immunohistochemistry and immunocytochemistry represent additional inseparable part of cancer and melanoma research. Such techniques enable visualization of antigen expression patterns and immune response across the tissue of cells. Immunofluorescence staining of intracellular cytokines as described in Flow cytometry section can be also analysed using regular or confocal fluorescence microscope. This technique does not allow sensitive and precise quantification but can provide additional information on morphology and identity of the cytokine producing cells.

3. Conclusions

Melanoma may serve as a model for tumour immuno-oncology. Melanoma spontaneous regression accompanied by tumour infiltration by T-lymphocytes suggest the active role of immune system in tumour management. Knowledge of immune system response to melanoma represents one of the key prerequisites for development of immunotherapies to manage cancers. Cytokines are the main regulatory molecules in the immune system. Significant progress in proteomics and other technologies enable monitoring of cytokine release with very high sensitivity. Simultaneous analysis of several molecules by multiplex techniques provides more global view of cytokine regulatory networks. Single-cell technologies open new ways of research towards functions of individual cells.

Further developments in multiplex and ultrasensitive techniques are expected in the next few years, including developments in antibodies and aptamers for cytokine molecule capturing or developments in sensing. However, careful testing should be applied to correctly quantify cytokines in real biological and patient samples by emerging analytical techniques. Inter-individual variability and inter-assay variability should be taken into account when summarizing results from various studies.

Knowledge gained from cytokine analysis is expected to facilitate identification of targets and monitoring of efficiency of melanoma immunotherapies.

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Abbreviations

bFGF	basic fibroblast growth factor
CAFs	cancer-associated fibroblasts
CCL	C-C motif chemokine
CTLA-4	T-lymphocyte-associated antigen 4
CXCL1	chemokine (C-X-C motif) ligand 1 (GROα)
DDA	data dependent acquisition
DIA	data independent acquisition
DIADI	direct immunoaffinity desorption/ionization
ECL	electrochemiluminescence
ELISpot	enzyme-linked immunospot assay
EVs	extracellular vesicles
G-CSF	granulocyte-colony stimulating factor
GDF15	growth/differentiation factor 15
GM-CSF	granulocyte-macrophage colony-stimulating factor
GROα	GRO1 oncogene
HGF	hepatocyte growth factor
HPLC	high-performance liquid chromatography
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
IL-10	interferon gamma-induced protein 10
IL1-ra	interleukin-1 receptor antagonist
IMR	immunomagnetic reduction
KGF	keratinocyte growth factor
MALDI	matrix-assisted laser desorption ionisation
MART-1	melanoma-associated antigen recognized by T cells 1
MCP-1	monocyte chemoattractant protein 1
M-CSF	macrophage colony-stimulating factor
MIF	macrophage migration inhibitory factor
MIP-1β	macrophage inflammatory protein-1β
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NK	natural killer
NRG-1	neuregulin-1
PBMCs	peripheral blood mononuclear cells
PD-1	programmed cell-death protein 1
PDGF	platelet-derived growth factor
PD-ligand	ligand of PD-1 receptor
PEA	proximity extension assay
PLA	proximity ligation assay
SCF	stem cell factor
Simoa	single molecule array
SISCAPA	stable isotope standards and capture by anti-peptide antibodies
SMC	single molecule counting

SRM	selected reaction monitoring
SWATH-MS	sequential windowed acquisition of all theoretical mass spectra
TAMs	tumour-associated macrophages
TARC	thymus and activation regulated chemokine (CCL17)
TGFβ	transforming growth factor-β
TNFα	tumour necrosis factor-α
Tregs	regulatory T-cells
VEGF-A	vascular endothelial growth factor A

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6 Závěr

Mým cílem v rámci doktorského studia bylo přispět k objasnění procesu spontánní regrese melanomu u prasat z linie MeLiM. Zaměřila jsem se především na vliv imunitního systému, a to z hlediska změn v specifické subpopulaci T lymfocytů i změn v hematologickém profilu, dalším pohledem na celou problematiku bylo vyhodnocení rozdílů v rámci mikrobiomu u jednotlivých kategorií prasat i nádorovou/zdravou tkání. Výsledků bylo dosaženo díky spolupráci se zaměstnanci z Ústavu živočišné fyziologie a genetiky AV ČR, Mikrobiologického ústavu AV ČR, České zemědělské univerzity v Praze a Univerzity obrany v Brně.

Z mé disertační práce jasně vyplývá, že stejně jako tumorigeneze je i spontánní regrese procesem komplexním, kde každá jednotlivá změna má své místo a dohromady tvoří soubor faktorů důležitých pro iniciaci SR. I když ještě všechny faktory nebyly popsány, tak i dílčí výsledky mají vliv pro budoucí výzkum. Nejdůležitější výsledky ukázaly, že se $CD4^+CD8^+$ T lymfocyty vyskytují ve zvýšené míře u regredujících zvířat a to v melanomové tkáni i periferní krvi, což naznačuje přímou souvislost se spontánní regresí. Jelikož byla jejich přítomnost potvrzena i u onkologických pacientů, tak je jejich možný prognostický význam neopomenutelný a v dalším výzkumu se budu zabývat charakterizací jejich funkčních znaků.

Sledování hematologických parametrů ukázalo, že se progredující zvířata v mnohých parametrech významně odlišují od zvířat regredujících i zcela zdravých. Pro další výzkum by bylo jistě zajímavé, kdyby se dále sledoval vztah mezi množstvím železa a průběhem onemocnění, tak aby bylo možné přesně určit, zdali se během progrese melanomu u MeLiM prasat jedná o nedostatek železa absolutní nebo funkční.

Vliv mikrobiomu na SR byl prokázán u mnohých nádorových onemocnění, avšak ještě nikdy nebyl studován mikrobiom prasečího melanomu. Výsledky ukázaly markantní rozdíly v mikrobiomu zdravé kůže a melanomu a v případě *Fusobacterium nucleatum* i jasný rozdíl mezi progredujícím a regredujícím nádorem. Další výzkum bude pokračovat analýzou střevního mikrobiomu a jeho vztahem k progresi/regresi melanomu u MeLiM prasat.

Během dalšího pokračování výzkumu by bylo vhodné doplnit dosavadní výsledky o souhrnné experimenty zahrnující komplexní cytokinový profil, přesnější fenotypizaci lymfocytů i genetické analýzy, kdy potvrzení přítomnosti dalších specifických markerů by mohlo vhodně rozšířit dosavadní skládačku vědomostí o spontánní regresi melanomu u MeLiM prasat.

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