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Dedicated to my parents Marion and Markus

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1 INTRODUCTION

1.1 The human immune system

Our body's integrity is constantly jeopardized by extrinsic and intrinsic threats such as microbiota and incipient cancer cells. As a result of evolutionary pressure, the human immune system has evolved into a complex network composed of different cell types, humoral factors and signaling pathways involved in the prevention and clearance of infective pathogens and in control of constant malignant transformation. A hallmark feature is the capability of discriminating "self" from "non-self" or "altered-self": foreign pathogens or cancer cells have to be recognized and eliminated while remaining tolerant to the body's own tissue and commensal microflora. Shifting this delicate balance in one direction or the other can lead to severe consequential damage such as neoplastic transformation, sepsis or autoimmune disease.

In all jawed vertebrates, the immune system is classically comprised of two branches: innate and adaptive immunity. The innate immune system, as a phylogenetically ancious and evolutionary conserved system, serves as a first-line host defense of every multicellular organism. In mammals, its cellular basis is largely reflected by phagocytes such as neutrophils, monocytes/macrophages and dendritic cells that sense microbial infection or engulf pathogens and induce a subsequent immune answer. Furthermore, innate immunity is indispensable as driver for acquired immunity. In contrast, the adaptive (or acquired) immune system shows delayed responses to pathogens but allows for immunological memory and tailored response. Whereas innate immunity is not a single entity, the adaptive immune system is mainly reflected by highly specialized T and B cells. This specificity is ensured by antigen-specific receptors expressed on the cell surface as the result of somatic hypermutation and individual recombination of receptor-encoding genes during lymphocyte maturation. As a result, this assembly process generates an enormous diversity of receptors each specific to a single antigen and expressed on the surface of its corresponding lymphocyte. Only receptors that bind their matching antigen induce clonal expansion of the corresponding lymphocyte.

In contrast to adaptive immunity, the molecular detection mechanisms of the innate immune system are mediated via germline-encoded and evolutionally highly conserved pattern recognition-receptors (PRR) [Zhang et al., 2010]. Thus, the specificity of any given PRR is genetically determined. Whereas the hereditability of such receptors is a decisive advantage, as no prior antigen exposure is necessary, the disadvantage is their limited diversity in comparison to the manifold repertoire of rearranged receptors used by the acquired system.

1.2 Pattern recognition receptor families

Pattern recognition receptors (PRRs) are responsible for sensing the presence of microorganisms by recognizing invariant microbial structures termed pathogen-associated molecular patterns (PAMPs). Even before they have been discovered, Charles Janeway postulated the presence of such receptors [Janeway, 1992]. PRRs have common characteristics: First, they are germ-line encoded, non-clonal and constitutively expressed on all cells of a given type. Second, they sense specific molecular patterns that share a common motif. In microorganisms, those structures are essential for survival thus highly conserved and difficult to alter during evolution. Third, receptor-binding elicits a rapid reaction and upregulation of genes involved in inflammatory responses. In 1996, Matzinger already hypothesized that the activation of PRRs is not limited to exogenous PAMPs but that they also sense endogenous molecules released after cell stress and tissue damage, termed damage-associated molecular patterns (DAMPs) (reviewed [Matzinger, 2002]). A concept that is nowadays well proven and clinically relevant in the pathogenesis of malignant, cardiovascular and autoimmune disease [Rakoff-Nahoum and Medzhitov, 2009; Frantz et al., 2007; Takeuchi and Akira, 2010].

Until now, five different classes of PRR families have been identified. These families include transmembrane proteins such as Toll-like receptors (TLRs) [Kawai and Akira, 2010], C-type-lectin receptors (CLRs) [Geijtenbeek and Gringhuis, 2009], scavenger receptors [Peiser et al., 2002], cytosolic helicases such as (RIG)-I-like receptors (RLRs) [Yoneyama and Fujita, 2007] and NOD-like receptors (NLRs) [Chen et al., 2009]. With regard to the enormous spectrum of abovementioned PRRs, the following sections are limited to the detailed description of TLRs and RLRs.

1.2.1 Toll-like receptors and their ligands

The best characterized innate immune receptors are Toll-like receptors. TLRs are responsible for sensing pathogens on the cell surface and in intracellular endosomes and lysosomes [Akira et al., 2006]. They are evolutionally conserved from the worm *Caenorhabditis elegans* to mammals [Beutler and Rehli, 2002; Hoffmann 2003}. Toll, the eponymous member of the TLR family was initially described in *Drosophila melanogaster* as a gene product regulating dorsoventral polarity in *Drosophila* [Nusslein-Volhard and Wieschaus, 1980; Hashimoto et al., 1988]. Later, Hoffmann and colleagues showed that Toll is also essential for antifungal response in flies [Lemaitre et al., 1996]. The first human homologue of the *Drosophila* toll protein - Toll-like receptor 4 - was shown to induce the expression of proinflammatory cytokines and co-stimulatory molecules [Medzhitov et al., 1997].

To date, fourteen TLRs have been identified among different species [Kawai and Akira, 2010]. Ten TLRs have been found in human and twelve in mice. TLR1-9 are conserved among mice and human, although TLR8 is non-functional in mice and human TLR10 lacks subsequent signaling due to gene disruption by insertion of an endogenous retrovirus. TLR10-13 are not expressed in humans. Recently TLR14 has been described in fish [Palti, 2011].

The cellular localization seems to play important roles in downstream signaling and maintaining tolerance to self-molecules such as nucleic acids. Some TLRs (TLR3, 7, 8 and 9) are exclusively located in intracellular compartments such as the endoplasmatic reticulum, endosomes and lysosomes where they sense nucleic acids. Another group of TLRs (TLR1, 2, 4, 5, 6, 11) is expressed on cell surfaces and mainly recognizes microbial membrane components such as lipoproteins and carbohydrates. While TLR1, 2 and 6 recognize ligands by forming heterodimers, the remaining receptors seem to function as homodimers.

Importantly, some TLRs have been shown to monitor the host's internal environment to detect endogenous abnormal self-antigens. For instance, numerous endogenous TLR ligands have been identified so far such as heat-shock proteins, high-mobility group box-1 protein (HMGB1), extracellular matrix components as well as endogenous nucleic acids [Asea et al., 2002; Park et al., 2004; Midwood et al., 2009; Kariko et al., 2004]. Endogenous TLR agonists are now thought to play an important role in regulating inflammation and seem to be involved in the pathogenesis of certain non-infectious disease such as autoimmune disorders, cancer and atherosclerosis [Piccinini and Midwood, 2010]. Table 1 gives an overview of different TLRs with their respective exogenous and endogenous ligands.

TLR	PAMP/DAMP	Origin	Reference
TLR1+2	Triacetylated lipopeptide	(Myco-)bacteria	[Takeuchi et al., 1999]
	β-defensin-3	Self	[Funderburg et al., 2007]
TLR2+6	Diacetylated lipopeptide	Mycoplasma	[Takeuchi et al., 2001]
	Zymosan	Fungus	[Ozinsky et al., 2000]
	HMGB-1	Self	[Park et al., 2004]
	Heat-shock proteins	Self	[Asea et al., 2002]
TLR3	dsRNA	Virus	[Alexopoulou et al., 2001]
	mRNA	Self	[Kariko et al., 2004]
TLR4	Lipopolysaccharide	Bacteria	[Poltorak et al., 1998]
	Envelope proteins	Virus	[Kurt-Jones et al., 2000]
	Mannan	Fungus	[Tada et al., 2002]
	Taxol	Plants	[Byrd-Leifer et al., 2001]
	Heat-shock proteins	Self	[Asea et al., 2002]
	HMGB-1	Self	[Park et al., 2004]
	Fibrinogen	Self	[Smiley et al., 2001]
TLR5	Flagellin	Bacteria	[Hayashi et al., 2001]

TLR7	ssRNA	RNA virus/self	[Vollmer et al., 2005]
	Imiquimod/Resiquimod	Synthetic	[Hemmi et al., 2002]
	Antiphospholipid antibody	Self	[Hurst et al., 2009]
hTLR8	ssRNA	RNA virus/self	[Heil et al., 2004]
	Antiphospholipid antibody	Self	[Doring et al., 2010]
TLR9	CpG DNA	Bacteria	[Hemmi et al., 2000]
	DNA	DNA virus	[Lund et al., 2003]
	Malaria hemozoin	Plasmodia	[Coban et al., 2005]
	IgG-chromatin complexes	self	[Leadbetter et al., 2002]
hTLR10	Unknown	Unknown	
mTLR11	Unknown	Uropath. bacteria	[Zhang et al., 2004]
	Profilin-like molecule	Toxoplasma	[Yarovinsky et al., 2005]
mTLR12	Unknown	Unknown	
mTLR13	Unknown	Unknown	

Table 1.1. Overview of Toll-like receptors and their main endogenous and exogenous ligands (modified from Ishii et al., 2006; Piccinini and Midwood, 2010; Takeuchi and Akira, 2010).

TLRs are differentially expressed depending on tissue and cell type. Moreover, most tissues express at least one TLR with predominance in cells associated with immune function. As an example, splenic cells and peripheral blood leukocytes express almost all TLRs as well as immune-associated tissue such as epithelial cells and fibroblasts [Zarember and Godowski, 2002]. Focusing on expression patterns in immune cells, myeloid cells constitutively express TLR1 and 6, whereas macrophages preferentially express TLR2, 3, 4 and 8. Cells of acquired immunity have been shown to express different TLRs, namely TLR2, 3, 5 and 9 on T cells [Kabelitz, 2007] and TLR1, 7, 9 and 10 on B cells [Bourke et al., 2003; Dasari et al., 2005]. Notably, the expression pattern of TLRs in dendritic cells significantly differs between mice and human: human myeloid DC express all TLRs with the exception of TLR9 while plasmacytoid DC express TLR1, 6, 7 and 9. In contrast, murine plasmacytoid DC express almost all TLR but TLR3 and 4. In addition, the expression level of TLR7 and 9 in murine DC is generally higher than in human DC [Muzio et al., 2000; Hornung et al., 2002]. These differences in expression patterns might explain why several studies using TLR7 and TLR9 agonists as adjuvants in cancer immunotherapy did not produce the expected results generated from preclinical studies performed in mice.

1.2.2 RIG-I-like receptors

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are a family of cytoplasmic DExD/H box RNA helicases that play a major role in sensing of RNA viruses to initiate and modulate antiviral immunity. The downstream signaling cumulates in the induction of a type I interferon response and antiviral gene expression. To date, three RIG-I-like helicases have

been discovered: RIG-I (retinoic acid-inducible gene I), which is the first and therefore best characterized RLR, MDA5 (melanoma differentiation associated factor 5) and LGP2 (laboratory of genetics and physiology 2) [Kang et al., 2002; Yoneyama et al., 2005]. RLRs are broadly expressed in most tissues and their expression is greatly enhanced with IFN exposure and after viral infection. Whereas in many cell types they play a dominant role in triggering antiviral immune defenses, plasmacytoid dendritic cells mainly use other RNA-sensors such as TLR3, 7 and for IFN production [Kato et al., 2005].

RIG-I and MDA-5 preferentially sense dsRNA either from dsRNA viruses or as an intermediate from ssRNA viruses. In addition, both are able to induce a potent IFN response following stimulation with synthetic polyinosinic:polycytidylic acid poly(I:C), especially when transfected intracellularily. In its low molecular form (0.2-1.0 kb) it mainly induces RIG-I activation, whereas high molecular weight poly(I:C) (1.5-8.0 kb) has been shown to be preferentially sensed by MDA-5 [Kato et al., 2008]. Furthermore, RIG-I detects RNA sequences marked with a 5'triphosphorylated (5'ppp) moiety which defines a non-self PAMP [Hornung et al., 2006]. Endogenous RNA also contains 5'ppp structures but is either capped or modified in the nucleus before it reaches the cytosol. Recent discoveries extended the spectrum of possible RIG-I substrates. As an example, Ablasser and colleagues showed a potent RIG-I response to dsDNA poly(dA:dT) from intracellular pathogens through recognition of a non-self product of polymerase III transcription [Kumar et al., 2006; Ablasser et al., 2009]. LGP2 is implicated in regulating the function of both of its family members depending on the type of RNA virus [Rothenfusser et al., 2005; Venkataraman et al., 2007]. Structurally, LGP2 is a homolog of RIG-I and MDA-5 but lacks the CARD domain and thus has no signaling ability but likewise has been shown to detect dsRNA [Li et al., 2009].

Notably, it is important to recognize that pathogens mostly do not activate one single PRR. The beginning of an innate immune answer and subsequent shaping of acquired immunity reflects an interplay between various PRRs in order to orchestrate a coordinated inflammatory response (reviewed in [Mogensen, 2009; Broz and Monack, 2013]. Accordingly, the current view on pathogen recognition has been shaped during the last two decades, initiated by Janeway's hypothesis and stimulated by the identification of over 100 PRRs to date. Unraveling the complex interplay between different PRRs and the network of innate and adaptive immunity is subject of intensive and ongoing research activity thus our understanding of this interplay might improve substantially in the next years.

1.2.3 Signaling of pattern recognition receptors

Downstream signaling of RIG-I-like helicases, TLRs, NOD-like receptors and C–type lectin receptors is mediated via some central proteins and transcription factors, the most popular being nuclear factor (NF-)κB, activated protein 1 (AP-1) and mitogen-activated protein kinases (MAPK) - together dozens of transcription factors are cooperating in the upregulation of inflammatory genes. More specifically, TLRs initiate common NFκB/AP-1 whereas others have shown to initiate the expression of type I IFN via interferon regulatory factor (IRF)3 and IRF7 [Kawai and Akira, 2006; Thompson et al., 2011]. CARD-domain containing helicases such as RIG-I and MDA-5 trigger the induction of interferons via the adaptor protein interferon promoter stimulator 1 (IPS-1). Nucleotide oligomerization domain (NOD)-like receptors (NLRs) also signal via NFκB but share a second innate immune pathway by activating inflammasomes [Martinon et al., 2002]. Dectin-1 as a prototype for C-type lectins is important in triggering antifungal immunity and activates NFκB. Figure 1.1 summarizes main signaling pathways of PRRs targeted in this work; details are given in the text.

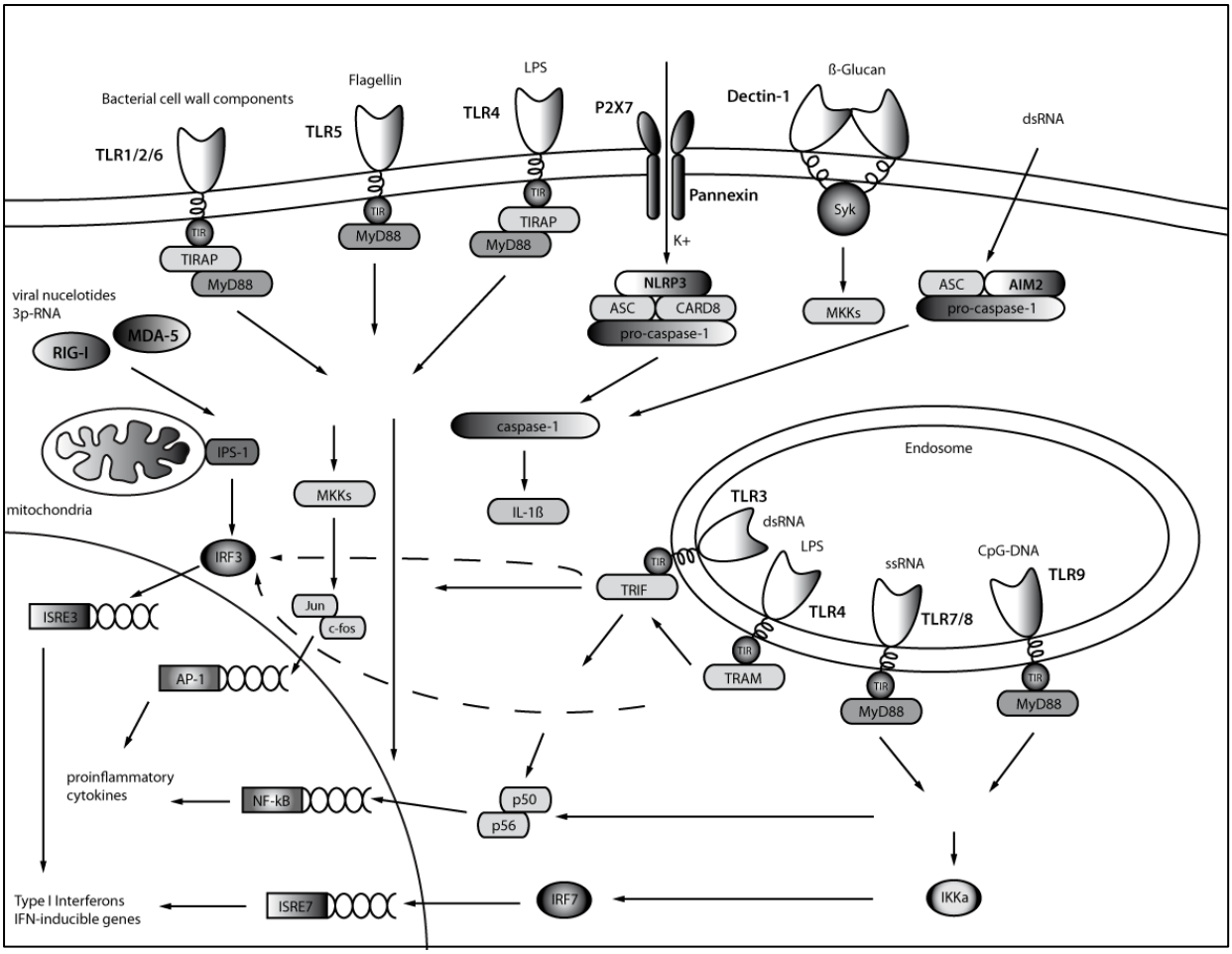


Figure 1.1. Major signaling pathways of TLRs and RLRs with main target transcription factors relevant in this work (own drawing, adapted from Kawai and Akira, 2010; Geijtenbeek and Gringhuis, 2009; Yoneyama and Fujita, 2007).

1.2.3.1 Signal transduction via Toll-like receptors

All TLRs are integral transmembrane glycoproteins that belong to a superfamily called the Toll/IL-1 receptor (TIR) family. The cytoplasmic TIR-domain shares considerable homology with Interleukin-1 receptors (IL-1Rs) and is required for mediating downstream signaling. The extracellular domain of TLRs contains variable leucine-rich repeats (LRR) motifs responsible for ligand recognition [Akira and Takeda, 2004]. TLRs share common and distinct signaling pathways: following LRR domain ligand recognition, TLRs dimerize and undergo conformational changes which is an essential step in the recruitment of cytoplasmic TIR-domain-containing adaptor molecules to the intracellular TIR domain of the activated TLR. Five adaptor molecules have been described yet: the myeloid differentiation primary response gene 88 (MyD88), TIR-containing adapter inducing IFN-β (TRIF), TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL), TRIF-related adapter molecule (TRAM) and Sterile-alpha and Armadillo motif-containing protein (SARM) [O'Neill et al., 2003; Oshiumi et al., 2003; Kawai and Akira, 2006]. Together these signaling pathways activate the transcription factors NFκB and AP-1, leading to the production of various pro-inflammatory cytokines and chemokines as well as the upregulation of co-stimulatory molecules in order to facilitate an adaptive immune response [Iwasaki and Medzhitov, 2004]. In addition, the inflammatory response is further amplified by recruiting innate immune cells such as monocytes, neutrophils and natural killer (NK) cells to the site of inflammation. They also lead to production of type I interferons via IRF3/7 upon activation of TLRs 3, 4, 7, 8, 9 and RLRs.

TLR signaling is divided into two pathways depending on the adaptor molecule used, MyD88 or TRIF. The MyD88-dependent pathway is used by all TLRs with the exception of TLR3. In detail, upon PAMP recognition, MyD88 associates with the cytoplasmic portion of TLRs though homophilic TIR-TIR domain interaction. TLR2 and TLR4 signaling additionally requires the adaptor protein TIRAP/MAL for bridging between the TLR and MyD88 [Horng et al., 2002]. Once activated, MyD88 recruits IL-1R associated kinases (IRAK)-1 and IRAK 4. Subsequent signaling eventually leads to the phosphorylation of the IκB kinase (IKK)-β and MAP kinase 6. The IKK complex (composed of IKKα, IKKβ and NFκB essential modulator [NEMO]), phosphorylates IκB, an inhibitory protein NFκB. IκB then undergoes degradation and releases NFκB for translocation into the nucleus. In turn, activation of the MAP kinase cascade activates another major transcription factor complex, AP-1. Both transcription factors elicit an upregulation of inflammatory genes leading to the induction of a variety of chemokines and cytokines such IL-1β, IL-6, IL-10, IL-12, IP-10, TNFα and IFNγ [Barr et al., 2007; Makela et al., 2009].

As mentioned before, TLR2 and 4 require the presence of MAL for MyD88-dependent downstream signaling. In addition, Kawai and colleagues have shown that MyD88-deficient mice do not suffer from septic shock following treatment with high doses of LPS [Kawai et al., 1999]. However, neither NFκB nor MAPK showed abrogated activity which suggests a second signaling pathway independent of MyD88. This MyD88-independent signaling pathway has been well described during the last decade and termed TRIF-dependent pathway. It gets activated via TLR3 as well as TLR4. In TLR4 signaling requires the recruitment of TRAM as a bridging adaptor to the TIR domain of TLR4 [Fitzgerald et al., 2003; Yamamoto et al. 2003]. In addition to the activation of the canonical NFκB pathway and the activation of MAP kinases, downstream signaling of TRIF induces phosphorylation of IRF3 and IRF7 which form homodimers, translocate into the nucleus and induce the transcription of type I interferons [Fitzgerald et al., 2003].

TLR-dependent type I IFN induction uses different signaling pathways and key molecules depending on the stimulated TLR, co-stimulatory effects of other PRRs and the cell-type stimulated. TLR4 as LPS-sensing molecule and TLR3 as sensor for dsRNA mainly activate IRF3 and 7 via the adaptor proteins TRAM/TRIF [Doyle et al., 2002]. TLR7, 8 and 9 in turn activate IRF5 and 7 in a TRIF-independent and MyD88-dependent manner with a central role for IRAK-1 downstream of MyD88 and IRAK-4 [Schoenemeyer et al., 2005; Uematsu et al., 2005]. IRF7 plays an essential role as transcription factor in the IFN induction following stimulation with CpG-DNA as TLR9 agonist and R848 as TLR7 agonist [Honda et al., 2005]. Notably, IFN induction upon TLR stimulation considerably differs between myeloid/conventional dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). Conventional DCs mainly use the TLR3/4-TRIF dependent pathway for the induction of IFNβ that leads to upregulation of IRF-7 in an autocrine manner [Au et al., 1998]. In addition, TLR7 and 9 expression levels as well as that of IRF7 as key transcription factor for IFNα production are significantly lower in cDCs. Honda and colleagues could unravel an additional mechanism, namely that CpG-DNA rapidly lysosomally degrades in cDCs, but is retained much longer in the endosome of pDCs thus facilitating the encounter between ligand and receptor-complex [Honda et al., 2005]. Interestingly, in human pDCs it has been shown that TLR9 exhibits a unique feature: knowing that TLR9 activation can result in a potent IFNα response and/or in triggering an adaptive immune response via IL-6 and TNFα secretion, this dual function has been attributed to the intracellular location where TLR9 encounters its ligand. Whereas in early endosomes TLR9 signaling primarily elicits the production of type I IFN, triggering TLR9 in late endosomes has been shown to mainly result in the production of inflammatory cytokines [Gilliet et al., 2008].

1.2.3.2 Signal transduction via RIG-I-like receptors

The RIG-I-like helicases MDA-5 and RIG-I recognize long dsRNA such as poly(I:C) and short dsRNA with 5'ppp moiety [Seth et al., 2005]. Once activated, both signal through homophilic interaction via CARD domains with a mitochondrial associated protein named IFNβ promotor stimulator 1 (IPS-1). IPS-1 has also been designated as mitochondrial anti-viral signaling (MAVS), CARD adapter inducing IFN-β (Cardif) or virus-induced signaling adapter (VISA)]Loo and Gale, 2011]. Downstream signaling results in a potent type I IFN response via TRAF3, TBK 1 and the already mentioned IRF3 and 7. Simultaneously, IPS-1-dependent signaling also mediates the nuclear translocation of NFκB via its non-CARD region which involves the activation of caspase-8. Nevertheless, the biological significance of that alternative pathway needs to be determined [Seth et al., 2005].

1.3 The human immune system and cancer

1.3.1 Cancer Immunology: *Immunosurveillance* and *Immunoediting*

Burnet and Thomas hypothesized 1957 that our immune system is capable of recognizing and eliminating nascent and continuously arising transformed cells; a concept which has been termed *immunosurveillance* [Burnet, 1957]. Already in 1909, Paul Ehrlich envisioned that our immune system is able to suppress and monitor neoplastic transformation. Notably, at that time Ehrlich already worked on sarcoma vaccination in rodents. Burnet's and Thomas' hypothesis gained further interest when the first tumor-associated antigens were discovered [Feldman, 1963; Old and Boyse, 1964; Rosenberg, 1999]. The concept of *immunosurveillance* ultimately gained acceptance in the 1990s with rising technological advances when DC vaccination with tumor-antigens and adoptive T-cell transfer showed significant immune responses against malignant cells and pivotal studies identified key effector molecules such as IFNγ [Kaplan et al., 1998; Shankaran et al., 2001] and perforin [van der Bruggen et al., 1991; Smyth et al., 2001] in protecting the host in both chemically induced and spontaneous tumors. Furthermore, extensive research of the last two decades revealed that *immunosurveillance* seems to be only one dimension in the complex relationship of cancer and our immune system [Smyth et al., 2001; Dunn et al., 2002; Dunn et al., 2004]. Tumor-immunologists learned that immunity not only protects the development of neoplastic lesions in terms of *immunosurveillance*, but also sculpts tumor immunogenicity and eventually can support tumor growth by selecting for more aggressive tumor escape variants with reduced immunogenicity - a concept that has been termed *immunoediting* [Dunn et al., 2004]. Cancer *immunoediting* resembles a three-step process: The first step is `the elimination phase' which resembles above described *immunosurveillance* by a cooperative action of innate and acquired immunity. The second phase has been named 'equilibrium phase'; genetically

unstable and rapidly mutating tumor cells that withstood the elimination's phase selection pressure enter this latency period as an intermediate between elimination phase to the emergence of clinically detectable malignant disease which defines the 'escape phase' as last phase (see below). Particularly, it is important to recognize that the process of *immunoediting* is not an inevitable linear process. Incipient malignant transformation can be cleared in the elimination phase and cancer cells that have entered the equilibrium phase can eventually still get eliminated by the immune system.

1.3.2 Tumor-escape mechanisms

To become clinically relevant in immunocompetent hosts, tumor cells must overcome innate and adaptive detection mechanisms. The failure of this immune recognition is arguably due to its inability to recognize cancer cells in an immunologic context which can be due to induction of immunogenic tolerance [Willimsky and Blankenstein, 2005] or by avoiding immune recognition; the concept of avoiding immune destruction has even pronounced as one of the emerging hallmarks of cancer [Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011].

First evidence for the role of defective immunological monitoring in the context of tumor formation was made by clinical epidemiologists in the late 1980s to 1990s when a striking increase of certain cancers such as Kaposi's sarcoma, Hodgkin's and non-Hodgkin's lymphoma in HIV/AIDS patients and immunocompromised patients following solid organ or bone marrow transplantation was observed [Farge, 1993; Lai et al., 1997; Vajdic and van Leeuwen, 2009]. However, the majority of these tumor entities were viral-induced cancers and given that over 80% of human malignancies are of non-viral etiology the conclusion of a general role of an over-alert immune system limiting the formation of nascent cancer cells into clinically evident tumors is not justified. Nevertheless, further research with genetically engineered mouse models as well as clinical evidence has warranted that our immune system indeed acts in limiting tumor formation and that neoplastic growth, in terms of a Darwinian fashion, triggers a variety of immunosuppressive features to avoid detection and to foster its own survival [Rabinovich et al., 2007; Hanahan and Weinberg, 2011].

1.3.2.1 Tumor cell-intrinsic traits

Tumor cells can alter distinct genes and signaling pathways to effectively avoid immune recognition. Several groups have shown that human cancer cells either downregulate or loose their HLA class I expression thus limiting the activation and cytotoxic effect of tumor-specific $CD8^+$ T cells [Ferrone and Marincola, 1995; Algarra et al., 2000]. Likewise, mutations in the

β2-microglobulin locus and loss-of-function mutations in the TAP1 locus lead to defects in signaling pathways involved in processing and presentation of antigens on tumor cells [Maeurer et al., 1996; Marincola et al., 2000; Rivoltini et al., 2002]. Other genetic lesions include the absence of IFNγ receptors on the surface which has been demonstrated for lung carcinoma cell lines [Kaplan et al., 1998] and melanoma cells [Wong et al., 1997] rendering them unresponsive to IFNγ-induced upregulation of HLA molecules.

Another striking mechanism is the expression of factors involved in modulating antitumor responses by negative co-stimulatory pathways with the programmed cell death protein 1 (PD-1) / programmed cell death protein 1 ligand (PD-L1) system as a prime example. PD-1 and PD-L1 are both membrane-bound proteins with PD-1 expressed on T cells and known for its regulatory effect on T cell receptor signaling. In turn, PD-L1 as its ligand has been shown to be expressed by almost all murine and a variety of human cancer cells playing a pivotal role in the escape from host immune response by blocking the effector phase of specific T cell antigen receptor mediated lysis of tumor cells [Blank et al., 2004]. Anti-PD-L1 antibodies were already evaluated in phase-I-trial for patients with selected advanced stage cancer [Brahmer et al., 2012].

Another mechanism contributing to immune evasion is the microenvironmental influence on amino acid metabolism. Uyttenhove and colleagues identified the enzyme indoleamine 2,3-dioxygenase (IDO) to induce T cell tolerance by oxidative breakdown of tryptophan [Uyttenhove et al., 2003]. Apart from its production in tumor cells, IDO has been found to be expressed by tolerogenic antigen-presenting cells (APCs) thus amplifying the suppression of T cell immunity and the recruitment of regulatory T cells (T_{reg}) [Prendergast, 2008].

A counterintuitive feature of cancer cells is using the immune system's own antitumoral defense mechanisms to evade immune destruction. Fas ligand or CD95L as a type II transmembrane protein is expressed by T cells and NK cells. By binding to its receptor FasR/CD95 it triggers an intracellular cascade leading to apoptotic cell death; a mechanism essential in mounting an effective T cell response in antitumor immunity [Siegel et al., 2000; Shanker et al., 2009]. Cancer cells twist around this feature by acquiring FasL on their cell surface making them capable of delivering death signals to Fas-positive cytotoxic T lymphocytes and NK cells. [Hahne et al., 1996; Andreola et al., 2002]. In accordance with this, the secretion of soluble CD95 in tumor patients abolishes antitumor response and significantly correlates with a poorer outcome [Ugurel et al., 2001; Igney and Krammer, 2002].

Finally, even highly immunogenic cancer cells may avoid immune destruction by overproduction of immunosuppressive factors such as transforming growth factor-β, IL-10, galectin-1 and PGE_2 [Yang et al., 2010]. IL-10 can impair adequate DC responses by impairing TAA cross-presentation [Gerlini et al., 2004]. TGFβ in turn has been shown to exert pleiotropic effects on tumor formation and tumor-associated immunosuppression. In early phases of tumor formation, TGFβ functions as a regulator of tissue homeostasis and acts as an inhibitor of tumor-progression via TGFβ Receptor II (TGFβ RII) and SMAD-dependent induction of apoptosis [Arteaga et al., 1990; Edlund et al., 2003]. That has been demonstrated in multiple mouse models and supported by clinical studies showing mutations of TGFβ RII and SMAD proteins in human lung, prostate, colon and breast cancer [Markowitz et al., 1995; Jakowlew, 2006]. On the other hand, numerous other studies outlined the detrimental effect of TGFβ in cancer biology. Alongside non-immunological and pro-tumoral mechanisms like activation of epithelial-to-mesenchymal transition [Ellenrieder et al., 2001; Drabsch and ten Dijke; 2012] and fostering tumor angiogenesis [Roberts et al., 1986], TGFβ is commonly known as a potent and naturally occurring suppressor of the immune system - a function generously used by tumor cells to facilitate their immune escape. Secreted by tumor or bystander cells of the microenvironment, TGFβ blocks the production of perforin, granzymes and IFNγ thus inducing T cell anergy of CTLs [Fukunaga et al., 2004; Thomas and Massague, 2005]. In addition, TGFβ shifts a T_H1-skewed immune response to a tumor-promoting T_H2 phenotype and participates in the induction of T_{regs} and recruitment and activation of myeloid-derived suppressor cells into the tumor-microenvironment [Chen et al., 2003; Li et al., 2012].

1.3.2.2 Tumor cell-extrinsic traits

In addition to intrinsic immune evasive mechanisms, more subtle mechanisms of immunosuppression in cancer operate together in order to recruit immunosuppressive bystander cells to the tumors immediate microenvironment and draining lymph nodes where tumor neoantigens scavenged by antigen-presenting cells are cross-presented to the adaptive immune system.

An increasing body of evidence shows that neoplastic immune escape is also mediated by regulatory T cells (T_{Regs}). Regulatory T cells have initially been described by Sakaguchi and colleagues as naturally occurring $CD4^+CD25^+$ forkhead box p3 (Foxp3) expressing cells in the context of immune homeostasis and in preventing autoimmunity by suppressing autoreactive T cells [Sakaguchi et al., 1995; Vieweg et al., 2007]. The proof-of-principle on the role of T_{Regs} in tumor immunity was made by Curiel in 2004 when he and colleagues found that tumor-associated T_{Regs} were recruited to tumor sites in a CCL22 dependent fashion and specifically

inhibited T cell-mediated antitumor immunity in patients with advanced stage ovarian cancer. In addition, on an individual base, they demonstrated that an increase in the amount of tumor-infiltrating T_{Regs} predicts for poorer survival [Curiel et al., 2004]. Since then, multiple other studies in mice and human cancer have provided mechanistic insights into the immunosuppressive capabilities of $T_{Regs.}$ On a cellular basis they inhibit antigen-presenting cells, NK cells and T cells in the tumor microenvironment or systemically. They do so via the expression of TGFβ, IL-10, IL-35 [O'Garra et al., 2004; Collison et al., 2007], induction of IDO-expression in APCs [Fallarino et al., 2003; Vignali et al., 2008], or via the expression of cytotoxic T lymphocyte antigen 4 (CTLA4) thus shutting down the costimulatory pathway in APCs by inhibitory binding to B7-H1 or B7-H2 [Waterhouse et al., 1995].

Dendritic cells (DCs) are critically important in mounting an effective antitumoral response via uptake and presentation of tumor-associated antigens to effector cells [Guermonprez et al., 2002]. Cancer patients, however, show decreased numbers of functionally active DCs in lymph nodes, spleen and peripheral blood [Almand et al., 2000; Hoffmann et al., 2002]. DCs loose their functionality during tumor progression as evidenced by studies in patients with prostate cancer, malignant glioma and breast cancer showing marked reduction in antigen-presentation and induction of IFNγ secretion by T cells [Pinzon-Charry et al., 2005]. Additionally, cancer cells induce expansion and accumulation of tolerogenic dendritic cells (tDCs). In 2003, Steinmann and colleagues described a physiologically occurring population of dendritic cells that bear an antigen-specific tolerogenic role in limiting autoimmunity and overwhelming immune responses by presenting antigens without concurrent co-stimulatory signals and simultaneous paracrine secretion of immunosuppressive molecules [Steinman et al., 2003]. Tolerogenic DCs, phenotypically matching pDCs, have been found in a variety of human malignancies and have been well studied in murine tumors models [Hartmann et al., 2003; Vermi et al., 2003]. By mechanisms depending on a tumor-induced differentiation blockage and increase in STAT3 expression, tDCs are kept immature and acquire immunosuppressive features such as IL-10, TGFβ and IDO-expression [Gabrilovich et al., 1996; Geissmann et al., 1999; Gabrilovich, 2004; Lob and Konigsrainer, 2008]. When T cells encounter tDCs that lack co-stimulatory receptors, surrounded by a suppressing cytokine milieu, they are either rendered anergic or even differentiate into regulatory T cells.

In addition to the above-mentioned immunosuppressive cells, other regulatory cell populations also contribute to impaired tumor surveillance including regulatory B cells (B_{regs}) [Mauri and Bosma, 2012], regulatory NK cells [Deniz et al., 2008], tumor-associated macrophages (TAMs) [Mantovani et al., 2002] and tumor-associated neutrophils (TANs) [Fridlender and Albelda, 2012]. Recently a population of immature myeloid cells termed

MDSC (myeloid derived suppressor cells) has been described which will be further discussed in detail below.

1.4 Myeloid-derived suppressor cells: Innate regulators of the immune system

Cancer-associated immunosuppressive myeloid cells were already described over 30 years ago [Duwe and Singhal, 1979; Young et al., 1987]. The appreciation of their functional importance only recently became apparent when researchers demonstrated that the administration of anti-Gr1 antibodies could significantly slow tumor growth by eliminating myeloid cells circulating in the blood of tumor-bearing mice [Seung et al., 1995] and when clinicians discovered $CD34^+$ cells in tumors and lymph nodes of patients with head and neck cancer [Pak et al., 1995]. Recently, these cells were generally termed myeloid-derived suppressor cells (MDSC) to reflect the abnormal nature of myelopoiesis during tumor formation [Gabrilovich and Nagaraj, 2009].

MDSC resemble a heterogeneous group of myeloid cells comprised of macrophage precursors, immature granulocytes and dendritic cells as well as myeloid cells at earlier stages of differentiation, all of which have been prevented from fully differentiating into myeloid cells by tumor-derived factors [Gabrilovich and Nagaraj, 2009]. By definition, this group shares two distinct features: their myeloid origin and potent immunosuppressive function. In addition, MDSC have been found in numerous human malignancies and in almost all murine cancer models tested [Nagaraj and Gabrilovich, 2010; Rodrigues et al., 2010; Sun et al., 2012]. MDSC have been detected in bone marrow, blood, spleen, tumor-draining lymph nodes as well as the tumor microenvironment of tumor-bearing mice and human cancer patients [Sinha et al., 2005; Serafini et al., 2006; Vincent et al., 2010]. Notably, a recent study by Jordan and colleagues showed MDSC accumulation in peripheral blood as an individual prognostic factor of poor outcome in patients with melanoma [Jordan et al., 2013].

MDSC employ multiple immunosuppressive features including their remarkable ability to suppress T cell responses as well as non-immunological functions such as promotion of angiogenesis, tumor invasion and metastasis. During the last decade, tumor immunologists started to further characterize MDSCs in the context of human and murine malignancies and begin to dissect molecular pathways regulating their expansion and activation thereby opening the doors for a new potential target in immunotherapy of malignant disease [Talmadge and Gabrilovich, 2013].

1.4.1 Origin of MDSC

Immature myeloid cells (iMCs) are an integral part of the normal process of myelopoiesis. Myelopoiesis in the bone marrow is a tightly regulated process and controlled by a complex network of soluble factors including cytokines such as IL-3, stem-cell derived factor (SDF), granulocyte/macrophage colony-stimulating factor (GM-CSF), FMS-related tyrosine kinase and a myriad of growth-factor receptors [Moore, 1979, Bender et al., 1987; Friedman, 2002]. Hematopoietic stem cells differentiate into common myeloid progenitor cells and then into immature myeloid cells. Normally iMCs constitute about 20 to 30% bone marrow cells, 2 to 4% splenic cells and approximately 0.5% of peripheral blood mononuclear cells, quickly migrating into peripheral organs and differentiating into macrophages, dendritic cells and mature granulocytes [Almand et al., 2001; Movahedi et al., 2008]. However, during trauma, inflammation and tumor formation they accumulate, become activated and migrate to sites of inflammation, alongside with further blockage of their differentiation. When these cells have finally acquired immunosuppressive properties they are further termed myeloid-derived suppressor cells (MDSC).

Notably, the importance of MDSC accumulation has also been described in a variety of other disease as cancer: bacterial, viral and parasite infections [Brys et al., 2005; Delano et al., 2007; Dietlin et al., 2007; De Santo et al., 2008], sepsis [Cuenca et al., 2011], chemotherapy [Angulo et al., 2000], traumatic stress [Makarenkova et al., 2006], autoimmunity [Zhu et al. 2007; Kerr et al., 2008] and in the setting of bone marrow transplantation [Highfill et al., 2010]. In cancer, MDSC play a detrimental role by immunosuppression of the host and simultaneously represent major obstacles for effective immunotherapy approaches. However, in the setting of hyperinflammation or autoimmunity such as autoimmune encephalitis, fulminant sepsis or graft-versus-host disease their presence might be ultimately beneficial by limiting immune-mediated damage to the host.

1.4.2 Phenotype and MDSC heterogeneity

In mice, MDSC are phenotypically characterized by the co-expression of the granulocyte differentiation antigen Gr-1 (constituted by the 2 epitopes Ly6G and Ly6C) and the αM-integrin CD11b or macrophage-1 antigen (Mac-1) [Kusmartsev and Gabrilovich, 2002]. MDSC are further divided into two major subpopulations with respect to their morphologic appearance and their differential expression of the Ly6G and Ly6C antigen: polymorphonuclear $Gr1^{+}CD11b^{+}Ly6G^{high}Ly6C^{low}$ MDSC (PMN-MDSC) and monocytic $Gr1^{+}CD11b^{+}Ly6G^{neg}Ly6C^{high}$ MDSC (MO-MDSC). Both subsets were found to potently inhibit antigen-specific T-cell responses *in vitro* and *in vivo*. Notably, PMN- and MO-MDSC further differ in their expression of specific enzymes involved in their immunosuppressive function:

While PMN-MDSC express high levels of arginase-1, MO-MDSC contain both arginase and inducible nitric oxide synthase (iNOS) (Figure 2.2).

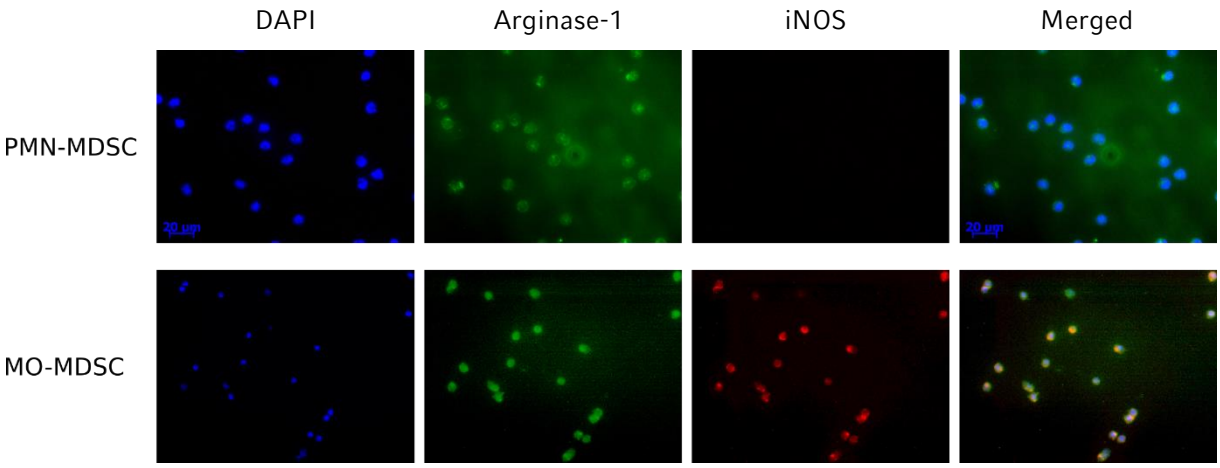


Figure 2.2. Immunofluorescence of Arg1 and iNOS of magnetically purified murine MDSC-subpopulations.

Other phenotypical markers have been described differing on murine MDSC-subpopulations such as CD71 (transferrin receptor), CD115 (macrophage colony-stimulating factor receptor/M-CSFR), CD31 (PECAM-1), CD1d which are more prominent or exclusively expressed on MO-MDSC [Movahedi et al., 2008].

Human MDSC were initially identified as $HLA^-DR^-CD33^+$ or $CD14^-CD11b^+$ populations, both of which were able to suppress T cell activity [Almand et al., 2001]. Currently, the human equivalents of MDSC are commonly defined as $CD34^+CD11b^+LIN^-HLA^-DR^-CD14^-$ cells, or more narrowly as cells expressing either one or both of the common myeloid markers CD33 or CD11b, lack lineage markers of mature myeloid or lymphoid cells (LIN^-) such as CD3, CD19, CD56 and CD13 and do not express the MHC class II molecule HLA-DR [Talmadge and Gabrilovich, 2013]. However, the expression of CD14 in human MDSC is still a matter of debate, as different groups reported the expression of CD14 on MO-MDSC [Zea et al., 2005; Filipazzi et al., 2007]. As human MDSC lack a phenotypical equivalent to the murine Gr1-antgen and likewise lack Ly6G/C equivalents, efforts have been made to differentiate between MO-MDSC and PMN-MDSC in the human setting either on the basis of their maturity or by phenotypical means [Dumitru et al., 2012]. So far, MO-MDSC have been found to have a $CD14^{high}$ phenotype whereas PMN-MDSC are $CD14^{neg/low}$. In addition, some groups used CD15 to discriminate between granulocytic $CD15^+$ and monocytic myeloid cells [Rodriguez et al., 2009]. Additional markers are under investigation such as differential expression of CD66b which seems to be more prominent on PMN-MDSC. Phenotypes of human and mouse MDSC and as well as variable mechanisms of immunosuppression of MDSC subpopulations are further reviewed here [Serafini et al., 2006; Gabrilovich and Nagaraj, 2009].

1.4.3 MDSC expansion and activation in cancer

Cancer patients show a four- to tenfold increase in peripheral MDSC numbers [Filipazzi et al., 2007; Hoechst et al., 2008; Diaz-Montero et al., 2009]. Likewise, MDSC levels in murine tumor models are significantly increased up to 70% of bone marrow cells, 10 to 20% peripheral blood mononuclear cells (PBMCs) and 5 to 40% of splenic leukocytes depending on the respective tumor model [Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009]. During tumor formation they also home to tumor-draining lymph nodes and represent a major cell type within the tumor microenvironment [Meyer et al., 2011].

In order to completely fulfill their immunosuppressive function, MDSC first need to be expanded and then activated [Gabrilovich and Nagaraj, 2009]. The past decade of research has failed to identify a single factor or signaling pathway that simultaneously mediates both necessary steps. Moreover, their accumulation is rather seen as a result of an interaction between different soluble molecules and receptors that are hypersecreted under pathological conditions, the lion's share being inflammatory mediators thereby linking MDSC with the old concept of chronic inflammation and cancer [Coussens and Werb, 2002]. Not surprisingly, the list of driver molecules for MDSC expansion seen in acute and chronic inflammatory conditions significantly overlaps with those identified in serum and tumor microenvironment of cancer patients and murine tumor models. This concept is supported by the fact that pharmacological inhibition of inflammatory mediators that drive and support tumor formation such as cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE_2), vascular endothelial growth factor (VEGF), IL-6, IL-1β and others, has concurrently shown to inhibit MDSC expansion and/or their activation [De Santo et al., 2005; Kusmartsev et al., 2008; Sumida et al., 2012; Zitvogel et al., 2012].

Several tumor-derived factors either directly secreted by tumor cells or released by microenvironmental bystander cells can induce a marrow expansion of MDSC and subsequently track them to the tumor site or other lymphoid organs such as tumor-draining lymph nodes and spleen [Gabrilovich, 2004]. The biological impact of these tumor-derived factors on myeloid cells was already shown in 1987, when researchers described that normal bone marrow cells gather an immunosuppressive phenotype and properties *in vitro* when cultured together with tumor-conditioned medium [Young et al., 1987]. Additional support came from studies in cancer patients revealing a significant decline in peripheral MDSC frequencies following cytoreductive surgery [Diaz-Montero et al., 2009]. More narrowly, GM-CSF has been shown to be a major promotor of MDSC accumulation in human as well as in murine cancer models [Pak et al., 1995]. In addition, complement factor 5a (C5a) contributes to tumor growth by inducing MDSC. C5aR-deficient mice showed impairment of T cell

suppression by MDSC accompanied by a greater influx of intratumoral CTLs. Myeloid progenitors express receptors for S100 calcium-binding protein family members. S100A8/9 proteins are released during inflammation and signal via TLR4 and the receptor for advanced glycation endproducts (RAGE) [Ehrchen et al., 2009; Leclerc et al., 2009] and have been linked to tumorigenesis [Ichikawa et al., 2011]. Notably, S100A9-deficient mice fail to increase MDSC numbers after challenging them with complete Freud's adjuvant [Cheng et al., 2008]. Complementarily, IL-1β as the first cytokine to be described, has been shown to mobilize and activate MDSC in a mouse model of gastric cancer [Tu et al., 2008]. Table 1.2. depicts soluble factors known to drive accumulation or activation of MDSC.

Tumor-derived factor	**Reference**
Granulocyte-colony stimulating factor (G-CSF)	[Sawanobori al., 2008]
Granulocyte/macrophage-colony stimulating factor (GM-CSF)	[Bronte et al., 1999; Filipazzi et al., 2007]
Vascular endothelial growth factor (VEGF)	[Kusmartsev et al., 2008]
Transforming growth factor beta (TGFβ)	[Terabe et al., 2003; Li et al., 2012]
Interleukin-1β (IL-1β)	[Tu et al., 2008]
Interleukin-6 (IL-6)	[Bunt et al., 2007]
Interleukin-10 (IL-10)	[Huang et al., 2006]
Interleukin-12 (IL-12)	[Li et al., 2004]
Interleukin-13 (IL-13)	[Terabe et al., 2003]
Interferon-γ (IFNγ)	[Gallina et al., 2006]
Tumor necrosis factor α (TNF-α)	[Zhao et al., 2012]
Stem cell factor (SCF)	[Pan et al., 2008]
Prostaglandins	[Sinha et al., 2007]
Complement factor 5a (C5a)	[Markiewski et al., 2008]
Matrix metalloproteinase 9 (MMP9)	[Melani et al., 2007]
S100A8 and S100 A9	[Cheng et al., 2008]
Heat-shock protein 72 (Hsp72)	[Chalmin et al., 2010]
CC-chemokine ligand 2 (CCL2)	[Fridlender et al., 2010]
CXC-chemokine ligand 5 (CXCL5)	[Toh et al., 2011]
CXC-chemokine ligand 15 (CXCL12)	[Obermajer et al., 2011]

Table 1. 2. Factors implicated in the expansion or activation of MDSC in cancer.

Upon differential activation, MDSC express a number of cytokines and proinflammatory molecules producing an autocrine feedback loop that triggers their own activation or sustains MDSC within the tumor microenvironment.

MDSC secreted cytokines	Reference
Transforming growth factor beta (TGFβ)	[Terabe et al., 2003]
Interleukin-1β (IL-1β)	[Bruchard et al., 2013]
Interleukin-10 (IL-10)	[Chen et al., 2001]
Interleukin-13 (IL-13)	[Gallina et al., 2006]
Tumor necrosis factor α (TNFα)	[Umemura et al., 2008]

Table 1. 3. Cytokines released by MDSC.

Most of the factors described above converge in common signaling pathways, some of which have been implicated in regulating MDSC expansion such as Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3). Engagement of cytokine receptors activates JAKs that subsequently induce phosphorylation of STAT protein family members [Rawlings et al., 2004]. Whereas constitutive activation of STAT3 in cancer cells and other tumor-infiltrating cells is well documented [Catlett-Falcone et al., 1999; Hu et al., 2014], Kusmartsev and colleagues could demonstrate a pivotal role for STAT3 in the expansion of MDSC by showing that STAT3 is persistently activated in MDSC and prevents myeloid cells from further differentiating [Kortylewski et al., 2005]. Blockage of STAT3 with selective inhibitors resulted in stronger antitumoral T cell responses and a significant decrease in MDSC frequency [Nefedova et al., 2007].

1.4.4 Mechanisms of immunosuppression exerted by MDSC

Myeloid derived suppressor cells use multiple mechanisms to suppress antitumor immunity. Their main feature is preventing the host from mounting an effective T cell response by inhibiting the proliferation of $CD8^+$ cytotoxic T lymphocytes, inducing T cell anergy or apoptosis, perturbing T cell activation or downregulation of IFNγ secretion by T cells. Most studies showed the necessity of direct cell-cell interaction with target cells implicating that MDSC function either via cell surface receptors and/or via the release of soluble short-lived mediators.

MDSC effectively suppress antigen-specific T cell responses by amino acid metabolism. In particular, both MDSC subsets express high levels of arginase 1 (Arg1) which allows them to metabolize L-arginine to L-ornithine and urea resulting in depletion of L-arginine from the microenvironment [Bronte and Zanovello, 2005; Rodriguez et al., 2009]. T cells in turn lack L-arginine and then fail to express CD3ζ-chain, which keeps them in the G_0-G_1 cell cycle phase thus repressing protein synthesis [Baniyash, 2004; Rodriguez and Ochoa, 2008]. Simultaneously, L-arginine is the substrate for another enzyme: inducible nitric oxide synthase (iNOS) that is mainly expressed by MO-MDSC and catalyzes the conversion of L-

arginine to L-citrulline and nitric oxide (NO). NO operates through various mechanism to suppress T cell function such as interfering with the IL-2 signaling pathway, preventing TCR activation and eventually leading to apoptosis [Fischer et al., 2001; Ferlito et al., 2006]. Another mechanism that has been recently described is the sequestration of cysteine. T cells lack the machinery to generate cysteine, thus it represents an essential amino acid for them. During activation, T cells especially require cysteine for protein synthesis and differentiation. Under steady-state conditions it is provided by antigen-presenting cells via direct import during antigen presentation. However, MDSC are unable to export cysteine thus depleting it from the environment.

Increased production of reactive oxygen species (ROS) has emerged as one of the main factors by which MDSC contribute to immunosuppression in tumor-bearing mice and patients with cancer [Schmielau and Finn, 2001; Kusmartsev et al., 2004]. ROS are derived from metabolism of cellular oxygen and include highly reactive species such as super oxide $O2^-$, hydrogen peroxide H_2O_2 and peroxynitrite ($ONOO^-$) as the product of NO and superoxide $O3^-$. ROS are involved in both cancer initiation as well as progression and directly associated with T cell unresponsiveness and immunosuppression in patients with advanced disease [Mantovani et al., 2003]. Peroxynitrite induces nitrosylation of amino acids of the TCR complex during cell-cell contact which blocks the formation of CD8/MHC-I complexes rendering T cells unresponsive to antigen-specific stimulation [Nagaraj et al., 2007]. Several known tumor-derived factors induce ROS generation by MDSC such as TGFβ, IL-10, IL-6 and GMCSF (Table 1.3).

Other described mechanisms of MDSC mediated immunosuppression include the downregulation of L-selectin, also known as CD62L, on naïve T cells. CD62L acts as a homing-receptor by promoting extravasation and tracking of naïve T cells to antigen-containing sites such as lymph nodes and the tumor mircroenvironment [Tedder et al., 1995]. L-selectin expression on T lymphocytes is inversely correlated with MDSC frequency in tumor-bearing mice and cancer patients. Additionally, co-culture of MDSC with $CD4^+$ and $CD8^+$ T cells results in downregulation of L-selectin expression [Hanson et al., 2009]. The induction of T_{regs} by MDSC as a contributing factor for tolerance to tumor-specific antigens *in vivo* has been described by several groups [Serafini et al., 2006; Movahedi et al., 2008]. However, due to conflicting reports, the physiological relevance and presence of this mechanism needs further scientific proof. Additional mechanisms described are the induction of NK cell anergy via a mechanism involving MDSC-bound TGFβ [Li et al., 2009]. Interestingly, Park and colleagues showed that a specific subset of $CD11b^+$ NK cells within the tumor microenvironment can be converted into $GR1^+CD11b^+$ MDSC *ex vivo* in a GM-CSF-dependent manner.

MDSCs also exhibit several non-immunological features to promote tumorigenesis. They foster tumor invasion and metastasis by expression of matrix metalloproteinases (MMPs) such as MMP9, a process that has shown to depend on the upregulation of hypoxia-inducible factor 1-α and microRNA-494 [Du et al., 2008; Liu et al., 2012]. Via secretion of VEGF, MDSC also promote tumor angiogenesis [Lechner et al., 2010]. Furthermore, one study demonstrates that splenic $Gr1^+CD11b^+$ cells can directly differentiate into endothelial progenitor cells and thereby contribute to *de novo* vasculogenesis of tumors [Yang et al., 2004].

Given the complexity of the described mechanism regulating MDSC accumulation, activation and the variety of immunosuppressive mechanism they exploit, it will be essential to determine which conditions and factors are dominant in order to specifically target MDSC-associated immunosuppressive features.

1.5 Immunotherapy of cancer

1.5.1 Overview

Most cancer patients are treated with a conventional combination of surgery, radio- and chemotherapy. Nevertheless, the primary failure in reducing cancer-related mortality is the insufficient control of advanced disease, metastatic spread and the presence of micrometastases or minimal residual disease which are not recognized by diagnostic imaging. As cancer becomes a chronic disease, another issue obtaining increasing attention is therapy-related mortality. In the last two decades multiple attempts have been made in order to eradicate clinically non-detectable disease and reducing therapy-related mortality. Meanwhile, besides surgery, chemotherapy and radiation, immunotherapy of cancer is already established as a fourth mainstay in clinical oncology.

Clinically established approaches include the administration of cytokines: a number of cytokines including GM-CSF, IL-7, IL-12, IL-15, IL-18 and IL-21 have been evaluated in preclinical studies and are now entering clinical trials for patients with advanced stage disease. To date, two cytokines have been FDA-approved, namely IL-2 for treatment of metastatic melanoma and renal cell cancer [Coppin et al., 2005] and IFNα for stage III melanoma and a number of hematologic neoplasms such as chronic myeloid leukemia [Talpaz et al., 1986; Golomb et al., 1986].

A second approach involves the use of monoclonal antibodies (mAb) targeting disease-related proteins for degradation. Monoclonal antibodies have achieved considerable success in recent years: over 30 mAbs are currently FDA-approved, the most popular in oncology being

rituximab, bevacizumab and trastuzumab [Scott et al., 2012]. Future trends facilitate the development of bifunctional antibodies such as bispecific T cell engagers (BiTEs) or trifunctional bispecific antibodies (trAb) aiming to bring cytotoxic T-cells, tumor cells and eventually innate immune effector cells into close proximity. Catumaxomab was the first trAb receiving FDA-approval in 2009 for treatment of malignant ascites [Heiss et al., 2005].

Considerable progress has been made in the last decade by using oncolytic viruses, adoptive T cell transfer or using cancer vaccines for cancer immunotherapy (reviewed by [Parato et al., 2005; Rosenberg et al., 2008]). Adoptive T cell transfer has being successfully studied in mice [Bourquin et al., 2010] and is now evaluated in various clinical trials in patients with advanced stage disease with our without dendritic cell vaccination (cp. http://www.clinicaltrials.gov/ct2/results?term=T+cell+transfer). Cancer vaccines are one of the latest strategies in immunotherapy. They include the use of tumor-associated antigens in combination with adjuvants for induction of a long-lasting and antigen specific immune response [Baxevanis et al., 2009]. Dendritic cell-based vaccines are currently under evaluation. Autologous DC are stimulated *ex vivo* and provided with the tumor-antigen via peptide-uptake, mRNA or cDNA. Autologous DC are a then reinjected into the patient with or without an additional adjuvant or disease specific therapy. Those approaches show promising results both in preclinical studies in mice as well as in clinical trials mice [Wurzenberger et al., 2009].

A novel therapeutic approach with remarkable antitumor potential that has been already evaluated in large scale clinical trials during the last years is targeting immune checkpoints. Tumors co-opt signaling pathways that normally avoid overactivation of T cells and maintain self-tolerance in healthy individuals. Many of these "immunological breaks" are initiated by distinct ligand-receptor interactions and can be targeted by so-called immune checkpoint inhibitors (ICIs). Thus far, these antibody-based treatment strategies target programmed cell death ligand 1 (PD-L1), programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA4). Importantly, ICIs have shown significant and durable responses not only in highly immunogenic malignancies such as malignant melanoma and renal cell carcinoma but also in a number of solid cancer entities that were previously not believed to be accessible to immune-based therapies [Brahmer et al, 2012; Brahmer and Pardoll, 2013].

The increasing knowledge in tumor immunology, namely the strong immunosuppressive paramalignant environment leading to inability of the immune system to recognize cancer cells, has led to the concept of activating the immune system in order to restore its functionality. Concurrent growing inside into the signaling and biological function of pattern recognition receptors and their central involvement in the initiation of innate and adaptive

immunity, has moved PRRs into the field of immunotherapy. However, there are conflicting reports questioning the use of PRR ligands, bearing in mind that TLRs and other PRRs are also expressed in tumor cells which might limit their clinical use in anticancer treatment [Huang et al., 2008].

1.5.2 Pattern recognition receptor ligands: a double-edged sword in cancer

Tumor cell signaling pathways that trigger essential malignant features such as uncontrolled proliferation, resistance to apoptosis, induction of angiogenesis and escape from immune evasion are partially understood. Recent work revealed that some of the signaling pathways coincide with those initiated by PRR ligands. Despite the use of PRR ligands in anti-cancer immunotherapy, various PRR receptors, especially TLRs are also expressed on a variety of tumors suggesting that TLR signaling may play important roles in tumor biology and thus limiting the effect of PRR-based immunotherapy.

1.5.2.1 PRR signaling and tumor progression

The initial idea that TLR stimulation may drive tumorigenesis came from reports demonstrating that systemic TLR4 and TLR5 activation unexpectedly augmented tumor growth in adoptively transferred murine tumor models [Pidgeon et al., 1999; Sfondrini et al., 2006]. Researchers from Mount Sinai Hospital and others have shown that human and murine cancer cell lines express multiple functionally active TLRs compared to the respective non-neoplastic tissue [Huang et al., 2005]. For instance, human lung, cervical and prostate cancer cells have been found to express high levels of TLR9 [Droemann et al., 2005; Ilvesaro et al., 2007; Lee et al., 2007], whereas TLR2, 3 and 4 expression has been found on laryngeal carcinoma, melanoma, multiple myeloma and leukemic cells [Bohnhorst et al., 2006; Molteni et al., 2006; Szczepanski et al., 2007]. Accumulating evidence now suggests that neoplastic transformation may usurp the signaling pathways used by TLRs and other pattern recognition receptors to favor cancer progression and facilitate the evasion of immune surveillance. In line with this, key transcription factors in TLR-dependent signaling pathways such as NFκB proteins, MyD88 and PI3K/AKT have been found to directly mediate survival, proliferation and increased angiogenesis during tumor progression [Karin and Greten, 2005; Rakoff-Nahoum and Medzhitov, 2007]. Notably, TLR signaling and NFκB activation may even play a more important role on the tumor microenvironment. By secreting inflammatory cytokines and chemokines, sometimes elicited by TLR activation, tumor cells attract non-malignant cells which foster local immunosuppression by upregulation of crucial factors like vascular-endothelial growth factor (VEGF) and immunosuppressive cytokines like IL-10 or TGFβ [Sato et al., 2009]. Given that tumor growth can mimic tissue damage, the studies discussed above

indicate TLRs might serve as a link between tissue repair, inflammation and tumorigenesis. Further dissection of associated signaling pathways and delineation of differences between microenvironmental versus systemic PRR stimulation should provide interesting insights into cancer development and the use of PRR ligands in immunotherapy.

1.5.2.2 Use of PRR ligands in the immunotherapy of cancer

Over a century ago, the New York surgeon William Coley showed that intratumoral injection of *streptococcus pyogenes* and later a mixture of heat-killed *streptococcus pyogenes* and *serratia marcescens* (Coley's toxin) can induce significant regression of soft tissue sarcoma [Coley, 1991]. Without understanding the underlying biologic mechanisms, he has set the cornerstone for modern immunotherapy. Today we know that such approaches function by stimulating PRR signaling and activate both innate and adaptive immune responses.

The idea of PRR agonists to provide a "danger signal" and break tolerance to tumor antigens has been well embraced by tumor immunologists. They make up attractive targets in cancer immunotherapy because of different reasons: in contrast to the application of distinct cytokines alone, PRR signaling induces a coordinated immune response, more resembling the natural situation. Furthermore, several PRR ligands have shown to induce co-stimulatory molecules (CD80, CD86, CD40) on dendritic cells and inflammatory cytokines such as TNFα and IL-12 both of which polarize a T_H1-skewed immune response. Finally, a number of PRR ligands generate a potent type I IFN response which is essential against intracellular pathogens and immunological defense against host tumor cells.

Because TLRs were the first PRRs to be described and their ligands and signaling pathways have been well elucidated, they were the first to be studied in anti-cancer immunotherapy and to enter clinical trials. Our group is especially interested in using different PRR ligands in murine tumors models. During the last years, we could show *in vivo* tumoricidal efficacy of different TLR and RLR ligands such as poly(I:C), R848, CpG-ODN and ppp-RNA that signal via TLR3/MDA-5, TLR7, TLR9 and RIG-I, respectively [Bourquin et al., 2006; Bourquin et al., 2009; Bourquin et al., 2011; Zoglmeier et al., 2011]. Whitmore and colleagues showed that administration of CpG-DNA complexed with cationic liposome-based lipoplexes elicits a potent induction of T_H1 cytokines (TNFα, IFNγ and IL-12) and showed tumoricidal NK cell activity in a murine model of pulmonary metastasis [Whitmore et al., 2001]. The same group reported in 2004 that dsRNA and CpG can synergistically enhance antitumor activity [Whitmore et al., 2004]. Multiple other studies during the last decade have proven that TLR ligands can have potent antitumor activity in murine tumor models [Okamoto et al., 2006; Sfondrini et al., 2006; Favaro et al., 2012; Stier et al., 2013]. Current efforts are mainly focused

on TLR7 and TLR9 [Lee et al., 2003; Pashenkov et al., 2006; Krieg, 2007]. However, the promise of TLR agonist-based immunotherapy remains to be realized in clinical practice. To date, there are only a few FDA-approved TLR agonists. As an example, bacillus Calmette-Guerin (BCG) and imiquimod are approved as monotherapy for urothelial carcinoma and basal cell carcinoma. Clinical trials have shown promising results for CpG-DNA in combination therapies for B-cell lymphoma [Brody et al., 2011], non-Hodgkin's lymphoma [Friedberg et al., 2005] and non-small cell lung cancer [Manegold et al., 2008]. Finally, recent reports also underline the role of Pattern recognition receptors in a process called immunogenic cell death (ICD). ICD is described as a cell death modality that stimulates an immune response against dead-cell antigens that function as damage-associated molecular patterns (DAMPs). Via TLR4, DAMPs induce a tumor-antigen specific immune response via TLR4 which requires cross-presentation of tumor antigens to DCs on MHC class I to generate CTLs [Apetoh et al., 2007]. Two years later the same group showed that NLRP3-dependent release of IL-1β links innate and adaptive immune responses against dying tumor cells [Ghiringhelli et al., 2009]. A current search on ClinicalTrials.gov using the key words "TLR" and "cancer" returned 35 listings. Despite promising results in some studies to date, some of which are still recruiting, quite a few have been withdrawn showing no benefit of conventional therapy versus conventional therapy plus the use of TLR agonists.

Other PRRs that gained interest as a target for tumor immunotherapy are the RLRs RIG-I and MDA-5. A prior study by Besch and colleagues showed potent induction of apoptosis when human melanoma cells were transfected with HMW-poly(I:C) as ligand for MDA-5 and pppRNA as ligand for RIG-I *in vivo*. Those results were confirmed in a humanized mouse model of lung metastasis in NOD/SCID mice showing potent antitumoral activity of both ligands by induction of apoptosis in line with a pronounced type I IFN response [Besch et al., 2009]. Researchers, including our own group now started to combine the effect of the ppp-moiety on RIG-I activation with siRNAs against known tumor-promoting factors such as B-cell lymphoma 2 protein (Bcl-2) or transforming growth factor beta (TGFβ) [Petrocca and Lieberman, 2008; Poeck et al., 2008].

These opposing roles of TLRs in immunotherapy delineate the need of research in order to elucidate how to overcome potentially harmful effects on PRR-expressing tumor cells while maintaining beneficial antitumoral activity and to minimize the therapy-limiting effect of a strong immunosuppressive environment.

1.6 Objectives

Overcoming the ability of malignant tumors to evade and suppress host immune responses is vital for successful immunotherapy approaches. Various immune suppressor cells have been identified as key mediators of such immune dysfunction in cancer patients. In particular, myeloid-derived suppressor cells (MDSC) have emerged as major regulators of immune tolerance, because they do not only enable and sustain malignant growth and metastatic disease, but also represent a major obstacle for effective immunotherapies. MDSC are a heterogenous population accumulating in cancer patients defined by their immature state due to a partial differentiation block. Little is known about the phenotypical difference of non-immunosuppressive immature myeloid cells in tumor-free hosts versus myeloid-derived suppressor cells in tumor-bearing hosts. Furthermore, it remains largely obscure if reversing this paramalignant differentiation block is feasible in order to enable a more vigorous anti-tumor immune response.

Activation of the innate immune system by stimulation of pattern recognition receptors has been evaluated as a potent approach in cancer immunotherapy protocols, thereby minimizing toxic side effects of chemotherapy alone. Specifically therapeutic oligonucleotides, such as CpG-ODN, have been intensively studied as immune adjuvants for cancer therapy. Previous studies, including work from our own group, have shown that innate immune activation with the TLR9 agonist CpG-ODN evokes efficient antitumor immune responses in mice with significant decrease in tumor load and prolonged survival [Heckelsmiller et al., 2002; Bourquin et al., 2008]. However, some studies suggest a pivotal role of Toll-like receptor activation in the expansion and/or activation of MDSC, calling the clinical utility of such approaches into question.

By using Toll-like receptor ligands to induce innate immune activation in tumor-free and tumor-bearing mice, this work was designed to answer the following questions:

(a) Does stimulation of TLR9 and other innate immune receptors *in vivo* affect phenotype and function of $Gr1^+CD11b^+$ immature myeloid cells?

(b) If so, does the same holds true for $Gr1^+CD11b^+$ myeloid-derived suppressor cells in tumor-bearing hosts?

(c) Which cellular or soluble factors following *in vivo* stimulation of pattern recognition receptors might be crucial for the observed effects?

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Technical equipment

Balance (LP 6209)	Sartorius, Göttingen, Germany
Confocal fluorescence microscope	Leica TCS SP5, Wetzlar, Germany
Cell culture CO2 incubator (BD 6220)	Heraeus, Hanau, Germany
Cell culture laminar flow	Heraeus, Hanau, Germany
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Centrifuge 5424	Eppendorf, Hamburg, Germany
DynaMag 15/50 magnet	Invitrogen Dynal, Carlsbad, USA
EasySep©Magnet	StemCell Technologies, Grenoble, France
FACS Canto II	BD Biosciences, San Diego, USA
Lab Water Purification Direct-Q 3 UV	Millipore, Billerica, Massachusetts, USA
Microscope Axiovert 25	Zeiss, Jena, Germany
MiniMACS, QuadroMACS, LS columns	Miltenyi, Bergisch Gladbach, Germany
Mithras LB940 multilabel plate reader	Berthold, Bad Wildbad, Germany
Multifuge 3L-R	Heraeus (Hanau, D)
Multifuge 4KR	Heraeus (Hanau, D)
Nanodrop ND-1000	NanoDrop, Wilmington, USA
Neubauer hemocytometer	Optik Labor Frischknecht, Balgach, Germany
pH meter	WTW, Weilheim, Germany
Refrigerators (4°C, -20°C)	Bosch, Gerlingen, Germany
Refrigerators (-80°C)	Thermo Scientific, Waltham, USA
Shaker	NeoLab, Heidelberg, Germany
Thermocycler T3	Biometra, Göttingen, Germany
Thermomixer	Eppendorf, Hamburg, Germany
Vortex VF2	Janke & Kunkel, Staufen, Germany

2.1.2 Chemicals, reagents and buffers

Agarose LE	Biozym, Hess. Oldendorf, Germany
Aqua ad injectabilia	Braun AG, Melsungen, Germany
Bovine serum albumine (BSA)	Sigma Aldrich, Steinheim, Germany
Collagenase D	Sigma Aldrich, Steinheim, Germany
Chloroform	Sigma Aldrich, Steinheim, Germany
DNAse I	Sigma Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Steinheim, Germany
Dulbecco's PBS (1x)	PAA, Pasching, Germany
Dynabeads® T-Activator	Invitrogen Dynal AS, Oslo, Norway
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, Steinheim, Germany
FACSFlow, FACSClean	Becton Dickinson, San Jose, USA
H_2SO_4	Sigma Aldrich, Steinheim, Germany
Heparin-Natrium 25000 I.E./5 ml	Braun AG, Melsungen, Germany
in vivo-JetPEI	Peqlab, Erlangen, Germany

Isoflurane (Forene®)	Abbott, Zug, Switzerland
Isopropanol (70 Vol%)	Apotheke Innenstadt, LMU Munich
PharmLyse (10x) RBS lysis	Becton Dickinson, Heidelberg, Germany
Paraformaldehyde (PFA)	Sigma Aldrich, Steinheim, Germany
Sodium azide (NaN_3)	Sigma Aldrich, Steinheim, Germany
Sodium chloride (NaCl 0.9%)	Apotheke Innenstadt, LMU Munich
Sulfuric acid H_2SO_4, 2N)	Apotheke Innenstadt, LMU Munich
Trypan blue Sigma	Aldrich, Steinheim, Germany
Tris-HCL (pH 7,4)	Sigma-Aldrich, Steinheim, Germany
Trypan blue	Sigma-Aldruch, Steinheim, Germany
Trypsin (10x)	PAA, Pasching, Austria
Tween 20 (pH 7,0)	Sigma-Aldrich, Steinheim, Germany

Erythrocyte lysis buffer
10% PharmLyse
in PBS

Dissection buffer (tumors)
1 mg/ml Collagenase D
0.05 mg/ml DNAse I
10% FCS
In RPMI

MACS buffer
10% FCS or 0.5 Vol% BSA
2 mM EDTA
in PBS

StemCell buffer Gr1-PE positive sort
2% FCS
1 mM EDTA
in PBS

FACS buffer
5% FCS
in PBS

Cell fixation buffer
2% PFA
in PBS

ELISA coating buffer 1
0.2 M sodium phosphate
in water
pH 6.5
(variations according to manufacturer)

ELISA coatin buffer 2
0.1 mM sodium carbonate
in water
pH 9.5
(variations according to manufacturer)

ELISA assay diluents
10% FCS
in PBS
pH 7.0
(variations according to manufacturer)

ELISA washing buffer
0.05% Tween 20
in PBS

2.1.3 Cell culture materials, reagents and media

β-Mercaptoethanol	Sigma-Aldrich, Steinheim, Germany
DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-3N-trimethylammonium-methylsulfate	Roche, Mannheim, Germany
Dulbeccos's modified Eagle's medium (DMEM), high glucose	PAA, Pasching, Austria
Dynabeads® Mouse T-Activator CD3/CD28	GibcoBRL, Karlsruhe, Germany
Fetal Calf Serum (FCS)	GibcoBRL, Karlsruhe, Germany
HEPES Buffer	Sigma Aldrich, Steinheim, Germany

Iscove's modified Dulbecco's medium	PAA, Pasching, Austria
L-glutamine 200 mM	PAA, Pasching, Austria
Lipofectamine™ 2000	Invitrogen, Karlsruhe, Germany
Minimum essential medium with non-essential amino acids (MEM-NEAA)	GibcoBRL, Karlsruhe, Germany
Opti-MEM® Reduced Serum Medium	Gibco, Life Technologies, Frankfurt
Phosphate buffered saline (PBS)	PAA, Pasching, Austria
Penicillin, Streptomycin (100x)	PAA, Pasching, Austria
Ciprofloxacin (2 mg/ml)	Bayer Pharma AG, Berlin, Germany
Rat serum	StemCell Technologies, Grenoble, France
Roswell Park Memorial Institute (RPMI) 1640 medium	PAA, Pasching, Austria
RPMI VLE	PAA, Pasching, Austria
Sodium pyruvate	Biochrom, Berlin, Germany

RPMI complete medium
10% FCS
2 mM L-glutamine
100 IU/ml penicillin
100 µg/ml streptomycin
1 mM sodium pyruvate
1% MEM-NEAA
3.75 x 10^{-4} β-mercaptoethanol
In RPMI 1640

T-cell medium (TCM)
10 % FCS
2 mM L-glutamine
100 IU/ml penicillin
100 µg/ml streptomycin
1 mM sodium pyruvate
1% MEM-NEAA
1 x 10^{-4} % β-mercaptoethanol
in RPMI 1640 VLE

DMEM complete medium
10% FCS
2 mM L-glutamine
100 IU/ml penicillin, 100 µg/ml streptomycin

Cryo medium
40% DMEM complete medium
50% FCS
10% DMSO

Bone marrow-derived macrophage medium (BMDM medium)
10% FCS
30% L929-cell-conditioned medium (LCCM)
0.5% Ciprofloxacin 100x
in RPMI

Disposable plastic materials for cell culture experiments were purchased from Becton Dickinson (Heidelberg, Germany), Bibby Sterrilin (Stone, Staffordshire, Great Britain), Corning (Corning, USA), Eppendorf (Hamburg, Germany), Falcon (Heidelberg, Germany), Greiner (Frickenhausen, Germany), Henke-Sass Wolf (Tuttlingen, Germany), Nunc (Rochester, USA) or Sarstedt (Nümbrecht, Germany).

2.1.4 PRR ligands, cytokines and growth factors

All following stimuli are displayed with used concentration *in vitro* or *in vivo* unless indicated otherwise. Special methodical remarks (e.g. complexation of RNA) are displayed below the respective list.

Recombinant cytokines

Cytokine	*In vitro* [c]	Distributor
IFNα, recombinant, human	1000 U/ml	Miltenyi, Bergisch-Gladbach, GER
IFNγ, recombinant, mouse	100 U/ml	eBioscience, San Diego, CA, USA
TGFβ$_1$, recombinant, mouse	20 ng/ml	R&D Systems, Wiesbaden, GER

Table 2.1. List of recombinant cytokines with concentrations used *in vitro*.

Pattern recognigtion receptor (PRR) ligands

Ligand	Receptor	*In vitro* [c]	*In vivo* [c]	Distributor
Pam3CSK	TLR1/2	2 µg/ml	-	InvivoGen, Toulouse, France
Poly I:C	TLR3	200 µg/ml	250 µg i.p.	InvivoGen, Toulouse, France
LPS E.coli K12	TLR4	1 µg/ml	100 µg i.p.	InvivoGen, Toulouse, France
Flagellin	TLR5	1 µg/ml	-	InvivoGen, Toulouse, France
R848	TLR7	2 µg/ml	-	InvivoGen, Toulouse, France
9.2dr RNA*	TLR7	-	20 µg i.v.	CureVac, Tübingen, Germany
CpG 1826	TLR9	5 µg/ml	100 µg s.c.	Coley, Langenfeld, Germany
5′-triphosphate 2.2 dsRNA*	RIG-I	1 µg/ml	50 µg i.v.	Eurogenentech, Köln, Germany
Poly dA:dT*	AIM2	2 µg/ml	-	Sigma Aldrich, Steinheim, GER
Curdlan	Dectin-1	20 µg/ml	-	InvivoGen, Toulouse, France

Table 2.2. List of pattern recognition receptor ligands, corresponding receptor and concentrations used for *in vitro* stimulation experiments or *in vivo* therapy. * indicated oligonucleotides are complexed with DOTAP, Lipofectamine™ 2000 or *in vivo*-JetPEI before *in vitro* or *in vivo* usage. For more detailed methodical explanations see chapter 2.2.3 and 2.3.4.

List of oligonucleotide sequences

Oligonucleotide	Nucleotide sequence 5′➔ 3′
CpG-B-ODN 1826 (CpG)*	TCCATGACGTTCCTGACGTT
9.2 double right RNA (9.2dr RNA)*	UGUCCUUCAAUGUCCAA
5′-triphosphate 2.2 dsRNA (3pRNA)*	GCAUGCGACCUCUGUUUGA
Murine siRNA against TGFβ$_1$	GAACUCUACCAGAAAUAUA
Control siRNA	GAUGAACUUCAGGGUCAGC

Table 2.3. List of selected oligonucleotide sequences shown from 5-prime to 3-prime end.

Indicated oligonucleotides (*) carry a PTO-modification. PTOs contain one sulfur atom instead of an oxygen atom in the internucleotidic linkage resulting in higher stability and less enzymatic degradation. siRNAs against TGFβ_1 were designed according to published guidelines including a 3´-dTdT overhang and were purchased from Eurofins MWG Operon, Ebersberg, Germany.

2.1.5 Tumor cell lines

Cell line	Background	Disease	Origin	Reference
B16-F10	C57Bl/6	Melanoma	Prof. T. Brocker, Institut für Immunologie, LMU Munich	(Fidler 1975)
C26	Balb/c	Colon cancer	CLS, Eppelheim, Germany	(Corbett, Griswold et al. 1975)
E.G7	C57Bl/6	Thymoma	ATCC, Manassas, USA	(Moore, Carbone et al. 1988)
EL-4	C57Bl/6	Thymoma	ATCC, Manassas, USA	(Ralph and Nakoinz 1973)
mGC8	C57Bl/6	Gastric cancer	Prof. W. Zimmermann, LIFE Center, LMU Munich	(Nockel, van den Engel et al. 2006)
RMA-S	C57Bl/6	Lymphoma	Dr. J. Charo, Max-Dellbrück- Center, Berlin	(Karre, Ljunggren et al. 1986)
Panc02	C57Bl/6	Pancreatic cancer	National Cancer Institute (NCI)	(Corbett, Griswold et al. 1975)

Table 2.4. List of tumor cell lines used in this work with listed mouse background, origin and reference.

2.1.6 Kits

Magnetic Cell Sorting

EasySep© Gr1-PE positive sort (StemCell Technologies, Grenoble, France)	Gr-1-PE-conjugated antibody, murine PE selection cocktail Magnetic NanoParticles
MDSC Isolation Kit, murine (Miltenyi Biotec, Bergisch Gladbach, GER)	Anti-Ly-6G- Biotin, murine Anti-Gr-1-Biotin, murine Anti-Biotin and Streptavidin MicroBeads FcR Blocking Reagent, murine
Anti-CD19 Microbeads, murine	Miltenyi Biotec, Bergisch Gladbach, GER
Anti-CD3-Biotin, murine	Miltenyi Biotec, Bergisch Gladbach, GER
Anti-Biotin-MicroBeads	Miltenyi Biotec, Bergisch Gladbach, GER
CD8 T-Cell Isolation Kit, murine	Miltenyi Biotec, Bergisch Gladbach, GER

Cytokine ELISA sets

IL-1β, murine	BD Biosciences, San Diego, USA
IL-6, murine	BD Biosciences, San Diego, USA
IL-10, murine	BD Biosciences, San Diego, USA
IL-12p40, murine	BD Biosciences, San Diego, USA
CXCL10 / IP-10, murine	R&D Systems, Wiesbaden, Germany
IFNγ, murine	R&D Systems, Wiesbaden, Germany

Cytokine ELISA antibodies

Detection of murine IFNα:	
Capture-Ab: Anti-IFNα (RMMA-1)	PBL, New Brunswick, USA
Detection-Ab: Anti-IFNα (polyclonal, rabbit anti-mouse)	PBL, New Brunswick, USA
HRP-conjugated F(ab´)2 fragments (donkey anti-rabbit)	Biomeda, Foster City, USA

MDSC Suppression Assay

Cell Proliferation ELISA, BrdU (chemiluminescent)	Roche Diagnostics, Mannheim, Germany

Apoptosis Assay

FAM Flica ™ Caspase 3 & 7 Assay Kit	ImmunoChemistry, Bloomington, USA

3p-RNA Transcription Kit

MEGAshortscript™ T7 Kit	Ambion, Austin, Texas, USA

2.1.7 Antibodies: FACS and Immunofluorescence

Flow cytometry antibodies

All antibodies used in this work were either purchased from BD/Pharmingen (Heidelberg, GER) or BioLegend (San Diego, CA, USA). Following fluorochromes were used depending on the experimental setup: FITC, PE, PeCy7, PerCP, APC, APC-Cy7, PacificBlue, PacificOrange.

Description	Isotype	Clone
Anti-CD3	Rat IgG_{2b}	17A2
Anti-CD4	Rat IgG_{2b}	GK 1.5
Anti-CD8a	Rat (LOU/Ws1/M) IgG_{2a}, κ	53-6.7
Anti-CD11b	Rat (DA) IgG_{2b}, κ	M1/70
Anti-CD11c	Armenian Hamster IgG_{1a}	N418
Anti-CD19	Rat, IgG_{2a}, κ	1D3
Anti-CD45/B220	Rat IgG_{2a}, κ	RA3-6B2
Anti-CD45	Rat IgG_{2b}	30-F11
Anti-CD80 (B7-1)	Armenian Hamster IgG_2, κ	16-10A1

Anti-CD86 (B7-2)	Rat (Louvain) IgG_{2a}, κ	GL1
Anti-F4/80	Rat IgG_{2a}, κ	BM8
Anti-Gr-1	Rat IgG_{2b}, κ	RB6-8C5
Anti-Ly-6C	Rat IgM, κ	AL-21
Anti-Ly-6G	Rat IgG_{2a}, κ	1A8
Anti-MHC I	Mouse IgG_{2a}, κ	$H\text{-}2D^b$
Anti-MHC II	Rat IgG_{2b}, κ	M5/114.15.2
Anti-Sca-1 (Ly6A/E)	Rat IgG_{2a}, κ	E13.161.7
Isotype controls		

Table 2.5. Fluorochrome-conjugated antibodies for flow cytometry with isotype and clone.

Fluorescence microscopy antibodies

Primary antibodies

Polyclonal rabbit anti-mouse CD3 (Abcam®)

Rat anti-mouse CD3 (clone 53-6.7) (BioLegend®)

Secondary antibodies

Cy3 Goat anti-rabbit IgG (H+L) (BioLegend®)

Polyclonal Cy2 Goat anti-Rat IgG Secondary Antibody (ThermoScientific®)

2.1.8 Software

Adobe Illustrator CS5	Adobe System, San Jose, USA
CellQuest	BD Biosciences, San Diego, USA
Endnote X2	Thompson Reuter, Carlsbad, USA
FlowJo	Tree Star, Ashland, USA
GraphPad Prism®	GraphPad Software, San Diego, CA, USA
Microsoft Office	Mircosoft, Redmond, USA

2.2 Animal experiments

2.2.1 Animals

Female Balb/c or C57BL/6 mice were obtained from Harlan-Winkelmann (Borchen, Germany). IFNα receptor (IFNαR)-deficient mice and Stem Cell Antigen-1 (Sca-1)-deficient mice were kindly provided by Dr. Z. Waibler (Paul-Ehrlich Institute, Langen, Germany) and Prof. William Stanford (Institute of Biomaterials and Biomedical Engineering, University of Toronto, ON, Canada). CEA424-Tag mice [Thompson et al., 2000], kindly provided by W. Zimmermann (LIFE Center, LMU Munich, Germany) were bred heterozygously from

transgenic male mice. Mice were 8 – 12 weeks of age at the onset of experiments. All animal studies were performed according to ethical guidelines and approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

2.2.2 Tumor induction and measurement

In order to study the phenotype and function of myeloid-derived suppressor cells we compared various tumor models and different PRR ligands. The main tumor model used in this work was the CT26 subcutaneously induced colon cancer model (Balb/c background) which yielded high numbers of MDSC following tumor engraftment and is a well established and analyzed tumor model in the setting of CpG therapy in our laboratory [Bourquin et al., 2006]. We also studied and compared our observations in other available mouse tumor models and various IFNα-inducing PRR ligands (see chapter 2.2.3), not least because some gene-deficient mice were only available on C57BL/6 background.

For induction of subcutaneous tumors, tumor cells were centrifuged, washed twice, suspended in 100 µl PBS and injected into the right flank of C57BL/6 mice or Balb/c mice (CT26). The concentration of tumor cells per 100 µl as well as the approximate date of tumor onset varied between the respective cell lines (see table 2.5 below). The day of tumor onset was defined as formation of a palpable nodule (5 – 8 mm in diameter) subcutaneously or in sacrificed control mice (orthotopic Panc02). For orthotopic Panc02 tumor development, the left flank of anaesthetized mice was opened and the spleen mobilized to access the pancreas. A total of 2 x 10^5 Panc02 cells were then injected into the pancreas.

Cell line	[c] of cells / 100 µl	~ day of tumor onset
B16-F10	1 x 10^6	Day 7 - 10
CT26	2,5 x 10^5	Day 7 - 10
E.G7	1 x 10^6	Day 7 - 10
EL-4	1 x 10^6	Day 7 - 10
mGC8	2 x 10^6	Day 15 - 18
RMA-S	1 x 10^5	Day 8 - 12
Panc02	1 x 10^6	Day 12 - 14
Panc02 orthotopic*	2 x 10^5	Day 10 - 12

Table 2.5. List of different tumor cell lines used for subcutaneous or orthotopic tumor induction (Panc02) with corresponding concentration for tumor induction and day of tumor onset. *Orthotopic tumor induction was kindly performed by co-workers of the research group of Prof. Dr. med. M. Schnurr.

CEA424-Tag mice [Thompson et al., 2000] bearing autochthonous gastric tumors were used at the age of 85 – 100 days for analysis of myeloid-derived suppressor cells.

For subcutaneous tumors, tumor area is expressed as the product of perpendicular diameters of individual tumors. The tumor volume was measured thrice per week unless indicated otherwise. The volume of CEA424-Tag-derived gastric tumors (SV40) was calculated as the product of 3 perpendicular diameters. When tumor volume exceeded 225 mm^2 or when signs of animal distress were observed twice in 48 hours, mice were anaesthetized and sacrificed by cervical dislocation.

2.2.3 Therapy with PRR ligands

In order to determine the role of systemic Toll-like receptor and RIG-I activation on phenotype and function of myeloid-derived suppressor cells as well as on Stem Cell Antigen-1 expression, mice were treated with different immunostimulants. Tumor-free and tumor-bearing mice underwent the same treatment protocol unless indicated otherwise. The different treatment protocols used in this work as well as sites of injection are depicted in Table 2.6. Treatment of tumor-bearing mice was initiated following establishment of tumors (tumor area $\geq$ 25 mm^2 or age of CEA424-TAg mice $\geq$ 85 days) unless indicated otherwise. Therapy of orthotopic Panc02 tumor-bearing mice started on day 12, when sacrificed control mice showed visible tumor nodules (5 – 8 mm diameter). In general, 100 µg LPS, 100 µg CpG-oligodesoxyribonucleotides (ODN) and 250 µg poly(I:C) were dissolved in 100 µl PBS and injected subcutaneously or intraperitoneally as indicated in table 2.6. Immunostimulatory RNA was injected intravenously after complexation with DOTAP - a liposomal transfection reagent - or *in vivo*-jetPEI in order to prevent degradation and to improve rate of transfection. In detail, for treatment with 3pRNA or siRNA, 8 µl of *in vivo*-jetPEI were mixed with 50 µg of nucleic acid at an N:P ratio of 8:1 (8 nitrogen residues of jetPEI per RNA phosphate) in a volume of 200 µl 5% glucose solution, incubated 15 minutes at room temperature and injected intravenously into the retro-bulbar venous plexus or tail vein [Poeck et al., 2008; Ellermeier et al., 2013]. For *in vivo* administration of 9.2dr RNA, 20 µg of RNA were incubated with 100 µg DOTAP and 40 µl PBS for 20 minutes and injected into the retro-bulbar venous plexus. Depending on the experimental setup, 24 – 48 hours after the last treatment, mice were sacrificed, organs removed and prepared for further analysis as described below (chapter 2.2.4).

PRR ligand	Injection site	Days of treatment		organ removal after last course
		Tumor-free	Tumor-bearing*	
LPS	intraperitoneally	0 – 3 – 7	-	24 hours
CpG-ODN	subcutaneously	0 – 3 – 6	1 – 4 – 7	24 hours
Poly I:C	intraperitoneally	0 – 3 – 6	1 – 4 – 7	24 hours
3p-RNA	intravenously	0 – 3 – 6	1 – 4 – 7	24 hours
9.2dr RNA	intravenously	-	0 – 2 – 4 – 6 – 8 – 12	48 hours
(3p-)siTGFβ	intravenously	-	12 – 14	24 hours

Table 2.6. Treatment protocols and injections sites for *in vivo* administration with PRR ligands unless indicated otherwise. * Day 1 is defined as first day of treatment depending on tumor volume in mm². Day 0 or 12 are defined as days after tumor inoculation.

2.2.4 Organ and single cell preparation

2.2.4.1 Blood collection and serum analysis

For analysis of MDSC phenotype with or without PRR ligand therapy, blood cells were analyzed via flow cytometry. Mice were anesthetized with isoflurane and sacrificed via cervical dislocation. In order to receive a maximum of blood, the left chamber of the heart was punctured with a heparin coated syringe right after cervical dislocation. The blood was then transferred into 15 ml Falcon tubes, washed with 13 ml PBS, centrifuged (400 x G, 7 minutes) and resuspended in 3 ml erythrocyte lysis buffer. After incubation for 5 min at room temperature, reaction was stopped with 14 ml PBS. Red blood cell debris was removed by a second centrifugation step. The resulting cell pellet was then resuspended in 1 ml FACS buffer for further analysis.

To determine serum levels of interleukins, 24 hours after PRR ligand therapy, the retro-bulbar venous plexus of mice was punctured with heparinized capillary tubes until a minimum of 500 µl blood was obtained. Blood was allowed to coagulate for 20 minutes and then centrifuged at 13.000 x G for another 20 minutes. The resulting serum in the supernatant was collected and stored at -20°C for subsequent evaluation of interleukin serum levels via ELISA.

2.2.4.2 Tumor removal and digestion

Mice bearing established subcutaneous tumors (tumor volume at least $\geq$ 100 mm²) were anesthetized with isoflurane and sacrificed via cervical dislocation. Tumors were dissected and the skin was carefully removed. The resulting tumor tissue was dissected into small

pieces and incubated in dissection buffer at moderate stirring for 30 – 45 minutes at 37°C. The digested tumor tissue was then passed through a 100 µm-pore cell strainer, followed by a second passage through a 40 µm-pore mesh. Single cell suspensions were then washed twice, resuspended in FACS buffer and analyzed via flow cytometry.

2.2.4.3 Isolation of splenocytes

Mice were anesthetized with isoflurane and sacrificed via cervical dislocation. Spleens were resected and passed with PBS through a 40 µm-pore cell strainer for mechanical tissue disintegration. The resulting single cell suspension was centrifuged (400 x G, 7 minutes) and resuspended in erythrocyte lysis buffer. After incubation for 3 minutes at room temperature, reaction was stopped by adding PBS and red cell debris was removed by a second centrifugation step. Splenocytes were then resuspended in either RPMI complete medium or FACS buffer for further analysis.

2.2.4.4 Preparation of lungs and lymph nodes

Mice were anesthetized with isoflurane and sacrificed via cervical dislocation. Lungs, peripheral lymph nodes (axillary or inguinal) or tumor-draining lymph nodes were removed. Single cell suspensions of lungs were prepared as described above (chapter 2.2.4.2) and analyzed via flow cytometry. Lymph nodes of individual mice were pooled and directly passed through a 40 µm-pore cell strainer. After washing twice with PBS, single cell suspensions were resuspended in FACS buffer and analyzed via FACS.

2.2.4.5 Bone marrow isolation

Mice were anesthetized with isoflurane and sacrificed via cervical dislocation. Femur and tibia were dissected bilaterally. The remaining muscle tissue was carefully removed with small scissors and scalpel followed by external cleaning with 70% isopropanol and complete RPMI medium. Each end of bone was then cut off and bone marrow was expelled using a 27G needle / 1 ml syringe filled with complete RPMI medium. After passage through a 40 µm-pore cell strainer, single cell suspensions were washed once with complete RPMI medium and resuspended in erythrocyte lysis buffer. After lysis, cells were washed again and resuspended in RPMI complete medium for later experiments.

2.3 Cell culture experiments

2.3.1 General culture conditions and cell viability

All cell culture experiments were performed with sterile technique and under sterile conditions using a laminar air flow hood. Culture of cell lines or primary cells was performed in incubators under 37°C, 5% CO_2/air mixture and 95% air humidity. Cell viability was tested using trypan blue exclusion test: whereas the outer membrane of dying cells loses its integrity and absorbs trypan blue, it cannot enter the cell through intact membranes. Thus, the cytoplasm of dead or dying cells stains dark blue, whereas viable cells remain golden under light microscopy. Thus, after appropriate dilution, cell numbers of viable cells could be determined by using a Neubauer hemocytometer.

2.3.2 Tumor cell cultures

Tumor cell lines were obtained as described above (chapter 2.1.5). In general, cell lines were cultured in T75-T175 culture flasks in cell culture medium according to the respective cell line (see table 2.7). Cells were continuously checked via light microscopy for level of cell growth, signs of cell death and bacterial contamination. Medium was replaced regularly every two to three days depending on cell growth. In addition, adherent cell lines were splitted at a ratio of 1:2 – 1:10 at least twice a week and transferred to new culture flasks according to level of cell confluence and rate of cell growth. Therefore, medium was discarded and cells were gently detached either with a cell-scraper or by using 1 % trypsin-EDTA solution. Cells were then washed twice with PBS and re-disseminated in appropriate medium at lower concentrations. Non-adherent cell lines were splitted continuously two to three times a week. For *in vivo* tumor engraftment, tumor cells were splitted one day before injection to ensure optimal growth conditions. On day of tumor inoculation, tumor cells were harvested as described, re-suspended in PBS and separated by flushing through a 27G needle for three times.

Cell line	Cell growth	Culture medium	Split frequency
B16-F10	adherent	10% FCS, 1% P/S, 1% L-glu in DMEM	2-3 x per week, 1:10
CT26	adherent	10% FCS, 1% P/S, 1% L-glu in DMEM	2-3 x per week, 1:2
E.G7	suspension	10% FCS, 1% P/S, 1% L-glu, 1% So-Pyr, 1% NEAA in DMEM	3 x per week, 1:3
EL-4	suspension	10% FCS, 1% P/S, 1% L-glu in DMEM	3 x per week, 1:3
mGC8	adherent	10% FCS (Gold), 1% P/S, 1% L-glu, 1% So-Pyr , 1% NEAA in DMEM	1 x per week, 1:2

RMA-S	suspension	10% FCS, 1% P/S, 1% L-glu, 1% So-Pyr, 1% NEAA in RPMI	2-3 x per week, 1:5
Panc02	adherent	10% FCS, 1% P/S, 1% L-glu in DMEM	2-3 x per week, 1:10
L929	adherent	10% FCS, 0.5 % Cipro, 1% So-Pyr in DMEM	1 x per week, 1:5

Table 2.7. List of used tumor cell lines in this work and appropriate medium and split frequency for *in vitro* culture. P/S = penicillin/streptomycin, L-glu = L-glutamine, So-Pyr = sodium pyruvate.

2.3.3 Generation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were generated as described previously with some modifications [Zanoni et al., 2009; Marim et al., 2010]. M-CSF is a lineage-specific growth factor that is responsible for the differentiation from myeloid progenitors into macrophages. We used M-CSF transfected L929 cells which secret high amounts of M-CSF into the supernatant in order to obtain enough M-CSF for BMDM generation *ex vivo* [Englen et al., 1995]. L929 cells were kindly provided by Prof. Veit Hornung (University of Bonn, Germany). For generation of L929-conditioned medium (LCM), 2 x 10^7 L929 cells were seeded in T175 tissue flasks in 20 ml L929 culture medium. After one week of culture, supernatant was collected, sterile filtered and stored at -20°C until usage. For generation of murine BMDM, bone marrow was prepared as described above (chapter 2.2.4.5). Following washing with PBS, bone marrow cells of individual mice were splitted in half, suspended in 25 ml of BMDM medium containing 30% LCM and seeded onto two T175 culture flasks, resulting in a concentration of approximately 1 x 10^6 cells/ml. After one week of culture, BMDM were harvested, washed twice in PBS and further used for *in vitro* stimulation with PRR ligands. Purity of BMDM was analyzed with flow cytometry via F4/80 expression and was typically over 90%.

2.3.4 *In vitro* stimulation with PRR-ligands

For *in vitro* stimulation of immature myeloid cells (iMC), myeloid-derived suppressor cells (MDSC) and bone marrow-derived macrophages (BMDM), single cells suspensions were prepared as described. Cells were then resuspended in RPMI complete medium and seeded on 96-well plates at a concentration of 2 x 10^5 MDSC/iMC per well or 1.5 x 10^5 BMDM per well in 200 µl medium. Experimental conditions were always performed in triplicates. Cells were allowed to attach to the well bottom for 1 hour before immunostimulants and cytokines were added to the culture. The respective cytokines and PRR ligands are described in chapter 2.1.4 with their respective concentrations unless indicated otherwise.

As mentioned in chapter 2.2.3, RNA has to be complexed before *in vivo* and *in vitro* usage in order to prevent degradation and ensure optimal transfection rate. For *in vitro* experiments, Lipofectamine™ 2000 - a polycationic lipid - was used for transfection of RNA. In this work, poly(dA:dT) and ppp-RNA had to be transfected for *in vitro* stimulation. Both, the appropriate amount of RNA as well as Lipofectamine were incubated in two separated tubes in the same volume of OptiMem at room temperature. After 5 minutes, both tubes were added together and incubated for another 20 minutes, before adding the desired amount into culture.

After addition of cytokines and PRR ligands, cells were regularly cultured at 37°C for 24 hours unless indicated otherwise. For measurement of cytokine induction, 96-well plates were centrifuged (400 x G, 7 minutes), supernatant was collected and stored at -20°C for later analysis via ELISA. In some experiments, the remaining cell pellet was washed twice with PBS and resuspended in FACS buffer for flow cytometric analysis.

2.3.5 MDSC isolation via magnetic-activated cell sorting

2.3.5.1 Principle of magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) is used for isolation of viable and functionally active cells via labeling with magnetic particles. The technique of magnetic cell separation was first described in 1990 by Miltenyi Biotec. A mixture of cells is incubated with supraparamagnetic particles (MicroBeads) of approximately 50 nanometers in diameter, which are composed of a biodegradable matrix. Thus, it is not necessary to detach them after the purification process resulting in minimal interference with subsequent experiments. These MicroBeads are coated with antibodies against a particular cell surface antigen that attach them to cells expressing the desired antigen. After incubation, the labeled cell solution is transferred in a column and placed in a strong static magnetic field induced by a permanent magnet. Antigen-expressing and bead-bound cells are retained in the column, whereas cells not expressing the particular antigen pass through the column and can be collected. The retaining cell population is eluted from the column after removal of the magnetic field. With this method, cell can be positively and negatively separated depending on the particular antigen. Positive selection means, that a target population of cells expressing the desired antigen is retained in the column, eluted and then used for further experiments. Negatively selected cells are isolated by depletion of undesired non-target cells, often via incubation with multiple non-target antibodies. Hence, only the non-magnetic and untouched target population rinses through and can be used for subsequent experiments.

In this work, two different purification methods were used for isolation of $Gr1^+CD11b^+$ MDSC and their respective subpopulations. Unless stated otherwise, materials and reagents from

StemCell Technologies were used to isolate the total population of $Gr1^+CD11b^+$ MDSC. In order to separate MDSC subpopulations from each other, the MDSC Isolation Kit from Miltenyi Biotec was used with some modification. For some proliferation assays, $CD8^+$ T Cells were purified with a CD8 T-Cell Isolation Kit (Miltenyi Biotec) and used as responder cells instead of splenocytes (see chapter 2.2.4). In general, magnetic cell separation technologies required fast working on ice and the use of pre-cooled solutions. Incubation periods were always performed at 4°C under gentle tilting. Rotation and centrifugation was done at 300 x G for 10 minutes, instead of the usual 400 x G for 7 minutes, all to ensure optimal cell viability.

2.3.5.2 Miltenyi protocol for isolation of MDSC subpopulations

As mentioned above (chapter 1.4.2), myeloid-derived suppressor cell subpopulations can be phenotypically distinguished via their expression of the Ly-6G antigen (Figure 2.1). Whereas polymorphonuclear MDSC (PMN-MDSC) are $Gr1^+CD11b^+Ly6G^{high}$, monocytic MDSC (MO-MDSC) are $Gr1^+CD11b^+Ly6G^{neg}$. In order to analyze and compare the function of MDSC subsets, we used a mixture of positive and negative selection methods for magnetic enrichment based on their differential expression of Ly-6G.

Therefore, we used the Miltenyi MDSC Isolation Kit with previous depletion of $CD19^+$ and $CD3^+$ cells (mainly B and T cells) to improve cell purity. After preparation of single-cell suspensions cells were adjusted to 10^8 per 350 µl MACS buffer. To prevent unspecific binding sites during the following incubation periods, cells were first incubated with 50 µl FcR Blocking Reagent for 10 minutes. After incubation with 50 µl anti-CD3-Biotin for another 10 minutes, cells were washed and readjusted to 350 µl starting volume. Then the cells were incubated simultaneously with 100 µl Anti-CD19- and 100 µl Anti-Biotin-Microbeads for 10 minutes and washed afterwards. LS columns were rinsed with 3 ml MACS buffer and loaded with labeled cells diluted in 500 µl MACS buffer. Columns were washed three times and the flow-through was gathered as CD3/CD19-depleted cell suspension.

For magnetic separation of PMN-MDSC, these cells were first diluted in 400 µl MACS buffer and incubated with 100 µl of Anti-Ly6G-Biotin for 10 minutes. After a washing step, cells were resuspended in 800 µl buffer and 200 µl Anti-Biotin-Microbeads were added. Following incubation for 15 minutes, cells were washed, diluted in 500 µl MACS buffer and added onto pre-rinsed LS columns. Columns were washed three times with 3 ml MACS buffer. The flow through was gathered as Ly-6G negative fraction. After removal of the magnetic field, $Ly\text{-}6G^{pos}$ PMN-MDSC were eluted by flushing the column with 2 ml MACS buffer by using the provided plunger. PMN-MDSC were centrifuged and kept in complete RPMI medium until further usage.

To purify MO-MDSC, the Ly-6G depleted flow through was centrifuged and diluted in 400 µl MACS buffer per total 10^8 cells. Cells were incubated with 100 µl Anti-Gr1-Biotin for 10 minutes, washed and further incubated with 100 µl Streptavidin MicroBeads in 900 µl MACS buffer for another 10 minutes. According to remaining cell numbers, either LS or MS-columns were pre-rinsed again with buffer. In most experiments, MS columns were rinsed, loaded, washed thrice and after removal of the magnetic field, the column was flushed with 1 ml of buffer. To increase the purity of the eluted $Gr1^+Ly6G^{neg}$ MO-MDSC, they were rinsed over a second MS column. MO-MDSC were centrifuged and kept in complete RPMI medium until further usage.

Cell purity was checked regularly via flow cytometry and always between 85 to 95% for PMN-MDSC and 75 to 85% for MO-MDSC (Figure 2.1 B). The purity of each subpopulation varied depending on MDSC numbers in the starting population, e.g. purity was always slightly higher in tumor-bearing mice with respect to higher amounts of MDSC in spleens or bone marrow.

2.3.5.3 Stem cell protocol for isolation of $Gr1^+CD11b^+$ MDSC

For isolation of the whole population of splenic or bone marrow MDSC, we used a novel separation method developed by StemCell Technologies. The difference between Miltenyi and StemCell protocols was that the latter did not require passage through magnetic columns. Instead, a FACS tube was placed in a strong magnet (EasySep®) with accumulation of magnetically labeled cells at the surrounding inner surface. According to the manufacturer, this techniques' advantages are higher cell viability and function due to less mechanical cell stress. In detail, single-cell suspensions of splenocytes or bone marrow cells were washed once with StemCell buffer and adjusted to a concentration of 10^8 per ml. After incubation with 25 µl/ml of Anti-Gr1-PE-conjugated antibody for 7 minutes at 4°C, cells were washed with StemCell buffer and readjusted to the starting volume. The Gr-1-PE labeled cell suspension was then incubated with 50 µl/ml PE-selection-cocktail for another 7 – 10 minutes at 4°C followed by direct addition of 25 µl NanoParticles. Following incubation for another 7-10 minutes at 4°C and volume adjustment up to 2.5 ml, the tube was placed into the EasySep® magnet for 5 minutes resulting in accumulation of $Gr\text{-}1^+$, NanoParticle-bound cells at the inner surface of the tube. For cell separation, the supernatant was discarded containing non-labeled, non-magnetic cells while the tube was still placed in the magnetic field. For further enrichment, previous steps were repeated two times with already purified target cells. Purified MDSC were centrifuged and kept in complete RPMI medium for subsequent experiments. The purity of $Gr1^+CD11^+$ cells was verified via flow cytometry and was typically over 90% (Figure 2.1 A).

2.4 Immunological methods

2.4.1 Fluorescent-activated cell sorting (FACS)

2.4.1.1 Principle

Flow cytometry or fluorescence-activated cell sorting (FACS) is a laser based method that allows the analysis of properties of single cell suspensions or particles suspended in a liquid phase. A suspension of cells is surrounded by a sheath fluid stream and aspirated with high velocity through a capillary into a flow chamber. By forcing the cells to enter a small nozzle one at a time, they are hydrodynamically focused and pass through a focused laser one by one. At the point where the laser is hitting the cell stream, the illuminating light is either scattered or absorbed. Simultaneously, cells can be stained with an antibody linked fluorescent dye, which absorbs light of a particular wavelength and emits light at a longer wavelength. The scattered light and emitted fluorescence are measured by a number of detectors. The detector in line with the light beam is called Forward Scatter (FSC). Several other detectors are arranged perpendicular to it - one that measures a parameter which is logged as Side Scatter (SSC) and multiple fluorescence detectors. FSC estimates the cell volume by analyzing the angle light that is scattered in straight direction. SSC correlates with inner cell complexity such as membrane structure and the amount of cytoplasmatic granules. Thus, SSC roughly serves as a proxy for cell granularity. The detected fluorescence and light scatter signals are amplified by multiple photodiodes and converted into electrical signals that are electronically processed. Unwanted light emission is blocked by several optical filters.

Each fluorochrome has to be excited by a laser of particular wavelength and measured by detectors appropriate for the emitted spectrum of light. All flow cytometric analyses were performed with a FACSCanto II (BD Biosciences) which allowed simultaneous examination of up to eight different antigens.

A

Dye	Ex^{max}	Em^{max}
FITC	495 nm	519 nm
PE	565 nm	575 nm
PerCP	490 nm	675 nm
Pe-Cy7	480 nm 565 nm 743 nm	767 nm
APC	650 nm	660 nm
APC-Cy7	650 nm	785 nm
PacBlue	410 nm	455 nm
PacOrange	410 nm	551 nm

B

Laser	Wave length	Dye
Blue	488 nm	FITC PE PerCP Pe-Cy7
Red	633 nm	APC APC-Cy7
Violet	405 nm	PacBlue PacOrange

Table 2.9. Dyes (A) and laser of FACSCanto II with corresponding excited dyes (B).

2.4.1.2 Analysis of cell surface antigens

For analysis of cell surface antigens, single cell suspensions were prepared as described previously (chapter 2.2.4). Depending on the experiment, cells were diluted at 2 x 10^5 up to 1 x 10^6 in 200 µl ice-cold FACS buffer or cell culture medium. Single cell suspensions were then incubated with the desired fluorochrome-conjugated antibodies for 30 minutes at 4°C with minimum light exposure (for overview of used FACS antibodies see Table 2.5). Cells were washed twice with PBS and re-suspended in 200 µl FACS buffer. If not analyzed immediately, cells were fixed in 200 µl fixation buffer and kept cold at 4°C until data acquisition. Each experimental condition was at least analyzed in triplets.

2.4.1.3 Flow cytometry and gating of MDSC

MDSC were analyzed from primary organs or following *in vitro* culture of purified MDSC or iMC, respectively. Given that MDSC subpopulations differ in their expression of the Ly-6G antigen, each staining contained at least the phenotypical marker Gr-1, CD11b and Ly-6G with additional maturation and differentiation markers (see chapter 1.4.2). The main gating strategy of MDSC subsets is illustrated below.

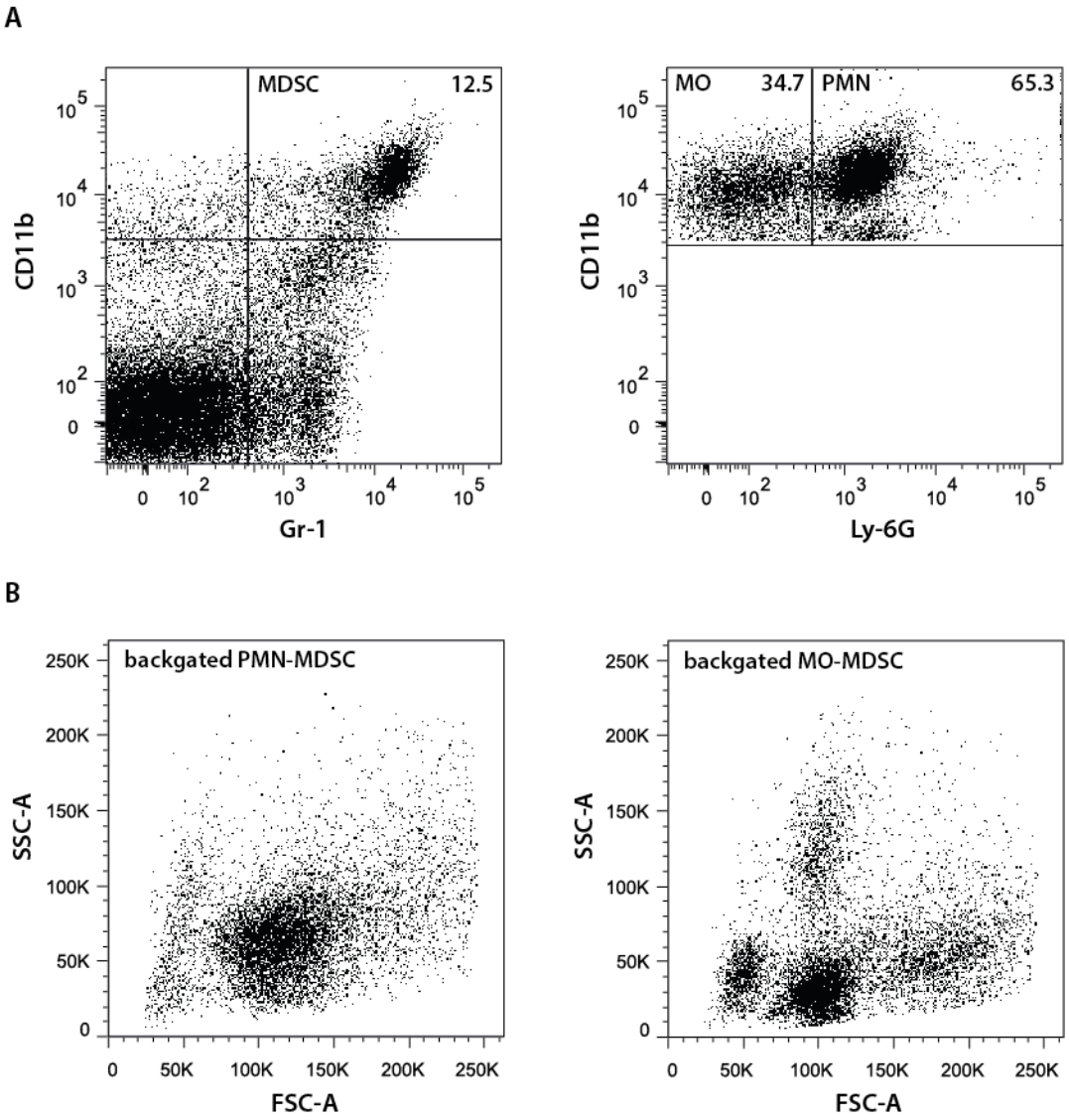


Figure 2.1. Representative flow cytometry data of MDSC with corresponding subpopulations. Dead splenocytes were excluded via scatter discrimination (not shown) and MDSC were gated on their status as $Gr1^{+}CD11b^{+}$ (left panel, A). MDSC were further divided into the major subpopulations of MO-MDSC and PMN-MDSC with respect to their differential expression of the Ly-6G antigen (right panel, A). B shows backgating of MDSC subsets and illustrates subset-related differences in cell size and granularity by variable appearance on forward (FSC) and sideward scatter (SSC) analysis.

2.4.1.4 Detection of apoptosis

For evaluation of apoptosis in myeloid-derived suppressor cells, the FAM FLICA ™ Caspase 3&7 Assay Kit (Immunochemistry) was used. Therefore, after staining of surface antigens, primary cells were diluted in 290 µl complete RPMI with addition of 10 µl FLICA (Fluorescent Labeled Inhibitors of Caspases) that covalently bind with active caspase enzymes. Cells were than incubated at 37°C for 45 minutes and washed twice with 2 ml 1 X Apoptosis Wash Buffer. After resuspension in 300 µl Apoptosis Wash Buffer, cells were ready for colorimetric analysis. In some experiments, propidium iodine (PI) was used to discriminate between early

(FLICA^{pos}PI^{neg}) and late apoptotic cells (FLICA^{pos}PI^{pos}) as well as exclusion of necrotic cells which than appear as FLICA^{neg}PI^{pos}).

2.4.2 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay, or ELISA, is a technique used to analyze a specific antigen in a liquid sample. The method is based on binding of enzyme-linked antibodies against the desired antigen leading to subsequent conversion of a colorless substrate to a colored reagent. The color change is detected and converted into a quantifiable electrical signal that correlates to the initial amount of antigen.

Different cytokine levels in serum of mice as well as from murine cell supernatants were measured in this work. The particular ELISA kits are listed in chapter 2.1.6. For cytokine detection, plates are coated with a capture antibody specific for the target cytokine. After addition of the sample, any given cytokine present is bound by the capture antibody. Plates are incubated again with biotinylated detection antibody which in turn is bound by streptavidin coupled to a peroxidase. By adding 3,3',5,5' – tetramethylbenzidine (TMB) as a substrate, this enzyme catalyzes a reaction that entails a blue color change. The reaction is stopped by addition of H_2SO_4 and read in a multiplate reader at 450 nm wavelength with a wavelength correction subtraction at 590 nm. All cytokines in this work except IFNα were measured by using commercially available kits according to the manufacturer's protocol. Depending on the expected amount of cytokines present in supernatant or serum, the samples were generally diluted 1:2; for the detection of IL-12p40 and IFN, samples were diluted 1:5 or 1:10.

Detection of IFNα was performed by using a set of antibodies listed in chapter 2.1.6: Plates were coated overnight at 4°C with 50 µl/well coating buffer containing 1 µg/ml of capture antibody. In order to block unspecific binding sites, plates were then blocked for 3 hours with 150 µl of assay diluent per well at room temperature. After intensive washing for several times with washing buffer, plates were incubated overnight with sample and standard protein at 4°C. The highest level of standard was generally set at 105 IU/ml, samples were loaded undiluted. After several washing steps, 50 µl/well of detection antibody at a concentration of 625 ng/ml was added and incubated again for 3 hours at room temperature. Following extensive washing, horseradish peroxidase (HRP)-conjugated F(ab')2 fragments were used at a concentration of 15 µg/ml and incubated for 3 hours in order to detect antigen-bound detection antibody. After extensive washing 50 µl of substrate solution were added to each well to induce a colored reaction. The reaction was stopped by addition of 2N H_2SO_4 and read in a multiplate reader at 450 nm with correctional subtraction at 590 nm.

2.4.3 Immunofluorescence of tumor slices

Immunofluorescence of tumor slices was performed together with Tina Adunka and Jonathan Ellermeier (AG Prof. Dr. med. M. Schnurr). Briefly, for evaluating influx of tumor-infiltrating cytotoxic T-lymphocytes (CTLs), immunofluorescence was performed on frozen tissue sections. Following removal of Panc02 tumors, tissue was immediately stored in cryotubes, shock frozen in liquid nitrogen and stored at -80°C. Consecutive cryostat tissue sections (5 µm) were mounted on glass slides and fixed in cold acetone (-20°C) for 10 minutes. Sections were blocked with 10% goat serum in PBS for 20 minutes followed by labeling with primary antibody overnight at 4°C (for antibodies see section 2.1.7). After washing with PBS, tumor slices were stained with the respective secondary antibodies diluted in 10% donkey serum for another 45 minutes and washed again with PBS. Slices were mounted with Aqua-PolyMount (Polysciences) and then stored at -20°C for analysis of tumor-infiltrating CTLs.

2.4.4 BrdU suppression assay

To access the immunoregulatory capacity of myeloid-derived suppressor cells on antigen-specific and -unspecific proliferation of T cells, we used a 5'Bromo-2'deoxy-uridine (BrdU) incorporation assay. The principle of measuring T cell proliferation in the presence of MDSC *in vitro*, as a surrogate for MDSC-mediated suppressivity, has been well described in literature (Dolcetti, Peranzoni et al. 2010). In detail, 1 x 10^5 splenocytes or 7.5 x 10^4 splenic T cells (Dynal Mouse Negative T Cell Isolation Kit, Invitrogen, Carlsbad, CA) from naïve mice were cultured with different ratios of MDSC in complete RPMI medium. Cultures were incubated in triplicate at 37°C and 5% CO_2 in flat-bottom 96-well plates. T cell proliferation was stimulated by the addition of anti-CD3/anti-CD28 coated microbeads (Invitrogen) at a bead-to-cell ratio of 1:75. Cells were co-cultured for 60 hours before 7.5 µM BrdU was added for another 12 hours. Cell culture plates were then centrifuged (300 x G, 10 minutes), medium was discarded and stored for further analysis before plates were dried with a hairdryer. Cell fixation, incubation with anti-BrdU-antibody and substrate reaction were done according to the manufacturer's protocol. BrdU incorporation was detected by chemiluminescence-based assay and was measured in relative light units (rlu) with a multiple plate reader.

Suppression of T cell proliferation in the presence of MDSC in increasing concentration was calculated relative to stimulated responder cells alone (MDSC-splenocyte-ratio 1:16, 1:8, 1:4, 1:2 with anti-CD3/CD28-stimulated SC or T cells as positive control). Percentage suppression of proliferation was calculated as

$$\left(1 - \frac{proliferation_{with_{MDSC}}}{proliferation_{without_{MDSC}}}\right) x\ 100.$$

Figure 2.2 shows a typical bar graph used in this work to display the results of a T cell proliferation assay with or without co-culture of T cell suppressing immature myeloid cells (iMC) or MDSC. As increasing concentration of iMC/MDSC in the culture are depressing T cell proliferation, the relative proliferation rate decreases. Negative controls are exemplary illustrated once and left out in other figures.

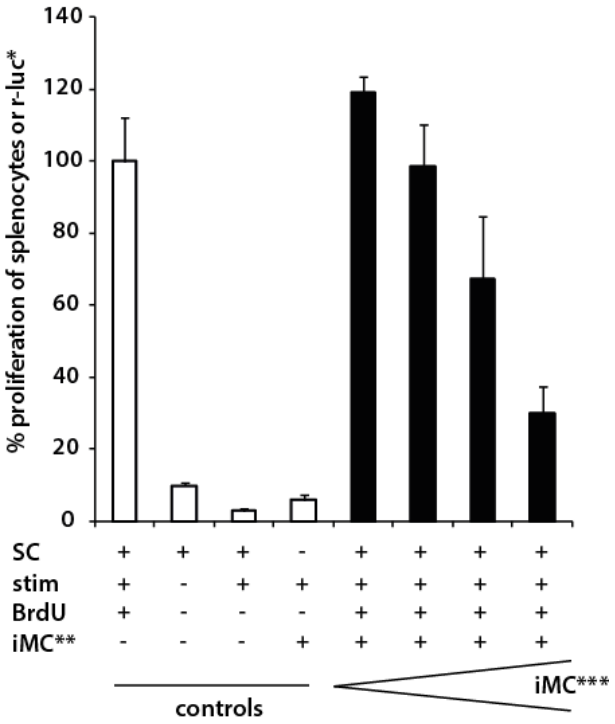


Figure 2.2. Example of a standard BrdU incorporation assay for measuring MDSC-mediated suppression of T cell proliferation *ex vivo*. The y-axis shows the relative percentage of T cell or splenocyte (SC) proliferation with or without co-culture of immature myeloid cells (iMC). Therefore, a 100% proliferation rate as positive control is defined as proliferation of anti-CD3/CD28-stimulated splenocytes alone. X-axis shows the different experimental conditions with internal controls (left side, white bars) and with co-culture of T cell suppressing immature myeloid cells or MDSC in ascending concentration (iMC-splenocyte-ratio 1:16, 1:8, 1:4, 1:2; black bars). SC = splenocytes; stim = anti-CD3/CD28-stimulation. * Relative light units (rlu) ** depending on the experimental setup MDSC-like immature myeloid cells or myeloid-derived suppressor cells were used *** iMC-splenocyte-ratio 1:16, 1:8, 1:4, 1:2 unless indicated otherwise.

2.5 Generation of ppp-RNA

In vitro transcription of ppp-RNA was performed using the MEGAshortscript™ T7 Kit (Ambion, Austin, Texas) according to the manufacturer's protocol. siRNAs against TGFβ_1 including a 3´-dTdT overhang and matching 5'-triphosphate-modified siRNA were kindly provided by Prof. Max Schnurr (Division of Clinical Pharmacology, LMU Munich). Respective oligonucleotide sequences are depicted in section 2.1.4.

2.6 Statistical Analysis

All data in this work is given as arithmetic mean values. Variance of mean values is expressed as standard error of the mean (SEM). Statistical significance in most experimental finding was calculated by independent two-tailed student's t-test. Comparison of multiple groups were performed using 1-way ANOVA with pair-wise Bonferroni posttest. Tumor sizes were analyzed using Mann-Whitney U test. Significance was considered at p levels ≤ 0.05, $p \leq 0.01$ and $p \leq 0.001$ and were then indicated with *, ** and ***. Statistical analyses and design were performed using GraphPad Prism 5 (GraphPad Software), Microsoft Office and Adobe Illustrator CS5 (Adobe System).

3 RESULTS

3.1 Effect of systemic CpG-DNA administration on Gr1$^+$CD11b$^+$ immature myeloid cells (iMC) in tumor-free mice

3.1.1 iMC numbers in different organs following systemic TLR stimulation

In our group, we previously demonstrated that activation of the innate immune system with the Toll-like receptor (TLR) 9 ligand CpG evokes efficient antitumor immune responses in mice bearing subcutaneous CT26 tumors [Heckelsmiller et al., 2002; Bourquin et al., 2008], despite a large number of highly immunosuppressive MDSC in this model [Gallina et al., 2006; Youn et al., 2008]. However, Vaknin and colleagues recently reported that chronic activation of innate immunity via TLR2, 4, 7 and 9 using a slow-release delivery system leads to immunosuppression in tumor-free mice attributed to the induction of Gr1$^+$CD11b$^+$ immature myeloid cells (iMC) or MDSC [Vaknin et al., 2008].

To address this issue, in a first experiment the capacity of CpG-DNA - our most intensively studied TLR ligand - was investigated for its ability to induce Gr1$^+$CD11b$^+$ iMCs *in vivo*. Therefore, mice were challenged with three repeated doses of 100 µg subcutaneous CpG at a 3-day interval; the dosage and application resembled typical anti-tumor treatment regimens. Indeed, when spleens were analyzed via flow cytometry for the number of Gr1$^+$CD11b$^+$ iMC, we saw a two-fold increase in iMC numbers compared to untreated mice (Figure 3.1 A,C). This effect was not restricted to splenic Gr1$^+$CD11b$^+$ cells, because the same percentage increase of iMCs was observed in other compartments such as blood and bone marrow indicating a systemic effect of TLR9 stimulation on iMC accumulation (Figure 3.1 B). Lymph nodes did only show minor iMC infiltration in untreated mice or in mice treated with CpG (data not shown). In a second step, the effect of other PRR ligands on iMC expansion was assessed by *in vivo* administration of poly(I:C), LPS and complexed triphosphate-RNA (3pRNA) using the same treatment protocol. TLR3 and TLR4 stimulation by poly(I:C) and LPS, respectively, induced splenic iMC accumulation comparable to CpG. However, systemic activation of the pattern recognition receptor RIG-I by 3pRNA did not increase the percentage of Gr1$^+$CD11b$^+$ iMCs in tumor free mice (Figure 3.1 C).

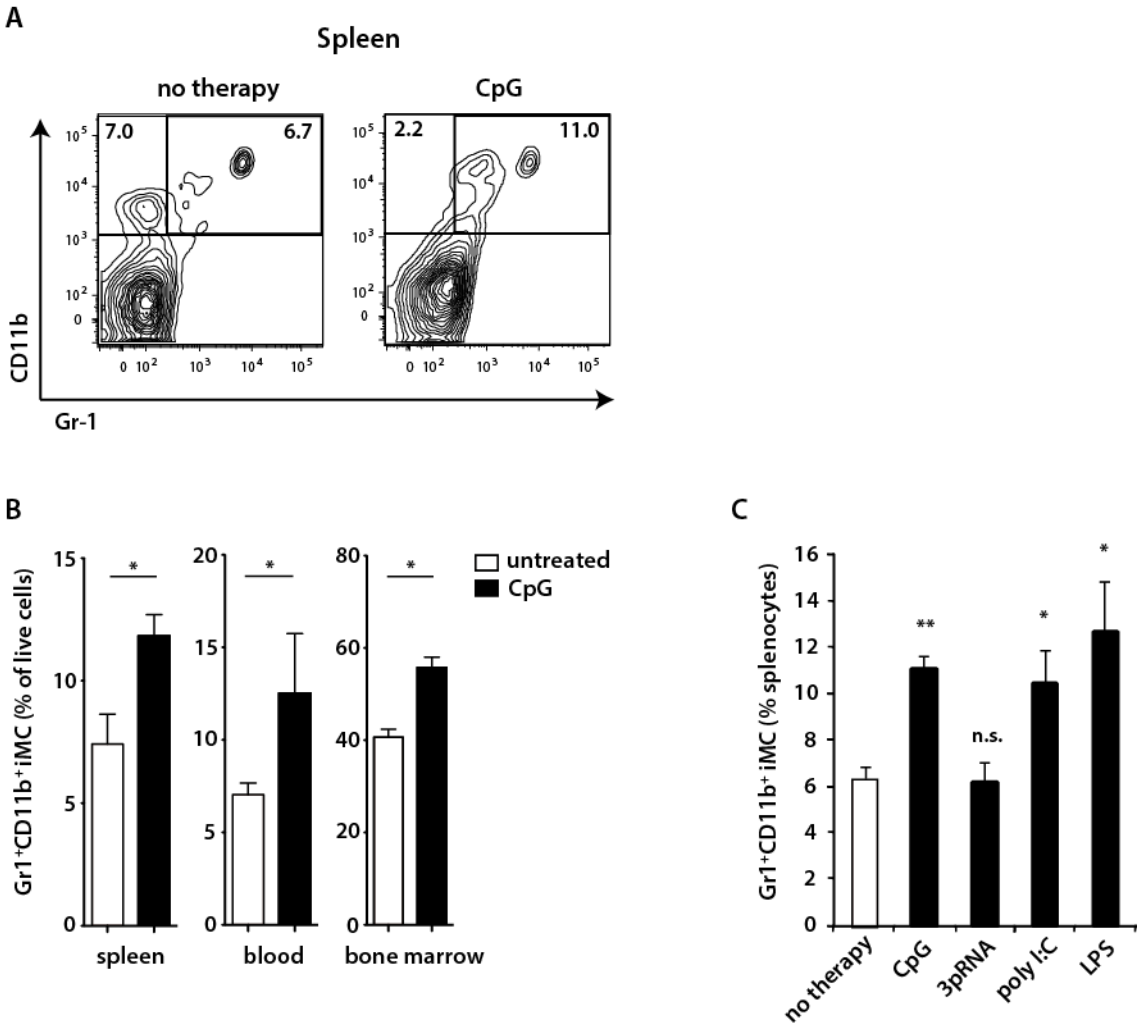


Figure 3.1. TLR therapy in tumor-free mice leads to expansion of MDSC-like cells. Naïve Balb/c mice received either 3 s.c. injections of CpG or PBS (no therapy) as a control at 3-day intervals (n = 5 per group). 2 days after the last injection mice were sacrificed and the number of $Gr1^+CD11b^+$ immature myeloid cells (iMC) was determined using flow cytometry. **A** Representative plot gated on viable splenocytes showing the total number of splenic iMC ($Gr1^+CD11b^+$) before and after CpG treatment. **B** Histograms showing total numbers of iMC in spleen, blood and bone marrow. **C** shows the percentage of $Gr1^+CD11b^+$ iMC in spleens of mice after therapy with different TLR- or RIG-I ligands (3p RNA). Treatment protocol was done as mentioned above. Data are the mean ± SEM from at least three independent experiments. *, $p \leq 0.05$; ** $p \leq 0.01$; paired Student's *t* test.

3.1.2 Effect of CpG treatment on iMC-mediated suppressivity

Immune suppression by MDSC is not only a result of their expansion, but in particular due to their activation [Gabrilovich et al., 2009]. A hallmark ability of MDSC is inhibiting T-cell proliferation by various mechanism (see chapter 1.4.4). Therefore, in a second step the ability of splenic $Gr1^+CD11b^+$ iMC to suppress the proliferation of T cells *ex vivo* was examined via a BrdU proliferation assay, which is a standard experiment for evaluating MDSC-mediated immunosuppression. Naïve mice were treated with CpG again as described above. One day after the last injection, spleens were removed and $Gr1^+CD11b^+$ cells were separated by magnetic sorting. Purified iMC from both untreated and CpG-treated mice showed a

moderately suppressive effect on CD3/CD28-stimulated T cells (Figure 3.2), but there was no difference when both groups were compared, which was validated in two other independent experiments. Thus, *in vivo* immune stimulation with TLR9 does not result in activation of MDSC-like $Gr1^{+}CD11b^{+}$, which contrasts the results observed by Vaknin and co-workers [Vaknin et al., 2008].

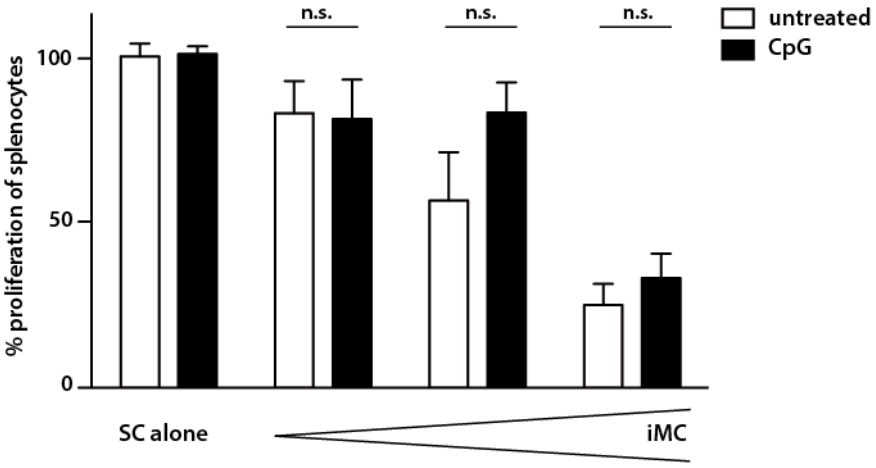


Figure 3.2. CpG treatment has no effect on suppressive function of iMC in tumor-free mice. Naïve Balb/c mice received either 3 s.c. injections of CpG or PBS (no therapy) as a control at 3-day intervals (n = 5 per group). 2 days after the last injection mice were sacrificed and iMCs were purified from spleens of untreated or CPG-treated mice by sequential MACS separation (magnetic activated cell sorting). Purity was verified via flow cytometry. Degree of suppressivity was assessed with a T-cell proliferation assay using CD3/CD28-stimulated splenocytes (SC) as responder cells co-cultured with MDSC-like immature myeloid cells for 48 hours (MDSC-splenocyte-ratio 1:8, 1:4, 1:2 with anti-CD3/CD28-stimulated SC as positive control). $p > 0.05$ was considered as not significant (n.s.).

3.1.3 Shifting of myeloid cell subset composition upon CpG-DNA therapy

As MDSC are comprised of two distinct subpopulations, the effect of TLR9 activation on the subset composition was analyzed. The most prominent subset in Balb/c as well as in C57BL/6 mice are polymorphonuclear iMC or $Ly6G^{high}$ iMC, respectively. When mice were challenged with CpG, we observed a strinking change on MDSC-defining markers on the surface: Ly6G, the PMN-MDSC defining marker, gets strongly downregulated which is accompanied by a significant upregulation of Ly6C (Figure 1 B). More narrowly, the relation of about 70% PMN-iMC to 30% MO-iMC is reversed when mice were challenged with CpG. This findings conclude that the induction of myeloid precursors by innate immune activation via TLR9 is mainly due to an increase in monocytic myeloid cells. In addition, this shift was not exclusive for splenic iMC, but was again seen in other organs (Figure 1 C).

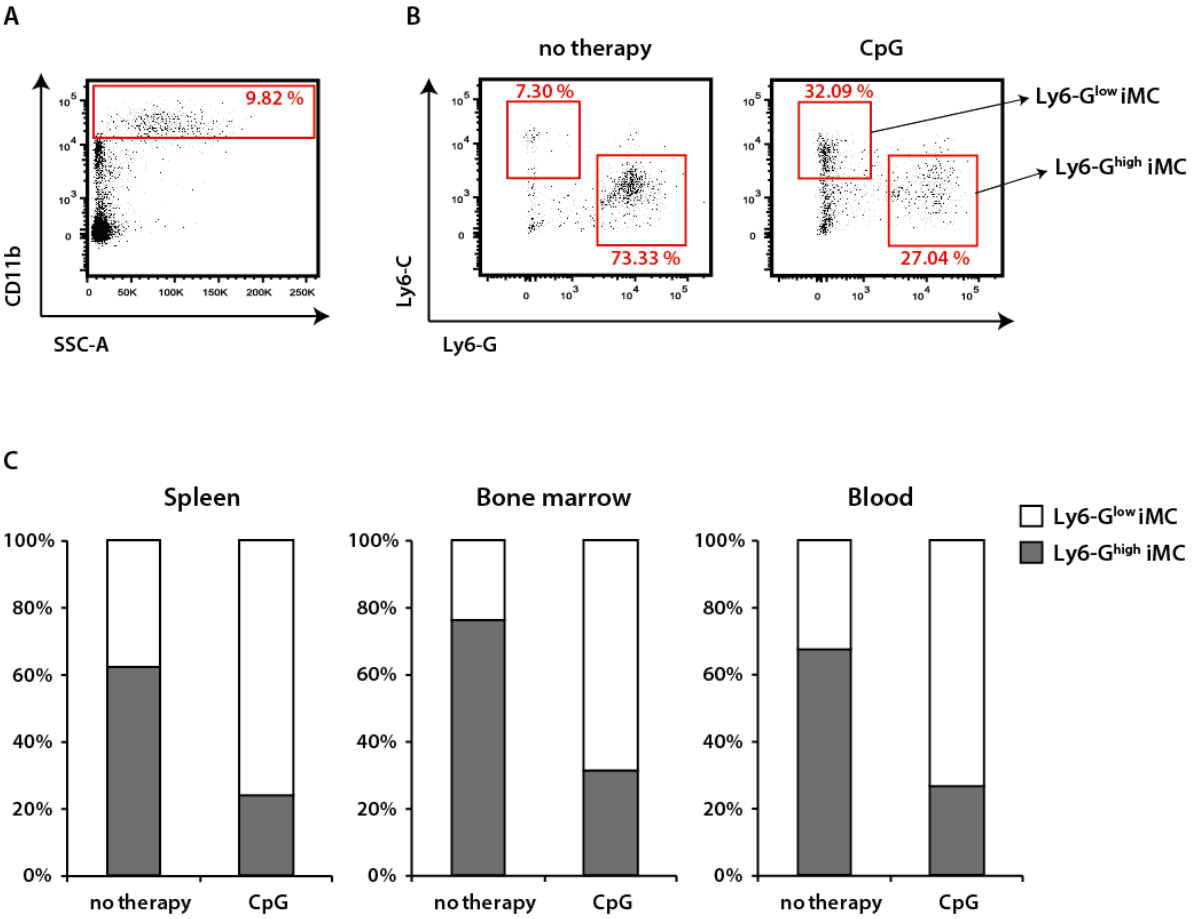


Figure 3.3. Treatment with CpG-ODN results in a shift of splenic iMC subpopulations. Naïve Balb/c mice received either 3 s.c. injections of CpG or PBS (no therapy) as a control at 3-day intervals (n = 5 per group). 2 days after the last injection mice were sacrificed and iMC in different organs were analyzed by flow cytometry. **A** Pre-gating of splenic myeloid cells was performed via Side-Scatter and $CD11b^{high}$ inclusion. As an example, the amount of $CD11b^{high}$-myeloid cells is depicted in the right upper corner. **B** $CD11b^{high}$ myeloid cells were further divided into two major subpopulations by using Ly6G and Ly6C marker expression: $Ly6G^{low}Ly6C^{high}$ monocytic iMC and $Ly6G^{high}Ly6C^{low}$ granulocytic iMC, respectively. A representative dot plot shows the shift of those two subpopulations before and after *in vivo* CpG-ODN treatment. **C** Bar graphs showing the effect of CpG therapy on iMC subpopulations in different organs. Data are the mean from at least three independent experiments.

3.1.4 Alteration of iMC phenotype through systemic TLR9 activation

One of the defining traits of MDSC compared to their physiologically occurring counterparts of immature myeloid cells is that MDSC are prevented from further differentiating whereas iMC rapidly mature and home to their target tissue (Kusmartsev and Gabrilovich 2006). To address the maturation status of naïve iMC versus iMC following *in vivo* treatment with CpG-ODN, splenic $Gr1^{+}CD11b^{+}$ cells from both groups were analyzed for maturation and differentiation marker expression by flow cytometry (Figure 3.4). The macrophage differentiation marker F4/80 as well as the DC differentiation marker CD11c and MHCII were significantly upregulated on iMC following TLR9 activation ($p \leq 0.05$). Additionally, there was

a trend towards higher expression of the co-stimulatory molecules CD80 and CD86, even though it did not reach statistical significance. Most of the markers analyzed were upregulated on both Ly6G^{low} and Ly6G^{high} subsets (data not shown). Unexpectedly, we observed a highly significant upregulation of stem cell antigen-1 (Sca-1) which is also known as Ly6A/E and typically expressed by murine hematopoietic stem cells. Moreover, when maturation marker expression was compared between in Sca-1^{+}Gr1^{+}CD11b^{+} iMCs versus Sca-1^{-}Gr1^{+}CD11b^{+} iMCs, Sca-1^{+} iMCs expressed significantly higher maturation and differentiation markers (data not shown). All together, these results show that CpG-ODN promotes maturation and differentiation of immature myeloid cells *in vivo*.

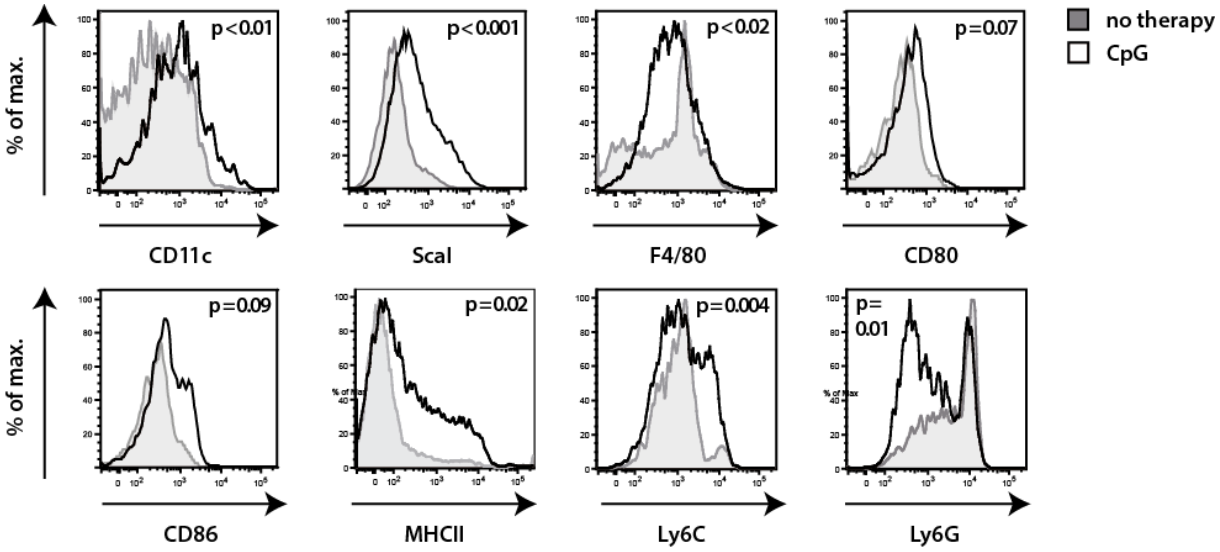


Figure 3.4. CpG therapy induces maturation and differentiation of iMC in tumor free hosts. Naïve Balb/c mice received either 3 s.c. injections of CpG or PBS (no therapy) as a control at 3-day intervals (n = 5 per group). 2 days after the last injection mice were sacrificed, spleens were harvested and examined by flow cytometry. Representative histograms show the expression of different surface and maturation markers on pre-gated splenic Gr1^{+}CD11b^{+} iMC with and without *in vivo* CpG administration. Data analysis was performed using paired Student's *t* test comparing mean fluorescent intensity (MFI) of each surface marker. $p \leq 0.05$ was considered as significant.

3.2 Myeloid-derived suppressor cells (MDSC) in tumor-bearing mice

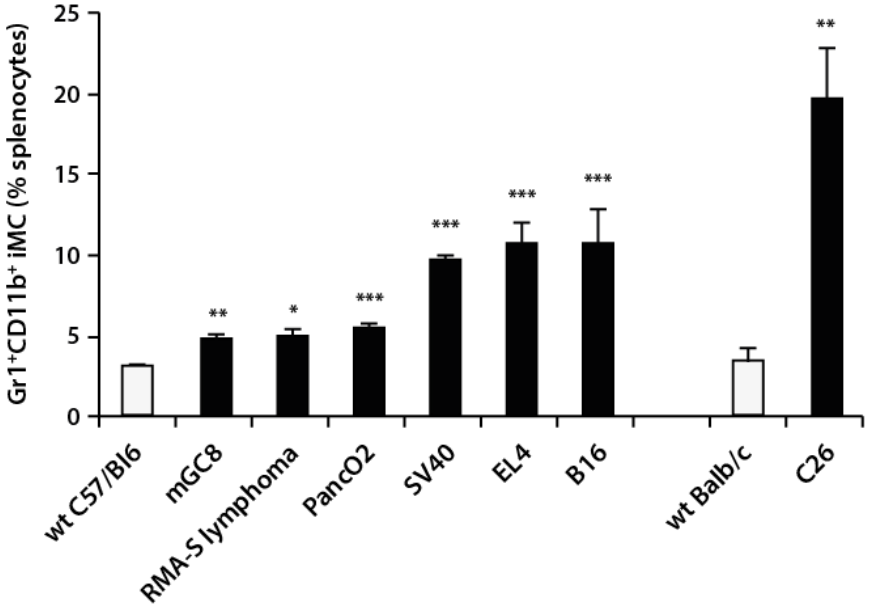
3.2.1 Comparison of MDSC in different mouse tumor models

Before starting to investigate the effect of innate immune stimulation on MDSC in tumor-bearing mice, we examined different subcutaneous murine tumor models on their ability to drive MDSC accumulation *in vivo*. Therefore, Balb/c or C57BL/6 mice were inoculated with respective tumor cell lines by subcutaneous injection into the left flank and spleens were analyzed for the presence of Gr1^{+}CD11b^{+} MDSC. To exclude an effect of tumor load on the number of peripheral Gr1^{+}CD11b^{+} cells [Pan et al., 2008], mice were sacrificed when tumor size reached 150 – 200 mm^2 or were 90 – 100 days of age in case of SV40-tumors in the

autochtonous CEA424-Tag-model. As expected, in all tumor models examined, the number of MDSC was significantly increased compared to naïve mice (Figure 3.5 A). More narrowly, in C57BL/6 or Balb/c mice about 3% of splenocytes were $Gr1^+CD11b^+$, which is consistent with current literature [Gabrilovich and Nagaraj, 2009]. Whereas comparatively low numbers of splenic MDSC were observed in mice bearing mGC8, RMA-S and PancO2 tumors ranging from 5.0 to 6.5%, MDSC accumulation was more pronounced in the autochtonous SV40 as well as EL4 and B16 tumor model (9.8 to 14.6%). Balb/c mice inoculated with CT26 colon carcinoma cells, exhibited the highest number of MDSC with around 21% of splenocytes (Balb/c background).

In a second step, $Gr1^+CD11b^+$ cells were further investigated for the composition of subpopulations by discriminating between $Ly6G^+$ PMN-MDSC and $Ly6G^-$ MO-MDSC. PMN-MDSC were the predominant subpopulation ranging from 40 to 70% in naïve mice and all tumor models examined, except for mice bearing subcutaneous B16 melanoma (Figure 3.5 B). Moreover, tumor induction resulted in significant increase of $Ly6G^+$ PMN-MDSC compared to naïve mice ($p \leq 0.05$). Notably, in CT26 tumor-bearing mice more than 90% of MDSC were granulocytic PMN-MDSC suggesting they play a major role in myeloid-cell associated immune suppression in this model.

A



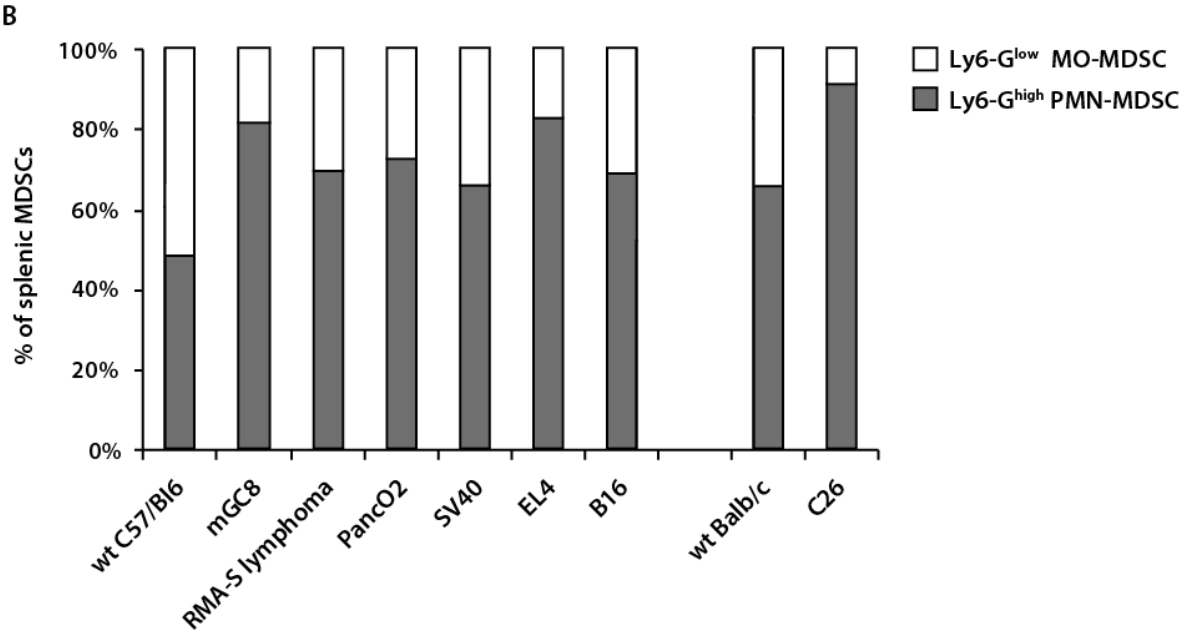


Figure 3.5. The amount of splenic MDSC and the distribution of subpopulations differs in several murine models of lymphoma, melanoma, gastric and colon carcinoma. Balb/c or C57/BL6 mice were inoculated s.c. with different tumor cells (n = 5 – 20 per group). Mice were sacrificed when subcutaneous tumor sizes reached 150 – 200 mm^2 or mice were 90 – 100 days of age (SV40). Spleens were then removed and $Gr1^+CD11b^+$ myeloid-derived suppressor cells (MDSC) quantified via flow cytometry. **A** Percentage of MDSC of viable splenocytes in Black/6 and Balb/c background. MDSC numbers of tumor bearing mice were compared with numbers of iMC in naïve mice. All data are expressed as mean ± SEM. *, $p \leq 0.05$; **, $p \leq 0.01$; *** $p \leq 0.001$, paired student's *t* test. **B** Pre-gated $Gr1^+CD11b^+$ MDSC were further divided into the two major subpopulations by discriminating via Ly6-G expression. The histograms show the percentage of Ly6-G^{low} monocytic MDSCs (MO) and Ly6-G^{high} polymorphonuclear/granulocytic MDSC (PMN). Data are the mean from at least 5 – 20 mice per group.

3.2.2 Surface marker analysis of MDSC in tumor-bearing mice versus iMC in naïve mice

As mentioned above, a hallmark of tumor-induced MDSC is their immature status. In previous experiments it was shown that TLR9 activation leads to maturation of immature myeloid cells in tumor-free mice (Figure 3.4). Thus we asked, if there are differences in wildtype compared to tumor-bearing mice regarding the maturation status of myeloid cells. There are only few studies which compared the expression of individual maturation markers between iMC in naïve mice and MDSC in tumor-bearing mice and no study that systematically compared a set of maturation markers. Thus, the expression of maturation and differentiation markers on splenic $Gr1^+CD11b^+$ iMC in naïve mice and $Gr1^+CD11b^+$ MDSC from CT26 tumor-bearing mice was analyzed (Figure 3.6).

As hypothesized, the expression levels of CD11c and F4/80 were significantly lower in tumor-induced MDSC as well as co-stimulatory molecules on the cell surface (CD80 and CD86). There was also a trend towards lower expression of MHCII even though it did not reach

statistical significance. In addition, the expression of Ly6G and Ly6C as subset-defining markers was changed contrary to the changes seen after TLR9 activation with upregulation of the Ly6G- and downregulation of the Ly6C antigen. That in turn reflects the substantial increase in PMN-MDSC and the relative decrease in MO-MDSC during tumorigenesis (Figure 3.5 B). Summarized, it is shown that MDSC are in fact less matured than their physiological counterparts which can objectified by analyzing a set of maturation markers. The same results were shown for MDSC and iMC in bone marrow, blood and lymph nodes, even though the downregulation of maturation markers in tumor-bearing mice was less pronounced in the bone marrow compared to peripheral compartments (data not shown).

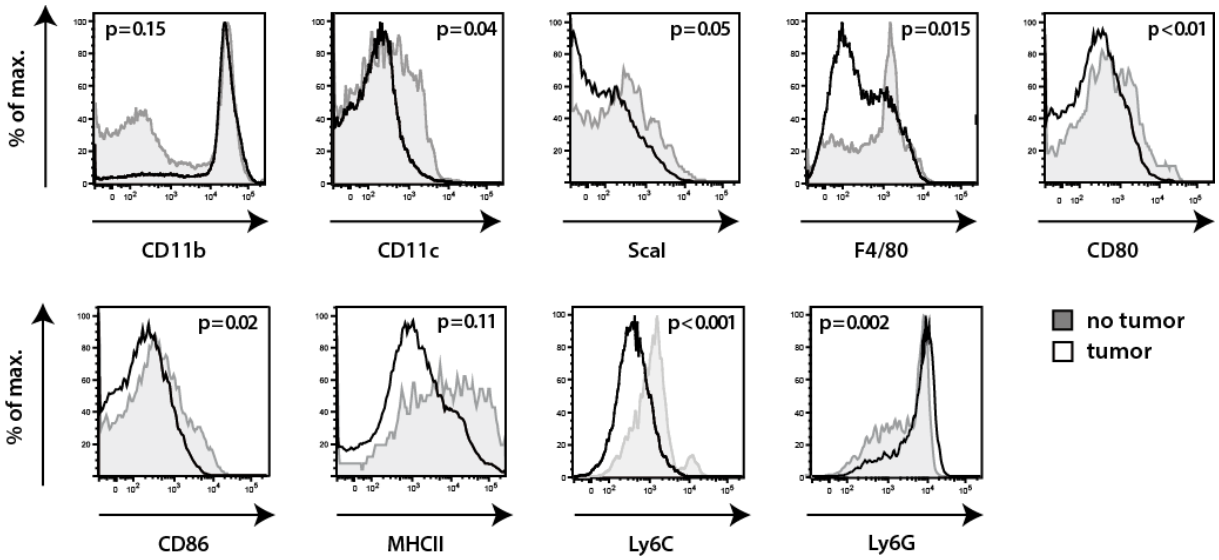


Figure 3.6. Tumor-induced MDSC exhibit less maturation markers than naïve iMC. Balb/c mice were inoculated subcutaneously with CT-26 colon carcinoma cells. When tumor size reached at least 150 mm², mice were sacrificed, spleens removed and splenocytes were analyzed by flow cytometry. The histograms show representatives of maturation marker expression (MFI) on pre-gated splenic $Gr1^+CD11b^+$ iMC in tumor free mice (grey curve) vs. $Gr1^+CD11b^+$ MDSC in CT26 colon cancer bearing mice (black curve). Statistics are calculated from at least 2 – 3 independent experiments (n = 5 mice per group in each experiment). Data analysis was performed using paired Student's *t* test comparing mean fluorescent intensity (MFI) of each surface marker. $p \leq 0.05$ was considered as significant.

3.2.3 Different immunosuppressive activity of MDSC subpopulations

With previous experiments showing that PMN-MDSC were the main population expanding during tumor growth, we decided to investigate the immunosuppressive effect of individual subpopulations. Therefore a magnetic-based technique to isolate $Ly6G^{low}$ MO-MDSC and $Ly6G^{high}$ MDSC was developed (see chapter 2.5.3.2). Mice bearing subcutaneous RMA-S lymphoma were sacrificed and subpopulations were isolated and separately co-cultured with CD3/CD28-stimulated splenocytes for 72 hours. T cell proliferation was analyzed using a BrdU proliferation ELISA.

Figure 3.7 shows that PMN-MDSC more potently suppress T cell proliferation than MO-MDSC. That was consistently observed throughout all experiments and was not restricted to the RMA-S lymphoma model but also shown for CT26 [Zoglmeier et al., 2011] and B16 tumor-bearing mice (data not shown). In addition, both tumor-derived MDSC subpopulations were significantly more suppressive than their respective counterparts from tumor-free mice (data not shown).

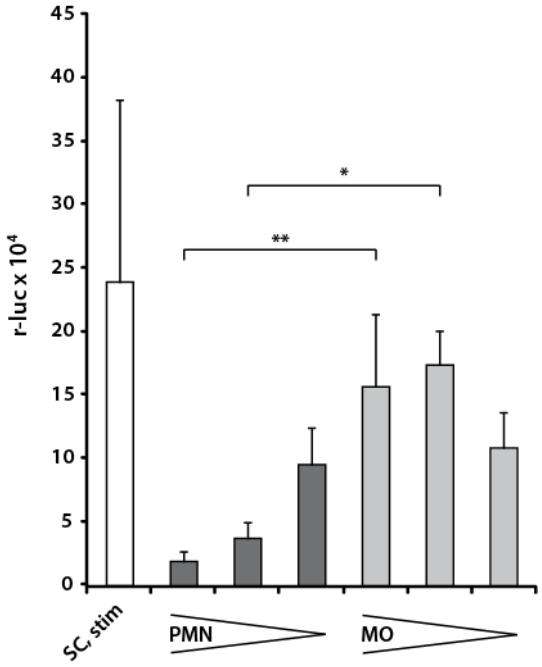


Figure 3.7. PMN-MDSC bear higher immunosuppressive capacities than MO-MDSC in tumor-bearing mice. Mice bearing subcutaneous RMA-S lymphoma (at least 150 mm² size) were sacrificed, spleens removed and MDSC subpopulations were purified by sequential MACS separation [PMN = polymorphonuclear MDSC; MO = monocytic MDSC]. Purity was verified via flow cytometry and was at least ≥ 85% in each group. Degree of suppressivity was assessed with a T-cell proliferation assay using CD3/CD28-stimulated splenocytes (SC) as responder cells cocultured with either PMN-MDSC or MO-MDSC for 72 hours (MDSC-splenocyte-ratio 1:8, 1:4, 1:2 with anti-CD3/CD28-stimulated SC as positive control). Data are expressed as mean ± SEM. *, $p \leq 0.05$; **, $p \leq 0.01$, paired Student's *t* test.

3.3 Effect of immunotherapy with pattern recognition receptor ligands on MDSC in tumor-bearing mice

3.3.1 MDSC numbers upon TLR ligand therapy in tumor-bearing mice

In previous experiments, we showed that innate immune activation with CpG-ODN and other TLR ligands increases the number of $Gr1^+CD11b^+$ immature myeloid cells and concurrently induces their maturation without further augmenting their immunosuppressive properties. Likewise, it was shown that tumor growth inhibits their differentiation and induces MDSC, especially the polymorphonuclear subset which is highly suppressive.

In a next step, the effect of innate immune activation on the number, phenotype and function of MDSC in tumor-bearing mice was examined. Based on prior experiments, the CT26 colon carcinoma model was selected due to high numbers of MDSC and rapid tumor development. Therefore, mice bearing subcutaneous CT26 were treated on day 14 and 17 with either subcutaneous poly(I:C), CpG or sodium chloride (NaCl) as control. Therapy with TLR ligands resulted in a potent tumor reduction compared to control mice (Figure 3.8 A). 48 hours after the last therapy, mice were sacrificed and organs were further analyzed by flow cytometry.

As expected, tumor growth expanded the number of splenic $Gr1^+CD11b^+$ MDSC compared to non-tumor bearing mice (Figure 3.8 D). Unexpectedly, mice treated with CpG-ODN showed no further expansion of splenic $Gr1^+CD1b^+$ cells compared to control mice with 12 to 14% splenic MDSC in both groups (Figure 3.8 C,D). Same results were obtained when mice were treated with poly(I:C). Likewise, no changes in absolute MDSC numbers exceeding the percentage seen in untreated mice could be observed in peripheral blood or bone marrow (data not shown).

Despite that IFNγ has been associated with MDSC expansion and activation in tumor-free mice [Greifenberg et al., 2009], a common surrogate marker for evaluating MDSC-mediated immunosuppression is the measurement of IFNγ serum levels, given the fact that MDSC directly inhibit IFNγ production by T cells. Additionally, IFNγ is crucial for mounting an effective immune response against malignant tumors [Shankaran et al., 2001] and reverses the immunosuppressive properties of tumor-associated macrophages [Duluc et al., 2009]. Consequently, beside a potent IFNα induction by CpG-ODN and poly(I:C) [Krug et al., 2001; Essers et al., 2009], measurement of serum cytokine levels showed that TLR9 activation with CpG-ODN restored the CT26-induced IFNγ-depletion (Figure 3.8 B). Because the number of MDSC was not altered by innate immune activation, we assessed the function of MDSC by *ex vivo* proliferation assay as described before. It showed that suppressivity of isolated $Ly6G^{high}$ MDSC on T-cell proliferation and IFNγ production was significantly inhibited by CpG therapy. For the $Ly6G^{low}$ subpopulation, suppressivity was also reduced by CpG treatment, although the effect was not significant [Zoglmeier et al., 2011].

Thus, we demonstrated that CpG-ODN do not further increase MDSC as key mediators of immunosuppression in tumor bearing hosts and, moreover, reverse their immunosuppressive capacities they exert on T cells. Moreover, TLR9 stimulation *in vivo* restores the tumor-mediated IFNγ-depletion.

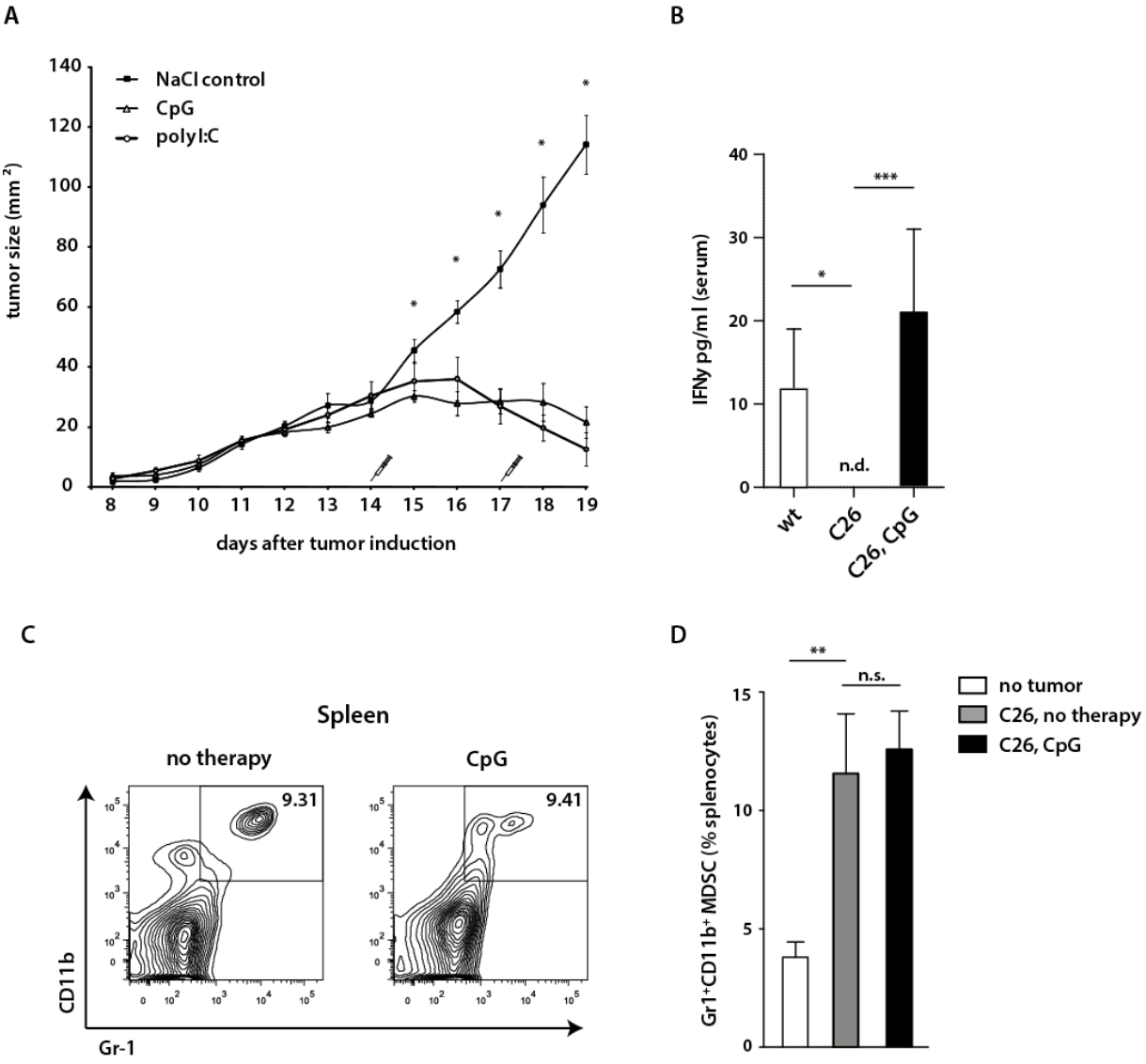


Figure 3.8. Treatment of established CT26 tumor-bearing mice with TLR ligands does not further induce of Gr1⁺CD11b⁺ MDSC. Balb/c mice were inoculated subcutaneously with CT-26 colon carcinoma cells. Mice bearing established tumors (area at least ≥ 25 mm²) were treated with CpG or poly(I:C) on day 14 and 17 after tumor induction (n = 5 per group). **A** Tumors were continuously measured with calipers every day in two dimensions. Mice were sacrificed two days after second therapy (day 19) and used for further analysis. **B** Serum levels of IFNγ in CT26 tumor-bearing mice before and after CpG therapy. Naïve Balb/c mice are shown as controls (wt, white bar). Mice were bled 24 hours after last therapy and serum levels of IFNγ levels were measured via ELISA. **C,D** Representative FACS plot and histograms showing the total number of splenic Gr1⁺CD11b⁺ MDSCs in untreated (PBS) and CpG-treated mice. The number of MDSC in non tumor-bearing mice is depicted as a control (white bar, C). $p \leq 0.05$ was considered as significant (paired Student's *t* test).

3.3.2 MDSC subset composition and phenotype following systemic PRR ligation

Previous studies showed contradicting results regarding the role of different MDSC subsets in tumor-mediated immunosuppression. Thus far, we showed that PMN- or Ly6G^{high} - MDSC are the most prominent subset induced during tumor growth in various murine tumor models. Compatibly, they seem to be the more potent suppressors of T cell proliferation (see chapter

3.2). Observing that treatment of tumor bearing mice with CpG-ODN not further induces myeloid-derived suppressor cells in total accompanied by a marked reduction in tumor size, in a next step we asked whether systemic Pattern recognition receptor ligation might influence the subset composition of tumor-induced MDSC.

Therefore, mice with established CT26 colon carcinoma, B16 melanoma or RMA-S lymphoma were challenged with 9.2RNA (TLR7), poly(I:C) (TLR3), CpG-ODN (TLR9) and ppp-RNA (RIG-I). Mice were than sacrificed and analyzed for MDSC subsets (Figure 3.9 A). As expected, in all three tumor models, systemic MDSC expansion was markedly due to a high number of $Ly6G^{high}$ PMN-MDSC. This was most prominent in spleens of CT26 colon carcinoma bearing mice, but also visible in RMA-S lymphoma and B16 melanoma. Furthermore, when mice were challenged with the respective Pattern recognition receptor ligand, we saw a strong and significant reduction of PMN-MDSC with augmentation of the $Ly6G^{low}$ MO-MDSC subset. The effect was mostly prominent for CpG but to a lesser extent also seen upon therapy with other PRR ligands. However, as described previously, the total number of MDSC remained unchanged. Again, the effect was not restricted to spleens alone but was likewise confirmed in bone marrow, blood and tumor-draining lymph nodes, even though less pronounced in bone marrow (data not shown).

Splenic $Ly6G^{high}$ PMN-MDSC and $Ly6G^{low}$ MO-MDSC subsets were than further analyzed for maturation and differentiation marker expression by flow cytometry. Given that one of the defining traits of MDSC is their immature status and that we could previously show that MDSC of tumor-bearing mice challenged with CpG exhibit less immunosuppressive capacities, we thus hypothesized that systemic TLR ligation alters their phenotype towards matured myeloid cells. Indeed, *in vivo* treatment with CpG led to an increase in expression of the macrophage differentiation marker F4/80, the DC differentiation marker CD11c and the co-stimulatory molecule CD80. As shown in wild-type mice before, we observed a highly significant upregulation of Sca-1 on both MDSC subsets again. Table 3 summarizes and compares different maturation, differentiation and activation marker in naïve and CT26 tumor-bearing mice with or without CpG therapy.

Marker	Naïve vs. CT26		Naïve vs naïve + CpG		CT26 vs CT26 + CpG	
	Change	(p)	Change	(p)	Change	(p)
Ly6C	-	≤ 0.001	+	≤ 0.001	+	≤ 0.001
Ly6G	+	≤ 0.01	-	≤ 0.05	-	≤ 0.01
CD11b	+	≤ 0.05	+	≤ 0.01	+	≤ 0.05
Sca-1	-	≤ 0.05	+	≤ 0.001	+	≤ 0.001
CD11c	-	≤ 0.05	+	≤ 0.01	+	≤ 0.05
MHCII	-	≤ 0.05	+	≤ 0.05	+	≤ 0.05
F4/80	-	≤ 0.05	+	≤ 0.05	(+)	0.08
CD80	-	≤ 0.01	(+)	0.07	+	≤ 0.01
CD86	-	≤ 0.05	(+)	0.09	(+)	0.059
CD88	(+)	0.088	n.t.	n.t.	-	≤ 0.01
CD124	(+)	0.088	-	≤ 0.05	=	n.s.
CD62L	=	n.s.	=	n.s.	=	n.s.
CD31	(+)	0.16	n.t.	n.t.	(-)	0.09

Table 3. Comparison of maturation, differentiation and activation marker on Gr1$^+$CD11b$^+$ myeloid-derived suppressor cells in naïve and tumor-bearing with or without treatment with CpG. n.t. : not tested, = : not significant (n.s.), - : significant downregulation, + : significant upregulation, (+) : trend towards upregulation, (-) : trend towards downregulation.

Thus, we demonstrated that challenging tumor-bearing mice with CpG-ODN and other TLR ligands, is able to overcome the tumor-induced differentiation block of myeloid cell differentiation [Gabrilovich and Nagaraj, 2009], restores expression levels of myeloid cell markers to those expressed in Gr1$^+$CD11b$^+$ cells in tumor-free mice and reverses the imbalance of Ly6G^{high}/Ly6G^{low} MDSC to a proportion seen in naïve mice.

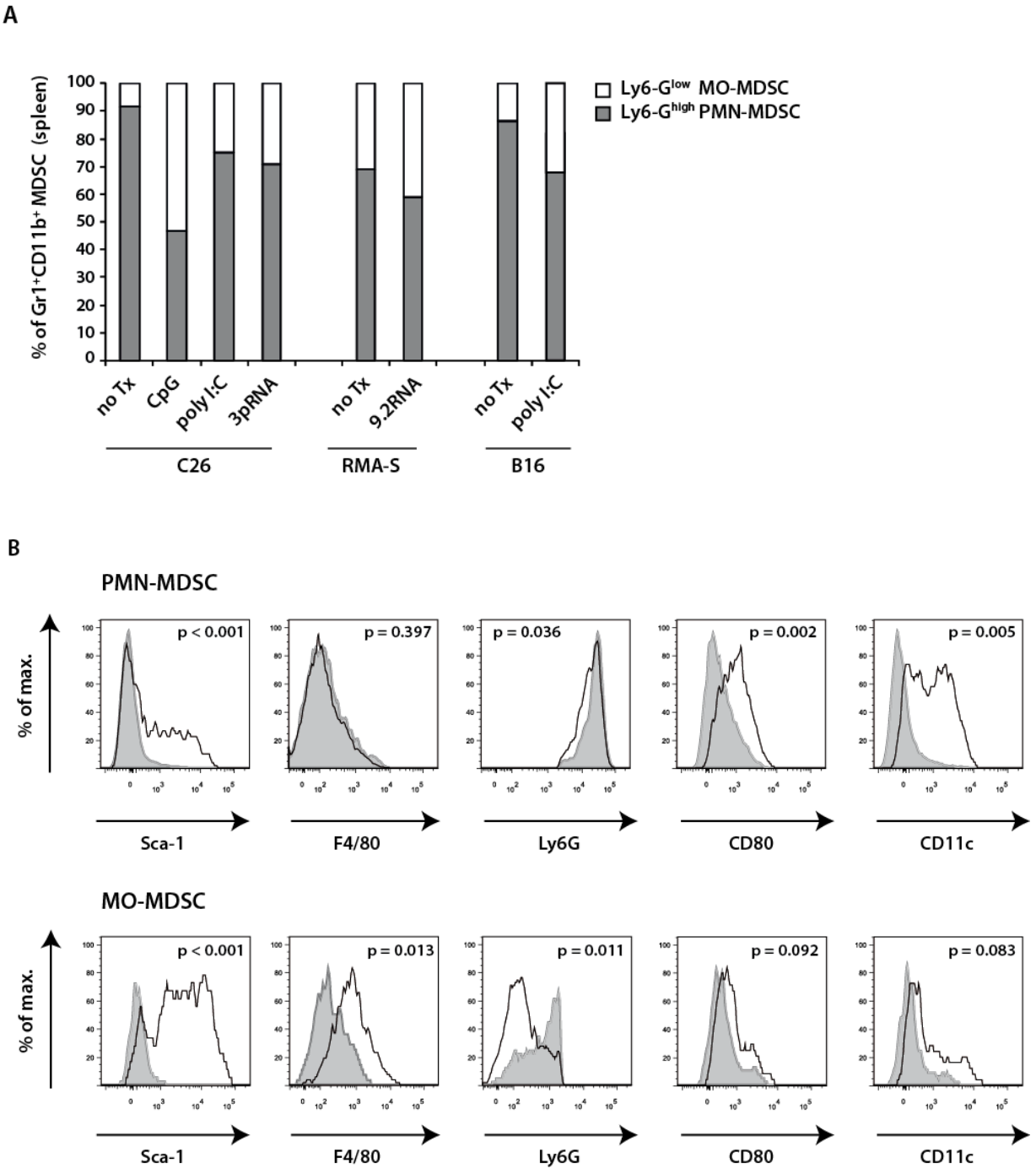


Figure 3.9. Systemic therapy with TLR ligands induces maturation and MDSC subset shifting towards MO-MDSC (Ly6G^{low}) in tumor-bearing mice. Subpopulations of splenic Gr1^{+}CD11b^{+} MDSC from mice with established C26 colon carcinoma, B16 melanoma or RMA-S lymphoma that were treated with different TLR / RIG-I ligands. MDSC subpopulations in spleens were analyzed via flow cytometry (n = 4 – 6 per group). **A** shows the percentage of splenic Ly6G^{low} monocytic MDSC (Ly6G^{low} MO) and splenic Ly6G^{high} polymorphonuclear MDSC (Ly6G^{high} PMN) with or without prior therapy in tumor-bearing mice. The total amount of MDSC in spleens is defined as 100%. **B** Histograms showing representatives of maturation marker expression (MFI) on pre-gated splenic MDSC-subpopulations in C26 tumor-bearing mice with (black curve) or without CpG therapy (grey curve). Data analysis was performed using paired Student's *t* test comparing mean fluorescent intensity (MFI) of each surface marker. $p \leq 0.05$ was considered as significant.

3.3.3 Impact of poly(I:C) therapy on intratumoral MDSC

MDSC not only play a pivotal role in tumor mediated general immunosuppression, but also resemble one of the most important players in the tumor microenvironment, where they can potently augment tumor growth, immune escape and where T cell suppression has the most profound effect [Umansky and Sevko, 2013]. Due to high practicability, most previous studies examined splenic MDSC. Thus, we next investigated, whether the effects on maturation and differentiation on tumor-induced MDSC after treatment with TLR ligands were also visible on tumor-infiltrating MDSC.

Therefore, established B16 melanoma tumors of mice received three subcutaneous injections of poly(I:C) as described previously. Tumors were explanted and compared with non-treated controls using flow cytometry. Analysis of tumor-infiltrating $Gr1^+CD11b^+$ MDSC revealed up-regulation of maturation and differentiation markers comparable with systemically expanded MDSC in bone marrow, blood and spleen after treatment with poly(I:C) (Figure 3.10). Higher expression of F4/80 and Ly6C resembles differentiation towards macrophages and mature monocytes following systemic TLR3 activation, respectively. There was also a trend towards DC-differentiation, indicated by upregulation of CD11c with the corresponding co-stimulatory molecule CD80, although it did not reach statistical significance. Comparable results were obtained when tumor-bearing mice were treated with CpG or ppp-RNA (data not shown). Nevertheless, the total amount of tumor-infiltrating MDSC after treatment with poly(I:C) or other pattern recognition receptor ligands was not altered (data not shown).

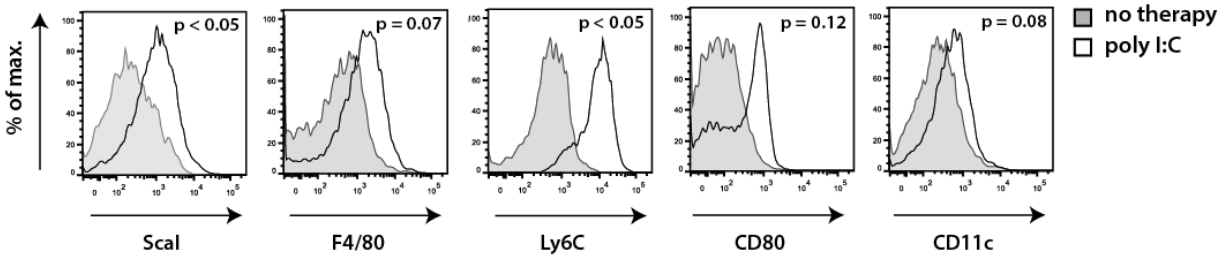


Figure 3.10. Poly(I:C) treatment induces maturation and differentiation of intratumoral myeloid-derived suppressor cells. C57/BL6 mice bearing established B16 tumors (area at least ≥ 25mm²) received 3 s.c. injections of poly(I:C) or PBS (no therapy). Two days after the last injection mice were sacrificed. The tumors were removed, kollagenase digested and analyzed via flow cytometry. Figure 10 shows differentiation and maturation marker expression on pre-gated intratumoral $Gr1^+CD11b^+$ MDSC. Histograms show representative data from individual mice. Data analysis was performed using paired Student's *t* test. Significances (p) indicate differences in the median marker expression between mice with or without prior poly(I:C) injections (n = 5 per group).

3.3.4 Phenotypical changes on MDSC upon systemic TLR stimulation are IFNα-dependent

After establishing that therapy with different TLR agonists and the RIG-I agonist 5'-triphosphate-RNA is able to induce maturation, differentiation and a population shift of myeloid-derived suppressor cell subsets, accompanied by a marked reduction in tumor size, we then asked what factors might contribute to the observed effects.

Maturation of MDSC as well as blocking their immunosuppressive properties was less prominent for the TLR7/8 agonist 9.2dr-RNA, R848 and the TLR4 agonist LPS (data not shown) and was most prominent for CpG-ODN (TLR9), poly(I:C) (TLR3) and 3p-RNA (RIG-I). When comparing differences and similarities of the systemically induced cytokine profile by these PRR ligands, a major difference of the latter is their greater ability to induce high amounts of IFNα [de Clercq, 1980; Krug et al., 2001; Hornung et al., 2006], suggesting IFNα as a major contributor for MDSC maturation and differentiation *in vivo*.

To test this hypothesis, we used the immunostimulatory RNA ligand poly(I:C) as one the strongest IFNα inducers throughout our experiments. Wildtype and type I IFN-receptor deficient mice ($IFN\alpha R^{-/-}$) were subcutaneously inoculated with B16 melanoma cells and treated with one dose of poly(I:C) or PBS as a control. Notably, the sole deficiency of type I interferon signaling in $IFN\alpha R^{-/-}$ mice was sufficient to induce a population shift towards higher numbers of monocytic $Ly6G^{low}$ MDSC, accompanied by a decrease in polymorphonuclear MDSC (Figure 3.11 A). There were no differences seen when comparing MDSC subset composition in wildtype mice bearing B16 tumors after one dose of poly(I:C), indicating that the effect of subset recomposition may take longer than the 24 hours. When performing maturation marker analysis, one dose of poly(I:C) resulted in phenotypic and functional changes in MDSC similar to those induced by CpG. Treatment with poly(I:C) was sufficient to significantly upregulate Sca-1 and other maturation markers (Figure 3.11 B). Strikingly, this upregulation was completely abolished in $IFN\alpha R^{-/-}$ mice, indicating that type I IFN is critically involved in MDSC maturation following innate immune activation.

Performing flow cytometry analysis of collagenase digested B16 tumors showed comparable results with intratumoral maturation and differentiation of tumor-infiltrating MDSC following IFNα-induction with poly(I:C), yet without altering the total number of MDSC infiltrating the tumor tissue (data not shown).

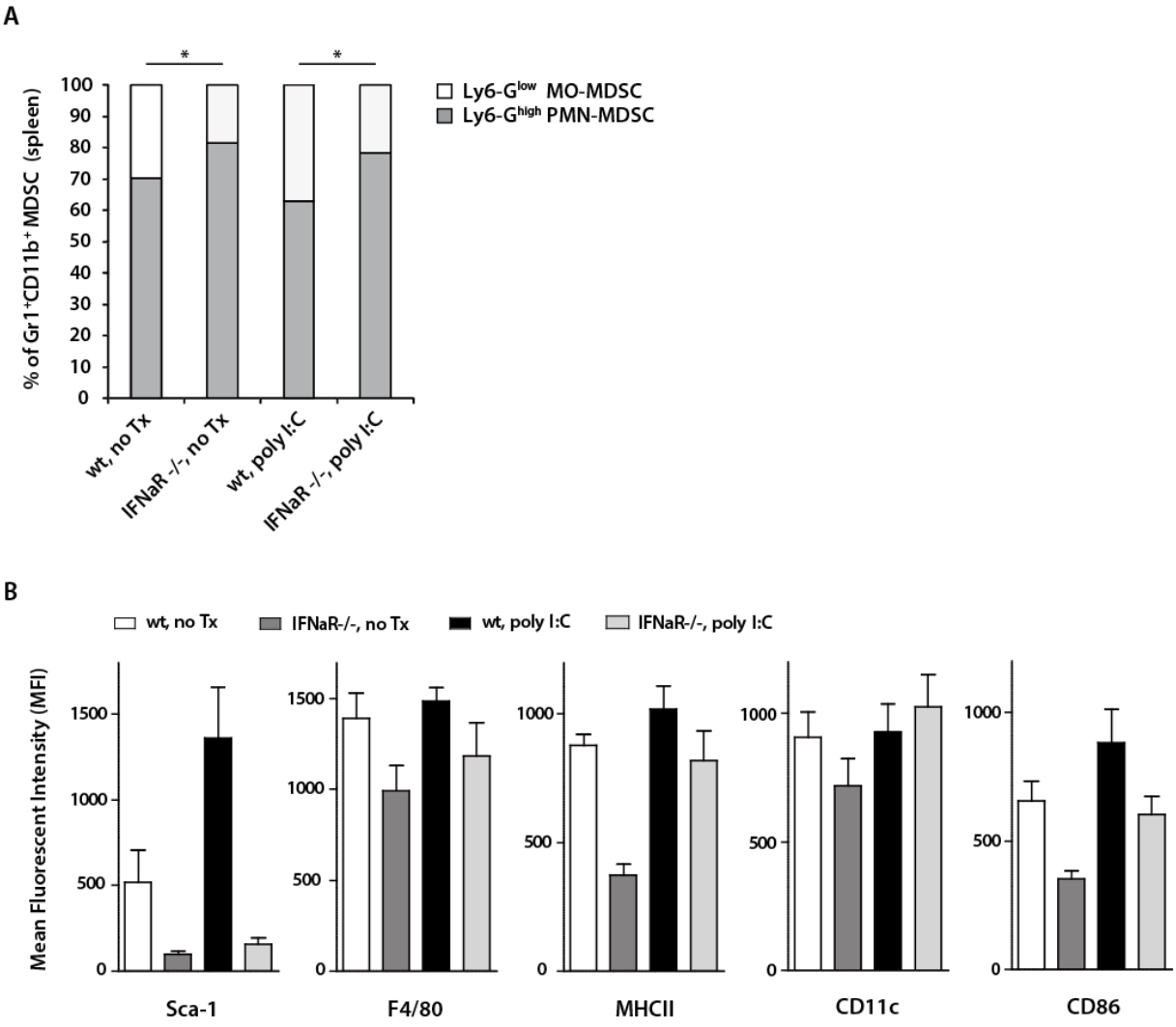


Figure 3.11. Maturation, differentiation and shifting of MDSC subpopulations after treatment with TLR ligands is IFNα-dependent. Wild type or type I IFN-receptor deficient (IFNαR$^{-/-}$) mice bearing s.c. B16 tumors (tumor area approximately 50 mm²) were treated once with poly(I:C) or PBS (no therapy). One day after injection mice were sacrificed. Organs were removed, tumors collagenase digested and analyzed via flow cytometry. **A** shows the percentage of splenic Ly6G^{low} monocytic MDSC (Ly6G^{low} MO) and splenic Ly6G^{high} polymorphonuclear MDSC (Ly6G^{high} PMN) with or without prior poly(I:C) therapy. The whole amount of MDSC (not shown) is defined as 100%. **B** shows differentiation and maturation marker expression on pre-gated splenic Gr1^{+}CD11b^{+} MDSC. Surface markers are demonstrated and compared in bar graphs as MFI (mean fluorescent intensity).

3.3.5 Role of ppp-RNA and TGFβ-silencing on MDSC in tumor-bearing mice

In preceding experiments we have observed that treatment of tumor-bearing mice with distinct pattern recognition receptor ligands, especially those that induce high amounts of IFNα such as CpG, poly(I:C) and 5'triphosphate-RNA, cause maturation and differentiation of systemic and intratumoral myeloid-derived suppressor cells. We showed that even though PRR ligation has pleiotropic downstream signaling effects, the effect of abolishing the maturation block, changing MDSC subset composition and attenuating their

immunosuppressive properties is largely dependent on type I IFN signaling [Zoglmeier et al., 2011].

However, numerous other tumor-derived factors and cytokines may lessen the beneficial effect of an IFNα-mediated MDSC maturation following innate immune stimulation. TGFβ has been emerged as one of the most crucial cytokines involved in MDSC accumulation in line with prevention of DC maturation and polarization of myeloid cells towards immune suppressive cells in the tumor microenvironment [Flavell et al., 2010]. In addition, Fridlender and colleagues showed that TGFβ-blockage results in polarization of tumor-associated neutrophils or $Ly6G^{high}$ MDSC towards an antitumoral neutrophil population.

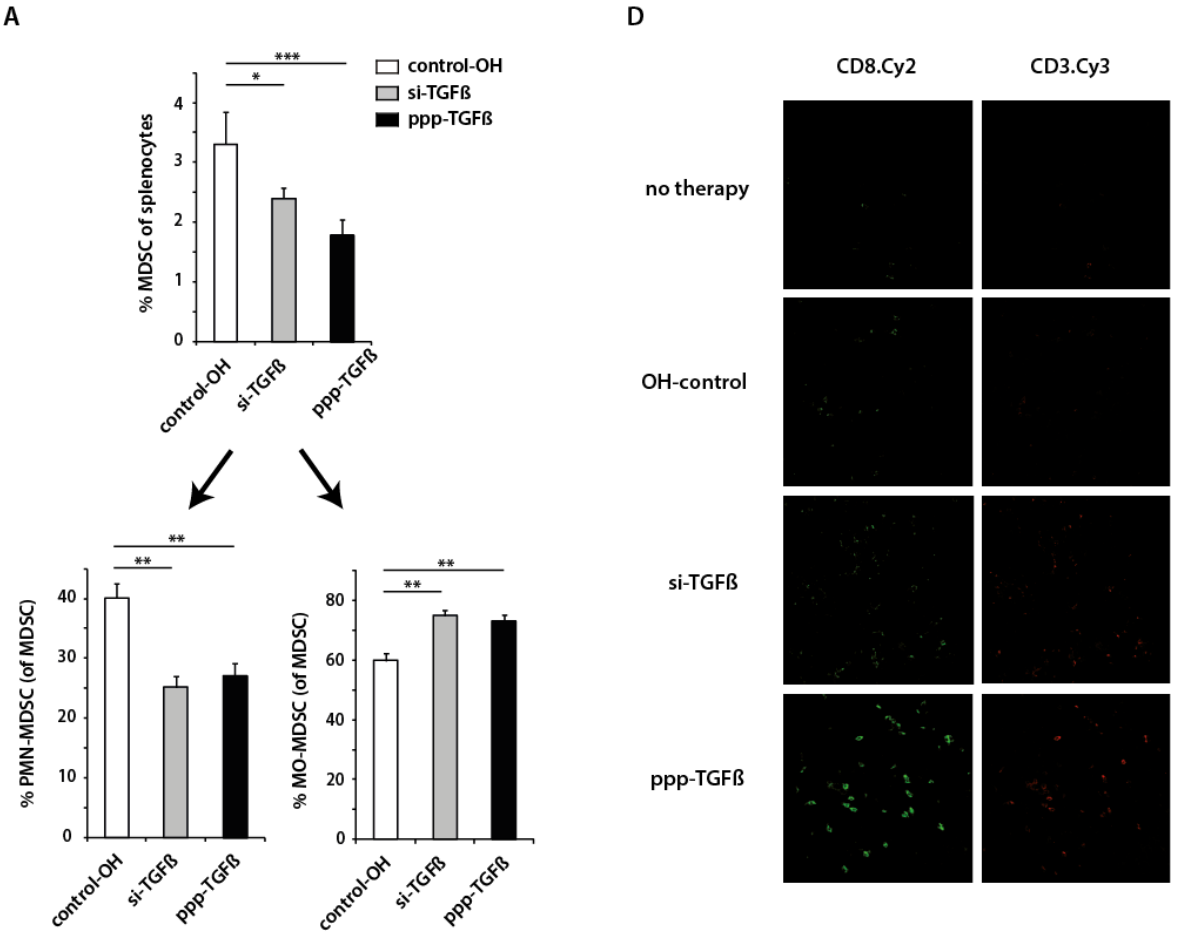
Hence, we wanted to investigate whether the maturation and differentiation of MDSC by systemic IFNα-induction following innate immune stimulation can be further augmented by concurrent blockage of TGFβ. Therefore a bifunctional ppp-siRNA was used that combines RIG-I activation for IFNα induction with gene silencing of $TGF\beta_1$ and its therapeutic efficacy was studied in the orthotopic Panc02 mouse model of pancreatic cancer [Ellermeier et al., 2013].

Treatment of PancO2-bearing mice with si-TGFβ or ppp-TGFβ led to a significant decrease of circulating myeloid-derived suppressor cells, illustrated by the percentage of $Gr1^+CD11b^+$ MDSC found in spleen (Figure 3.12 A). This effect was partially depending on TGFβ-blockage, although there was a trend towards further decrease in circulating MDSC with the triphosphate moiety and subsequent RIG-I activation. As hypothesized, downregulation of TGFβ by siRNA resulted in a highly significant change in subset composition with a decrease of polymorphonuclear $Ly6G^{high}$ MDSC and a relative increase of the monocytic subset. A trend towards this change in subset composition could also be demonstrated for 3p-RNA alone, albeit it lacked significance (data not shown, also see Figure 3.9 A). Again, by combining RIG-I activation and TGF-blockage with bifunctional ppp-TGFβ-siRNA, the shift in myeloid cell subset composition could not be further augmented. When analyzing apoptosis on an individual cell basis via measurement of Caspase-9 activity (FLICA), splenic $Gr1^+CD11b^+$ MDSC showed a significantly higher rate of apoptotic cell death compared to untreated mice. A more detailed analysis of MDSC subpopulations revealed, that the induction of apoptosis in this experimental setting on PMN-MDSC is largely dependent on TGFβ-blockage, whereas apoptosis in the monocytic subset is primarily induced by RIG-I activation via the 5'-triphosphate moiety (Figure 3.12 B).

To examine whether TGFβ-blockage alone or in combination with RIG-I activation can also promote MDSC differentiation in tumor bearing mice, $Gr-1^+CD11b^+$ MDSC were assessed on

their expression of CD80, CD11c, Ly6C and Sca-1 (Figure 3.12 C). Knockdown of TGFβ via siRNA was sufficient to induce monocytic cell differentiation in MDSC illustrated by upregulation of Sca-1 and Ly6C [Serbina et al., 2009]. However, DC differentiation with higher expression levels of CD11c and the co-stimulatory molecule CD80 was solely induced via the accessory triphosphate moiety with subsequent RIG-I activation.

MDSC are major inhibitors of cytotoxic T lymphocytes, inducing T cell anergy and negatively correlate with the influx of $CD8^+$ tumor-infiltrating cytotoxic T lymphocytes [Gabrilovich and Nagaraj, 2009; Curran et al., 2010]. By analyzing tumor slices via fluorescence microscopy we examined the influx of cytotoxic $CD3^+CD8^+$ T lymphocytes (CTLs) following therapy with bifunctional ppp-siRNA against TGFβ (Figure 3.12). We saw a significant increase in CTL-infiltration following TGF-beta blockage with either siTGFβ or ppp-TGFβ compared to control OH-RNA (Figure 3.12 D,E). Systemic RIG-I activation via a –ppp moiety was not able to further increase CTL influx, although a trend was visible. This might serve as a proxy of the observed pro-apoptotic and differentiating effects of TGFβ blockage and RIG-I activation on MDSC in this setting. Nevertheless, a casual link between both has yet to be determined.



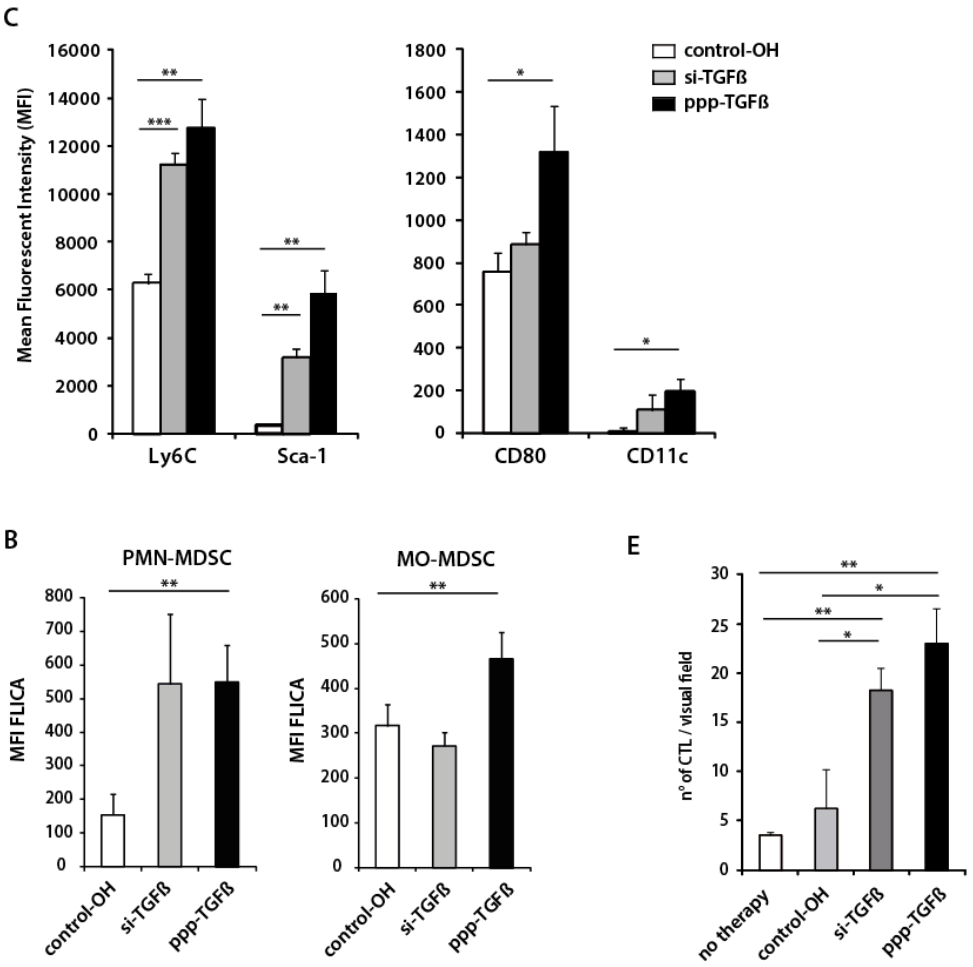


Figure 3.12. Increased apoptosis and maturation of MDSC by TGFβ blockage and IFNα induction via bifunctional 5'triphosphate-siTGFβ RNA. Mice bearing orthotopic PancO2 tumors were subsequently treated intravenously with 50 µg RNA on days 12 and 14 with unspecific RNA (control-OH; white bar), small-interfering RNA directed against $TGF\beta_1$ (siTGFβ; grey bar) or bifunctional 5'triphophosphate-siTGFβ RNA (ppp-TGFβ; black bar). Spleens and tumors were removed on day 15 and further analyzed by flow cytometry and immunofluorescence. **A** Percentage of $Gr\text{-}1^+CD11b^+$ MDSC (gated on live cells) and respective subpopulations (gated on MDSC). **B** Degree of apoptosis in MDSC was assessed by measuring Caspase-9 (FLICA) activity via flow cytometry in PMN-MDSC or MO-MDSC. **C** Maturation and differentiation of $Gr\text{-}1^+CD11b^+$ MDSC as assessed by mean fluorescence intensity (MFI) of CD80, CD11c, Ly6C and Sca-1, respectively. **D** Tumor slices were stained with Cy2 anti-CD8 (green) and Cy3 anti-CD3 (red). Representative pictures of $CD3^+CD8^+$ tumor-infiltrating cytotoxic T-lymphocytes (CTLs) into tumor tissue. **E** shows the average number of CTLs per visual field. Cell infiltration was averaged measuring at least 15 visual fields per sample out of 3-5 different tumor samples. Pooled data of 4 – 6 mice per group are shown as means ± SEM. * indicates $p \leq 0.05$. ** indicates $p \leq 0.01$. *** indicates $p \leq 0.001$. All data are expressed as mean ± SEM. A two-tailed p-value of ≤ 0.05 was considered significant.

3.4 Characterization of stem cell antigen-1 (Sca-1) on MDSC

Following innate immune stimulation and therapy of tumor-bearing mice with different PRR ligands, the Ly6 protein family member Sca-1 (Ly6A/E) has been shown to be one of the most significantly upregulated proteins on MDSC among the surface proteins throughout our

experiments. Stem cell antigen-1 (Sca-1) or lymphocyte activation protein-6A (Ly6A) is a GPI-anchored protein and is localized to lipid rafts of the plasma membrane [Curran et al., 2010]. It has been originally identified as an antigen upregulated on activated lymphocytes more than 30 years ago [Yutoku et al., 1974].

In prior experiments we had shown for the first time that Sca-1 is strongly upregulated by MDSC in response to CpG-ODN, poly(I:C) or 5'triphosphate-RNA and that it represents a highly sensitive activation/differentiation markers for this cell population. Furthermore, its upregulation inversely correlates with the suppressive activity of MDSC. Therefore, we aimed to further dissect the role of Sca-1 on MDSC mediated immunosuppression and tumor development.

3.4.1 Expression of Sca-1 in different tissues and MDSC subpopulations

Using flow cytometry, we first started to analyze the expression of Sca-1 on $Gr1^+CD11b^+$ immature myeloid cells (iMC) in non-tumor-bearing mice. Mean fluorescence intensity of Sca-1 on iMC was lowest in bone marrow with induction of its expression after peripheral homing of immature myeloid cells into spleen, lung and peripheral lymph nodes with the highest measurable Sca-1 expression in lymphoid tissue (Figure 3.13 A). Surprisingly, when comparing tumor-induced splenic MDSC and tumor-associated MDSC in CEA424-TAg mice bearing spontaneous gastric tumors, tumor-associated MDSC showed three-fold higher expression levels of Sca-1 in comparison to splenic MDSC (Figure 3.13 C). In a next step, we compared Sca-1 expression on splenic immature myeloid cells in tumor-free mice versus tumor-induced myeloid derived suppressor cells showing that MDSC express significantly lower levels of Sca-1 than their naïve counterparts. In addition, when comparing MDSC subpopulations on their differential expression of Sca-1, monocytic $Ly6C^{high}$ MDSC express higher amounts of Sca-1 on their cell surface than granulocytic PMN-MDSC (Figure 3.13 B).

We thus show that Sca-1 is upregulated during homing of $Gr1^+CD11b^+$ myeloid cells into peripheral organs and even tumor tissue. Furthermore, Sca-1 seems to be downregulated on MDSC via tumor-derived factors, which complements previous results showing Sca-1 upregulation via TGFβ-blockage (Figure 3.12 C). In addition, when performing detailed flow cytometry analysis of $Sca\text{-}1^+Gr1^+CD11b^+$ cells compared to $Sca\text{-}1^-Gr1^+CD11b^+$ cells, the expression of Sca-1 strongly correlated with a more mature and differentiated MDSC phenotype in both naïve and tumor-bearing mice (data not shown).

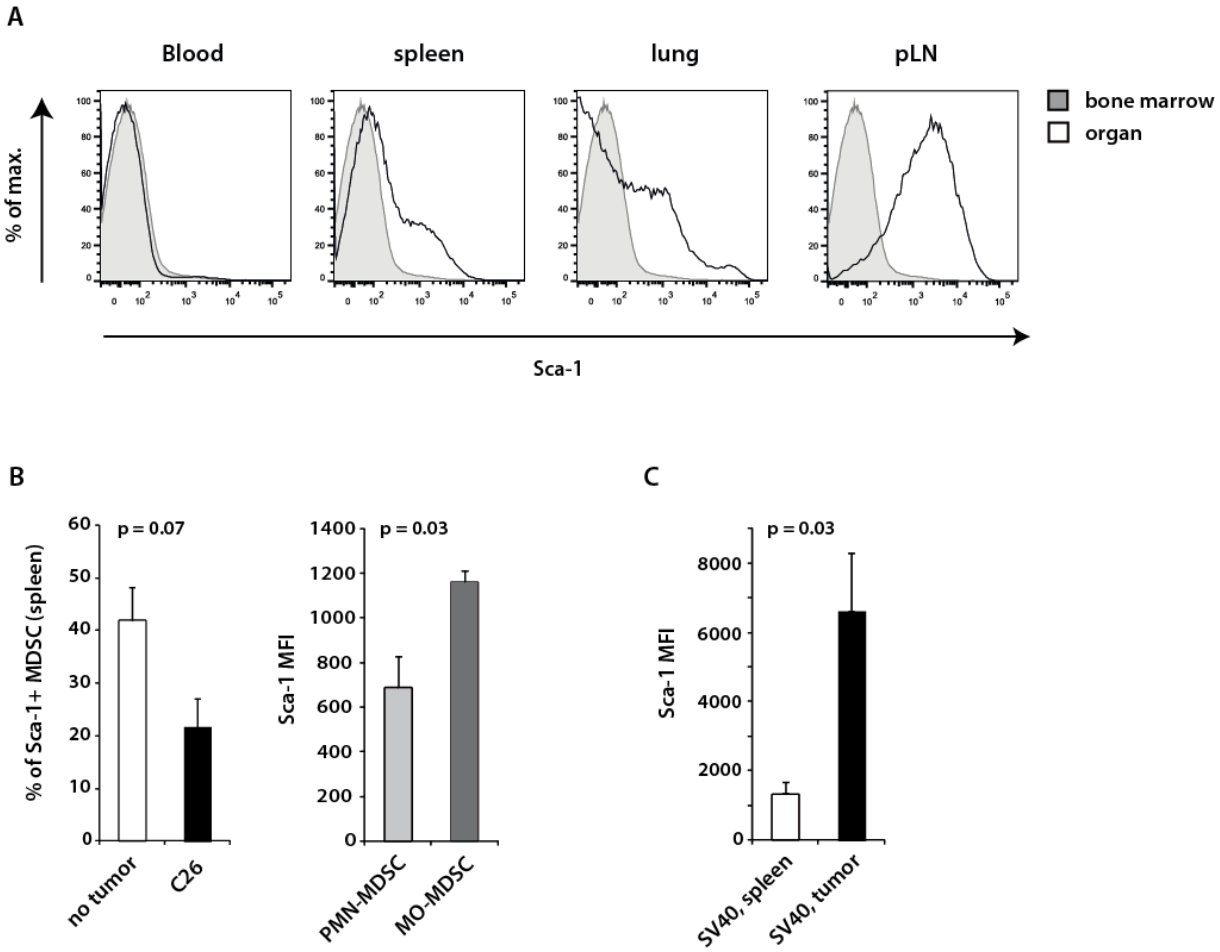


Figure 3.13. Sca-1 expression on MDSC is tissue-dependent and differs between naïve iMC, MDSC-homing site and MDSC subpopulations. A Naïve Balb/c mice (n = 3) were sacrificed and Sca-1 expression on $Gr1^+CD11b^+$ iMCs was analyzed via flow cytometry in indicated tissues (pLN: peripheral lymph nodes). Histograms display an example of representative individual mice. **B** Balb/c mice were inoculated subcutaneously with CT26 colon carcinoma cells. Mice bearing established tumors (area at least $\geq$ 50 mm²) are compared with non-tumor bearing mice (n = 5 per group). Bar graphs show the amount of $Sca\text{-}1^+$ MDSC in spleen (left) and Sca-1 expression on MDSC subpopulations (right) displayed as Mean Fluorescent intensity (MFI). **C** MDSC in CEA424-TAg mice bearing spontaneous gastric tumors (SV40; mice aged 90 – 100 days; n = 3 per group) were analyzed via flow cytometry. Bar graphs show the mean fluorescent intensity (MFI) of Sca-1 on splenic and intratumoral $Gr1^+CD11b^+$ MDSC. Analysis was performed using paired Student's *t* test. Significances are depicted in the left upper corners.

3.4.2 Role of Sca-1 in tumor development and MDSC-mediated immunosuppression

To further evaluate the role of Sca-1 in tumor formation and to evaluate a functional role in MDSC-mediated immune escape, wild type C57/BL6 mice and mice deficient in stem cell antigen-1 ($Sca\text{-}1^{-/-}$) were subcutaneously inoculated with PancO2 cells. Tumor formation was then continuously measured. When mice were sacrificed, we purified $Gr1^+CD11b^+$ MDSC from spleens as described previously and performed a T cell suppression assay. Based on

prior experiments, we hypothesized that Sca-1 may act as a negative regulator of MDSC-mediated tumor tolerance, thereby promoting a more efficient immune answer.

To our surprise, when we compared Sca-1$^{-/-}$ MDSC with wild type MDSC on their capability to suppress CD3/CD28-activated splenocyte proliferation *ex vivo*, MDSC lacking Sca-1 were significantly less suppressive than their naïve counterparts (Figure 3.14 B). In addition, Sca-1$^{-/-}$ mice showed a trend towards slower tumor growth, even it did not reach statistical significance ($p = 0.11$). In contrast to what we had expected, we thus show for the first time that Sca-1 functionally fosters the capability of myeloid-derived suppressor cells to suppress T cell proliferation and thereby might attenuate immune surveillance of malignant tumors.

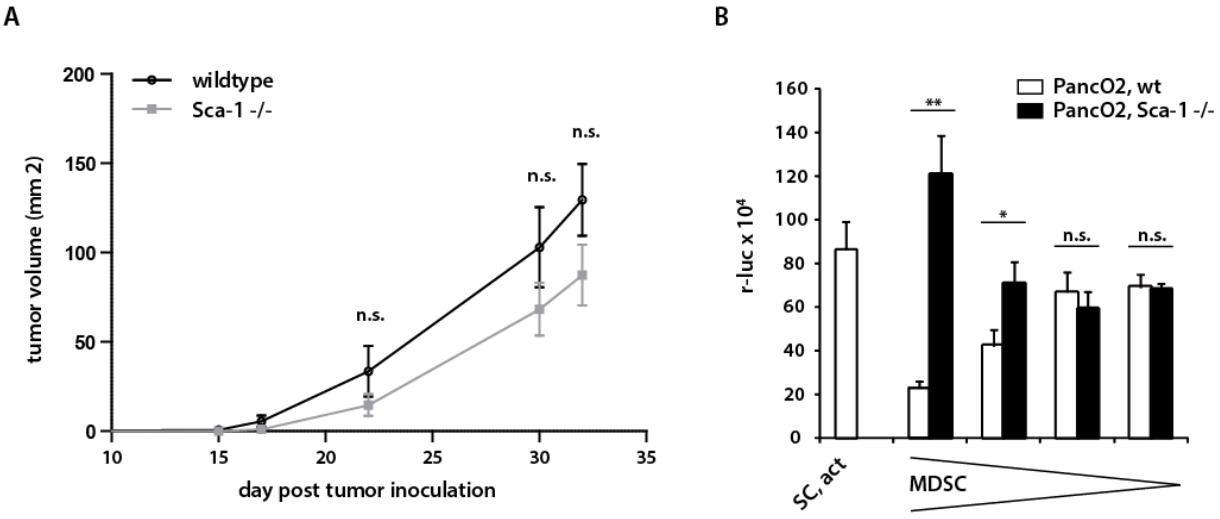


Figure 3.14. Sca-1$^{-/-}$ mice have less immunosuppressive MDSC without significant alteration of tumor growth. A C57/BL6 and Sca-1$^{-/-}$ mice were inoculated subcutaneously with PancO2 pancreatic carcinoma cells (n = 5 – 8 per group). Tumors were continuously measured with calipers in two dimensions. Mice were sacrificed on day 32 after tumor inoculation when tumor size reached at least ≥ 75 mm^2 in each group. **B** MDSC were purified from spleens of wildtype and Sca-1$^{-/-}$ PancO2-bearing mice by sequential MACS separation. Purity was verified via flow cytometry and was ≥ 80% in each group. Degree of MDSC-mediated suppressivity was assessed with a T-cell proliferation assay using CD3/CD28-stimulated splenocytes (SC) as responder cells cocultured with MDSC for 48 hours [MDSC-splenocyte-ratio 1:16, 1:8, 1:4, 1:2 with anti-CD3/CD28-stimulated SC as positive control (SC, act)]. $p \leq 0.05$ was considered as significant.

3.4.3 Induction of Sca-1 on MDSC via TLR ligands *in vivo* and *in vitro*

Preceding experiments using immunotherapy with different TLR ligands in vivo showed a significant upregulation of Sca-1 on MDSC in tumor bearing mice, concurrent with an inverse relation of Sca-1 expression with immunosuppressive properties of Gr1$^+$CD11$^+$ MDSC. Throughout our experiments, especially PRR ligands known for their potential to induce high amounts of type I interferons after receptor ligation such as CpG-ODN (TLR9), poly(I:C) (TLR3) and 3p-RNA (RIG-I) were the most efficient inducers of Sca-1 on MDSC *in vivo*.

To validate this finding, CT26 tumor-bearing mice received three repeated injections of CpG-ODN and poly(I:C) again, as we and others have shown a major type I interferon response *in vivo* following treatment with these immunostimulatory oligonucleotides [de Clercq, 1980; Krug et al., 2001]. Indeed, Sca-1 expression on myeloid derived suppressor cells was upregulated four- to tenfold as compared to untreated mice (Figure 3.15 A).

In a next step, we used B16 melanoma-bearing IFNαR$^{-/-}$ mice and injected poly(I:C) as it appeared to be the strongest inducer of IFNα with subsequent upregulation of Sca-1 on Gr1^{+}CD11b^{+} cells (Figure 3.15 B). Whereas poly(I:C) treatment of wild type mice led to Sca-1 upregulation on splenic as well as intratumoral MDSC, its expression was completely abrogated in IFNαR$^{-/-}$ mice. This is supported by recent literature by Essers and colleagues, showing that Sca-1 is inducible via IFNα in a STAT1 dependent fashion *in vivo* [Essers et al., 2009].

In parallel experiments we found that abrogation of MDSC suppressivity, their maturation and Sca-1 induction following CpG treatment, is mainly mediated by type I IFN induction via plasmacytoid dendritic cells, rather than a direct effect of TLR9 activation on MDSC [Zoglmeier et al., 2011]. To test whether direct stimulation of Gr1^{+}CD11b^{+} immature myeloid cells with different Toll-like receptor ligands is able to induce Sca-1 expression, we purified Gr1^{+}CD11b^{+} immature myeloid cells from bone marrow via magnetic sorting. Cell purity was greater than 98% which was validated via flow cytometry. As hypothesized, IFNα induces the highest Sca-1 expression on immature myeloid cells, at the same time proving that iMC/MDSC express a functional type I interferon receptor (Figure 3.15 C). Neither CpG-ODN or poly(I:C) were able to induce Sca-1 expression *in vitro*. In contrast, LPS (TLR4) and R848 (TLR7) as well as the combination of Pam3CysK (TLR2) and Curdlan, a beta-1,3-glucan-polymer binding to Dectin-1, significantly induced Sca-1 expression in immature myeloid cells. Neither Pam3CysK nor Curdlan alone were able to upregulate Sca-1 alone, which supports findings of Ferwerda and colleagues in 2008, showing that dectin-1 amplifies TLR2-dependent induction of cytokines [Ferwerda et al., 2008].

Thus, we show that upregulation of Sca-1 on Gr1^{+}CD11b^{+} immature myeloid cells upon stimulation with CpG and poly(I:C) *in vivo* is mainly mediated via indirect induction of IFNα as direct stimulation of their corresponding Toll-like receptor on iMC did not result in a significant upregulation of Sca-1. Concurrently, we show that immature myeloid cells express functional TLR2, TLR4, TLR 7 and Dectin-1 and to a certain extent, Sca-1 expression is also inducible via their direct activation.

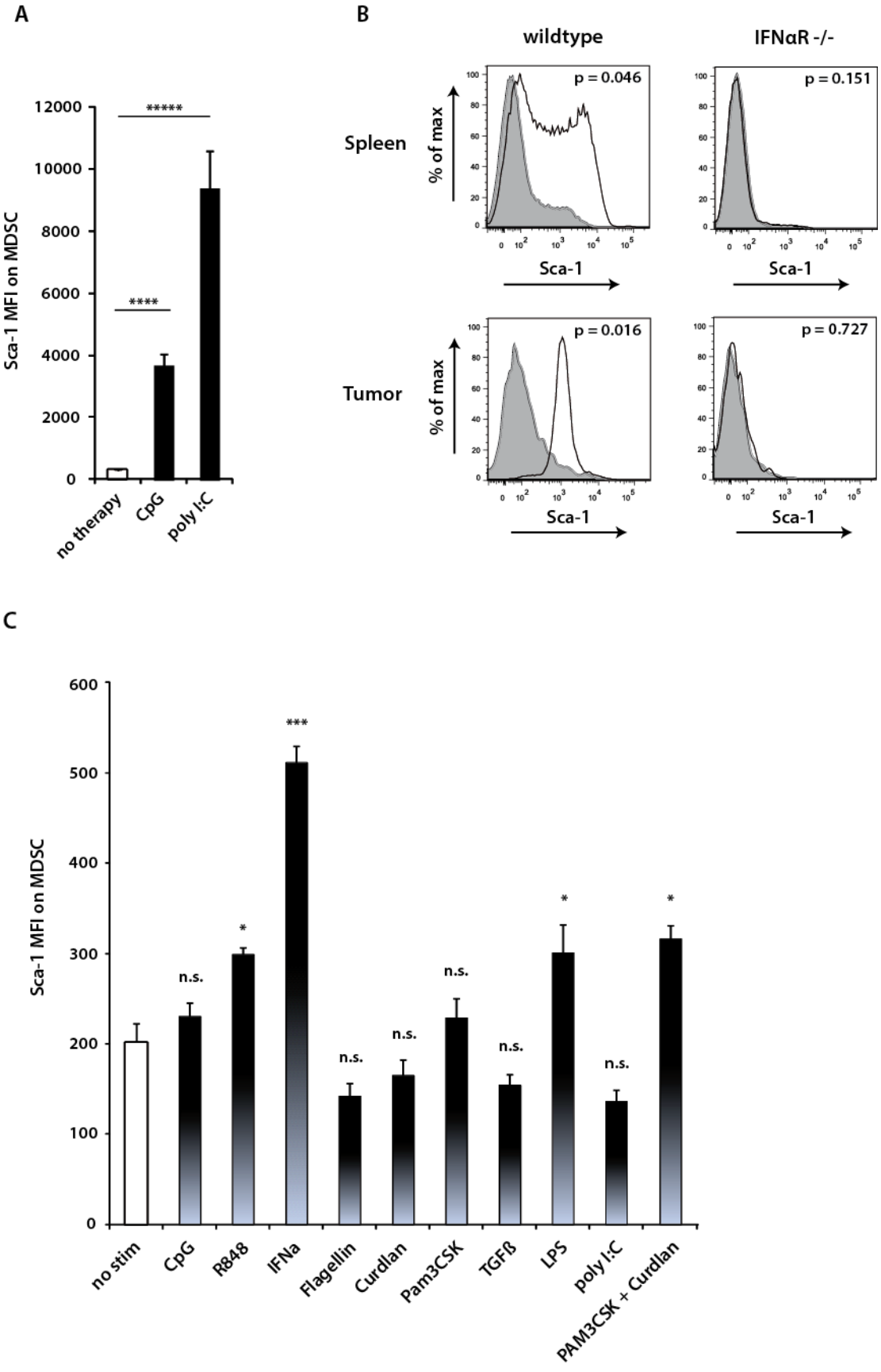


Figure 3.15. TLR ligands induce Sca-1 on MDSC *in vivo and in vitro* with an essential role for IFNα. **A** CT26 tumor-bearing mice received 3 s.c. injections of CpG, poly(I:C) or PBS (no therapy). Two days after the last injection mice were sacrificed and Sca-1 expression on splenic myeloid-derived

suppressor cells was analyzed via flow cytometry (MFI = mean fluorescent intensity). Pooled data of 4 – 6 mice per group are shown as means ± SEM. **** indicates $p \leq 0.0001$. ***** indicates $p \leq 0.00001$. **B** Wildtype or IFN-I receptor deficient ($IFN\alpha R^{-/-}$) mice bearing s.c. B16 tumors (tumor area approximately 50 mm^2) were treated once with poly(I:C) or PBS (no therapy). Organs were removed, tumors kollagenase digested and analyzed via flow cytometry. Histograms show the changes in the expression of Sca-1 on splenic and intratumoral MDSC two days after treatment; an example of representative individual mice is depicted [gray, no therapy; black line, poly(I:C)]. Significances (p) indicate differences in median expression (n = 5 per group). **C** $Gr1^+CD11b^+$ iMC from bone marrow of naïve C57/BL6 mice were purified, plated in 96-well and cultured *in vitro* with indicated stimuli. Purity of $Gr1^+CD11b^+$ was greater than 98% (validated via FACS). Sca-1 expression (MFI) on iMC was evaluated after 24 hours of stimulation via flow cytometry. [CpG:TLR9; R848:TLR7; IFNα:IFNαR; Flagellin:TLR5; Curdlan:Dectin-1; Pam3CysK:TLR2; TGFβ:TGFβR; LPS:TLR4; poly(I:C):TLR3]. Significances (p) indicate differences in the median marker expression (n = 3 per group). A two-tailed p-value ≤ 0.05 was considered significant (Student's *t* test).

3.4.4 Analysis of cytokine induction in Sca-$1^{-/-}$ mice upon PRR ligation

On the one hand we found out that the induction of IFNα via innate immune receptors is critically involved in maturation, differentiation and attenuation of MDSC suppressivity, along with a highly significant increase in the expression of stem cell antigen-1. In addition, we unexpectedly found that myeloid-derived suppressor cells deficient for Sca-1 exhibit less immunosuppressive properties in tumor-bearing mice in conjunction with a trend towards slower tumor growth *in vivo*. On the other hand, increasing evidence in the literature implicated that Sca-1 might not only be a bystander protein indicating T cell activation or serving as a phenotypical marker for lineage determination in haematopoietic stem cells [Ma et al., 2002; Holmes and Stanford, 2007], but may also play a functional role in distinct signaling pathways, e.g. type I interferon and TGFβ signaling [Essers et al., 2009, Upadhyay et al., 2011].

Based on that findings and in order to dissect functional differences between Sca-$1^{-/-}$ and wildtype mice, C57/BL6 and Sca-$1^{-/-}$ mice received one injection of poly(I:C). Twenty hours later mice were bled and serum was tested for cytokine levels. Based on findings of other groups that Sca-1 may prohibit downstream type I interferon signaling, we hypothesized lower levels of IFNα upon systemic TLR3 activation via poly(I:C). To our surprise, serum levels of IFNα were comparable in wild type and Sca-$1^{-/-}$ mice (Figure 3.16 A, left column). When we further screened for serum levels of other cytokines, no differences in the induction of IL-6, IL12p70, TNFα or IL1β could be detected (data not shown). Incidentally, we detected high levels of IP-10/CXCL-10 in Sca-1 deficient mice upon therapy with poly(I:C) (Figure 3.19 A, right column). IFNγ-inducible protein of 10 kDa or IP-10/CXCL10 is an early response gene mainly induced by Interferon-γ but also type I interferons, LPS or CpG-ODN [Neville et al., 1997; Imaizumi et al., 2013] and is critically involved into effector T cell trafficking to sites of

inflammation [Dufour et al., 2002]. Experimental models also demonstrated that CXCL10/IP-10 has antitumorigenic properties and reduces angiogenesis [Narvaiza et al., 2000].

Next, we wanted to evaluate if this pronounced induction of IP-10 *in vivo* is either an indirect effect of TLR3 activation via other soluble mediators/cytokines and likewise, if stimulation of other pattern recognition receptor pathways also triggers a more pronounced IP-10 induction. Therefore, bone marrow-derived macrophages (BMDM) from wild type and Sca-$1^{-/-}$ mice were generated as previously described [Manzanero, 2012], cultivated, plated in 96-well plates and stimulated with various pattern recognition receptor ligands available in our laboratory. BMDM were used because they resemble a common tool when analyzing innate immune receptor signaling and cytokine production [Kobayashi et al., 2002].

Direct stimulation of wild type macrophages with poly(I:C), LPS, poly(dA:dT), 5'ppp-RNA and IFNα/γ induced moderate levels of IP-10 secretion into the supernatant (Figure 3.16 B). In this experimental setting, IP-10 induction via IFNγ at 100 U/ml was lower as expected after 24 hours of stimulation. When we repeated the same experimental setting with higher concentrations of IFNγ (up to 500 U/ml), IP-10 induction was markedly increased in a dose dependent manner (data not shown). When we measured IP-10 levels in the supernatant of pattern recoginiton receptor stimulated Sca-$1^{-/-}$ BMDM, a highly significant increase in its production compared to wildtype mice was observed. Especially stimulation of TLR3, TLR4, the AIM2-inflammasome and RIG-I resulted in a three- to sixfold increase in IP-10 production as compared to macrophages posessing the Sca-1 antigen. Comparable results (only less pronounced) were obtained when bone marrow, splenocytes and magnetically sorted $Gr1^{+}CD11b^{+}$ immature myeloid cells from wild type and Sca-$1^{-/-}$ mice were used in the same experimental setting (data not shown).

Thus, we show for the first time that mice deficient for Stem cell antigen-1 show a pronounced IP-10 production following innate immune stimulation with various PRR ligands *in vivo* and *in vitro*. Whether that observation has a biological significance, or might even explain reduced MDSC suppressivity of Sca-$1^{-/-}$ mice, needs to be elucidated in further studies.

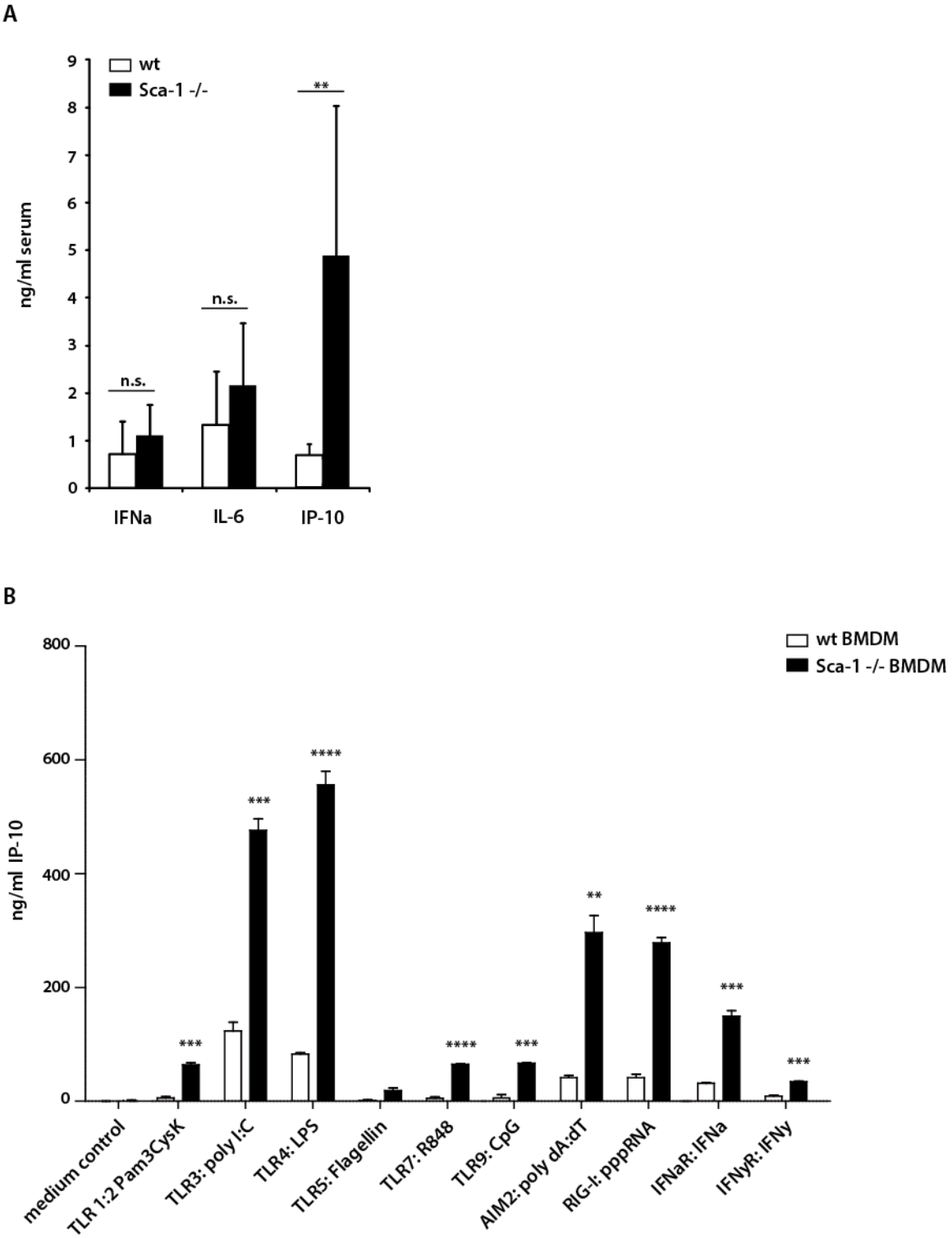


Figure 3.16. Pronounced IP-10 induction *in vivo* and *in vitro* following PRR ligation in Sca-1$^{-/-}$ mice. **A** Naive C57/BL6 and Sca-1$^{-/-}$ mice (n = 5 per group) received one s.c. injection of 250 µg poly(I:C). 20 hours later mice were bled and serum was analyzed for the amount of IFNα, IL-6 and IP-10 via ELISA. **B** Bone marrow-derived macrophages (BMDM) from wildtype (wt) and Sca1$^{-/-}$ mice were cultured, plated in 96-well and stimulated for 24 hours with indicated PRR ligands. Supernatant was measured for the amount of IP-10 via ELISA. ** indicates $p \leq 0.01$. *** indicates $p \leq 0.001$. **** indicates $p \leq 0.0001$. All data are pooled of w wells per group and expressed as mean ± SEM. A two-tailed p-value of ≤ 0.05 was considered significant.

4 DISCUSSION

4.1 MDSC subpopulations

4.1.1 Differentiated view on MDSC subpopulations in tumor-bearing hosts

In recent years, the role of MDSC in tumor-associated immune suppression has been established in a large number of clinical and preclinical studies. It quickly became apparent that MDSC do not represent a single cell population, but are rather comprised of myeloid cells at different stages of cell differentiation [Almand et al., 2001: Bronte, 2009]. Tumor-induced MDSC are further dichotomized into two major subpopulations, in mice based on their differential expression of Ly6G and Ly6C on the cell surface: polymorphonuclear $Gr1^{+}CD11b^{+}Ly6G^{high}Ly6C^{low}$ MDSC and monocytic $Gr1^{+}CD11b^{+}Ly6G^{neg}Ly6C^{high}$ MDSC (MO-MDSC) [Movahedi et al., 2008]. However, with more studies on MDSC biology, it becomes more and more apparent that this classical dichotomy is more complex than originally thought. Based on their differential expression of Gr-1, CD11b, scatter-appearance, other surface expression markers such as CD115 and morphologic appearance, Greifenberg and colleagues could identify six different populations in the $Gr1^{+}CD11b^{+}$ compartment with varying immunosuppressive properties [Greifenberg et al., 2009]. Other groups made attempts to further subdivide polymorphonuclear and monocytic MDSC in more or less immunosuppressive phenotypes by using additional surface markers such as CD49d, CD115 or CD124/IL-4Rα [Mandruzzato et al., 2009; Haile et al., 2010].

Due to feasibility and the lack of clearly defined additional subpopulations of myeloid-derived suppressor cells other than $Ly6G^{high}$ PMN and $Ly6G^{low}$ MO-MDSC, we have adopted the classical division of MDSC in this work. All tumor models examined in this study showed a variable but significant increase in the whole population of MDSC. Intriguingly, PMN-MDSC were the most abundant cell type induced by tumor growth *in vivo*. This finding is supported by the literature showing the granulocytic subset as a predominant population of MDSC in tumor-bearing mice [Youn et al., 2008; Gabrilovich and Nagaraj, 2009]. However, in other pathologic conditions, such as experimental autoimmune encephalitis, a mouse model of multiple sclerosis, a significant increase in the number of $CD11b^{+}Ly6G^{-}Ly6C^{high}$ cells resembling monocytic MDSC was observed that limited the course of disease. At the bottom line, the basis for this subset dichotomy remains elusive as the exact nature of tumor-derived factors that drive MDSC accumulation and define the expansion of a specific subset.

Likewise, in the context of tumor development, there is still ongoing debate whether granulocytic or monocytic MDSC exploit more immunosuppressive features. In this work, we consistently observed that the $Ly6G^{high}$ fraction (when purified and compared to $Ly6G^{low}$

MDSC) was substantially more suppressive. This was validated in the murine B16 melanoma, CT26 colon carcinoma, RMA-S lymphoma and orthotopic SV40 gastric carcinoma model. In contrast, a study using EL-4 lymphoblastic lymphoma, MO-MDSC have shown to inhibit T cell proliferation more potently than PMN-MDSC [Movahedi et al., 2008]. Likewise, another study by Dolcetti and colleagues proposed an inverse correlation of Gr1-brightness to the immunosuppressive power of MDSC on Ag-stimulated $CD8^+$ T cells in a C26-GM and 4T1 tumor model [Dolcetti et al., 2010]. On the contrary, our data is strengthened by a recent study analyzing MDSC subpopulations from mice bearing 3LL, MCA-38 and EL-4 tumors that shows that PMN-MDSC had higher ability to impair proliferation and expression of effector molecules in activated T cells, thus inducing T cell anergy [Raber et al., 2014]. Regarding human MDSC, a recent NIH study by Tim Greten and colleagues analyzed monocytic ($CD14^+$) and granulocytic ($CD15^+$) MDSC in patients with gastrointestinal malignancies and found the granulocytic fraction to be more suppressive than their counterparts [Duffy et al., 2013].

However, defining the exact differences between MDSC subsets in mice and particularly in human has not reached a consensus yet. For the future studies of MDSC biology it will be crucial to further dissect molecular pathways and mechanisms of immunosuppression which are partially substantially different between MDSC subsets. Even though, these variations are not overly surprising since the factors driving MDSC accumulation vary between different tumor models and human cancers: If a tumor of a given type will produce a particular set of MDSC driver molecules that induce a MDSC phenotype of a given type, another tumor entity will produce other driver molecules that induce a different MDSC phenotype.

In addition, variations between different study groups regarding MDSC suppressivity and their behavior following pharmacologic therapy may be explained by different isolation techniques used for MDSC purification as well as the functional readout used for estimation of their immunsosuppressive properties. In this study we used different magnetic-bead based technologies to sort MDSC subpopulations with high purity. Nevertheless, by histologic evaluation, purified $Ly6G^{high}$ MDSC were exclusively polymorphonuclear, whereas the nuclear morphology of $Ly6G^{low}$ MDSC was more heterogeneous, comprising both monocyte-like and polymorphonuclear cells [Zoglmeier et al., 2011]. That may limit the direct transfer of our data when compared to studies in which other isolation techniques have been utilized. Furthermore it may clarify that these cells do not represent a defined subset of cells but a group of phenotypically heterogenous myeloid cells sharing common biological activity rather than specific phenotypical characteristics.

4.1.2 Shift in subset composition upon TLR activation

In non-tumor bearing mice, the proportion of polymorphonuclear Ly6G^{high} to monocytic Ly6G^{low} immature myeloid cells is relatively stable varying from 1:1 up to 3:2 in favor of granulocytic myeloid cells with minor distinctions between different mouse strains. *In vivo* treatment with the TLR ligands CpG and poly(I:C) mainly increases the frequency of monocytic immature myeloid cells in naive mice, whereas the total number of PMN-MDSC remains relatively stable, ultimately resulting in a so-called "shift" in subset composition with the majority of Gr1^{+}CD11b^{+} cells being monocytic in their nature. As discussed before, granulocytic MDSC outnumber monocytic MDSC in numerous mouse tumor models and our data show them to be the major players of immunosuppression in tumor-bearing mice. In addition, when tumor-bearing mice were treated with CpG, poly(I:C) and to a lesser extent with 3pRNA, a strong reduction of PMN-MDSC accompanied by a significant increase of MO-MDSC could be observed, resulting in a proportion of MDSC subsets observed in naïve mice. This shift was almost completely abrogated in IFNα-deficient mice and nor could we observe it in tumor-bearing mice treated with LPS as it lacks potent type I IFN induction *in vivo* without prior priming [Richez et al., 2009].

As hypothesis, this may be a reflection of the effects of IFNα on (1) the induction of monocyte differentiation and proliferation [Paquette et al., 1998; Svane et al., 2006] and (2) the negative influence of type I interferons on myelo-/granulopoiesis [Koren and Fleischmann, 1993; Paquette et al., 1998] thus shutting down the pool of subsequent granulocytic MDSC from the bone marrow. That hypothesis is further strengthened by our observation that the decrease of PMN-MDSC in tumor bearing mice is equally observed in bone marrow. In addition, PMN-MDSC are not capable of proliferating and have a substantial shorter lifespan than MO-MDSC [Condamine et al., 2014].

Interestingly, work by the group of Dimitry Gabrilovich recently assessed one of the underlying mechanisms of PMN-MDSC abundance in tumor-bearing hosts [Youn et al., 2013]. By performing detailed fate-mapping experiments in tumor-bearing mice, their results suggest that in cancer, a large proportion of MO-MDSC can convert into PMN-MDSC instead of differentiating into macrophages and dendritic cells. This process was tightly regulated by epigenetic transcriptional silencing of the retinoblastoma gene (RB). Our data, illustrating a decrease in PMN-MDSC with a relative increase in more mature and differentiated MO-MDSC, suggests that systemic activation of distinct TLRs might overcome this fate decision of pathologic myeloid differentiation in cancer. Of note, induction of the RB protein by Interferon-α has been described more than 20 years ago, although it was shown in human Burkitt lymphoma cells rather than in the myeloid compartment [Kumar and Atlas, 1992].

If the individual proportion of PMN-MDSC to MO-MDSC displays a prognostic or even predictive value upon therapy remains elusive as well as a potential interplay within MDSC subsets as no studies exist that have investigated the effect of granulocytic MDSC on monocytic MDSC and vice versa. If at all, the significance of our finding may only be conferred to tumor models in which PMN-MDSC display the major immunosuppressive subset as well.

4.2 Myeloid-derived suppressor cells and pattern recognition receptor ligands

4.2.1 Expansion and activation of MDSC by Toll-like receptor ligands

Myeloid-derived suppressor cells accumulate under certain pathological conditions in bone marrow, blood, peripheral lymphoid organs, in case of malignancy at the tumor site itself or even in premalignant lesions [Tu et al., 2008]. Whether the stimulus for MDSC expansion concurrently triggers their activation to gain fully immunosuppressive MDSC remains questionable. In fact, research in the last years suggests a so-called "two-signal-model" proposing that MDSC expansion and activation is mediated by several different factors with molecules driving MDSC accumulation and other factors involved in direct activation of MDSC [Gabrilovich and Nagaraj, 2009; Condamine and Gabrilovich, 2011].

The significance of Toll-like receptor activation and other pattern recognition receptors in MDSC activation has not been fully elucidated yet. If MDSC expanded by innate immune stimulation acquire immunosuppressive properties remains largely elusive. In a model of polymicrobial sepsis, Delano and colleagues demonstrated that MDSC expansion is dependent on the TLR adaptor protein MyD88 but does not require TLR4, suggesting other PRRs/TLRs to be responsible for MDSC accumulation during systemic inflammation [Delano et al., 2007]. The above described "two-signal-model" with LPS as TLR4 agonist involved in the expansion of MDSC, has been nicely demonstrated by Greifenberg and colleagues. They showed that the combined treatment of mice with LPS and IFNγ activating the JAK/STAT axis enhanced immunosuppressive MDSC functions and impaired the capacity of $Gr1^{+}CD11b^{+}$ to mature into dendritic cells. Recently, membrane bound heat-shock protein 72 (Hsp72) expressed on tumor-derived exosomes has shown to be involved in triggering MDSC expansion in a STAT-3 and TLR2-dependent manner [Chalmin et al., 2010]. In fact, evidence for an activation of MDSC suppressivity has so far only been provided for TLR2 and TLR4, which may therefore hold a unique position among TLRs.

In 2008, with the use of a slow-delivery system, Vaknin and colleagues showed that chronic immune stimulation with TLR2, 3, 4, and TLR 9 ligands leads to systemic expansion of MDSC-like cells [Vaknin et al., 2008]. They proposed that TLR stimulation in this setting also delivers

the second signal required to activate MDSC suppressivity. Because TLR9 ligands are used in cancer vaccines, where MDSC activation would be deleterious for the clinical outcome, we investigated the effect of systemic TLR stimulation on accumulation and suppressive function of MDSC in naïve mice.

Indeed, our own data support the expansion of $Gr1^+CD11b^+$ immature myeloid cells via systemic activation of Toll-like receptors *in vivo*. More specific, TLR9 activation with CpG-ODN, TLR3 activation with poly(I:C), TLR7 activation with R848 (data not shown) and TLR4 activation with LPS resulted in a significant increase of iMC in naïve mice. This induction was systemic as it has been shown in different organs. We propose it is probably due to a higher proliferation rate and systemic induction of myelopoiesis, given that we also found a two- to threefold increase of iMC numbers in the bone marrow. This consequence upon TLR-stimulation is not overly surprising, as short term TLR stimulation mimics an acute infection of the host and triggers the mobilization of myeloid cells, in particular neutrophils and monocytes, from the bone marrow to infected tissue. This process in particular has been termed "emergency myelopoiesis" [Takizawa et al., 2012]. In addition, we could not see induction of myelopoiesis and immature myeloid cells upon RIG-I stimulation via 5'ppp-RNA *in vivo*. Early studies on the suppression of myelopoiesis by type I IFNs and work published in 2008 demonstrate a critical role of RIG-I by negatively regulating granulopoiesis, support this finding [Platanias et al., 1999; Zhang et al., 2008].

It is important to note that in the context of acute infection or a short immunogenic stimulus, an increase in the generation or recruitment of immature myeloid cells does not necessarily reflects an expansion of an immunosuppressive MDSC population. Albeit we observed a reproducible and significant increase in immature myeloid cells upon TLR stimulation, CpG- and poly(I:C)- expanded cells were not activated towards higher suppressivity. Thus, whereas expansion of immature myeloid cells in tumor-free mice can be triggered by several TLR ligands, activation of suppressivity may be limited to selected TLR ligands or the duration of immune stimulation. Especially in the case of endosomal TLRs such as TLR3, 7 and TLR9, no convincing study has yet shown the accumulation of immunosuppressive MDSC via induction and concurrent activation by the same TLR ligand.

4.2.2 Pattern recognition receptor expression on MDSC

The fact that myeloid-derived suppressor cells and immature myeloid cells can be induced by systemic activation of Toll-like receptors does not necessarily mean that MDSC are directly expanded by ligand-receptor activation. Several known factors and cytokines which have been shown to be involved in MDSC accumulation such as IL-1β, IL-6, IL-10, IL-12, IFNγ, TNFα and others are also effector molecules of innate immune stimulation by PRR ligands.

Besides a number of studies analyzing TLRs in the context of MDSC biology, Dectin-1 and the NLRP3 inflammasome as other innate immune receptors have been attributed to play a role in regulating the immunosuppressive function of MDSC [Li et al., 2010; Bruchard et al., 2013]. Regarding the function of Toll-like receptors on MDSC, by using transcriptomic analysis, Fridlender and colleagues could show an upregulation of Toll-like receptor signaling genes in granulocytic MDSC when compared to neutrophils in naïve mice [Fridlender et al., 2012]. Nevertheless, the expression Toll-like and other innate immune receptors on myeloid-derived suppressor cells has not been systematically assessed yet. In fact, only TLR2, TLR4 and TLR5 have been described to be expressed in MDSC [Bunt et al., 2006; Ostrand-Rosenberg and Sinha, 2009; Chalmin et al., 2010; Rieber et al., 2013].

By using rtPCR, the work of former doctoral students in our laboratory confirmed that MDSC express high levels of TLR4 [Zoglmeier et al., 2011]. On the contrary, when we stimulated isolated MDSC with CpG, we did not observe changes in their ability to suppress T cell proliferation nor a change in maturation marker expression, suggesting that CpG cannot directly activate MDSC. Likewise, TLR9 on MDSC was only expressed at minor levels when compared to plasmacytoid dendritic cells. This contrasts a recent work by Shirota and colleagues; they showed that monocytic MDSC express TLR9 and can be actively maturated into tumoricidal macrophages by direct TLR9 activation with CpG *in vitro*. Of interest, they also described TLR2, 3, 4, 7 and 8 expression by monocytic MDSC [Shirota et al., 2012]. By measuring Sca-1 expression levels on $Gr1^{+}CD11b^{+}$ cells following stimulation with a set of pattern recognition receptor ligands *in vitro*, we could show significant upregulation of Sca-1 by Pam3CysK, LPS, R848 and Dectin-1. Of course, that finding does not necessarily exclude the presence of other TLRs and PRRs on immature myeloid cells nor it addresses the issue of up- or downregulation of pattern recognition receptor ligands on MDSC in the course of disease, though it demonstrates that immature myeloid cells at least express functional TLR2, TLR4, TLR 7 and Dectin-1.

Taken together at least for the majority of TLRs it appears probable that MDSC induction is rather mediated by secondary effector molecules than a consequence of direct TLR stimulation on MDSC in an *in vivo* experimental setup. Nevertheless, more systematic studies are needed to clarify direct and indirect effects of pattern recognition receptor activation and their relevance for MDSC function in the context of disease.

4.3 MDSC in the context of cancer immunotherapy with PRR ligands

Current efforts bringing TLR agonists in the focus of cancer immunotherapy are mainly focused on TLR3, TLR7 and TLR9 [Krieg, 2007; Nicodemus et al., 2010, Hotz et al., 2012].

During the last years, our own group could demonstrate potent antitumoral activity for the TLR3/MDA-5 ligand poly(I:C), TLR7 ligand R848 and the TLR9 ligand CpG in murine tumor models [Bourquin et al., 2006; Bourquin et al., 2009; Bourquin et al., 2011].

MDSC strongly rely on the local NF-κB driven inflammatory milieu for their suppressive capabilities [Karin and Greten, 2005]. In addition, proinflammatory cytokines, in particular TNFα, IL-1 and IL-6, drive MDSC accumulation and further lead to NF-κB activation [Gabrilovich and Nagaraj, 2009]. On the other hand, NF-κB as a center molecule in TLR-signaling cascades and its activation by TLR ligands *in vivo* leads to the induction of many proinflammatory cytokines, most of which are also known for MDSC induction [Medzhitov, 2001; Gabrilovich and Nagaraj, 2009]. Thus, it may seem counterintuitive to use TLR and other innate immune receptor ligands to break MDSC induced tumor tolerance and immunosuppression. Indeed, we demonstrated that *in vivo* activation of TLRs with poly(I:C), R848 and CpG resulted in a potent induction of MDSC-like cells. But in contrast to recent findings by Vaknin and colleagues they lacked immunosuppressive properties [Vaknin et al., 2008].

Nevertheless, tumor formation alters the local and systemic cytokine-milieu, enabling MDSC accumulation as well as their attraction to the tumor-site. For a number of murine tumor models, we and others could demonstrate that MDSC numbers are already substantially higher than in naïve mice. For example, the use of a monoclonal antibody binding IL-6 has been shown to eliminate MDSC in mice and ovarian cancer patients [Coward et al., 2011; Sumida et al., 2012]. We hypothesize that innate immune sensing in tumor-bearing mice induces additional antitumoral cytokines (such as IFNα and IL-12 which will further discussed below) that were not present in the tumor-induced local and systemic cytokine profile and thus break MDSC-mediated immunosuppression. In that case, the additional induction of "bystander" cytokines such as IL6 or TNFα might be dispensable.

4.3.1 MDSC as a target of cancer immunotherapy with CpG

Specifically in mice bearing subcutaneous CT26 tumors we could previously demonstrate that activation of the innate immune system with the TLR9 ligand CpG evokes efficient antitumor immune responses, despite the presence of large numbers of highly immunosuppressive MDSC in this model [Gallina et al., 2006; Youn et al., 2008]. By showing that systemic therapy with CpG, and to a lesser extent with poly(I:C), cannot further increase immunosuppressive MDSC populations and mitigates their T-cell suppressive activity in line with initiating their maturation, we demonstrate that immune modulators such as CpG and poly(I:C) are capable

of converting the immunosuppressive tumor microenvironment and are able to overcome MDSC-mediated immunosuppression.

Our findings are complemented by a recent study of Shirota and co-workers, demonstrating that intratumoral injection of CpG oligonucleotides results in differentiation of monocytic MDSC in line with a decrease of their immunosuppressive properties [Shirota et al., 2012]. We show that CpG induced maturation and especially the loss of suppressivity is most pronounced on the $Ly6G^{high}$ polymorphonuclear subset of MDSC, that displays the dominant population associated with immunosuppression in our murine tumor models. Instead, Shirota and colleagues show monocytic MDSC as the main target population of TLR9 activation. One explanation for these divergent results may be different isolation techniques as well as differences in the experimental setup regarding the analysis of MDSC subsets versus the entire MDSC population. For *in vitro* experiments we used the whole population of $Gr1^+CD11b^+$ magnetically sorted MDSC, whereas Shirota and colleagues used FACS-sorted monocytic MDSC. Of note, by analyzing PMN-MDSC they could only detect minor expression of TLR9 and failed to elicit further maturation of PMN-MDSC by direct stimulation with CpG *in vitro*. Given that we assessed the maturation potential and suppressive activity in the total population of MDSC *in vitro*, a possible effect on monocytic MDSC may be superimposed by PMN-MDSC that make up to 90% of $Gr1^+CD11b^+$ cells in CT26 tumor-bearing mice.

Although we did not find direct effects of CpG on tumor-associated MDSC [Zoglmeier et al., 2011], another group described a direct effect of TLR agonists affecting influenza-associated MDSC differentiation [De Santo et al., 2008]. In their work, the TLR3 agonist poly(I:C), TLR7/8 agonist R848 and the TLR9 agonist CpG, were all individually able to attenuate the suppressive capacity of isolated MDSC on T cells. Interestingly, this could be further augmented by co-incubation with invariant NKT (iNKT) cells, thereby maximizing IL-12 production. Notably, whereas we used splenic MDSC, De Santo and colleagues used bone marrow-derived MDSC by culturing bone marrow with GM-CSF followed by depletion of $CD11c^+$ cells and positive selection via $Gr1^+$ purification. If these cells represent tumor-associated MDSC, that have been "*in vivo* – generated" by a variety of tumor-derived factors, remains questionable. Besides, an oncolytic adenovirus engineered for increased TLR9 stimulation in a xenograft model of lung cancer has recently achieved similar TLR9-dependent, MDSC-modulating and T-cell immunity-stimulating results. Furthermore, in mice bearing orthotopic renal cell carcinomas, CpG was used as an adjuvant for a TRAIL-encoding recombinant adenovirus, CpG modulated phenotype and function of MDSC and enabled a more vigorous antitumoral response [Cerullo et al., 2012; James et al., 2014].

Despite ambiguities regarding a direct or indirect effect of TLR9 activation on MDSC in tumor-bearing mice, our data as well as work by others clearly support the role of systemic TLR9 activation in order to overcome MDSC mediated immunosuppression.

4.3.2 MDSC as a target of cancer immunotherapy with poly(I:C)

Our data also demonstrate comparable results for the TLR3/MDA5 ligand poly(I:C) in maturation, differentiation and attenuation of MDSC-mediated immune suppression upon *in vivo* therapy of tumor-bearing mice. In addition, poly(I:C) treatment induced maturation of intratumoral MDSC. Due to methodical difficulties regarding the purification of intratumoral MDSC we could not confirm a lack of suppressivity as it has been demonstrated for splenic MDSC upon poly(I:C) treatment. Data on the effect of TLR3 activation on myeloid-derived suppressor cells are even more seldom than with TLR9 activation. A study by Liu and colleagues in 2011 showed that in addition to GM-CSF and IL-4, poly(I:C) can induce bone marrow precursors to differentiate into cells with MDSC-like properties [Liu et al., 2011]. However, the acquisition of these results is restricted to *in vitro* studies and lacks validation in a clinically relevant setting *in vivo*. In contrast, our data are further strengthened by unpublished data presented by Forghani and Waller at the annual ASH meeting in 2014; in a 4T1 murine breast cancer model they show a poly(I:C)-induced polarization of MDSC into M1 macrophages. As proof of principle, by performing *in vitro* studies they demonstrate antigen-presenting properties of splenic MDSC when stimulated with poly(I:C). In addition, they also observe a significant reduction in tumor-infiltrating MDSC upon TLR3 activation *in vivo*. This could not be validated by our data – despite intratumoral MDSC differentiation we did not see a reduction in B16 melanoma-infiltrating MDSC upon poly(I:C) treatment which might be explained by different murine tumor models used.

4.3.3 MDSC as a target of cancer immunotherapy with 5′-triphosphate-RNA

The innate cytosolic immune receptor RIG-I has recently gained interest as a target for tumor immunotherapy. Importantly, in contrast to TLRs, RIG-I is expressed in all nucleated cells in the body, including tumor cells. By sensing double-stranded RNA structures it serves as a sensor of viral infection of the host cell, inducing apoptosis accompanied by a pronounced type I IFN induction in immune, non-immune and tumor cells [Yoneyama et al., 2004; Kawai et al., 2005]. The hallmark activating structure of RIG-I, a short and blunt-ended 5′-triphosphate dsRNA (3pRNA), has been used *in vivo* in a humanized mouse model of melanoma where it induced high amounts of type I interferons and IL-12, thereby mediating an effective antitumor immunity [Poeck et al., 2008].

Previous work in our laboratory showed that *in vivo* treatment of tumor-bearing mice with 3pRNA is able to overcome MDSC-mediated immunosuppression on T cells (data unpublished). When we identified IFNα as key player for Toll-like receptor induced maturation and differentiation of MDSC, we were interested whether 3pRNA may be equally able to induce maturation and differentiation of MDSC *in vivo*. By using bifunctional siRNA with RIG-I triggering and silencing of TGFβ (which will further be discussed below) (Ellermeier et al., 2013], we could indeed see maturation of MDSC by introducing a triphosphate group at the 5' end of siRNA. In addition, we observed that ppp-TGFβ-siRNA reduced the frequency of $Gr1^+CD11b^+$ MDSC more significantly that TGF-blockage alone. That finding could not be demonstrated for other IFN-inducing TLR ligands *in vivo*. Surprisingly, we also found that the RIG-I triggering part of the bifunctional siRNA, was able to induce apoptosis in PMN- and MO-MDSC. Interestingly, by downregulating the Fas-signaling pathway, MDSC have been shown to have a higher apoptotic resistance than their non-inflammatory counterparts [Ostrand-Rosenberg et al., 2012]. Only few studies addressed the induction of apoptosis in myeloid-derived suppressor cells. Cimetidine has been shown to suppress lung tumor growth in mice through the induction of apoptosis in MDSC and the anthracycline doxorubicine has been shown to deplete MDSC by mediating their apoptosis in a ROS-dependent fashion [Zheng et al., 2013].

We thus demonstrate a potential novel antitumoral mechanism of 3pRNA by inducing maturation as well as apoptosis in tumor-associated myeloid-derived suppressor cells. Our data on the IFNα-dependent differentiation of MDSC upon TLR activation support the hypothesis that RIG-I induced maturation of MDSC is as well caused by the strong type I IFN induction upon 3pRNA therapy *in vivo*. Whether the induction of apoptosis is caused by direct RIG-I activation in $Gr1^+CD11b^+$ cells or a secondary result via Interferon-dependent apoptotic pathways remains to be determined in future studies.

4.4 Maturation of myeloid-derived suppressor cells

Myelopoiesis is a tightly regulated hierarchical process of cell lineage commitment. This process is altered in cancer, skewing myeloid differentiation to the expansion and activation of myeloid-derived suppressor cells. Most importantly, a hallmark feature of MDSC is their immaturity as they lack specific markers expressed by mature monocytes, macrophages or dendritic cells. Early studies showed that only a minor portion of tumor-induced MDSC have the ability to form myeloid-cell colonies *in vitro* and about one third can differentiate into major macrophages and dendritic cells upon appropriate cytokine stimulation *in vitro* and *in vivo* [Bronte et al., 2000; Kusmartsev and Gabrilovich, 2002; Li et al., 2004]. It is assumed that MO-MDSC and PMN-MDSC develop along different pathways involving

monocyte/macrophage and granulocyte progenitors, respectively. Increasing evidence additionally suggests, that even this process of tightly controlled myelopoiesis in naïve mice, is altered and disturbed in tumor-bearing hosts with MO-MDSC preferentially differentiating into PMN-MDSC, thus resembling an even more complex system that might be targeted for therapeutical benefit [Youn et al., 2013].

Maturation of myeloid cells involves the upregulation of known differentiation markers for macrophages and dendritic cells. F4/80 (with its human homologue EMR1) is a typical macrophage differentiation marker in mice [Austyn and Gordon, 1981]. CD11c serves as a marker for myeloid differentiation towards dendritic cells in mice and human, although it has been shown to be expressed by neutrophils, macrophages and some B cells as well [Corbi and Lopez-Rodriguez, 1997]. Ly6C is differentially expressed on activated circulating monocytes [Gordon and Taylor, 2005]. Some other maturation markers used to phenotypically assess the maturation status of MDSC in this study were the co-stimulatory molecules CD80 and CD86, that are both indispensable for an effective T cell priming [Acuto and Michel, 2003], MHC II as an antigen-presenting molecule and indicator of immature to mature dendritic cell conversion [Shin et al., 2006] and Stem cell antigen-1 which will further be discussed below.

Indeed, when we compared immature myeloid cells and tumor-induced myeloid-derived suppressor cells, these and other markers assessed for evaluating the maturation status were significantly downregulated on tumor-associated myeloid cells, indicating a tumor-induced differentiation block and its measurability by these surface molecules. In contrast, here we show for the first time, that systemic activation of distinct Toll-like receptors (TLR3/9) and RIG-I in tumor-bearing mice is able to overcome altered features of myelopoiesis by reversing the population shift as well as promoting differentiation and maturation of MDSC into antigen-presenting and functionally non-immunosuppressive cells. Notably, we observed a comparable upregulation of CD11c, CD80, Ly6C and to a lesser extent F4/80 on intratumoral MDSC following systemic TLR activation. This may seem counterproductive as Ly6C^{high} monocytes have been suggested to represent tumor-associated macrophage precursors may tipping the balance back towards tumor promotion [Movahedi et al., 2010]. However, our data regarding loss of suppressivity and the practical outcome of efficient tumor regression demonstrate that microenvironmental changes are maintained in favor of antitumor immunity. Supporting evidence comes from more recent studies, showing that the emergence of Ly6C^{high} inflammatory myeloid cells in a murine model of colon cancer, using cyclophosphamide/anti-CD25-mAb and IL-12 as chemoimmunotherapy, coincides with the emergence of effector T cells into the tumor microenvironment [Medina-Echeverz et al., 2011]. In addition, the group around Laurence Zitvogel discovered intratumoral

$CD11b^{+}CD11c^{+}Ly6G^{high}$ myeloid cells, which displayed dendritic cells and granulomonocytic precursors as crucial mediators in anthracycline-induced immunogenic cell death and priming of antitumoral T cells [Ma et al., 2013].

Not only MO-MDSC, with further capabilities of differentiating into macrophages and dendritic cells, but also PMN-MDSC express co-stimulatory molecules such as CD80 and CD86 [Movahedi et al., 2008; Talmadge et al., 2013]. Interestingly, we observed an upregulation of the dendritic cell differentiation marker CD11c as well as the co-stimulatory molecules CD80, CD86 and MHC class II on PMN-MDSC following TLR stimulation *in vivo*. Especially maturation of PMN-MDSC was associated with decreased immunosuppressive properties, as $Ly6G^{high}$ cells from non-treated tumor-bearing mice represented the major suppressors of T cell stimulation in our tumor models. This may seem counterintuitive, because PMN-MDSC as granulocytic cells are principally thought to be terminally differentiated [Gabrilovich and Nagaraj, 2009; Condamine et al., 2014]. Accordingly, F4/80 expression was not altered indicating that redifferentiation into monocytic cells, as it has been demonstrated vice versa [Youn et al., 2013], was not occurring following systemic TLR stimulation. Based on phenotypical assessment of PMN-MDSC, alongside with abolishment of their immunosuppressive function exerted on T cells, our results reinforce the notion that neutrophils (and in that case granulocytic MDSC) can acquire antigen-presenting cell (APC) - like properties. In accordance with this, neutrophils have shown to act as antigen-presenting cells via co-expression of MHC II and upregulate CD80 and CD86 in the presence of appropriate cytokines and bystander cells [Radsak et al., 2000; Ashtekar and Saha, 2003]. In the presence of GM-CSF, immature and mature neutrophils can differentiate into a "hybrid" population exhibiting phenotypical und functional features of both neutrophils and dendritic cells [Matsushima et al., 2013]. Thus, upregulation of co-stimulatory molecules on PMN-MDSC and their interaction with corresponding ligands on T cells may impart a loss of immunosuppressive properties.

Of course, more work is needed to distinguish between "immature" and "mature" PMN-MDSC in tumor-bearing hosts and to further dissect antigen-presenting properties of IFNα- or TLR ligand-stimulated PMN-MDSC in an appropriate *in vivo* setting. Currently there is no reliable marker such as morphologic appearance or flow cytometry analysis to distinguish PMN-MDSC from their natural counterparts. As naïve neutrophils and PMN-MDSC share distinct morphological features, PMN-MDSC might just represent a different functional state, rather than a separately occurring cell subset. Here we show, besides significant maturation of MO-MDSC, that at least a part of tumor-associated PMN-MDSC further differentiates into granulocytes with an antigen-presenting phenotype following *in vivo* stimulation with type I

IFN inducing pattern recognition receptor ligands. Thus, we propose a new mechanism by which CpG, and other type I IFN-inducing TLR ligands, exert their anti-tumoral effect *in vivo*.

4.4.1 Interferon-α as key effector molecule in differentiation and maturation of MDSC upon stimulation of pattern recoginition receptor ligands

As inhibition of MDSC suppressivity and maturation by stimulation with CpG could not be observed *in vitro* [Zoglmeier et al., 2011], we tried to identify factors that might be crucial for the seen effects. A hallmark of stimulation through TLR3 and TLR9 is, however, the induction of high levels of IFNα [Uematsu and Akira, 2007; Matsumoto and Seya, 2008]. In addition, 3pRNA that is known for its potent induction of type I Interferons results in differentiation and maturation of MDSC when administered to tumor-bearing mice as well. Indeed, the positive effects on MDSC maturation and subset recomposition by poly(I:C) could not be observed in IFNαR$^{-/-}$ mice. When we compared B16-melanoma induced MDSC in wild type and IFNαR$^{-/-}$ mice, MDSC already expressed lower baseline levels of common maturation markers such as CD80, CD86 and MHC II, thus indicating that endogenous Interferon-α may serve as a baseline stimulus for MDSC maturation and differentiation. Indeed, one of the myriad of immunological functions of IFNα is the activation of dendritic cells with upregulation of MHC proteins and co-stimulatory molecules such as CD80 and CD86 as well as their maturation from monocytes [Santini et al., 2000; Baccala et al., 2005]. Interestingly, IFNα has been reported to synergistically cooperate with all-trans retinoic acid to induce maturation and differentiation of promyelocytic blasts in acute promyelocytic leukemia [Gallagher et al. 1987; Nason-Burchenal et al., 1996]. Our observations are complementary to findings by Jablonska and colleagues that showed endogenous Interferon-β as major negative regulator of GR1^{+}CD11b^{+} granulocytic MDSC driven tumor angiogenesis. Considering that Interferon-β and Interferon-α as type I Interferons bind to the very same receptor (IFNαR), we would expect comparable results following a potent Interferon-α induction. In adoptive transfer experiments, granulocytic MDSC deficient for the IFNα-receptor dramatically increased tumor growth in contrast to PMN-MDSC from wild type mice. Simultaneously, this proves the expression of a functional IFNαR on Gr1^{+}CD11b^{+} and thus matches our results showing that co-incubation of purified MDSC with IFNα significantly upregulates Sca-1 as an interferon-response gene [Bamezai et al., 1995; Pietras et al., 2014]. Although Jablonska and colleagues did not analyze the maturation status of L6G^{high} MDSC, nor did we examine tumor angiogenesis, their results strengthen our data as a proof of principle [Jablonska et al., 2010].

Further validation of interferon-induced maturation of MDSC was gained by other students in our laboratory demonstrating that stimulation of isolated MDSC with recombinant IFNα

induces a significant upregulation of F4/80. Accordingly, short-term treatment of tumor-bearing mice with IFNα significantly reduced MDSC-mediated immune suppression accompanied by their differentiation [Zoglmeier et al., 2011]. We could further identify plasmacytoid dendritic cells as the major source of Interferon-α upon stimulation of TLR9 with CpG [Siegal et al., 1999], closing the gap between TLR9, type I interferons and maturation of myeloid-derived suppressor cells *in vivo*.

Several biological functions of type I IFNs, including their modulating function of innate and adaptive immunity as well as their proapoptotic and antiangiogenic effects, make them an attractive molecule in anti-cancer immunity. Indeed, type I IFNs have been used with success in a number of human cancers, including hematological malignancies and solid tumors [Ferrantini et al., 2007; Moschos and Kirkwood, 2007]. However, the mechanism of its antitumor effect remains largely elusive. Interesting results come from the group of Robert Schreiber, identifying IFNα/β as critical components in cancer *immunoediting* [Dunn et al., 2005]. Surprisingly, not tumor cells, but hematopoietic and in particular IFNα/β-responsive NK cells were the main targets of type I IFNs and sufficient for mediating tumor rejection.

A recent study revealed interferon-regulatory-factor-8 (IRF-8) as a critical transcriptional component in the control of tumor-induced MDSC development [Waight et al., 2013]. IRF-8 enhancement ameliorated granulocytic MDSC accumulation and slightly increased monocytic MDSC in IRF8-transgenic mice which was accompanied by a significant reduction of tumor-size. Notably, the same effects were notified when tumor-bearing mice were injected with CpG, poly(I:C) or Interferon-α. In addition, several tumor-induced factors inhibited IRF-8 expression leading to expansion of granulocytic and, to a lesser extent, monocytic MDSC. IRF-8 also seems to act as a positive regulator of IFN signaling in the murine system. In addition, $IRF8^{-/-}$ mice display a general defect in pro-inflammatory cytokine induction and upregulation of co-stimulatory molecules such as MHCII and CD40 in splenic DCs stimulated with CpG [Tsujimura et al., 2002; Tsujimura et al., 2003]. Since IRF-8 is tightly involved in the TLR- and interferon response of the host, these findings provide an interesting cross-point between our results and possible mechanistic actions of TLR stimulation and IFNα treatment on MDSC maturation and subset shifting *in vivo*.

STAT3 has been generally accepted as one of the main transcription factors that regulate expansion and activation of MDSC [Gabrilovich and Nagaraj, 2009; Rebe et al., 2013; Vasquez-Dunddel et al., 2013] and has been recognized as a negative regulator of type I IFN responses *in vivo* [Kirkwood et al., 1999; Vasquez-Dunddel et al., 2013]. In turn, systemic Interferon-α treatment leads to STAT3-inactivation, which has been demonstrated in melanoma precursor lesions. Interestingly, a number of studies has shown that MDSC perturb

endogenous anti-tumor immunity effects of IFNα by downregulation of IFNα-induced STAT1 phosphorylation in effector immune cells. This has been demonstrated in mice as well as in human malignancies [Mundy-Bosse et al., 2011; Mundy-Bosse et al., 2011]. Notably, the ability of MDSC to interfere with IFN responses, suggests the importance that IFN-α might have in converting their immunosuppressive phenotype and function.

Taken together, our findings suggest that IFNα plays a major role in mediating the functional and phenotypic changes in MDSC following TLR activation. Here we describe a new potential antitumor-mechanism of type I IFNs and TLR3/9-mediated IFNα-induction, namely by maturating and differentiating myeloid-derived suppressor cells hence mitigating their immunosuppressive properties. Regarding the underlying mechanism, definitely further studies are needed to dissect associated signaling pathways that may be involved.

4.4.2 MDSC maturation is not due only to IFN-α

Like IFNα, the cytokine IL-12 can potently induce MDSC differentiation and promote tumor immunity. IL-12 triggers a programmatic change in MDSC maturing them into antigen-presenting cells as displayed by upregulation of costimulatory markers such as CD80, CD86 and differentiation markers F4/80 and MHC-II [Steding et al., 2011] and forces them to support rather than suppress proliferation of $CD8^+$ cytotoxic T lymphocytes [Kerkar et al., 2011]. The immunostimulatory effect of IL-12 is further enhanced by a MDSC-reducing combinatory treatment with oxaliplatin in a murine model of liver metastasis [Hernandez-Alcoceba and Berraondo, 2012]. When used together with cyclophosphamide, IL-12 even shows the ability of converting MDSC into immunostimulating myeloid cells that facilitate tumor rejection [Medina-Echeverz et al., 2011].

TGFβ is an essential mediator of immunosuppressive effects within the tumor microenvironment, allowing tumors to usurp its homeostatic effect for promoting tumor growth, invasion and metastasis [Massague, 2008]. As a naturally occurring suppressor of the immune system, tumors alienate its function to facilitate their immune escape. A number of studies has tightly linked TGFβ as a crucial cytokine involved in MDSC-mediated immunosuppression. For instance, depletion of $GR1^+CD11b^+$ cells in a murine mammary model of breast cancer, diminishes the antitumor effect of TGFβ neutralization [Li et al., 2012]. Fridlender and colleagues showed that tumor-associated inflammation polarized granulocytic MDSC from an anti-tumoral G1 to a pro-tumoral G2 phenotype. By inhibiting the TGFβ-receptor with a kinase inhibitor, SM16, they could reprogram PMN-MDSC from G2 to G1 [Fridlender et al., 2009]. Accordingly, anti-TGFβ antibody can drive a shift from the M2 to M1 monocytic/macrophage phenotype in myeloid cells [Gong et al., 2012]. Moreover, in

murine colon adenocarcinoma and murine glioma, tumor-infiltrating MDSC are the main producers of TGFβ within the tumor microenvironment [Umemura et al., 2008].

When we found IFNα as the main driver of MDSC maturation in TLR3 and TLR9 stimulated hosts, we were interested whether this is an exclusive mechanism for IFNα or might also be achieved by targeting factors detrimentally involved in MDSC-mediated immune evasion of tumors, as it has been shown for TGFβ. Indeed, siRNA-mediated knockdown of TGFβ resulted in differentiation of $GR1^+CD11b^+$ MDSC *in vivo*, though the effect was not as significant as seen with additional RIG-I activation and subsequent type I IFN induction [Ellermeier et al., 2013]. Interestingly, TGFβ gene silencing led to a significant decrease in circulating MDSC and resulted in a comparable shift in MDSC subset composition as seen after treatment of tumor-bearing mice with CpG or poly(I:C), respectively. In accordance with our findings, TGFβ partially exerts its pro-tumoral activity by preventing DC maturation within the tumor-microenvironment, thus limiting an efficient $CD8^+$ T cell response [Flavell et al., 2010]. Although Interferon-α plays a crucial role in mediating maturation of MDSC upon pharmacological therapy with distinct TLR ligands, overcoming the tumor-induced differentiation block of myeloid cells can also be achieved by targeting other cytokines such as TGFβ.

4.5 Stem cell antigen-1 (Sca-1) as a possible marker for MDSC maturation

4.5.1 General aspects of Sca-1

The Ly6 protein family member Sca-1 (Ly6A/E), has been originally identified on activated lymphocytes [Yutoku et al., 1974]. Since then, for more than 20 years it has been widely used as a marker for hematopoietic stem cells (HSCs) and their enrichment *ex vivo* [Spangrude et al., 1988]. Its expression during hematologic maturation is regulated in a complex fashion: When HSCs further differentiate into myeloid/lymphoid progenitors, Sca-1 expression is downregulated [Akashi et al., 2000], becoming upregulated in colony-forming unit progenitors, mature thymocytes and peripheral T cells again [Bamezai et al., 1995]. Outside the hematopoietic system, Sca-1 is similarly expressed by stem- and progenitor cells as well as differentiated cell types in a variety of tissues and organs as well as on some murine tumors including retinoblastoma, mammary gland tumors and prostate cancer [Seigel et al., 2005; Xin et al., 2005; Batts et al., 2011].

Nonetheless, little is known about its biological function. Recent studies suggest that it might be more than just a convenient marker for enrichment of adult murine HSCs [Holmes and Stanford, 2007]. Interestingly, Sca-1 is a glycosyl phosphatidylinositol–anchored protein (GPI-AP) and thus lacks an individual cell signaling pathway upon ligand-receptor interaction.

In addition, no specific Sca-1 ligand could be identified yet. As GPI-APs are localized to lipid rafts of the plasma membrane, Sca-1 rather seems to affect signaling pathways of other cell surface receptors [Stefanova et al., 1991; Lingwood and Simons; 2010]. Indeed, Upadhyay and colleagues could demonstrate that Sca-1 attenuates GDF10-dependent TGFβ signaling by disrupting TGFβ-receptor heterodimerization [Upadhyay et al., 2011]. In addition, Sca-1 seems to be involved in myeloblast/cardiomyogenic differentiation as mice lacking Stem cell antigen-1 display mild defects in self-renewal of hematopoietic and stem cell progenitor cells [Ito et al., 2003; Mitchell et al., 2005]. Notably, Sca-1 has been linked to tumorigenesis; in the prostate, selective AKT overexpression in Sca-1^{+} cells initiates tumorigenesis and cancer progression correlated with increased Sca-1-positivity. Similarly, repression of Sca-1 in mammary gland tumor affected proliferation and migration of malignant cells and high Sca-1 levels in tumor cells were significantly associated with a more aggressive phenotype [Katz et al., 1994; Xin et al., 2005; Batts et al., 2011]. However, none of the studies depicted above has evaluated the role of Sca-1 on tumor-associated bystander cells or systematically analyzed its function on tumorigenesis in an appropriate *in vivo* model.

4.5.2 Sca-1 induction via innate immune receptor activation

We demonstrated that Sca-1 is highly upregulated on Gr1^{+}CD11b^{+} myeloid-derived suppressor cells following innate immune stimulation with different Toll-like receptor ligands. However, whereas LPS, R848, Dectin-1, Curdlan as well as IFNα significantly induced Sca-1 expression *in vitro*, CpG and poly(I:C) did not. In contrast, the most significant Sca-1 induction *in vivo* was seen following treatment with poly(I:C), CpG and to a lesser extent 3pRNA. Notably, upregulation of Sca-1 upon TLR3 activation with poly(I:C) *in vivo* was abolished in IFNαR$^{-/-}$ mice, suggesting a pivotal role for type I Interferons in Sca-1 induction. Indeed, early work and some newer studies suggested Sca-1 as an Interferon-response gene [Ma et al., 2001; Pietras et al., 2014]. Moreover, in a widely acclaimed work by Essers and colleagues in 2009, they not only confirm our results of impaired Sca-1 induction following poly(I:C) treatment in IFNαR$^{-/-}$ mice, but also demonstrate that IFNα-induced proliferation of HSCs is also dependent on Sca-1 itself [Essers et al., 2009]. However, our results and work by other groups support the notion that Sca-1 can also be induced by other cytokines and innate immune receptors as shown for LPS and *E. coli* bacteremia [Shi et al., 2013].

4.5.3 Sca-1 as a possible differentiation marker on myeloid-derived suppressor cells

Throughout our experiments, we observed a highly significant upregulation of Stem cell antigen-1 (Sca-1) on myeloid-derived suppressor cells following innate immune activation with TLR and RIG-I ligands in naïve as well as tumor bearing mice. Sca-1 is known to be

highly expressed on HSCs as highly undifferentiated cells and immaturity is a hallmark of MDSCs, this may seem counterintuitive. But given the complexity of Sca-1 up- and downregulation during hematopoietic ontology, branding Sca-1 as an overall marker for undifferentiated cells may be oversimplified. One could also hypothesize, that this upregulation may just be a result of IFN-induced gene expression on target cells, including Sca-1 with no further use in drawing conclusions from phenotype to function.

Nevertheless, we hypothesize Sca-1 can serve as a highly sensitive marker for MDSC maturation. First and foremost, tumor-associated MDSC express significantly lower levels of Sca-1 than their naïve, non-immunosuppressive counterparts. This finding is further supported by Movahedi and colleagues that could not detect measurable Sca-1 expression on tumor-induced untreated mice [Movahedi et al., 2008]. Second, upon TLR stimulation, the strength of Sca-1 expression was inversely correlated with their immunosuppressive activity on T cells. Third, Sca-1 expression also correlated to the specific "homing-site" - whereas immature myeloid cells in bone marrow and blood showed minor levels of Sca-1, its expression was significantly upregulated during homing into peripheral organs and lymph nodes, where undifferentiated myeloid cells will be without use. In line with this, recent work by a German group showed that Sca-1 expression on plasmacytoid dendritic cells defines their developmental stage as Sca-1$^-$ pDC are mainly found in the bone marrow and represent an early developmental stage, whereas Sca-1$^+$ pDC are mainly located in secondary lymphoid organs and show higher MHC class II expression [Niederquell et al., 2013]. Accordingly, when we performed detailed flow cytometry analysis, Sca-1$^+$ MDSC had significantly higher expression levels of other maturation/differentiation marker such as CD11c, F4/80, CD80 and CD86 when compared to Sca-1$^-$ MDSC. Finally, knock-down of TGFβ in PancO2 tumor-bearing mice by siRNA, also led to upregulation of maturation and differentiation marker, including Sca-1. Although we did not test for MDSC suppressive activity in this experimental setting, given the crucial role for TGFβ in mediating immune suppression via MDSC (as discussed previously), we would expect a loss of their immunosuppressive potential as it has been demonstrated previously [Fridlender et al., 2009].

Although we propose Sca-1 as a new marker for less immunosuppressive and more maturated MDSC, it does not necessarily reflect an anti-tumoral role of Sca-1 in tumor-bearing mice. Moreover, when we challenged Sca-1$^{-/-}$ and wildtype mice with syngenic PancO2 tumor cells, we unexpectedly found that myeloid-derived suppressor cells deficient for Sca-1 exhibit less immunosuppressive properties in conjunction with a trend towards slower tumor growth *in vivo*. Thus, in addition to prior studies that have demonstrated a detrimental role for Sca-1 expression on tumor cells, we find that Sca-1 and seem to foster the capability of myeloid-derived suppressor cells to suppress T cell proliferation and might attenuate immune

surveillance of malignant tumors. Unravelling the underlying mechanism will be work of future projects and should include bone-marrow chimeras in order elucidate relevant *in vivo* effects of tumor-associated and tumor-infiltrating myeloid cells.

When increasing evidence also suggested a role for the involvement of Sca-1 into distinct signaling processes, as it has been suggested for TGFβ and IFNα, respectively [Essers et al., 2009; Fridlender et al., 2009; Upadhyay et al., 2011] we aimed to further dissect functional differences between Sca-$1^{-/-}$ and wildtype mice treated with the TLR3 ligand poly(I:C). Incidentally, we detected high levels of IP-10/CXCL10 in Sca-$1^{-/-}$ mice upon therapy with poly(I:C), whereas other cytokine levels, especially Interferon α, remained stable. Comparable results were observed *in vitro* with a pronounced IP-10 induction following stimulation of bone marrow-derived macrophages with various PRR ligands. IFNγ-inducible protein of 10 kDa or IP-10/CXCL10 is an early response gene, induced by type I and type II interferons, LPS or CpG-ODN, attracts effector T lymphocytes to infectious sites and serologically serves as a proxy for a TH_1 skewed immune response [Neville et al., 1997; Dufour et al., 2002]. Sca-1 obviously dampens IP-10 induction *in vivo*. Our *in vitro* studies suggests that this effect is mediated on cellular basis. These results are hard to interpret as there is scarce literature on how Sca-1 might be involved in cell signaling pathways and nothing is yet published about a relationship of Sca-1 and IP-10/CXCL10 [Holmes and Stanford, 2007]. Given the anitumoral properties of IP-10 [Narvaiza et al., 2000], this pronounced IP-10 response might explain why tumor formation in Sca-$1^{-/-}$ might be slower than in wild type mice. Interestingly, MDSC have shown to significantly affect IP-10/CXCL10 mediated tumor infiltration of CTLs [Fujita et al., 2011]. However, these results have to be carefully interpreted and validated (1) for their reproducibility in other tumor models and mouse strains other than C57/BL6 and (2) for their biological significance.

4.6 MDSC as therapeutic targets in cancer

Since MDSC are still poorly defined and the knowledge of their molecular mechanisms of action is still in its infancy, they are difficult to target. Significantly, only two clinical trial have launched yet that selectively target myeloid-derived suppressor cells in cancer patients, albeit various approaches that have undertaken in murine tumor models and cancer patients aiming to boost the host's immune response by targeting MDSC [Greten et al., 2011]. Our findings in this work provide further evidence for the concept that overcoming the maturity block of MDSC is a highly promising approach to abrogate MDSC-related immunosuppression and to improve the success of cancer vaccines.

Apart from multiple strategies that target MDSC-associated immunosuppression in cancer including depletion, inactivation and inhibition of their development, a major strategy pursued by cancer immunologists is initiating MDSC differentiation. Besides individual molecules/cytokines that have been studied in preclinical murine studies, a number of commercially available drugs including all-trans retinoic acid (ATRA), 25-hydroxy-vitamin D (D3) and 5-Aza-2'-deoxycytidin (Vidaza®) lead to maturation and hence less-immunosuppressive MDSC in human [Lathers et al., 2004; Mirza et al., 2006; Daurkin et al., 2010]. ATRA, that showed groundbreaking results in facilitating the differentiation of leukemic promyelocytes in acute promyelocytic leukemia, has already entered randomized phase II trials that employ ATRA to modulate MDSC (NCT0060179, NCT00618891). Interestingly, in some studies murine as well as human MDSC morphologically matched promyelocytes [Greten et al., 2011; Youn et al., 2013]. Vitamin D3 has also shown efficacy in human studies by increasing HLA-DR expression on PBMC and decreased $CD34^+$ circulating myeloid cells in patients with HNSCC [Wiers et al., 2000]. Tyrosine-kinase inhibitors used for the therapy of a variety of human cancers have also shown to decrease the number of circulating MDSC and to block their T cell suppressive activity. Sunitinib, used for the treatment of renal cell carcinoma, leads to a decline of $CD33^+HLA\text{-}DR^-$ and $CD15^+CD14^-$ MDSC paralleled by an increase in IFNγ-release by T cells stimulated with anti-CD3 antibodies *ex vivo* [Rodriguez et al., 2009]. In addition, Vemurafenib, a selective blocker of the serine/threonine-protein kinase B-Raf decreases both PMN- and MO-MDSC as well as reverses their suppressing effect on autologous T cell proliferation in patients with malignant melanoma [Schilling et al., 2013]. Even conventional chemotherapy drugs such as doxorubicin, gemcitabine and 5-FU reduced the number of circulating MDSC in both murine tumor models and cancer patients [Le et al., 2009; Vincent et al., 2010; Alizadeh et al., 2014]. Some compounds that are already FDA approved (e.g. ATRA, PDE5 inhibitors, COX-2 inhibitors and bisphosphonates) will likely be the first to enter late phase clinical trials to test their ability to suppress MDSC and improve the efficacy of other immunactivating agents. However, the precise mechanisms leading to abnormal differentiation of myeloid progenitor cells into MDSC remain largely elusive and will provide a goal and more tailored pharmacological starting point for future studies. Table 4.1 features some selected available strategies and interventions targeting MDSC in murine and human cancer.

Therapeutic agent	**Effect on MDSCs (mouse / human)**	**Reference**
ATRA	Differentiation of immature myeloid cells to mature leukocytes, partially via ROS neutralization (m/h)	[Mirza et al., 2006; Nefedova et al., 2007]
5-Aza-2'-deoxycytidin (Vidaza®)	Differentiation into APCs (m)	[Daurkin et al., 2010]
Vitamin D3	Differentiation and inhibition of expansion (m/h)	[Lathers et al., 2004; Wiers et al., 2000]

Interleukin-12	Differentiation and reduction of tumor-infiltration (m)	[Steding et al., 2011; Kerkar et al., 2011]
Paclitaxel	Differentiation in small doses, inhibition of immunosuppression in higher doses (m/h)	[Naiditch et al., 2011; Sevko et al., 2013]
Sunitinib	Inhibition of proliferation, STAT-3 inhibition, reduction of tumor-infiltration, induction of apoptosis? (m/h)	[Rodriguez et al., 2009; Ko et al., 2009; Xin et al., 2009]
N-Bisphosphonates	Inhibition of expansion, decreased immunosuppression via prenylation of MMP9 (m)	[Heissig et al., 2002; Melani et al., 2007]
STAT-3 inhibitors	Differentiation, reduction of MDSC numbers, inhibition of apoptosis, proliferative and pro-angiogenic gene expression (m/h)	[Nefedova et al., 2005; Lu et al., 2012]
Doxorubicin	Reduction of MDSC numbers (m/h)	[Alizadeh et al., 2014; Diaz-Montero et al., 2009]
Gemcitabine	Reduction of MDSC numbers by apoptosis, direct mitigation of T cell suppression (m/h)	[Le et al., 2009; Suzuki et al., 2005]
5-Fluoruracil	Reduction of MDSC numbers by apoptosis (m)	[Vincent et al., 2010]
PDE-5 inhibitors	Downregulation of IL4Rα and reduced immunosuppressive function (m)	[Serafini et al., 2008]
COX-inhibitors	Inhibition of iNOS and Arg2 (m)	[Sinha et al., 2007; Rodriguez et al., 2005]
CXCR2/4 antagonists	Altered recruitment to the tumor site (m/h)	[Porvasnik et al., 2009]
PPARγ inhibitor	Induces proliferation of MDSC (m)	[Wu et al., 2012]
Very small size proteo-liposomes	Differentiation of MDSC into mature APCs, changes in MDSC subset distribution (m)	[Fernandez et al., 2014]

Table 4.1. Selected pharmacological agents targeting MDSC in murine and human tumors. Adapted from [Gabrilovich et al., 2007; Greten et al., 2011; Talmadge et al., 2013; Wesolowski et al., 2013].

Although MDSC have gained increasing interest by cancer immunologists and oncologists, their biological significance in other disease has also been recognized. They are intimately involved in regulating immune responses during viral, bacterial and parasitic infections, sepsis and trauma. Notably, given their immunosuppressive nature, they do not exclusively exert detrimental effects on the host but possibly bear a distinct therapeutic potential under certain pathological conditions for which dampening the immune response will be of benefit. Indeed, a number of studies implicated beneficial effects of MDSC in preclinical models of multiple sclerosis, inflammatory bowel disease, systemic lupus erythematosus and described their occurrence and prognostic impact in human autoimmune disease [Drujont et al., 2014]. Furthermore, recent work using therapeutic administration of MDSC in mouse models of multiple sclerosis, rheumatoid arthritis and type I diabetes has shown promising results in delayed onset or reduction of disease severity [Chou et al., 2012; Ioannou et al., 2012; Fujii et al., 2013].

Interestingly, endosomal TLRs such as TLR3, 7 and 9 as well as type I IFNs have been closely connected to the pathogenesis of both systemic and organ-specific autoimmune disease. [Baccala et al., 2007; Theofilopoulos, 2012]. Congruent findings implicate the innate immune system as culprit for subsequent auto-antibody and T cell-mediated tissue damage of the host. Via recognition of self-antigens, especially when sensed by nucleic acid-sensing TLRs, elicit a strong production of IFNα. Moreover, IFNα in lupus patients promoted the maturation of monocytes to efficient antigen-presenting cells [Blanco et al., 2001]. Although previous studies did not analyze the occurrence and significance of MDSC in these patients and experimental models of autoimmunity, based on our data it is tempting to speculate that TLR- and type I IFN-elicited maturation of MDSC may play a disease-perpetuating and -promoting role. Future studies will provide additional insights regarding a potential interplay between MDSC, Toll-like receptors and type I IFNs in autoimmune diseases. Nevertheless, our findings emphasize the role of MDSC as crucial disease modulators and the promotion of their maturation as a promising goal in order to restore *immunosurveillance* of malignant tumors. Likewise, our work underlines the potential of distinct Toll-like receptor ligands such as CpG and poly(I:C) in cancer immunotherapy.

4.7 Summary

Neoplastic growth triggers the induction of highly immunosuppressive myeloid cells that have been termed myeloid-derived suppressor cells (MDSC). MDSC disrupt local and systemic *immunosurveillance*, enable and sustain malignant growth and represent a major obstacle for effective cancer immunotherapy. A hallmark of MDSC is their immaturity due to a tumor-induced differentiation block reflecting the abnormal nature of myelopoiesis in cancer. Restoring a disrupted myelopoiesis and blocking MDSC-related immunosuppression in cancer patients might attenuate disease progression and open the path for more effective immunotherapy protocols. Existing immunotherapy protocols use Toll-like receptor ligands such as the TLR3 ligand poly(I:C) or the TLR9 ligand CpG-DNA. However, some studies suggest a pivotal role of Toll-like receptor activation in the expansion and activation of MDSC, calling the clinical utility of such approaches into question.

Using flow cytometry and *ex vivo* T-cell suppression assays, in the first part of this study we could demonstrate that *in vivo* treatment of tumor-free mice with CpG or poly(I:C) indeed causes systemic accumulation of immature myeloid cells, but these cells lack immunosuppressive features. In tumor-bearing mice, we show that PMN-MDSC are the most abundant MDSC subset in nearly all tumor models examined and exhibit significantly stronger immunosuppressive properties than MO-MDSC. Moreover, *in vivo* treatment with CpG and poly(I:C) leads to a significant reduction of PMN-MDSC, reverses the paramalignant differentiation block and reestablishes the subset composition to a proportion seen in naïve mice, thus enabling a more vigorous anti-tumor immune response. Using IFNαR$^{-/-}$ mice, we could further identify IFNα as a key effector molecule upon stimulation with TLR ligands *in vivo*. In addition, by performing siRNA experiments *in vivo,* we could demonstrate that TGFβ might be another detrimental cytokine rendering MDSC in an immature immunosuppressive state. Finally, we show that stem cell antigen-1 (Sca-1) serves as a highly sensitive maturation marker on MDSC, its upregulation significantly correlating with maturation and differentiation status and inversely correlating with their suppressive activity as measured by their capacity to suppress T cell proliferation *ex vivo*.

Taken together, our findings provide further evidence for the concept that targeting the disrupted myelopoiesis during tumorigenesis and overcoming the maturation block of MDSC is a highly promising approach in cancer immunotherapy. Here we describe a new potential antitumor-mechanism of type I IFN and of TLR3- and TLR9-mediated IFNα-induction, namely by maturating and differentiating myeloid-derived suppressor cells hence mitigating their immunosuppressive properties. Thus, our work further underlines the potential of distinct Toll-like receptor ligands such as CpG and poly(I:C) in cancer immunotherapy.

4.8 Zusammenfassung

Die Unterdrückung der immunologischen Tumorabwehr wird wesentlich durch die Induktion sogenannter myeloider Suppressorzellen (MDSC) bedingt. Durch Umgehung der Immunüberwachung des Wirtes fördern MDSC nicht nur progredientes malignes Wachstum und die Entstehung einer metastasierenden Erkrankung, sondern stellen auch ein wesentliches Hindernis für effektive Immuntherapien dar. Bedingt durch einen tumor-induzierten Differenzierungsblock, ist die hämatologische Unreife ein zentrales Merkmal myeloider Suppressorzellen. Die Wiederherstellung einer gestörten Myelopoese mit konsekutiver Aufhebung der MDSC-assoziierten Immunsuppression bietet daher einen attraktiven Ansatz für effektivere Immuntherapien. Ein Element experimenteller Tumorimmuntherapien ist der Einsatz von *Toll-like* Rezeptor-Liganden wie dem TLR3-Ligand poly(I:C) oder dem TLR9-Ligand CpG-DNA. Einige Studien schreiben *Toll-like* Rezeptoren jedoch eine zentrale Rolle in der Aktivierung und Expansion myeloider Suppressorzellen zu, was deren klinischen Nutzen in Frage stellt.

Im ersten Teil dieser Arbeit konnten wir mittels Durchflusszytometrie und *ex vivo* T-Zell Suppressions-Assays zeigen, dass eine CpG- und poly(I:C)-vermittelte *Toll-like*-Rezeptor-Aktivierung in tumorfreien Mäusen zu einer Akkumulation von myeloiden Vorläuferzellen führt, diesen jedoch immunsuppressive Eigenschaften fehlen. Weiterhin stellten tumor-assoziierte PMN-MDSC nicht nur zahlenmäßig die dominierende Subpopulation dar, sondern wiesen auch eine signifikant höhere antiproliferative Wirkung auf T-Zellen als MO-MDSC auf. Die *in vivo* Therapie tumortragender Mäuse mit CpG oder poly(I:C) führte sowohl zur signifikanten Reduktion von PMN-MDSC, als auch zur Aufhebung des paramalignen Differenzierungsblockes. Mittels IFNαR$^{-/-}$ Mäusen, ließ sich Interferon-α als ein Schlüsselmolekül der *Toll-like* Rezeptor-induzierten Maturierung von MDSC identifizieren. Eine entgegengesetzte Rolle konnten wir durch siRNA-basierte *in vivo* Experimente TGF-β als differenzierungsblockierendem Molekül myeloider Zellen zuschreiben. Zuletzt konnten wir zeigen, dass *stem cell antigen-1* (Sca-1) einen hochsensitiven Differenzierungsmarker für die Ausdifferenzierung myeloider Suppressorzellen darstellt, dessen durchflusszytometrisches Expressionslevel hochsignifikant mit dem Maturierungsstatus von MDSC, sowie invers mit deren antiproliferativer Wirkung auf T-Zellen korreliert.

Zusammengefasst unterstützen die Ergebnisse dieser Arbeit, dass die Wiederherstellung einer normalen Hämatopoese und das Überwinden des Differenzierungsblockes myeloider Suppressorzellen einen vielversprechenden Ansatz in der Tumorimmuntherapie darstellen. Weiterhin unterstreichen unsere präklinischen Daten den potentiellen Nutzen einer TLR3- und TLR9-Liganden vermittelten IFNα-Induktion zur Überwindung einer tumorassoziierten Immunsuppression sowie deren klinisches Potenzial.

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6 APPENDIX

6.1 Abbreviations

3p	Triphosphate
3pRNA	5′-triphosphate 2.2 dsRNA
5-FU	5-Fluoruracil

A

AIM2	Absent in melanoma 2
AKT	Protein kinase B
AP-1	Activated protein 1
APC	Allophycocyanin
APC	Antigen-presenting cell
Arg1	Arginase 1
ASH	American society of hematology
ATRA	All-trans retinoic acid

B

B7-H1	B7 homolog 1
BCG	Bacillus Calmette-Guerin
Bcl-2	B-cell lymphoma 2 protein
BiTE	Bi-specific T-cell engager
BMDM	Bone marrow-derived macrophages
BrdU	5-bromo-2′-deoxyuridine
B_{reg}	Regulatory B cell

C

C5a	Complement factor 5a
CARD	Caspase recruitment domain
Cardif	CARD adapter inducing IFN-β
CCL	Chemokine C-C motif ligand
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDNA	Copy-desoxyribonucleic acid
CLR	C-type lectin receptor
COX-2	Cyclooxygenase-2
CpG	Oligonucleotide with cytosine-(phosphate)-guanine motifs
CTL	Cytotoxic T lymphocyte

CTLA4	Cytotoxic T-lymphocyte antigen 4
CXCL10	C-X-C motif chemokine 10

D

DAMP	Damage-associated molecular pattern
DMEM	Dulbecco's modified Eagle´s medium
DNA	Desoxyribonucleid acid
DOTAP	Dioleoyl trimethylammonium propane
dsRNA	Double-stranded RNA
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay

F

F4/80	Macrophage-differentiation marker
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isocyanate
FLICA	Fluorochrome-labeled inhibitor of caspases
Foxp3	Forkhead box p3
FSC	Forward scatter

G

GM-SCF	Granulocyte/macrophage colony-stimulating factor
GPI-AP	Glycosyl phosphatidylinositol–anchored protein
Gr-1	Granulocyte-differentiation antigen-1

H

H_2SO_4	Sulfuric acid
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HMGB1	High-Mobility-Group-Protein B1
HMW	High molecular weight
HNSCC	Head and neck squamous cell carcinoma
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
HSP72	Heat-shock protein 72

I

ICD	Immunogenic cell death

ICI	Immune checkpoint inhibitor
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFNαR	Interferon α receptor
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1β	Interleukin-1β
iMC	Immature myeloid cell
iNOS	Inducible nitric oxide synthase
IP10	Interferon gamma-induced protein 10
IPS-1	Interferon promoter stimulator 1
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
IRF-8	Interferon regulatory factor 8
IκB	NFκB inhibitor

J

JAK2	Janus kinase 2

K

k.o.	Knockout
kDa	Kilodalton

L

LCM	L929-conditioned medium
LGP2	Laboratory of genetics and physiology 2
LMW	Low molecular weight
LPS	Lipopolysaccharide
LRR	Leucin-rich repeat
Ly6G/C	Lymphocyte antigen 6G/C

M

mAb	Monoclonal antibody
MACS	Magnetic Activated Cell Sorting
MAL	MyD88-adaptor-like
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial anti-viral signaling
MDA5	Melanoma differentiation-associated factor 5
MDSC	Myeloid-derived suppressor cell

MHC	Major histocompatibility complex
MMP9	Matrix metalloproteinase 9
MO-MDSC	Monocytic myeloid-derived suppressor cell
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88

N

NEMO	NFκB essential modulator
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NK cell	Natural killer cell
NLR	NOD-like receptor
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain

O

ODN	Oligodesoxyribonucleotide
$ONOO^-$	Hydrogen peroxide H2O2 and peroxynitrite

P

PacBlue	Pacific blue
PacOrange	Pacific orange
PAM3CSK4	Synthetic triacylated lipopetide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood monocytes
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PD-L1	Programmed cell death protein 1 ligand
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cyanine dye 7
PerCP	Peridinin chlorophyll protein
PGE	Prostaglandine E2
PI	Propidium iodine
PI3K	Phosphoinositide 3-kinase
PMN-MDSC	Polymorphonulcear myeloid-derived suppressor cell
poly dA:dT	Poly(deoxyadenylic-deoxythymidylic) acid
Poly(I:C)	Polyinosinic:polycytidylic acid
PPARγ	Peroxisome proliferator-activated receptor gamma
PRR	Pattern recognition receptor

PTO	Phosphorothioate

R

R848	Resiquimod
RAGE	Receptor for advanced glycation endproducts
Rb	Retinoblastoma
RIG-I	Retinoic acid inducible gene
RLR	RIG-I like receptor
rlu	relative light units
RNA	Ribonucleic acid
ROS	reactive oxygen species
rtPCR	Real-time PCR

S

s.c.	Subcutaneous
SARM	Sterile-alpha and Armadillo motif-containing protein
Sca-1	Stem cell antigen-1
SDF	Stem-cell derived factor
SEM	Standard error of the mean
siRNA	Small-interfering RNA
SSC	Sideward scatter
ssRNA	Single-strand RNA
STAT	Signal transducer and activator of transcription
SV40	Simian vacuolating virus 40

T

TAA	Tumor-associated antigen
TAM	Tumor-associated macrophage
TAN	Tumor-associated neutrophil
TAP-1	Transporter associated antigen 1
TBK 1	TANK-binding kinase 1
TCR	T cell receptor
tDC	Tolerogenic dendritic cell
TGF-β	Transforming growth factor beta
$T_H1/2$	T helper cell 1/2
TIR	Toll/IL-1 receptor
TIRAP	TIR-associated protein
TLR	Toll-like receptor

TMB	3,3′,5,5′ – tetramethylbenzidine
TNFα	Tumor necrosis factor α
trAb	Trifunctional bispecific antibody
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adapter molecule
T_{reg}	Regulatory T cell
TRIF	TIR-containing adapter inducing IFN-β

V

VEGF	Vascular endothelial growth factor
VISA	Virus-induced signaling adapter

6.2 Publications

6.2.1 Original publications

1. Zoglmeier, C., H. Bauer, **D. Noerenberg**, G. Wedekind, P. Bittner, N. Sandholzer, M. Rapp, D. Anz, S. Endres and C. Bourquin (2011). "CpG blocks immunosuppression by myeloid-derived suppressor cells in tumor-bearing mice." Clin Cancer Res 17(7): 1765-1775.

> Commented by: Lechner M, Epstein A. A new mechanism for blocking myeloid-derived suppressor cells by CpG. Clinical Cancer Research 2011 Apr 1;17(7):1645-8.

2. Bourquin, C., C. Hotz, **D. Noerenberg**, A. Voelkl, S. Heidegger, L. C. Roetzer, B. Storch, N. Sandholzer, C. Wurzenberger, D. Anz and S. Endres (2011). "Systemic cancer therapy with a small molecule agonist of toll-like receptor 7 can be improved by circumventing TLR tolerance." Cancer Res 71(15): 5123-5133.

3. Pinilla S, Weckbach LT, Alig SK, **Noerenberg D**, Singer K, Tiedt S (2013). „Blogging medical students: a qualitative analysis.“ GMS Z Med Ausbild 30(1):Doc9.

4. Ellermeier, J., J. Wei, P. Duewell, S. Hoves, M. R. Stieg, T. Adunka, **D. Noerenberg**, H. J. Anders, D. Mayr, H. Poeck, G. Hartmann, S. Endres and M. Schnurr (2013). "Therapeutic efficacy of bifunctional siRNA combining TGF-beta1 silencing with RIG-I activation in pancreatic cancer." Cancer Res 73(6): 1709-1720.

6.2.2 Book chapter

1. Trenkwalder C, **Nörenberg D**, Trenkwalder T. Aus- und Weiterbildung in der Neurologie. Aus- und Weiterbildung in der klinischen Medizin. Didaktik und Ausbildungskonzepte. M.G. Krukemeyer. Schattauer Verlag 2011.

6.2.3 Oral presentations

Nörenberg, D. CpG effectively blocks immunosuppressive activity of myeloid-derived suppressor cells. Förderprogramm für Forschung und Lehre (FöFoLe) Retreat, 2010, Herrsching, Germany.

Nörenberg, D. The impact of therapeutic oligonucleotides on myeloid-derived suppressor cells. 1st Autumn Schoool Current Concepts in Immunology, 2009, Bad Schandau, Germany.

6.2.4 Poster presentations

1. **Nörenberg, D.**, Wedekind G., Wurzenberger C., Sandholzer N., Endres S., Bourquin C., Zoglmeier C. The impact of innate immune activation on the distribution and phenotype of myeloid-derived suppressor cells. 3rd Tegernsee Conference on immunotherapy of Cancer, 2009, Starnberg, Germany.

2. **Nörenberg D.,** Bauer H., Wedekind G., Bittner P., Sandholzer N., Zimmermann W., Endres S., Bourquin C, Zoglmeier C. The impact of TLR ligands on the function and phenotype of myeloid-derived suppressor cell subpopulations. 8th World Congress on Trauma, Schock, Inflammation and Sepsis, TSIS 2010, Munich, Germany.

3. **Nörenberg D**, Zoglmeier C, Bauer H, Wedekind G, Bittner P, Sandholzer N, Endres S, Bourquin C. In vivo treatment of tumor-bearing mice with CpG reduces suppressivity and promotes maturation of myeloid-derived suppressor cells. World Immune Regulation Meeting IV, 2010, Davos, Switzerland.

4. **Nörenberg D**, Frick M, Cuoronné L, Lenze D, Baldus CD, Bastard C, Okosun J, Fitzgibbon J, Dörken B, Schmitt CA, Zenz T, Hummel M, Bernard OA, Damm F. NFKBIE mutations occur in 15% of GCB DLBCL and in various other lymphoid malignancies. 20th Congress European Hematology Association (EHA), 2015, Vienna, Austria.

6.3 Curriculum vitae

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Eidesstattliche Versicherung

Nörenberg, Daniel

Ich erkläre hiermit an Eides statt,
dass ich die vorliegende Dissertation mit dem Thema:

„The effect of innate immune activation on maturation of myeloid-derived suppressor cells“

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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Berlin, den 20.08.2016

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Daniel Nörenberg