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ENHANCEMENT OF TOLEROGENIC OR IMMUNOSTIMULATORY POTENTIAL OF DENDRITIC CELLS FOR THERAPEUTIC GOALS

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A thesis submitted to the Nanyang Technological University in partial fulfillment of the requirement for the degree of
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Summary

Dendritic cells are the heterogeneous and versatile regulators of immune system. They possess an inherent plasticity allowing them to either prime and boost the immune responses or induce immunological tolerance. They are the most potent APCs, specialized to capture, process and present antigens to T cells. Increased knowledge about their development and function will give us more opportunities to influence the immune system to our benefit. On one hand boosting of immunity towards multitude of infectious agents as well as against cancers is of high priority while on the other hand dampening or tolerizing the specific immune responses is highly desirable in many autoimmune diseases, clinical transplantations and in gene therapy settings.

The goal of this project was to engineer 'tailor made' DCs with either "highly tolerogenic" or "immunostimulatory" properties through genetic and cellular manipulations, which could be used as tools for cellular therapies. To do this, we exploit the recent findings that the forced expression of HOXB4 homeo domain containing protein fused to NUP98 (NUP98-HOXB4) in hematopietic stem cells and primitive progenitors increases their self-renewal capacity in culture. This system permits us to maintain and grow *in vitro* multipotential hematopoietic progenitors for extended periods and therefore, provides us a wide window for time consuming genetic manipulations and selection procedures like cell sorting. The manipulated progenitors and their normal counterparts were then differentiated to desired lineages of immature DCs or to their precursors for *in vivo* and *in vitro* studies.

For generation of tolerogenic DCs we exploited the inhibitory potentials of some members of B7 family of co-regulatory molecules, including PD-L1, PD-L2 and B7-H4 and also HVEM a member of TNFR superfamily. BM cells immortalized by NUP98-HOXB4 were transduced with inhibitory molecules and differentiated to DCs for further studies. We also utilized our system for sequential transduction of hematopoietic stem cells with different combinations of two inhibitory molecules. DCs derived from these modified HSC progenitors showed robust overexpression of respective molecules *in vitro* and *in vivo*. Furthermore, DCs overexpressing inhibitory molecules PD-L1, PD-L2 or their combinations, didn't affect the T cell proliferation *in vitro*, but partially reduced the T cell proliferation, abrogated their cytokine (e.g. IFN-γ) production *in vivo* and rendered them more apoptotic. This manipulation led to

induction of T cell exhaustion by our modified DCs. DCs overexpressing HVEM were able to partially dampen the T cell proliferation *in vivo* while not affecting their cytokine production ability.

We also observed that PD-L2 overexpression (mainly by DCs and Macrophages), led to a dramatic depletion and decrease in B cell repertoire *in vivo* and high levels of PD-L2 expression on B lymphocytes rendered them highly apoptotic.

On the other hand, to achieve our second goal, "enhancement of immunostimulatory potential of DCs", we exploited the immunostimulatory capacity of the growth factor GM-CSF. GM-CSF is known for its multifunctional properties ranging from cellular differentiation, proliferation, and activation to inhibition of apoptosis and boosting inflammation. Our remarkable experimental observation was a potent up-regulation of IL-1 production (in particular IL-1β) by DCs and macrophages upon their treatment with exogenous GM-CSF in combination with TLR agonists, *in vitro*. IL-1β is a potent pyrogenic cytokine and a key modulator involved in the regulation of immune responses against a variety of microbes as well as several acute and chronic inflammatory disorders, such as autoimmunity and septic shock. The IL-1β production is tightly regulated by a three step process involving; (i) the priming of the pro-IL-1β, (ii) its processing into a mature active IL-1β and (iii) the subsequent release as a biological active form which is ultimately controlled by a multiprotein complex called inflammasome. Our findings reveled that GM-CSF boosts this process by hyper-activation of inflammasome in an NF-κB- dependent fashion. Through a series of experiments using mice deficient in GM-CSFR we also showed that GM-SCF tightly regulates the inflammasome activation *in vivo*, as absence of its signaling impairs this process, and renders mice resistant to LPS induced endotoxic shock and dampens the IL-1 production. Collectively, our findings suggest that GM-CSF could be a potential candidate to boost immunostimulatory capacity of DCs *in vitro* and *in vivo*. Those DCs could be used in different therapeutic approaches to strengthen and modulate the immune responses. The proposed role for GM-CSF in IL-1 production suggests that combining anti IL-β1 therapies with GM-CSF blockade would be of potential therapeutic benefits for auto-inflammatory disorders.

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List of Publications

1. Christiane Ruedl, **Hanif Javanmard Khameneh** and Klaus Karjalainen. 2008. Manipulation of immune system via immortal bone marrow stem cells. *International Immunology*. 20:1211-1218.

2. **Hanif Javanmard Khameneh**, Siti Aminah Bte Mohammad, Lin Min, Fam Wee Nih and Christiane Ruedl. 2011. GM-CSF signalling boosts dramatically IL- production. *PLoS ONE*. (In Press).

Abbreviations

Ab: Antibody

Ag: Antigen

APC: Antigen Presenting Cell

APC: Allophycocyanin

ASC: Apoptosis associated Speck-like protein Containing a caspase recruitment domain

ATP: Adenosine Triphosphate

BCR: B Cell Receptor

BMDC: Bone Marrow-Derived Dendritic Cell

BMDM: Bone Marrow-Derived Macrophage

BSA: Bovine Serum Albumin

BTLA: B and T lymphocyte Attenuator

CARD: Caspase Recruitment Domain

CD: Cluster of Differentiation

CFSE: 5-(and-6)-CarboxyFluorescein di-acetate Succinimidyl Ester

CRP: C Reactive Protein

CTL: Cytotoxic T-lymphocyte

CTLA-4: Cytotoxic T-lymphocyte Antigen-4

DAMPs: Danger Associated Molecular Patterns

DC: Dendritic Cell

DMSO: Dimethylsulfoxide

ELISA: enzyme-linked Immunosorbant assay

FACS: Fluorescent-Activated Cell Sorter

FCS: Fetal Calf Serum

FITC: Fluorescein Isothiocyanate

Flt3-L: Fms-like Tyrosine kinase 3- Ligand

GFP: Green Fluorescence Protein

GVHD: Garft-Versus-Host Disease

GM-CSF: Granulocyre-Macrophage Colony-stimulating Factor

GM-CSFR: Granulocyre-Macrophage Colony-stimulating Factor Receptor

HRP: Horse Raddish Peroxidase

HOXB4: HomeoBox 4

HSC: Hematopoietc Stem Cell

HVEM: Herpes Virus Entry Mediator

IFNγ: Interferon Gamma

Ig: Immunoglobulin

IL: Interleukine

i.p: Intarperitoneal

i.v: Intraveneous

IRES: Internal Ribosome Entry Site

ITIM: Immunoreceptor Ttyrosin-based Inhibitory Motif

ITSM: Immunoreceptor Ttyrosin-based Switch Motif

LN: Lymph Node

LPS: Lypopolysaccharide

mAb: Monoclonal Antibody

MAPK: Mitogen-Activated Protein Kinase

M-CSF: Macrophage Colony Stimulating Factor

MHC: Major Histocopmatibility Complex

MFI: Mean Fluorescence Intensity

mL: Milliliter

MSU: Mono-Sodium Urate

NF-κB: Nuclear Factor-kappa B

NLR: NOD-Like Receptor

NUP98: Nucleoporin 98

OVA: Ovalbumin

PAGE: Polyacrylamide Gel Electrophoresis

PAMPS: Pathogen-associated Molecular Patterns

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PD-1: Programmed Death 1

PD-L1: Programmed Death1-Ligand 1

PD-L2: Programmed Death1-Ligand 2

PDGF: Platelet Derived Growth Factor

PE: Phycoerythrin

RAG: Recombination Activation Gene

ROS: Reactive Oxygen Species

RT: Room Temperature

TCR: T Cell Receptor

TGF-β: Transforming Growth Factor β

Th1: T Helper 1

Th2: T Helper 2

TLR: Toll-Like Receptor

TNF-α: Tumor Necrosis Factor-Alpha

WT: Wild Type

1. Introduction

1.1 Dendritic cells, master regulators of immunity

Dendritic cells are the most potent and efficient antigen presenting cells (APCs) which were first described by Steinmann and Cohn in 1973 as large, stellate cells. They further initiated a series of experiments to reveal the functional characteristics of these cells [1-4].

DCs develop from hematopoietic stem cells through several steps of differentiation and can exist in two functional states, the immature and mature state. Only mature DCs can prime the immune response. They can sample antigen in the peripheral tissues and then migrate to regional lymphoid organs where naïve T cells can encounter the peripheral antigens and promote the immune response. Because of this, DCs have been described as "Natural Adjuvants" [5] which are able to initiate the primary immune response and boost the immune reactivity. DCs in non-lymphoid tissues neighboring mucosal surfaces are able to sample the environment and sense the danger signals from infectious agents like bacteria, parasites, etc. These cells are phenotypically immature and have the ability to capture antigen with different strategies (e.g. phagocytosis, endocytosis, macropinocytisis) mediated by interactions with a variety of cell surface receptors [6]. These receptors include immunoglobulin superfamily members, lectins, Fc receptors, heat shock proteins (HSP) and Toll-like receptors (TLRs) [7].

DCs represent a heterogeneous cell population virtually present in all tissues except central cornea and brain. Murine DCs can be divided to conventional (myeloid and lymphoid DCs), plasmacytoid DCs and inflammatory monocyte-derived DCs. Different DC subtypes show different patterns of localization, migration, expression of surface markers and functional characteristics.

The issue of origin of DCs has been controversial. It has been shown that both common lymphoid and common myeloid progenitors (CLP and CMP) in mouse and human have the ability to generate all DC subtypes [8-10]. Peripheral blood monocytes were recently been shown to have the capacity to fully differentiate to DC-SIGN/CD209^{+} DCs, upon microbial stimulation [11].

Upon stimulation by inflammatory signals, like PAMPs (pathogen-associated molecular patterns) or a variety of cytokines, DCs become activated and migrate through the afferent lymph or circulation into the T cell areas of secondary lymphoid organs where they present antigenic peptides in the context of MHC class I or II

complexes to naive $CD8^+$ and $CD4^+$ T cells, respectively, and thereby induce an efficient antigen-specific T cell-mediated immunity [5]. This switching process from antigen capturing and processing to antigen presentation is called “maturation” [5, 12]. Endogenous proteins are degraded into small peptide fragments within DCs and are presented to $CD8^+$ CTLs in context of MHC class I molecules whereas exogenous antigen will be presented to $CD4^+$ T cells in context of MHC class II molecules. DCs possess the unique capability to present the exogenous antigens to $CD8^+$ T cells in the context of MHC class I molecule, a process called “cross-presentation” [13]. T cell receptor recognizes the peptide/MHC complex and this event provides the signal I, required for T cells activation. TLR/PAMPS ligation (e.g. TLR4/LPS) [14], CD40 ligation [15, 16] and some inflammatory cytokines such as TNFα [17] are known to induce DC maturation. The phenotypic criterion of maturation includes high expression of co-stimulatory molecules CD40, CD80, CD83 and CD86 as well as adhesion molecules, and translocation of MHC class II molecules from lysosomal compartments to the plasma membrane [18-21]. DCs expressing co-stimulatory molecules like CD80 and CD86 interact with CD28 molecule on T cells and further provide the T cell with the co-stimulatory signal, which is necessary for T cell activation (Signal II) [22]. Furthermore, mature DCs will be able to conduct the Th1 or Th2 polarization of activated T cells [23, 24](**Figure 1- 1**)

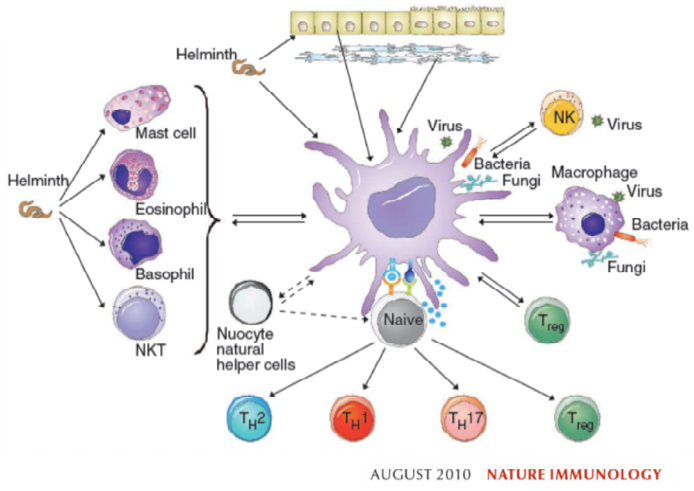

Figure 1- 1 Network of DC interaction. DC interaction with other cells of immune system or microorganisms bridges innate and adaptive immunity [25]. The Ag-specific DC:T cell interactions prime the naïve T cells towards different fates, depending on the tissue microenvironment, physiologic status of cells, signaling networks and presence of certain cytokines/soluble factors.

As mentioned, DCs can act as “cellular switches” to either promote the immune response and inflammation or to induce the immune tolerance depending on various cellular and molecular determinants, which help DC to make final decision in this regard.

Here we discuss the role of DCs in promotion of tolerance (section 1.1.1) and also in stimulation of inflammatory responses through production of IL-1 family of cytokines (section 1.1.2). In each section, we describe the theoretical background of the strategies we exploited for enhancement of tolerogenic or immunostimulatory potential of DCs *in vitro* and *in vivo*.

1.1.1 Immune Tolerance and role of DCs

The immune system protects the organism from pathogens, distinguishes self from non-self and prevents catastrophic and self-destructive immune responses through central and peripheral tolerance. Tolerance can be operationally defined by different mechanisms such as absence of antigen-specific autoimmunity, or the acceptance of an allograft, attributable of antigen accessibility (ignorance), absence of Ag-specific T cells (deletion) [26] or lack of sufficient activation and co-stimulatory signals (anergy or unresponsiveness) [27, 28] , suppression or regulation of T cell responses by T regulatory cells [29], or a combination of two or more of these mechanisms. The central tolerance mechanisms mediate deletion of self-reactive T cells and B cells to eliminate most of the potentially auto-reactive cells, but large number of self proteins are not sufficiently expressed in thymus to induce negative selection and deletion of the respective self-reactive T cells [30]. Hence, peripheral negative regulatory pathways are also playing a crucial role in suppressing potentially pathogenic or self-reactive lymphocytes that escape central tolerance [31, 32].

Despite having an important role in initiation and promoting the Ag-specific cell-mediated immunity, DCs play an important role in dampening or regulating the immune responses as well. DCs are involved in induction of both central and peripheral tolerance within the immune system. In context of central tolerance, thymic DCs together with thymic epithelial cells, have shown to mediate negative (but not positive) selection by deletion of MHC class I or II-restricted auto-reactive T cells [33-36]. These DCs are located exclusively within the thymus medulla and don’t seem to traffic [37].

Peripheral DCs play an important role in induction of peripheral tolerance to peripheral tissue antigens. It's been proposed that activation state of DC is relevant to the final decision for induction of tolerance or boosting the immune response [38]. Targeting of the protein antigen to the immature DCs via scavenger receptor CD-205 showed that these antigen-loaded immature DCs were able to tolerize $CD8^+$ Ag-specific T cells, while CD40-activated DCs didn't show such activity but instead activated the T cells [39-41]. Probst *et al.* also showed the Ag-specific tolerization of $CD8^+$ T cells by resting DCs (but not activated DCs), a process which showed to be mediated by synergistic effects of inhibitory molecules CTLA-4 and PD-1 [42, 43]. As another strategy to promote the peripheral tolerance DCs have been shown to induce the differentiation of regulatory T cells (either $CD4^+$ $Foxp3^+$ T_{regs} or IL-10 producing Tr1 cells), a process which is regulated by immunosuppressive cytokines IL-10, TGF β or retinoic-acid [44-49]. DCs have also been shown to be responsible for maintenance of T_{reg} cell homeostasis through cell-cell contacts (CD40 ligation) and production of IL-2 [50], which further emphasizes the role of DCs in induction and maintenance of tolerance to peripheral self-antigens. Several reports have documented the presence of tolerogenic antigen presenting DCs in mucosal tissues, which express CD103 and are able to induce regulatory T cells. These cells were reported to exist in small intestine lamina propria [47, 49] as well as lungs, while in skin dermis-derived $CD103^-CD11b^+$ counterparts can induce the T_{reg} generation [51]. Mechanical disruption of E-cadherin bonds between DCs was also shown to result in an alternate maturation pathway, which renders DCs tolerogenic, by affecting the β-catenin signaling pathway [52, 53], but the exact mechanism of this phenomenon has not been clearly understood yet.

1.1.1.1 Generation of Tolerogenic DCs, exploiting inhibitory potentials of B7 family members

BM-derived or *ex vivo* isolated DCs are not tolerogenic *in vitro*, hence several strategies have been used to make the DCs express a tolerogenic phenotype *in vitro*, and when transferred, induce donor-specific tolerance *in vivo* (**Figure 1- 2**). Immature DCs are good candidates for induction of tolerance but this doesn't mean that tolerogenic DCs should necessarily be immature, as these cells could be immature, maturation-resistant or alternatively activated DCs that express low amounts of MHC

molecules on surface and are defective in production of Th1-inducing cytokines (such as IL-12p70) [54]. Tolerogenic DCs are able to present the antigen to T cells but fail to provide the T cells with co-stimulatory signals (or signal via co-inhibitory molecules) which leads to T cell unresponsiveness to antigen (anergy), T cell death or the induction of regulatory T cells [54]. DCs have been exposed to (or manipulated to express) various immunosuppressive or anti-inflammatory molecules like IL-10, TGF-β, inducers of cyclic AMP (cAMP) such as Prostaglandin E_2, histamine, neuropeptides, β_2 agonists, the vitamin D_3 metabolite 1α,25-dihydroxy vitamin D_3 and its analogues, glucosamine, the antioxidant *N*-acetyl-L-cysteine, ligands for inhibitory immunoglobulin-like transcript receptors (such as MHC class Ib molecule HLA-G), and cobalt protoporphyrin (to induce hem oxygenase-1 expression) [54, 55] in order to render them tolerogenic.

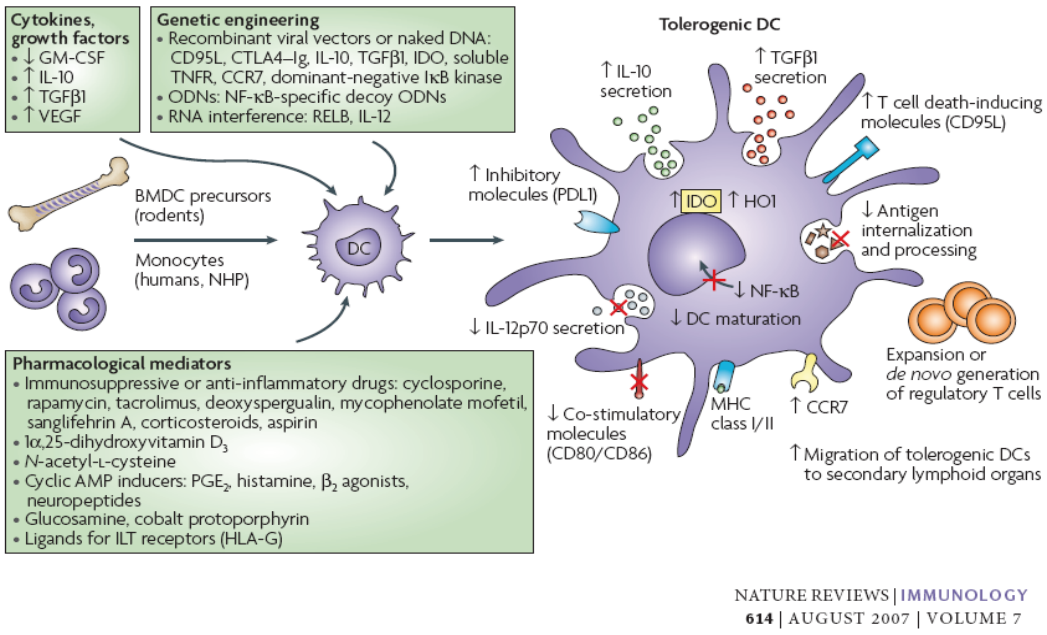

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Figure 1- 2 Strategies for generation of tolerogenic DCs in vitro.

One of the interesting approaches to generate tolerogenic DCs is to engineer them to deliver inhibitory signals to T cells. B7 family is the key player in regulation of TCR-mediated T cell responses, and its members (co-signaling molecules) determine the outcome of signaling events in the immunological synapse, which results in either immune-reactivity or immune tolerance.

B7 family consists of molecules, which play either co-inhibitory or co-stimulatory roles. Members of B7 family – CD80, CD86, ICOS-L, PD-L1, PD-L2 and B7-H4- share 21-27% amino acid identity and a structural organization that consists of a signal sequence, IgV- and IgC-like and transmembrane domains and a short cytoplasmic tail (**Figure 1- 3**) The most common interaction which provides the signal II for the T cell activation is the engagement of CD80 (B7.1) and CD86 (B7.2) on APCs by CD28 receptor on T cells [56]. CD80 can deliver co-inhibitory signal when engaged by another receptor on T cells, called cytotoxic T-lymphocyte antigen-4 (CTLA-4). This interaction down-regulates cytokine production and cell-cycle progression and eventually, inhibits T cell expansion and differentiation, which is a potent mechanism for induction of tolerance [57] (**Figure 1- 4**).

The other important signaling axis mediated by B7 family members is PD-1/PD-1 ligands pathway, which has been extensively studied for last couple of years. Engagement of PD-1 immunoreceptor by its ligands B7-H1 (PD-L1) or B7-DC (PD-L2) has been shown to switch on downstream signaling pathways which lead to inhibition of T cell activation and proliferation, thus playing an important role in switching off the auto-reactive T cells as well as in induction and maintenance of peripheral tolerance [58, 59]. B7-H4, a new member of B7 family and its putative unknown receptor, are also known to form another signaling axis which delivers co-inhibitory signals, to negatively regulate the T cell immunity [60]. The last two co-inhibitory pathways are further described in detail in following sections.

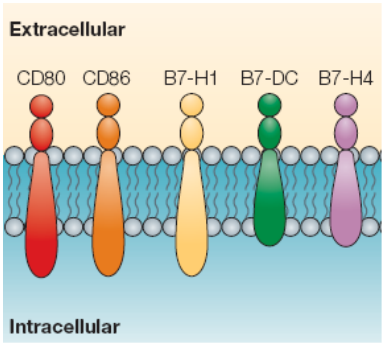

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Figure 1- 3 Structural organization of B7 family. Members of B7 family have an IgV-like domain, an Ig-c like domain, extracellular domain and a short cytoplasmic tail.

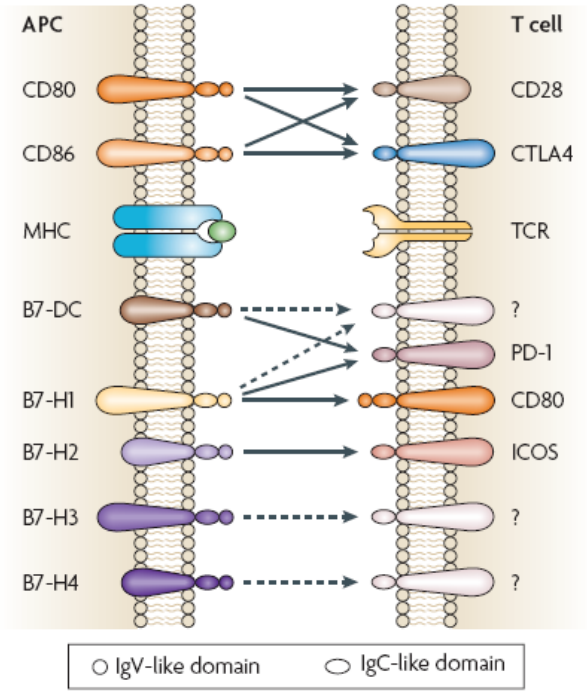

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Figure 1- 4 Co-regulatory molecular interaction between DCs and T cells. Co-inhibitory axis of B7 family includes CD80/CD86-CTLA4, PD-L1/PD-L2-PD-1 and B7-H4 putative receptor.

1.1.1.2 PD-1 and its ligands PD-L1 and PD-L2

PD-1 (CD279) is a co-inhibitory molecule of CD28 gene family which was first described by Ishida *et al.* [61] in an attempt to identify a gene that induces programmed cell death. This molecule is expressed on double negative αβ and γδ T cells in thymus and its expression is induced on T, B and myeloid cells upon activation[62, 63]. The structural analysis revealed that PD-1 exists on cell surface as well as in solution in a monomeric form [64]. It is a 50-55-kDa type I transmembrane glycoprotein consisting of a single immunoglobulin variable-like (IgV) domain in the extracellular region and two tyrosin-based signaling motifs in cytoplasmic domain; the immunoreceptor tyrosin-based inhibitory motif (ITIM) and immunoreceptor tyrosin-based switch motif (ITSM) [65, 66]. These motifs are able to recruit the *src* homology 2-domain-containing tyrosine phosphatase 2 (SHP2) after ligand engagement and inhibit TCR signaling [67]. Studies on a murine B-cell line expressing various mutants

of PD-1 showed that PD-1 exerts inhibitory role on BCR signaling via its ITSM motif but not ITIM [68]. PD-1 signaling has been shown to inhibit Akt phosphorylation by preventing CD28-mediated activation of phosphatidylinositol 3-kinase (PI3K) in B cells [69], regulate the germinal center formation and control the quantity and quality of long-lived plasma cells [70].

In case of T cells, PD-1 engagement leads to phosphorylation of ITSM and recruiting of both SHP-2 and SHP-1 which de-phosphorylate downstream effector molecules ZAP70 and CD3ζ [71]. Previous studies showed that PD-1 induces T cell tolerance in the absence of strong positive co-stimulation. Inhibition of PD-1 signaling suppressed the expression of Grail, Itch and Cbl and induced the expression of effector-specific transcription factors GATA3 and T-bet, resulting in augmented proliferation and secretion of effector cytokines [72]. PD-1 is also known to be required for induction of tolerance by resting dendritic cells [42]. It's also recently documented that PD-1 is capable of induction of $CD8^+$ T cell anergy *in vivo* through suppression of cell autonomous IL-2 production [73]. Mechanistically it's shown that PD-1 upregulation upon T cell activation is partly regulated via recruitment of NFATc1 to a novel regulatory element on *pdcd1* locus [74]. The T cell inhibition by PD-1 could be mediated by up-regulation of a basic leucine transcription factor, ATF-like (BATF) on $CD8^+$ T cells [75].

The functional significance of PD-1 was first highlighted by the autoimmune phenotype of $PD1^{-/-}$ mice ($pdcd1^{-/-}$). PD-1 deficient mice demonstrate multiple autoimmune features such as hyperactivation of immune system (e.g splenomegaly), glomerulonephritis (a lupus-like syndrome), arthritis and dilated cardiomyopathy depending on their MHC background [76-78]. Also several single-nucleotide polymorphisms (SNPs) in human PD-1 gene were shown to be associated with development of various autoimmune disorders [58].

PD-1 immunoreceptor has two known ligands; programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2). These ligands are type I transmembrane glycoproteins of Ig superfamily, which were identified by database searches according to their homologies with other members of B7 family [79-82]. Both of these ligands consist IgC- and IgV-type extracellular domains and short cytoplasmic tails (with no known motif for signal transduction) [79-82]. PD-1/PDLs interactions have been demonstrated to negatively regulate cytokine production and proliferation of both $CD4^+$ and $CD8^+$ T cells [79, 83-85].

PD-L1 (also known as B7-H1 or CD274) shows a broad expression pattern. It's expressed on resting B, T, myeloid and dendritic cells and upregulated upon activation. [79, 80, 86]. It's also expressed on a range of non-hematopoietic cells including microvascular endothelial cells and non-lymphoid organs including heart, lung, pancreas and muscle [86-88]. The expression of PD-L1 in immune-privileged sites such as placenta and the eye protects these sites from destructive immune responses [89-93]. Yamazaki *et al*. have reported up-regulation of PD-L1 expression on DCs by various stimuli including anti-CD40, IFNγ, IL-4, IL-12 and GM-CSF, on T cells with anti-CD3 antibody and on macrophages with anti-CD40 antibody, LPS, IFNγ and GM-CSF [94]. PD-1 engagement by PD-L1 on T cells has been shown to inhibit anti-CD3-mediated activation of human T cells [79]. The PD-L1 expression in Tolerogenic DCs was reported to be regulated in a MAPK/cytokine/STAT-3 manner, as blocking STAT-3 blocked the PD-L1 expression on these cells [95]. Recently, Senju *et al*. showed that forced-expression of PD-L1 on embryonic stem cell-derived DCs (ES-DC) down-modulates the proliferative response of co-cultured allogenic T cells via the interaction of PD-L1 with PD-1 on the T cells [96]. It's also reported that PD-L1 interactions by its receptor PD-1 can regulate the induction and maintenance of invariant NKT cell anergy [97] and induce the IL-10 production by monocytes [98]. The most striking recent discovery regarding PD-L1's mechanism of action was the report by Sharpe *et al* who showed that, PD-L1 is involved in development, maintenance and function of $Foxp3^+$ iT_{regs} [99]. This observation was further validated *in vivo* as PD-L1 and PD-L2 expressions on mesenteric lymph node DCs were shown to be essential for generation of Ag-specific T_{regs} [100].

In contrast to broad expression pattern of PD-L1, the expression of PD-L2 (also known as B7-DC or CD273) is restricted to macrophages and DCs and can be upregulated with IFNγ, GM-CSF and IL-4 [94]. Preliminary studies using dimeric Ig fusion proteins showed that PD-L2 has a two- to six-fold higher affinity to PD-1, comparing to PD-L1 [64]. Engagement of PD-1 by PD-L2 dramatically inhibits TCR-mediated proliferation and cytokine production by $CD4^+$ T cells. In this scenario, PD-1/PDLs interactions have shown to block the cell cycle progression by arresting cells in G_0/G_1, not by increasing the cell death [81]. APCs from PD-$L2^{-/-}$ mice are able to strongly activate T cells *in vitro* and upon oral immunization with ovalbumin, these mice exhibited increased activation of $CD4^+$ and CD8+ T cells *in vivo* compared to WT animals, suggesting the important role PD-L2 in negative regulation of T cell

responses as well as induction of oral tolerance [101]. PD-L2 has been shown to attenuate the Th2 T cell responses mostly by decreasing IL-13 and IgE levels but this effect was not mediated by PD-1 suggesting possible function of an still unknown receptor for PD-L2 [102].

Several groups have reported positive co-stimulatory effects of PD-L1 and PD-L2 on T cells [80, 82, 103-105]. It's not known whether these positive effects of PDLs are due to inhibition of negative signaling or there is another receptor rather than PD-1 for these ligands, which might exert co-stimulatory effects. A second unknown receptor for PDLs has been suggested [106], but at least a recent study of Steinberger *et al.* dismisses any evidence for dualism in receptors for PD-L2 on human T cells [107]. It's already shown that ITSM motif of PD-1 is capable of mediating co-inhibitory as well as co-stimulatory signals, depending on the presence of another adaptor molecule (SH2D1A). These opposing results mediated by PD-1 ligands suggest that maybe they have different signaling patterns in different experimental settings and also in various *in vivo* situations which leads to playing inhibitory roles in some scenarios and stimulatory roles in others. Outcome of these signaling network might depend on the activation status of T cells as well as the status of APCs as it seems that inhibitory effects of PD-L1 are mediated predominantly by resting or virus-treated DCs but not by activated DCs [42, 59, 108]. Also, the interfering signals from PD-L2 may abolish the inhibitory effects of PD-L1 upon engagement by PD-1. Different cytokines including common γ-chain family of cytokines (such as IL-2, IL-7, IL-15 and IL-21), were reported to be able to induce the expression of PD-1 immunoreceptor and its ligands [109].

Two ligands of PD-1 have also been reported to play distinct roles *in vivo*. They influence the auto-reactive T cells in murine experimental autoimmune encephalomyelitis (EAE) (mouse model of multiple sclerosis), in different fashions depending on genetic background of mice. For instance, in myelin oligodendrocyte glycoprotein (MOG)-induced EAE in C57BL/6 mice, blockade of PD-L2, but not PD-L1 significantly enhanced disease severity [110, 111]. In BALB/c mice, blocking PD-L1, but not PD-L2 significantly increased disease manifestations [111]. In 129Sv mice, both PD-L1 and PD-L2 blockade resulted in severe clinical EAE [101, 112]. These differential functions of PD-1 ligands could be originated from distinct expression and regulatory mechanisms. It's also possible that they might be selectively used or recruited to the immunological synapse in which a broad range of other

regulatory signals might influence their potency. In attempts to identify other binding partners of PD-L1, a recent findings suggest that it is able to bind to CD80 as well, exert it's inhibitory functions and induce T cell tolerance [113-115]. These finding to some extent were able to address some questions regarding the functions of PD-L1 shown not to be mediated via its interactions with other receptor PD-1. However these data should be interpreted carefully as the PD-L1/CD80 interactions have not been yet well documented in all physiological and pathologic conditions.

1.1.1.3 B7-H4, a new member of B7 family with negative regulatory effects

B7-H4 also known as B7-S1 and B7x is a B7-like molecule, which shows about 25% amino acid homology in the extracellular portion with other members of B7 family. The extracellular region of this molecule contains both IgV and IgC-like domains. It's not constitutively expressed on peripheral tissues but the expression could be induced on T cells, B cells, macrophages and DCs. Previous studies confirmed the presence of a putative receptor for B7-H4 on activated DCs and dismissed its binding to other receptors of B7-family like PD-1 or CTLA-4 [116]. It has been proposed that B and T lymphocyte attenuator (BTLA) could be the receptor for B7-H4 since Th1 cells from wild type mice bound to B7-H4 protein but no binding observed with cells from BTLA-deficient mice [117]. But a recent study showed BTLA doesn't directly bind to B7-H4 but may mediate the appearance of B7-H4 putative receptor on Th1 cell surface [118].

In vitro T cell assays and *in vivo* blockade experiments have indicated that B7-H4 inhibits $CD4^+$ and $CD8^+$ T-cell proliferation, cell cycle progression, cytokine production, and generation of CTLs, in an IL-2 dependent fashion [60, 116, 119]. Further studies indicated that B7-H4 exerts its inhibitory effects on T cells by cell cycle arrest, while induction of apoptosis is minimal, showing a similar mechanism of activity with PDLs [116].

Zou *et al.* showed that T regulatory cell–induced production of IL-10 by APCs stimulates B7-H4 expression on APCs and render them immunosuppressive and capable of negative regulation of T cell response [120].

1.1.1.4 HVEM, a versatile regulator of T cell responses

Herpes virus entry mediator, also known as TNF-receptor-like molecule 2 (TR2)[121], herpes virus entry mediator A (HveA) [122] another TNFR-associated factor-associated receptor (ATAR)[123] or LIGHT receptor (LIGHTR), was first described as a molecule which is required for HSV1 entry into Chinese hamster ovary (CHO) cells [124]. This process was shown later to be mediated by HVEM interactions with glycoproteins D and H (gD and gH) of HSV1, using HEp-2 cell line [124]. HVEM is a cell surface monomeric protein, a member of tumor necrosis factor receptor (TNFR) superfamily (Tnfrsf14), which shows a broad pattern of tissue distribution with high expression levels documented in lung, liver and kidney and lower levels of expression in brain, pancreas, heart, placenta and skeletal muscle [124]. HVEM is widely expressed on cells of immune system and other cell types. It can be found on surface of T and B-lymphocytes, DCs, monocytes, neutrophils and NK cells [121, 125, 126]. HVEM expression shows fluctuating patterns on different cell types, varying based on activation state of the cell (**Figure 1- 5**). Immature dendritic cells express high levels of HVEM but the level starts to decline once the cells get activated, with lowest expression seen on mature DCs [126]. T cells downregulate the HVEM expression upon activation but the levels raise while the cells enter the resting or memory phase [118, 121, 126, 127]. Human naïve and memory B cells express high levels of HVEM but mouse naïve B-lymphocytes show a low expression pattern [121, 125, 127]. It's been suggested that as a general phenomenon, activated cells express low levels of HVEM due to interaction with LIGHT, a known receptor for HVEM (discussed later) but resting or naïve cells have higher expression levels[128].

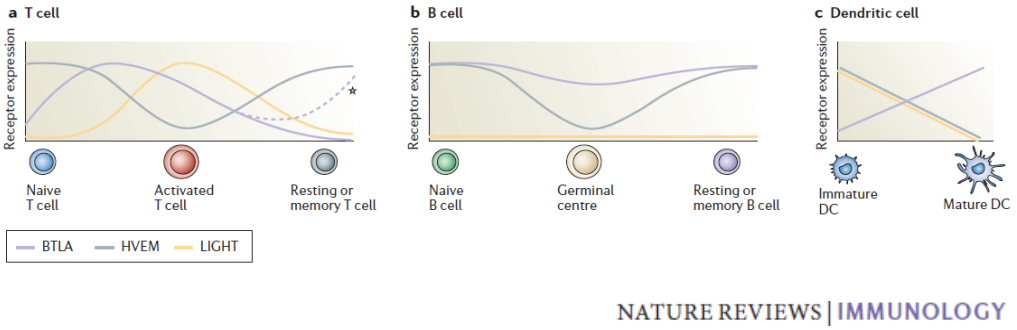


Figure 1- 5 Expression patterns of HVEM, BTLA and LIGHT on DCs, T cells and B cells [129]. HVEM generally shows high levels of expression on cells of immune system during resting state, while the expression declines upon activation. BTLA, on the contrary is upregulated upon activation.

1.1.1.4.1 HVEM binding partners

Before identification of its binding partners, HVEM was an orphan member of TNFR superfamily. On top of its interactions with HSV1 gD, HVEM has been shown to interact at least with four other known binding partners including: lymphotoxin-α (LTα), LIGHT (lymphotoxin-like, inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes), CD160, and B and T lymphocyte attenuator (BTLA) (**Figure 1- 6**). The first ligand reported for HVEM was LTα, which was identified through its precipitation with HVEM-Fc fusion protein [130]. LTα is a secreted homotrimer that can interact with other members of TNFR superfamily including TNFR1 and TNFR2 as well as lymphotoxinβ receptor (LTβR) [131]. LIGHT, the other lignad, is a type II transmembrane protein which was shown to bind to both HVEM and LTβR [130]. High levels of LIGHT expression on immature DCs decrease when they are activated [132]. On T cells LIGHT shows a reciprocal expression pattern comparing to HVEM, as it is upregulated on activated T cell and declines afterward (**Figure 1- 5**)[133, 134].

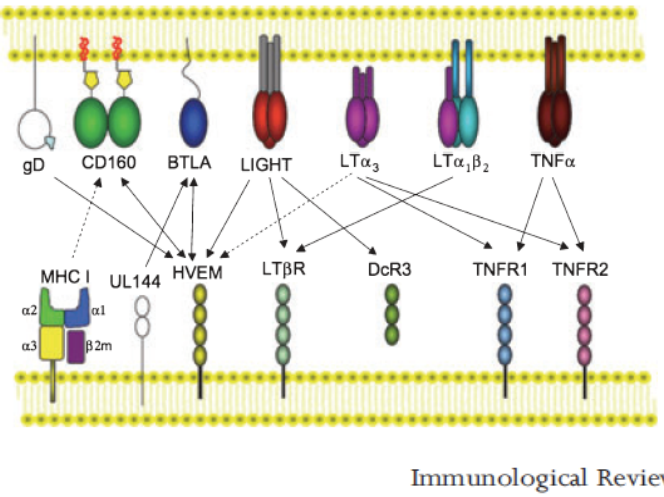


Figure 1- 6 The multiple receptor-ligand binding patterns of HVEM. HVEM has been shown to interact with 5 ligands, namely, HSV1 gD, BTLA, LT-α, LIGHT and CD160 [135].

1.1.1.4.2 Co-stimulatory effects of HVEM is mediated by its interactions with LIGHT

HVEM interaction with LIGHT has been shown to result in co-stimulation of T cells [136]. This has been highlighted in case of human T cells as HVEM has 10 folds higher affinity to bind to LIGHT than to BTLA, the ligand that is responsible for co-inhibitory effects [137]. On the other hand, in mice HVEM shows more affinity towards binding to BTLA than for LIGHT. The HVEM-LIGHT interaction strongly costimulates T cell proliferation, as highlighted by impairment of T cell activation, expansion and cytokine secretion in LIGHT-deficient mice [138-140]. Administration of anti-HVEM antibodies that block its interaction with LIGHT, has led to inhibition of T cell proliferation and cytokine secretion which further supports the co-stimulatory role for LIGHT-HVEM axis [121]. It was also shown that LIGHT deficiency would reverse the cardiac graft rejection modulated by HVEM-LIGHT interactions and prolongs the graft survival [141]. Engagement of LIGHT with HVEM would activate the NF-κB and MAPK pathways [132, 134, 136]. All these experimental evidences support the co-stimulatory outcome of HVEM-LIGHT interactions.

1.1.1.4.3 Co-inhibitory properties of HVEM

The inhibitory functions of HVEM are shown to be mediated through its interaction with other two ligands, BTLA and CD160. BTLA was first described by Watanabe *et al.* as an inhibitory receptor which binds to B7-H4 [117], but later studies showed there is no evidence of functional binding between B7H4 and BTLA[118]. It's a transmembrane monomeric glycoprotein of CD28 family, which is expressed on many cell types such as T and B cells, DCs, macrophages and NK cells. On T cells, it's expressed in low levels on naïve T cells but highly upregulated on activated T cells (**Figure 1- 5**) [142].

The first clue, which suggests BTLA might have inhibitory effects comes from the fact that its cytoplasmic domain has three highly conserved tyrosin motifs, of which two of them are the same a tyrosine motifs of ITIMs [143, 144] which are involved in inhibitory function of PD-1 and CTLA4 [117]. Mechanistically it has been shown that similar to PD-1 signaling, upon BTLA engagement the two tyrosin motifs become phosphorylated and this results in recruitment of the tyrosin phosphatases SHP1 and SHP2 [145], however mutations of those phosphateses didn't seem to abrogated the BTLA function suggesting the presence of another signaling mechanisms, through which BTLA might function [146]. The downstream targets of BTLA engagement in T cell signaling have not been fully revealed [147], although a report suggests that it forms co-clusters with CD3ζ, hence inhibits TCRζ phosphorylation [148]. By using agonistic anti-BTLA antibodies Krieg *et al.* have shown that BTLA exerts its inhibitory effects on T cells by down-regulation of activation marker CD25, as well as partial reduction of IL-2 secretion [149].

BTLA-deficient lymphocytes show enhanced proliferation upon activation [117, 150]. It was shown that BTLA regulates the homeostatic proliferation of $CD4^+$ and $CD8^+$ T cells in an Ag-independent manner, as BTLA-deficient cells were more efficient in generating memory pools [151]. *In vivo,* BTLA has been also shown to be involved in induction and maintenance of peripheral tolerance, as BTLA-deficient mice were resistant to induction of T cell tolerance to an oral antigen [152]. BTLA deficiency escalates inflammatory responses *in vivo*, as shown in models of EAE [117] and airway hypersensitivity [153]. In context of transplantations, BTLA was also reported to be a key regulator of partially MHC class II-mismatched cardiac grafts as BTLA deficient mice acutely rejected the graft much earlier than did WT counterparts [154].

CD160 has been also recently reported to bind to HVEM and inhibit T cell activation [155]. CD160 is a GPI-anchored protein lacking the transmembrane and intracytoplasmic domains and is expressed on $CD8^+$ cells, a small subset of $CD4^+$ cells, NK cells but not on myeloid cells or B cells [135]. CD160 previously was reported to engage with TCR complex through binding to protein tyrosine kinase p56lck and tyrosine phosphorylated zeta chains, hence delivering co-stimulatory signals to anti-CD3 activated T cells [156]. But further reports highlighted its potent co-inhibitory effects when engaged with HVEM.

CD160 is also shown to be one of the markers which is co-upregulated in exhausted CD8 T cells together with other known markers of exhaustion such as PD-1, CTLA-4 and lymphocyte activation gene-3 (LAG-3) [157, 158], suggesting that CD160 belongs to molecular signature of exhausted T cells during viral infections and therefore HVEM-CD160 axis might also play a role in functional exhaustion of T cells.

Collectively, the reports suggest that HVEM is a “master switch” that can act as a versatile co-regulator of T cell responses through bi-directional signaling. These effects might be co-inhibitory or co-stimulatory depending on nature and abundance of HVEM binding partner and strength of interactions.

1.1.2 DCs and stimulation of inflammatory immune responses through IL-1 family of cytokines

As discussed earlier DCs, as professional APCs are able to initiate, regulate and boost the immune responses in various conditions. DCs play important roles in induction of inflammatory responses through cell-cell interactions as well as production of inflammatory mediators. IL-1 family of cytokines are among the most important mediators of inflammation, released by DCs and some other cell types. As a part of our strategic plan for enhancement of immunostimulatory potential of DCs, we exploited the stimulatory capacity of the growth factor GM-CSF to boost the production of IL-1 family of cytokines by these cells. Here we discuss the nature of IL-1 family, and regulatory mechanisms of their production through inflammasomes as well as GM-CSF and its roles in inflammatory responses and regulation of DC function.

1.1.2.1 IL-1 family of Cytokines, major regulators of inflammation

IL-1 family of cytokines consists of 11 **(Table. 1)** so far identified members which play pivotal roles in regulating innate immune system as well as adaptive immunity. They have been reported to exert different effects ranging from activtory to inhibitory/suppressive [159].

New Name	Other Name	Property
IL-1F1	IL-1α	Agonist
IL-1F2	IL-1β	Agonist
IL-1F3	IL-1Ra	Receptor antagonist
IL-1F4	IL-18; IFN-γ-inducing factor	Agonist
IL-1F5	FIL1δ	Anti-inflammatory
IL-1F6	FIL-1ε	Agonist
IL-1F7	IL-1H4, IL-1ζ	Anti-inflammatory
IL-1F8	IL-1H2	Agonist
IL-1F9	IL-1ε	Agonist
IL-1F10	IL-1Hy2	Receptor antagonist (?)
IL-1F11	IL-33	Agonist

Table -1. Eleven identified memebrs of IL-1 family, exhibit different funtional properties [159].

Among the memebers, IL-1α (IL-1F1), IL-1β (IL-1F2), IL-1 receptor agonist (IL1-Ra or IL-1F3) and IL-18 (IL-1F4) have been extensively studied for their functions in regulation of inflammatory responses. The family members lack a signal peptide which might be a conserved evolutionary hint for them [160]. Here we explain in detail characteristics of 3 key members of the family involved in inflammatory processes, IL-1α , IL-1β and IL-18.

1.1.2.1.1 IL-1α

Early experiments in 1940's revealed that supernatants from PBMCs when injectied into rabbits were showing pyrogenic activities, causing fever [161, 162]. In search for the molecules involved in pyrogenic activity of leukocyte supernatants, Dinarello and his colleauges identified IL-1α together with the other major culprit, IL-1β [163]. The murine IL-1α cDNA was first isolated from $P388D_1$ macrophage line and cloned into *E.coli* in 1984 [164].

IL-1α is synthesized as an immature form that could be cleaved and processed by Calpains into a mature form [165], however it's been shown that its precursor form is transcriptionaly active without being processed [166], moreover under physiologic conditions or even with stimulation of blood PBMCs, the proform cytokine is not processed and secreted as mature form [167, 168]. Unlike IL-1β, IL-1α is shown to be constitutively present in different primary cell lines without any stimulation [169, 170]. The cytokine exists in two forms; membrane-bound IL-1α and intracellualr IL-1α (icIL-1α). The membrane-bound form is the functional bioactive cytokine which is known to be involved in inflammatory procecess. The intracellular form is hypothesized to act as a autocrine growth factor in normal cellular differentiation which could be shuttled into nucleus, bind to DNA and act as a transcription factor as well [159, 160]. Experimental reports have suggested that icIL-1α could be traslocated to the nucleus in conjunction with its receptor or other molecules [166, 171, 172], as it harbours a KVLKKRRL nuclear localization signal [173]. Hammerberg *et al.* have shown that there is a tightly controlled balance of icIL-1α and the IL1Ra in normal and psoriatic skin cells which could be a strategy employed by the skin cells to neutralize the effects of extra IL-1α by IL-1 receptor agonist [174].

The IL-1α deficient animals have no obvious defects in growth and development. The mice show minor effects upon fever induction compared to normal mice suggesting that IL-1α is a less important pyrogen comparing to IL-1β [175]. However, it should be noted that these animals are not totally deficient as the cells have shown to retain the propiece of the cytokine which can act as a nuclear factor, hence exerting some effects. Kamari *et al.* have studied the role of IL-1α in artherogenesis by high-choletsterol diet and lipid metabolism. They have reported a milder phenotype in IL-1α deficient mice comparing to WT animals as the aortic lesion sizes were smaller and concentrations of SAA (serum amyloid A), as an indication of inflammatory response, were lower as well [176].

The inflammatory properties of necrotic cells have been attributed to IL-1α, as these cells contain considerable amounts of bioactive IL-1α [177].

1.1.2.1.2 IL-1β

IL-1β (IL1-F2) has been extensively studied for its well characterized pyrogenic activity. It was first described as "endogenous pyrogen" because of its distinct and robust function in induction of fever [178]. The first insights into discovery of IL-1β as explained earlier, came from attempts to describe the know-hows about the pyrogenic properties of PBMC supernatants. The unknown molecule was called LAF (lymphocyte activating factor) due to its ability to activate lymphocytes and induce their proliferation. The pioneering work of Dinarello and his colleauges resulted in isolation of the IL-1β cDNA from human monocytes and its cloning in 1984 [179].

IL-1β is one of the earliest cytokines which is released by different cells of immune system (mainly monocytes, macrophages, DCs and to less extent B cells and NK cells), upon bacterial, fungal or viral infections, to support the antimicrobial host defence [180-184]. This cytokine initiates a cascade of inflammatory events including recruitment of inflammatory phagocytic cells and boosting production of othere inflammatory mediators to clear the microbial infections. IL-1β has also been shown to induce T cell responses against pathogenic infections, which are skewed toward Th2 and Th17 phenotypes [185-188]. It has been shown recently, that IL-1β plays a key regulatory role in adaptive anti-tumor immunity as well [189].

Microbial and non-microbial factors have been reported to induce the transcription of IL-1β gene which will be further regulated through complex mechanisms. Non-microbial factors namley; stress (e.g. hypoxia, hyperosmolarity), neuroactive substances (e.g. methamphetamine), inflammatory substances (e.g. complement component C5a, Factor H, retinoic acid, urate crystals, CRP), cellular matrix (e.g collagen, fibronectin), lipids, cytokines and growth fctors (e.g. IL-1 itself, IL-2, TNF, SCF, PDGF) are known to induce the transcription but not necessarily the IL-1β protein syntheis [160]. This transcription seems to be happening rapidly within 15 min post stimulation and later starts to decline in a 4 hr period of time. This might be due to presence of an instability element in the coding region of the gene [190, 191], mRNA half-life (about 2hr) or possible action of regulatory microRNAs. Microbial products known as pathogen associated molecular patterns (PAMPs) which act as TLR agonists such as LPS can trigger the stabilized transcription and further translation of IL-1β. Dinarello and colleauges have shown that IL-1 itself is able to further induce its own production, as recombinant IL-1α was able to augment the IL-1β production *in*

vitro and *in vivo* [192, 193]. Recently, it was revealed that one of the regulators of NF-κB pathway, Inhibitor of κB β (IκBβ) can directly bind to IL-1β promoter, in complex with with NF-κB subunits RelA/c-Rel, and induce it's transcription [194]. This finding further proves the regulatory role of NF-κB pathway in IL-1β gene transcription.

IL-1β exerts its effects by binding to two known membrane-bound receptors; IL-1 receptor type 1 and IL-1 receptor accessory protein (IL-1RAcP). The interaction with IL-1R1 is direct with high affinity. IL1-R1 is expressed on several cell types, including T cells [195, 196]. IL-1RAcP forms a trimeric complex with IL-1β and IL-1RI which results in proper intracellular IL-1 signaling [197, 198] that eventually activates NF-κB and MAPK pathways. Two naturally occuring inhibitors of IL-1β are responsible for regulating the IL-1β signaling; (i) IL-1R agonist (IL-1Ra) which competitively binds to IL-1R1 [199] and (ii) IL-1RII which binds to IL-1β as well as IL-1α but lacks a TIR domain, hence is unable to transmit signal [200]. All IL-1 receptors do exist in soluble form as well, which provides a broader regulatory mechanism for controlling IL-1β actions *in vivo* [201].

The result of IL-1β mRNA translation is a 31-kDa pro-form protein, which is biologicaly inactive. Cystein protease, caspase-1 (formerly called Interleukin-1 converting enzyme, ICE) can further cleave the inactive pro-IL-1β and process it into 17-kDa bioactive form [202-205]. Proteolytic cleavage happens between Asp^{116} and Ala^{117} within the cytoplasm and results in active IL-1β [206]. Caspase-1 was shown to be essential and playing a dominant role in this process as caspase-1-deficient mice exhibit a severe defect in processing of immature IL-1β into its active form [207, 208]. Caspases are cysteinyl aspartate-specific proteases responsible for various cellular mechanisms including programmed cell death, apoptosis and inflammation. In human, so-called "inflammtory caspases" are caspase-1, caspase-4 and caspase-5, while in mice they consist of 3 members; caspases-1, -11 and -12 [209, 210].

Caspase-1 is synthesized as a zymogen consisting of p10 and p20 subunits and a CARD domain. It is synthesized as inactive form, hence for exerting its function, needs to be proteolitycally cleaved. This is the critical stage where the conversion of IL-1β into active form is tightly regulated. IL-1β is a pro-inflammatory cytokine which production and secretion of it's bioactive form must be tightly regulated during health and disease. This process is very important, as unwanted release of active IL-1β

in normal physiologic conditions will result in extensive tissue damage and catastrophic auto-inflammatory processes. The activation of caspase-1, cleavage of pro-IL-1β and further release of bioactive cytokine is regulated by a proteolytic, large multi-protein complex (~700 kDa) , called inflammasome [211].

1.1.2.2 Inflammasomes

The core component of inflammasome complexes is a member of NOD-like receptor (NLR) family of cytosolic sensors. These heteromeric proteins consist of three main domains; a C-terminal leucine-rich repeat domain with ligand-sensing and autoregulatory properties, a centrally located NOD or NACHT domain which can bind to ribonucleotides and mediate the self-oligomerization and inflammasome assembly, and a N-terminal effector domain. Depending on the type of inflammasome the N-terminal domain could be a Pyrin domain (PYD), a CARD domain or a baculovirus inhibitor of apoptosis repeat domain (BIR) [212]. Schematic structure of NLR domain organizations is shown in **Figure 1- 7**.

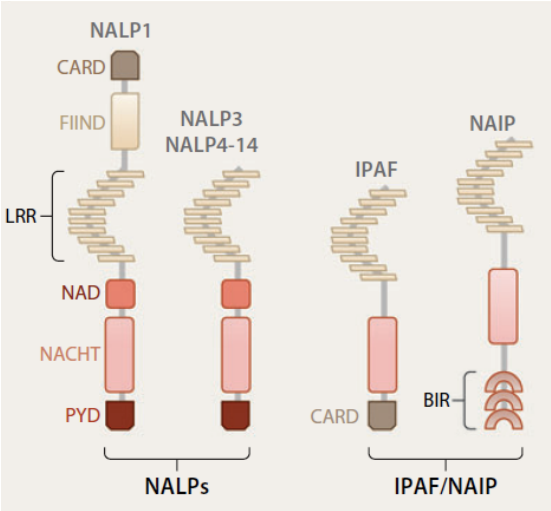


Figure 1- 7 Domain organization of NLRs in different inflammasomes [212] .

The N-terminal domains are responsible for protein-protein interactions with downstream signaling molecules which leads to activation of caspases.

Inflammasmes provide a proteolytic scaffold for activation of inflammatory caspases, namely caspase-1. NLRP3 (NALP3) inflammasome (cryopyrin) is one the best

characterized inflammasomes that is composed of Nlrp3, adaptor protein ASC (apoptosis associated speck-like protein containing a caspase recruitment domain) and caspase-1. ASC contains a Carboxy-terminal CARD domain, which can interact with pro-caspase-1 and recruit it to the inflammasome complex [211, 213]. This recruitment would lead to caspase-1 autocleavage and activation. The amino terminal PYD domain of this protein is known to interact with NALPs through PYD-PYD interactions [214], hence ASC is an important functional linker for proper inflammasome assembly. The schematic structure of assembled NALP3 inflammasome is shown in **Figure 1- 8**.

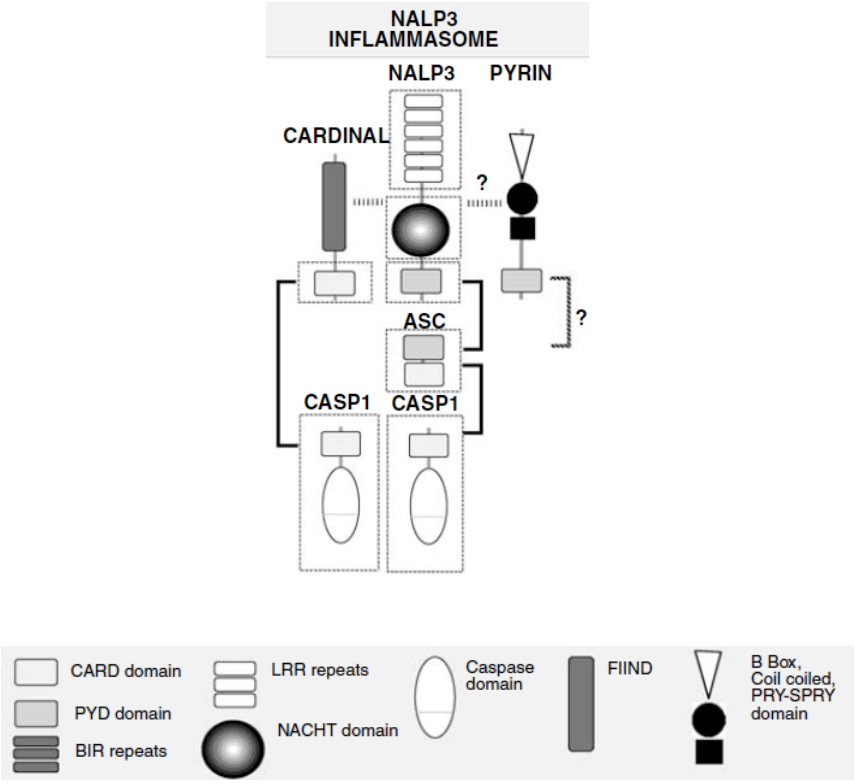


Figure 1- 8 The schematic model of NLRP3 inflammasome assembly. The inflammasome is composed of NALP3, ASC adaptor protein and caspase-1 [210].

1.1.2.3 Models of NLRP3 inflmmasome activation

The concept of inflammasome activation have been the core element of extensive debate and review. A broad range of exogenous and endogenous signals have been shown to be sensed and activate the NLRP3 inflammasome. This includes a lot of different pathogenic microorganisms like *sendai* and *influenza A* viruses [215]

adenoviral particles [216], fungi, such as *candida albicans* [217], pathogen associated molecular patterns (PAMPs) like lypopolysaccharide (LPS), muramyl dipeptide (MDP), bacterial RNA, dsRNA analog poly(I:C) and imidazoquinoline compounds R837 and R848 can activate the NLRP3 inflammasome [215, 218-222].

The accumulating evidence suggests that inflammasomes can sense a broad range of damage-associated molecular patterns (DAMPs), which later initiate the activation process. Extracelluar ATP [223], some bacterial pore-forming toxins such as α-toxin from *Staphylococcus aureus* [223], aerolysin from *Aeromonas hydrophila* [224], listeriolysin O from *Lysteria monocytogenesis* [225], streptolysin O from *Streptococcus pyogenes* [226], malarial hemozoin [227], nigericin, particulate structures such as uric acid crystals, monosodium urate (MSU), causative agent of gout), calcium phosphate dehydrate (causing agent of pseudogout) [228], silica and asbestos [229, 230], amyloid-β fibrils (within barain plaque of Alzheimer's patients) [231], islet amyloid polypeptide (IAPP, in pancreatic tissue of type 2 diabetes patients) [232] and the adjuvant aluminum hyroxide (Alum) [233-235] are among the danger signals proved to have the capability of strongly activating NALP3 inflammasome.

Three major mechanisms have been suggested to be involved in activation process, as discussed and reviewd by Tschopp and Schroder and illustrated in **Figure 1- 9** [236].

The first proposed model focuses on the importanat role of potassium (K^+) efflux which is triggered via ATP engagement by purinergic receptor P2X7. This event gradually recruits a hemi-channel called pannexin-1, which eventually forms pores allowing the NALP3 activators to reach the cytosol, being sensed and activate inflammasome [237, 238]. However, it is not clearly understood whether other NALP3 agonists are also able to induce the K^+ efflux. Moreover, some evidence suggests that K^+ efflux could not be sufficient to activate NALP3 inflammasome as alterations in Na^+ concentrations could abrogate the activation state [239].

The second model highlights the role of crystaline and particulate structures such as silica, asbestos and alum in causing a lysosomal damage upon their uptake. This damage leads to release of lysosomal protease, cathepsin B, which could somehow be sensed by NALP3 inflammasome [231, 233], this model is also not proven to be very accurate as cathepsin B-deficient mice show no significant defects in IL-1β release as a result of NALP3 inflammasome activation [227].

The third model emphasizes on the role of reactive oxygen species (ROS) in activation of NALP3 inflammasome. ROS include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH). It's been shown that many agonists of inflammasome including ATP, crystaline structures and hemozoin are able to induce the generation of ROS and ROS blockade with various strategies abrogated their potency in inflammasome activation [183, 227, 230, 240, 241]. There are controversies regarding this model as well, namely the ambiguous source of ROS. It's also reported that superoxide dismutase-1 can inhibit the caspase-1 activation which has been speculated to be a negative feed-back loop for caspase activation [242, 243]. A recent report by Zhou *et el.* suggests that NALP3 inflammasome can sense the mitochondrial damage and mysfucntion which leads to ROS generation and eventually inflammasome assembly and activation [244].

It is worth mentioning that the proposed mechanisms discussed above might not be mutually exclusive. It's possible that the activation of NALP3 inflammsome is the outcome of a regulated interaction between all three mechanisms defined above, nonetheless we can't rule out the importance of other yet-to-be identified mechanisms in regulation of inflammasome activation.

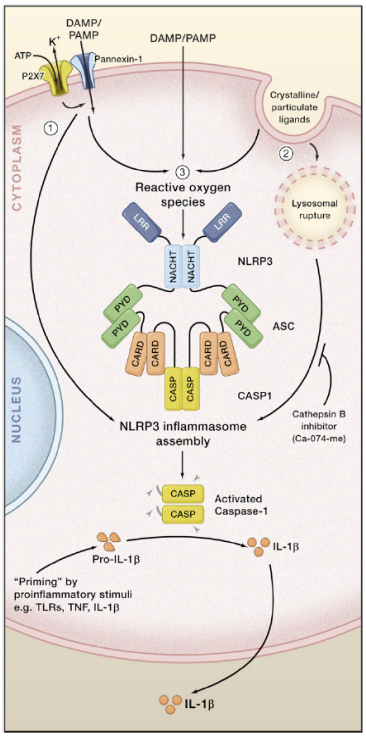


Figure 1- 9 Schematic presentation of 3 proposed models for NALP3 inflammasome activation. (1) ATP-mediated potassium efflux, (2) lysosomal damage by pore-forming toxins and crystals (3) ROS mediated activation [236].

Since IL-1β plays a key role in regulation of many inflammatory conditions, inflammasome activity is linked to a broad range of auto-inflammatory disorders such as periodic fever syndrome, vitiligo and Crohn's disease [245]. The mutations in NALP3 gene have been documented to be associated with some auto-inflammatory disorders like familial cold auto-inflammatory syndrome, Muckle Well's syndrome and neonatal-onset multi-systemic inflammatory disease [246, 247].

1.1.2.4 Mechanisms of IL-1β release

Since IL-1β lacks a secretory signal peptide, its secretion pathway doesn't follow the common ER-to-golgi pathway [248]. The nonclassical pathway of IL-1β has always been a matter of debate and it's not clearly deciphered. Five models have been proposed for IL-1β release, as reviewed by Claudia Eder [249]. These models include: (i) Exocytosis of IL-1β-containing secretory lysosomes (ii) shedding of IL-1β-containing plasma membarane microvesicles (iii) exocytosis of IL-1β-containing exosomes (iv) IL-1β release via plasma membrane transporters and (v) IL-1β release upon cell lysis.

1.1.2.5 IL-18

IL-18 (IL-1F4) was first cloned in 1995 by Okamura and his colleauges as a "new cytokine that induces IFNγ production by T lymphocytes" [250]. IL-18 has been thought to play partial role in immunopathogenesis of several inflammatory disorders such as type 1 diabetes, rheumatoid arthritis, garft-versus-host disease (GVHD), psoriasis, systemic lupus erythematosus (SLE) and Crohn's disease [159]. IL-18 protein, similar to IL-1β, is synthesized as an immature pro-form of 24 kDa, which is a substrate for active caspase-1. The proteolytic cleavage by caspase-1 releases bioactive 18 kDa IL-18. Its mRNA is constitutively expressed in healthy PBMCs in steady state [251]. The IL-18 levels and its signaling is regulated and balanced with a naturally occuring antagonist, IL-18 binding protein (IL-18BP) to avoid autoimmune

inflammatory reactions caused by extra amounts of active IL-18 [252, 253]. As release of bioactive IL-18 is mediated by caspase-1, this member of IL-1 family is aslo tightly regulated by inflammasome activation [251].

1.1.2.6 Granulocyte-macrophage colony stimulating factor (GM-CSF) and its role in inflammation

GM-CSF is a 24 kDa glycoprotein which was first described due to its role to promote differentiation of granulocytes and macrophages from murine bone marrow hematopoietic precursors [254] but GM-CSF doesn't seem to be the only factor mediating myelopoietic differentiation as GM-CSF-deficient mice don't show major perturbations of hematopoiesis or defects in myeloid-cell differentiations. The only visible phenotype of these animals is the development of a characteristic lung pathology and proteinosis, which is due to impaired fucntion of alveolar macrophages [255]. It's also reported that aged GM-CSF-deficient mice show a SLE-like disorder marekd with immue complex-mediated glomerulonephritisa and the high levels of anti-dsDNA autoantibodies which is associated with impaired phagocytosis of apoptotic cells [256]. GM-CSF can be produced in many tissues by various cell types including: macrophages, activated lymphocytes, fibroblasts and endothelial cells, keratinocytes, tumor cells, NK cells and some other cell types [257, 258]. It is a multifunctional cytokine invloved in cellular activation, proliferation, differentiation and inhibition of apoptosis [259]. However the physiologic concentrations of GM-CSF in serum is not very high (20-100 pg/ml) [260], suggesting that it might exert its function in concert with other molecules.

Due to its role in supporting myelopoiesis, GM-CSF has been extensively used in laboratries as growth factor in combination with other factors to derive dendritic cells with different functional and phenotypic features [261]. The best growth factor combination that have been used for years to support the DC generation *in vitro* has been the combination of GM-CSF and IL-4 [14].

GM-CSF exerts its biological function upon binding to its receptor, GM-CSFR. The receptor is composed of two subunits, low afinity binding 60- to 80-kDa α subunit, which binds to cytokine and high affinity 120- to 140-kDa β subunit, which is responsible in signal transduction and is shared with IL-3 and IL-5 receptors. The receptor complex is expressed broadly on $CD34^+$ hematopietic progenitors,

neutrophils, eosinophils, basophils, monocytes, macrophages, microglia, lymphocytes, enothelial and mesenchymal cells [261-263]. The expression of receptor on hematopoietic cells is at very low levels (100-1000 per cell). The engagement of GM-CSF with its receptor initiates several downstream pathways including Jak2/STAT5, MAPK and PI3K/Akt pathways, which are responsible for multifunctional outcome of GM-CSF signaling [264, 265]. The mice deficient in GM-CSFR β chain exhibit the similar manifestations as GM-CSF-deficient mice and develop lung pathology and exhibit low basal number of eosinophils [266, 267]. GM-CSF has aslo been shown to activate NF-κB through interactions of GM-CSFR with IκB kinase β (IKKβ) which leads to degradation of IκB [268].

As a multifunctional growth factor, GM-CSF has been suggested to be involved in inflammation and autoinflammatory processes [269, 270]. The evidences for this scenario come from observation of elevated levels of GM-CSF at sites of inflammation. Skin biopsies of allergic patients has shown elevated levels of GM-CSF mRNA expression [271]. Synovial fluids of joints of patients suffering from rheumatoid arthritis has also shown to contain elevated, measurable levels of GM-CSF [272].

The second line of evidence suggesting a regulatory role for GM-CSF in inflammation comes from studies showing that exogenous administration of GM-CSF induces inflammatory responses including inflammatory cell recruitment and priming of pro-inflammatory mediators [273-275] and exacerbates the autoinflammatory disrorders [276-278]. In line with these findings the transgenic mice over-expressing GM-CSF has shown a hyperactive immune system marked with accumulation of large and activated macrophages in the peritoneal and pleural cavities [279], up-regulation of plasminogen activator (PA) and some inflammatory cytokines [280], and a 50-100-fold increase in peritoneal macrophages numbers due to excessive proliferation [281]. The lines of evidence provided here, highlight an elusive regulatory role for GM-CSF in inflammation and autoinflammatory diseases. Apart from its role in supporting DC generation and development, GM-CSF has been suggested to be an activation factor for DC function [282-284]. Lin *et al*. have recently showed that CD8 T cell derived GM-CSF is a potent licensing factor for DC activation which, up-regulates the co-stimulatory molecules on DCs efficiently and primes them for release of proinflammatory cytokines, hence boosting their immunostimulatory potential [285].

The global gene profiling of curdlan-primed and GM-CSF-treated DCs in our lab showed a signature of upregulated molecules. Of note, the IL-1β message was dramatically increased in these cells comparing to non-treated cells.

1.2 Unlimited expansion of hematopoietic stem cells *in vitro*

Hematopoietic stem cells (HSCs) are the multipotent stem cells, which can rise to all blood cell types including myeloid (e.g. macrophages, monocytes, dendritic cells, megakaryocytes/platelets) and lymphoid (T, B and NK) lineages. The major source of HSCs is the bone marrow. These cells have been widely used for HSC transplantations. HSCs possess two major features, multipotency and self-renewal; hence, they have the ability to self-renew *in vivo* and *in vitro* and eventually support the lympho-myeloid hematopoiesis in irradiated recipients upon transplantation. [286-288].

Genetic and cellular manipulations of HSCs are long, time-consuming processes and long-term expansion of these cells in culture causes rapid loss of their self-renewal and multipotency. In order to avoid this problem, different approaches have been tried to expand the HSC ex vivo. One of the recent attentions, has been focused on Homeodoamin (Hox) family of transcription factors. Dysregulation of these genes has been linked to different types of malignancies, which influences hematopoietic proliferation and differentiation [289-291]. Previous studies have showed that ectopic retroviral expression of *HOXB4* significantly enhanced the regeneration of HSC *in vivo* and *in vitro* [292-295]. Sauvageau *et al.* also demonstrated that the repeated delivery of exogenous HOXB4 to the HSCs (in form of a TAT-HOX fusion protein) significantly enhanced their self-renewal and expansion [296]. Furthermore, it's been shown that forced over-expression of HOXB4 fused to Nucleoporin98 (NUP98-HOXB4), augments expansion of HSCs in culture as well as *in vivo* [297, 298]. NUP98 is a component of nuclear pore complex which is involved in mRNA/protein export/import from/to the nucleus and it's gene is often found to be translocated to several members of *HOX* gene family in acute myelogenous leukemais [299]. Although, forced expression of many of *HOX* genes or *HOX-NUP98* fusion products has shown to induce leukemia *in vivo* [291, 292, 300, 301] but *HOXB4* and *NUP98-HOXB4* have the ability to increase the expansion and self-renewal capacity of HSCs without disrupting their normal differentiation and induction of leukemia. Hence, we

used BM cells retrovirally transduced with the construct encoding NUP98-HOXB4 fusion protein as bulk cells in our studies [302]. This system allows us to keep these cells in culture for long periods, which is required for introduction of genes into the cells and long-term selection and expansion steps. A medium including IL-6 and stem cell factor (SCF) is used to maintain and grow NUP98-HOXB4 transduced BM cells in culture.

1.3 Generation of DCs

1.3.1 *In vitro* generation

The relative number of DCs *in vivo* is low compared with most other cell types and only a limited number of DCs could be isolated and purified from tissues, which makes it a difficult and burdensome process. Therefore, an important, useful alternative approach for *in vivo* DCs is the *in vitro* generation of DCs from blood monocytes or bone marrow progenitors with the appropriate heamtopoietic growth factors such as GM-CSF, Flt3-L and IL-4.

Flt3 (Fms-like tyrosine kinase-3) is a receptor tyrosin kinase with homology to the kinases c-Kit and c-Fms. Its ligand, Flt3-L is able to induce differentiation of both pDCs and cDCs from hematopoietic progenitors *in vitro* [303, 304]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is another growth factor used for generation of myeloid DCs from bone marrow progenitors [305-307]. A recent study of Xu *et al*. showed distinct morphological and functional properties of GM-CSF-derived and flt3-L-derived DCs. GM-CSF-derived DCs produced more inflammatory mediators including TNF-α, IL-10, CCL-2 and NO, than Flt3-L-derived DCs upon TLR ligation. On the other hand, Flt3-L-derived DCs exhibited more efficient migration to draining lymph nodes after sub-cutaneous injection and seem to be a better representative of the steady-state resident DCs [308].

1.3.2 *In vivo* generation

Hematopietic stem cell transplantation has been an efficient method for reconstitution of bone marrow repertoire in animals (and in humans for therapeutic aims) over past few decades [309, 310]. High doses of myeloablative radiation (total body irradiation,

TBI) kill all HSCs, but later, infusion of bone marrow HSCs rescues this lethal situation by reconstitution of all lineages in hematolymphoid system. HSCs which play the crucial role in repopulating the bone marrow repertoire are multipotent cells and possess the unique ability of self-renewal [311]. There is not any unique phenotypic marker available to distinguish the HSCs in BM cells but nowadays the murine HSCs are characterized by KTLS phenotype ($c\text{-}kit^{+}Thy1.1^{low}Lin^{-/low}Sca\text{-}1^{+}$) [312, 313] or with the help of SLAM (signaling lymphocyte activation molecule) family of receptors ($CD150^{+}CD244^{-}CD48^{-}$) [314]. Unlike human, mice after TBI completely accept the allogenic mismatched transplants without post-transplant immunosupression, which provides a convenient system to make the chimeric mice with genetically modified HSCs. These multi-potent cells can give rise to dendritic cells through hematopoiesis [315], hence provide an efficient source of genetically-manipulated DCs *in vivo*, which could be used as potent cells in cellular-therapy settings.

Myeloablation via total body irradiation is a risky protocol in terms of clinical applications, which can cause severe side effects and transient deficiencies in immune cell repertoire and function. Hence, one approach aimed for clinical use of modified HSCs would be the bone marrow reconstitution without myleoablation. There are some evidences, confirming the concept that myeloablation is not strictly obligatory, at least for syngenic HSC transplantation. Stewart *et al.* showed that non-myeloablated female mice injected with $40x10^{6}$ male cells per day for 5 consecutive days, exhibit 5-46% of reconstitution with donor cells, 3-9 months post-transplantation [316, 317]. It's not clearly known whether the low-percentage of reconstitution with donor cells in non-myeloablated recipients is a result of space shortage for homing of donor HSCs or lack of their proliferation [318, 319]. It seems that there is a competition between donor stem cells and host counterparts, so the donor cells could even home in non-myeloablated recipients but the final outcome and their contribution to hematopoiesis is strictly dependent to this competition. As the ratio of donor cells is much less compared to host cells in irradiated hosts, administration of high HSC doses seems to increase the potency of donor cells in competition with host counterparts [311, 316, 317].

1.4 Aims of this thesis

As stated in the introduction, DCs play a pivotal role in regulation of immune responses in health and disease. As major players of immune system, DCs can make the very important decision of either promoting and boosting of an immune response or inducing the immune tolerance and immune non-responsiveness. In this thesis we aimed to exploit this unique feature of DCs to design and generate “tailor made” DCs with different immunoregulatory properties.

Immortaized BM HSCs (NUP98/HOXB4-transduced BM cells) provided us with a reliable source of progenitors which could be easily manipulated to over-express different molecules of interest. Co-regulatory molecules of B7 family, namely PD-L1, PD-L2 and B7-H4, together with TNFR superfamily member, HVEM have shown a broad range of co-inhibitory effects, highlighted in literature as discussed before. Therefore they are potentially valuable candidates to be used for induction of tolerance. As experimental strategy, we chose to over-express these molecules on BM progenitors and further *in vitro* differentiate them into desired lineages of DCs. Then we examined the tolerogenicity of these modified lines in different in vitro settings. As DCs are professional APCs which activate T cells in an Ag-specific manner, we followed the fate of T cells after Ag-specific stimulation by our “tailor made” DCs, overexpressing different molecules of interest or their combinations. In this context, we evaluated the T cells proliferation and cytokine production after activation by DCs. We also generated the *in vivo* counterparts of those modified DCs by reconstitution of lethally-irradiated mice in order to investigate their *in vivo* tolerogenicity as well.

On the other hand boosting the immune response by DCs is of great interest in several therapeutic settings such as vaccine design. Hence we decided to exploit the immunostimulatory potential of GM-CSF and render DCs further immunogenic. As our primary observation revealed a potent synergistic effect between TLR agonists and GM-CSF in boosting the IL-1 production by DCs and macrophages, we designed and performed a series of *in vitro* and *in vivo* experiments to prove the sound effect of GM-CSF in boosting IL-1 production in LPS-primed cells. Since bioactive IL-1 production is tightly regulated by inflammasome complex, we also performed several

experiments to investigate the possible contribution of GM-CSF in this process. Finally a series of *in vivo* experiments were carried out on WT and GM-CSFR-deficient mice to further examine the role of GM-CSF in promotion and regulation of inflammation *in vivo*.

Collectively, our main goal was to generate "Tailor made" DCs through different cellular and genetic manipulations and experimentally assess their capabilities as either "tolerogenic" or "immunogenic" DCs *in vitr*o and *in vivo*. Those modified DCs could be further characterized in different models of immune-mediated diseases in future investigations as candidates for DC-based immunotherapies.

2. Methods and Materials

2.1 Molecular cloning and cell transductions

2.1.1 PCR amplification of target sequences and their cloning

mRNA sequences of all genes were identified from the NCBI database and they were ultimately cloned into the pMYc-IRES-GFP retroviral vector. pMYc-IRES-GFP is a bisistronic retroviral vector which contains an internal ribosome entry site (IRES), followed by gene coding the green fluorescent protein (GFP), ampiciline resistance gene cassette and the multiple cloning site. The full length mouse PD-L1 (B7-H1) cDNA was amplified by PCR with PFU polymerase from mouse spleen cDNA with the sense primer (5'- AGGAATTCAAACATGAGGATAT-3') and the antisense primer (5'- ACCTCGAGTCGAATTGTGTATC-3'), PD-L2 (B7-DC) were amplified with the sense primer (5'- AAGAATTCATGCTGCTCCTGCTGCCGAT-3') and the antisense primer (5'- TTCTCGAGCTAGATCCTCTTTCTCTGGA-3') from PCR-PD-L2-TOPO vector, PD-1 with the sense primer (5'-GGGAATTCAGCATGTGGGTCCGGCAGGT-3') and the antisense primer (5'-GACTCGAGTCAAAGAGGCCAAGAACAATG-3') from mouse thymus cDNA. All fragments were cloned into vector EcoRI and XhoI sites. B7-H4 was amplified with sense primer (5'- ACGGATCCACCATGGCTTCCTTGGGGCA-3') and antisense primer (5'- AGCTCGAGTCATCTTAGCATCAGGCAAC-3') from mouse ovary cDNA, and cloned into vector BamHI and XhoI sites. HVEM was amplified from mouse spleen cDNA using sense primer (5'-TGGGATCCAAAATGGAACCTCTCCCAGGA-3') and antisense primer (5'-GACTCGAGTCAGTTGGAGGCTGTCTCC -3') and cloned into pMYc-IRES vector between BamHI and XhoI sites. PD-L2 was amplified from mouse spleen cDNA with the sense primer (5'- TGCCATGGATGCTGCTCCTGCTG -3') and antisense primer (5'- AGGTCGACCTAGATCCTCTTTCTCTGGAT -3') and cloned into psR-LP-TREtight inducible vector between NcoI and Sal1 sites. PCR reactions were performed using a Biometra T3000 thermocycler with the following conditions: 1 min enzyme activation at 95°C, followed by 35 cycles of: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and 72°C for 2 minutes.

The PCR products were visualized by ethidium bromide by G:BOX Gel documentation system (SYNGENE) after separation over a 1% agarose gel at 100-

140V. PCR products were purified using PureLink™ Quick Gel Extraction Kit (Invitrogen, Germany). Ligation of DNA fragments was carried out by T4-DNA ligase (NEB) for 2 hr at RT. The plasmids were electroporated into competent bacteria for 5 ms at 1800V in an Eppendorf Multiporator system (Eppendorf). Plasmid minipurification was performed using QIAprep Spin Miniprep Kit (QIAGEN, USA) and plasmid maxipurification was done using PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, Germany). The design of all primers resulted in cloning of cDNAs downstream of the Kozak sequence. The nucleic acid sequences of genes were then verified by DNA sequencing.

2.1.2 Cloning of secreted chicken ovalbumin (OVA_s) as a model antigen

The full-length chicken ovalbumin (OVA) was amplified by PCR with PFU polymerase from pAc-neo-OVA vector (kindly provided by Ton Rolink, University of Basel, Switzerland). This vector is a mammalian expression vector which complete OVA cDNA has been subcloned into it using BamH1 and HindIII sites, under control of the human β-actin promoter [320]. Primers used for PCR amplification of OVA were as follows: the sense primer (5'-ATCTCGAGCTTTTTAAGGGGAAACACATC-3') and antisense primer (5'-ATGGATCCGACAACTCAGAGTTCACCATG-3'). Resulting fragment subcloned into pMYc-IRES-GFP at BamH1 and XhoI sites. The nucleic acid sequence of OVA [321] was then confirmed by DNA sequencing.

2.1.3 Cloning of transmembrane form of ovalbumin (OVA_{tm})

For cloning of transmembrane form of OVA (OVA_{tm}) the full length chicken OVA PCR amplified with PFU polymerase from pAc-neo-OVA vector by sense primer (5'-ATCTCGAGCTTTTTAAGGGGAAACACATC-3') and antisense primer without stop codon (5'-ATCTCGAGAGGGGAAACACATCTGCCAA-3'), and subcloned into pDisplay vector at BamHI and SalI sites, upstream of myc and platelet-derived growth factor receptor transmembrane region (PDGFR TM). DNA sequence of OVA was confirmed by DNA sequencing. Further, the OVA_{tm} fragment was removed from

pDisplay vector by digestion with BamHI and XhoI and subcloned into the multiple cloning site of pMYc-IRES-GFP at the mentioned sites.

2.1.4 Calcium phosphate transfection of Phoenix ectotropic packaging cell line

Phoenix cells (~70% confluent) were plated on 75 cm^2 tissue culture flasks. 5 minutes prior to transfection, the medium replaced by 8 ml Iscove's modified Dulbecco's medium (IMDM) with 10% fetal calf serum (FCS, Biowest) containing 25 μM Chloroquine. Chloroquine is known to enhance the transfection efficiency by avoiding lysosomal degradation of DNA. 20 μg of retroviral DNA diluted in H_2O, proper volumes of 2 M $CaCl_2$ and 2x HBS added and the mixture vigorously aerated for about 15 seconds. 2x HBS contains 50mM HEPES, 10mM KCl, 12mM Dextrose, 280mM NaCl and 1.5 mM Na_2HPO_4 (pH 7.05).
The 2 ml mixture added to chloroquine medium on phoenix cells. 8 Hours later the medium changed to IMDM containing 10% FCS. The viral supernatant collected on days 2, 3 and 4 post-transduction, filtered with 0.45 μm filter and stored at 4°C.

2.1.5 Transduction of HOXB4 bone marrow cells using fibronectin-coated plates

60mm bacteriological petri dishes (Costar) were coated with 10 μg/ml of recombinant H296 fragment of fibronectin overnight at 4°C. The next day supernatant was decanted and plates were washed twice with PBS. Next, the petri dishes were incubated for 30 min with viral supernatant at 37°C. After incubation, the viral supernatant removed and 10^6 BM cells (which were already transduced by pMYc-NUP98-HOXB4-IRES-PURO to over-express NUP98-HOXB4 fusion protein) resuspended in IMDM containing IL-6 and SCF with 2% FSC and added to each plate. This procedure was repeated with new fibronectin pre-coated dishes with 5 hrs of intervals up to 4-5 times. Transduction efficiencies were assessed by determining the proportion of GFP positive cells or by specific Ab staining, using FACS analysis and fluorescent microscopy as well. After 36-48 hours cells were collected and expanded. After adequate expansion of cells, GFP positive or mAb-stained positive cells were

sorted by cell sorter (FACS-Aria BD, USA) and placed in culture in aforementioned medium. Sorted cells were pelleted and resuspended in FCS containing 10% DMSO in a small volume (0.5ml) in cryotubes, cooled down at -80°C and transferred to liquid nitrogen 24 hrs later for long term storage.

2.1.6 Inducible gene expression using Tet-On system

The inducible gene expression system (induction via tetracycline), was first developed by Gossen *et al*. [322, 323]. In *E. coli*, Tet repressor protein (TetR) can regulate the tetracycline resistance genes on its operon. In absence of tetracycline TetR binds to tet-operator (tetO) sequences and blocks the transcription. This is the basis for Tet-off system by which the bacteria repress the transcription. The Tet-On system (Clontech Laboratories, Inc) consists of two plasmids, the regulatory plasmid, which contains rtTA, a reverse Tet repressor that is a transactivator protein. It's also fused to herpes simplex virus activation domain, VP16, which converts the repressor to activator of transcription. The second element of the system is the response plasmid, pSR-LP-TREtight that expresses the gene of interest under control of TRE (tetracycline-response element). TRE is located upstream of a minimal CMV promoter. The minimal promoter is modified and lacks the strong enhancers to reduce the background levels of gene expression. Once the cells transduced with both plasmids, rtTA can bind to TRE and activate the gene transcription in presence of doxycycline (a tetracycline derivative) as illustrated in **Figure 2- 1**. This system is a very reliable and tightly regulated approach for inducible gene expression in a specific manner with very low background expression levels. The expression levels could be regulated by varying doxycycline concentrations *in vitro* and *in vivo*.

We transduced the NUP cells with pSR-rtTA-A-pgk-iner5 plasmid, which contains histidinol dehydrogenase *(hisD)*-resistance gene. The transduced cells were selected by L-histidinol and expanded for transduction with second plasmid pSR-LP-TREtight, which carries gene of interest (in this case PD-L2). The cells were treated with 1μg/ml dox and 24 hr later the PD-L2^{hi} cells were sorted using a monoclonal anti-PD-L2 antibody by FACSAria cell sorter. For *in vivo* induction, mice received 2 mg dox (i.p) and the drinking water of animals was also supplemented with 1mg/mL dox as well, to maintain and increase the induction levels.

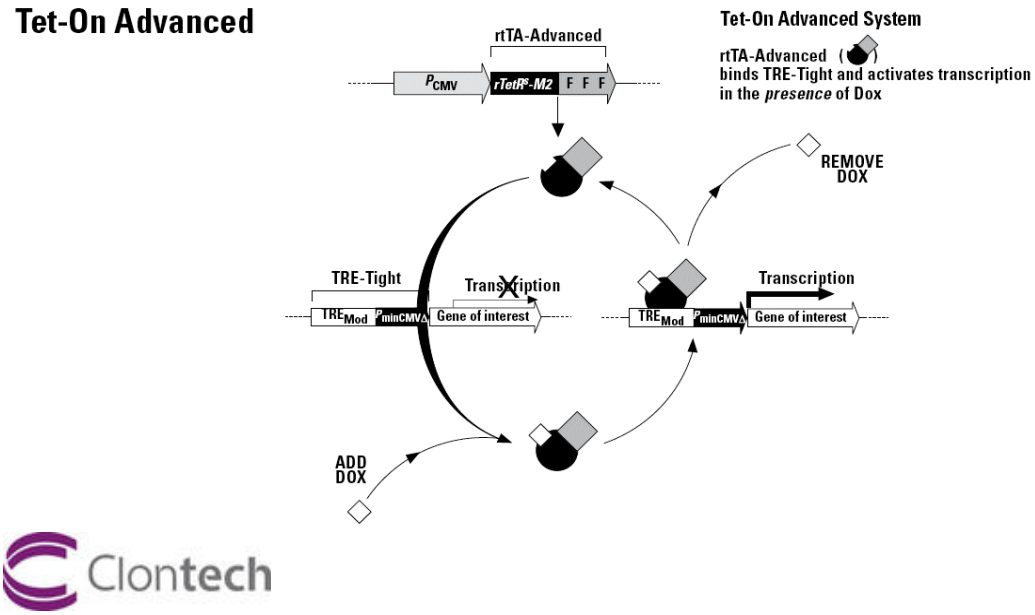


Figure 2- 1 Tet-On gene expression system. rtTA binds to TRE in presence of doxycycline and activates the transcription.

2.2 Cell cultures, mice and cellular methods

2.2.1 Generating of BM-derived dendritic cells and macrophages

For preparation of BM cells, mice were euthanized by CO_2 and the tibia and femurs were isolated and bone cavities containing the marrow, flushed with PBS containing 2% FCS. The erythrocytes were lysed by 0.89% Ammonium chloride solution. Cells were pelleted and resuspended in them IMDM with 2% FCS containing appropriate growth factor for DC or macrophage differentiation.

2.2.1.1 GM-CSF culture

10^6 BM cells resuspended in IMDM with mouse GM-CSF (100 ng/ml), plated on 100mm cell culture Petri dishes and cultured at 37°C in 7% humidified CO_2. After 36-48 hr of culture, the fresh medium was added. DCs were harvested on day 6-7.

2.2.1.2 Flt3-L Culture

10^7 BM cells resuspended in IMDM with Flt3-L (100ng/ml) and plated on cell culture Petri dishes. The fresh media were added on days 3 and 6. DCs were collected on day 9. For use in inflammasome experiments the Flt3-L derived DCs were purified with

mouse CD11b immunomagnetic microbeads and LS columns according to manufacturer's protocol (Miltenyi Biotec).

2.2.1.3 L929 Macrophages

3-6 x 10^6 BM cells were resuspended in IMDM medium containing 2% FCS and 30% L929 cell-conditioned medium. Cells were incubated for 5-7 days at 37°C (7% CO_2) until uniform monolayers of macrophages were established. Adherent cells were detached by Trypsin-EDTA solution (Gibco).

2.2.1.4 M-CSF Macrophages

3-6 x 10^6 BM cells were resuspended in IMDM medium containing 2% FCS and 20 ng/ml rmM-CSF (Miltenyi Biotec). Cells were incubated for 5-7 days 37°C (7% CO_2) until uniform monolayers of macrophages were established. Adherent cells were detached by Trypsin-EDTA solution (Gibco).

2.2.2 Mice

Age and sex-matched C57BL/6 wild-type mice were purchased from CARE, National University of Singapore. C57BL/6 OTI /OT II TCR transgenic mice bearing T cells reactive with immunodominant peptides of chicken ovalbumin (MHC I restricted $OVA_{257-264}$ for OTI and MHC II restricted $OVA_{323-339}$ for OTII) [324], and B6.129S1-$Csf2rb^{tm1Cgb}$/J (GM-CSFR-deficient mice) [325], C57BL/6-Tg(Actb-OVA)916Jen/J [326] and $RAG2^{-/-}$ [327] mice were obtained from Jackson Laboratories, USA. All mice were raised, maintained and studied in common pathogen free conditions at SBS animal house according to institutional guidelines of SBS, NTU. Mice were 6-8 weeks old at the beginning of each experiment.

2.2.3 Reagents

TLRgrade® LPS from *E. coli* was from Enzo Life Sciences (Alexis, Lausen, Switzerland). Brewer's Thioglycolate medium, Collagenase-D, Chicken Egg Albumin (OVA), Bay 11-7082, Wedelolactone, Aluminum hydroxide, Pam3Csk4, poly(I:C), and Imidazoquinoline were from Sigma (St Louis, MO, USA). MSU, ATP and

Nigericin were purchased from InvivoGen (San Diego, CA, USA). [H^3] Thymidine was purchased from PerkinElmer.

2.2.4 Isolation and purification of antigen-specific T cells

OT I / OT II transgenic mice were sacrificed, spleen and lymph nodes (mesenteric, inguinal, axillary, popliteal and superficial cervical) were isolated and single cell suspensions prepared. For single cell suspension preparation, organs were gently smashed by pressing with the flat surface of a syringe plunger through a 70 μM stainless steel sieve. Erythrocyte lysis was done using 0.89% NH_4Cl for 10 min at room temperature. $CD4^+$ or $CD8^+$ T cells were purified using CD4 or CD8 microbeads (Miltenyi Biotec, CA, USA) through LS immunomagnetic MACS columns. In cases which naïve OTI $RAG^{-/-}$ mice used for T cell preparation, T cells were >90% $CD8^+$ as determined by flow-cytometry.

2.2.5 Isolation of peritoneal macrophages

WT mice were i.p injected with 1ml of sterile 4% Brewer's Thioglycolate Medium (Sigma) and were sacrificed 4 days later. The peritoneal cavities were flushed with 8ml ice-cold PBS under sterile conditions. Cells were washed once with PBS, centrifuged and resuspended in IMDM medium with 2% FCS and counted with a hemocytometer. 10^6 Cells were plated in each well of a 6-well cell culture dish and macrophages allowed to settle down and adhere. After 2 hr non-adherent cells were removed and adherent macrophages (>95% purity, determined by MACI staining) were harvested for downstream experiments.

2.2.6 Preparations of murine peripheral blood mononuclear cells (PBMCs)

The mice were bled through retro-orbital plexus and the blood was collected in tubes containing PBS and 20% sodium citrate as anti-coagulant. The cells were isolated by Ficoll-Hypaque (Sigma) density centrifugation. Briefly, after adding Ficoll, the blood was centrifuged at 800 x g for 30 min. The cloudy ring at PBS/Ficoll interface containing mononuclear cells was carefully collected with a Pasteur pipette and

transferred to a new tube and filled up with PBS. Cells were centrifuged and resuspended in PBS containing 2% FCS for staining and flowcytometric analysis.

2.2.7 Preparation of ALU-OVA

OVA was dissolved in PBS and adsorbed to Aluminum Hydroxide Gel (Sigma) in presence of 0.9% NaCl salt (pH: 7.4). The mixture was stirred overnight at 4° C. On the next day, the mixture was centrifuged and the white pellet dissolved in PBS for injection into mice.

2.2.8 CFSE labeling of T cells

5-(and-6)-carboxyfluorescein di-acetate succinimidyl ester (CFSE) is an intracellular fluorescent dye, which incorporates into cell cytosol and allows monitoring of lymphocyte proliferation and migration *in vivo* and *in vitro*. Cell division results in sequential halving of fluorescence, and up to 8 divisions can be monitored before the fluorescence is decreased to the background fluorescence of unstained cells. Enriched T cells incubated for 10 min at 37°C in 5 µM CFSE (Molecular Probes, Invitrogen, USA) in PBS. The reaction was quenched by addition of 1ml of FCS. Cells were washed with excess of PBS and resuspended in fresh medium (IMDM with 2% FCS). Efficient CFSE-labeling of T cells was confirmed by fluorescence microscopy or FACS.

2.2.9 Antibodies and flow-cytometric analysis

Cells were stained with single or combinations of different mAbs. Antibodies diluted in PBS containing 2% FCS at the recommended dilutions. For extracellular staining, cells resuspended in either diluted antibody or PBS alone, and incubated at 4°C for 20 min, washed and analyzed on FACS (FACSCalibur, BD Biosciences). For some stainings, to reduce non-specific antibody binding and background signal on FACS, cells were treated with anti-FcR mAb (2.4G2) for 15 min at 4°C prior staining.

For intracellular staining, cells obtained from *in vitro* cultures were incubated for 4 hours with phorbol 12-myristate 13-acetate (PMA) (1ng/ml) and ionomycin (1mg/ml)

or 6 hr with immunodominant peptide $OVA_{323-339}$;ISQAVHAAHAEINEAGR. Brefeldin A (10μg/ml) (Sigma) was added for final 2 hr. Cells were kept in 96-well tissue culture plates at 37°C. Cell surfaces were stained for 20 min with the appropriate cocktail of fluorescence-labeled antibodies and after surface staining, the cells were fixed with fixation buffer (4% paraformaldehyde in PBS) for 30 min at room temperature, permeabilized with permeabilization buffer (0.5% Saponin in PBS with 2% FCS) and stained with antibodies diluted in permeabilization buffer for 30 min at RT. Anti-mouse monoclonal antibodies used for staining, stimulation, blockade or ELISA were as follows;

Name	Clone	Isotype	Source
CD3	145-2C11		
CD4	GK1.5	Rat IgG2b	Biolegend
CD8α	53-6.7	Rat IgG2a	Biolegend
CD11b	M1/70	Rat IgG2b	Biolegend
CD11c	N418	Armenian hamster IgG	Biolegend
CD19	6D5	Rat IgG2a	Biolegend
CD90.2 (Thy1.2)	30-H12	Rat IgG2b	Biolegend
Ly6C	HK1.4	Rat IgG2c	Biolegend
Ly6G	1A8	Rat IgG2a	Biolegend
CD40	3/23	Rat IgG2a	Biolegend
IFN-γ	XMG1.2	Rat IgG1	Biolegend
XD279 (PD-1)	J43	Armenian hamster IgG	eBioscience
B7-H1 (PD-L1)	MIH5	Rat IgG2a	eBioscience
B7-DC (PD-L2)	TY25	Rat IgG2a	Biolegend
B7S1 (B7-H4)	Clone9	Rat IgG1	R&D Systems
CD270 (HVEM)	LH1	Armenian hamster IgG	eBioscience
CD272 (BTLA)	6F7	Mouse IgG1	eBioscience
CD28	37.51	Syrian hamster IgG	eBioscience
CD5	53-7.3	Rat IgG2a	Biolegend
CD80	16-10A1	Armenian hamster	Biolegend

		IgG	
CD86	GL-1	Rat IgG2a	Biolegend
B220	RA3-6B2	Rat IgG2a	Biolegend
I-A/I-E (MHC II)	M5/114.15.2	Rat IgG2b	Biolegend
GM-CSF	MP1-22E9	Rat IgG2a	Biolegend
IgG2c		Rat IgG	Southern Biotech
IgG2b	RMG2b-1	Rat IgG	Biolegend
IgG1	RMG-1	Rat IgG	Biolegend
IgE	RME-1	Rat IgG1	Biolegend

Data collected from flowcytometer were analyzed with FlowJo software (Mac version 9.3.1,TreeStar).

2.2.10 ELISA

IFN-γ production from the supernatants of co-cultured cells was determined by standard ELISA. The purified rat anti-mouse IFN-γ and biotinylated rat anti-mouse IFN-γ antibodies were purchased from BD Pharmingen (San Diego, CA). IL-1α, IL-1β, TNF-α and IL-6 ELISA Kits were purchased from Biolegend and ELISA was performed according to manufacturer's protocol. For measurement of Ig isotypes in blood sera, serial dilutions of sera were prepared in plates coated with 10ug/ml OVA, and were analyzed by standard ELISA using Biotin-conjugated anti IgG1 anti IgG2b, anti IgG2c and anti IgE followed by HRP-labelled streptavidin. Briefly, OVA-coated plates were washed with 0.05% TWEEN20/PBS, and blocked with 1% BSA for 1hr at RT. The plates were washed 2 times and diluted sera from mice were added to the wells. Plates were incubated for 2hr at RT followed by 4 times washing. Biotin-conjugated antibodies were added and plates were incubated for 1hr at RT followed by 4 washes. The plates were incubated with Streptavidin-HRP (Biolegend) for 30 min and washed 5 times. Finally, TMB substrate (Biolegend) was added and the O.D of resulting blue color product was measured at 405nm with a Multiskan Spectrum ELISA reader (Thermo Scientific).

For measuring the NF-κB subunits p65, p50 and p52, the equal amounts of cell nuclear extracts were analyzed for DNA binding activity of the subunits using a DNA-

binding ELISA kit (TransAM NF-κB transcription factor kit, Active Motif) according to manufacturer's manual.

2.2.11 Western blotting

Cells were harvested, centrifuged, washed once with ice-cold PBS and lysed using a lysis buffer containing 0.1% Nonidet-P40 supplemented with Complete protease inhibitor cocktail (Roche, Basel, Switzerland) and 2mM DTT. Lysates were supplemented with SDS-PAGE sample buffer and boiled for 5min. Equal amounts of proteins were loaded into the 10-15% polyacrylamide gel and resolved by electrophoresis for approx. 25 min at 80V followed by 1-2 hr at 120 V. The proteins were transferred to nitrocellulose membranes (BioRad). Blots were blocked for 1hr in RT with 3% non-fat dry milk in PBS/0.5% TWEEN20, followed by overnight incubation with proper dilutions of primary antibodies, anti-pro-IL-1β (R&D), Caspese-1 p20, anti-c-Rel, USF-2 (Santa Cruz Biotechnology), anti-Caspase-11 (Biolegend), anti-NALP3 (Alexis, Enzo Life Sciences), anti-IκB-α, anti-phospho-IκB-α (Ser32), anti-IκB-β (Cell Signaling), diluted in 2% milk solution. The blots were washed 4 times next day in wash buffer (PBS containing 0.5 % TWEEN-20) and incubated for 1hr with respective HRP conjugated secondary antibodies, HRP Goat anti Rat IgG, HRP Donkey anti Rabbit IgG and HRP Goat anti-Mouse IgG (diluted 1:5000 in 2% milk solution), followed by 4 times washing. The blots were developed by Western Lightning *Plus*-ECL Enhanced Chemiluminescence Substrate (PerkinElmer) and visualized by LAS-4000 system (FujiFilm). The supernatants of cell cultures were concentrated by centrifugation using Amicon Ultra-4 centrifugal filters with NMWL (nominal molecular weight limit) of 3000k (Millipore, Ireland), the protein concentrations were measure by Bradford assay using Bio-Rad Protein Assay Dye Reagent (BioRad, CA) in MULTISKAN SPECTRUM spectrophotometer (Thermo).

2.2.12 BM reconstitution and generation of chimeric mice

6-weeks old wild type C57BL/6 mice were lethally gamma-irradiated at 900 rads or 9 Gy (divided into two doses of 450 rads with 4 hrs of interval) by Cs137 source

(Biobeam 8000, Germany), and injected intravenously with 2-5x10^6 BM cells on the following day. Mice drinking water was supplemented with antibiotic (800 µg/L Neomycin sulfate, Sigma-Aldrich) for the first 10 days after irradiation. For peripheral blood screening of the chimeric mice and evaluation of reconstitution efficiency, the chimeras were bled through retro-orbital plexus vein 4-6 weeks after irradiation, blood mononuclear cells were separated from erythrocytes using density centrifugation after adding of Ficoll-hypaque to blood samples. The cells were then stained with proper cocktail of various monoclonal antibodies to visualize T (Thy1.2$^+$) cells, B (CD19$^+$) cells, macrophages (CD11b$^+$) and granulocytes (Gr1$^+$).

For generation of chimeric mice over-expressing PD-L2 or HVEM on myeloid cells only, the lethally irradiated mice were co-injected with 2-5x10^6 NUP98-RAG$^{-/-}$ PD-L2 or NUP98-RAG$^{-/-}$ HVEM BM cells with 2x10^5 wild type BM cells isolated from WT B6 mice (to reconstitute the lymphoid compartments).

2.2.13 Visualization of apoptotic cells using CaspACE FITC-VAD-fmk staining

After erythrocytes lysis, splenocytes were resupended in PBS containing 10µM of FITC-VAD-fmk (Promega) and incubated for 20 min at 30°C in the dark. Cells were washed and resuspended in PBS and the green fluorescence intensity was analyzed by flowcytometry.

2.2.14 Septic shock model

Mice were intraperitoneally injected with 50ug/g/body weight. LPS from *E. coli* (055:B5, Sigma) corresponding to the mouse LD50 dose, and were monitored for survival over a 7 d period. 3 hr post-LPS injection mice were anesthetized with isoflurane and bled through retro-orbital plexus vein. The sera were separated from blood cells by centrifugation of whole blood in BD Microtainer serum separator tubes (B, NJ, USA). The sera were collected for cytokine measurements and stored at -20° C.

3. RESULTS (I) Enhancing Tolerogenic Potential of DCs

3.1 *In vitro* and *in vivo* Presentation of OVA by genetically modified cells

To confirm the ability of OVA_s transduced BM cells to express ovalbumin as antigen which could be captured and presented by APCs, leading to activation of the OVA-specific transgenic T cells, $2x10^7$ NUP98-HOXB4-OVAs BM cells were transferred intravenously into C57BL/6 mice without irradiation. $5x10^6$ OVA specific OT-I T cells were purified, labeled with CFSE and injected intravenously into mice 2 weeks later. Mice were sacrificed after two days, single cell suspensions obtained from spleens, and the intensity of CFSE fluorescence was analyzed in the $CD4^+$ and $CD8^+$ subpopulations of lymphocyte in flow cytometer. As shown in **Figure 3- 1** (A) the CFSE labeled $CD8^+$ T cells became activated and proliferated several times due to presentation of OVA by APCs in spleen. These results confirm that ovalbumin is expressed and presented efficiently by transduced cells, which have given rise to APCs.

The same experiment was performed to show the biological activity of trans-membrane form of OVA, expressed by OVA_{tm} transfected cells, but the proliferation of CFSE-labeled T cells was hardly detectable on FACS (data not shown). This could be a result of low expression of antigen or inefficient presentation of antigen on the cell surface.

Presentation of OVA peptide in the context of MHC class II molecules by NUP98-HOXB4 OVA_s BM-derived DCs was investigated *in vitro* as well. OVA-specific T cells were prepared from spleen and LNs of OTI or OTII mice. Proliferative response of the OVA specific T cells was monitored based on CFSE dilution during co-culture with OVA_s expressing DCs. As shown in **Figure 3- 1** (B) secretion and presentation of OVA by DCs efficiently induced proliferation of either $CD4^+$ or $CD8^+$ OVA peptide-reactive T cells *in vitro*. No proliferative response was observed when OVA specific T cells were co cultured with wild type NUP98-HOXB4 BM-derived DCs.

In order to evaluate the levels of IFN-γ production by T cells primed with OVA presenting DCs, ELISA performed with supernatants from co-cultured cells. ELISA results, showed potent IFN-γ production by the activated OT I/OT II T cells, comparing to T cells co-cultured with control DCs, which further confirms activation

and proliferation of OVA-specific T cells by DCs genetically engineered to present ovalbumin (**Figure 3- 2**).

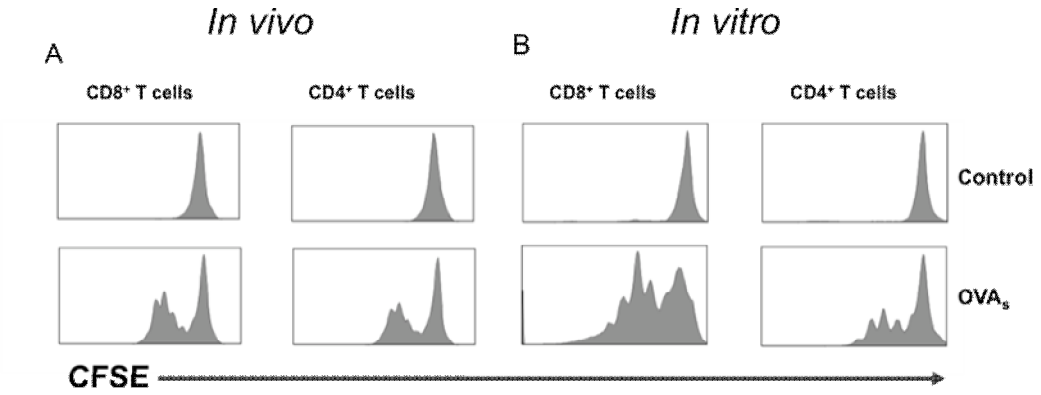


Figure 3- 1 Efficient *in vivo* presentation of OVAs (soluble form of OVA) and induction of T cell proliferation by DCs. **(A)** *In vivo* proliferation of CFSE-labeled $CD8^+$ and $CD4^+$ OVA-specific T cells was monitored in WT mice adoptively transferred with OVAs expressing BM cells. **(B)** *In vitro* proliferation of OVA-specific $CD8^+$ and $CD4^+$ T cells co-cultured with OVAs-BMDCs, assayed by CFSE dilution in gated populations of $CD4^+$ or $CD8^+$ cells. Each peak corresponds to one cycle of lymphocyte proliferation.

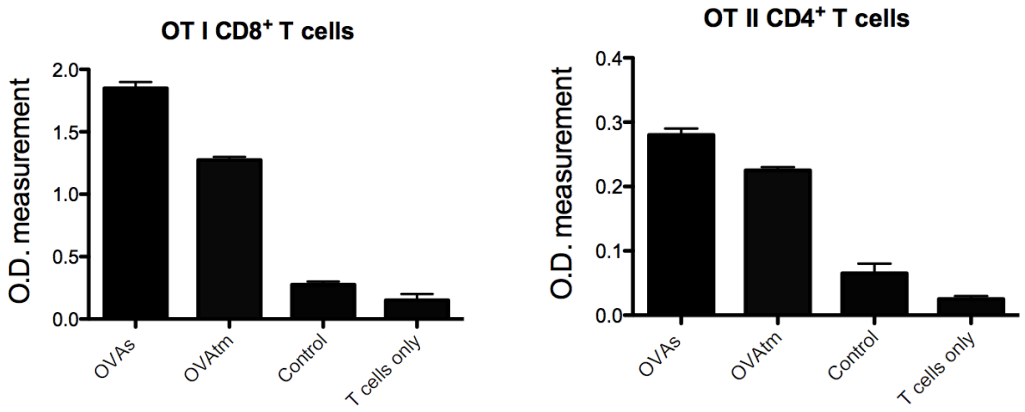


Figure 3- 2 Ag-specific IFN-γ production by DC-stimulated T cells *in vitro*. Enzyme-linked immunosorbant assay of IFN-γ production by OT I or OTII cells (800,000 cells), co cultured with OVA_s , OVA_{tm}, WT control BMDCs or without any APCs ($2.5x10^4$ cells) for 48hrs. Bar graphs represent the O.D. measurements. Data are mean ± SD (n=3).

3.1 Monitoring expression of PD-L1, PD-L2, and B7-H4 on Transduced BM cell lines

To confirm the over-expression of PD-L1, PD-L2 and B7-H4 molecules on transduced cell lines, NUP98-PD-L1, NUP98-PD-L2, NUP98-PD-L1/2 and NUP98-B7-H4 BM cell lines were stained with respective antibodies and analyzed by FACS. Expression levels of each molecule were monitored on GFP positive cells compared to wild type control cells. Results confirmed the obvious over-expression of mentioned molecules on transduced NUP98-HOXB4 BM cell lines, while no expression was detected on WT control BM cells (

Figure 3- 3).

3.2 Sequential transduction of HOXB4 BM cells

In order to examine the effect of simultaneous over-expression of inhibitory molecules in generating a tolerogenic phenotype on DCs, we designed an experimental approach to double-transduce the NUP98-HOXB4 BM cells with different combinations of inhibitory molecules. In the first case, the NUP98-HOXB4-PD-L2 BM cell line, which had already been transduced to over-express PD-L2, was transduced with pMYc-PD-L1-IRES-GFP retroviral vector. We repeated the retroviral infection of cells 4-6 times to reach a high percentage of transduction efficiency. As the primary cell line used for double transduction was already GFP positive, we used monoclonal anti-PD-L1 Ab to monitor the PD-L1 expression and transduction efficiency on FACS. When a sufficient percentage of cells tranduced to overexpress the PD-L1, cells were expanded in culture, stained with anti-PD-L1 antibody and $GFP^+/PD\text{-}L1^+$ double positive cells were sorted on cell sorter (FACS-Aria, BD). Resultant single or double-transduced BM cells were also subjected to differentiation to DCs in GM-CSF culture. The overexpression of both PD-L1 and PD-L2 on double transduced BM cells or DCs was confirmed by flow-cytometric analysis (

Figure 3- 3). The same experimental design was used to generate NUP98-HOXB4-PD-L1/B7-H4 and NUP98-HOXB4-PD-L2/B7-H4 cell lines which over-express B7-H4 molecule together with either PD-L1 or PD-L2.

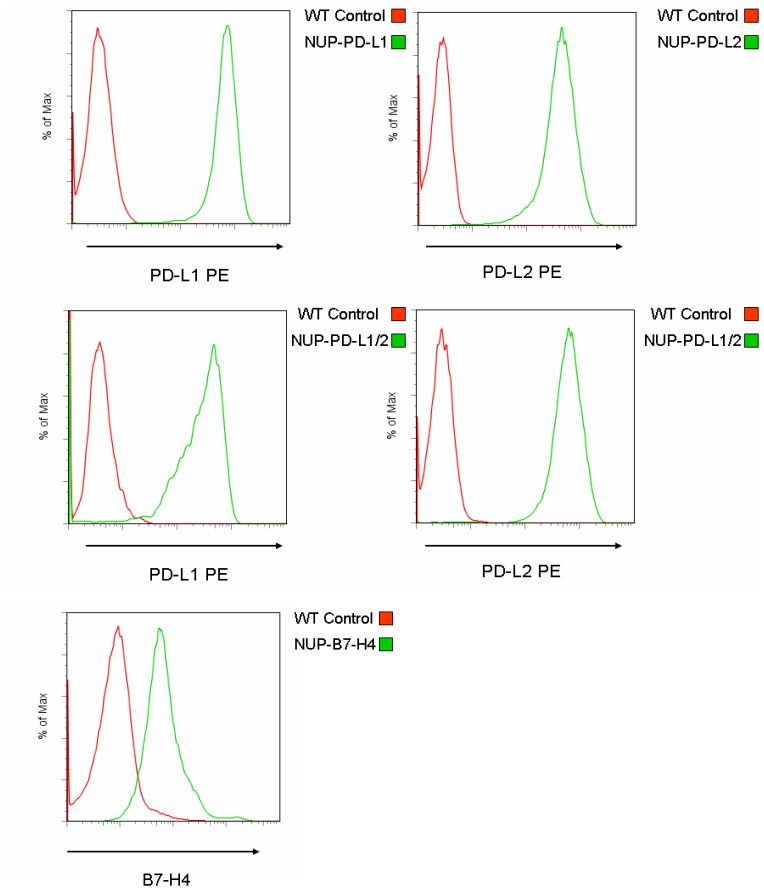


Figure 3- 3 Analysis of overexpression by modified BM cells. FACS analysis of surface expression of PD-L1, PD-L2 and B7-H4 on single and double-transduced NUP98-HOXB4 BM cells. Histograms represent expression of surface molecules with gate of GFP^+ cells.

3.3 Overexpression of PD-L1, PD-L2 and, B7-H4 on BM-derived DCs doesn't interfere with DC development

The over-expression of PD-L1, PD-L2 and B7-H4 on NUP98-HOXB4 BM-derived DCs (generated via GM-CSF or Flt3-L treatment) was confirmed using monoclonal anti-PD-L1, anti-PD-L2 and anti-B7-H4 antibodies respectively, comparing to the expression pattern of the wild type control (**Figure 3- 4** and **Figure 3- 5**). BM-derived DCs were stained with anti-CD11c together with anti-PD-L1 or anti PD-L2 or anti-B7-H4 antibodies. To monitor the expression of co-stimulatory molecules of B7 family, DCs were also stained with anti-CD11c together with anti- CD80 (B7.1) and anti-CD86 (B7.2). As shown in **Figure 3- 4** DCs generated via GM-CSF treatment exhibit a mature phenotype, expressing higher levels of B7.1 and B7.2 molecules. No significant difference was observed in the level of surface expression of co-stimulatory

molecules among control DCs and DCs overexpressing PD-L1, PD-L2, or B7-H4. Thus, forced overexpression of PD-L1, PD-L2 or B7-H4 doesn't seem to have a major influence on the differentiation and maturation of BM-derived DCs. However, on the other hand expression levels of co-stimulatory molecules (CD80 and CD86) were clearly lower in Flt3-L derived DCs comparing to GM-CSF-derived DCs (**Figure 3- 4** and **Figure 3- 5**).

Based on this important observation, we decided to choose Flt3-L derived DCs for all the downstream experiments. The low expression of co-stimulatory molecules is a key feature, making these cells better candidates for induction of T cell anergy and tolerance.

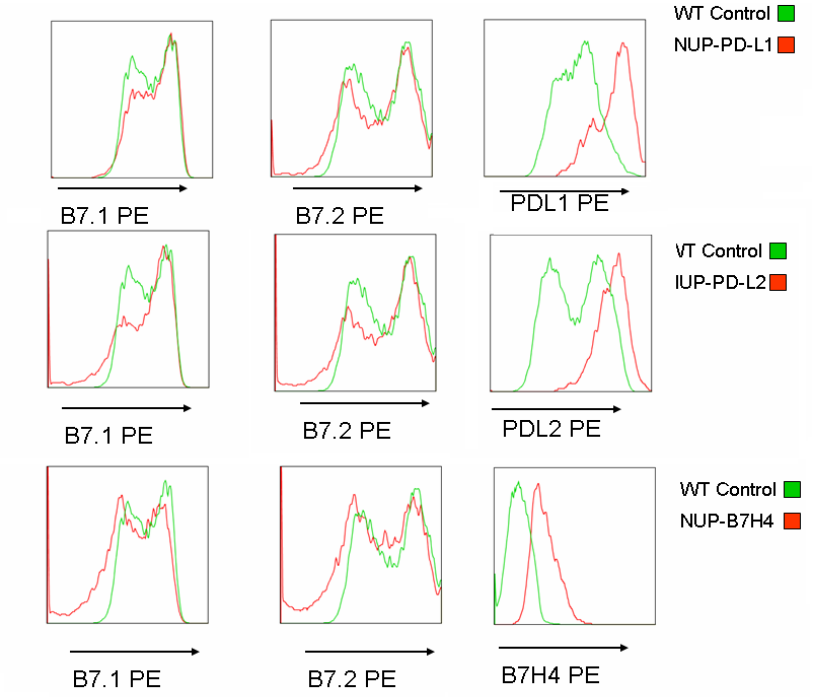


Figure 3- 4 Phenotypic analysis of modified GM-CSF-derived BMDC lines. BMDCs cultured in presence of GM-CSF, harvested at day 7 and stained with mAbs specific for CD11c together with indicated cell surface markers. Histograms represent stainings gated on $CD11c^{+}$ cells.

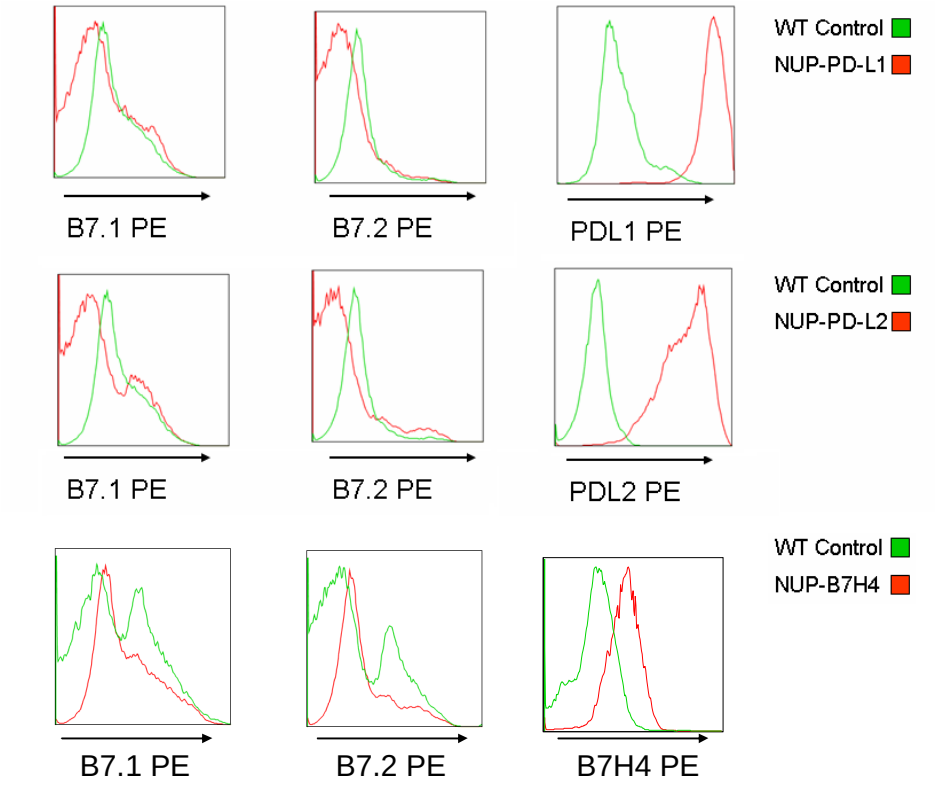


Figure 3- 5 Phenotypic analysis of modified Flt3L-derived BMDC lines. BMDCs cultured in presence of Flt3-L, harvested at day 9 and stained with mAbs specific for CD11c together with indicated cell surface markers. Histograms represent stainings gated on $CD11c^{+}$ cells.

3.4 *In vivo* over-expression of PD-L1 and PD-L2

In order to confirm the over-expression of molecules PD-L1, PD-L2 and double transfectant PD-L1/2 on DCs, B cells and T cells *in vivo*, C57BL/6J mice were lethally irradiated and reconstituted with NUP98-HOXB4-OVAs BM cells transduced with the respective molecules. Mice were sacrificed after 5-6 weeks, single cell suspensions were prepared from spleens, digested with 400 U/ml collagenase-D for 1 hr at 37°C and stained with anti-CD11c antibody together with anti-PD-L1, anti-PD-L2 mAbs or both. The undigested fraction was also stained with anti-CD19 for detection of B cells, anti-Thy1.2 for detection of T cells in combination with anti-PD-L1, anti-PD-L2 mAbs or both. As illustrated in **Figure 3- 6**, DCs, T cells and B cells from reconstituted mice clearly over-express ligands PD-L1 (in mouse reconstituted with NUP98-PD-L1 BM cells) or PD-L2 (in mouse reconstituted with NUP98-PD-L2 cells) comparing to wild type control. These cell types already express intermediate to

high levels of PD-L1 in WT spleens, but almost don't express PD-L2. Hence, our over-expression strategy induced high levels of PD-L2 expression *in vivo* while augmenting the constitutive levels of PD-L1 expression on spleen cells. Flow cytometric analysis of lymphoid and myeloid cells from the mice reconstituted with PD-L1/2 double-transfectant line, showed over-expression of both molecules comparing to wild type control (**Figure 3- 6**).

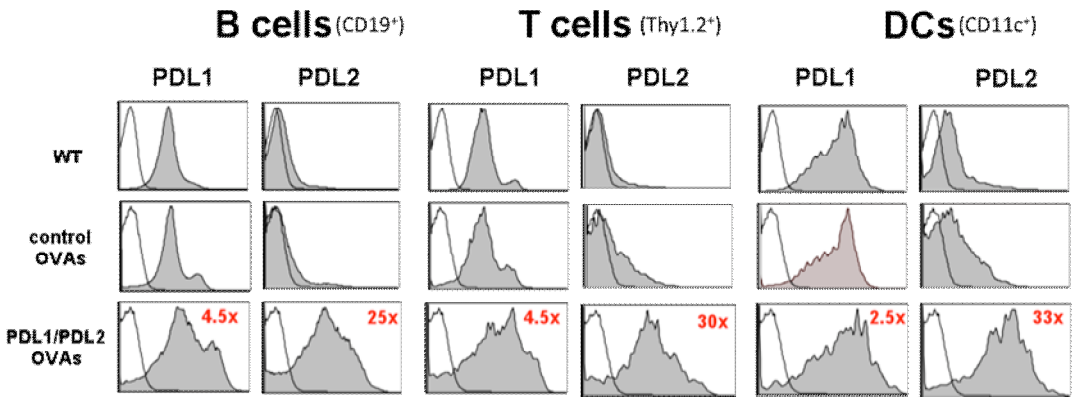


Figure 3- 6 PD-Ls expression on splenic DCs, T and B lymphocytes of BM chimeric mice. Splenocytes were stained with anti-CD11c, CD19, Thy1.2 (for visualizing DC, B and T cells respectively) and either anti-PD-L1 or anti-PD-L2 and analyzed by flow cytometry. Histograms represent staining gated on $CD19^{+,}$ $Thy1.2^{+}$ and $CD11c^{+}$ cells respectively (and GFP^{+} cells for PD-L1, PD-L2 and PD-L1/2). Numbers indicate the fold increase in mean fluorescence intensity (MFI).

3.5 Expression of PD-1 on T cell subsets

To assess the expression pattern of the inhibitory receptor, PD-1 on two subsets of T cells, we enriched $CD4^{+}$ and $CD8^{+}$ T cells from mice spleens through immunomagnetic columns and activated them on anti-CD3/anti-CD28 pre-coated plates. The expression of PD-1 monitored on day 0 (freshly isolated cells) and days 1, 2 and 3 after activation by staining cells with biotinylated anti-PD-1 antibody followed by PE-conjugated streptavidin and either anti-CD4 APC or anti-CD8 APC and analyzed by FACS. As shown in **Figure 3- 7** only a small number of naïve T cells express PD-1 on their surfaces, but on day 1 after activation with anti-CD3/CD28, the majority of $CD4^{+}$ and $CD8^{+}$ T cells start to express high levels of PD-1 and this level increases on next days. As we observed, the number of naïve $CD4^{+}$ T cells which

express PD-1 is slightly more than $CD8^+$ T cells, therefore it seems that the onset of PD-1 expression on $CD4^+$ T cells is earlier than on $CD8^+$ T cells (**Figure 3- 7**). Intracellular staining didn't show intracellular expression of PD-1 (data not shown).

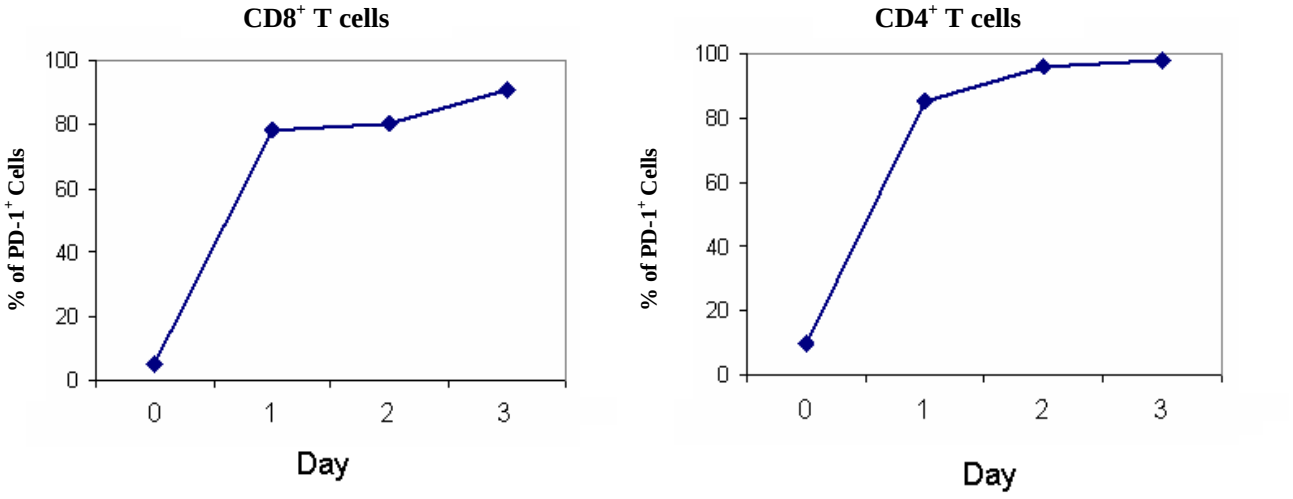


Figure 3- 7 *In vitro* expression of PD-1 on T cells during activation process. Purified $CD4^+$ or $CD8^+$ T cells were stimulated with plate-bound anti-CD3/anti-CD28. Cells harvested on days 0,1,2 and 3 after activation, stained with anti-PD-1 antibody and analyzed by FACS.

3.6 Monitoring surface expression of PD-1 on T cells *In vivo*

In order to confirm our *in vitro* observation about the PD-1 expression profile *in vivo*, $3x10^6$ CFSE-labeled OT II $CD4^+$ T cells were injected into B6-OVA transgenic mice (in which cells constitutively express OVA), intravenously. Mice were sacrificed on days 1, 2 and 3 after injection and splenocytes were stained with anti-CD4 and anti-PD-1 antibody. As shown by FACS analysis (**Figure 3- 8**), more than half of the CFSE-labeled $CD4^+$ T cells showed surface expression of inhibitory receptor PD-1 on day 1 after injection. FACS analysis on days 2 and 3 revealed progressive increase in the percentage of positive cells, as almost all $CD4^+$ T cells expressed PD-1, which is consistent with *In vitro* results. These results confirm the PD-1 expression on activated T cells, which usually occurs 24 hours after activation.

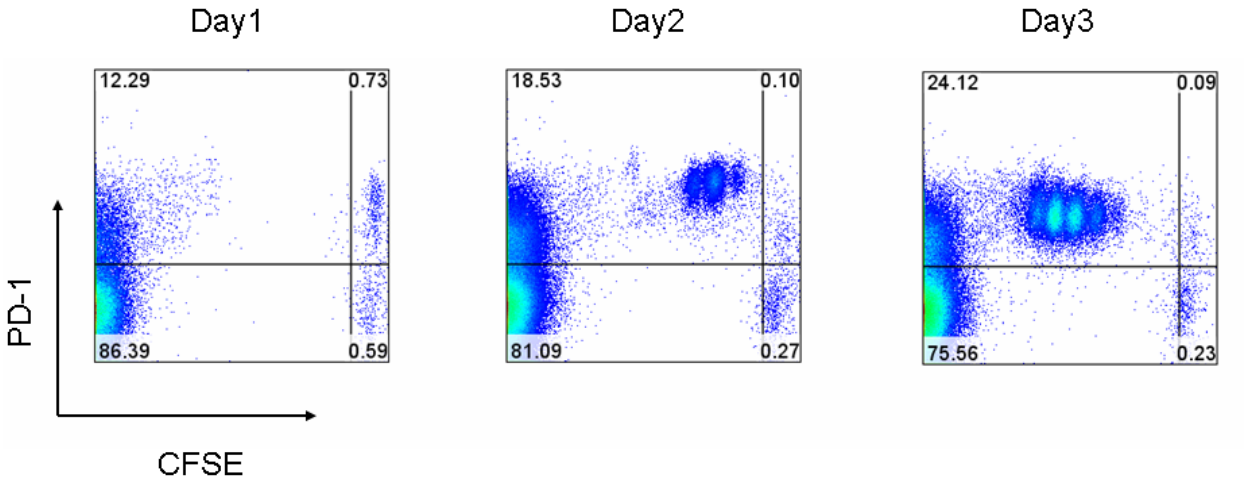


Figure 3- 8 *In vivo* PD-1 expression on OTII $CD4^+$ T cells. Mice were injected with CFSE-labeled OTII $CD4^+$ T cells, and sacrificed on days 1, 2 and 3 after injection. Single cells suspension prepared from spleens and stained with ani-CD4 and anti-PD-1 and analyzed by flowcytometry. Plots represent staining gated on $CD4^+$ cells.

3.7 Overexpressed inhibitory receptors on DCs modulate primary T cell response

To study the property of various inhibitory receptors during a primary T cell response (e.g. proliferation and cytokine secretion), genetically modified NUP98-HOXB4-OVA BM cells were *in vitro* differentiated into DCs by Flt3L treatment.

DCs were incubated with naïve CFSE-labeled $CD4^+$ T cells. The decrease in green fluorescence intensity, a parameter for cell proliferation, was assessed by FACS after 3 days co-culture and, at the same time, IFNγ content in the supernatant was measured by ELISA.

No difference was observed in T cell proliferation, since both control DCs as well as inhibitory receptor-expressing DCs vigorously induced T cell divisions as shown in **Figure 3- 9**. However, a clear discrepancy in induction of cytokine secretion was detected between control and inhibitory receptor expressing DCs. IFNγ levels in the culture supernatants were clearly lower in the co-cultures when the inhibitory receptors were involved. In particular, the combination PD-L1/PD-L2 on DCs reduced by 3-fold the secretion of IFNγ during the primary T cell response (**Figure 3- 9 B**).

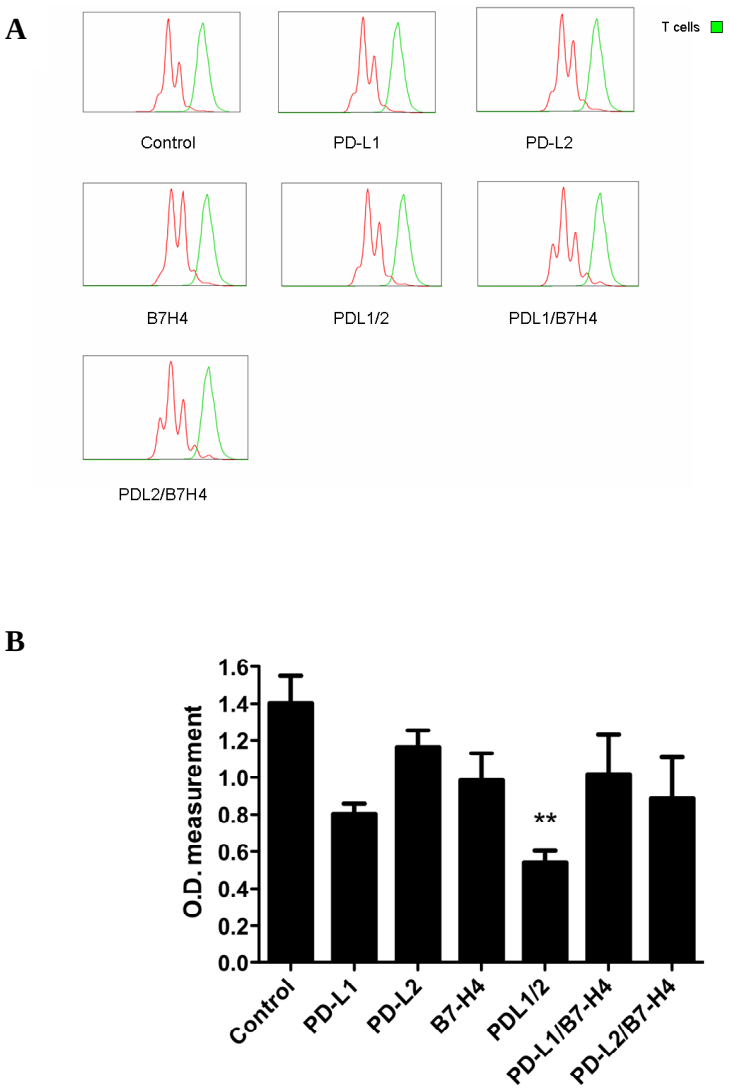


Figure 3- 9 Effects of modified DCs on T cell proliferation and cytokine production *in vitro*. **(A)** No difference was observed in T cell proliferation after 72 hr of co-culturing OT II T cells with modified DCs overexpressing inhibitory ligands or their control counterparts. **(B)** IFNγ measurement by ELISA in co-culture supernatants revealed some differences in secretion between T cells stimulated by modified DCs and T cells stimulated by WT control DCs. Data are mean±SD (n=3).

3.8 Overexpression of inhibitory receptors on APCs inhibits effector T cell function *in vivo*

We generated various chimeric mice overexpressing several inhibitory receptors with the aim to monitor their effects on T cell proliferation and function *in vivo*.

NUP98-HOXB4 lines derived from OVA-expressing BM cells were genetically modified with PD-L1, PD-L2, B7H4 and several combinations (PD-L1/PD-L2, PD-L1/B7-H4 and PD-L2/B7-H4). After successful enrichment by cell sorting and cell transplantation into lethally irradiated mice, we obtained chimeric mice expressing the gene(s) of interest. These genetically modified mice were subsequently used to investigate the fate of adoptively transferred OVA-specific $CD4^+$ $Ly5.1^+$ ($C45.1^+$) T cells. 10 days after T cell transfer, animals were sacrificed and the number of $Ly5.1^+$ T cells present in the spleen as well as their capacity to secrete effector cytokines, such as IFNγ, was assessed. To study the influence of the DC maturation stage on T cell function *in vivo* in presence of the overexpressed inhibitory receptors, one set of mice was injected with an agonistic stimulatory anti-CD40 antibody, known to trigger DC maturation.

All chimeric mice expressing different sets of inhibitory receptors showed reduced numbers of transferred T cells when compared to the control mice pointing to a negative effect of the inhibitory receptors on T cell survival (**Figure 3- 10A**). In particular, the combination PD-L1/PD-L2 was most efficient in inhibition of T cell proliferation (6% versus 14% in control mice).

At the same time, the capacity of cytokine production by $Ly5.1^+$ transferred T cells, was monitored by intracellular staining as well as by ELISA upon Ag specific stimulation *in vitro*. Approximately 10% of OVA-specific T cells obtained from control chimeric mice secreted high amounts of IFN-γ, data were also confirmed by the measurement of the cytokine in the supernatant (**Figure 3- 10B**). However, in the case of T cells obtained from chimeric mice overexpressing PD-L1, PD-L2 and their combinations, the property to secrete IFNγ was almost completely abolished (only 1% producing cells).

However, the scenario changed when DCs were activated by the treatment with anti-CD40 antibody. The number of adoptively transferred T cells was still reduced when compared to $Ly5.1^+$ T cells of the control chimeric mouse, but the IFN-γ secretion

capacity was restored as shown by intracellular staining and INF-γ measurement in the culture supernatant (**Figure 3- 10B**).

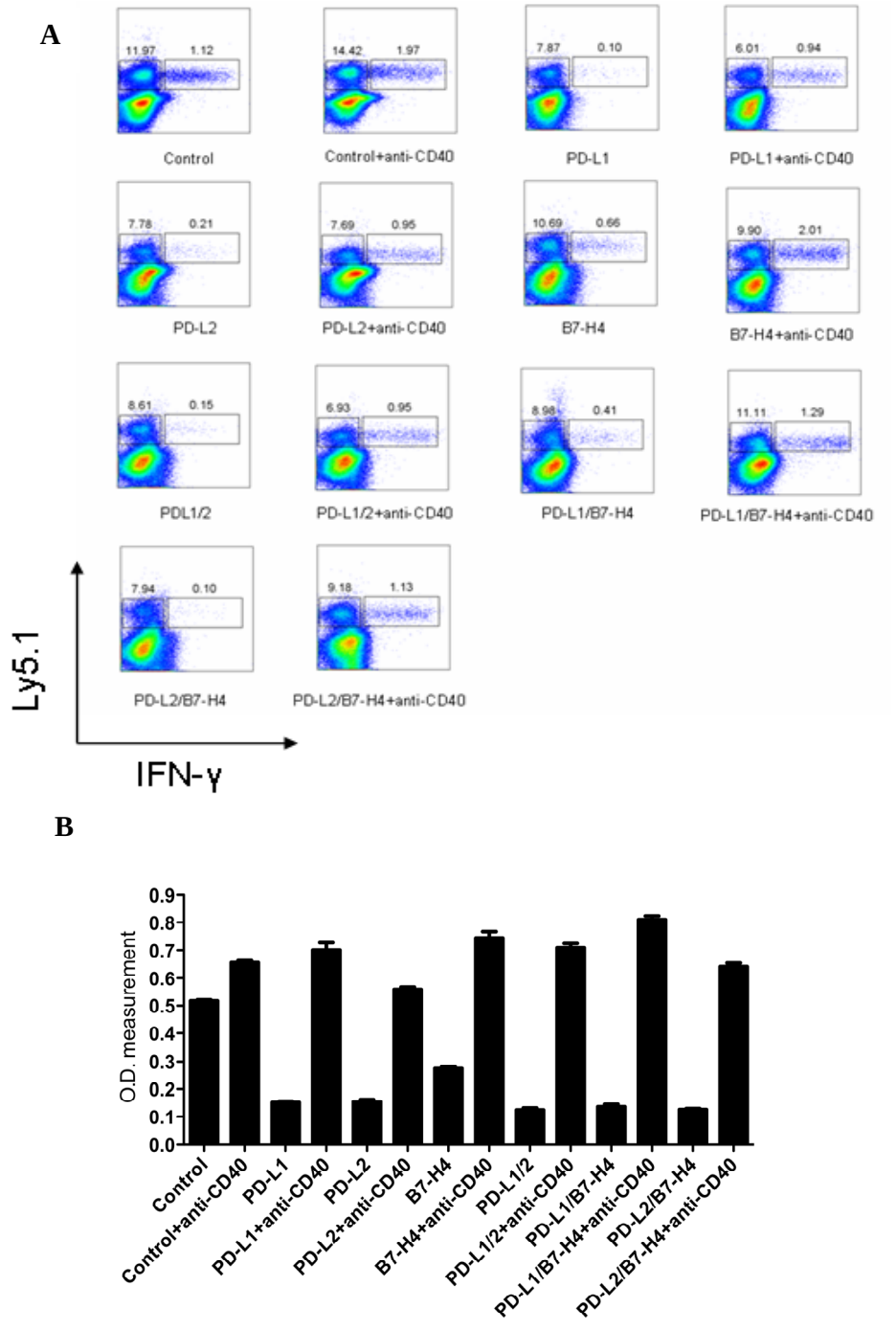
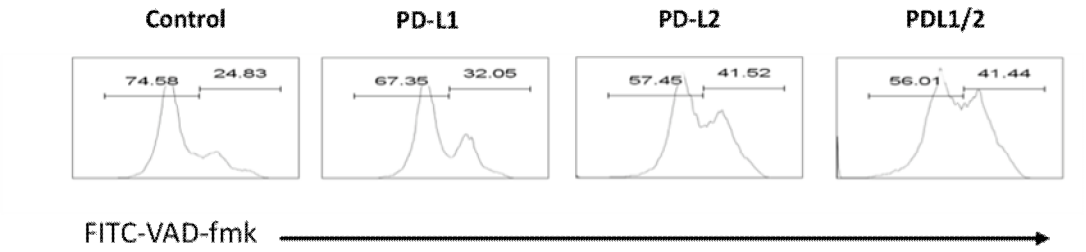


Figure 3- 10 Abrogation of IFN-γ by overexpression of PDLs and B7-H4 *in vivo*. **(A)** Intracellular production of IFN-γ by OVA-specific OT II Ly5.1^{+} T cells transferred to chimeric mice over-expressing various inhibitory molecules of b7 family. Splenocytes were cultured for 6 hrs in presence of 10^{-9} M $OVA_{232-339}$ peptide and then, fixed, permeabilized and stained with anti-Ly5.1 conjugated to PE antibody and anti- IFN-γ-APC. **(B)** IFN-γ levels in supernatants of murine splenocyte cultures, measured by ELISA. Data are mean±SD (n=3).

We also noticed that the transferred CD4 T cells showed elevated rates of apoptosis when transferred into PD-L1, PD-L2 or PD-L1/PD-L2 overexpressing chimeric mice. The splenocytes were stained with anti-ly5.1, anti-CD4 together with CaspACE FITC-VAD-fmk *in situ* marker which is a cell permeable caspase inhibitor that binds to activated caspases in the cells. As shown in **Figure 3- 11**, a bigger fraction (41% vs 24%) of T cells isolated from chimeric mice overexpresing PDLs show high levels of activated caspases comparing to WT control, indicating a higher percentage of cells which are undergoing apoptosis after encounter with APCs overexpressing PDLs.

Figure 3- 11 Overexpression of PDLs on APCs induced higher levels of apoptosis on transferred T



cells *in vivo*. Splenocytes from chimeric mice injected with $CD4^+$ $Ly5.1^+$ T cells were stained with anti-CD4, anti-Ly5.1 and FITC-VAD-fmk *in situ* marker and analyzed by FACS. Histograms represent stainings gated on $CD4^+Ly5.1^+$.

3.9 Transmembrane form of OVA as a model low-dose antigen failed to prime the T cells efficiently *in vivo*

The previous observations in chimeric mice over-expressing regulatory PD-1 ligands showed no pronouncing effect on proliferation of adoptively transferred OVA-specific T cells (**Figure 3- 10**). Hence, we asked the question whether the amount of antigen *in vivo* could affect the outcome of those experiments. In BM chimeras derived from OVAs-expressing NUP98-HOXB4 cells the ovalbumin is continuously released via secretion. This massive amount of Ag is accessible for all APCs in different tissues and could be taken up, processed and presented to the OVA-specific T cells throughout bystander APCs efficiently, leading to their activation and robust proliferation. In order to avoid the massive Ag presentation via bystander APCs, in this system *in vivo*, we generated NUP98-HOXB4 BM lines overexpressing PD-L1, PD-L2 or their combination (PD-L1/2) together with the model antigen

transmembrane-OVA. The purpose here was to avoid secretion of high amounts of antigen at *in vivo* environment. The chimeic mice were generated with respective BM lines, as described before. Adoptive transfer of OTII CD4+ T cells into chimeric hosts resulted in their optimal proliferation as monitored in short term (after 3 days via CFSE dilution pattern) as well as long term (after 12 days). We detected no difference in numbers of T cells 12 days post transfer between 4 experimental groups tested (**Figure 3- 12A and B**). We also assessed the IFN-γ production capability of these cells upon co-culture of splenocytes of chimeric mice with different concentrations (10^{-3}-10^{-6} M) of $OVA_{323-339}$ peptide. Exposure to peptide didn't lead to any detectable production of IFN-γ as examined by intracellular cytokine staining. (**Figure 3- 13**) This observation led us to the conclusion that the dose of antigen in the present system (provided as transmembrane from of OVA [OVA_{tm}]) is not potent enough to prime the transferred Ag-specific T cells efficiently, hence they failed to respond to secondary activation upon interaction with Ag-loaded APCs in splenocyte culture.

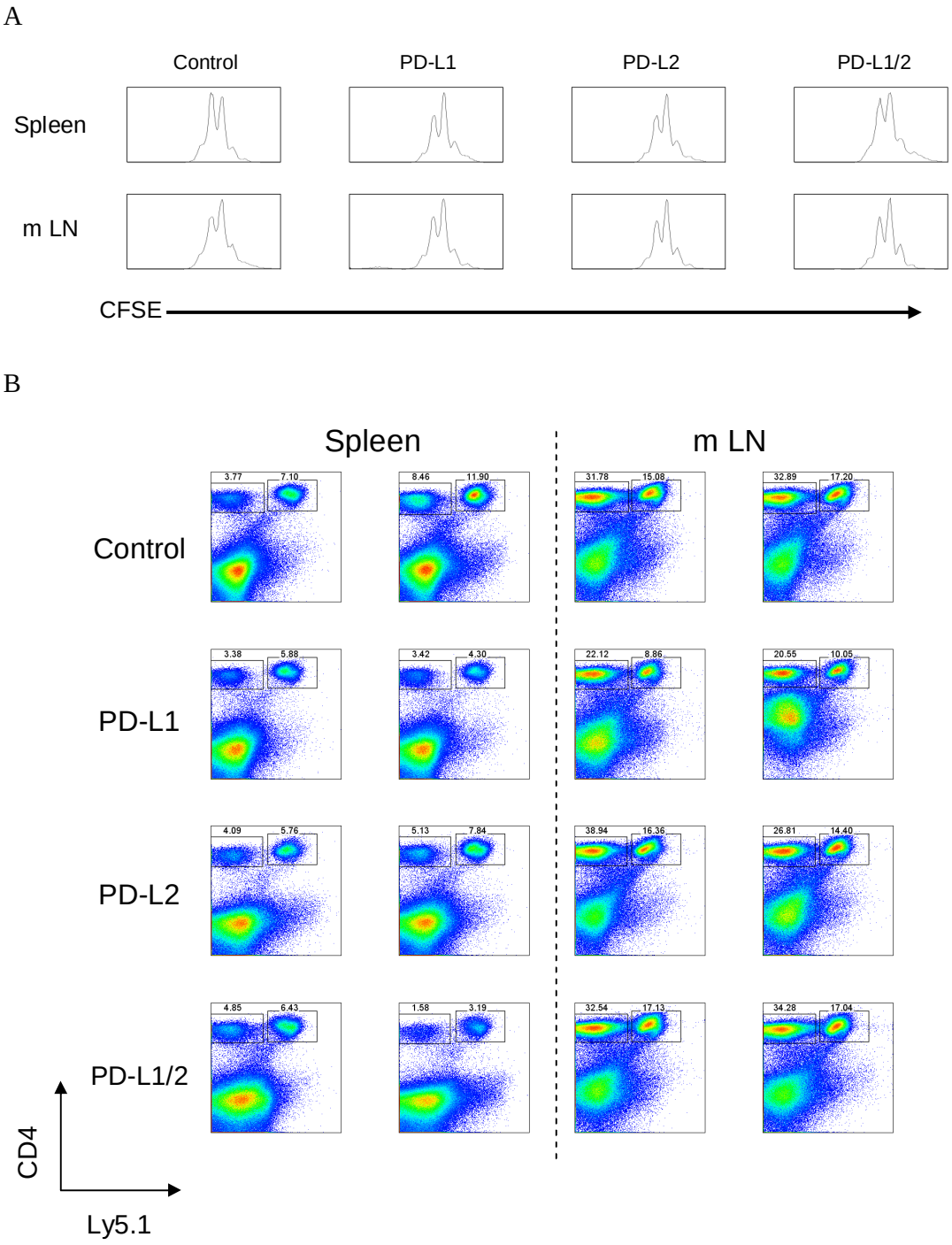


Figure 3- 12 OVAt expression *in vivo* is sufficient to prime T cell proliferation. **(A)** 3 days post transfer, no significant difference in terms of T cell proliferation and numbers were observed among different OVAt-expressing chimeric mice adoptively transferred with OVA-specific $Ly5.1^{+}CD4^{+}$ T cells. **(B)** Proliferation pattern of adoptively transferred CFSE-labeled OT II T cells in spleens and mesenteric lymph nodes (mLN) monitored by CFSE dilution 12d post-transfer. Gatings represent the percentages of transferred T cells 12 days post transfer.

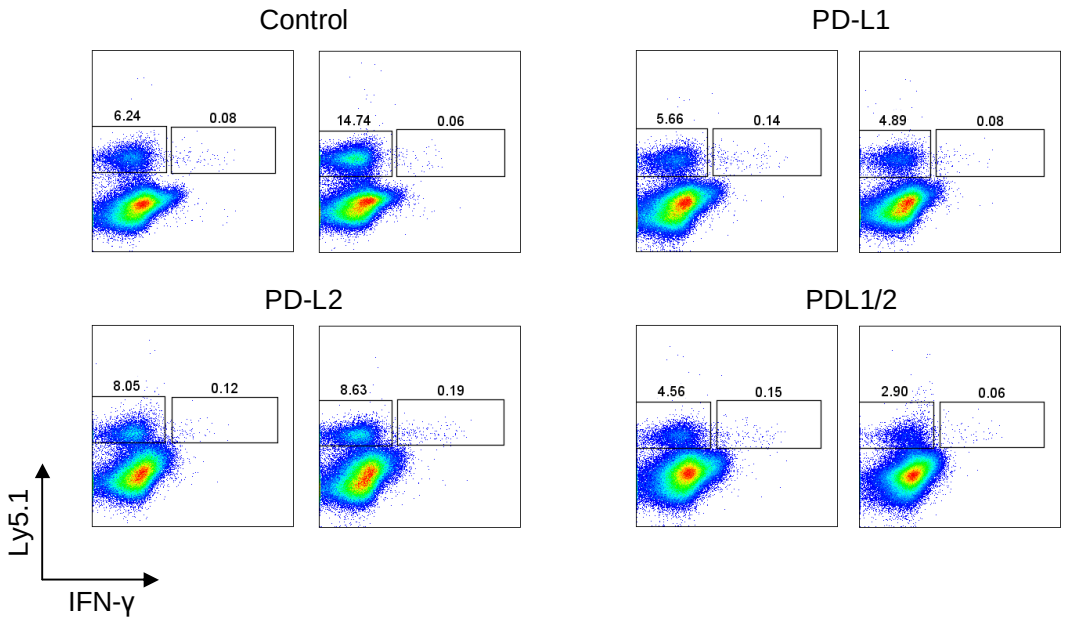


Figure 3- 13 OVAt-stimulated T cells fail to produce IFN-γ. Intracellular cytokine staining revealed no significant levels of IFN-γ production by transferred T cells 6 hrs after incubation of splenocytes with $OVA_{323-339}$ peptide.

3.9.1 PD-L2 over-expression in bone marrow chimeras leads to dramatic decrease in size of B cell repertoire

In order to evaluate the multi-lineage reconstitution of chimeric mice reconstituted with different modified NUP98-HOXB4 lines we assessed the blood phenotypes 6 weeks after infusion of BM cells. T cell:B cell ratios in blood samples were assessed with flowcytometry as determined by staining of blood mononuclear cells with anti-Thy1.2 and anti-CD19 mAbs to visualize T and B lymphocytes, respectively. While the T:B cell ratios in the WT control and PD-L1 overexpressing chimeras were almost normal, surprisingly the PD-L2 and PD-L1/PD-L2 mice showed an altered phenotype in this regard with a dramatic decrease in B cell proportion. However the T cell proportions remained almost comparable to WT mice (**Figure 3- 14A**). The granulocyte proportions were also remained unchanged (data not shown).

In order to investigate whether the altered phenotype reflects the changed ratios in peripheral lymphoid organs as well we carried out the phenotyping on spleens and lymph nodes of chimeric mice. Results indicated the same decrease in B cell proportions in lymphoid tissues of PD-L2-overexpressing animals (**Figure 3- 14B**).

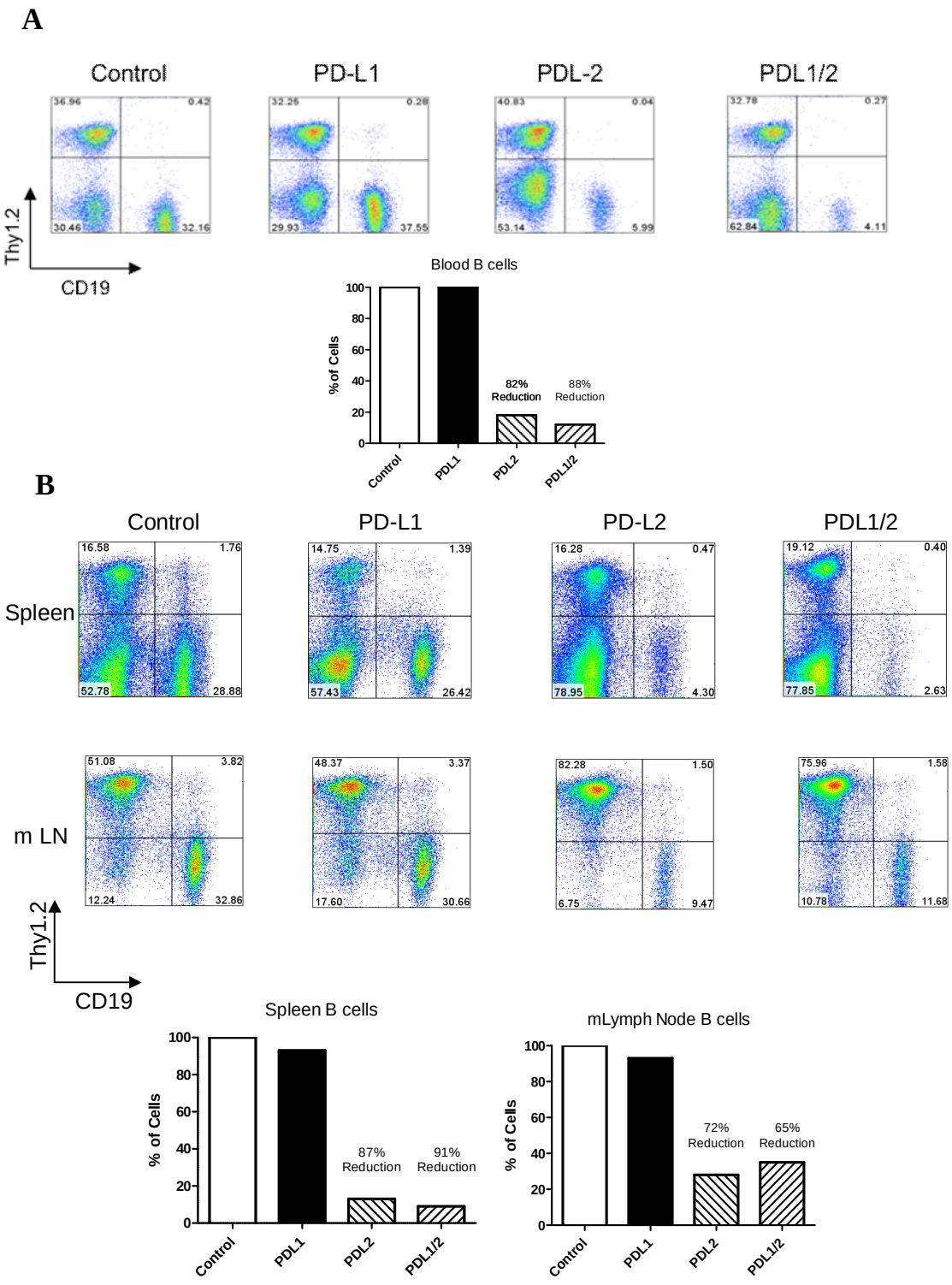


Figure 3- 14 Altered T:B cell ratios in chimeric mice over-expressing PD-L2 molecule. **(A)** Blood, (**B)** spleen and LN phenotypes. T:B ratios determined by staining the cells with anti-Thy1.2 and anti-CD19 mAbs to visualize T and B lymphocytes respectively, and analyzed by FACS. Graphs show the percentage of B cells of indicated organs comparing to wild type chimeric mice. Data shown refer to one representative mouse/group.

Our further analysis of BM cells from WT and PD-L1 chimeric mice revealed an apparent increase in proportion of $B220^{+}CD19^{-}$ population suggesting that the PD-L2-expressing B cells fail to further maturate and express terminal differentiation marker, CD19 and stop at early time points after maturation (**Figure 3- 15**).

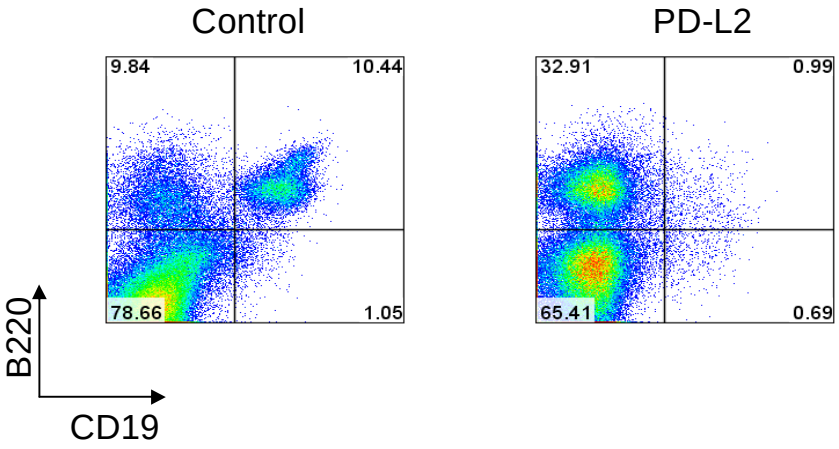


Figure 3- 15 Impaired BM phenotype in PD-L2 overexpressing chimeras. Absence of terminally differentiated $B220^{+}CD19^{+}$ B cells in BM of PD-L2-overexpressing chimeric mouse. After erythrocyte lysis, BM cells were stained with anti-CD19 and anti-B220 mAbs and analyzed by FACS.

3.9.2 Inducible over-expression of PD-L2 leads to a decrease in B cell pool *in vivo*

Since the dramatic loss of B cells observed in BM chimeras was a result of constitutive overexpression of PD-L2 we asked the question whether inducible over-expression of PD-L2 on B cells and other cells after their normal differentiation *in vivo* might affect the B cell repertoire as well and lead to their death/deletion. To address this question we generated BM chimeric mice in which the expression of PD-L2 was inducible by i.p injection of doxycyclin. As shown in **Figure 3- 16**, these animals show normal proportions of B cells in blood 5 weeks post-reconstitution. 3 days after induction of PD-L2 expression via doxycycline treatment, almost 50% of blood cells expressed PD-L2 and this led to about 40% drop in B cell percentage (calculated as average number of 3 individual mice). Mice were again bled and analyzed 6 days after induction of PD-L2 expression and as it is shown the B cell proportions were back to normal levels. This could be due to continuous refilling of periphery by new B cells coming from BM as well as the fact that the doxycyclin which is present *in vivo* is

diluted out over time and cells start to down-regulate the induced PD-L2 expression as the inductive signal drops.

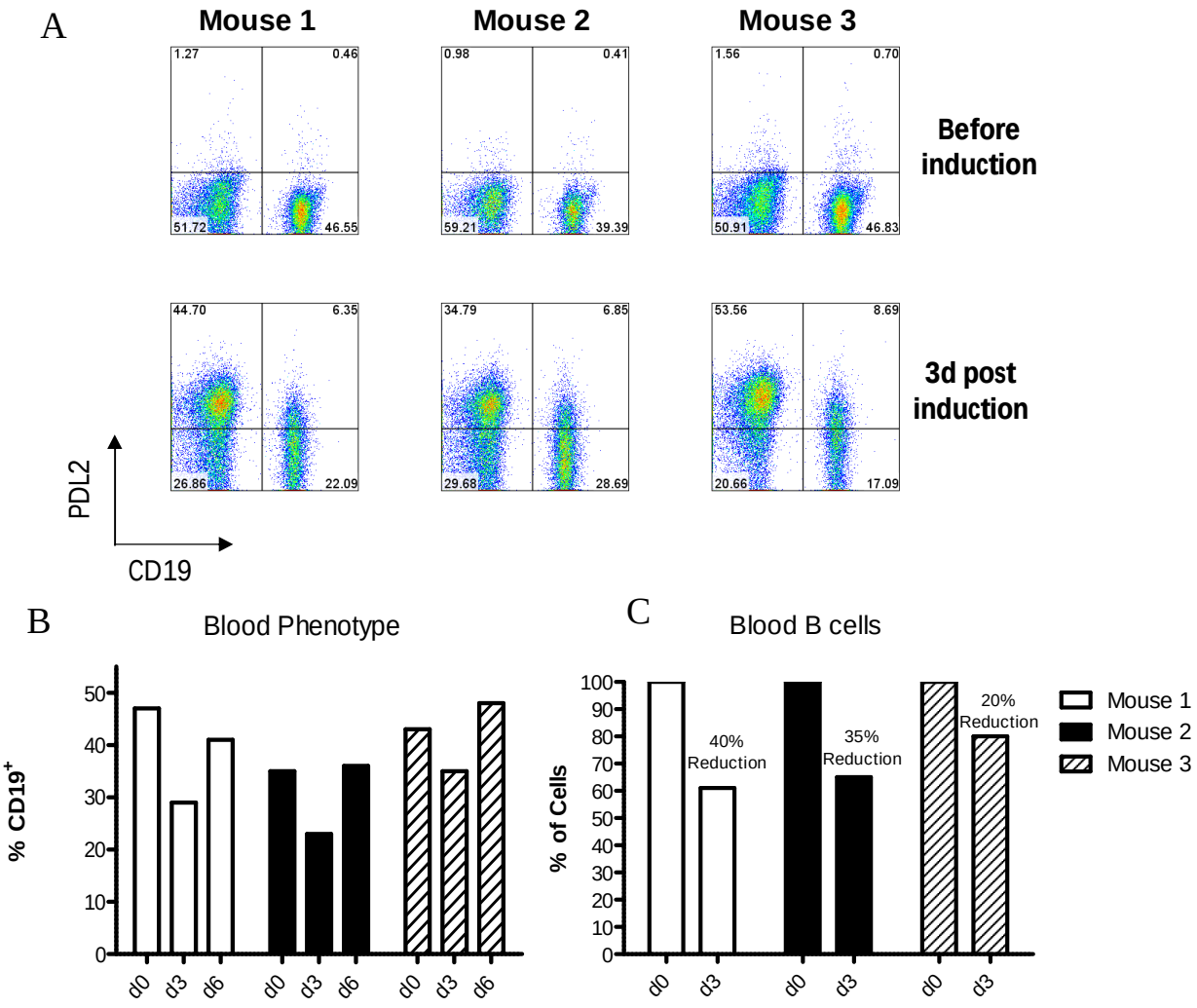


Figure 3- 16 Induction of PD-L2 expression results in partial B cell depletion *in vivo*. **(A)** The level of PD-L2 expression on $CD19^{+}$ Blood B-lymphocytes before induction of PD-L2 expression and 3 days post-induction. **(B)** Percentages of B cells in blood before and after in induction of PD-L2 expression. **(C)** The graph shows the percentage of B cells 3 days after induction of PD-L2 expression comparing to d0 (before induction). Numbers indicate % reduction in B cell compartment.

3.9.3 B cells expressing high levels of PD-L2 exhibit a more apoptotic phenotype

The wide range of PD-L2 expression on B cells after induction also provided us with opportunity to look further into apoptotic phenotype of these remaining cells based on different expression levels. As it's shown in **Figure 3- 17**, high levels of PD-L2 on B cells renders them more apoptotic as shown by staining with FITC-VAD-fmk, a flurochrome-labeled caspase inhibitor which visualizes expression of activated caspases in cells. This observation shows that high levels of PD-L2 on surface of B cells increase the level of apoptosis in these cells.

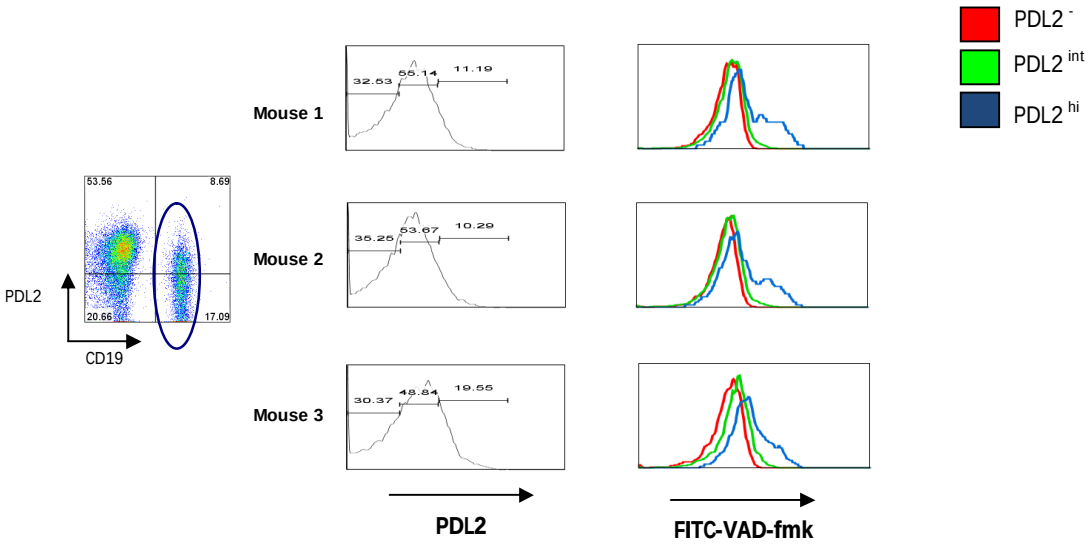


Figure 3- 17 Elevated rate of apoptosis on B cells expressing high levels of PD-L2. Blood B cells were co-stained with FITC-VAD-fmk and CD19 and were analyzed on FACS.

3.9.4 Overexpression of PD-L2 on myeloid cells decreases the B cell numbers *in vivo*

One of the possible mechanisms by which PD-L2 expression might lead to B Cell death/deletion could be the engagement of PD-1 or another unknown molecule on B cells by PD-L2 over-expressed on other cells such as myeloid cells. To investigate this hypothesis we generated BM chimeras over-expressing PD-L2 only on myeloid cell repertoire, namely DCs and macrophages while the T cells and B cells and other cell

types don't express it. This has been achieved by reconstituting the lethally irradiated mice with RAG2$^{-/-}$ PD-L2 overexpressing BM cells. In order to reconstitute the rest of the repertoire including the lymphoid cells mice also received wild type bone marrow cells. As illustrated in **Figure 3- 18**A in these chimeric mice PD-L2 overexpression is restricted to myeloid cells while other cells do not express the molecule. Further analysis of blood T:B ratios revealed an average of 60% reduction in B cells percentage comparing to WT chimeras, while the T cell proportions were not changed in individual mice evaluated (**Figure 3- 18B**).

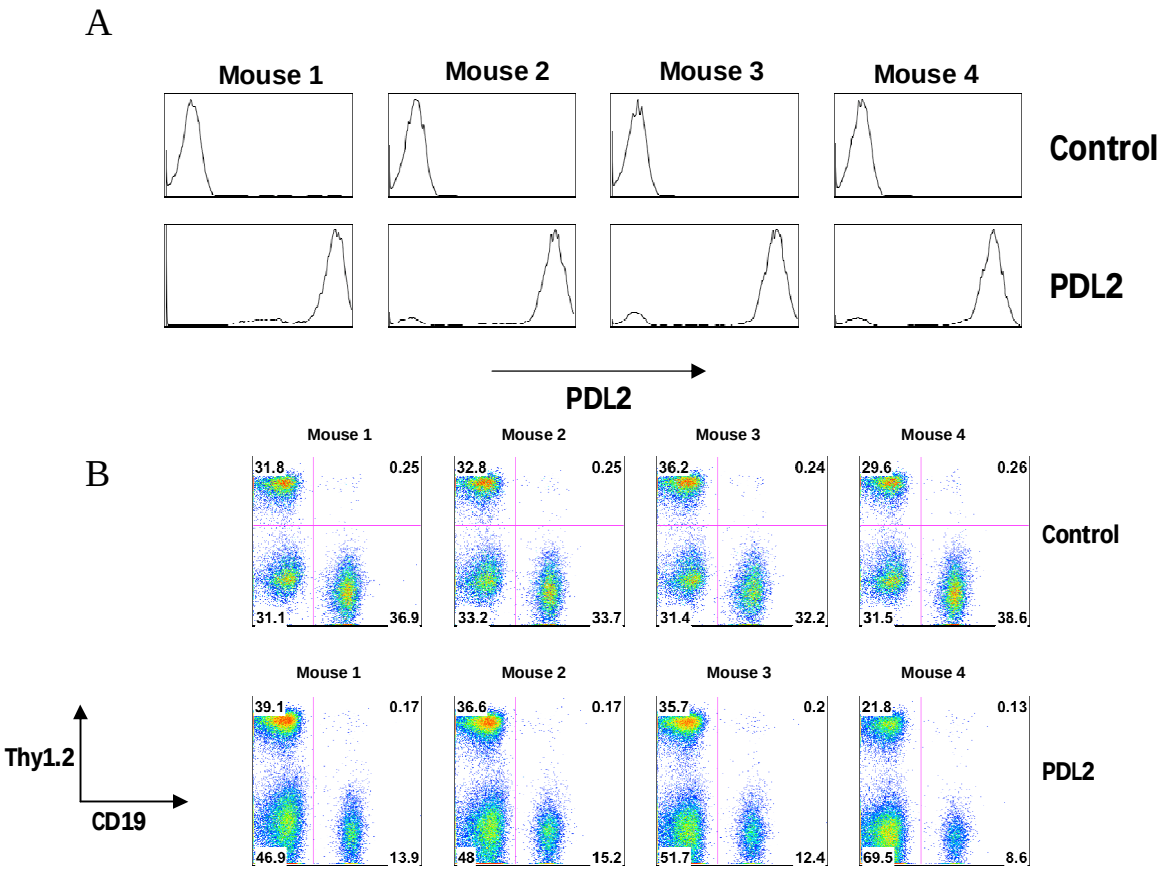


Figure 3- 18 PD-L2 overexpression by myeloid cells reduces the B cell numbers *in vivo* **(A)** PD-L2 overexpression on myeloid cells within blood of chimeric mice reconstituted with RAG KO PD-L2 BM cells. Histograms represent the gatings on MAC1/Gr.1$^{+/+}$ cells. **(B)** T:B cell ratios within blood of WT and mixed RAG$^{-/-}$ chimeras.

3.9.5 Inducible overexpression of PD-L2 *in vivo* leads to decreased numbers of B cells in vivo

We also repeated the same type of experiments (as in 3.9.3), using chimeras over-expressing PD-L2 on myeloid cells only upon induction. As shown in **Figure 3- 19A** the doxycycline treatment of these chimeric mice leads to overexpression of PD-L2 on myeloid cells but not on other cells e.g. B cells. The results showed almost 36% reduction in B cell percentage 5 days post induction of PD-L2 expression on myeloid cells (**Figure 3- 19B**). In these mice the T cells compartment within blood was not changed after induction of PD-L2 expression as shown in Fig. 30 C. Together these data suggest that inducible PD-L2 overexpression on DCs and macrophages can lead to deletion and/or death of B cells.

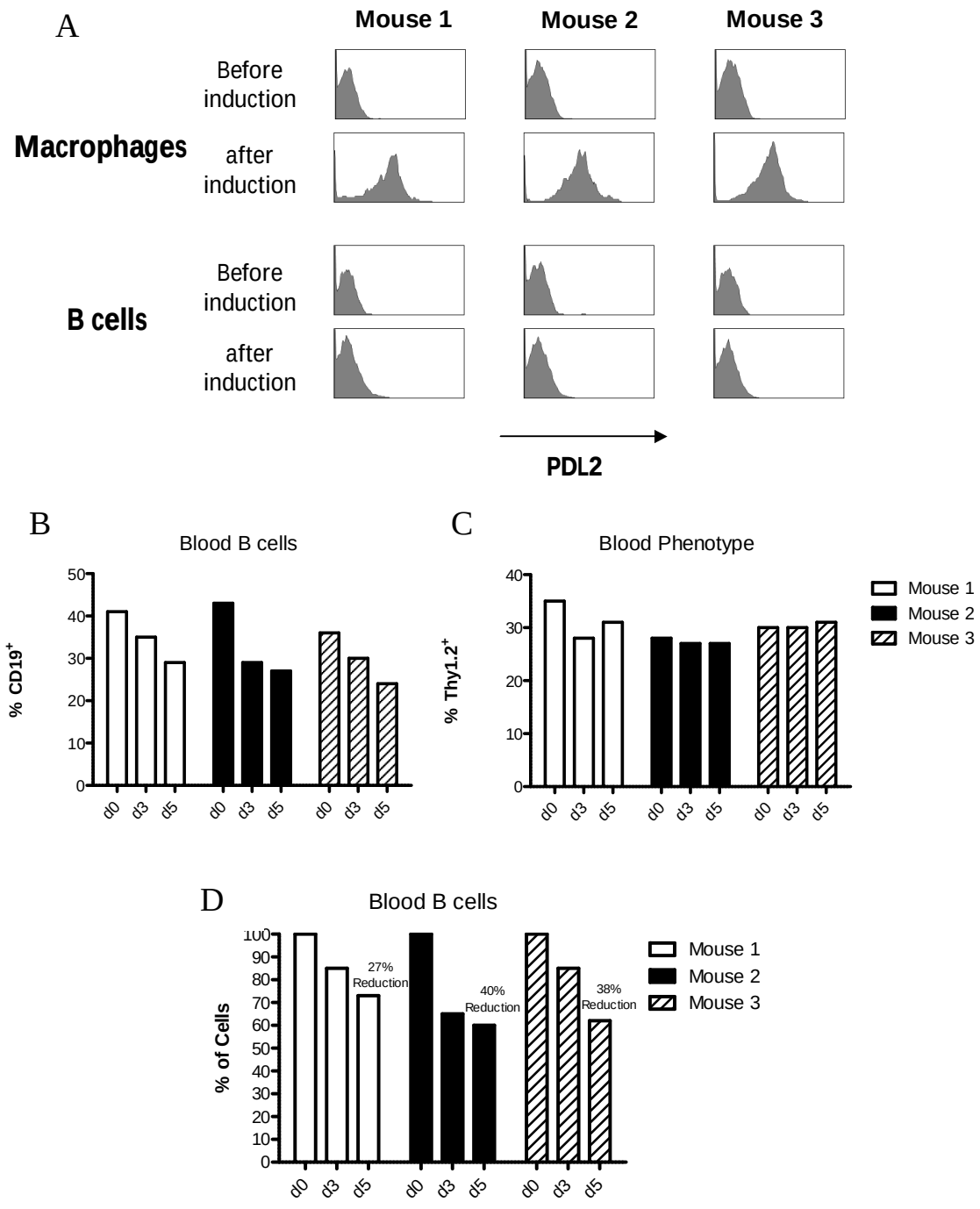


Figure 3- 19 Induced PD-L2 overexpression by myeloid cells reduces the B cell numbers *in vivo* **(A)** PD-L2 expression on blood myeloid cells of RAG2$^{-/-}$ PD-L2 inducible mixed BM chimeric mice after induction via i.p injection of 2 mg Doxycycline. Histograms represent gatings on MACI^{+} cells for macrophages and CD19^{+} cells for B cells. **(B,C)** Percentages of B (B) and T (C) cells within blood on day 0 (before induction of PD-L2 expression) and days 3 and 5 post induction. **(D)** The graph shows the percentage of B cells 3 and 5 days after induction of PD-L2 expression comparing to d0 (before induction). Numbers indication % reduction in B cell compartment.

3.9.6 PD-1 might not be responsible for PD-L2 mediated loss of B cell repertoire *in vivo*

Since we noticed that DCs and macrophages expressing high levels of PD-L2 are able to ablate significant numbers of B cells *in vivo* we wondered whether this effect could be mediated through engagement of PD-1 on B cells. To address this question we treated a group of $RAG^{-/-}$ PD-L2 mice with blocking anti-PD1 antibody and followed the percentages of B and T cells on a daily basis for 3 days post-PD-1 blockade. As shown in **Figure 3- 20** The PD-1 blockade did not rescue the loss in B cell repertoire (even further reduced the b cell numbers), suggesting that the observed phenomenon might not be due to PD-L2 engagement with its known receptor PD-1.

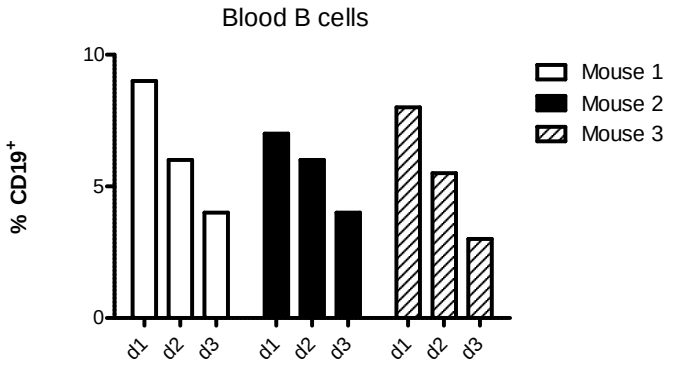


Figure 3- 20 PD-1 blockade does not rescue the loss of B cells mediated by PD-L2 overexpression *in vivo*. 3 mice were i.v injected with 110 µg anti-PD1 blocking antibody. The percentage of CD19+ B cells in peripheral blood was monitored on days 1, 2 and 3-post injection by flowcytometric analysis.

3.10 Overexpression of HVEM on *in vitro* generated BMDCs

To analyze the effects of over-expression of HVEM on DC phenotype, we generated Flt3L-derived DCs from WT NUP98-HOXB4 BM cells or NUP98-HOXB4 BM cells overexpressing HVEM. The Flt3-L derived BMDCs were analyzed for their surface expression of HVEM as well as a group of co-stimulatory molecules, including CD80, CD86, CD40 and MHC Class II by flowcytometry. The expression of HVEM was evidently increased (almost 50 fold increase) on Flt3-L derived DCs while not affecting the expression of costimulatory markers comparing to wild type controls DCs (**Figure 3- 21**).

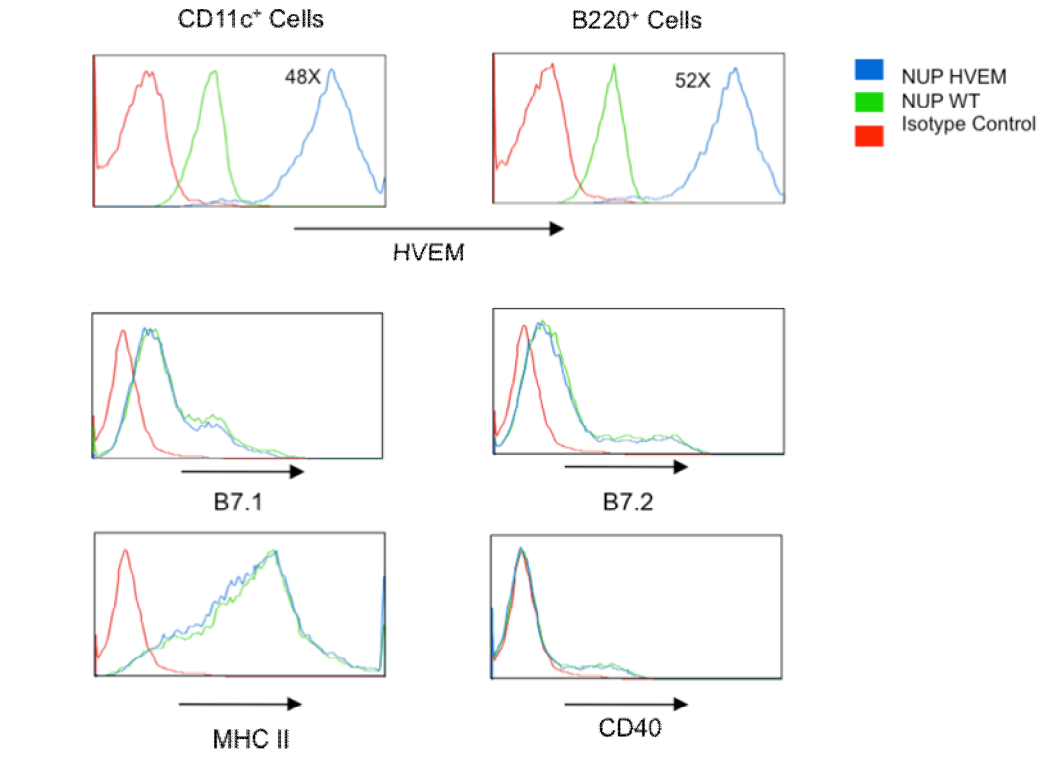


Figure 3- 21 Phenotypic analysis of HVEM overexpressing BMDCs. Overexpression of HVEM on Flt3L BMDCs doesn't affect their surface expression of costimulatory markers. Flt3L-derived BMDCs were analyzed on d9 for their expression of HVEM, CD80, CD86, CD40 and MHC Class II by flowcotometry. Numbers indicate fold increase in MFI.

3.11 Overexpression of HVEM by BMDCs has a minor effect on Ag-specific T cell proliferation *in vitro*

To evaluate the the regulatory effects of HVEM overexpressing BMDCs on T cell proliferation we sorted the γ-irradiated Flt3L-derived OVA-expressing BMDCs (d8) into $CD11c^{+}B220^{-}$ and $CD11c^{+}B220^{+}$ fractions and co-cultured them with purified OTII $CD4^{+}$ T cells for 3 days and T cell proliferation was measured by [H^{3}] thymidine incorporation assay. As shown in **Figure 3- 22**, the HVEM-overexpressing DCs efficiently primed the $CD4^{+}$ T cells for Ag-specific proliferation, however the proliferation rate induced by these cells, was lower comparing to T cells stimulated by WT BMDCs.

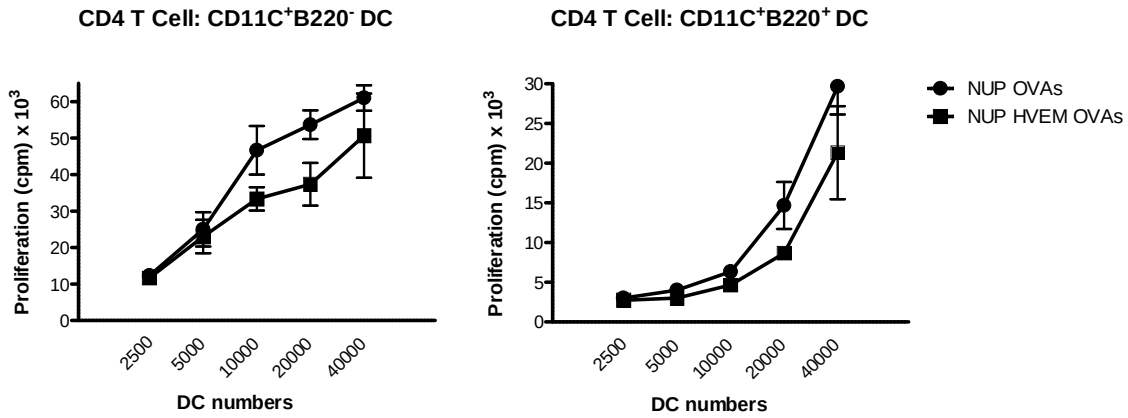


Figure 3- 22 HVEM overexpressing DCs slightly decrease the T cell proliferation *in vitro*. The OVAs FLt3L-derived BMDCs were sorted into two fractions ($CD11c^+B220^-$ and $CD11c^+B220^+$) by cell sorter and tirated numbers of cells were co-cultured with purified OTII $CD4^+$ T cells for 72 hr. Cells were incubated with 1μCi [H^3] Thymidine for the last 16hr. The thymidine incorporation by T cells was quantified by scintillation counting. Data are mean±DC (n=3).

3.12 Activated CD4 T cells upregulate the HVEM receptor, BTLA *in vitro*

To make sure that the HVEM receptor, BTLA (which exerts co-inhibitory effects) is expressed and upregulated on activated T cells we thought to determine the kinetics of BTLA expression upon T cell activation, we stimulated the $CD4^+$ T cells with anti-CD3 antibodies and followed their pattern of BTLA expression over a 5d period by flowcytometry. As shown in **Figure 3- 23**, T cells started to upregulate the BTLA as early as 24hr-post stimulation with anti-CD3 antibodies, the peak of expression was observed in day 2 and started to decline later on but it was still detectable at lower levels on d5 post-stimulation.

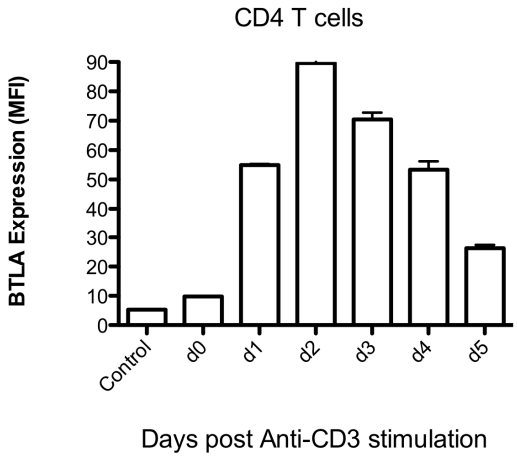


Figure 3- 23 The kinetics of BTLA expression on *in vitro*-activated CD 4^+ T cells. The $CD4^+$ T cells were immunomagnetically purified from spleens of B6 mice and plated in anti-CD3-coated cell culture plates. The BTLA expression on cells was monitored on d0 and over a 5d period post activation using anti-BTLA mAb and was analyzed by flowcytometry. The bars represent the MFI of BTLA expression on $CD4^+$ T cells.

3.13 Over-expression of HVEM by myeloid cells inhibits T cell proliferation

Since HVEM overexpressing DCs slightly decreased T cell proliferation *in vitro* we asked the question whether they could affect the proliferation rate *in vivo*. To address this question we generated BM chimeric mice over-expressing HVEM on myeloid compartments (DCs and macrophages) by reconstitution of lethally γ-irradiated WT mice with RAG2-deficient BM cells overexpressing HVEM. Since RAG-deficient progenitors can only give rise to myeloid cells we co-injected the mice with WT BM cells which replenish the lymphoid repertoire by giving rise to T, B and NK cells. 8 weeks post-reconstitution the mice were analyzed for overexpression of HVEM on splenic DCs by flowcytometry. As shown in **Figure 3- 24**, although WT immature DCs already express decent levels of HVEM, DCs of chimeras reconstituted with

RAG$^{-/-}$ HVEM cells expressed almost 20-fold higher levels of HVEM *in vivo* comparing to the animals reconstituted with RAG$^{-/-}$ cells or WT B6 mice splenic DCs.

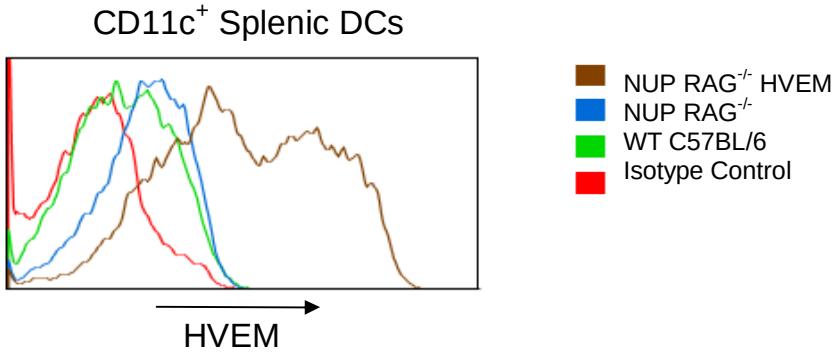


Figure 3- 24 HVEM overexpression on splenic DCs of chimeric mice. The spleens from RAG$^{-/-}$ or RAG$^{-/-}$ HVEM BM chimeric mice or WT C57BL/6 mouse were digested with 400 U/ml collagenase-D for 1h in 37° C followed by single cell preparation. The splenocytes were stained with anti-CD11c and anti-HVEM antibodies and analyzed by FACS. Histograms represent stainings gated on CD11c^{+} cells.

To examine functional capacities of these DCs, we adoptively transferred 10^6 purified Ly5.1^{+} CD4^{+} OT II T cells into chimeric mice. The animals were sacrificed 12 d later and the T cell proliferation was assessed by determining the proportion of transferred Ly5.1^{+}CD4^{+} T cells within spleen and mesenteric lymph nodes, via flow cytometry (**Figure 3- 25**). We observed an approx. 40-50% decrease in percentage of proliferated T cells in spleen as well as mLNs of HVEM overexpressing chimeric mice comparing to control chimeras.

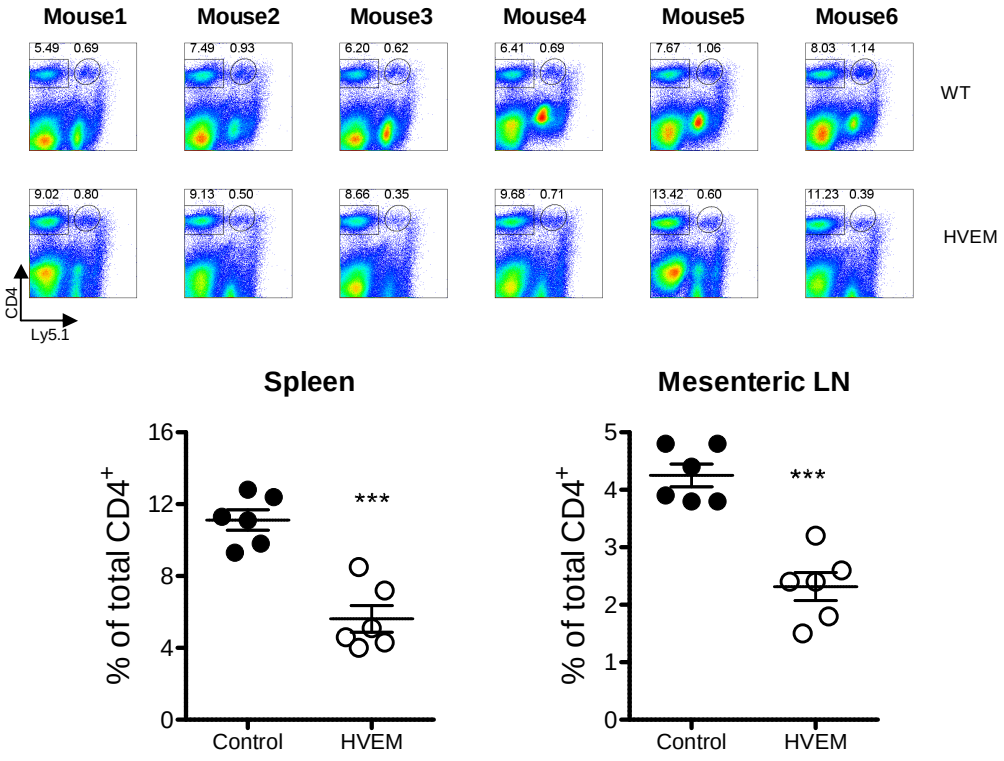


Figure 3- 25 Overexpression of HVEM partially inhibits T cell proliferation *in vivo*. The $RAG^{-/-}$ or $RAG^{-/-}$ OVAs HVEM chimeric mice were adoptively transferred with 10^6 purified $Ly5.1^+CD4^+$ OTII T cells. 12d later the mice were sacrificed and single cell preparations were prepared from spleens and mesenteric lymph nodes (mLNs). Cells were stained with anti-Ly5.1 and anti-CD4 mAbs to visualize the presence of transferred T cells. (A) Dot plots represent the percentages of endogenous $CD4^+$ cells as well as transferred $Ly5.1^+$ $CD4^+$ cells within spleens of 6 individual mice per group. (B) The graphs show the percentages of transferred CD4 cells out of total $CD4^+$ compartments in spleen and mLNs. Data are mean±SD (n=3), P<0.0001.

3.14 *In vitro*-generated DCs overexpressing HVEM can partially dampen the T cell proliferation when transferred *in vivo*

Since *in vivo* generated myeloid cells over-expressing HVEM were able to dampen the T cell proliferation we asked the question whether the *in vitro* generated DCs could behave the same way when transferred *in vivo*. To address this query, we adoptively transferred $5x10^6$ $Ly5.1^+CD4^+$ OTII T cells to wild type B6 recipients followed by two times i.v injections of $6x10^6$ Flt3L-derived OVAs BMDCs (WT or HVEM-overexpressing) on day 1 and day 5. On day 10, mice were sacrificed and single cell preparations were prepared from spleens and mesenteric lymph nodes (mLNs). As

shown in **Figure 3- 26**, activation of transferred T cells by HVEM overexpresssing resulted in an impaired proliferation reflected by their proportions and absolute numbers in spleen and mLNs, compared to WT controls. This reduction was similar to the reduction of T cell proliferation we observed in chimeric mice overexpressing HVEM on myeloid cells (nearly 40-50 % reduction).

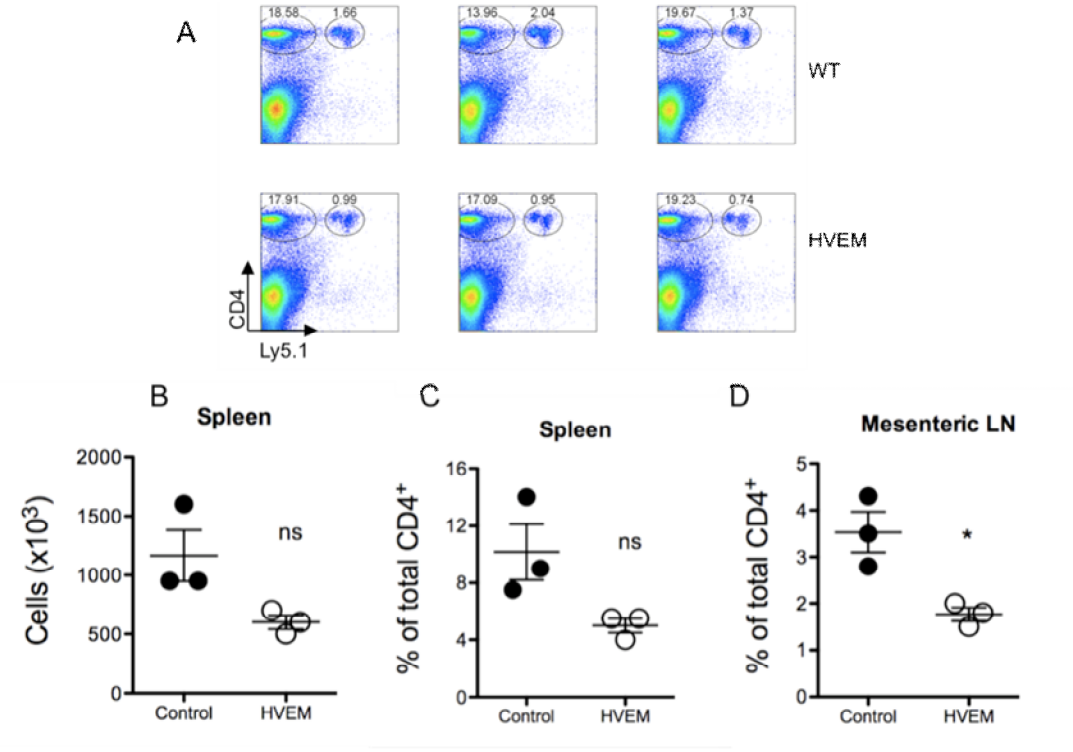


Figure 3- 26 HVEM overexpressing BMDCs dampen the Ag-specific CD4 T cell response when transferred *in vivo*. WT B6 mice (3 mice/group) were injected with $Ly5.1^+$ $CD4^+$ T cells followed by two injections of OVAs WT or HVEM BMDCs on days 1 and 5. Mice were sacrificed on day 10 and single cell suspensions from spleen or mesenteric lymph nodes were stained with anti-Ly5.1 and anti-CD4 mAbs to visualize the presence of transferred T cells. (A) Dot plots represent the percentages of endogenous $CD4^+$ T cells as well as transferred $Ly5.1^+$ $CD4^+$ cells within spleens of 3 individual mice per group. The graphs show the absolute numbers of transferred CD4 cells in spleens (B) and their percentages out of total $CD4^+$ fraction in spleens (C) and mLNs (D). Data are mean±SD (n=3), $p<0.05$).

We also examined the IFN-γ production capacity of transferred T cells in spleens. As shown in **Figure 3- 27**. The same proportion of transferred CD4 T cells from spleens of mice received WT or HVEM DCs were able to produce IFN-γ when stimulated *in vitro* with $OVA_{323-339}$ peptide. We have not detected any IL-10 production by these cells upon stimulation (data not shown).

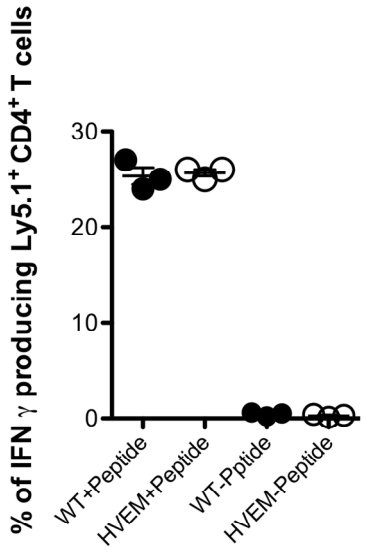


Figure 3- 27 HVEM overexpressing DCs don't affcet IFN-γ production by activated T cells. The splenocytes from the mice received WT or HVEM BMDCs and $CD4^+$ T cells, were stimulated *in vitro* in presence or absence of $2x10^{-6}$ M $OVA_{323-339}$ peptide for 6 hr followed by 2 hr Brefeldine A treatment. The IFN-γ production by $Ly5.1^+$ cells was determined by intracellualr cytokine staining and visualized by FACS. The graph represnts the percentage of IFN-γ^+ cells out of total transfered $Ly5.1^+$ T cells.

4. Discussion (I) Enhancement of Tolerogenic Potential of DCs

Dendritic cells are one of the most proficient regulators of the immune system. These cells are able to efficiently capture the antigens, process and present them to Ag-specific T cells in context of MHC molecules [5]. Mature DCs can express high levels of co-stimulatory molecules of so-called B7 family which furnishes T cells with the second signal, required for activation and expansion [12, 22]. Furthermore DCs can polarize T helper cells towards Th1, Th2, Th17 or T_{reg} fate, hence promoting different types of immune reactions [23, 24].

Despite their important role as "nature's adjuvants" [5] in boosting innate and adaptive immune responses, DCs have been shown to precisely modulate the immune responses and induce the tolerance to self-antigens. Central tolerance during thymic selection eliminates most of potentially auto-reactive cells [33-35], but not all self-proteins can be expressed in thymus [30], thus the peripheral tolerance strategies have been developed within immune system to suppress those cells which escape the central arm of immune tolerance. DCs contribute to negative selection of auto-reactive T cells in context of central tolerance and also play important roles in induction of peripheral tolerance via different mechanisms such as priming of regulatory T cells [29, 31, 32].

The term "Tolerogenic" refers to immature, maturation-resistant or alternatively activated DCs expressing low levels of co-stimulatory molecules and are defective in production of Th-1-inducing cytokines [54]. These cells fail to prime T cells due to lack of co-stimulation or as a result of inhibitory signaling.

As the bone marrow derived or *ex vivo* isolated dendritic cells are not tolerogenic, various experimental approaches have been used to render DCs tolerogenic *in vitro* and *in vivo* [55]. An interesting and efficient approach for doing so, is to manipulate DCs in a way that they deliver co-inhibitory signals to T cells, hence dampening the Ag-specific cell-mediated immunity. B7 family of co-stimulators has been at the center of attention recently, for designing of such experiments. Inhibitory molecules of this family form co-signaling axis and deliver the inhibitory signals to the T cells via receptor-ligand interactions.

Programmed death 1 (PD-1) engagement by its ligands PD-L1 and PD-L2 on APCs have shown in many studies to inhibit T cell activation [58, 59]. Although there are some pieces of data demonstrating co-stimulatory effects of both PD-L1 and PD-L2 [80, 82, 103-105], it's not clearly understood whether these effects are mediated by another receptor rather than PD-1, or it is a consequence of difference in nature of signaling events in different experimental settings. Furthermore, the potency of PD-

1/PDLs pathway in immunomodulation might depend on physiological status of both DCs and T cells *in vitro* and *in vivo*, which could influence the outcome of signaling network in favor of either co-inhibition or co-stimulation. B7-H4 is another molecule of B7 family with inhibitory effects on T cell proliferation, cell cycle progression and generation of CTLs [60, 116, 119, 328].

The molecules, PD-L1, PD-L2, B7-H4 and HVEM share the potential to down-regulate T cell-mediated immunity and might play important roles in induction of tolerance. Hence, we chose to study their role in context of DC:T cell interactions. Our approach consists of forced-retroviral over-expression of inhibitory molecules on HSC progenitors, which could be further differentiated into various hematopoietic cells including DCs.

The NUP98-HOXB4 transduced immortalized BM progenitors provided us with a unique and reliable system to generate modified progenitors, perform the time-consuming genetic modification and selection processes, and eventually differentiate them into desired DC lineages.

As mentioned earlier in introduction, the so-called inhibitory molecules of B7 family have been shown to act as versatile tools in terms of regulating the immune response (co-stimulation vs. co-inhibition). In fact recent data corroborates the idea that there is another putative receptor rather than PD-1 which in different situations could compete with PD-1 by binding to its ligands PD-L1 and PD-L2. Most probably this alternative receptor-ligand engagement switches on a signaling pathway, which results in positive regulation of T cell responses.

Over-expression of co-inhibitory ligands on DCs as an experimental strategy for induction of T cell exhaustion

Although, our genetically engineered DCs over-expressing PD-L1, PD-L2, B7-H4 or their different combinations didn't show significant inhibitory effects on T cell proliferation in context of primary immune responses, their interactions with T cells prime a so-called anergized or exhausted T cell state (**Figure 3- 9** and **Figure 3- 10**). In such condition, T cells are capable of proliferation but downregulate the ability to produce the major cytokines. Previous studies by Ahmed *et al.* as described earlier, reveled that the PD-1/PD-L pathway restrains virus-specific $CD8^+$ T cells in an exhausted state, which this phenomenon could be reversed by blockade of the pathway via antibodies to ligands. It has recently been proved that PD-L1 and PD-L2

expression on DCs directly regulated the state of $CD8^+$ T cell exhaustion as siRNA silencing of these two molecules on DCs enhanced T-cell proliferation and cytokine production specially production of IL-2 and IFN-γ by T cells [329]. This further highlights the functional importance of PDLs on DCs suggesting that the induced T cell exhasution phenomeon we observed in our chimeric animals overexpressing PDLs is mainly mediated via dendritic cells. Kuipers *et al.* have showed that forced over-expression of PD-L1 or PD-L2 on embryonic stem (ES) cell-derived DCs didn't inhibit the T cell proliferation but slightly decreased production of IL-2 by T cells co-cultured with these DCs [330] but in our experiments the DCs overexpressing PDLs caused a reduction in IFN-γ production by T cells while not affecting the IL-2 levels significantly. Kuiper's group also didn't provide any *in vivo* evidence regarding the function of DCs over-expressing PDLs, while we provide *in vivo* evidence that overexpression of PD-L1 or PD-L2 leads to functional exhaustion of CD4 T cells *in vivo* (**Figure 3- 10**).

Many studies such as Freeman *et al.*'s [83] work have confirmed that blockade of PDLs on DCs would activate the T cells and rescue their cytokine production capability.

On the other hand, the previous reports, including Ahmed's pioneering studies [331, 332] have documented the PD-1/PDLs-mediated $CD8^+$ T cell exhaustion while here, we report the functional exhaustion of $CD4^+$ T cells, induced by PDLs overexpression *in vivo*. Although recent reports suggest that PD-L1 and PD-L2 are involved in induction and maintenance of $Foxp3^+$ regulatory T cells [99, 100] , we haven't observed any significant induction of $Foxp3^+$ T_{regs} by PD-L1 or PD-L2 overexpression in our *in vitro* or *in vivo* experiments (data not shown). This might be due to low concentartions of endogenous TGF-β, required for induction of T_{regs} or other cell-intrinsic mechanisms regulating T_{reg} induction.

During 2002-2006 periods, Pease *et al*. have published a series of papers claiming that cross-linking PD-L2 with a human monoclonal antibody (B7-DCXAb) resulted in activation of dendritic cells, activation of cytotoxic T cells, induction of tumor immunity, modulation of allergic responses, breaking tolerance in the RIP-OVA diabetes model, and the reprogramming of Th2 and T regulatory cells. Those results were very impressive suggesting a key role for PD-L2 in modulation of many immunologic events. To examine their claims we conducted some experiments using

several anti-PD-L2 monoclonal antibodies, but we couldn't observe any of the functional outcomes reported by Pease's group (data not shown). Later on, in 2010 a series of retractions in *PNAS* [333], *Journal of Immunology* [334-336], *Journal of Allergy and Clinical Immunology* [337], *Journal of Experimental Medicine* [338] and *Cancer Research* [339] shocked the scientific community, reporting that none of those previously described observations with B7-DCXAb were reproducible and the reports were all subjects of scientific misconduct. These events indeed made us and many others to reconsider and rethink about the functional importance of PD-L2 in immunologic responses.

A novel role for PD-L2 in B cell development and survival

As an accidental observation in PD-L2-over-expressing chimeric mice, we found out that these animals lack a significant percentage of B cell repertoires in peripheral blood and lymophoid tissues as well (**Figure 3- 14**). As PD-L2 is not constitutively expressed on B cells we aimed to figure out through which mechanisms PD-L2 over-expression on B cells leads to their early depletion *in vivo*. Apoptosis studies revealed that high expression of PD-L2 on B lymphocytes leads to their complete eradication while PD-L2$^{Low/int}$ populations tend to survive more comparing to high-expressors. Several mechanisms might be involved in the generation of phenotype observed in PD-L2 chimeric mice (**Figure 4-1**), including an intrinsic, *cis* effect of PD-L2 on B cells which could lead to their death. This indeed requires auto-signaling of PD-L2 molecule on B cells, which so far is poorly documented, as no signaling function has been reported for short cytoplasmic tale of this molecule. The observed phenotype could also be the result of interaction of PD-L2 with PD-1 or another unknown receptor on surface of either the same cell or another immunocytes or non-immune cells. We generated RAG-deficient BM chimeric mice in which only myeloid cells over-expressed PD-L2. The analysis of peripheral blood showed the same pattern of B cell loss as observed if a full chimera, suggesting that PD-L2 expression by other cells, not B cells only, is sufficient to eradicate the B cell pool (**Figure 3- 18**). Hence this might be a result of engagement between PD-L2 and unknown molecule(s) on surface of B cells leading to apoptotic decline in their numbers. PD-1 is not thought to play major role in this process, as PD-1 blockade didn't rescue the B cell deficiency in chimeric animals overexpressing PD-L2 (**Figure 3- 20**). However, we can't totally rule out the contribution of PD-1in this phenomenon, as PD-1 blockade might not have

been effective or sufficient in terms of treatment strategy to fully block the PD-1 activity.

Since we observed an impaired B cell maturation in BM of PD-L2 overexpressing chimeric mice (marked with absence of $CD19^+$ and accumulation of $B220^+$ cells), we wondered whether PD-L2 overexpression only affects the B cell precursors undergoing maturation or could also deplete already existing fully differentiated B cells, *in vivo*. We exploited the inducible gene expression system to test this hypothesis. We showed that inducing PD-L2 overexpression on all hemopoietic cellular compartments, or on myeloid cells only, was able to decrease the B cell percentages in peripheral blood of chimeric mice (**Figure 3- 16** and **Figure 3- 19**). This proves that the observed phenotype in normal chimeras was not only due to a defect in B cell development as terminally differentiated B lymphocytes in blood was also affected by inducible expression of PD-L2. The reduction in B cell pool after PD-L2 induction was lower compared to what we observed in full chimeras constitutively overexpressing the molecule. This might be due to the fact that, intensity of PD-L2 overexpression (as defined by MFI) and the proportion of cells over-expressing it following induction, never tend to reach as high as constitutive over-expression levels. Hence, it might not be powerful enough to eradicate a bigger proportion of B cells *in vivo*.

Collectively, our data is suggestive of a mechanism(s) by which overexpressed PD-L2 on different cell types leads to its engagement with unknown molecule(s) on surface of B cells causing their depletion, possibly, mainly through induction of apoptosis. As mentioned earlier in introduction chapter, there are experimental clues suggesting another receptor(s) for PD-L2 but by today attempts to identify those molecules haven't been successful. Our own preliminary experiments using PD-L2-Ig fusion protein for detection of binding partners on B cells failed to provide us with any further information regarding that elusive engagement pattern (data not shown). Hence, further experiments using new sensitive techniques with high accuracy are required to decipher the potential binding partners for PD-L2 on B cell surfaces. It's also possible that PD-L2 acts in a soluble form (as suggested for some other members of B7 family) by shedding its extra-cellular domain which can engage the B cells through surface receptors, however the presence of PD-L2 in soluble form is hypothetical and needs to be documented. The observed effect of PD-L2 on B cell survival opens a new era for investigating PD-L2 function in areas beside T cell co-

regulation. Since PD-L2 deficient mice don't exhibit any obvious defects in B cell repertoire, the effect of PD-L2 on B cell pool could be rather an indirect effect through unknown interactions when PD-L2 is highly expressed in tissue microenvironments. The elucidation of the mechanisms involved in this process would also guide us through potential applications for targeted depletion of B cells in therapeutic settings (e.g. autoreactive B cells) and further updates our understanding of mechanisms underlying regulation of B cell homeostasis.

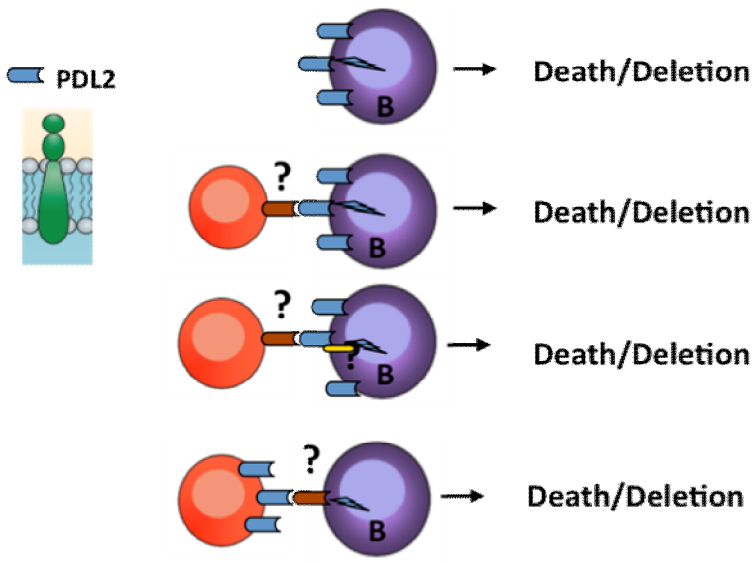


Figure 4-1 Possible mechanisms for PD-L2 mediated B cell death/depletion. **(A)** Intrinsic autosignaling by accumulation of PD-L2 on B cells **(B)** Engagement of PD-L2 molecules on B cells by PD-1 or an unknown ligand on the other cells causes cell death **(C)** the engagement shown in **(B**) could be mediated by a signaling complex on B cells including other co-receptors **(D)** PD-L2 overexpression on other cell types (e.g. DCs and macrophages) could engage PD-1 or an unknown molecule on B cells and lead to apoptotic B cell death.

HVEM over-expressing DCs dampen T cell proliferation in vitro and in vivo

HVEM is a member of TNFR superfamily, which shows a complex pattern of binding partners. It can bind to HSV1 gD, LIGHT, CD160, LT-α and BTLA [135]. HVEM is an interesting molecule, since in different physiologic situations and cellular microenvironments it can bind to a different ligand and exhibit a broad range of paradoxical functional outcomes. Engagement of LIGHT on T cells by HVEM has been shown to result in co-stimulation and activation of NF-κB while its binding to BTLA or CD160 is responsible for co-inhibitory functions. These known facts about HVEM proves it is a molecular switch capable of co-regulating T cell responses. As our strategic framework for generation of tolerogenic DCs was focused on

overexpression of transmembrane proteins with known inhibitory function, we decided to further exploit and investigate the co-inhibitory capacities of HVEM by its forced overexpression on DCs. Firstly, our phenotypic analysis of the DCs overexpressing HVEM showed no obvious differences in terms of expression of co-stimulatory molecules, comparing to WT BMDCs (**Figure 3- 21**). This observation suggests that HVEM overexpression doesn't affect the DC function and maturation through regulation of known co-stimulatory molecules such as B7.1 and B7.2. Although immature DCs express relatively high levels of HVEM but our overexpression strategy was able to further dramatically elevate those levels over 50 folds. We investigated the co-regulatory function of HVEM overexpressing BMDCs *in vitro* and *in vivo*. *In vitro*, HVEM DCs efficiently induce Ag-dependent $CD4^+$ T cells proliferation, although the proliferation rate was slightly lower comparing to proliferation pattern of T cells stimulated with WT BMDCs (**Figure 3- 22**). These observations *in vitro* were not so surprising. BTLA, the molecule that is thought to mediate the major co-inhibitory functions of HVEM as its ligand, is indeed rapidly upregulated during T cell activation but as we discussed earlier in introduction, HVEM can exert co-stimulatory effects (thorough binding to LIGHT, for instance) as well. So its possible that other binding partners of HVEM can also engage with overexpressed molecule and fine tune the outcome of stimulation in a regulatory fashion.

By transferring OVA-specific CD4 T cells to chimeric animals over-expressing HVEM on myeloid cells, we observed a consistent near half reduction in proportion of proliferated T cells *in vivo* 12 days post transfer (**Figure 3- 25**). Considering that *in vivo* HVEM chimeric mice express high levels of HVEM on myeloid cells, especially DCs as professional APCs, the findings suggest that *in vivo* microenvironment favors the HVEM interaction with its inhibitory binding partners such as BTLA and CD160. Nevertheless, the inhibition couldn't reach higher levels probably due to HVEM interaction with its other binding partners which can balance the regulatory function as well as presence of other co-regulatory pathways in T cells which can overpower and diminish the strong inhibitory functions of HVEM.

We confirmed that *in vivo,* HVEM DCs are sufficient enough to cause the observed partial inhibition of T cell proliferation (**Figure 3- 26**). To determine whether the proliferated T cells in HVEM group show an exhausted phenotype or not (lack of IFN-γ production) we tested their ability to produce IFN-γ upon Ag-specific activation *in*

vitro. Those T cells were able to secret IFN-γ in comparable fashion to T cells of control group (**Figure 3- 27**). This observation suggests that mechanism of inhibitory function by HVEM might not be similar to what observed in PD1/PDLs pathway. We also didn't observe any IL-10 production by those stimulated T cells, ruling out IL-10 as key suppressive player in this scenario (data not shown).

Since the exact molecular and cellular mechanisms by which HVEM dampens T cells responses have not been understood, further investigations are required to explain the mechanistic function of HVEM. As we observed only partial inhibition of Ag-specific T cell responses by HVEM over-expression on DCs, we suggest combining this strategy with other tools in order to achieve full inhibition of T cell responses in therapeutic settings. Nevertheless, the partial inhibition observed *in vivo* could of potential significance and importance in many pathological conditions where down-modulation of escalated T cell responses would ameliorate the catastrophic manifestations.

4.1 Conclusion

Our strategy to over-express several molecules with inhibitory functions on immortalized BM progenitors provided us with the opportunity to select, expand and eventually differentiate them to DCs *in vitro* or use them to generated BM chimeric mice to study DC functions *in vivo*. Over-expression of PD-L1, PD-L2 and B7-H4 singularly, or in combinations didn't affect the T cell proliferation but managed to exhaust the T cells as they failed to produce IFN-γ as an important effecter cytokine in response to Ag-specific stimulation. HVEM-over-expressing DCs, on the other hand, partially dampened the T cell proliferation without affecting their cytokine production (**Figure 4- 2**). These strategies are suggested to be used in a combined fashion in order to effectively suppress the unwanted T cell responses in autoimmune conditions.

PD-L2 over-expression on B cells reveled another surprising new function of this molecule affecting B cell survival *in vivo*. The massive depletion of B cells upon their encounter with PD-L2 over-expressing cells suggests the presence of another molecule(s) on B cell surface, than can engage with PD-L2 and initiate a signaling pathway which leads to B cell death. This phenomenon needs to be further

investigated in detail in order to divulge the mechanistic role of PD-L2 in B cell survival and homeostasis.

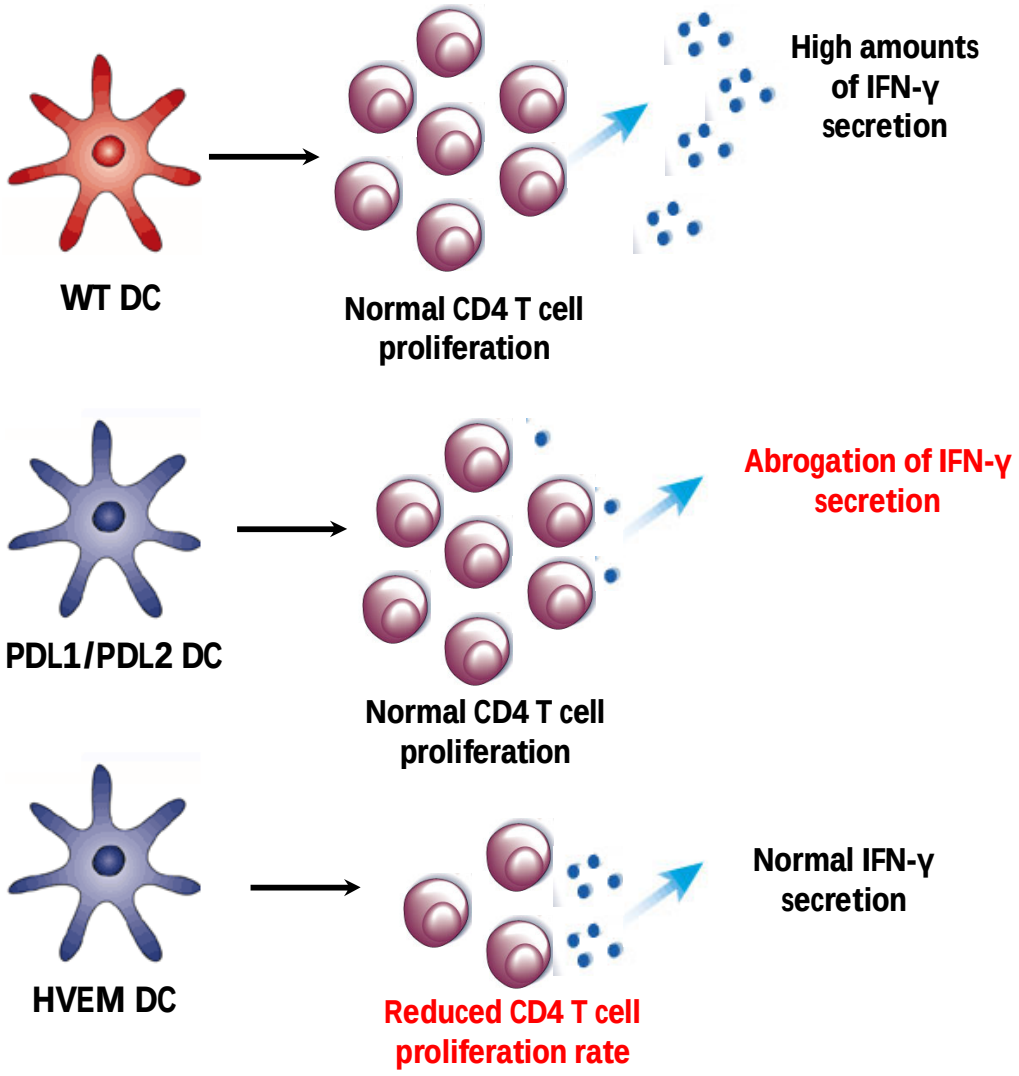


Figure 4- 2 Functional model for genetically modified tolerogenic DCs. DCs over expressing PD-L1, PD-L2 or their combination don't affect $CD4^+$ T cell proliferation while abrogating their IFN-γ production capability. On the other hand, HVEM-overexpressing DCs dampen the CD 4^+ T cells proliferation but don't influence their IFN-γ secretion.

5. RESULTS (II) Enhancement of Immunostimulatory Potential of DCs

5.1 GM-CSF boosts the production of IL-1β by Macrophages and Dendritic cells

In order to examine the role of GM-CSF on production of IL-1β by macrophages and dendritic cells, WT or GM-CSFR$^{-/-}$ BMDCs (either GM-CSF derived or Flt-3L derived) and BMDMs (L929-conditioned medium -derived or , M-CSF-derived) were pre-treated for 16 hours with 100 ng/ml LPS as TLR agonist (Signal I) in presence or absence of 5 ng/ml recombinant mouse GM-CSF. The cells were further incubated with different danger signals (ATP, MSU, Alu, nigericin) for aditional 6 hours (Signal II). The supernatants were harvested and IL-1β concentrations were determined by ELISA. As shown in **Figure 5- 1**, addition of GM-CSF strongly boosted the production of IL-1β by DCs and Macrophages. The level of production was in average 5-10 times higher when cells were treated in presence of LPS and GM-CSF. GM-CSF alone or danger signals alone failed to prime the cells for producing IL-1β. The GM-CSFR deficient cells were not responsive to co-treatement with GM-CSF and LPS which shows the observed increase in this cytokine is mediated through GM-CSF signaling. Among different danger signals applied for the above experiments we always observed strong production of IL-1β using ATP and nigericin, while lower amounts of IL-1β were detected when cells treated with other danger signals, MSU or alum which seem to be weaker stimulators in this experimental setup.

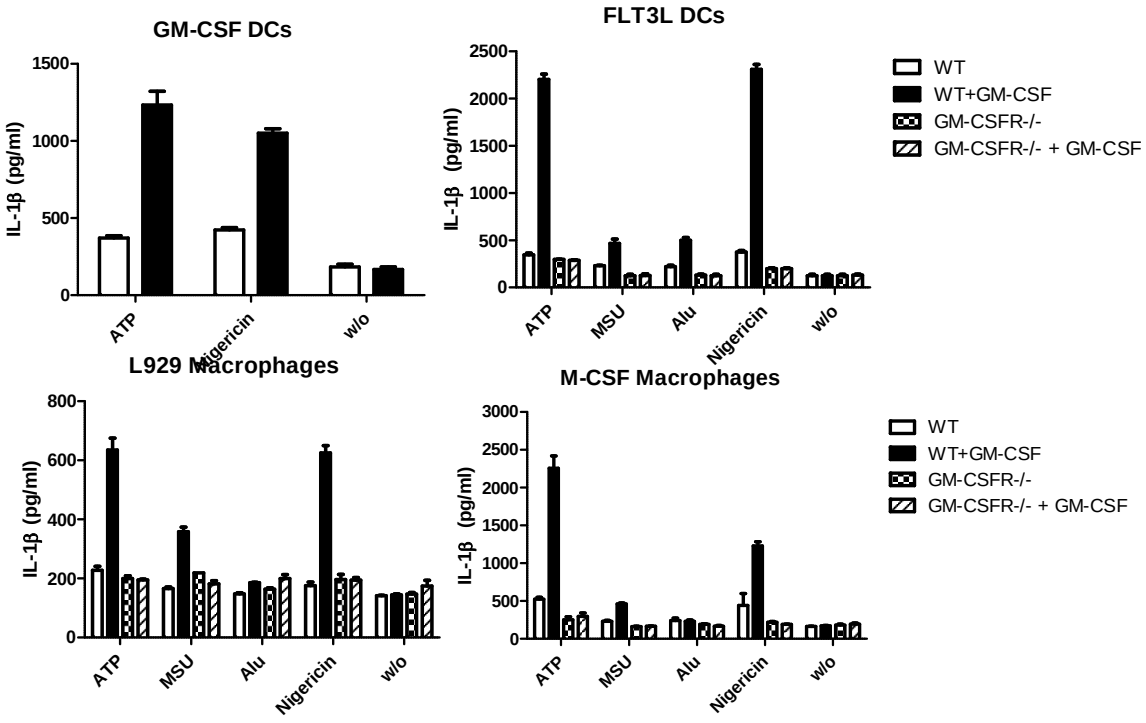


Figure 5- 1 GM-CSF boosts the production of IL-1β by LPS-primed DCs and Macrophages. BM-derived DCs or Macrophages were primed with LPS in presence or absence of GM-CSF overnight followed by 6hr incubation with different danger signals. ATP: 4mM, Monosodium Urate (MSU): 100μg/ml, Aluminium Hydroxide (Alu): 200μg/ml and Nigericin: 1μM (a pore-forming toxin from bacteria).

In order to define the limiting dilution of GM-CSF needed for the observed escalation of IL-1β levels we titrated the GM-CSF concentrations in culture from 2-50 ng/ml. As shown in **Figure 5- 2**, presence of GM-CSF at concentrations as low as 2 ng/ml was still able to dramatically boost the IL-1β production by the cells.

5.2 GM-CSF, but not M-CSF, boosts IL-1 production

M-CSF (also known as CSF-1) is a growth factor known as a potent activator of functions of monocytes and macrophages. We asked the question whether the role of GM-CSF in boosting IL-1β release is unique and specific or the other growth factor; M-CSF is also capable of exerting similar effects. To address this question we incubated LPS-treated macrophages or DCs with various concentrations of recombinant mouse M-CSF (rmM-CSF) or GM-CSF followed by treatment with ATP

as danger signal. We noticed that M-CSF had no effect on IL-1β production by cells even in concentration as high as 50ng/ml (**Figure 5- 2**) as determined by ELISA.

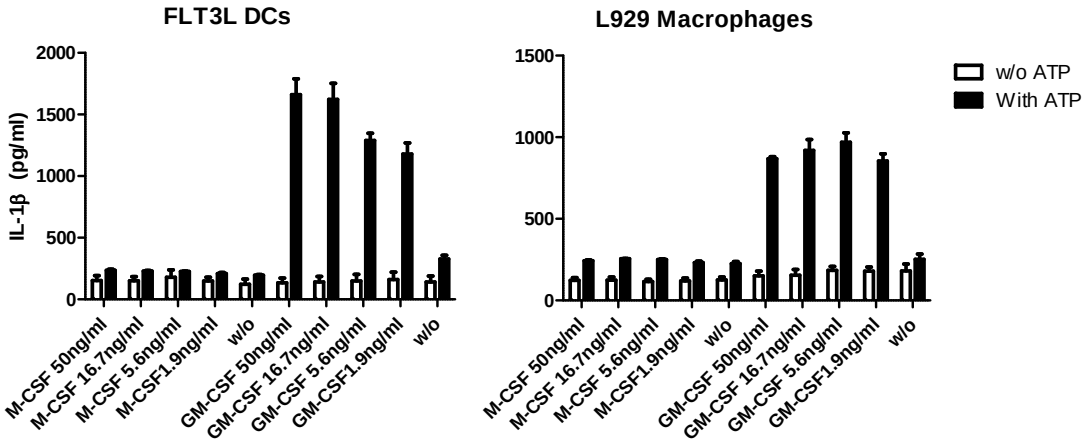


Figure 5- 2 GM-CSF but not M-CSF boosts the production of IL-1β by DCs or macrophages. The BM-derived DCs or macrophages were primed overnight by LPS in presence or absence of various concentrations of GM-CSF or M-CSF +/-ATP treatment. The IL-1β concentration in supernatants was measured by ELISA.

5.3 GM-CSF boosts the IL-1β production by DCs primed with different TLR agonists

We used LPS treatment as an example of TLR agonist for TLR4 to deliver the priming signal I to the cells but our further experiments proved that GM-CSF was able to exert the same effect once the cells were stimulated with different TLR agonists, namely CpG (TLR9 agonist), Pam3CSK4 (TLR1/2 agonist), Imidazoquinoline (TLR7 agonist), poly (I:C) (TLR3 agonist) (**Figure 5- 3**). As expected, GM-CSFR$^{-/-}$ DCs or macrophages failed to secret elevated amounts of IL-1β in response to combination of LPS and GM-CSF.

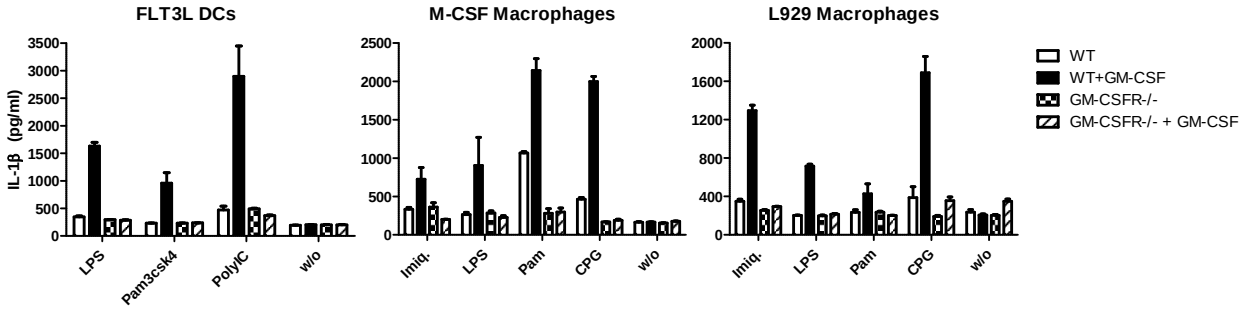


Figure 5- 3 DCs and Macrophages primed with different TLR agonist produce high amounts of IL-1β upon exposure to GM-CSF. WT or GM-CSFR$^{-/-}$ cells were primed overnight with different TLR agonists followed by 5mM ATP treatment and IL-1β concentrations in culture supernatants were determined by ELISA. LPS: 100ng/ml, poly (I:C): 50 µg/ml, Pam3Csk4: 1 µg/ml, CpG: 1uM, Imidazoquinoline: 10 µg/ml.

5.4 GM-CSF blockade abrogates the boosting of IL-1 β production by exogenous GM-CSF

To further prove that the observed effect in boosting IL-1β production was directly mediated by GM-CSF we treated the GM-CSF-derived, LPS-primed DCs with various concentrations of blocking anti-GM-CSF antibody in presence of GM-CSF. The blockade of GM-CSF fully abrogated the IL-1β production by DCs, proving the unique role of GM-CSF in this process (**Figure 5- 4**).

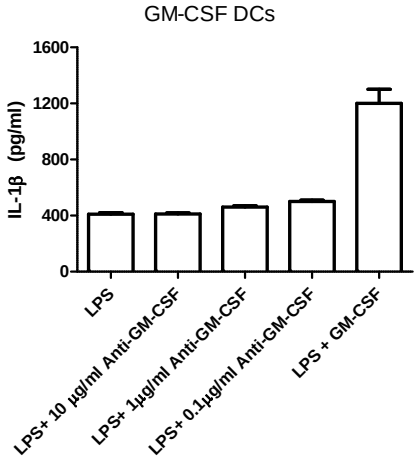


Figure 5- 4 Blocking GM-CSF activity abrogates the GM-CSF-mediated IL-1β production. GM-CSF-derived DCs were primed with LPS and GM-CSF overnight in presence or absence of different concentrations of blocking Anti-GM-CSF antibody followed by 6hr ATP treatment. The IL-1β concentrations were determined by ELISA.

5.5 A short exposure of DCs to GM-CSF primes them for production of high levels of IL-1β

Although in our experiments we chose to co-incubate DCs with LPS and GM-CSF overnight but further experiments showed that even a shorter exposure of DCs to GM-CSF during overnight LPS priming is sufficient to prime the cells for augmented production of IL-1β following treatment with danger signals. As shown in **Figure 5- 5**, shorter time points of GM-CSF treatment, ranging from 3-12 hours during overnight LPS priming was able to boost the IL-1β productions. This further shows that brief exposure of cells to GM-CSF could efficiently prime them for synthesis and release of high amounts of IL-1β.

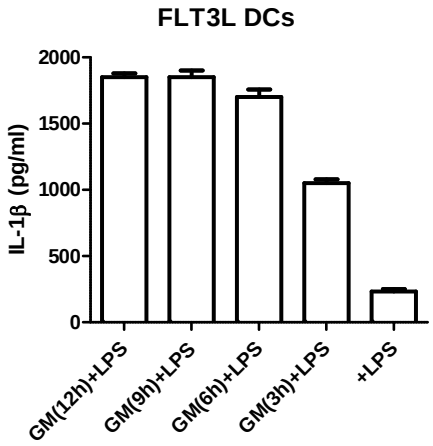


Figure 5- 5 Short exposure to GM-CSF can prime DCs for IL-1β production. Flt3-L derived $CD11b^+$ BMDCs were incubated overnight with LPS and different periods of GM-CSF treatment (3-12 hr). GM-CSF was washed away after designated time point incubations, while LPS was kept in culture as priming signal. Cells were incubated with ATP for additional 6hr and the IL-1β production level was determined in supernatants by ELISA. Data are mean±SD (n=3).

5.6 GM-CSF boosts the production of IL-1α and IL-18

Our data also showed that the production of the other member of IL-1 family of cytokines, IL-1α, which plays an important role during inflammation, was also dramatically increased upon treatment of Flt3-L derived BMDCs with LPS and GM-CSF (**Figure 5- 6**).

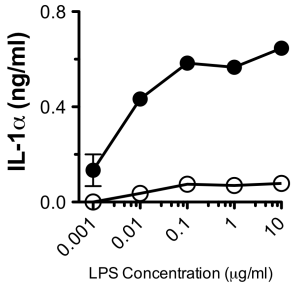


Figure 5- 6 Increased production of IL-1α by DCs upon GM-CSF treatment. FLT3-L derived $CD11b^+$ BMDCs were primed overnight with various concentrations of LPS, followed by 6 hr incubation with ATP. The IL-1α secretion to the supernatants was determined by ELISA. Data are mean±SD (n=3).

IL-18 is another member of IL-1 family of cytokines, its production and release is also mediated via inflammasome activation. Bioactive IL-18 is a product of immature IL-18 cleaved by caspase-1. To examine the role of GM-CSF in IL-18 production we measured the levels of this cytokine in supernatants of LPS-stimulated DCs treated in presence or absence of GM-CSF and incubated with nigericin as danger signal. GM-CSF strongly increased IL-18 levels as well (**Figure 5- 7**), suggesting that it is able to boost the production of IL-18 through activation of inflammasome in a similar fashion to IL-1β.

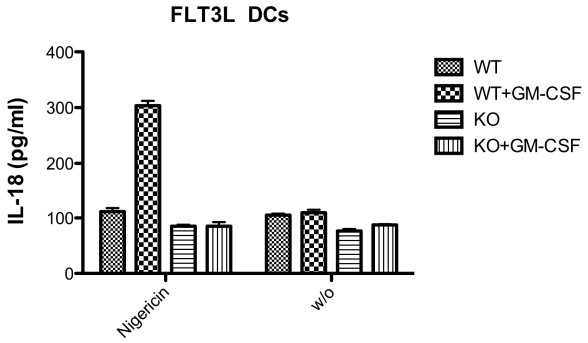


Figure 5- 7 GM-CSF augments the IL-18 production by DCs. FLT3-L derived $CD11b^+$ BMDCs were stimulated overnight with LPS in presence or absence of GM-CSF followed by 6 hr ATP treatment. The IL-18 levels in culture supernatants were measured by ELISA. Data are mean±SD (n=3).

5.7 GM-CSF dramatically boost IL-1 production by DCs and macrophages while augmenting the production of other pro-inflammatory cytokines

A distinct hallmark of inflammasome activation is the increased secretion of bioactive IL-1β by the cells. We aimed to determine whether the effect of GM-CSF is solely restricted to IL-1 family of cytokines or it affects the production of other inflammatory cytokines as well. We defined the levels of different cytokines including IL-1β, TNF-α and IL-6 released by distinct DCs or macrophages treated with increasing concentrations of LPS and further incubated with ATP. As shown in **Figure 5- 8**, GM-CSF distinctively boosted the production of IL-1β while the increase of the other two cytokines TNF-α and IL-6 was not as dramatic as observed for IL-1 cytokines. TNFα and IL-6 productions were already increased by LPS alone, as these cytokines are regulated independent of inflammasome. In contrast, the cells treated with LPS only showed very low levels of IL-1β.

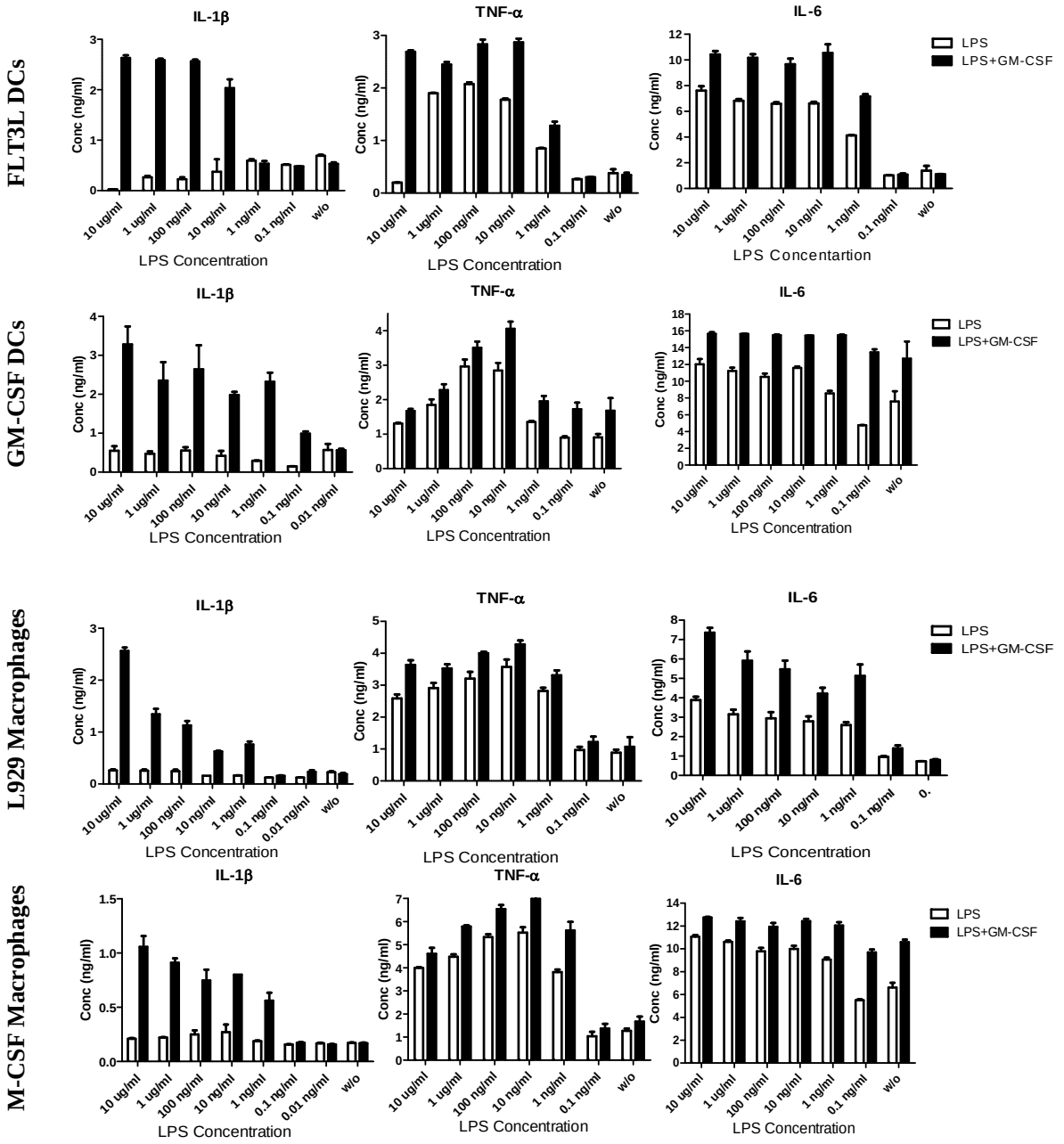


Figure 5- 8 GM-CSF dramatically boosts IL-1 production, but not TNF-α and IL-6, by DCs and macrophages. Flt3L-derived $CD11b^{+}$ BMDCs or L929-derived macrophages were primed with different concentrations of LPS in presence or absence of GM-CSF followed by ATP treatment. The presence of IL-1β, TNF-α and IL-6 in culture supernatants was determined by ELISA. Data are mean± SD (n=3).

5.8 GM-CSF boost IL-1β production by *ex-vivo* isolated peritoneal macrophages

To examine whether *ex vivo* isolated cells were able to show the same effect in terms of IL-1β release upon treatment with GM-CSF we treated *ex vivo* isolated thioglycolate-elicited peritoneal macrophages with LPS in presence or absence of GM-CSF, followed by 6 hr treatment with different danger signals. As expected the LPS-primed *in vivo* counterparts of *in vitro* generated macrophages, also did produce large amounts of IL-1β when treated with GM-CSF (**Figure 5- 9**), We were not able to include the GM-CSFR-deficient peritoneal macrophages in this experiment as these animals fail to recruit macrophages to the peritoneal cavity once elicited with thioglycolate.

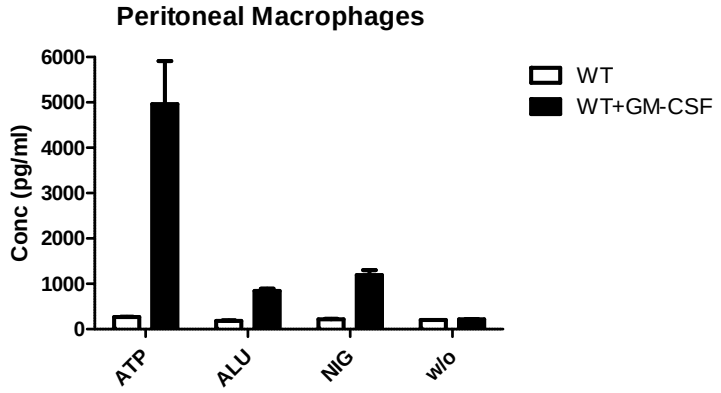


Figure 5- 9 GM-CSF boost the IL-1β production by *ex vivo* isolated macrophages. WT C57BL/6 mice were treated with 4 % Brewer's Thioglycolate Medium (i.p) and 4 days later peritoneal cavities were lavaged, peritoneal macrophages were harvested and plated for stimulation with 100ng/ml LPS in presence or absence of 5ng/ml GM-CSF followed by 4hr treatment with ATP, Nigericin, Alum or MSU. IL-1β production by these cells was monitored by ELISA. Data are mean± SD (n=3).

5.9 GM-CSF increases the Pro- IL-1β protein level

The observed effect of GM-CSF on IL-1β release was indicative of increased levels of bioactive IL-1β. The question is whether GM-CSF also affects the synthesis of inactive pro-form IL-1β as well. To address this question, we treated the WT or GM-CSFR$^{-/-}$ macrophages with LPS in presence or absence of GM-CSF for 16hr and the presence of intracellular pro-IL-1β in cell lysates was determined via western blotting.

As shown in **Figure 5- 10**, cells treated with GM-CSF showed a distinct accumulation of pro-IL-1β in cytoplasm compared to cells received LPS only (5 fold increase).

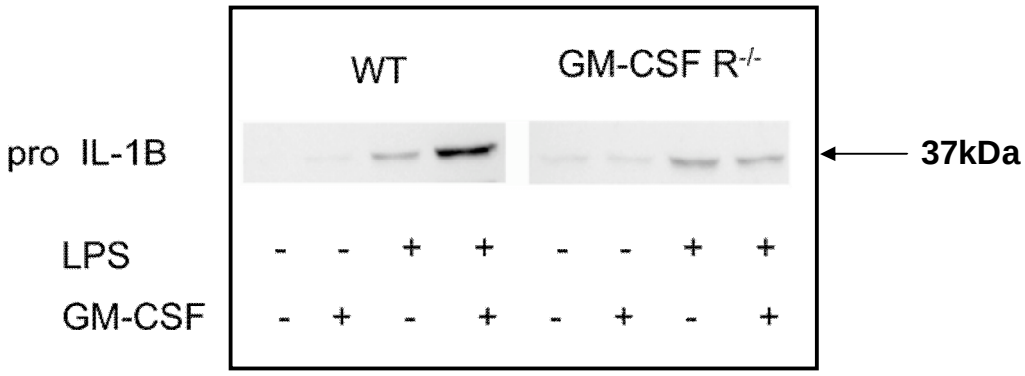


Figure 5- 10 Augmented production of pro-IL-1β upon treatment of LPS-primed cells with GM-CSF. WT or GM-CSFR$^{-/-}$ L929 BMDMs were pre-treated with 100 ng/ml LPS with or without 5ng/ml rGM-CSF for 16 hr. Cell lysates were subjected to an immunoblot analysis with Abs against pro- IL-1β.

Next, we additionally treated the cells with nigericin as danger signal, and determined the release of bioactive IL-1β p17 in the supernatants of the cells and as well as pro-IL-1β in cell lysates in western blots. As shown in **Figure 5- 11**, bioactive p-17 IL-1β was strongly released into supernatants of macrophage cultures treated with LPS and GM-CSF but was weakly detectable in supernatants of cells stimulated with LPS only. This increase was validated by ELISA results showing 5-fold increase in secreted IL-1β in cells treated with GM-CSF. The cleavage of activated caspase-1 was also evident in samples treated with LPS and nigericin as demonstrated by the presence of cleaved p20 subunit of caspase-1 in supernatants. However, addition of GM-CSF didn't have any significant effect on caspase-1 p20 protein levels.

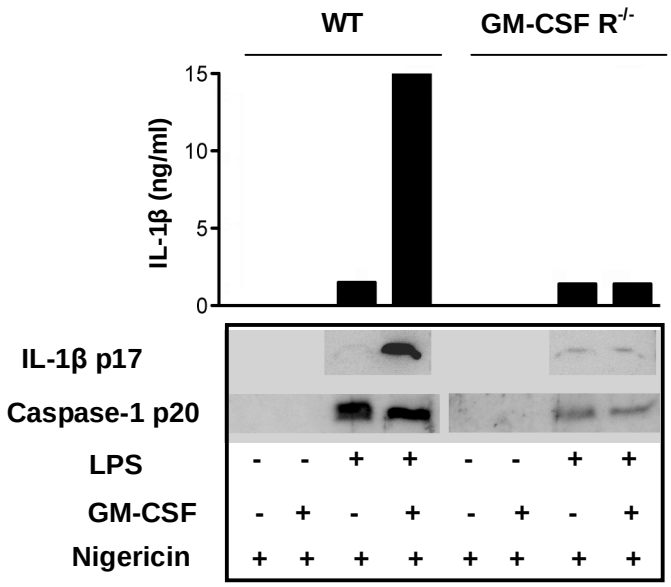


Figure 5- 11 Efficient release of bioactive IL-1β upon treatment of primed cells with GM-CSF and danger signal. WT or GM-CSFR$^{-/-}$ L929 BMDMs were pre-treated with 100 ng/ml LPS with or without 5ng/ml rGM-CSF for 16 hr (A) followed by 20 min additional treatment with 1uM nigericin. Culture supernatants were concentrated with Amicon filter tubes and subjected to an immunoblot analysis with Abs against IL-1β p17 and Caspase-1 p20 subunit. The secreted IL-1β levels in supernatants were measured by ELISA.

5.10 GM-CSF did not affect the protein levels of NALP3 and other inflammasome components

Since release of IL-1β is tightly regulated by inflammasome complexes we thought to investigate the possible changes in levels of inflammasome components in cells treated with GM-CSF. As shown in **Figure 5- 12**, as already described, LPS increases the levels of NALP3 but the levels of pro-caspase 1 and NALP3 proteins were not affected by combination of LPS and GM-CSF.

Caspase-1 activation is dependent on another inflammatory caspase, caspase-11. Caspase-11 deficient mice fail to produce IL-1β in response to LPS [340]. Therefore we also investigated the protein level of caspase-11 as an upstream regulator of caspase-1 upon treatment of LPS-primed cells with GM-CSF. Although LPS clearly up-regulates the caspase-11 levels but we didn't observe any striking difference in caspase-11 protein levels in presence or absence of GM-CSF.

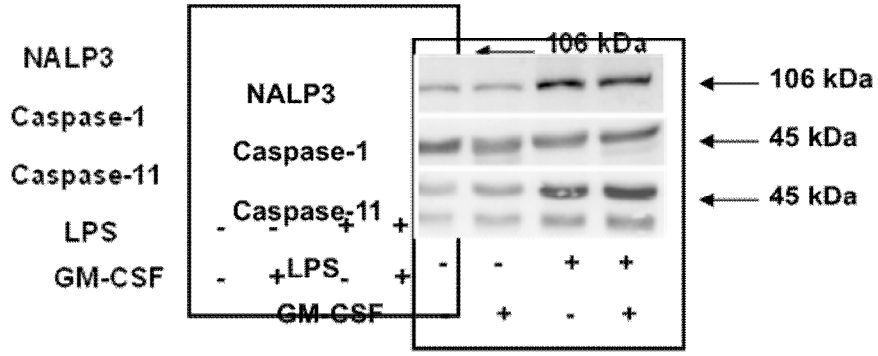


Figure 5- 12 GM-CSF does not affect the levels of inflammasome components. WT L929 BMDMs were pre-treated with 100 ng/ml LPS with or without 5ng/ml rGM-CSF for 24hr and cell lysates were subjected to an immunoblot analysis with Abs against NALP3, pro-caspase-1 and caspase-11.

As shown earlier GM-CSF in combination with LPS was able to augment the synthesis of pro-form IL-1β. We were interested to know if higher concentrations of LPS alone would increase the pro-IL-1β levels to similar levels produced by synergistic effect of GM-CSF and LPS. To test this possibility, we stimulated the BMDMs with 3 different concentrations of LPS in presence or absence of GM-CSF. After overnight stimulation, we determined the protein contents of pro-IL-1β, as well as NALP3 and pro-caspase-1 in cell lysates by western blotting. As shown in Fig. **Figure 5- 13**, even in presence of a 100 fold less concentrated LPS (1ng/ml), GM-CSF was still able to increase the pro-IL-1β synthesis. On the other hand, increasing LPS concentrations by 100-fold was not able to exert the boosting effect of LPS combined with GM-CSF. We also noticed that increasing LPS concentration in combination with GM-CSF didn't affect the protein contents of two key inflammasome components, pro-caspase-1 and NALP3.

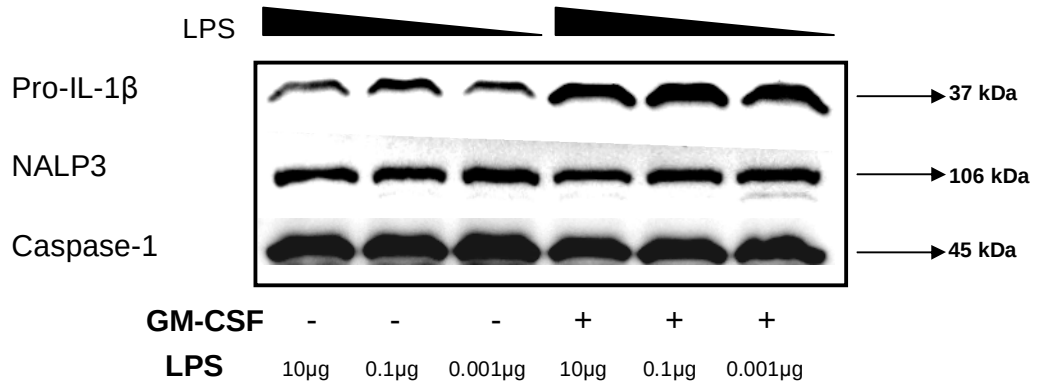


Figure 5- 13 Low dose LPS efficiently primes pro-IL-1β synthesis. WT L929 BMDMs were pre-treated with 3 different concentrations of LPS (10, 0.1, 0.001 μg/ml) with or without 5 ng/ml rGM-CSF for 24hr and cell lysates were subjected to an immunoblot analysis with Abs against NALP3, pro-caspase-1 and pro-IL-1β.

Quantitative PCR data have shown a big increase in mRNA levels of inflammasome components after co-treatment of cells with LPS and GM-CSF (data not shown). Next, we wondered if the protein level of NALP3 component would also be higher with GM-CSF and LPS treatment at earlier time point post stimulation. Indeed, this wasn't the case as macrophages treated with LPS and GM-CSF didn't show any difference in protein content of NALP3 after 6 hr stimulation, compared to LPS-only treated cells (**Figure 5- 14**). We also analyzed the pro-IL-1β content after 6 hr of stimulation and as shown in **Figure 5- 14**; at this earlier time point the pro-IL-1β synthesis is already increased by GM-CSF when combined with LPS.

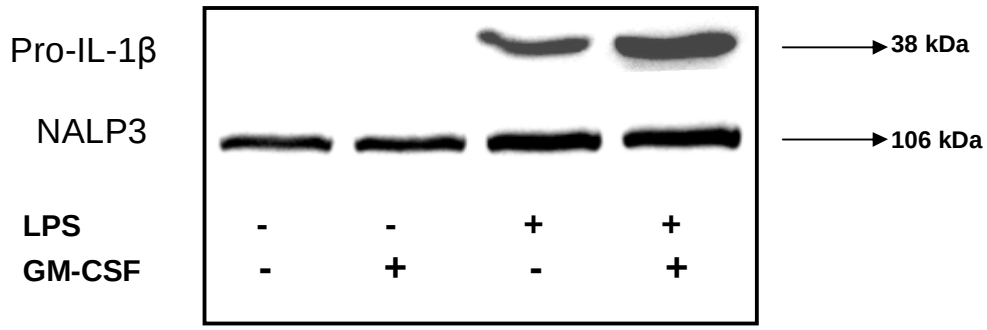


Figure 5- 14 The protein contents of NALP3 and pro-IL-1β 6 hr post stimulation of macrophages. WT L929 BMDMs were pre-treated with 100 ng/ml LPS with or without 5ng/ml rGM-CSF for 6hr and cell lysates were subjected to an immunoblot analysis with Abs against NALP3 and pro-IL-1β.

5.11 GM-CSF-mediated boosting of IL-1β production is caspase-dependent

Although we haven't observed any detectable increase in levels of caspases-1 and -11 in LPS+GM-CSF treated macrophages but the GM-CSF mediated augmentation of IL-1β production was dependent to these two caspases. Blockage of caspases-1 and -11 with specific inhibitors resulted in abrogation of GM-CSF-boosted IL-1β secretion by DCs in a dose-dependent manner (**Figure 5- 15**).

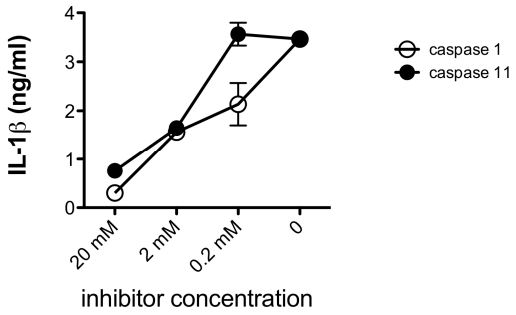


Figure 5- 15 Inhibition of caspases-1 and -11 abrogates the effect of GM-CSF in boosting IL-1β production. Flt3L-derived $CD11b^+$ DCs were incubated for 30 min at 37°C with decreasing concentrations of YVAD-cmk (caspase-1 inhibitor) or Z-LEVD-fmk (caspase-11 inhibitor). Cells then were washed with medium and primed overnight with LPS in presence or absence of 5ng/ml GM-CSF followed by 6hr 5mM ATP treatment. The levels of IL-1β release into culture supernatants were measured via ELISA. The data are mean±SD (n=3).

5.12 GM-CSF boosts the IL-1β production by cells in an NF-κB dependent manner

Transcription and production of bioactive IL-1β is known to be regulated via NF-κB pathway. We were interested to see if GM-CSF-mediated augmentation of IL-1β by DCs is also dependent on NF-κB or is an independent effect. To investigate this matter we treated the LPS-stimulated, GM-CSF treated DCs in presence or absence of increasing concentrations of any of the two known inhibitors of NFκB, Bay 11-708 [341] or Wedelolactone [342], which selectively inhibit IκB-α phosphorylation. The results clearly show that the inhibitors of NF-κB were able to abrogate the GM-CSF effect on IL-1β production in a dose-dependent manner (**Figure 5- 16**). This observation proves that the GM-CSF exerts its effect on IL-1β production through NF-κB pathway.

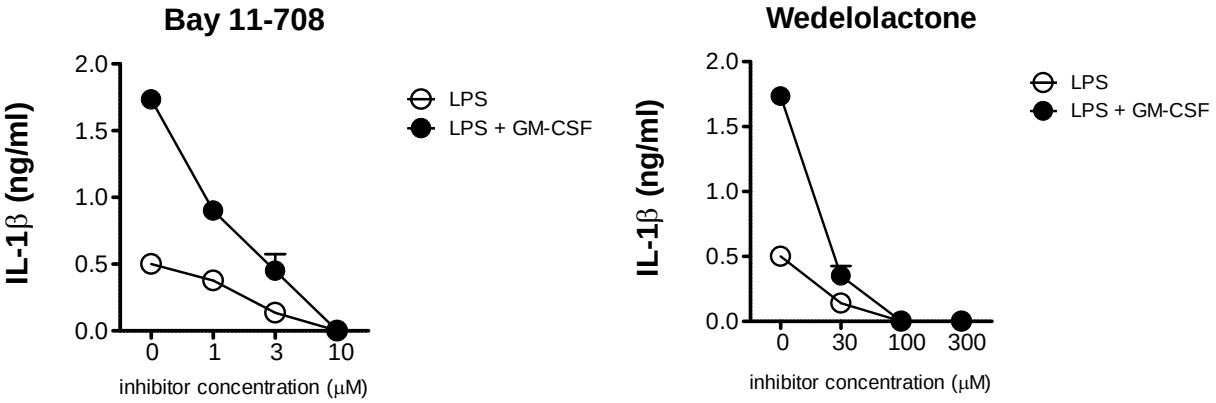


Figure 5- 16 GM-CSF augmentation of IL-1β secretion is an NF-κB- dependent effect. The $CD11b^{+}$ Flt3L-derived BMDCs were treated with different concentrations of NF-κB inhibitors, Bay 11-708 or Wedelolactone for 30 min. The cells were then washed, and primed overnight with LPS in presence or absence of GM-CSF followed by 6hr ATP treatment. The levels of IL-1β release into culture supernatants were measured by ELISA.

In order to further investigate the effect of GM-CSF in modulation of NF-κB we decided to look at the pattern of degradation and phosphorylation of IκBα, a cytoplasmic regulator which binds to NF-κB family members and sequester them, resulting in inhibition of NF-κB. We also investigated the degradation pattern of the other member of IκB family, IκBβ. This molecule has recently been suggested to form a complex with NF-κB subunits in cytoplasm and shuttle them to the nucleus where it can directly interact with IL-1β promoter. As shown in **Figure 5- 17** upon LPS stimulation, the molecule starts to degrade within 30 min and is re-synthesized later on, as it reaches the pre-stimulation levels within 2 hrs. Phosphorylation is rapid and takes place after initial 30 min of stimulation. The combination of GM-CSF with LPS didn't affect neither the kinetics of LPS-induced IκBα degradation nor its phosphorylation over the 2hr analysis period. We also didn't observe any significant changes in degradation pattern of IκBβ when cells were co-incubated with GM-CSF and LPS comparing to LPS alone.

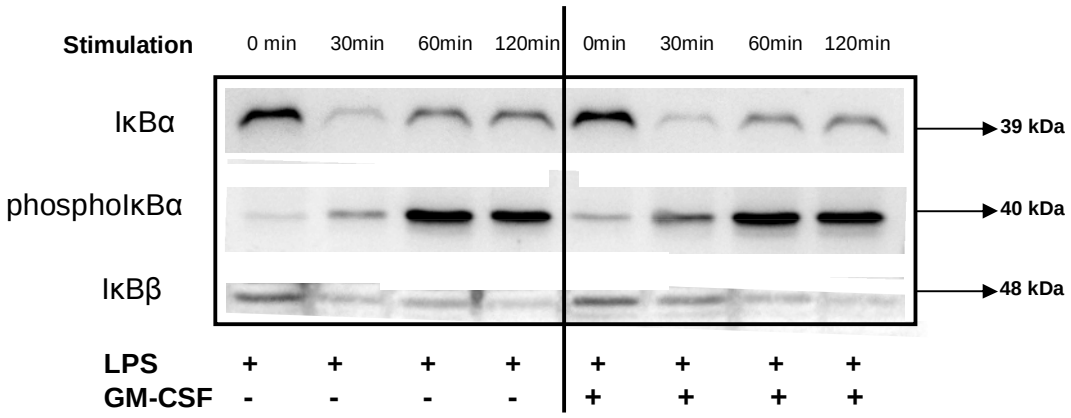


Figure 5- 17 Co-stimulation of cells with LPS and GM-CSF does not alter the kinetics of IκBα and IκBβ expression. WT L929 BMDMs were pre-treated with 100 ng/ml LPS with or without 5ng/ml rGM-CSF for different timepoints (0-120min) and cells lystaes were subjected to an immunoblot analysis with Abs against IκBα, pIκBα and IκBβ.

5.13 GM-CSF enhances the nuclear translocation of NF-κB subunits in LPS-primed DCs

Upon NF-κB activation, its different subunits, stabilized in cytoplasm, will form various complexes which will be shuttled to the nucleus and initiate the NF-κB-specific gene transcription process. We asked the question whether GM-CSF could affect this nuclear translocation process to mechanistically hyper-activate the NF-κB pathway. To answer this question, we treated the DCs with LPS in presence or absence of GM-CSF and monitored the DNA binding activity of NF-κB subunits p65, p50 and p52 by DNA-binding kit as well as nuclear translocation of other component, c-Rel, by western blotting. The results (**Figure 5- 18**) indicated an increased DNA binding of p65, p50 and p 52 and significantly more of c-Rel translocation into the nucleus when DCs were co-incubated with LPS and GM-CSF comparing to LPS alone. This finding is direct proof showing that GM-CSF can amplify the NF-κB pathway, hence boosting the transcription of gene products, including IL-1.

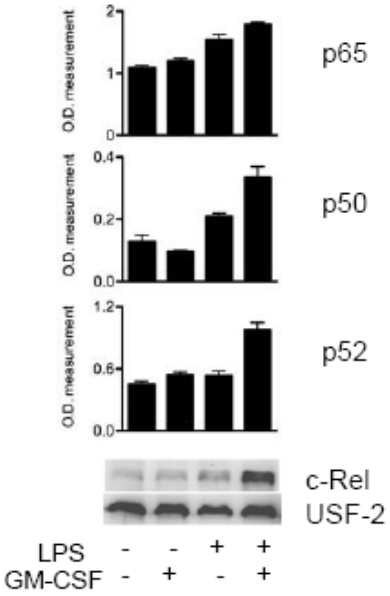


Figure 5- 18 GM-CSF hyperactivates NF-κB pathway. The Flt3-L-derived BMDCs were stimulated with 100ng/ml LPS in presence or absence of 5ng/ml GM-CSF for 24hr. The cells were lysed, nuclear and cytoplasmic fractions were separated and nuclear fractions were subjected to an immunblot analysis with antibodies against c-Rel as well as USF-2 as an internal control. The nuclear fraction was also analyzed for DNA binding activity of NF-κB subunits p50, p52 and p65, using a DNA-binding ELISA kit. The O.D measurements are representatives of DNA binding capacity of each subunit.

5.14 Inflamasomme-related antibody response to antigen is impaired in GM-CSFR-deficient mice

Aluminum Hydroxide (Alum) is one of the commonly used adjuvants in boosting the immune response upon vaccination. It's been well documented that Alum is able to activate the NALP3 inflammasome [235], hence promoting IL-1 production. This process leads to elevated production of Th-2 biased immunoglobulin isotypes IgG1 and IgE while not affecting the Th-1 associated isotypes, IgG2a, IgG2b and IgG2c [186, 234, 235, 343] . To test whether absence of GM-CSF activity would compromise the Th-2 skewed Ab response *in vivo*; we intraperitoneally vaccinated the WT or GM-CSFR-deficient mice with ALU-OVA. 10 days post vaccination animals were bled and the presence of 4 isotypes of anti-OVA Ig in serum was evaluated by ELISA. As observed (**Figure 5- 19A**), the sera of vaccinated GM-CSFR-deficient mice contained lower titres of IgG1 and IgE, the inflammasome-related Igs, comparing to WT counterparts. The Th-1-related Ig isotypes in B6 mice, IgG2b and IgG2c, were not affected in GM-CSF-R deficient mice, and their levels were comparable to WT

controls. The OVA-specific IgG1 titre was elevated after 2nd boost of animals with alum, in both WT and GM-CSFR-deficient mice. The IgE levels dropped after the boost, although the titres were still clearly lower in GM-CSFR-deficient mice comparing to WT controls (**Figure 5- 19B**). These results highlight the role of GM-CSF in *in vivo* activation of inflammasome.

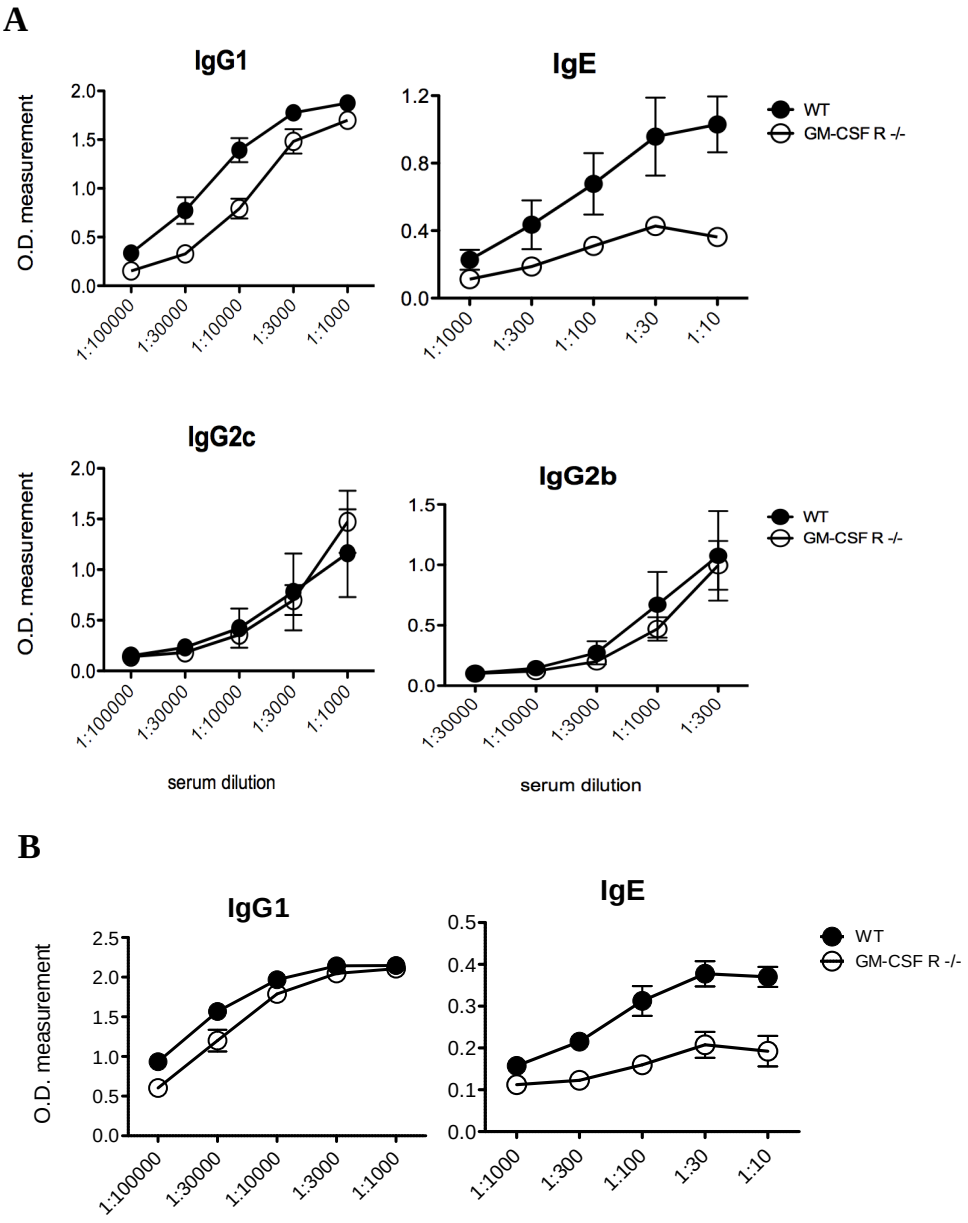


Figure 5- 19 The Th-2 biased antibody response to antigen following vaccination with Alum adjuvant is impaired in GM-CSFR-deficient mice. WT or GMCSF-R$^{-/-}$ mice were intreaperitoneally injected with 10 μg ALU-OVA. Mice were bled through retro-orbital plexus 10 days post vaccination and OVA-specific serum Ab titers of different isotypes were measured by ELISA **(A)**. The mice were boosted on d10 with 10 μg ALU-OVA and bled on d18 to analyze the secondary Ab response **(B).** Date are mean±SD (n=3).

5.15 The inflammatory cell recruitment to peritoneal cavity upon *in vivo* inflammasome activation is fully impaired in absence of GM-CSF function

In order to further investigate the role of GM-CSF in *in vivo* activation of inflammasome we monitored the inflammatory cell recruitment to peritoneal cavity upon injection of mice with ALU-OVA. Alum as an adjuvant activates inflammasome, which consequently recruits a large number of different inflammatory mononuclear cells to the site of injection [186]. We i.p injected the WT or GM-CSFR-deficient mice with OVA absorbed to alum and monitored the inflammatory cell influx into the peritoneal cavities of mice 24 hr post injection. As observed (**Figure 5- 20**), alum injection led to a massive inflammatory cell recruitment to the peritoneal cavity of WT mice including neutrophils, eosinophils and monocytes. But clear differently, the cellular influx was dramatically impaired in GM-CSFR-deficient animals suggesting the key role of GM-CSF in mediating this process via regulation of inflammasome activation *in vivo*. The total cell numbers as well as differential cell counts were massively decreased in peritoneal lavage fluids of GM-CSFR-deficient mice upon alum injection while the eosinophils were almost absent, as these mice reported to have a defect in eosinophil homeostasis [267].

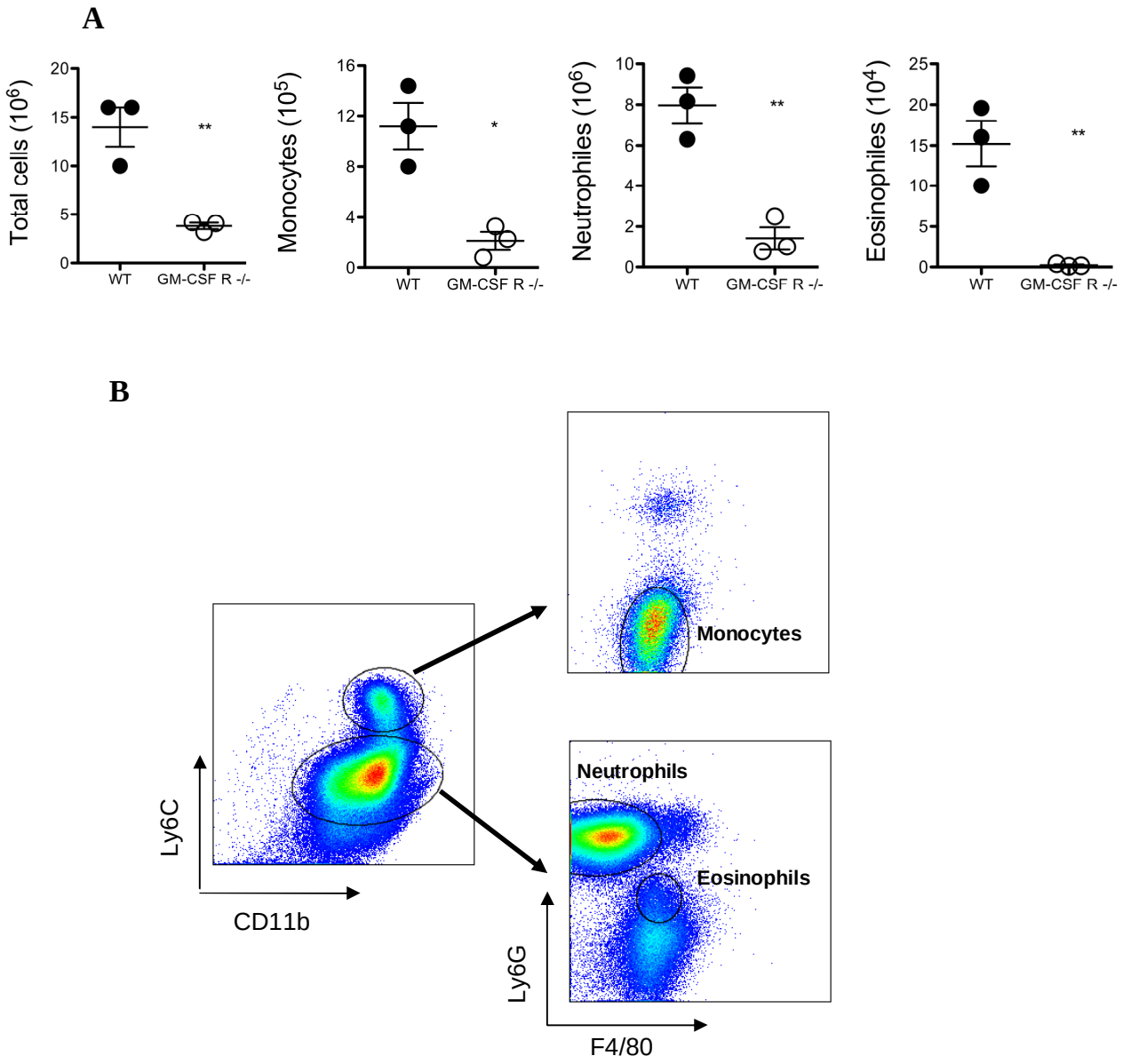


Figure 5- 20 Inflammatory cell recruitment to the peritoneal cavities of Alum-injected GM-CSFR-deficient mice is severely impaired. The WT or GM-CSFR-deficient mice (3 animals/group) were i.p injected with 10ug OVA absorbed to Alum, the mice were sacrificed 24 hr later and peritoneal cavities were flushed with PBS, the cellular content of lavage fluid was stained with a cocktail of antibodies to quantitatively differentiate different cell types and analyzed by FACS. Neutrophils ($Ly6G^+$, $F4/80^-$, and $CD11b^+$ cells), eosinophils ($F4/80^{dim}$, $Ly6G^+$, and $CD11b^+$ cells), and inflammatory mononuclear cells ($CD11b^+$, $F4/80^+$, and $Ly6C^+$ cells). **(A)** The graphs represent the differential counts of cell recruited to peritoneal cavity. Data are mean±SD (n=3). **(B)** A representative scheme of FACS analysis of neutrophils, eosinophils and monocytes in peritoneal lavage fluid of Alum-injected WT mice.

5.16 GM-CSFR-deficiency renders mice resistant to LPS-induced septic shock with diminished levels of IL-1 secretion

Septic shock is induced by high doses of endotoxins such as LPS. To investigate the role of GM-CSF in septic shock and consequent release of inflammatory cytokines, especially IL-1, we i.p injected WT or GM-CSFR-deficient animals with high dose LPS (50μg/g body weight) and analyzed the survival of mice over a 7 d period. In line with previous findings, we observed that GM-CSFR-deficient mice well tolerated the LPS-induced shock, as these mice showed the shock symptoms at the first 24-36 hours post LPS injection such as lethargy and fever but 10 out of 12 mice recovered afterwards and survived over a monitored period of 7 d. On the other hand 80% of the WT mice died because of the lathal shock (**Figure 5- 21A**). We bled the mice 3 hr post LPS injection and monitored the levels of pro-inflammatory cytokines IL-1α, IL-1β, TNF-α and IL-6 in the sera by ELISA. As shown in **Figure 5- 21B**, the sera of GM-CSFR-deficient mice contained lower levels of IL-1 (α andβ) and, to lower extent, TNF-α and IL-6.

These findings once again highlight the importance of endogenous GM-CSF levels in regulating the inflammatory processes such as septic shock, which we believe, is mostly regulated through priming the secretion of IL-1 family of cytokines.

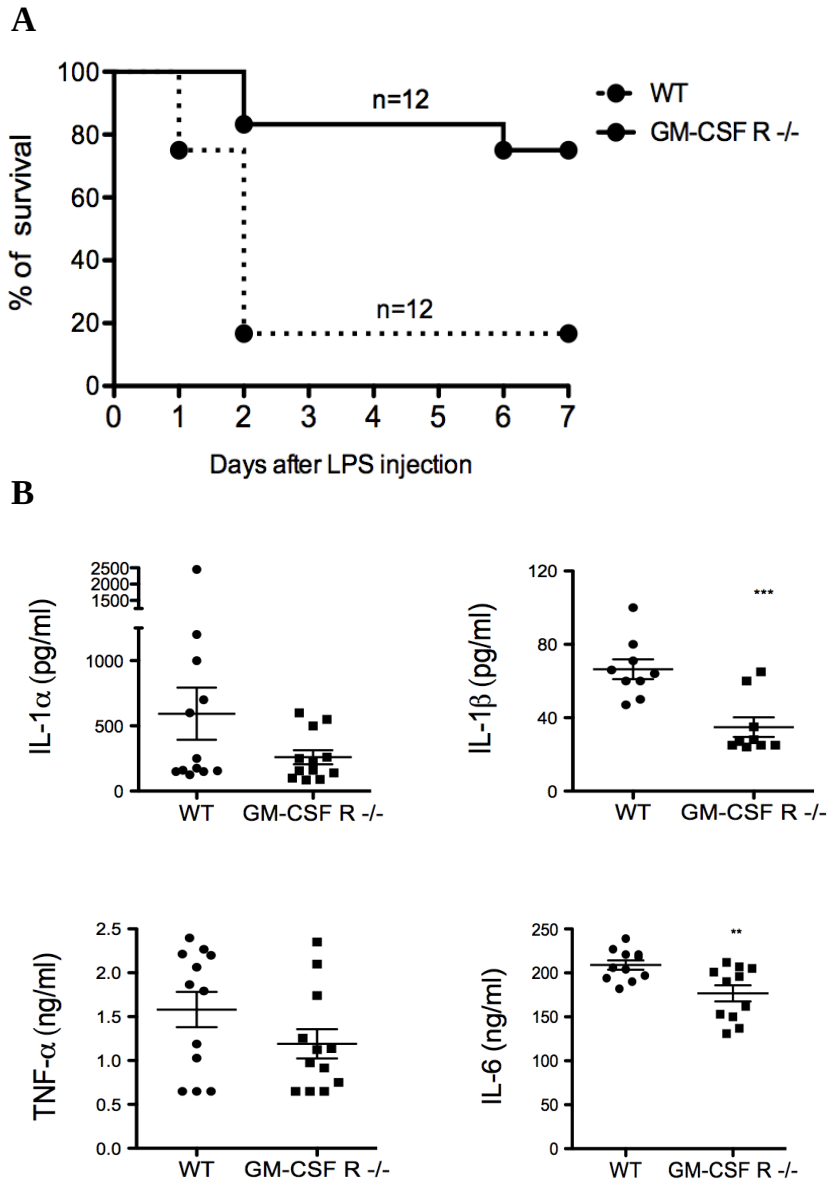


Figure 5- 21 GM-CSFR-deficient mice are resistant to septic shock, and exhibit lower levels of inflammatory IL-1 release. 12 WT or GM-CSFR-deficient mice were i.p injected with 50ug/g LPS and were followed for survival over a week period. The results were analyzed using survival curve analysis **(A)**. The mice were bled through retro-orbital plexus vein 3hr post LPS injection and levels of pro-inflammatory cytokines were measured in sera by ELISA **(B)**.

6. Discussion (II) Enhancement of Immunostimulatory Potential of DCs

GM-CSF regulates the inflammatory processes, a candidate for therapeutic intervaentions

GM-CSF is an "old" growth factor, well characterized for its multifunctional features in regulating immune system, ranging from involvement in cellular activation, proliferation, differentiation, homeostasis and inhibition of apoptosis [259]. This growth factor has been extensively used *in vitro* for generation of DCs from bone marrow progenitors [305]. *In vivo* it has also been administered in many therapeutic settings because of its adjuvanticity for eliciting humoral and cellular anti-tumor immune responses because it promotes differentiation, proliferation and activation of cells of immune system, including macrophages, neutophils, DCs and to some extent, T cells [344].

GM-CSF has been extensively used in experimental and clinical therapies due to its anti-tumor properties. In mouse model, irradiated tumor cells engineered to secret GM-CSF have been shown to elicit an strong long-lasting anti-tumor immune response marked with increase in local recruitment and activation of DCs. These DCs later present the tumor antigens to the T cells and promote their proliferation and function [345, 346]. The GM-CSF secreting tumor cells also activate the macrophages and iNKT cells to further boost the immune response [345, 347]. However, this strategy has been challenged by development of $CD11b^{+}$ $Gr1^{+}$ myeloid derived suppressor cells (MDSCs) after GM-CSF administration [348]. These cells are associated with tumor and could dampen the immune response to cancer. It's been suggested that cumulative levels of serum GM-CSF mobilizes the MDSCs from bone marrow to tumor site, while minimal concentrations would promote the anti-tumor immunity [349].

In human cancers GM-CSF secreting autologous GM-CSF-secreting tumor cells have been used for vaccination in some cancers such as melanoma [350], lung cancer [351], renal cell carcinoma [352], genitourinary malignancies [353] and prostate cancer [354]. All these studies have led to various degrees of increase in anti-tumor immune activities mainly local infiltration of immune cells including DCs, macrophages, T cells and granulocytes. GM-CSF protein has been also used in combination with other factors to elicit anti-tumor immune responses in lymphoma [355], melanoma [356-360], metastatic carcinoma [361] and has showed some clinical benefits.

GM-CSF has been also administered for HIV patients in several clinical trials and has been shown to increase the total WBC count and CD4+ cells of the patients and

reduced the plasma viral load [362-364]. But it's suggested that GM-CSF should be used in combination with anti-retroviral therapies to potentially reduce the viremia and strengthen the immune system [365].

GM-CSF has also been used in several cases as an immunosuppressive factor for autoimmune inflammatory conditions. As mentioned before GM-CSF administration, especially at high doses, might led to formation MDSCs which could abrogate the anti-tumor activity and promote immune suppression through several mechanisms such as induction of nitric oxide (NO) synthesis [366]. The human homologues of these cells have not been fully identified. In renal cancer the $CD11b^{+}$ $CD14^{-}$ $CD15^{+}$ cells were shown to suppress the anti-tumor T cells responses in an arginase-dependent manner [367], these cells could be the human version of murine MDSCs. But in carcinoma patients treated with GM-CSF, CD14+ myeloid cells were shown to be the suppressive population which function independent from iNO and arginase [368].

In mice, GM-CSF-induced DCs can activate the T_{reg} cells and promote the expansion of regulatory IL-10 secreting T cells, hence suppress the experimental autoimmune thyroiditis [369, 370].

GM-CSF has been also suggested to be a potential therapeutic factor for suppressing the severe inflammation in chronic obstructive pulmonary disease (COPD) [371]. In murine model of lung inflammation neutralizing GM-CSF activity by administration of anti-GM-CSF antibodies suppresses innate immunity. This treatment causes reduced macrophage and nuetrophil accumulation and proliferation and suppressed the Erk1/2, AKT, AP-1 and NF-κB [372, 373].

It is speculated that immunosuppressive function of GM-CSF and its contribution to tolerance is partly mediated through its induction of a molecule called milk fat globule epidermal growth factor protein-8 (MFG-8). This protein is absent in GM-CSF-deficient DCs and macrophages and is involved in uptake of apoptotic cells, hence those deficient cells are impaired in ingestion of apoptotic cells. The MFG-8 function leads to stimulation of T_{regs} and suppression of Th1 and Th17 responses [374]. As a supportive evidence for this idea the GM-CSF-secreting tumor line which also secretes a mutant non-functional MFG-8 was able to completely eliminate the established tumors [375].

IL-1β as a key player in inflammation; therapeutic applications

Our current understanding of GM-CSF function has been further renewed by several recent reports suggesting that this growth factor plays an exclusively activating role in regard to myeloid cells, in particular, dendritic cells [282-285]. Since we were interested to apply strategies for boosting "immunostimulatory" potential of dendritic cells, it was worth it to focus in detail on immunostimulatory effects of GM-CSF on DCs. The first observation that guided us through these studies, was the fact that GM-CSF treated DCs were shown to up-regulate several immunostimulatory molecules, such as pro-inflammatory cytokines. Of note, the LPS-activated DCs exposed to exogenous GM-CSF showed dramatic upregulation of mRNA levels of an important inflammatory mediator, IL-1β. IL-1β was identified decades ago and its function as endogenous pyrogen in health and disease has been extensively studied.

IL-1β plays key role in the severe immunopathology observed in some inflammatory disorders, hence blocking IL-1β activity either through administration of IL-1Ra or blocking anti-IL-1β antibodies has been applied as a strategy to reduce the inflammatory manifestations of these diseases. In case of Rheumatoid Arthritis (RA), which is an autoimmune inflammatory disorder, blocking IL-1β has shown to have some clinical benefits. In murine model of RA, collagen induced arthritis (CIA), IL-1β was found to play a key dominant role with high levels and its blockade profoundly suppressed the disease[376]. In humans, increased expression of IL-1β has been documented in synovial and non-synovial tissues [377], lymph draining from joints [378] as well as synovial fluid [379] of RA patients and correlates with pathology [380]. The human monoclonal blocking anti-IL-1β antibody ACZ885 administration has been shown to completely reverse the joint inflammation and cartilage destruction in human IL-1β-induced arthritis in mice and a significantly reduced the RA disease Score in patients [381].

The inflammatory events in type 2 diabetes (T2D) were also shown to be linked with IL-1β . Donath *et al*. have documented the presence of IL-1β in pancreatic tissues of T2D patients, produced by beta cells, which strongly correlates with disease progression [382]. This was also reported in another study showing elevated IL-1β mRNA in beta cells of T2D patients [383]. IL-1β was also shown to increase the risk of developing T2D in healthy individuals [384]. Recently XOMA052 (gevokizumab),

a novel anti-IL-1β monoclonal antibody has reported to prevent the insulin resistance and abrogate the β cell apoptosis in a high fat diet induced T2D in mice together with another anti-inflammatory properties [385, 386]. The early stage phase I clinical trial observations after administration of this antibody for T2D aptients have been promising and it's being used for larger clinical trials [387].

The other anti-IL-1β monoclonal antibody; Ilaris (canakinumab) is currently being used in clinical trials for treatment of Cold Autoinflammatory syndrome which is a inflammasome mediated disease marked by large amounts of IL-1β production [387]. Collectively, these evidences suggest that IL-1β blockade could be of clinical benefit for treatment of inflammatory conditions in different disorders.

IL-1β is a unique cytokine in terms of synthesis, processing and release and shows different characteristics in comparison to other known classical pro-inflammatory cytokines such as IL-6 and TNF-α. The synthesized IL-1β exists in an inactive, immature pro-form, which is not able to conduct any biological activities before proteolytic processing. Caspase-1 has been identified as the unique molecule responsible for proteolytic cleavage of immature IL-1β and formation of bioactive cytokine, which is able to signal upon binding to its known receptors. Caspase-1 is one of the inflammatory cytokines that itself exists as inactive pro-form and needs to be cleaved and activated to be able to catalyze the IL-1β conversion process. The discovery of inflammasomes, the large multi-protein complexes involved in caspase-1 activation was a remarkable milestone in further understanding the biology of IL-1β. This discovery shed light on the ambiguities regarding the IL-1β processing and production *in vitro* and *in vivo*. Hence, we decided to conduct a detailed investigation of IL-1β secretion and inflammasome activation by treating DCs or macrophages with exogenous GM-CSF.

Synergistic effect of GM-CSF with TLR agonists in boosting IL-1 production

As the first step, we thought to evaluate the effect of GM-CSF on IL-1β production by myeloid cells. The experimental setup for inducing DCs or macrophages to secrete bioactive IL-1β requires two distinct signals to be delivered to the cells. The first signal is a stimulus which would engage the pattern recognition receptors e.g TLRs and via agonism leads to mRNA transcription and synthesis of pro-IL-1β within the cells. Many PAMPs as TLR agonists, have been shown to promote this process while

engaged with their respective receptors on cell surface or within the cytosol. As the product of this stimulation, pro-form IL-1β, needs to be processed into bioactive form through inflammasome as an activating platform for caspase-1, a second signal is clearly required to be delivered to the cells primed with signal I. Danger-associated molecular patterns (DAMPs) such a extracellular ATP, pore-forming toxins like nigericin, the adjuvant Alum, crystalline particulate structures like MSU, asbestos and silica and many others have been shown to have the capacity to activate the inflammasome complexes leading to activation of caspase-1 and consecutive release of bioactive IL-1β.

Based on these facts, we exploited the standard experimental protocol where overnight priming of DCs and macrophages with TLR agonists (signal I), followed by a short exposure of cells to various danger signals (signal II), results in secretion of bioactive IL-1β into the culture supernatants. As an experimental intervention, we co-primed the LPS-treated cells with exogenous GM-CSF before delivering the danger signal. The striking result was the huge difference between the amounts of IL-1β, released by DCs or macrophages when treated in presence or absence of exogenous GM-CSF. While LPS alone were able to prime the cells to produce modest levels of IL-1β, after treatment with ATP as danger signal, addition of GM-CSF to the cultures resulted in boosting the production almost 5-10 folds (**Figure 5- 1**). The nanogram-range concentration of released IL-1β is of great attention, considering the strong immunogenic functions of this cytokine in cellular environment. In majority of previous reports the reported concentrations of IL-1β in *in vitro* assays are in picogram ranges, while GM-CSF treatment in combination with TLR agonist increased these levels to nanogram ranges.

We also showed that not only ATP-treated but the cells treated with a range of other so-called danger signals with different natures, such as MSU, alum and nigericin responded in a same way to GM-CSF by elevation of IL-1β production to different extents (**Figure 5- 1**). The next approach was to show that the cells treated with various priming signals, including different TLR agonists were able to strongly upregulate IL-1β levels, once treated with GM-CSF. To prove this point we used other TLR agonists such as CpG, Imidazoquinoline, poly (I:C) and Pam3Csk4, instead of commonly used LPS to prime the cells overnight. As expected those differently-primed cells did secret elevated levels IL-1β when treated in presence of GM-CSF

followed by danger signal, comparing to TLR-agonist-only treated cells (**Figure 5- 3**). This suggests that the observed effect of GM-CSF on IL-1β is not exclusive to a specific TLR simulation.

It should also be stressed that augmented production of IL-1β is dependent on the presence of all three key players, namely priming signal, GM-CSF and danger signal, as LPS alone, GM-CSF alone or ATP alone, were not able to boost the production of IL-1β suggesting that GM-CSF is not there to replace any of signals I or II required for inflammasome activation, but rather acts as an additional potent inflammatory signal (signal III) to boost the process effectively. This effect was exclusive to GM-CSF as the other tested growth factor M-CSF with some shared functions with GM-CSF were not able to induce production of IL-1β even in concentrations as high as 50 ng/ml (**Figure 5- 2**). On the contrary GM-CSF concentrations as low as 2 ng/ml were able to efficiently boost the production of IL-1β suggesting that this observation could be of functional relevance in physiologic conditions *in vivo* as many inflammatory pathogenic events are able to elevate the GM-CSF concentrations *in vivo,* hence affecting the production of IL-1 cytokines. Endogenous levels of GM-CSF were shown to be important in regulating inflammasome activation and IL-1β release, as absence of efficient GM-CSF signaling compromised this aspect *in vivo*.

As a proof of concept, cells deficient in GM-CSFR β chain which lack the GM-CSF signaling ability, failed to respond to GM-CSF and augment IL-1β production indicating the direct involvement of GM-CSF signaling through it's receptor in this phenomenon. One might argue that the GM-CSFR-deficient cells might have some other unknown functional defects which would compromise their ability to activate inflammasome and release IL-1β. To rule out this possibility, we blocked the GM-CSF activity by treatment of the WT BMDCs in presence of blocking anti-GM-CSF antibodies and monitored their response to exogenous GM-CSF. As expected LPS-primed DCs, which were treated with blocking Ab, followed by ATP treatment, failed to boost the IL-1β secretion in response to exogenous GM-CSF (**Figure 5- 4**).

We also observed that shorter exposure time points (as shorts as 3hrs) when treating LPS-primed cells with GM-CSF was sufficient to promote the elevated production of IL-1β by DCs (**Figure 5- 5**). This should be noted carefully as *in vivo*, elevated amounts of GM-CSF in pathologic conditions don't tend to persist for a long period and declines over a fast period of time. So, as we showed, transient exposure of cells

to GM-CSF *in vitro* led to production of significant and decent amounts of IL-1β compared to cells treated in absence of this growth factor.

GM-CSF –mediated augmentation of IL-1α

Next we also showed that the levels of IL-1α, the other member of IL-1 family, was also strongly increased in response to GM-CSF treatment *in vitro,* in a same manner (**Figure 5- 6**). The levels of other pro-inflammatory cytokines which are not related to IL-1 family in terms of evolutionary relationship, regulation and secretion, such as IL-6 and TNF-α was modestly increased in response to GM-CSF treatment but it should be noted that these cytokines were already primed in response to LPS stimulation and GM-CSF was able to further increase their production while in case of IL-1 cytokines LPS alone failed to prime the cells efficiently for the production of IL-1α or IL-1β. But addition of exogenous GM-CSF, as documented here, dramatically boosted their production in an efficient manner.

The increase observed in IL-6 and TNF-α levels upon GM-CSF treatment could be secondary to immunostimulatory effects of GM-CSF as the state of high inflammation marked by remarkable increase in IL-1 levels could further boost the production of other inflammatory mediators. On top of that, GM-CSF is known to activate the NF-κB pathway, so it's not a surprise that GM-CSF will induce further production of IL-6 and TNF-α, which are among known targets of NF-κB activation.

Although the IL-1α production was also boosted by GM-CSF treatment in our *in vitro* assays, but the concentrations of IL-1α was much lower than values observed for IL-1β. This could be, to some extent, due to the regulatory function of IL-1β over IL-1α [159] or other regulatory mechanisms involved in regulation of IL-1α levels *in vitro* and *in vivo*, considering the fact that this cytokine is not regulated through inflammasomes. As GM-CSF greatly enhances the IL-1β through activation of inflammasome, its effect on IL-1α production is not expected to be as strong as observed for IL-1β.

GM-CSF exerts its effect at the level of IL-1β synthesis

We were able to show that GM-CSF exerts its effect on the level of IL-1β synthesis as GM-CSF treated cells showed marked accumulation of pro-IL-1β levels in cytoplasm. We noticed that the synergistic effect between LPS and GM-CSF which leads to augmented intracellular pro-IL-1β synthesis was still functional in presence of lower concentration of LPS (as low as 0.001μg/ml) (**Figure 5- 13**). This fact suggests that GM-CSF is potent enough to boost the synthesis of proform-IL-1β even when the cells receive a lower threshold of activation by TLR agonism. This increase in pro-form cytokine was observed in earlier time points as early as 6hr post stimulation with LPS and GM-CSF and the pro-IL-1β seems to accumulate during the stimulation period as the difference in protein content was the highest (compared to LPS-only) when cells were stimulated for 24 hrs. We also noticed this time-dependent increase in mature cytokine as longer incubation of LPS-primed cells with GM-CSF led to further increase of IL-1β release, peaking at 24 hr post stimulation.

Although these observations show that GM-CSF exerts its stimulatory effects already on IL-1β synthesis stage, but we still cannot rule out its involvement in the intricate and elusive pathways of IL-1β secretion. Since the exact mechanism of IL-1β secretion hasn't yet been elucidated, it's not easy to investigate in detail the possible role of GM-CSF in IL-1β secretion process. Therefore, this issue needs to be addressed in future investigations. A recent study by Becker *et al.* has revealed Rab39a, a member of Rab GTPase family (which are involved in protein secretion) is able to bind to caspase-1 hence, regulate the IL-1β secretion. Knocking down this protein diminished of IL-1β secretion and its over-expression caused an enhancement of IL-1β secretion [388]. Since this protein is directly involved in IL-1β secretion in a caspase-1 dependent manner, it is worth to investigate possible effect of GM-CSF on its expression and function in future studies in order to examine the involvement of GM-CSF in IL-1β secretion pathways.

GM-CSF boosts the IL-1 production in an inflammasome-dependent manner

We believe and provided some evidence that GM-CSF boosts IL-1 production in an inflammasome-dependent fashion. It was clear that blocking caspase-1 a core element of NALP3 inflammasome abrogated the GM-CSF's ability to boost the IL-1β production (**Figure 5- 15**). The upstream caspase, caspase-11, which is shown to be necessary for caspase-1 activation was also crucial for this process as blocking

caspase-11 with specific inhibitor, abrogated the IL-1β release after GM-CSF treatment in the same fashion. We also investigated the components of NALP3 inflammasome, namely caspase-1, caspase-11 and NALP3 in terms of protein levels upon GM-CSF treatment but we didn't observe any significant increase in their protein quantity in GM-CSF treated macrophages (**Figure 5- 12**). However this finding should be interpreted carefully as we assessed protein levels 24 hours post treatment with GM-CSF and there is a possibility that protein levels were somehow regulated at earlier time-points and later were adjusted to lower levels through possible auto-regulatory feedback mechanisms. To assess this possibility we monitored the protein content of NALP3 6hr post stimulation, but to our surprise the protein levels were the same in presence or absence of GM-CSF in LPS-primed cells (**Figure 5- 14**). It should be noted that, GM-CSF was found to strongly upregulate NALP3 and caspase-11 at mRNA levels (data not shown) so we still don't know the mechanisms leading to balancing the protein expression levels following GM-CSF treatment.

The possible cooperation between GM-CSF and NF-κB pathways in regulation of inflammasome activation

The inflammasome components are upregulated via activation of NF-κB signaling pathway upon LPS-priming, therefore NF-κB plays a key role in regulating inflammasome activation. On the other hand it is well documented that GM-CSF can activate NF-κB pathway in different cell types including DCs [194, 268, 373, 389-391]. In line with these facts, we showed that the observed boosting of IL-1β production by DCs or macrophages is totally dependent on NF-κB pathway as inhibition of this pathway with selective inhibitors, Wedelolactone or Bay11-708 abrogated the IL-1β level augmentation by GM-CSF in a dose-dependent manner. This observation further suggests that GM-CSF regulates the inflammasome complex by hyper-activating NF-κB signaling pathway.

Upon GM-CSF engagement with it's receptor several downstream signaling pathways pathways including Jak2/STAT5, MAPK and PI3K/Akt are activated [264, 265]. The β chain of receptor is mainaly associates with JAK2 [392] and causes its transphosphorylation of both βc and JAK2 leading to intiation of multiple signaling events and biological activity [393]. It has been shown that this event takes place during assembly of GM-CSFR complex into a dodecamer with two hexmers which

their interactions brings β and α chains to close proximity for functional dimerization [394].

GM-CSF has been shown in several reports to activate the NF-κB pathway [373, 389, 395, 396]. The exact mechanism underlaying this co-operative effect is not yet elucidated. One possible link between two pathways could be the signaling molecule STAT5. STAT 5 can be phosphorylated by activated JAK2 which leads to its dimerization and translocation into the nucleus [397]. It has been shown years ago by Nakamura *et al.* that GM-CSFR-induced activation of STAT5 can increase the DNA binding activity and transactivational potential of NF-κB. This was confirmed by the experiments showing overexpression of constitutively active STAT5 hyper-activates NF-κB pathway [391].

Another report by Meads *et al.* [398]suggest that TNFR-associated factor 6 (TRAF6) links the GM-CSFR β chain to NF-κB activation. TRAF6 is a member of TRAF family of adaptor molecules which is required for TLR and TNFR dependent of NF-κB signal transduction [399]. Meads and his colleagues have demonstrated a direct binding between TRAF6 and βc, and upon mutating TRAF6 binding motif on βc, the GM-CSF-mediated NF-κB activation was abrogated [398]. The crosstalk points mentioned above could be further investigated in future with molecular and cellular techniques to reveal whether they are involved in GM-CSF mediated hyper-activation of NF-κB signaling which leads to massive production of IL-1β in combination with TLR agonists.

One of the other possible mechanisms of GM-CSF and NF-κB crosstalk could be involvement of members of IκB family of regulatory molecules such as IκBα and IκBβ. These molecules could be phosphorylated by a functional IKK (inhibitor of kappaB kinase) complex, composed of IKKα and IKKβ, leading to their ubiquitination and degradation and activation of NF-κB [400]. To further investigate the contribution of GM-CSF to NF-κB signaling in this scenario, we monitored the kinetics of degradation and re-synthesis of IκBα and IκBβ. The degradation patterns of both molecules in LPS-primed cells were similar in presence or absence of GM-CSF treatment. Therefore, we could rule out that GM-CSF-mediated augmentation of NF-κB signaling is due to its effect on IκB family members, IκBα and IκBβ.

IKKβ could be a potential link between GM-CSF and NF-κB signaling pathways as it was shown that GM-CSF activated the NF-κB pathway through direct interactions

between GM-CSFR α chain and IKKβ which leads to IκB degradation [268]. IκBβ is thought to act as versatile regulator as it can support both pro- and anti-inflammatory responses. It can form a stable complex with NF-κB subunits p65 and c-Rel to translocate to the nucleus and support the expression of certain genes such as TNF-α [401]. A recent study by Scheibel *et al*. implicates a direct role for IκBβ in supporting IL-1β transcription. IκBβ was shown form a complex with c-Rel and RelA and after translocation to the nucleus directly interact with IL-1β promoter [402]. We haven't observed any further up-regulation of this molecule upon co-treatment of cells with LPS and GM-CSF comparing to LPS-only treated cells (**Figure 5- 17**).

Although the IκB family of regulatory proteins were not influenced by GM-CSF treatment, we were able to show that the endpoint event of NF-κB activation which is the translocation of NF-κB subunits (p50, p52, and c-Rel) to the nucleus was clearly escalated by synergistic effect of GM-CSF and LPS (Khameneh, LinMin *et al*, Submitted) (**Figure 5- 18**). This is a direct proof and major hallmark to illustrate the involvement of GM-CSF in amplification of NF-κB signal. One of the major products of this amplification within the NF-κB gene signature is IL-1β which was validated by us to be greatly boosted by GM-CSF and LPS synergism. We still need to investigate in detail, the intracellular events which occur after engagement of GM-CSF by its receptor on DCs or macrophages to elucidate the unknown crosstalk between NF-κB and GM-CSF signaling pathways.

GM-CSF mediates Th-2 –skewed Ab response to antigen

We exploited Alum as an adjuvant known to induce NALP3 inflammasome activation *in vivo* in our studies. The alum injection has been shown to elicit a Th-2 biased Ab response to antigens marked with preferential increase of IgG1 and IgE isotypes, as a result of inflammasome activation. If GM-CSF is involved in process of inflammasome activation *in vivo* it should affect this phenotype, hence we i.p vaccinated the WT or GM-CSFR-deficient mice with OVA absorbed to alum and investigated the specific Ab titers in blood sera 10 days post-vaccination. As expected absence of GM-CSF signaling abrogated the Th-2 –skewed Ab response to antigen, as levels of IgG1 and IgE were markedly decreased in GM-CSFR-deficient animals while the Th-1 related isotypes IgG2b and IgG2c were not affected (**Figure 5- 19**). Using an inflammasome-independent adjuvant, CFA (complete Freund's adjuvant),

for vaccination didn't show any difference in Ab response between WT and GM-CSFR-deficient animals (data not shown), further proving the specific role of GM-CSF in modulating inflammasome-mediated Ab responses. This observation suggests that GM-CSF plays an important role in activation of inflammasome following Alum administration, as it affects the functional outcome of this event, Th-2-biased Ab response to antigen.

GM-CSF involvement in inflammatory cell influx

Alum administrtion *in vivo* also causes massive infiltration of inflammatory cells to the infusion site as result of inflammasome activation. This cellular influx is a hallmark of inflammasome activation. To address the possible involvement of GM-CSF in this process we immunized WT or GM-CSFR-deficient mice i.p with Alum and monitored the inflammatory cell recruitment to the peritoneal cavity 24 hr later. As expected the WT mice exhibited a massive influx of inflammatory cells including neutrophils, eosinophils and monocyte in peritoneum. But surprisingly, this recruitment was dramatically impaired in GM-CSFR-deficient mice highlighted with poor infiltration of cells into peritoneum. Particularly, eosinophils were almost absent in KO mice in line with previous reports that these mice have a defect in eosinophil homeostasis and show low basal levels of eosinophils. The numbers of DCs recruited to peritoneum was also greatly decreased in GM-CSFR-deficient animals following alum treatment (**Figure 5- 20**).

Altogether these data suggests that GM-CSF tightly regulates the extent of inflammasome activation *in vivo* since the absence of its signaling causes severe deffects of hallmark inflammatory responses to inflammasome activation *in vivo*. This further highlights the involvement of GM-CSF in regulation of innate and adaptive immune reactions to foreign stimuli. Hence, endogenous levels of GM-CSF, although relatively low, play a key role in orchestrating inflammatory responses and could not be easily neglected by mild phenotype of GM-CSFR-deficient or GM-CSF-deficient mice.

GM-CSF potently regulates endotoxic shock

Another inflammatory event in which inflammasome activation plays a key role is the endotoxic shock caused by high doses of endotoxin. To test whether GM-CSF also plays a role in LPS-induce septic shock we injected the WT or GM-CSFR-/- mice with

high dose LPS and monitored their survival rate over 7 days period. As expected, the WT mice were sensitive to high dose LPS and died because of shock within 24-36 hr post-injection, but GM-CSFR-deficient animals managed to survive the shock (**Figure 5- 21**). These animals showed the symptoms of shock in the first 72hr but started to recuperate later on. The previous report have shown that GM-CSF-deficient mice are resistant to LPS-induced septic shock and their peritoneal macrophages secret less IL-1α, NO but comparable levels of TNF-α [403] while another reports suggest decreased levels of TNF-α and IL-6 as well [404]. GM-CSF is thought to play a pivotal role in this process as administration of neutralizing anti-GM-CSF antibodies profoundly suppresses the LPS-induced inflammation in WT animals [373]. In line with those reports, we also observed lower amounts of IL-1α, IL-1β and IL-6 in GMCSFR-deficient mice blood sera 3hr post LPS treatment (**Figure 5- 21**). Since the production of IL-1 is tightly regulated by inflammasome activity, the lower levels of this cytokine in absence of GM-CSF signalling highlight impairment in inflammasome activation. Our observations in GM-CSFR-deficient mice was reminiscent of the same phenotype (resistance to LPS shock) reported in animals lacking the inflammasome components or their regulators such as cryopyrin [223], ASC[405], caspase-1 [207, 208], caspase-11 [340] and IκBβ [194].

This consistency further suggests the presence of a tightly regulated crosstalk between GM-CSF and the inflammasome complex. In this study we tried to investigate the components of NALP3 inflammasome as one of the widely studied inflammasome complexes in terms of their regulation by GM-CSF. However, further investigations are required to show whether GM-CSF is also involved in regulation and activation of other inflammasomes such as IPAF, NALP1 and NLRC4 inflammasomes..

6.1 Conclusion

Collectively, our findings provide us with new insights into GM-CSF function in regulation of innate and adaptive immune responses during health and disease. GM-CSF has been shown here to strongly boost the IL-1β production by DCs. It also boosts the production of other members of IL-1 family, IL-α and IL-18. This effect can be observed when DCs primed with a broad range of different stimuli including TLR agonists and is mainly due to hyper-activation of NF-κB pathway. GM-CSF was

shown to greatly affect the extent of inflammasome activity and inflammatory responses *in vivo*, hence playing an important role in promoting inflammation.

The proposed role of GM-CSF in the inflammasome activity and production of IL-1 cytokines could be further investigated to elucidate the intricate mechanisms involved in regulation of inflammasomes, which have been under broad investigation due to their growing importance. The unknown crosstalk between GM-CSF and NF-κB signalling pathways should be extensively investigated to further explain how GM-CSF boosts the NF-κB activity mechanistically.

GM-CSF treated DCs have the capability to release massive amounts of IL-1 upon receiving signals I and II for inflammasome activation. This strategy could be applied in several therapeutic interventions including cell-based therapies, combination therapies, antibody treatments and vaccine design.

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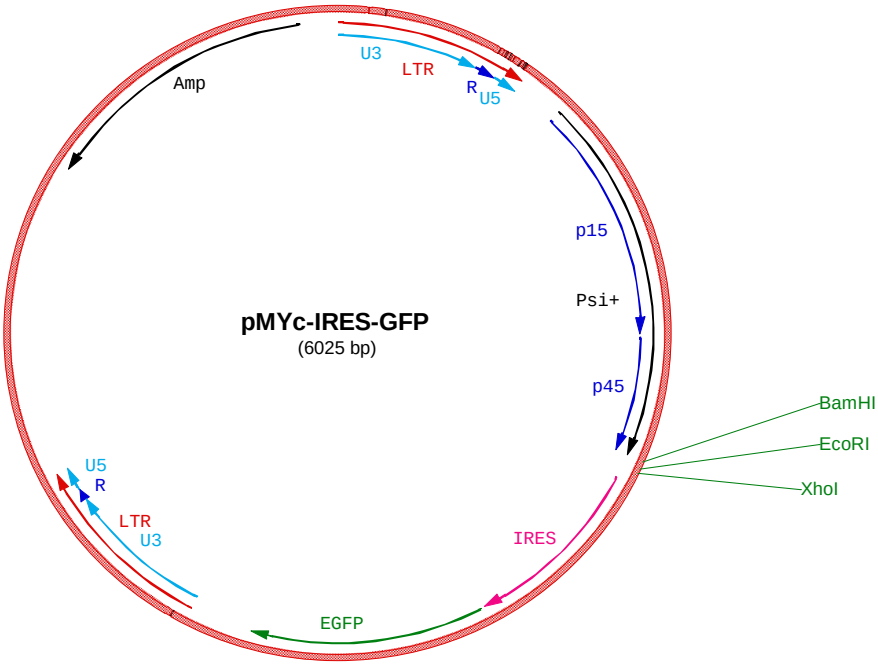
Appendix I. Commonly used media and buffers

Name	Composition
Electrophoresis running buffer 10x	100mL 1% SDS, 144.13g Glycine, 30.3g Tris base ad. 4L ddH_2O
ELISA blocking solution	1% (w/v) BSA in PBS
ELISA washing solution	0.05% (v/v)TWEEN20 in PBS
FACS buffer	PBS / 2% (v/v) FCS
Fixation Buffer	4% (w/v) Paraformaldehyde in PBS
Freezing Medium for cells	10% DMSO in PBS
Cell culture medium (IMDM 2% FCS)	IMDM Powder (1x10L) with L-glutamine and 25mM HEPES, 34.25g Sodium bicarbonate, 100mL Pen-Strep, 100mL MEM NEAA, 10mL 5mg/mL Insulin, 10mL β-mercaptoethanol, 30mL 10% Primatone. ad. ddH_2O 10L. pH adjusted to 7.0, filtered. Supplemented with 2% FCS.
LB Medium LB Medium + Ampicilin LB Agarose plates	10g Bacto-tryptone, 5g Yeast Extract, 10g Nacl ad. 1L ddH_2O; pH 7.0, autoclaved LB Medium + 100μg/mL Ampicilin (Ampicilin stock: 10mg/mL in ddH_2O, pH 8.4 filtered and stored at -20°C). LB Medium + 15g Agar; autoclaved
Lysis Buffer	10% Glycerol, 150mM NaCl, 20 mM HEPES (pH 7.9), 0.9% Triton X-100, 0.1% NP-40, 0.2mM EDTA, 0.5mM PMSF. Supplemented with a Protease inhibitor cocktail tablet (Roche Diagnostics) prior to use.
PBS (Phosphate Buffered Saline)	8g NaCl, 0.2g KCl, 1.15g

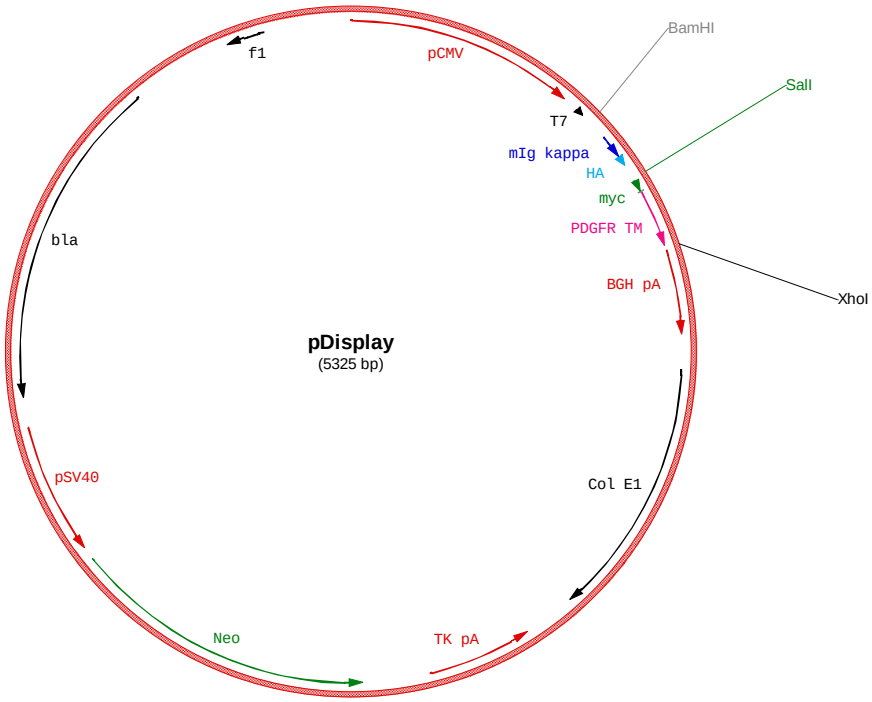
	$Na_2HPO_4.2H_2O$, 0.2g KH_2PO_4, 1.67 $CaCl_2.4H_2O$, 0.1g $MgCl_2.6H_2O$, 0.1g BSA, ad. 1L ddH_2O; pH adjusted to 7.2
Permeabilization Buffer	0.5 % (w/v) Saponin in PBS
Resolving gel (10%)	3.3mL 30% Acrylamide/Bis Solution 29:1 (3.3% C), 2.5mL 1.5M Tris-Cl (pH 8.8), 0.05mL 20% (v/v) SDS, 0.05mL APS, 0.004mL TEMED, 4.1mL H_2O
SDS Loading Buffer (1X)	50mM Tris-HCl (pH 6.8), 100mM Mercaptoethanol, 2% (w/v) SDS, 0.1% (w/v) Bromophenol blue, 10% (w/v) Glycerol
Stacking Gel	1.7mL 30% Acrylamide/Bis Solution 29:1 (3.3% C), 2.5mL 1M Tris-Cl (pH 6.8), 0.05mL 20% SDS, 0.05mL APS, 0.01mL TEMED, 6.9mL H_2O
TAE (50x)	2.42g Tris, 57.1mL Citric acid, 100mL 0.5M EDTA, ad. 1L ddH_2O; pH adjusted at 8.0
TE Buffer	10mM Tris-HCl, 1mM EDTA in ddH_2O; pH adjusted at 8.0
Transfer Buffer (10x) Transfer Buffer (1x)	116g Tris base, 580g Glycine, ad. 4L ddH_2O 100mL 10x Transfer Buffer, 200mL Methanol, ad. 700mL ddH_2O
Western blot blocking solution	3% (w/v) non-fat dry milk in PBS/0.05%TWEEN20
Western blot wash buffer	PBS/0.05%TWEEN20

Appendix II. Plasmids

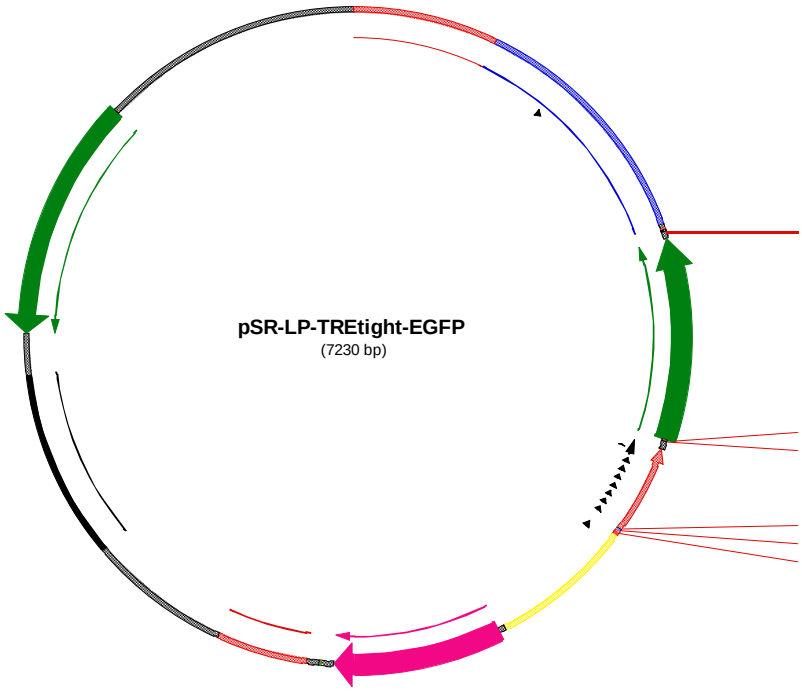
1. pMYc-IRES-GFP



2- pDisplay



3- pSR-LP-TREtight-EGFP



4- pSR-rtTA-A-pgk-iner5