INFLUENCE OF CD137L REVERSE SIGNALING ON MYELOPOIESIS IN ACUTE AND CHRONIC INFLAMMATION

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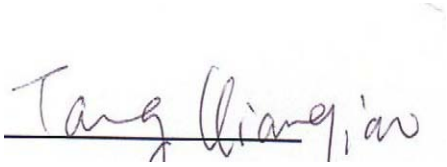
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

CD137 is a costimulatory molecule expressed on activated T cells. The signaling of CD137 into T cells upon ligation by its ligand, CD137L expressed on antigen presenting cells (APC), can potently enhance the activation of T cells. Reversibly CD137 can also induce signalling into APC via CD137L to promote activation and proliferation. By investigating the role of CD137L on myelopoiesis under inflammatory condition in vitro and in vivo, it was shown that CD137L reverse signaling represents a novel and potent growth and differentiating factor for murine myeloid cells during acute and chronic inflammation. In acute peritonitis and chronic aging model CD137L reverse signaling promotes myeloid cell proliferation and accumulation. Further investigations revealed the driving force behind the observed myelopoiesis as CD137+CD4+ T cells and absence of CD137L reverse signaling leads to accumulation of undifferentiated progenitor cells.

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List of Abbreviation

AML	Acute myeloid leukemia
APC	Antigen presenting cells
APC (dye)	Allophycocyanin
BCG	Bacillus Calmette–Guérin
BM	Bone marrow
BMM	Bone marrow-derived macrophage
BrdU	Bromodeoxyuridine
CD137L	CD137 ligand
CDP	Common DCs progenitor
CFSE	Carbosyfluorescein diacetate, succiniidyl ester
CFU-G	Colony forming unit-granulocyte
CFU-GM	Colony forming unit-granulocyte/macrophage
CFU-M	Colony forming unit-macrophage
CLP	Common lymphoid progenitor
CPM	Count per min
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediamine tetraaccetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
Fc	Fc portion of antibody
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HSC	Hematopoietic stem cell
IDO	Indoleamine 2,3-dioxygenase

IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
ILA	Induced by lymphocyte activation
i.p	Intraperitoneal
IRB	Institutional review board
i.v.	Intravenous
KO	Knockout
LCMV	Lymphocytic Choriomeningitis Virus
LPS	Lipopolysaccarides
MACS	Magnetic activated cell sorting
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Monocyte colony stimulating factor
MDSC	Myeloid derived suppressor cell
MEP	Megakaryocyte-erythroid progenitor
MLR	Mixed lymphocyte reaction
MPP	Multipotency progenitor
MFI	Mean fluorescence intensity
MLR	Mixed lymphocyte reaction
NK	Natural killer
NO	Nitride Oxide
PBS	Phosphate buffered saline
PBST	PBS+0.05% Tween-20
PE	Phycoerythin
PE-Cy	PE-Cyanine
PercP	Peridinin-chlorophyll-protein complex
PFA	Paraformaldehyde

RBC	Red blood cell
ROS	Reactive oxygen species
SD	Standard deviation
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFRSF	Tumor necrosis factor super family
WT	Wild type

Chapter 1 Introduction

1.1 Hematopoiesis

Hematopoiesis is the process of generating blood cells from hematopoietic stem cells (HSCs). The rapid turnover of blood cells such as erythrocytes and neutrophils requires a steady supply from the bone marrow, the primary hematopoietic organ in adult mammals. A small pool of undifferentiated, self-renewing hematopoietic stem cells are responsible of giving rise to all the downstream blood cells. Classical viewing of hematopoiesis is usually composed of a cascade of differentiation, starting from HSCs at the top of the pyramid and ending with terminal differentiated cells that enter peripheral tissues. Except for T cells, B cells and certain tissue specific macrophages, the relationship of the differentiation state and self-renewing ability is reciprocal, meaning that as the more differentiated the cells are, the lesser proliferative they are.

Moreover, only the rare population of HSCs retains the ability of differentiating to all lineages of blood cells. The potential of committing to other lineages diminishes as the cells progress through several stages of intermittent phenotypes. For example, once the HSCs are committed to the fate of common myeloid progenitor cells (CMP), they have lost the potential of lymphopoiesis. When the stem cells have to make a decision of which lineage to commit to, they must

choose between the destiny of lymphoid lineage or myeloid lineage depending of the signals received from the external environment.

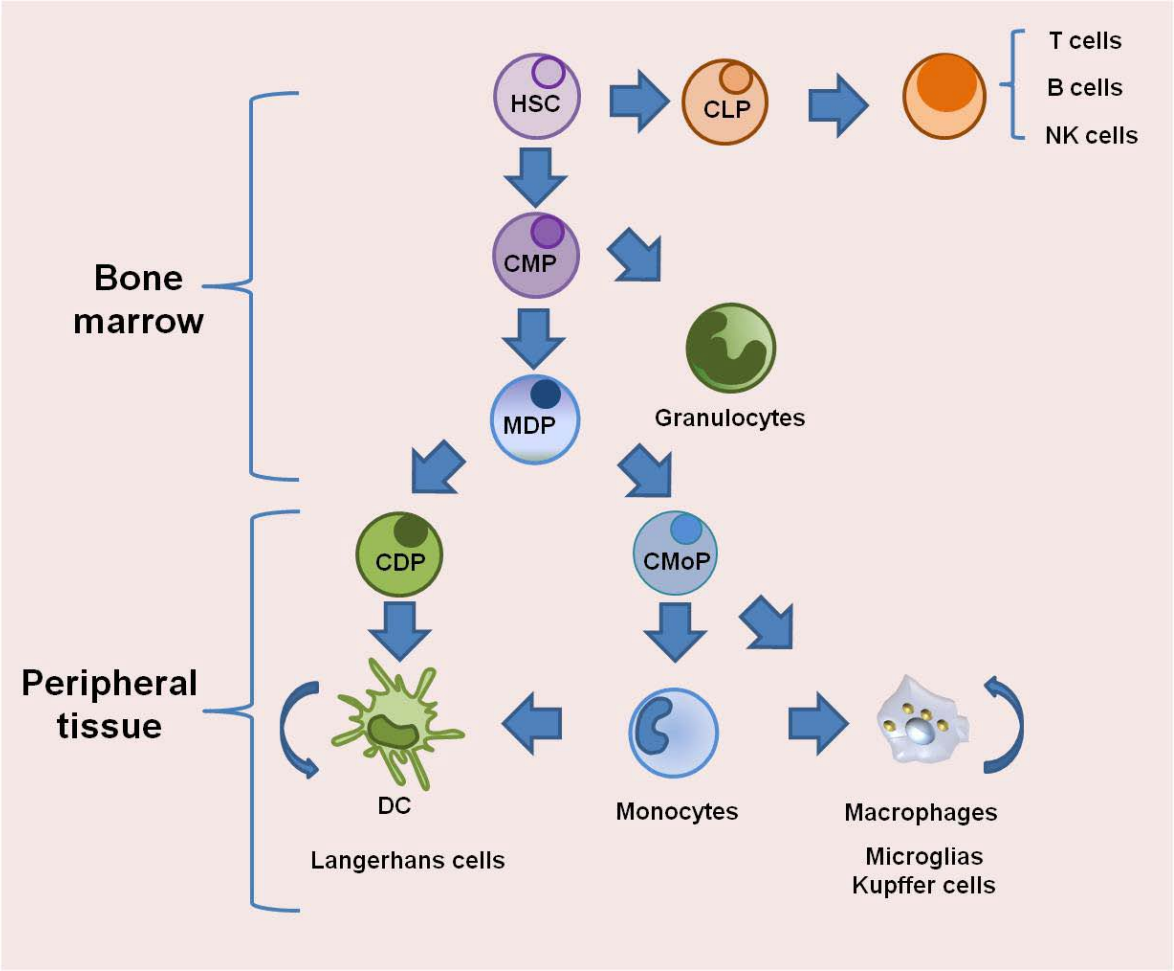


Figure 1.1 Overview of hematopoieis . Differentiation of blood cells start with long term HSCs and move down the cascade of a series of intermittent progenitor cell types. HSC: hematopoietic stem cells. CLP: common lymphoid progenitor cells. CMP: common myeloid progenitor cells. MDP: macrophage-dendritic cell progenitor cells. CDP: common dendritic cell progenitor cells. CMoP: common monocytes-macrophage progenitor cells. NK cells: natural killer cells.

1.2 Myelopoiesis during steady state

Myeloid cells consist of a variety of cells from both innate and adaptive immunity, including neutrophils, macrophages, monocytes, myeloid dendritic cells

(DCs) and the less concerned eosinophils, mast cells and basophils (Fogg et al. 2006, Geissmann et al. 2010). Understanding the underlying mechanism of myelopoiesis during steady and disease state can significantly contribute to elucidation of the potential pathogenic or regulatory role of each cell population.

1.2.1 DC

First identified in mouse spleen by Ralph Steinman in 1973 (Steinman 2007), DCs are probably the most studied myeloid cells due to its central role of antigen presentation. Early studies on monocytes biology suggest that monocytes are the precursor of DCs when they migrate towards lymphoid tissue to present antigen to T cells (Randolph, Beaulieu et al. 1998). However, many of these studies were performed under the situation of inflammation and the origin of DCs during steady state were not well identified. Adoptive transfer and in vivo labeling reveal that while monocytes, macrophages and DCs may share a group of common myeloid progenitor cells in the bone marrow term macrophage-DC progenitor (MDP), DCs precursors become separated from the other two cell populations at the level of pre-DCs (Fogg et al. 2006, Liu and Nussenzweig 2010). They have the plasticity of differentiating to most of DCs found in lymphoid organ but not monocytes. Therefore, instead of being the offspring of monocytes, DCs has its own precursor termed common DCs progenitor cells (CDP). However, not all the DCs are of HSCs origin. Langerhans cells, the skin-residing DCs, are proven to arise from yolk sac (YS) myeloid

progenitor cells and fetal liver monocytes early in the embryonic development. The cells retain self-renewal ability and do not require a contribution of blood monocytes for replenishment at steady state (Hoeffel, Wang et al. 2012).

1.2.2 Macrophage

Aside from DCs, studies also show that local macrophages population such as microglia are resistant to radiation, indicating that some of the macrophages populations are able to self renew and be independent of HSCs origin . Geissman suggested that at least a few groups of myeloid macrophages, including microglia, may arise from YS origin instead of monocytes at homeostatic state (Geissmann, et al 2010; Schulz, et al 2012). Indeed, studies show that myeloid progenitor cells from YS arrive at the embryo and seed in the brain and skin, which gave rise to microglia. The local microglia population is self-sustained throughout life without a contribution from blood monocytes. When the blood-brain-barrier is not disrupted, local microglia population is able to expand on its own (Ajami, Bennett et al. 2007). Moreover, a group of macrophages expressing a high level of F4/80, the classical macrophage marker, is found to originate independently from hematopoietic stem cells, suggesting two routes of myelopoiesis (Schulz, et al 2012, Gomez Perdiguero, et al. 2013, Kierdorf, et al. 2013).

Similary to microglia, Kuffper cells, peritoneal macrophages and splenic macrophages were once thought to arise from blood monocytes after they extravasate from circulation. However, studies showed that this scenario holds true only in the condition of inflammation or when the local macrophage population is depleted. The only macrophage population that is steadily replenished by blood monocytes is the macrophages in lamina propria where there is a constant low degree of inflammation (Yona, et al 2012). In conclusion, blood monocytes are dispensable for tissue DC and macrophage population during steady state and they only transiently differentiate to DC and macrophage when inflammation occurs.

1.2.3 Monocytes

As discussed above, monocytes were once considered the direct precursors of both DCs and macrophages and replenished both populations after they extravasate from the circulation. However, the identification of common DC progenitor cells (CDP) in the bone marrow suggested that monocytes may have distinct progenitors from DCs during steady state. Indeed although in the bone marrow the monocytes and DC can be derive from monocytes-DC progenitor cells (MDP, the differentiation program diverge at this stage (Hettinger, et al 2013). Downstream of MDP are two distinct populations of progenitor cells: CDP and common monocytes progenitor cells (CMoP). CMoP express Ly6C and exclusively give rise to $Ly6C^{+}$ blood monocytes but not DCs.

The revolutionary findings of DC and macrophages help to elucidate the lineages of different myeloid cells. However new questions arise, if monocytes do not differentiate to DCs and macrophages after they extravasate, what type of cells will they commit to?

Contradictory to previous dogma where the default differentiation program of monocytes is to become either DC or macrophage, Jakubzick reported that the degree of differentiation of monocytes after they infiltrate non-inflammatory tissue is minimal and the gene expression profile remain largely similar to blood monocytes but not to tissue macrophage and DCs, both of which are capable of self-renewal. Furthermore, the extravasated blood monocytes exit the tissue and migrate to lymph node and cross present antigen, a function exhibited by DCs (Jakubzick, et al 2013). The study revises the picture where monocytes were obliged to differentiate to macrophages or DCs after diapedesis and identify them as a unique mononuclear phagocyte population distinct from tissue macrophages and DCs.

1.3 Altered myelopoiesis during inflammation

Under normal circumstance the output of myeloid cell is kept at a steady rate to replace the constant loss of cells in the periphery. However, the rate of proliferation

as well as the nature of the downstream myeloid cells can be considerably altered by various disease states.

1.3.1 Proliferation

Pathogen invasion functions as an alarming signal to the immune system. In time supply of large number of phagocytes is necessary to keep the pathogen invasion in check before the adaptive immune response can take action. As the hematopoietic system is the major factory of immune cells, deficiencies in hematopoiesis can become the cause of deleterious diseases. For example, patients with neutropenia have low number of neutrophils and hence they suffer from recurrent infections (Lieschke et al. 1994, Ancliff et al. 2003, Catenacci and Schiller 2005, Cheung et al. 2007, Panopoulos and Watowich 2008, Bugl et al. 2012). Under certain circumstances hematopoietic system can be manipulated by tumors to produce high number of immunosuppressive myeloid cells termed myeloid derived suppressor cells (MDSC) that infiltrate tumors and facilitate metastasis by suppressing T cell proliferation and function (Gabrilovich and Nagaraj 2009, Van Ginderachter, Beschin et al. 2010).

Due to the self-renewing ability of HSCs; it is not surprising that the hematopoietic system is also prone to malignant disease where intrinsic mutation occurs and progenitor cells undergo uncontrolled growth. Numerous leukemic

diseases have been related to dysregulated hematopoiesis. Take acute myeloid leukemia (AML) as an example. Instead of continuing the journey towards mature myeloid cells, the committed progenitor cells fail to exit as mature cells and become arrested in an undifferentiated stage. As the degree of differentiation is reciprocal to the capability to proliferate these undifferentiated myeloid progenitor cells are prone to malignant growth (Catenacci and Schiller 2005, Licciulli et al. 2010, Sexauer et al. 2012).

1.3.2 Differentiation

Not only the proliferation rate but also the phenotype of the myeloid cells commit to can be influenced by the different disease states and in turn the differentiated myeloid cells can substantially shape the progress of the disease.

In the periphery, although monocytes do not contribute to the homeostasis of DCs and majority of the tissue macrophages during steady state, they remain the bona fide population that quickly replenish phagocytes lost in the periphery during infection and infiltrate inflammatory tissue (Geissmann, et al. 2003, Serbina, et al. 2003, Leon, et al. 2007, Bosschaerts et al. 2010). During bacterial infection of L.monocytogenesis, monocytes from the blood are recruited to the spleen and differentiate to DCs. The production of Nitrite Oxide (NO) of these monocytes-

derived DCs is essential for pathogen clearance and mice with reduced number of monocytes are more susceptible to infection-induced death. The ability of phagocytosis and production of microbiocidal peptides and NO deem them as one of the essential defense mechanism of innate immunity (Serbina et al. 2003, Bosschaerts et al. 2010, Chong et al. 2011). Similarly, viral infection induces rapid differentiation of blood monocytes to antigen-presenting DC within 18 hours ex vivo. (Hou, et al 2012). The transition from monocytes to the highly effective antigen presenting DCs during infections makes them an ideal targeting population for ex vivo DCs generation. Therefore, understanding the biology of monocytes during infection can contribute considerably to the future development of immunotherapy.

Under certain circumstances, however, the infiltration and differentiation of monocytes can become detrimental to the health of the animals. Numerous inflammatory diseases are known to recruit and drive the differentiation of inflammatory leukocytes at the site of inflammation. Taking colitis as an example, the disease progress is linked to uncontrolled proliferation of inflammatory neutrophils and macrophages in the bone marrow and spleen due to a skewing towards myelopoiesis by the hematopoietic progenitor cells in the inflammatory environment (Griseri et al. 2012, Oduro et al. 2012). Moreover, it is reported that the differentiation program of monocytes is frequently switched from anti-inflammatory macrophage to inflammatory DCs by local environment (Rivollier, He et al. 2012).

During development of experimental autoimmune encephalomyelitis (EAE), inflammatory monocytes from peripheral blood also migrate to central nervous system (CNS) and give rise to myeloid DCs and microglia, which are responsible for presenting antigen to pathogenic $CD4^+$ T cells and demyelination (Mildner et al. 2007, King et al. 2009). In another instance, monocytes are recruited to site of plague and differentiate to macrophages and DCs, contributing to the formation of atherosclerotic lesion (Tacke, Alvarez et al. 2007).

Therefore, studying the biology of monocytes differentiation during inflammation and identify the terminal phenotype that the cells have committed to enables development of novel therapeutic tool to block the actions of pathogenic DCs and macrophages.

1.4 Factors that influence myelopoiesis during inflammation

During infection, granulocytes and monocytes exit the circulation and infiltrate tissues to eliminate invading pathogen, causing a rapid drop in the number of granulocytes and monocytes in the peripheral blood. Unlike lymphocytes which can be further expanded, granulocytes are postmitotic and do not undergo further proliferation. Peripheral monocytes can undergo proliferation and replenish the local population but the rate of expansion is still low compare to lymphocytes. To prevent

spread of the pathogen, bone marrow must quickly expand the myeloid population which is termed emergency myelopoiesis (Boiko and Borghesi 2012). When receiving signal of pathogen invasion, on one hand, bone marrow mobilizes T cells and B cells into the circulation to free up the limited space for expansion of the myeloid population, on the other hand the progenitor cells population experiences a shift from lymphopoiesis to myelopoiesis (Chandra, Villanueva et al. 2008, Oduro, Liu et al. 2012)

Not all pathogens are disseminated to the bone marrow. How can a pathogen invading peripheral tissue deliver a message to the distant primary hematopoietic tissue and influence the output of myeloid cells? One possibility is through a cytokine level change in the serum. When granulocytes and macrophages fail to control local infection, pathogens spread and induce a systemic infection, causing a systemic reaction and release of proinflammatory cytokines from immune cells as well as epithelial cells (King and Goodell 2011). A number of cytokines have been reported to differentiate progenitor cells to myeloid cells. GM-CSF, M-CSF, IL-3 and G-CSF are the first few cytokines that are noted to support myeloid cells differentiation. Later, more cytokines, particularly those that are highly induced during inflammation, are reported to promote myelopoiesis. One example is IFN-gamma. Produced by Th1 T cells in large quantities, IFN-gamma is critical for clearance of intracellular bacteria, parasites and virus by inducing production of ROS in macrophages. Although in vitro

IFN-gamma has been found to limit the colony forming ability of human HSCs, during infection by intracellular bacteria, IFN-gamma was found to induce monopoiesis in the bone marrow in the case of E. muris, and L.monocytogenesis. Similarly, another member of the IFN family, IFN-beta, is also reported to induce myelopoiesis during acute inflammation (Diamond et al. 2011, MacNamara et al. 2011, Wilkison et al. 2012, Buechler et al. 2013). Besides proinflammatory cytokines, component of bacteria and virus are also shown to induce myelopoiesis in the bone marrow. Toll-like receptors have been shown to be expressed on HSCs, and binding of LPS to TLR-4 on HSC can activate HSC and skew hematopoiesis towards myelopoiesis during acute and chronic exposure (Boettcher, et al 2012).

Aside from acting on progenitor cells to promote differentiation to downstream granulocytes and monocytes, some proinflammatory cytokines and TLR ligands can shape the phenotype of cells. For instance, IFN-gamma is a maturation factor of classical inflammatory macrophages which is beneficial for pathogen clearance while the IL-4- induced alternative activated regulatory macrophages can suppress ongoing inflammation (Classen, et al. 2009). It is also reported that upon exposure to the milieu created by $CD8^+$ T cells and DCs interaction that contains high level of IFN-gamma, IL-1, IL-6, IL-12p70p40 and TNF-alpha, monocytes differentiate to Tip DCs (Chong, et al. 2011). TLR signaling can also trigger rapid differentiation of monocytes to DCs and macrophages and the phenotype of cells that

monocytes commit to can significant shape the outcome of the disease (Krutzik, et al 2005).Therefore, the phenotypes of the infiltrating myeloid cells strongly depend on the environmental cues that they encounter and identifying the factors involved in these processes allows efficient monitoring of myeloid cell differentiation at the site of infection.

1.5 Biological function of CD137

One of the proinflammatory molecules that have received much attention in the last decade is the inducible T cell coactivator CD137 (TNFRSF9, 4-1BB), a member of the TNF receptor super family (TNFRSF). When first discovered, it was found on activated T cells (Kwon and Weissman 1989, Pollok et al. 1993, Schwarz et al. 1993). As a very potent coactivator of T cells, CD137 enhances T cell proliferation, protects T cells from superantigen-induced cell death and reinforces proinflammatory cytokine production (Schwarz et al. 1995). The primary role of CD137 activation in T cells leads to activation of $CD8^+$ T cells which is the major force to eradicate intracellular bacteria and viruses (Shuford et al. 1997, Lee et al. 2002). Anti-CD137 antibody is found to be able to reject established carcinoma, mastoma, and melanoma in animal models (Melero et al. 1997, Ju, Lee et al. 2005). The effect is so prominent that an agonistic anti-CD137 antibody has been developed for cancer therapy. Currently the antibody-based therapy in melanoma has already completed phase II clinical trials. (NIH reference number: NCT00612664)

Interestingly, activation of CD137 on T cells surprisingly suppresses the progression of a number of autoimmune diseases. Administration of agonistic CD137 antibodies shows preference on IFN-gamma producing $CD8^+$ T cell expansion but suppress $CD4^+$ T cells activation. In autoimmune disease models including collagen Type II arthritis and EAE, expansion of this $CD8^+$ T cell population surprisingly ameliorates the disease progression (Foell et al. 2004, Seo, Choi et al. 2004, Kim, Choi et al. 2011). IFN-gamma dependent expansion of IDO-mediated immune suppression is the prime mechanism while CD137-mediated suppression of pathogenic Th17 T cells also partly contributes to the disease suppression.

As more and more attention was drawn towards CD137, studies continued to report expression of CD137 on a wide range of immune cells including monocytes, B cells, NK cells and DCs. With a few exceptions the activities of the molecule on the cells are mainly activating. In DCs, CD137 provides a survival signal as well as activation signal to enhance costimulatory molecule and cytokine production (Futagawa et al. 2002, Choi et al. 2009). In non-immune cells CD137 is found on epithelial cells and endothelial cells and ligation of the molecule reinforces cytokine and chemokine production that enhance leukocyte infiltration (Quek et al. 2010, Teijeira et al. 2012).

1.6 CD137/CD137L bi-directional signaling system

A central feature of TNFRSF is that both receptor and ligand can transduce signaling into the cells they are expressed on, respectively (Domonkos et al. 2001). Not only can CD137 transduce a signal into T cells upon crosslinking, its ligand, CD137L, can also signal into cells on which it is expressed. Expression of CD137L is mainly found on antigen presenting cells (APCs) including DCs, macrophages and B cells (Bossen et al. 2006, Yang et al. 2008). The signaling through CD137L is termed reverse signaling to distinguish it from the signaling through CD137. Expression of CD137L is, however, not found exclusively on APC. In the past decade, the molecule has been also reported to be present on T cells, endothelial cells, microglia and hematopoietic progenitor cells (Jiang 2008, Jiang et al. 2008).

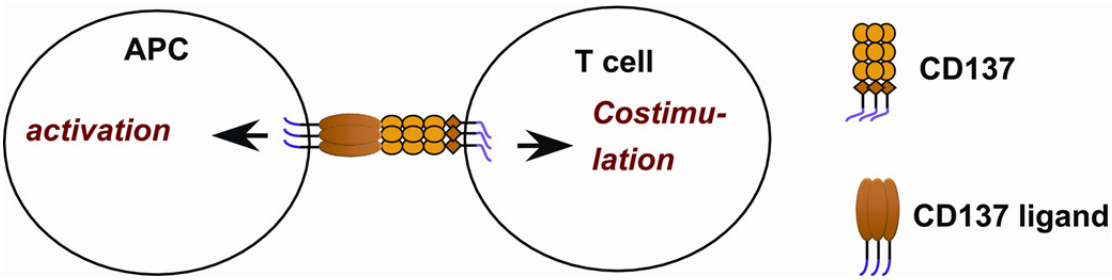


Figure 1.2 Illustration of bidirectional signaling of CD137 and CD137L on APC and T cells. APC: Antigen Presenting Cells (Shao and Schwarz 2011).

1.7 Reverse signaling of CD137L on APC

1.7.1 Importance of understanding DC biology

APCs are a group of immune cells that can present specific antigen to T cells and hence bridge the innate and adaptive immunity. The adaptive immunity against a certain pathogen largely depends on how efficient the antigen is presented. DCs are probably the best known APC since 1973 reported by Steinman (Steinman 2007). The high level of MHC-II and costimulatory molecules expressed on DCs makes them the most efficient APC. These properties of DCs are determinants of the following immune responses. DCs subsets have been categorized based on surface markers, function and tissue location in the human and murine system, and it was agreed that different DCs subsets have their own preference of T cell stimulation, and eventually determining the following immune responses. For example studies focusing on DCs ontogeny have identified a particular subset of $CD103^+$ DCs responsible for tolerance in the gut. On the other hand the $CD103^-$ DCs is the pathogenic DCs subset that drives the Th17 response in intestinal inflammation (Matteoli et al. 2010, Rivollier et al. 2012). Therefore, by studying the different phenotypes and functions of DCs subset, it helps to identify therapeutic targets in disease models for manipulations.

Since the discovery of DCs and their powerful roles in antigen presentation, the cells have been of the central interest for immunotherapy. Researchers are keen to

develop antigen-specific DCs vaccines that can target tumor antigen. However, this approach requires ex vivo expansion of DCs. In man, GM-CSF plus IL-4 or Flt-3 ligand have proven to be effective in differentiating monocytes from cord blood or peripheral blood mononuclear cells (PBMC) to DCs (Romani et al. 1994, Sallusto and Lanzavecchia 1994). In the murine system, bone marrow is a more readily available source as it contains relatively high number of hematopoietic progenitor cells and monocytes compared to peripheral blood. Immature DCs usually have low level of MHC-II and costimulatory molecule and hence a weak antigen presenting ability, properties that may induce T cell anergy and tolerance instead of activation (Sallusto et al. 1995, Van Gool et al. 1996). The reagents used for maturation can also influence the phenotype of DCs. Therefore, in order to derive fully functional DCs that can effectively deliver antigen to T cell, maturation is an essential step. LPS, IFN-gamma, IL-1 and TNF-alpha are some of the common reagents to induce DCs maturation. In spite of the potent maturation effect of these reagents, when it comes to clinical application, it is more desirable if fewer reagents are required for the culture while maintaining the same activation state to minimize complication.

1.7.2 Reverse signaling of CD137L on human monocytes and immature DCs

In the pioneering study on CD137L reverse signaling, Schwarz et al showed that human monocytes proliferate upon the treatment of recombinant CD137 protein, partly by inducing production of M-CSF in an autocrine and/or paracrine manner

(Langstein et al. 1998, Langstein et al. 1999). Later it was demonstrated that activation of CD137L on immature DCs derived from human cord blood monocytes enhances expression of costimulatory molecules and IL-12p70 production (Kim et al. 2002, Lippert et al. 2008). The CD137L-matured DCs can also induce higher T cell proliferation than its control, suggesting that CD137L can function as a maturation agent in the process of DCs differentiation. Another study on CD137L reverse signaling on APC showed that when CD137L is expressed in DCs by a vector, costimulatory molecules such as CD80, CD86 and CD40 are also upregulated (Yurkovetsky et al. 2006).

Taking a step further, Kwajah et al found that crosslinking of CD137L on human blood monocytes induces differentiation to a distinct type of DCs (Kwajah and Schwarz 2010). Although these CD137L-DCs have reduced HLA-DR and IL-12p70, both of which are essential for a Th1 response, they can nonetheless potently induce T cell proliferation in allogenic mixed lymphocyte reaction (MLR), proving their capability in antigen presentation and marking them as a potential tool for DCs therapy. The T cells primed by CD137L-DCs also have higher cytotoxic activity than classical DCs. Moreover, unlike classical DCs which need maturation by LPS, the property of CD137L-DCs is not further enhanced by LPS, indicating that CD137L alone can function as both a differentiating factor as well as maturation factor. These findings promise clinical potential particularly for DCs-based immunotherapy when a

large number of DCs expanded ex vivo is desirable. Compared to classical DCs which need three factors to become fully mature, CD137L-DCs require only one factor to derive a functional inflammatory DCs and hence can significantly reduce the cost and potential complications.

1.7.3 Reverse signaling of CD137L on macrophages

Macrophages are another important group of myeloid APC involved in both innate and adaptive immunity. Although the majority of the macrophages possess the basic function of phagocytosis , ROS production and release of cytokines, they are a highly heterogeneous group categorized based on the function and tissue they reside in. For example, while the major function of red pulp macrophages in the spleen is to engulf aged red blood cell, osteoclasts in the bone marrow perform bone resorption. Studies up to date have shown a functional role of CD137L reverse signaling on a wide range of macrophages, including microglia, peritoneal macrophages and osteoclasts (Shin et al. 2006, Shin et al. 2007, Jeon et al. 2010, Yeo et al. 2012). On bone marrow derived macrophages, CD137L reverse signaling prolongs survival of the macrophages and enhances cytokine production (Kim et al. 2009). The interaction of CD137 and CD137L is also essential for macrophages mobilization because in the peritoneal cavity influx of macrophages is impaired in $CD137^{-/-}$ mice due to a reduced level of IL-10 (Shin et al. 2007). Stimulation of CD137L in macrophages can be also involved in pathological conditions. For instance, activation of microglia in the brain

via CD137L can induce ROS production and proinflammatory cytokine release which lead to apoptosis of oligodendrogcytes. In brain section of mice with experimental autoimmune encephalomyelitis (EAE), expression of CD137L and activation of microglia were simultaneously increased (Yeo et al. 2012).

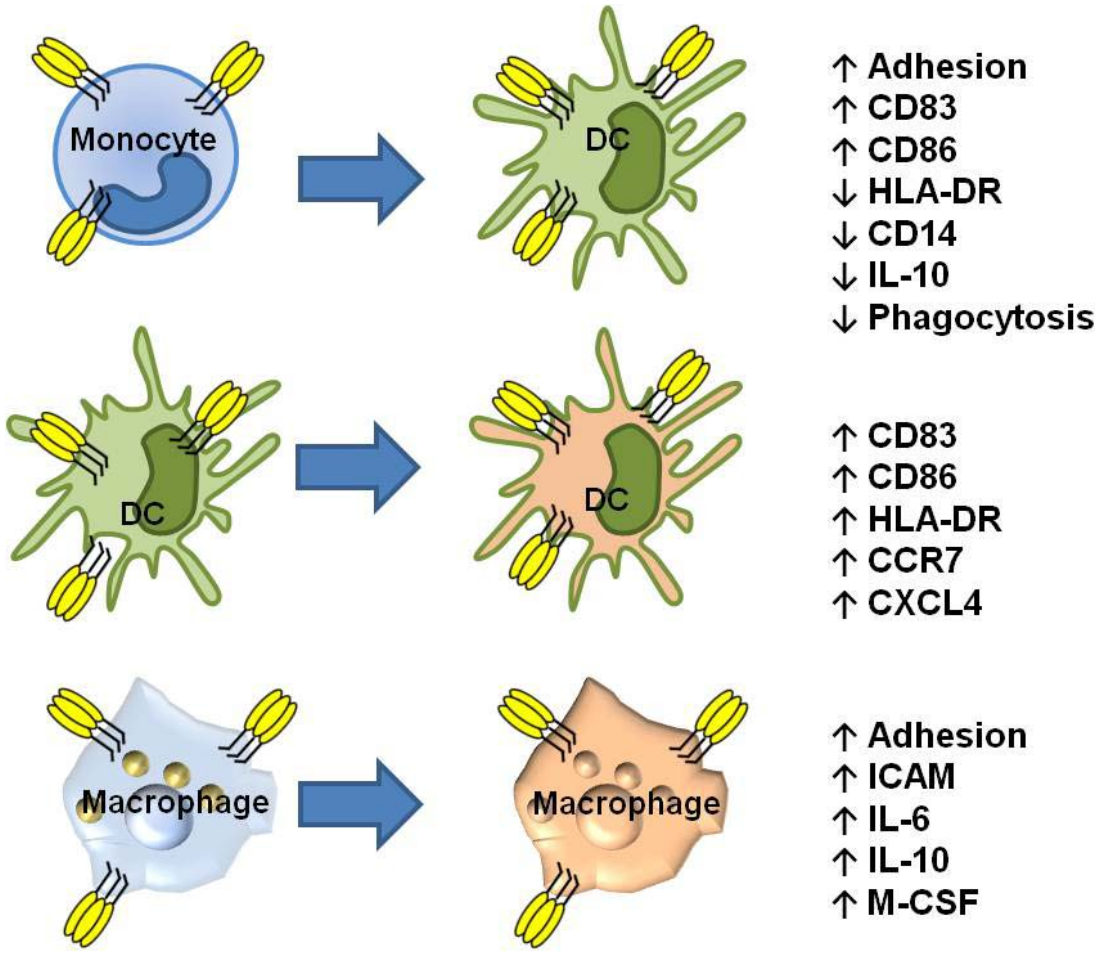


Figure 1.3: Illustration of reverse signaling of CD137L on monocytes, DCs and macropahges.

1.8 Effect of CD137 on hematopoietic stem cells

In recent years, it was gradually recognized that the expression of CD137L reaches far beyond the territory of differentiated immune cells. The expression of

CD137L is found on a small subset of human cord blood $CD34^+$ hematopoietic progenitor cells as well as on murine Lin^- progenitor cells (Jiang 2008, Jiang et al. 2008). Crosslinking of CD137L reverse signaling on the HSCs induces proliferation of the cells and enhances colony forming. The treated cells also undergo extensive morphological changes, a sign of activation and differentiation. Functional studies found that the cells exhibit characteristic of macrophages. On the more committed progenitor cells that have restricted potential in differentiation, CD137L reverse signaling selectively induces proliferation and differentiation of common myeloid progenitor (CMP) cells other than granulocytes-macrophages progenitor (GMP) cells (Jiang and Schwarz 2010). While CD137L supports bone marrow cell proliferation and differentiation just like other hematopoietic factors, it antagonizes the effect of G-CSF in the fate decision of monocytic and granulocytic lineage by biasing differentiation towards the monocytic lineage.

1.9 Biphasic role of CD137L reverse signaling

Although the majority of the studies have focused on the activating properties of CD137L, it should be noted that CD137L can exhibit regulatory roles at different stages of the immune response.

1.9.1 Monocytes

In vitro coculture of PBMC on culture plates coated with recombinant CD137 protein showed that in early phase of monocytes activation, CD137L reverse signaling induces ROS that caused apoptosis of T cells (Kwajah, Mustafa et al. 2011). However, if monocytes are exposed to CD137 for a longer period, the cells become inflammatory DCs that stimulate T cell proliferation (Kwajah and Schwarz 2010). This biphasic role of CD137 represents a tightly regulated mechanism: when the exposure to CD137 is transient and at the early phase of infection, the signal functions as a regulatory signal to remove potential pathogenic T cells. It is also possible that depletion of excess T cells with low specificity by monocytes in the early phase of an immune response is to empty the repertoire for future expansion of antigen –specific T cells. If the exposure is prolonged, the signal becomes a persistent inflammatory response and indicates prolonged pathogen invasion. Therefore the reverse signal of CD137L on monocytes switches to an activating signal to induce DC differentiation and eventually an augmented immune response.

1.9.2 B cells

Not only does CD137L on monocytes exhibit a biphasic function, stimulation of CD137L on B cells also showed a similar phenomenon where in vitro stimulation of CD137L on B cells induces proliferation and costimulation (Pauly et al. 2002, Zhang et al. 2010) but constitutive expression of CD137L on APC of transgenic mice

leads to B cell deletion as the mice age, suggesting that a persistent signal of CD137L is deleterious for B cells (Zhu, Flies et al. 2001). Additionally aged CD137L$^{-/-}$ mice have a higher tendency of developing B cell lymphoma and CD137L is found to regulate hyperproliferation of B cells in lymph nodes (Middendorp et al. 2009). Again the data suggested that CD137L reverse signaling can have significant distinct impacts on APC depending on the length of exposure time.

1.10 Conflicting findings for CD137L reverse signaling

The biphasic role of CD137L reverse signaling suggests that the CD137/CD137L system is more complicated than expected. In spite of the progress in understanding the CD137/CD137L reverse signaling system, conflicting data exist among different research groups.

1.10.1 Anti-tumor effect

Taking the anti-tumor effect mediated by CD137 as an example. With the impressive potency to reject established tumor by agonistic anti-CD137 antibody, one may expect mice lacking the gene for CD137 have poorer immune protection from tumor development. Indeed in the first report by Kwon et al, CD137$^{-/-}$ mice showed higher mortality and larger tumor size when inoculated with melanoma cells (Ju, Lee et al. 2005). However, years later, the same groups utilizing a different set of tumor

cell lines, and reported completely opposite findings that WT mice are more susceptible to tumor growth than $CD137^{-/-}$ mice (Choi et al. 2010). The discrepancy between the two reports probably has its root in the different properties of the tumor cell lines that have differential sensitivity to $CD8^+$ T cells and NK cells killing. In the latter study the authors find that IFN-gamma by $CD8^+$ T cells actually leads to depletion of periphery NK cells, rendering the mice more susceptible to tumor growth. The striking difference in response suggested that, depending on the type of tumor, the anti-tumor effect of CD137 may be detrimental instead of beneficial.

1.10.2 Autoimmune disease

In autoimmune disease models, similar conflicts were also noted. While administration of agonistic anti-CD137 antibodies suppresses development of autoimmune disease (Kim et al. 2011), $CD137L^{-/-}$ mice are protected from EAE. The resistance of $CD137L^{-/-}$ mice is largely due to reduced VCAM-1 expression which in turn leads to lower number of leukocytes infiltration and demyelination of the spinal cord (Martinez Gomez et al. 2012). One possible explanation to resolve the conflict is that agonistic antibodies tend to overactivate T cells beyond the physiological situation and the large amount of IFN-gamma released by $CD8^+$ T cells leads to IDO-mediated immune suppression. Simultaneously the agonistic antibodies prevent the reverse signaling of CD137L which is required for leukocytes infiltration in the CNS for disease progression. In the case when $CD137L^{-/-}$ mice are employed, signaling

through both the ligand and the receptors are abolished. Even without the IFN-gamma from $CD8^+$ T cells, removing CD137L reverse signaling alone is sufficient to prevent the disease progression.

1.10.3 Osteoclastogenesis

Osteoclasts are macrophages derived from hematopoietic stem cells and mainly involved in bone resorption. Differentiation of osteoclasts largely depends on M-CSF and RANKL secreted by stromal cells. During inflammation bone abnormalities like periodontitis and osteomyelitis are frequently reported and complicate disease progress. Proinflammatory cytokines upregulated during infections including IFN-gamma and IL-12p70 are found to inhibit osteoclast differentiation (Wilkison, Gauss et al. 2012).

The influence of CD137 on osteoclastogenesis was firstly reported by Saito and colleague that osteoblasts upregulate CD137 when infected with E.coli, BCG and streptococcus and suggested that the expression of CD137 may influence the lineage commitment of bone marrow macrophages (BMM) to osteoclasts since BMM express CD137L. Indeed in the presence of recombinant CD137 immobilized on culture plate number of mature osteoclasts were significant reduced compared to control culture.

The findings are further confirmed by two more studies from Choi and colleague that differentiation of BMM to osteoclast by M-CSF and RANKL are inhibited by crosslinking of CD137L (Shin et al. 2006, Shin et al. 2006). The two latter studies also elucidated that the underlying mechanism of CD137L-mediated inhibitions are dependent on increase level of Type I IFN−beta and IL-10 from WT mice because depletion of either cytokines partly rescued the differentiation of osteoclasts. The last study by Senthikumar and colleague also reported similar findings that CD137L reverse signaling inhibits osteoclastogenesis (Senthilkumar and Lee 2009). Importantly this study found that the inhibition depends on cell-cell interaction between $CD137^+$ T cells and $CD137L^+$ osteoclast progenitor cells, indicating potential involvement of $CD137^+$ T cells in myeloid cell differentiation.

Interestingly conflicting data are reported regarding the CD137/CD137L reverse signaling on osteoclast. A study argued that expression of CD137 enhances osteoclasts differentiation due to cis expression of both receptor and ligand on the osteoclast precursors and both signaling through CD137 or CD137L can positively regulate osteoclast differentiation(Yang et al. 2008). The study present a very strong piece of evidence that WT mice have lower bone density than the $CD137^{-/-}$ mice, characteristic of more actively osteoclastogensis and bone resorption. Although the authors suggested that the potential discrepancy may lie in different strains of mice used (C57BL/6 vs Balb/c) as well as the length of culture time, the conflicts would be

better resolved in model using $CD137L^{-/-}$ mice or double knock out mice of CD137 and CD137L where the involvement of CD137L can be completely excluded. Moreover the level of osteoclastogenesis in mice should be assessed during inflammation state because pathogen invasions are known to influence osteoclast differentiation.

1.10.4 NK cells

The majority of the studies on CD137 have been conducted in the murine system. However, it should be noted that a discrepancy exists between the human and murine system on the effects of CD137. The difference is best illustrated in NK cells. When CD137 is activated on murine NK cells, the signal is stimulatory and favors a better cytotoxicity for malignant cell clearance. However, researchers also found that in cocultures of human AML cell lines with human NK cells, the effect of CD137 is surprisingly inhibitory, suggesting that in the human system, activation of CD137 on NK cells is probably undesired for an anti-tumor effect (Baessler et al. 2010). Such species differences should be taken note off as they may significantly impact the interpretation of data derived from murine models and affect future study designs.

1.10.5 Myelopoiesis

More recently CD137L has also been found on HSCs and microglia (Jiang, 2008 a b; Yeo, 2011;). Signaling of CD137L into these cells induces proliferation, activation and differentiation. The wide range of cells that CD137L is expressed on and numerous functions suggest an essential role of the molecule in the immune system. Its expression on HSCs particularly triggered interest in whether CD137L reverse signaling may regulate hematopoiesis.

When $CD137^{-/-}$ mice were first generated, it was found that in spite of the normal development naïve $CD137^{-/-}$ mice have nonetheless higher numbers of myeloid progenitor cells and colony forming units than WT mice. BrdU incorporation also indicates a higher turnover rate of the progenitor cells, suggesting that in the absence of CD137 myeloid progenitor cells are undergoing a more extensive process of proliferation (Kwon et al 2002). Later, Lee and colleague showed that while the absence of CD137 or CD137L has no effect on the number of long-term and short-term stem cells, both $CD137^{-/-}$ and $CD137L^{-/-}$ mice have higher numbers of myeloid progenitor cells during steady state and progenitor cells from the two knock out strains have better reconstitution ability in irradiated mice than WT, particularly in the myeloid and B cell lineage, confirming the previous finding (Lee, Park et al. 2008). The researchers continued to identify which downstream myeloid populations are most affected and found that the number of DCs in the lung was decreased in WT

compared to $CD137^{-/-}$ and $CD137L^{-/-}$ mice. Hence, it was concluded that CD137 limits myelopoiesis particularly DCs development during steady state.

Contradictory, Jiang et al reported that in both the human and murine system crosslinking of CD137L by recombinant CD137 protein on hematopoietic stem cells induced morphological change and activation (Jiang 2008, Jiang, Yue et al. 2008). Proliferation assays further shows that the progenitor cells proliferate when crosslinked by CD137. Functional studies on the CD137-treated progenitor cells shows that the differentiated cells exhibit a phenotype that is highly similar to macrophages but not DCs evidenced by an enhanced phagocytic activity but reduced T cell stimulation in allogenic MLR. Furthermore, CD137L-treated human monocytes induce differentiation towards DCs that have strong T cell stimulation ability, clearly contrasting the report that CD137L limits DCs development in the lung during steady state (Kwajah and Schwarz 2010). In another study where Zhu and colleague selectively expressed CD137L in APC, the level of $CD11b^{+}$ cells were increased by 3-fold (Zhu, Flies et al. 2001, Kuang, Weng et al. 2012). How can the seemingly conflicting data be resolved?

It should be noted that there are fundamental differences in the experimental set up between the two groups' work. While Lee and colleagues utilized naïve $CD137^{-/-}$ and $CD137L^{-/-}$ mice (Lee et al. 2008), Jiang and Kwajah investigated the

effect of CD137L in vitro where high level of CD137 recombinant protein was used (Jiang 2008, Jiang et al. 2008, Kwajah and Schwarz 2010). The latter experiment set up is closer to the scenario where CD137 protein is induced on T cells and endothelial cells by pathogen infection. To better resolve the conflict it is necessary to perform study in vivo with inflammation.

1.11 The role of T cells in maintaining myelopoiesis

The study by Senthikumar found that $CD137^+$ T cells and $CD137L^+$ osteoclast progenitors interact and mediates signaling through both directions, suggesting potential involvement of $CD137^+$ T cells in lineage decision of progenitor cells. Studies in bone marrow transplants indicated that T cells are essential for successful bone marrow engraftment and reduced graft failure risk (Kaufman et al. 1994). Since hematopoietic stem cells and monocytes express CD137L, can T cells be a source of CD137 in vivo?

1.11.1 Presence of T cells in bone marrow

Accumulations of T cells have been reported in human and murine bone marrows (Slifka et al. 1997, Price and Cerny 1999, Di Rosa and Pabst 2005, Herndler-Brandstetter et al. 2011). The majority of the T cells are mainly effectors and memory T cells (Mazo et al. 2005). It is believed that the major role of these T cells in the

bone marrow is to combat malignant cells and infections that have been disseminated to the bone marrow (Slifka et al. 1997). The role of these T cells in hematopoiesis, however, remains poorly unknown.

1.11.2 Primary myelopoiesis

In the past few years it has become clear that T cells, though player in the adaptive immune system can influence the output of myeloid lineage cells in hematopoiesis. Monteiro et al shown that athymic mice have reduced matured myeloid cells and reconstitution of $CD4^+$ T cell but not $CD8^+$ T cell restore the normal myelopoieisis (Monteiro et al. 2005). While the colony forming units of granulocytes and macrophages are reduced, the normal myelopoiesis clearly depends on the growth factors released by T cells. The T cells taking part in this process are activated T cells that have reduced CD62L but increased levels of CD69, concluding that it was the antigen-experienced $CD4^+$ T cells in the bone marrow that are the key players of hematopoieisis.

Apart from maintaining normal hematopoiesis T cells are also found to enter the bone marrow during infection of LCMV and transient production of IFN-gamma by these T cells is essential to enhance monopoiesis over granulopoiesis. In IFN-gamma$^{-/-}$ mice, the number of monocytes is considerably reduced compared to WT

mice when infected with LCMV (de Bruin et al 2012). This preference of monocytes production instead of granulocytes production supports a greater influx of monocytes to peripheral tissue to eliminate intracellular pathogen.

1.11.3 Extramedullary myelopoiesis

Not only can T cells influence primary myelopoiesis but also secondary myelopoiesis in the spleen which is also termed extramedullary myelopoiesis. Effector T cells in the spleen are demonstrated to promote extramedullary myelopoiesis through production of GM-CSF and IL-3. On the other hand, $Foxp3^{+}$ regulatory T cells suppress the extramedullar myelopoiesis by suppressing the differentiation of naïve T cells to hematopoietic factor producing T cells, mainly through the production of TGF-beta (Lee et al. 2009).

1.11.4 Mechanism of T cell mediated myelopoiesis

Piecing together the evidence it is strongly suggested that activated T cells are a driving force of myelopoiesis to contain infections. Then what is the underlying mechanism of the T cell mediated myelopoiesis?

One possible route is through soluble factor. Upon activation T cells produce IL-3, stem cell factor, GM-CSF and G-CSF all of which can support hematopoiesis, particularly myelopoiesis. Involvement of IFN-gamma and Type-I IFN in monocytes production further proves that T cells can modulate myelopoiesis (Deonarain et al. 2003, MacNamara et al. 2011).

Apart from releasing cytokines such as GM-CSF and G-CSF which support proliferation and differentiation of myeloid cells, another possible mechanism of T cell mediated myelopoiesis is through interaction of surface molecule in close contact with progenitor cells. Since CD137 is expressed most abundantly on activated T cells while its ligand is found on hematopoietic stem cells and monocytes, it is postulated that T cells may promote myelopoiesis both through surface-bound CD137 and soluble cytokines.

1.12 Immuneaging, inflammation and myelopoiesis

Aging is a process accompanied by low grade chronic inflammation. In both man and mice remodeling of hematopoiesis occurs through a shift from lymphopoiesis to myelopoiesis. Accumulated numbers of myeloid cells are observed in aged adults with diminishing numbers of lymphocytes. Not only do the numbers of myeloid progenitor cells exceed lymphoid progenitor cells but also do hemtaoptoitic

stem cells tend to develop a bias towards myeloid lineage differentiation (Morrison et al. 1996). The enhanced myelopoiesis is also evidenced by an increased number of myeloid cells in the bone marrow. This phenomenon can be viewed as the aging of immune system because of the diminishing output of lymphocytes of adaptive immune system.

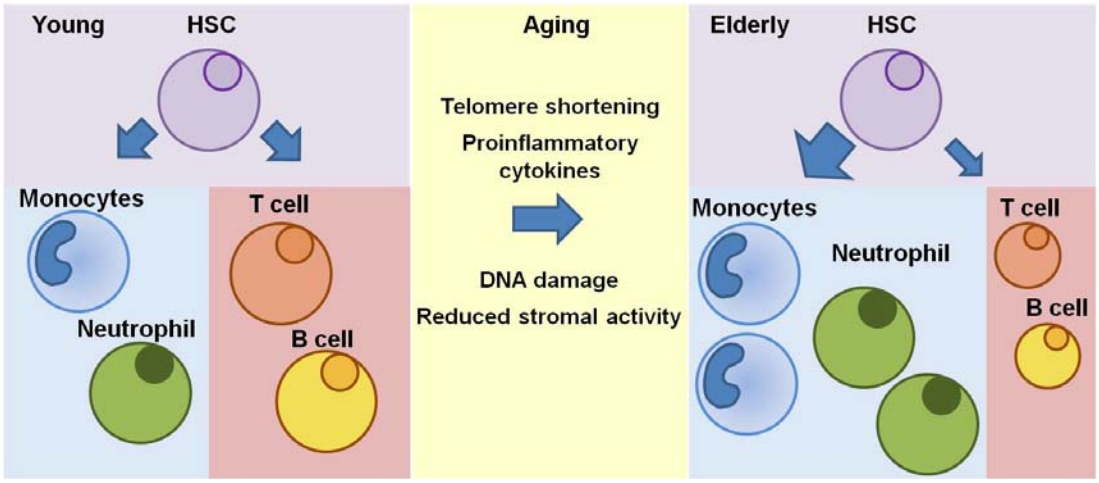


Figure 1.4 Illustration of the changed balance between lymphopoiesis and myelopoiesis during aging.

1.12.1 Mechanism and consequence of immuneaging

Various mechanisms have been suggested to explain the imbalance of lymphopoiesis and myelopoiesis in aged humans and animals. DNA damage and shortened telomerase in hematopoietic stem cells have been reported to be related to a weaning potential of lymphoid differentiation (Lieber and Karanjawala 2004, Rossi, Bryder et al. 2007, Nalapareddy et al. 2008). Changes in the microenvironment of the bone marrow including reduced support from stromal cells and increased levels of

inflammatory cytokines have been reported to contribute to the dysregulated hematopoiesis. Persistent chronic inflammatory signals such as tumor and autoimmune disease that developed during aging are also potential driving forces of myelopoiesis because many of the proinflammatory cytokines including INF-gamma, Type-I IFN and IL-6 have been reported to modulate myelopoiesis during infection (Deonarain et al. 2003, MacNamara et al. 2011).

The consequence of imbalanced hematopoiesis can have severe complications in elderly as the myeloid population is expanding at the expense of the lymphoid population which is the central player of adaptive immunity. With a shrunk repertoire of lymphoid progenitor cells, the adaptive immune system becomes less efficient to respond to vaccination and to eliminate malignant cells, which explains the reduced vaccination efficacy and higher tumor incidents in elderly (Goodwin et al. 2006). With growing aging populations around the world, it would be of clinical importance to understand the disrupted hematopoiesis in elderly so that novel therapeutic tools can be developed to monitor the process.

1.12.2 Intervention of immuneaging

Recently researchers have managed to reboot the lymphopoiesis in mice, particularly the production of naïve B cells from bone marrow by removing

circulating B cells. It is hypothesized that circulating memory B cells exhaust the repertoire of the naïve B cell compartment and send a negative signal to hematopoietic stem cells for B cell lymphopoiesis. Therefore, by removing existing B cells the hematopoietic system may sense a need for producing more B cells. Experiments that deplete B cells using anti-CD19 antibodies showed that the output of naïve B cell in the bone marrow is restored when the aged mice have undergone more than one round of B cell depletion. Most importantly, the treated aged mice had an enhanced response to antigen stimulation (Keren, Naor et al. 2011).

It is possible, however, to rejuvenate the hematopoiesis from another aspect by understanding the mechanism of skewed myelopoiesis, particularly at the level of the progenitor cells. By identifying the molecules that are driving the myeloid bias, it may be possible to rekindle the lymphopoiesis by blocking the myelopoiesis signaling pathway in HSCs by the responsible molecules. The proinflammatory property of CD137L reverse signaling makes it a potential influential factor of immuneaging. Detection of CD137L on HSCs also suggests that it may play a role in the lineage decision making. However, it remains unclear whether this influence is only transient during emergency like infection or is a sustained, accumulative force that acts on hematopoietic progenitor cells in the long term such as aging.

1.13 Aim and scope

Reports on CD137L reverse signaling suggest that CD137L may promote myeloid proliferation, activation and differentiation of APCs and HSCs. However, contrasting data from different groups indicate that CD137L reverse signaling may have different activities depending on the respective physiological conditions. Species difference may also exist between human and murine cells for their response to CD137L reverse signaling. This project aims at resolving several conflicts and adding knowledge of CD137L reverse signaling during acute and chronic inflammation.

The project aims at answering the following four questions:

1. Can CD137L reverse signaling in murine monocytes induce DCs differentiation?
2. Does CD137L reverse signaling promote myelopoiesis during acute inflammation like infection?
3. What is the role of CD137L reverse signaling in myelopoiesis during chronic inflammation in the context of aging?
4. What is the underlying mechanism of CD137L-mediated myelopoiesis?

By answering these questions, it is hoped to evaluate the potential of testing CD137L-DCs in murine model and also to resolve the conflicts existing in the role of CD137L reverse signaling in myelopoiesis. Eventually this project is aimed at elucidating the underlying mechanism and cell populations responsible for the CD137L reverse signaling on myelopoiesis under various circumstances to provide knowledge for developing novel therapeutics tools.

Chapter 2 Materials and Methods

2.1 Mice

Female BALB/c and C57BL/6 mice between 8 and 16 weeks of age were used as a source of bone marrow cells and femur bones. Animals were specific pathogen free, and kept with free access to food and water in the animal care facility at the National University of Singapore under the institutional guidelines for usage of experimental animals. $CD137^{-/-}$ and $CD137L^{-/-}$ mice were generated as described previously (Kwon 2002).

In the aging model gender and age matched mice of WT, $CD137^{-/-}$ and $CD137L^{-/-}$ origins were maintained until the age of 12 months. Mice with visible skin lesion or dermatitis due to fighting were excluded from the study. 3-months old mice were used as young controls.

2.2 Infection of mice

BALB/c mice were each infected intranasally with 10^6 colony forming units (CFU) of the wild type *Bordetella pertussis* BPSM strain in 20 µl as described previously. Mice were sacrificed 1, 3 and 7 days post-infection and femur bones were isolated for immunohistochemistry (IHC).

C57BL/6 mice (6 - 8 weeks old) were infected intranasally with 10^7 *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) per mouse. After 3 weeks mice were euthanized and femur bones were collected for IHC.

8 - 10 week old C57BL/6 WT and CD137$^{-/-}$ mice were intraperitoneally injected with 10^6 CFU *Escherichia coli*. Mice were sacrificed 2 days later and bone marrow cells were isolated.

8 - 10 week old C57BL/6 mice were infected intranasally with 5 PFU *Influenza A* virus (PR8, H1N1) per mouse. Uninfected naïve mice were used as control. Mice were sacrificed 4 days post-infection and femur bones were collected for IHC.

8 - 10 week old C57BL/6 mice were i.p. injected with 100 μl of 1 mg/ml LPS (*Escherichia. coli* 0111:B4, Sigma-Aldrich, St Louis, MO, USA). Naïve mice that received PBS injection were used as control.

2.3 Preparation of bone marrow cells and splenocytes

Mice were euthanized by CO_2 inhalation. The femur bones were dissected and the bone marrow was flushed out aseptically with phosphate-buffered saline (PBS), 2 mM EDTA using a 10 ml syringe and 27G needle. Total bone marrow cells were passed through a 30 µm filter (Miltenyi Biotec, Bergisch Gladbach, Germany), washed with PBS containing 2 mM EDTA and resuspended in RPMI1640 medium (Sigma-Aldrich, St Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin and 2.1 Mm L-glutamine.

Spleens were aseptically removed from the abdominal cavity and minced through a 40 µm nylon cell strainer (Becton Dickinson, Franklin Lakes, New Jersey, USA) with a 5 ml syringe core in 10 ml of PBS. Red blood cells were depleted with Tris-NH_4Cl lysis buffer. Splenocytes were washed with PBS containing 2 mM EDTA and resuspended in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin and 2.1 Mm L-glutamine.

2.4 Isolation and culture of bone marrow monocytes

Murine monocytes were isolated from8-10 weeks old mouse bone marrow by negative enrichment using EasySep™ Mouse Monocytes Enrichment Kit (Stem Cell Technologies, Vancouver. Canada). In brief, bone marrow cells were labeled with a

cocktail of biotinylated antibodies against T cells, B cells, progenitor cells, erythrocytes and granulocytes in PBS with 5% normal rat serum at 4 ° C for 15 minutes. The labeled cells were washed by PBS with 5% normal rat serum and centrifuged at 300 g for 10 minutes. Cells were further labeled by secondary anti-Biotin antibodies. Unlabeled monocytes were collected by pulling out the cell solution while in a magnetic field.

The enriched monocytes were seeded on culture plate coated with recombinant CD137-Fc protein or Fc fragment at 4ºC overnight. The monocytes were cultured for 7 days and harvested by 10 mM EDTA in PBS. 1 μg/ml LPS was added for the last 24 h to mature the cells.

2.5 ^{3}H-thymidine proliferation assay

Cell proliferation was determined by ^{3}H-thymidine incorporation. Cells were pulsed with 0.5 μ Ci of ^{3}H-thymidine (PerkinElmer, Boston, USA) for the last 16 hr of the culture period. The cells were then harvested onto a Packard Unifilter Plate using a MicroMate 196 Cell Harvester and counted using a TopCount Microplate Scintillation Counter (Packard Instruments, Meriden, USA).

2.6 CFSE proliferation assay

Cells were stained with 5 μM CFSE (Invitrogen, Carlbad, USA) in PBS with 0.1% BSA at 37 °C for 10 min. Incorporation of CFSE dye was stopped by adding 5 volumes of ice-cold RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and 2.1 Mm L-glutamine and incubation on ice for 5 min. Cells were then washed with the same medium and centrifuged at 300g for 10 min for three times. Dilution of CFSE dye was determined by flow cytometry on Cyan flow cytometer (Dako, Denmark) or BD LSRFortessa cell analyzer (BD Bioscience). Mean fluorescence intensity (MFI) of CFSE dye at FITC channel was used as measure for cell proliferation.

2.7 Phagocytosis assay

Yellow-green fluorescent carboxylated-modified microspheres (FluroSpheres, Molecular Probes) were added at a ratio of 50 beads per cell at 37°C in the dark. Cells undergoing the same treatment but without the additions of beads served as negative controls. After 1 hr incubation, the reaction was arrested by addition of 1 ml ice-cold PBS. Cells were then washed in PBS and trypsin-EDTA was added to dislodge surface adherent beads. Cells were washed again with PBS and resuspended in FACS buffer for flow cytometry analysis.

2.8 ELISA

The concentrations of IL-10, IL-12p70 , GM-CSF, M-CSF, MCP-1 and IL-1 beta in cell supernatants were determined by mouse IL-10 (R&D) , IL-12p70 p70 (R&D), GM-CSF (R&D), IL-1 beta (Perprotech), M-CSF (Perprotech), G-CSF (Perprotech), MCP-1 (Perprotech) according to the manufacturer's instructions. All measurements were done in triplicate.

2.9 Allogeneic mixed lymphocyte reaction

$CD11b^+$, $Ly6G^-$ monocytes were isolated from the femur bones of 6-week old BALB/c mice by negative enrichment according to manufacturer's instruction (Stemcell Technologies). $2x10^5$ monocytes per well were cultured in 24-well plates that had been precoated with 10 μg/ml of Fc or CD137-Fc. In parallel, cells were treated with 100 ng/ml GM-CSF + 25 ng/ml IL-4 (Peprotech) to generate immature classical DCs. Maturation of DCs and CD137-treated monocytes was induced on day 6 by addition of 1 μg/ml LPS (Sigma). Subsequently, cells were harvested by incubation with 1x PBS, 10 mM EDTA at RT for 10 min and washed twice with PBS, and served as stimulator cells. T cells were isolated from splenocytes of 6-week old C57BL/6 mice by magnetic selection using the Pan T cell isolation kit (Miltenyi Biotec). 10^5 T cells were cocultured with stimulator cells at a 10:1 ratio in 96-well round bottom plates for 3 days and pulsed with 0.5 μCi ^{3}H-thymidine per well for the

last 16 hr. The rate of T cell proliferation was determined with a scintillation counter as described in 2.5 (Packard, Meriden, CT, USA).

2.10 Isolation of T cells from splenocytes

Splenic T cells were isolated by MACS using the mouse Pan T Cell isolation kit (Miltenyi Biotec). Briefly, the fresh splenocytes were labeled with a cocktail of biotin-conjugated antibodies against CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119 expressed on non-T cells (B cells, NK cells, DCs, macrophages, granulocytes and erythroid cells), followed by anti-biotin microbeads. The cell suspension was passed through a LS column in a strong magnetic field, and the untouched T cells were collected in the effluent.

Splenic $CD4^+$ T cells were isolated by MACS using the CD4 microbeads (Miltenyi Biotec). Splenocytes were labeled with microbeads and passed through LS column in a magnetic field. Positive selected $CD4^+$ T cells were later flushed out using a plunger.

2.11 Antibodies and flow cytometry

Low endotoxin, azide-free anti-mouse CD3 (clone 17A2), CD28 (clone 37.51), PE conjugated anti-mouse CD11b (clone M1/70), Gr-1 (clone RB6-8C5), TER-119 (clone TER-119), B220 (clone RA3-6B2), CD19 (clone 6D5), CD3 (clone 145-2C11), CD11c (clone N418), F4/80 (clone BM8), CD137 (17B5), CD69 (H1.2F3), APC conjugated anti-CD3 (17A2), Ly6C (clone HK1.4), Sca-1 (clone D7), FITC conjugated anti-CD44, BrdU, CD117 (clone 2B8), PE-Cy7 conjugated anti-CD62L (MEL-14), Ly6G (clone RB6-8C5), eFluor450 conjugated anti-CD4 (GK1.5), eFluor710 conjugated anti-CD8 (53-6.7) and their isotype controls rat IgG2a (clone RTK2758), rat IgG2b (clone RTK4530), Armenian hamster IgG (clone HTK888) were obtained from eBioscience and Biolegend (San Diego, CA, USA).

2 - 3 × 10^5 cells were stained with respective fluorochrome-conjugated antibodies in PBS containing 0.5% FBS and 0.1% sodium azide (FACS buffer) together with mouse FcR blocker (Miltenyi Biotech) for 1 hr at 4°C in the dark. Cells were then washed twice and resuspended in 500 µl of FACS buffer. If fixation was required, the cells were fixed with 1% PFA for 1 hr at 4ºC. Flow cytometry was performed either on a FACSCalibur (BD Biosciences, San Diego, CA, USA) with CellQuest data acquisition and analysis software, or on a Cyan flow cytometer (Dako, Denmark) with Summit software v4.3. Flow cytometry was also performed using the

BD LSRFortessa cell analyzer (BD Bioscience) and analyzed with Flowjo. Nonspecific staining was controlled by isotype-matched antibodies.

Countbright Absolute Counting Beads (Invitrogen) were added to samples for flow cytometry when calculation of absolute cell number was performed. Cell number in each sample was calculated based on following formula:

$$\frac{\text{Number of cells in the gate}}{\text{Number of beads in the gate}} = \frac{\text{Concentration of cells in the sample}}{\text{Concentration of beads in the sample}}$$

Figure 2.1 Calculation of absolute cell number. Concentration of cells in the sample was calculated based on the ratio of number of cells and counting beads acquired in respective gate during flow cytometry.

2.12 Immunohistochemistry

B. pertussis-infected BALB/c mice or Influenza A (PR8, H1N1) infected or *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) C57BL/6 mice were sacrificed 3, 4 and 21 days post-infection for harvesting femurs respectively. Naïve mice were used as negative control. The femur bones were harvested, fixed with 10% formalin followed by decalcification with 10% EDTA in H_2O for 14 days. The decalcified bones were fixed and embedded in paraffin. After deparaffinization in Xylene, and dehydration in a graded series of alcohol, the slides were pretreated with citrate buffer (Dako, Denmark) in a pressure cooker at 109ºC for 15 min, or were incubated in 20 µg/ml Proteinase K (Sigma Aldrich, St Louis, MO, USA) in PBS at 37 °C for 1 hr for

antigen retrieval. Endogenous peroxidases were inactivated by 3% hydrogen peroxide for 15 min. Unspecific staining was blocked by 3% dry milk in PBS for 30 min. 0.5 μg/ml of biotinylated goat anti-mouse CD137 polyclonal antibody (R&D Systems, MN, USA) or its isotype control (biotinylated goat IgG, R&D systems) in TBS were used as primary antibodies and hybridized overnight. The secondary Strepavidin-HRP (Sigma-Aldrich, St Louis, MO, USA) was added for 30 min, followed by DAB^+ substrate (Dako, Denmark). The entire procedure was carried out at room temperature and after each step the samples were washed three times with PBS. Finally, the tissue sections were counterstained with hematoxylin (Sigma-Aldrich, St Louis, MO, USA) and mounted.

2.13 Transfer of *in vitro* activated T cells to WT mice

WT or $CD137^{-/-}$ splenic T cells were isolated as previously described and activated by α-CD3/CD28 antibodies (Biolegend, USA) for 48 h. Cells were stained with 5 μM CFSE at 37°C for 10 min. Uptake of CFSE dye was stopped by addition of 5 volumes of ice-cold RPMI-1640 medium with 10% FBS. Cells were incubated on ice for 5 min and washed in completed medium three times. After the wash cells were resuspended in PBS at a concentration of 10^6 cells/ml and intravenously injected into mice. 24 hr after the injection, mice were euthanized by CO_2 inhalation. Spleen, draining lymph node and bone marrow were harvested. Single cells solution of each organ was prepared and stained for CD3. Numbers of $CFSE^+$ cells were detected by

flow cytometry on a Cyan flow cytometer (Dako, Denmark) with Summit software v4.3..

2.14 Isolation of Lin$^-$ progenitor cells from bone marrow and coculture of CFSE-labeled bone marrow/ Lin$^-$ progenitor cells with activated T cells

Lin$^-$ progenitor cells were isolated by magnetic cell sorting using the mouse lineage cell depletion kit (Miltenyi Biotec). Briefly, the fresh bone marrow cells were labeled with a cocktail of biotin-conjugated antibodies against CD5, CD45R, CD11b, Gr-1, Ter-119 and 7-4, followed by anti-biotin microbeads. The cell suspension was passed through a LS column in a strong magnetic field and the lineage negative progenitor cells were collected in effluent.

Bone marrow cells and Lin$^-$ progenitor cells were resuspended at 10^6 cells/ml in PBS with 0.1% BSA. Cells were stained with 5 μM CFSE, and 10^5 CFSE-labeled bone marrow and Lin$^-$ progenitor cells were then cocultured with CD4$^+$ T cells prectivated by CD3 and CD28 antibodies for 48 hr at a ratio of 1:2. On day 6 of coculture the cells were harvested and stained for CD4, CD11b, Gr-1, Ly6G, Ly6C and analyzed by flow cytometry.

2.15 Cell Viability Count

Numbers of viable cells were counted by using Sphero Accucount Blank Particles (Spherotech) and 7-AAD (BD Pharmingen) staining on flow cytometry. Cells were first stained for 7-AAD and 5 μl of counting beads were added to each sample before flow cytometry. Cells were gated for 7-AAD^{-} population and number of cells were calculated. Each sample was performed in triplicates.

2.16 BrdU Incorporation

1 mg of BrdU was injected i.p into WT and CD137$^{-/-}$ mice 6 hr before euthanization. The staining of BrdU protocol follows manufacturer's instruction (BD Pharmingen). Briefly, bone marrow cells were fixed and permeabilized by Cytofix/Cytoperm Buffer on ice for 30 min. Cells were washed by 1x Perm/Wash Buffer. Cells were incubated with Cytoperm Permeabilization Buffer Plus on ice for 10 min to permeabilized the nucleus membrane. After the wash the cells were refixed with Cytofix/Cytoperm Buffer on ice for 5 min. DNA was digested by incubation of cells in PBS with 300 μg/ml of DNase for 1 hr at 37°C. Then BrdU incorporated was stained by addition of FITC conjugated anti-BrdU antibodies for 20 min at RT. The stained cells were washed and prepared in FACS buffer for flow cytometry analysis.

2.17 Microscopy

Morphological changes of cells were documented by using a Zeiss Axiovert 40 inverted microscope (Zeiss, Göttingen, Germany) and Canon PowerShot G6 digital camera.

Images of IHC stainings were documented using a Leica DM 2000 microscope, a Leica DFC 490 digital camera, and the Leica application suite (version 2.8), (Leica Microsystem, Wetzlar, Germany). Four to five representative areas were selected from each slides and number of positive cells were counted in each area.

2.18 Colony formation assay

Bone marrow cells from 3-months and 12-months old mice were harvested as previously described. Cells were resuspended in IMDM at the concentration of 1 million/ml. 300 µl of cell solution were added to 3 ml of Methocult 3434 (Stemcell Technologies) for myeloid progenitor detection. 1.1 ml of medium was dispensed to treated culture dish and incubated at 37°C for 7 to 10 days. Types of colonies were determined based on manufacturer's instruction. Duplicates of plates were prepared for each mouse and at least three mice per strain were analyzed.

2.19 Statistics

Quantitative data are presented as mean ± SD. Statistical significance was determined by two-tailed unpaired Student's *t*-test or Mann-Whitney Test when stated. P values less than 0.05 were considered statistically significant.

Chapter 3 Results

3.1 CD137L reverse signaling in murine monocytes

Studies from our laboratory have shown that CD137L reverse signaling induces proliferation, activation and differentiation of human and murine progenitor cells in vitro (Jiang 2008 a, Jiang et al. 2008 b). In the human cells, crosslinking of CD137L also promotes differentiation of peripheral blood monocytes to DCs that efficiently elicit allogenic T cell proliferation as well as enhance cytotoxic T cell activity (Kwajah and Schwarz 2010). These findings inspired us to test whether CD137L reverse signaling has similar influence on murine monocytes. It will be beneficial if CD137L can differentiate murine monocytes to DCs as it does in the human cells so that it is possible to investigate the efficacy of CD137L-DCs in vivo.

3.1.1 CD137L reverse signaling induces morphological change in murine monocytes

Purified murine bone marrow monocytes were grown on plates that had been coated with a recombinant protein (CD137-Fc) consisting of the extracellular domain of CD137 fused to the constant domain of human IgG1 (Fc). Wells coated with an equal concentration of the Fc protein served as negative controls.

In response to CD137L reverse signaling the monocytes attached to the plates, spread and formed lamellipodia (**Figure 3.1.1**), similar as it has been shown for human monocytes (Langstein et al., 1998). For comparison classical immature DCs differentiation induced by GM-CSF and IL-4 showed cluster formation and a substantial proportion of the cells remained in suspension. On the other hand, only a few of the Fc-treated monocytes attached to the plate and the majority of the attached cells lacked lamellipodia formation. The overall density of cells upon Fc treatment was also lower than that upon CD137-Fc treatment.

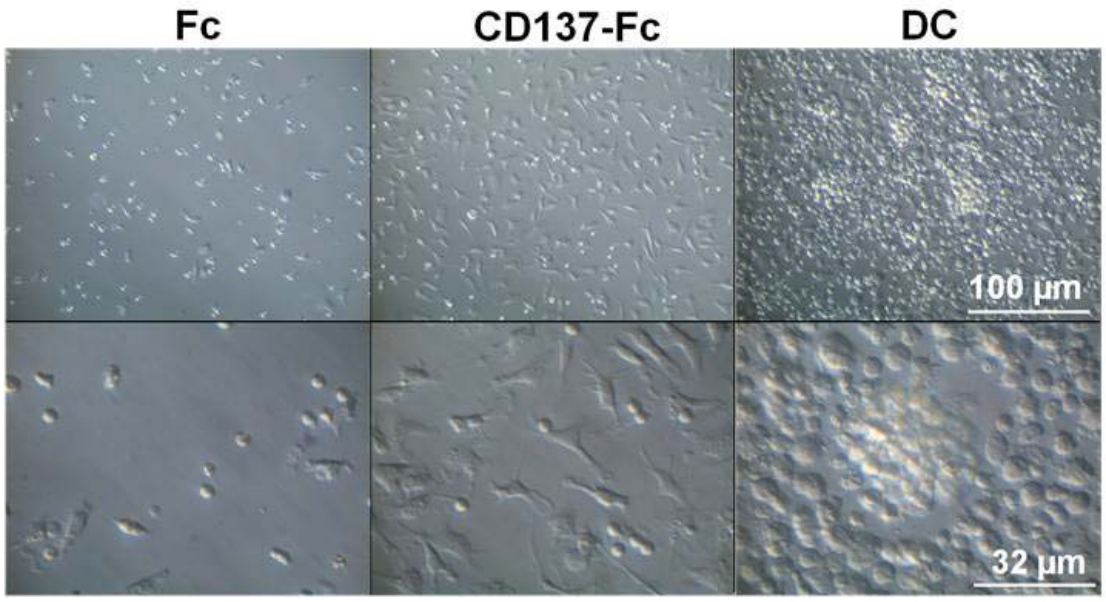


Figure 3.1.1 CD137-Fc induces morphological change of murine bone marrow monocytes. Murine bone marrow monocytes were isolated by negative depletion and treated with recombinant CD137-Fc or Fc for 6 days. Classical immature DCs were generated by GM-CSF and IL-4 for comparison. Morphology was recorded on day 6 at 20x (upper panel) and 63x (lower panel). Data were representative of three independent experiments.

3.1.2 CD137L promotes survival and proliferation of murine monocytes

Since the cell density in Fc treatment groups appeared to be lower, it was possible that crosslinking of CD137L promoted cell survival. Cells were harvested by 10 mM EDTA in 1x PBS after a 7 day culture period. Dead cells were stained by 7-AAD and gated out by flow cytometry. Counting beads were added to the samples to determine the number of live cells. The data showed that the numbers of live cells in the CD137-Fc coated wells were five times higher than those in Fc coated wells (**Figure 3.1.2.A**).

The increased number of live cells resulting from treatment with CD137-Fc protein could be the result of prolonged cell survival, induction of proliferation or a combination of both effects. Indeed, ^{3}H-thymidine proliferation assay showed that CD137L reverse signaling induced a significant proliferation of monocytes while control cultures did not proliferate (**Figure 3.1.2.B**). These data demonstrated that similar as in the human cells CD137L reverse signaling induced adherence, morphological changes and proliferation in murine monocytes.

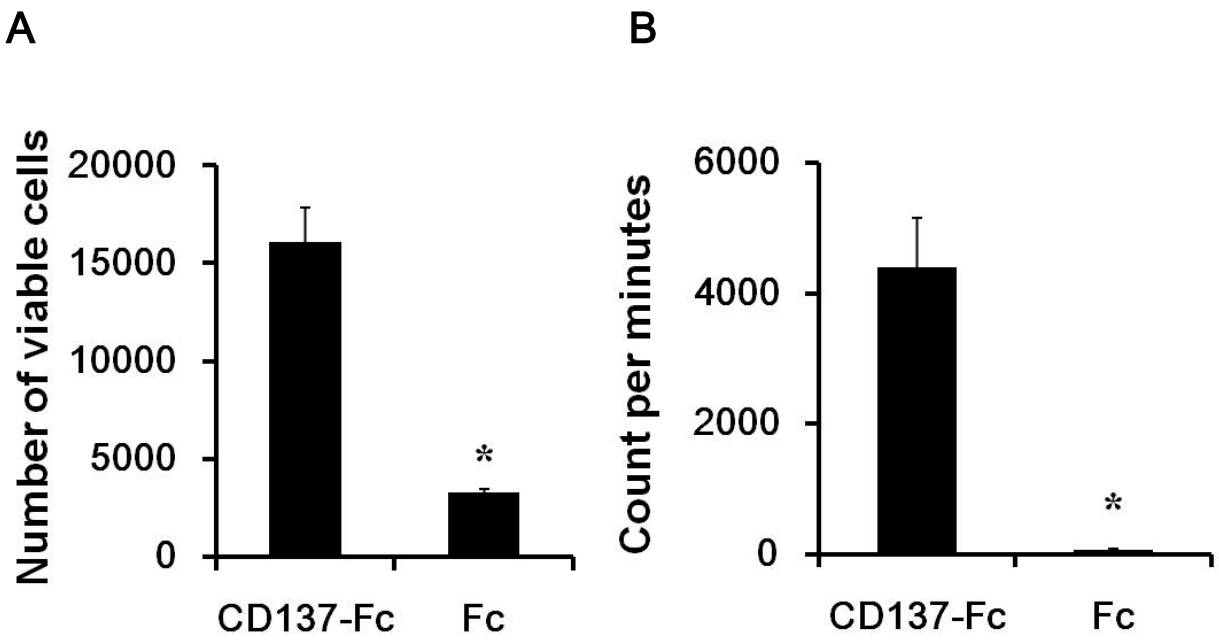


Figure 3.1.2 CD137L promotes survival in murine monocytes. Monocytes were isolated from bone marrow and treated with CD137-Fc or Fc as previous described. (A) Number of viable cells treated by CD137-Fc and Fc. Murine monocytes treated by CD137-Fc or Fc were stained by 7-AAD and analyzed by flow cytometry. Numbers of viable cells (7-AAD$^-$) were determined using counting beads. (B) Proliferation of monocytes measured by ^{3}H thymidine assay. Cells were pulsed with 0.5 μ Ci ^{3}H-thymidine for 16 hr. Triplicates were performed for each treatment. *$p<0.05$. Data were representative of three independent experiments.

3.1.3 Costimulatory molecules are absent in CD137-treated monocytes

In human monocytes CD137L reverse signaling induces strong expression of costimulatory molecules including CD80 and CD86 which are further enhanced by DCs maturation signals such as LPS and IFN-gamma (Lippert et al., 2008; Ju et al., 2009, Kwajah and Schwarz 2010). The presence of costimulatory molecules is a strong indication that cells exhibit a DCs phenotype. Hence, the expression of two

major costimulatory molecules, CD80 and CD86, was examined on CD137-Fc treated monocytes.

High levels of CD80 (79.5%) and CD86 (58.7%) were expressed by the classical immature DCs induced by GM-CSF and IL-4. However, in murine monocytes treated by CD137, CD137L reverse signaling only marginally induced CD80 and CD86 with percentages of positive cells of 9.6% and 14.8 %, respectively. Addition of LPS had no effect on the expression of CD80, but slightly increased the level of CD86 to 26.7%. Both costimulatory molecules were expressed at very low levels on Fc-treated monocytes, regardless of the addition of LPS (**Figure3.1.3**).

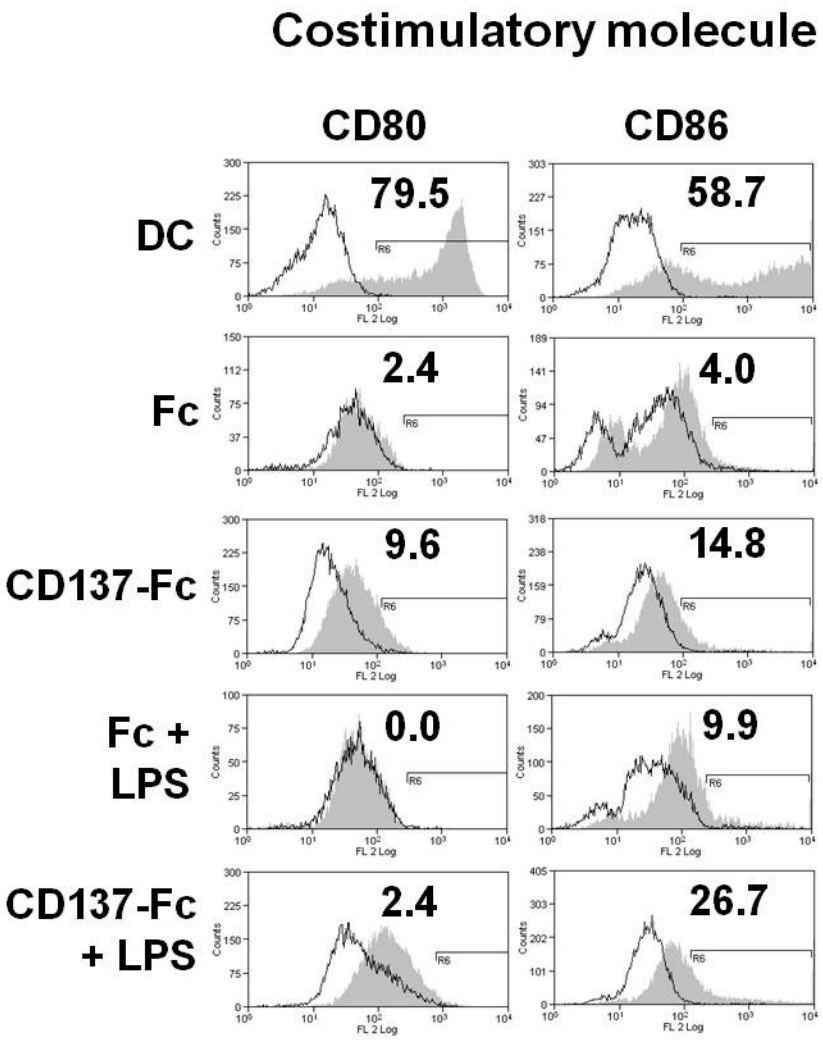


Figure 3.1.3 Expression of costimulatory molecules in CD137-Fc treated monocytes. Murine monocytes were treated with CD137-Fc or Fc for 7 days. Cells were matured by LPS for the last 24 h. Murine monocytes treated with GM-CSF and IL-4 served as classical immature DCs. Expression of CD80 and CD86 was examined by flow cytometry. Open histograms represent cells stained with an isotype control. Grey histograms represent cells stained with an antibody specific for the indicated surface marker. Numbers in each graph represent percentages of cells stained positive for the antigen relative to the isotype control. Data were representative of three independent experiments.

3.1.4 DCs markers and MHC-II molecule are absent on CD137-treated monocytes

A central property of DCs is their ability to present antigens to T cells. Hence, it is important to have high levels of MHC-II, the key antigen presenting molecule on the DCs. DCs also express relatively high levels of CD11c which is one of the earliest marker used to describe DCs by Steinman (Steinman, 1973; O'Doherty, 1994). Therefore, costaining of CD11c and MHC-II provides a first glimpse on the phenotype of the cell. As expected, classical mature DCs derived from murine monocytes treated with GM-CSF, IL-4 and LPS expressed high level of MHC-II and about 46% of the cells expressed both CD11c and MHC-II. However, in spite of the substantial upregulated expression of CD11c on CD137-treated monocytes (about 10-fold, from 3.1 to 29.3%), only a few (6%) of these $CD11c^+$ cells expressed also MHC-II and only at low levels. As in the case of costimulatory molecule expression, the addition of LPS had no effect on enhancing the expression of MHC-II. Compared to CD137-Fc treated monocytes Fc control cells were almost devoid of both MHC-II and CD11c, with only 1.0% of the cells staining double positive.

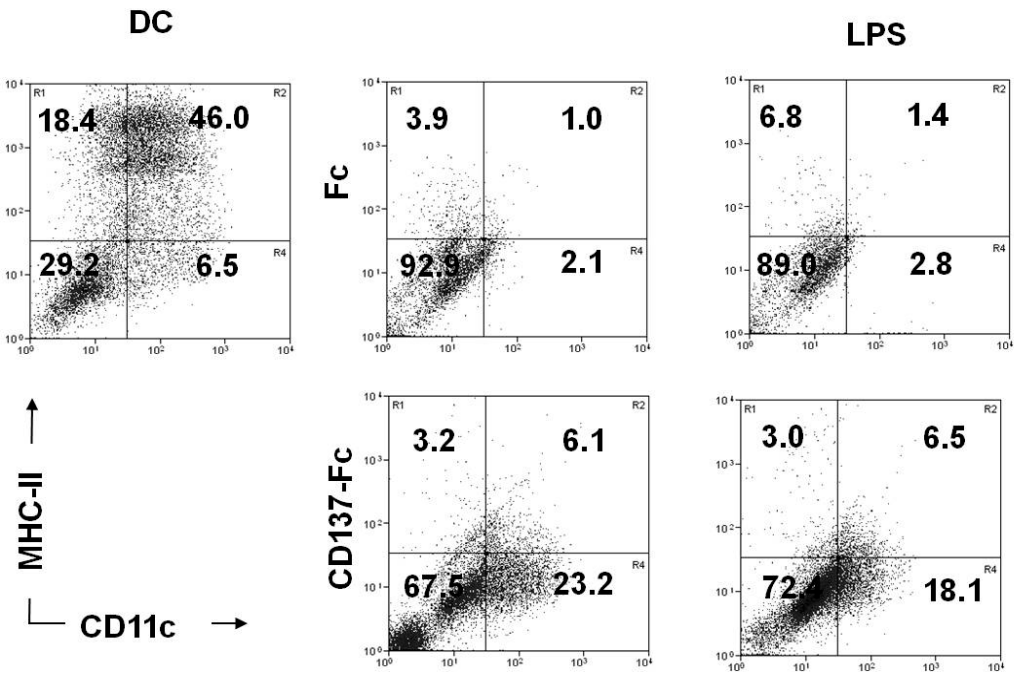


Figure 3.1.4 CD137-treated monocytes lack DCs marker and antigen presenting molecules. Expression of CD11c and MHC-II on murine monocytes were determined by flow cytometry. Murine monocytes were treated with Fc or CD137-Fc for 7 days. LPS were added to mature the cells for last 24 hours. Murine monocytes treated with GM-CSF, IL-4 and LPS were used as classical mature DC controls. Numbers in the graphs represent percentages of cells in each quadrant. Data were representative of three independent experiments.

3.1.5 CD137-treated monocytes have a low IL-12p70/IL-10 ratio

Classical mature DCs have been reported to have a very high IL-12p70/IL-10 ratio, a typical phenotype of a Th1 immune response because IL-12p70 is the essential stimulus for Th1 T cell proliferation and differentiation. On the other hand, IL-10 is an immunosuppressive cytokine and typically expressed by regulatory T cells and Th2 cells (Blanco et al 2008). Usually inflammatory DCs/macrophages have a high IL12/IL10 ratio while regulatory or suppressive DCs/macrophages have a low

IL12/IL/10 ratio. Therefore, the levels of IL-12p70 and IL-10 reflect the phenotype of CD137-treated monocytes.

As expected classical mature DCs secreted high level of IL-12p70 while the level of IL-10 was undetectable (**Figure 3.1.5**). In the absence of LPS, CD137 triggered a low level of IL-10 in murine monocytes but not IL-12p70. Both cytokines were undetectable in Fc-treated monocytes.

The addition of LPS slightly upregulated IL-12p70 production in Fc-treated monocytes but had no effect on CD137-treated monocytes. While stimulation by LPS had little effect on expression of CD80, CD86, MHC-II and IL-12p70, it synergized with CD137L reverse signaling to reinforce the IL-10 secretion, increasing the concentration of this cytokine from less than 100 pg/ml to more than 1600 pg/ml **(Figure 3.1.5)**. Although LPS also enhanced IL-10 production in Fc-treated monocytes to 400 pg/ml, the level was 4 times lower than that of CD137 treatment.

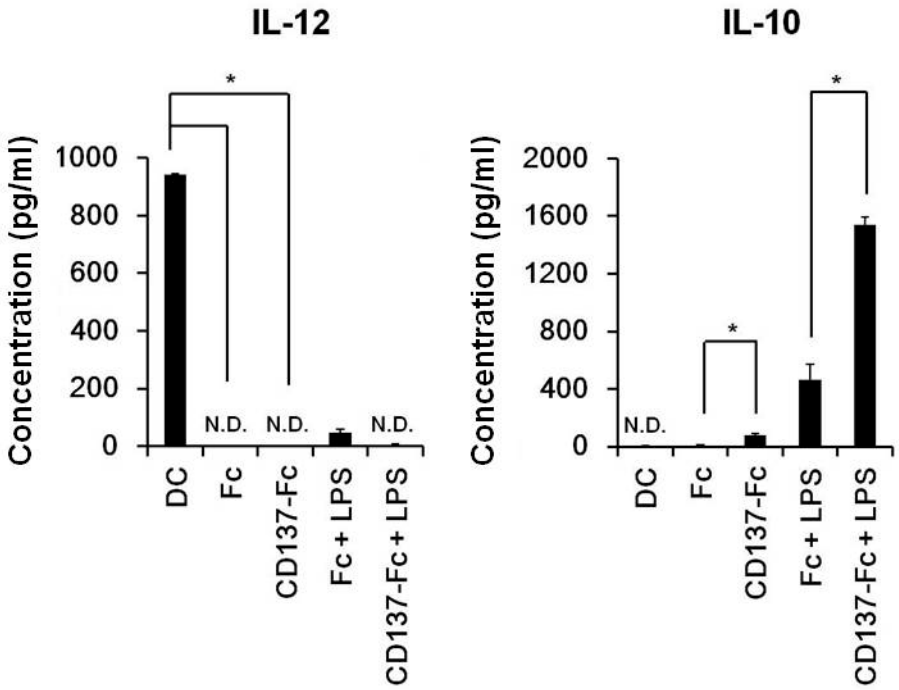


Figure 3.1.5 Cytokine production in CD137-treated monocytes. Murine monocytes were treated with Fc or CD137-Fc for 7 days. LPS were added to mature the cells for last 24 hours. Murine monocytes treated with GM-CSF, IL-4 and LPS were used as classical mature DC controls. Concentrations of IL-10 and IL-12p70 in the supernatants of monocytes with indicated treatments were determined by ELISA. N.D. indicates that the level of the cytokine is under the detection limit. * $p<0.05$. Data were representative of three independent experiments.

3.1.6 CD137-treated monocytes cannot stimulate T cells in an allogenic mixed lymphocytes reaction

Because CD137L reverse signaling enhances neither costimulatory molecule expression nor the production of IL-12p70, the gold-standard to determine the phenotype of DCs, allogenic mixed lymphocyte reaction, was employed to assess whether CD137-treated murine monocytes exhibited DCs properties.

In an allogenic MLR CD137-Fc or Fc treated monocytes from BALB/c mice were cocultured with T cells from C57BL/6 mice for 3 days. Classical immature DCs and mature DCs were used as negative and positive controls.

Even without maturation by LPS, DCs can stimulate a certain level of T cell proliferation, which was increased up to twofold if the DCs were further matured by LPS (from 20000 cpm to 40000 cpm). T cell proliferation was hardly detectable in all other conditions where monocytes were treated by either CD137-Fc or Fc, regardless of addition of LPS.

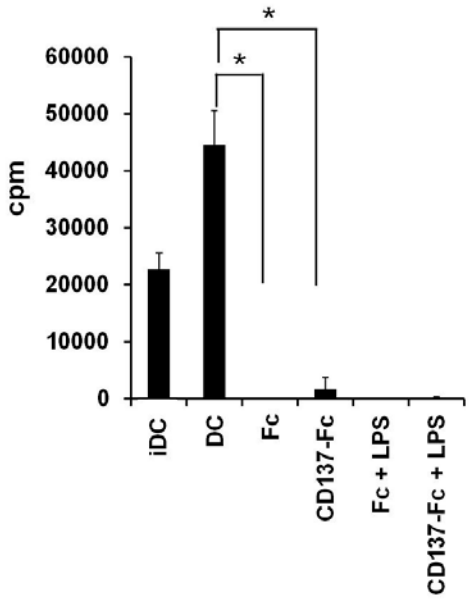


Figure 3.1.6 Allogenic mixed lymphocytes reaction by CD137-treated monocytes. Murine bone marrow monocytes were treated as indicated for 6 days and further matured by LPS for the last 24 hr. The treated monocytes were cocultured with T cells from C57BL/6 mice at a ratio of 1:10 for 3 days. ^{3}H-thymidine was added for the last 16 hr and proliferation was measured as count per min (cpm). *$p<0.05$. Depicted are means ± standard deviations of triplicate. Data were representative of three independent experiments.

3.1.7 CD137L reverse signaling upregulates macrophage markers on murine monocytes

Along the differentiation journey of monocytes during inflammation, the cells can become either DCs or macrophages dependent on the signals they receive. Since CD137-Fc treated monocytes did not exhibit typical markers and cytokine profile of classical DCs, it was investigated whether the cells have taken an alternate path to become macrophages.

To answer this question, the expression levels of CD14 and F4/80, two markers commonly found on differentiated macrophages, were examined. Staining of CD14 and F4/80 showed that CD137-Fc substantially upregulated the expression of both molecules compared to Fc treatment on murine monocytes. CD14 was totally absent in Fc-treated monocytes but expressed by 59.4% of cells treated with CD137-Fc. Compared to classical DCs, CD137-treated monocytes had 15% more cells expressing CD14. The effect of CD137L reverse signaling in inducing macrophage markers became more evident for F4/80, which was upregulated from 51.1% to 80.9% in CD137-Fc treated monocytes compared to Fc treatment. As expected, only 18% of the classical DCs expressed F4/80, almost 4 times lower than CD137-treated monocytes.

Addition of LPS slightly enhanced the expression of F4/80 in CD137-Fc treated monocytes, bringing up the level by 7% (80.9% to 87.5%). The increase was more prominent for CD14, where the expression level was increased by 20% to 80.9%. However, LPS did not further increase the expression level of either F4/80 or CD14 of Fc-treated monocytes.

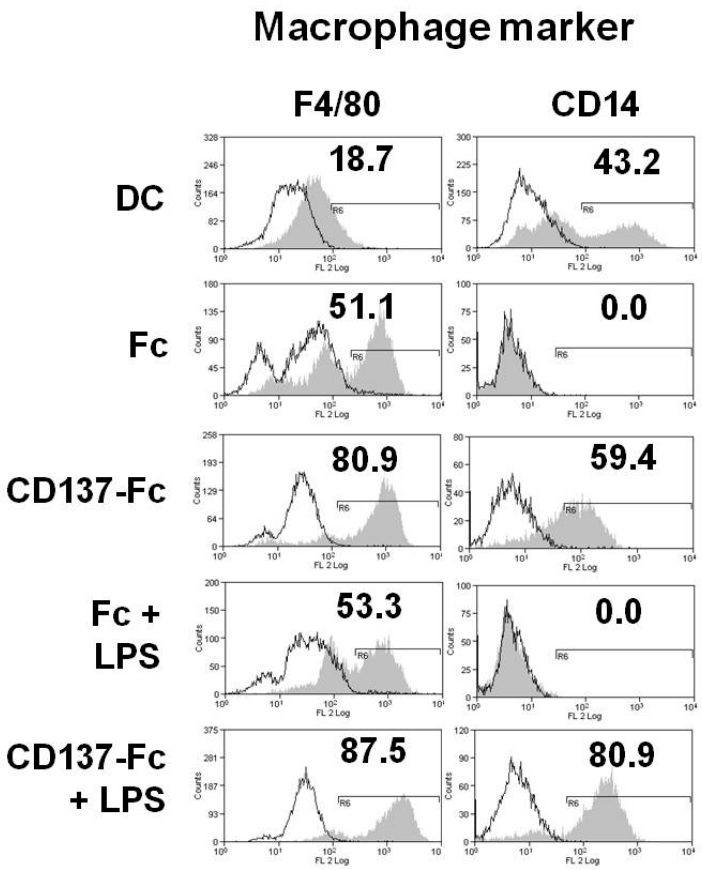


Figure 3.1.7 CD137 upregulates macrophage markers in monocytes. Murine monocytes were treated with Fc or CD137-Fc for 7 days. LPS was added to mature the cells for last 24 hours. Murine monocytes treated with GM-CSF, IL-4 and LPS were used as classical mature DC controls. After 6 days of culture, cells were harvested and stained for the two macrophage markers, F4/80 and CD14. The expression levels were analyzed by flow cytometry. Open histograms represents cells stained with isotype controls. Grey histograms represent cells stained with antibodies against the indicated surface marker antigen. Numbers in the graphs represent percentages of cells that have shifted relative to the isotype controls. Data were representative of three independent experiments.

3.1.8 CD137-treated monocytes have enhanced phagocytic activity

One major characteristic that distinguishes classical DCs from macrophages is the level of phagocytosis. As the major APCs, DCs have high levels of endocytosis but very low levels of phagocytosis (Kwajah and Schwarz, 2010). In macrophages, however, the situation is reversed as the core function of macrophages is to clear pathogens and debris. To determine whether CD137-treated monocytes were macrophages the level of phagocytosis was examined by flow cytometry.

Monocytes treated by CD137-Fc or Fc were given fluorescent beads at 37°C for 1 hr. To confirm that the observed phagocytotic activity was not a result of random adherence to the cell surface, trypsin was added to the harvested monocytes to cleave off beads binding to surface proteins. The percentages of cells that have phagocytosized fluorescent beads and numbers of fluorescent beads taken up by each cells (expressed as mean fluorescent intensities) were measured by flow cytometry.

Upon treatment by CD137-Fc, monocytes became highly phagocytic as 71.8% of the cells have taken up the fluorescent beads while only 51.1% of the Fc-treated cells have become phagocytic. Among the phagocytic cells, CD137-Fc treated monocytes were on average more phagocytic as their MFI was considerably higher than that of Fc treated monocytes (1930 vs 794).

Trypsinization significantly reduced the non-specific binding of beads to Fc-treated monocytes by reducing the percentage of phagocyotic cells from 62.4% to 51.1%, and the MFI from 2031 to 794. In the CD137-treated monocytes, however, the percentage of phagocytotic cells as well as the MFI was hardly affected, meaning that the CD137-treated monocytes were truly phagocytic.

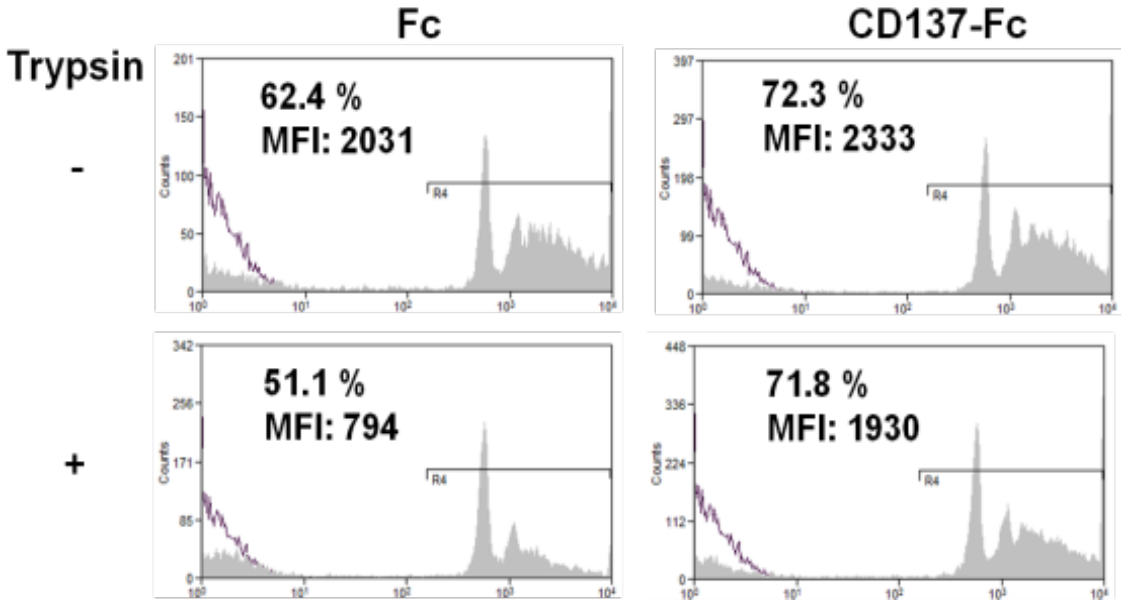


Figure 3.1.8 CD137L reverse signaling upregulates the phagocytic activity of monocytes. Monocytes were treated with CD137-Fc and Fc protein for 6 days. Fluorescent beads were added to the culture for 1 hr at 37°C in dark. Monocytes were harvested by trypsin and analyzed by flow cytometry. Open histograms represent cells that have not internalized the fluorescent beads. Grey histograms represent cells that have internalized the fluorescent beads. Numbers in the graphs indicate the percentages of cells that have internalized fluorescent beads and mean fluorescent intensities (MFI). Data were representative of three independent experiments.

3.1.9 CD137-treated monocytes exhibit cytokine profile similar to macrophage upon stimulation by LPS

To confirm the phenotype of CD137-treated monocytes as macrophage, the cytokine profile of CD137-treated monocytes was compared to that of bone marrow derived macrophage (BMM) generated by M-CSF. Unlike classical DCs, BMM typically have a low IL-12p70/IL-10 ratio, particularly when stimulated by LPS.

As shown in **Figure 3.1.9** CD137-treated monocytes had a very similar cytokine profile as BMM. Without addition of LPS, both cell types had low level of IL-10 but no IL-12p70 at all. However, upon the addition of LPS, the level of IL-10 increased significantly while the level of IL-12p70 remained undetectable.

In the human cells, crosslinking of CD137L on peripheral blood induces release of M-CSF to support survival and activation of macrophage. In the case of murine monocytes, however, M-CSF was not detectable in all conditions (data not shown).

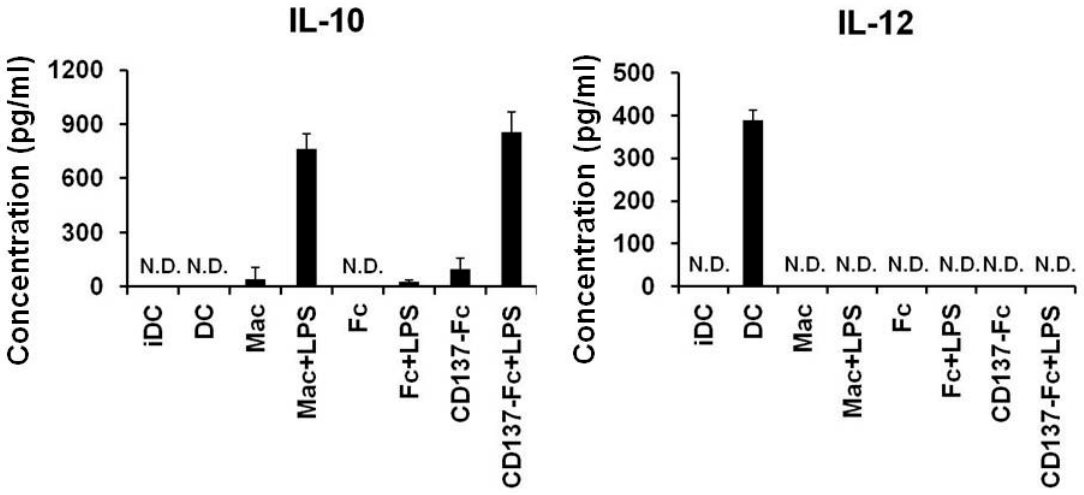


Figure 3.1.9 CD137-treated monocytes exhibit property of macrophage. Murine monocytes were treated with Fc or CD137-Fc for 7 days. LPS were added to mature the cells for last 24 hours. iDC: classical immature DCs: generated by a 7 day culture with GM-CSF + IL-4. DCs: classical mature DCs generated by a 7 day culture with GM-CSF + IL-4, and stimulated by LPS for the last 24 h. Mac: BMM generated by a 7 day culture with M-CSF. Levels of IL-10 and IL-12p70 in the supernatants of DCs, macrophages and CD137-Fc treated monocytes were determined by ELISA. N.D: non-detectable. Depicted are means ± standard deviations of duplicates *$p<0.05$. Data were representative of three experiments.

3.1.10 CD137L reverse signaling does not induce maturation in murine DCs

Previous studies have reported that CD137L reverse signaling upregulates MHC-II and costimulatory molecules CD86 by more than two fold in cord-blood derived DCs in the human cells (Kim, Li et al. 2002). Although treatment of CD137 induces macrophage instead of DC differentiation in murine monocytes, it remains possible that CD137L reverse signaling can mature immature DCs in the murine cells.

Classical immature DCs were generated by GM-CSF and IL-4 for 6 days in vitro. The cells were harvested and stimulated by CD137-Fc or Fc immobilized on plates for 24 hr. LPS was used as a positive control for DC maturation. Cells were then stained for costimulatory molecules: CD40, CD80 and CD86.

As expected classical immature DCs treated by LPS upregulated all three costimulatory molecules: CD40 (8.9% to 33.7%), CD80 (56.7% to 59.8%), CD86 (21.0% to 61.0%). However, the levels of costimulatory molecules remained unaltered on DCs stimulated by CD137-Fc compared to classical immature DCs treated by PBS. The expression of CD40 was only 5.6% in CD137-Fc treated DCs. Although CD80 on CD137-Fc treated DCs was similar to mature DCs, the latter one had higher expression on a subpopulation of DCs based on the higher MFI. Expression of CD86 on CD137-Fc treated DCs was only 22.1% compared to 60.1% of mature DCs. The expression levels of all three costimulatory molecules were similar between CD137-Fc and Fc-treated DCs.

It was also reported in previous studies that upon maturation by CD137 protein human DCs increased production of proinflammatory cytokines including IL-12p70 and TNF-alpha (Kim, Li et al. 2002). Examinations of the levels of four proinflammatory cytokines, IL-12p70, TNF-alpha, IL-1 and IL-6, in the supernatants of CD137-treated murine DCs showed that CD137L reverse signaling was able to

induce low levels of IL-12p70, TNF-alpha, and IL-1 in the classical immature DCs. In spite of the low level of cytokine release, these concentrations were significantly lower than in DCs matured by LPS. CD137 was only one-tenth as effective as LPS in inducing IL-12p70 (462.5±145 vs 39.5±10.5 pg/ml) and TNF-alpha (1279.3±40.8 vs 242±15 pg/ml) release in DCs. Additionally, it completely failed to induce IL-6 production in immature DCs.

The low level of costimulatory molecules and proinflammatory cytokines suggested that CD137L reverse signaling on murine DCs might induce a low degree of activation but the cells remained largely immature. The data were further confirmed by allogenic MLR. It shows that the CD137-treated DCs were poor APCs because they induced considerably lower level of T cell proliferation than classical mature DCs. Most importantly there were no difference in T cell stimulation among classical immature DCs treated by CD137-Fc and Fc.

A

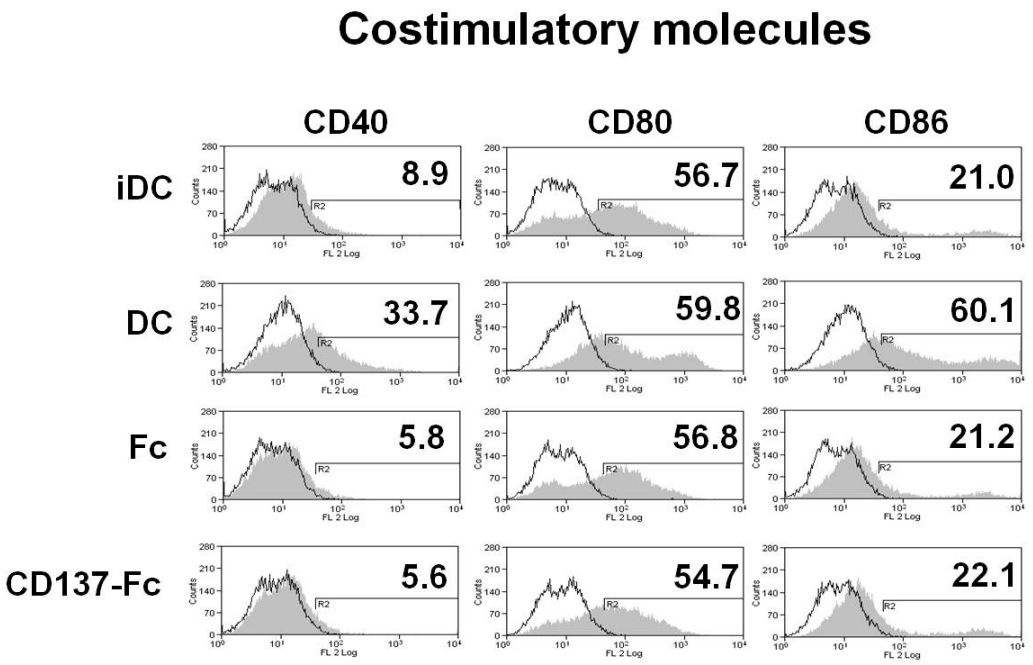


Figure 3.1.10 CD137-Fc does not mature murine DCs. Classical immature DCs from BALB/c mice were generated by GM-CSF + IL-4 for 6 days. Immature DCs were harvested by 1x PBS, 10mM EDTA and treated by CD137-Fc and Fc immobilized on plate for the last 24 hr. Classical mature DCs were induced by addition of 1 μg of LPS for the last 24 h. iDC: Classical immature DC treated with PBS. DC: Classical immature DC induced by LPS. (A) Expression of costimulatory molecules was determined by flow cytometry. Numbers in the graphs represent the percentages of populations that had shifted compared to isotype control (open histograms). Cells were stained for CD40, CD80 and CD86 and analyzed by flow cytometry. Data were representative of two independent experiments.

B

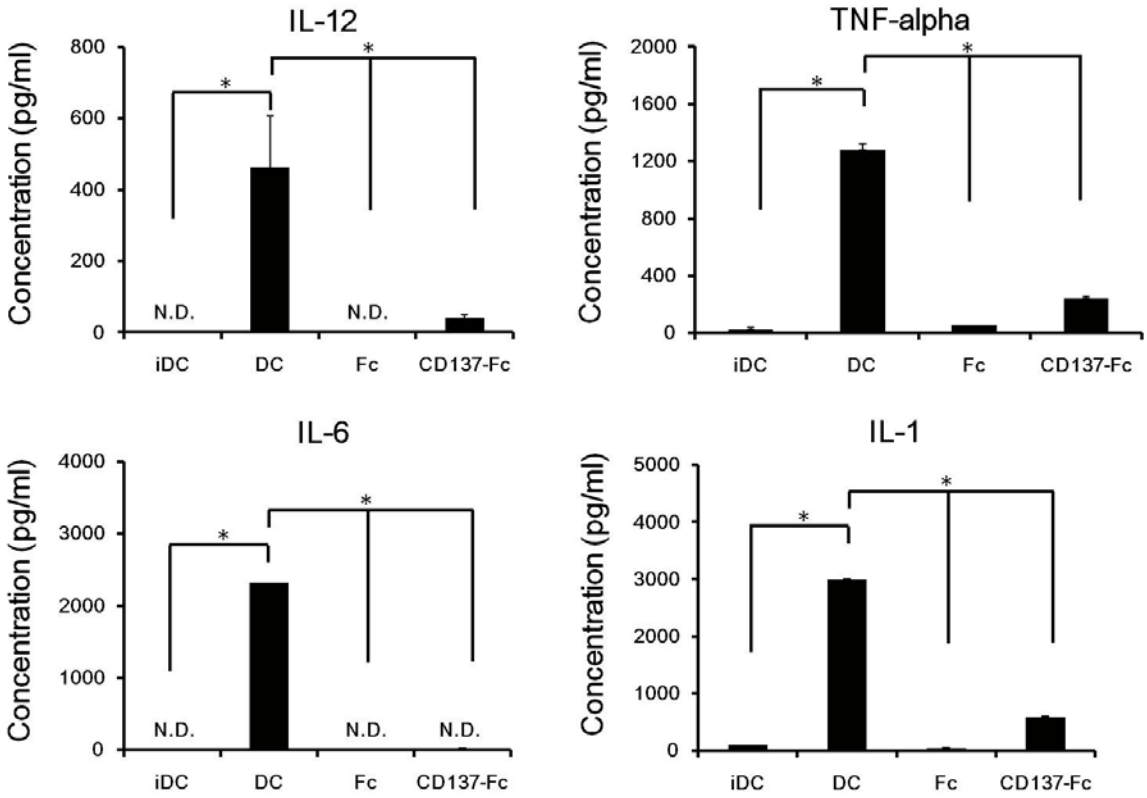


Figure 3.1.10 (B) Levels of IL-12p70, TNF-alpha, IL-6 and IL-1beta were examined in the supernatants of CD137-Fc and Fc-treated DCs. N.D: non-detectable. Depicted are means ± standard deviations of duplicates. Data were representative of two independent experiments.

C

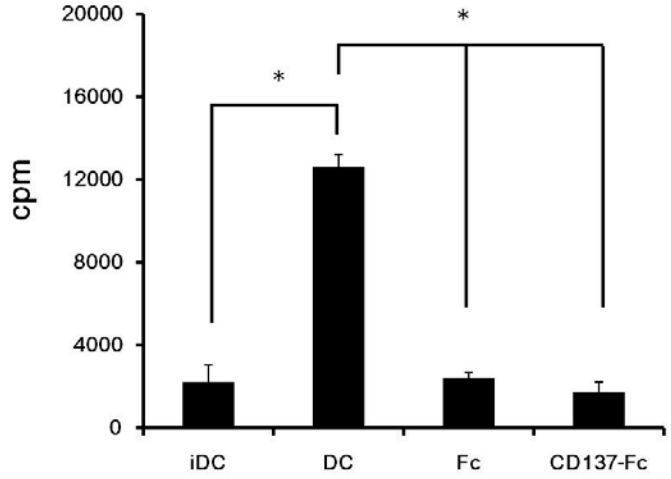


Figure 3.1.10 (C) Bone marrow derived immature DCs generated from Balb/c mice treated with LPS, CD137-Fc or Fc were cocultured with splenic T cells from C57BL/6 mice for 3 days. ^{3}H thymidine was added for the last 16 hr and proliferation was measured as count per min (cpm). Depicted are means ± standard deviations of triplicates. *$p<0.05$. Data were representative of two independent experiments.

3.1.11 CD137L reverse signaling on murine monocytes is unique and distinct from that by other members of TNF receptors

Monocytes can differentiate to macrophage after they exit from the circulation and enter the tissue during inflammation. Component of extracellular matrix such as fibronectin have been shown to elicit differentiation of monocytes to macrophage in vitro (Sudhakaran, Radhika et al. 2007). Also, in vitro, surface proteins and other biomolecules passively adhere to surfaces through hydrophobic and ionic interactions and the resulting attachment can induce low degree of differentiation of monocytes to macrophage. It is possible that attachment itself is a

differentiation signal for monocytes, regardless of the material as either ECM or culture dish surface.

Since the culture plates are coated with a high concentration of CD137-Fc or Fc protein, it may mimic an environment of ECM and the observed differentiation is simply a result of random binding of surface proteins to culture plate surface coated with protein. To exclude such possibilities, murine monocytes were treated with two other recombinant proteins, TNFRI and OX40, from the TNF receptor family which may exhibit similar properties as CD137. If the observed differentiation is caused by random interactions between surface proteins and culture plate, a similar morphological change and differentiation in monocytes should be observed when they were treated with TNFRI and OX40.

As shown by light microscopy (**Figure 3.1.11A**), CD137-Fc induced the greatest degree of morphological changes with lamellipodia formation and the highest degree of cell density in murine monocytes. TNFRI-Fc could also induce a certain level of morphological change in the monocytes and lamellipodia formation but to a much lesser extent than CD137-Fc. The cell density also appeared to be lower in the TNFRI-treated monocytes. Among all three recombinant proteins, OX40-Fc elicited the lowest morphological changes with the majority of the cells adopting a round, seemingly undifferentiated morphology.

Based on the morphological changes of the treated cells, it was observed that the cell density appeared to be the highest in CD137-Fc treatment (**Figure 3.1.11B**). 7-AAD staining further confirmed that the CD137-Fc treatment group had the highest number of viable cells among all treatment groups even though it was not statistically significant higher than TNFRI and OX40.

Aside from the morphological changes, CD137-Fc, TNFRI-Fc and OX40-Fc had also different effects on cytokine production (**Figure 3.1.11C**). Monocytes treated with CD137-Fc , TNFRI-Fc or OX40-Fc protein had distinct cytokine profiles. None of the molecules induced IL-12p70 production. TNFRI-Fc treated monocytes had the highest level of IL-10 and MCP-1 while OX40-Fc treated monocytes had the lowest of all.

A

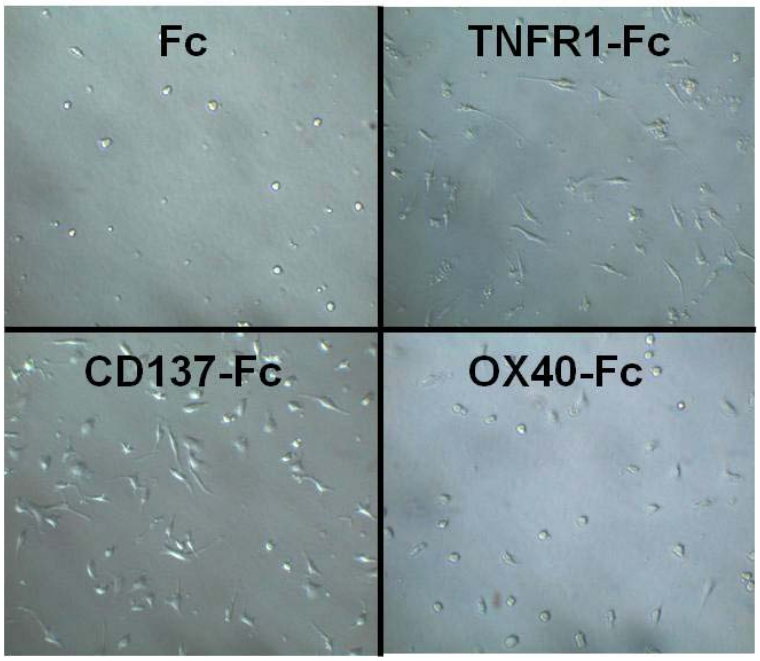


Figure 3.1.11 Morphological change, survival and cytokine productions of murine bone marrow monocytes treated by TNFR family members.
(A) Monocytes were treated with CD137-Fc, OX40-Fc, TNFRI-Fc and Fc. Morphological changes were recorded by light microscopy. Data were representative of two independent experiments.

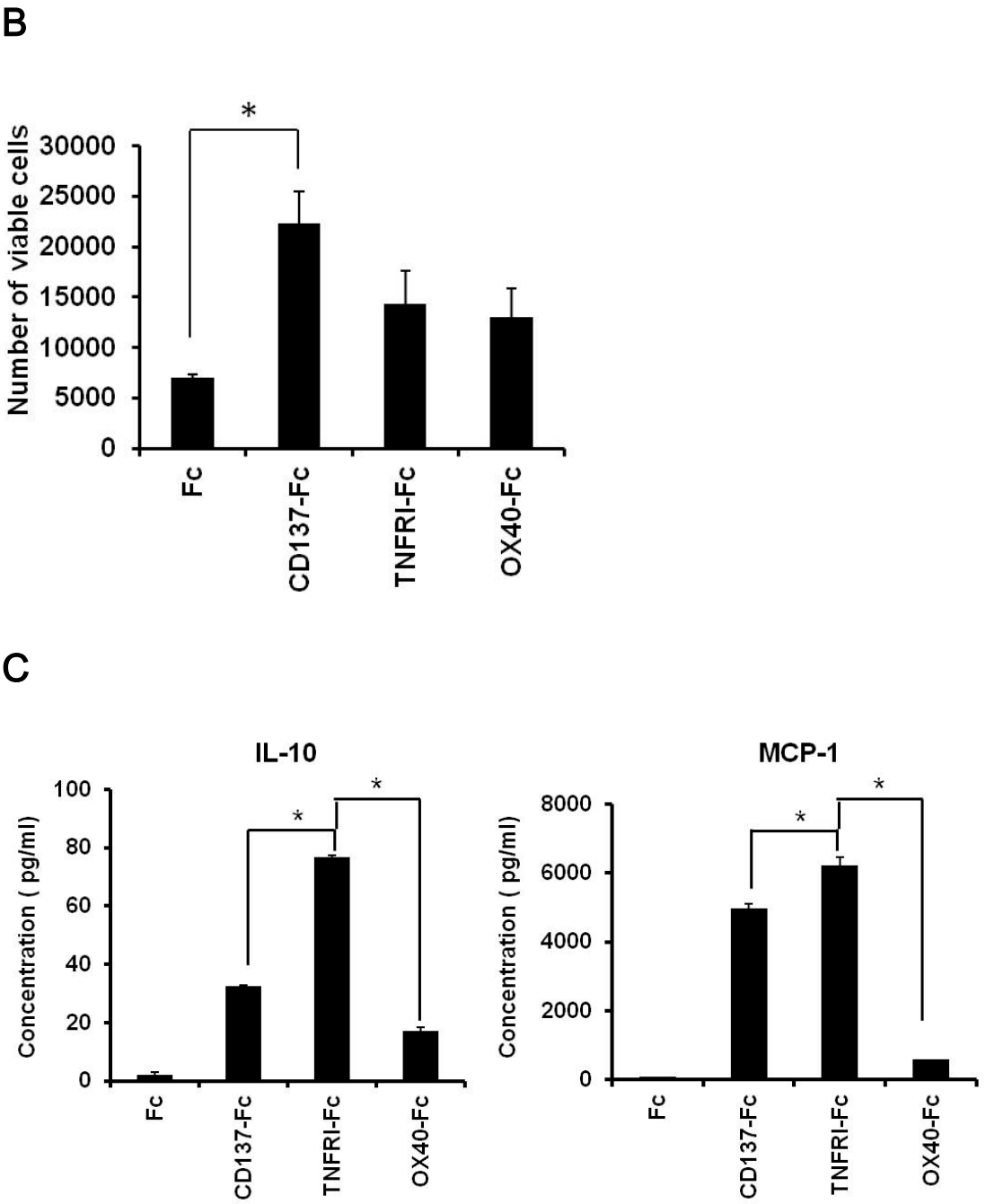


Figure 3.1.11 (B) Numbers of viable cells were determined by 7-AAD staining and flow cytometry. Depicted are means ± standard deviations of triplicate measurements. Data were representative of two independent experiments. (C) Monocytes were treated with CD137-Fc, TNFRI-Fc, OX40-Fc and Fc for 6 days. Concentrations of IL-12p70, IL-10 and MCP-1 were determined by ELISA. Depicted are means ± standard deviations of duplicates *p< 0.05. Data were representative of two independent experiments.

3.2 CD137L reverse signaling induces myelopoiesis during inflammation in vivo

In Section 3.1 it was shown that CD137L reverse signaling induces macrophage differentiation and the effect is caused solely by interaction of CD137 and CD137L. The data indicate a species difference between human and murine cells of more differentiated myeloid cells in response to CD137 stimulation. This finding restricts the further usage of murine models for testing CD137L-DCs in vivo.

However, CD137L still remains a potential myeloid differentiating factor because the less differentiated progenitor cells show similar response in human and murine cells (Jiang et al 2008 a; Jiang et al 2008 b). The response of progenitor cells is particularly important during infection due to the urgent need for large number of leukocytes to clear pathogen. Understanding the influence of CD137L in myelopoiesis especially on progenitor cells will contribute significantly to development of biological agents to combat infections. The work shown so far has been done in vitro and the high concentration of CD137 recombinant protein used simulates a highly inflammatory state. It would be of physiological relevance if a similar situation can be recapitulated in vivo. In this section the question whether CD137L reverse signaling can promote myelopoiesis during acute infection is addressed.

3.2.1 Percentage of myeloid cells during naïve state

Previously Lee and colleagues have reported that CD137 limits myelopoiesis during steady state (Lee et al. 2008). Examination of the percentages of the major myeloid markers CD11b, in the bone marrow of naïve WT and CD137$^{-/-}$ mice revealed that during steady state CD137$^{-/-}$ mice indeed exhibited significantly higher percentages of myeloid cells. The percentages of CD11b were 5% more in the CD137$^{-/-}$ mice than WT mice (**Figure 3.2.1**).

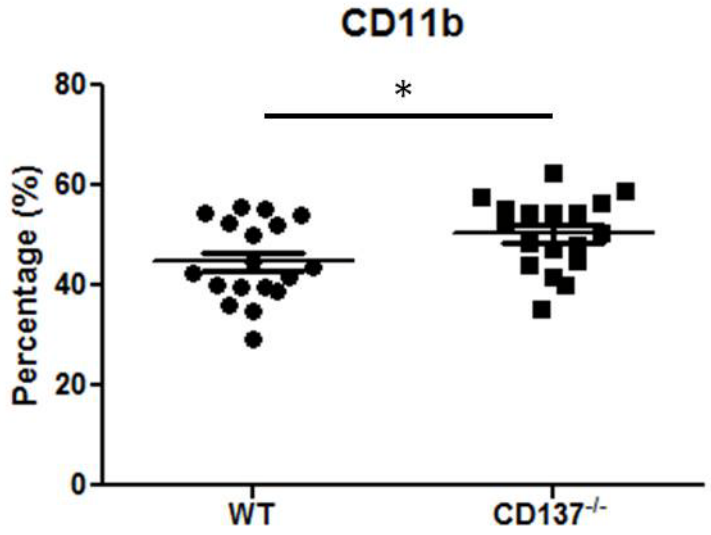


Figure 3.2.1 Myelopoiesis during steady state of WT and CD137$^{-/-}$ mice. Bone marrow of naïve WT and CD137$^{-/-}$ mice were stained for CD11b. Each dot represents one animal. Mann-Whitney test was performed for statistic. * $p<0.05$. Data were pooled from three independent experiments. Each experiment consisted of at least five mice per strains.

3.2.2 CD137 is upregulated in bone marrow during infection

If CD137L reverse signaling plays a role in myelopoiesis during inflammation, the level of CD137 must increase in the first place to crosslink the ligand. Although

the expression of CD137 on a small population of bone marrow cells of healthy mice has been reported by Jiang (Jiang er al 2008), the level is very low. In order to gain a better understanding of the function of CD137 in the bone marrow during inflammation, the level of CD137 expression in the bone marrow was determined by IHC under different infection states, including bacteria and viruses. Naïve mice were used as basal control.

Intratracheal infection of C57BL/6 WT mice with 5 PFU Influenza A (H1N1/PR8) significantly increased the number of CD137-expressing cells in the bone marrow 4 days post-infection as determined by IHC (**Figure 3.2.2 A**). Similarly, nasal infection with 10^6 CFU M. bovis BCG led to an increase in CD137-expressing cells in the bone marrow after 3 weeks (**Figure 3.2.2 B**). Also, 3 days post-infection there were more CD137-expressing cells in the bone marrow of mice that were nasally infected with 10^6 CFU Bordetella pertussis than in the bone marrow of naïve mice (**Figure 3.2.2 C**). The data were quantified by counting cells staining positive for CD137 in at least four areas each slide. (**Figure 3.2.2 D**) Quantification data showed that in each infection model, the numbers of $CD137^+$ cells were increased by 3 to 4 times.

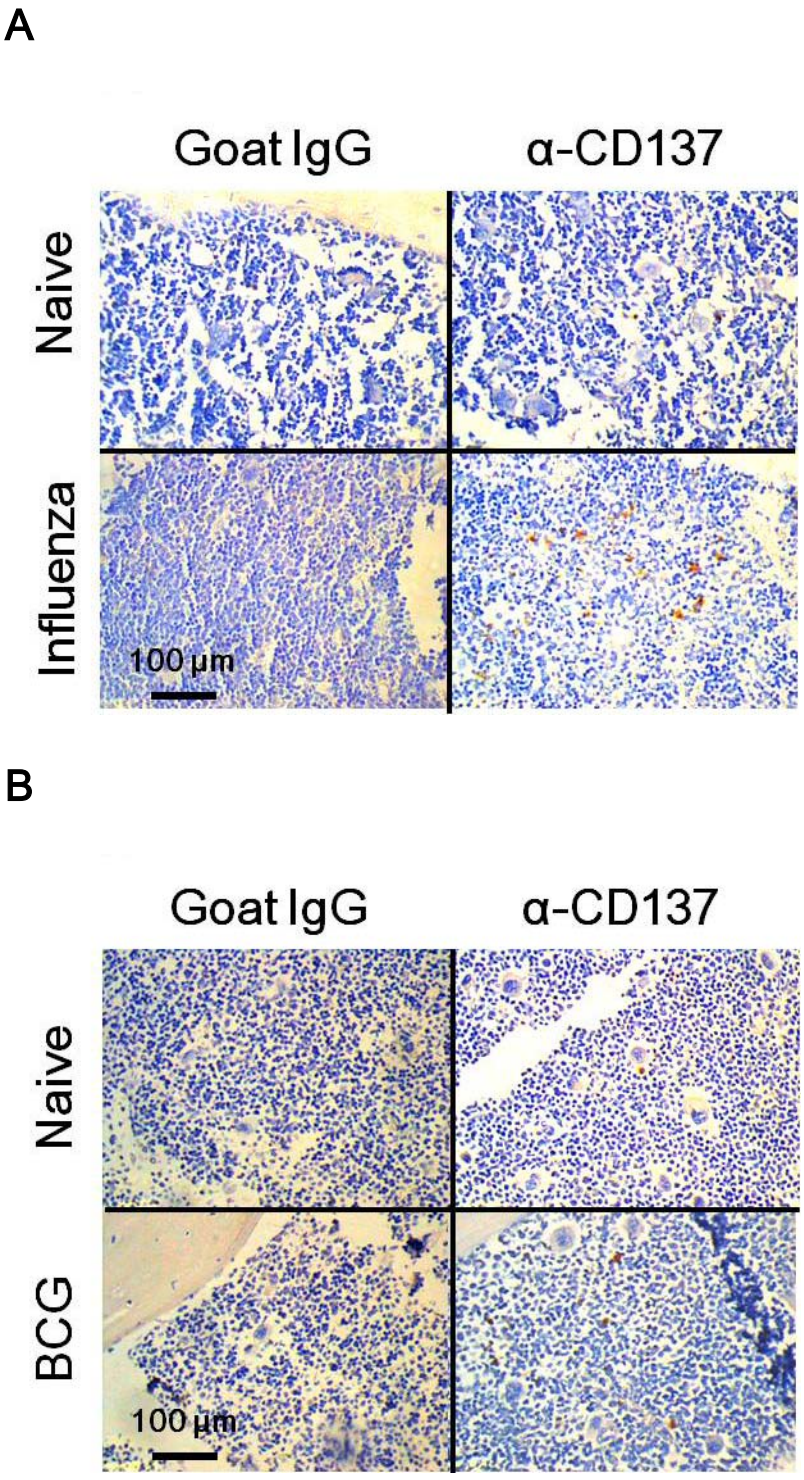
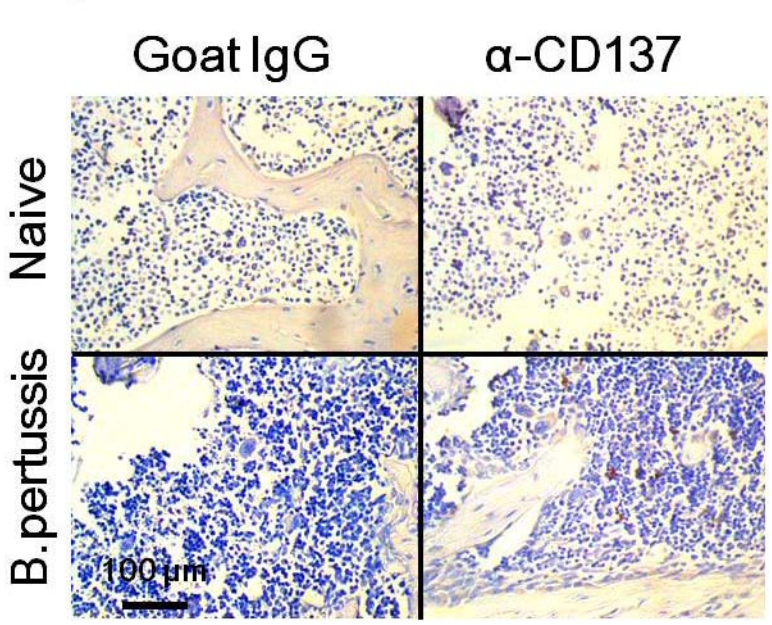


Figure 3.2.2 Increased CD137 expression in bone marrow during infections. (A) C57BL/6 mice were infected with 5 PFU of Influenza A virus (H1N1) and femur bones were harvested 4 days later. (B) C57BL/6 mice were infected with 10^6 CFU BCG and femur bones were harvested 3 weeks later.

C



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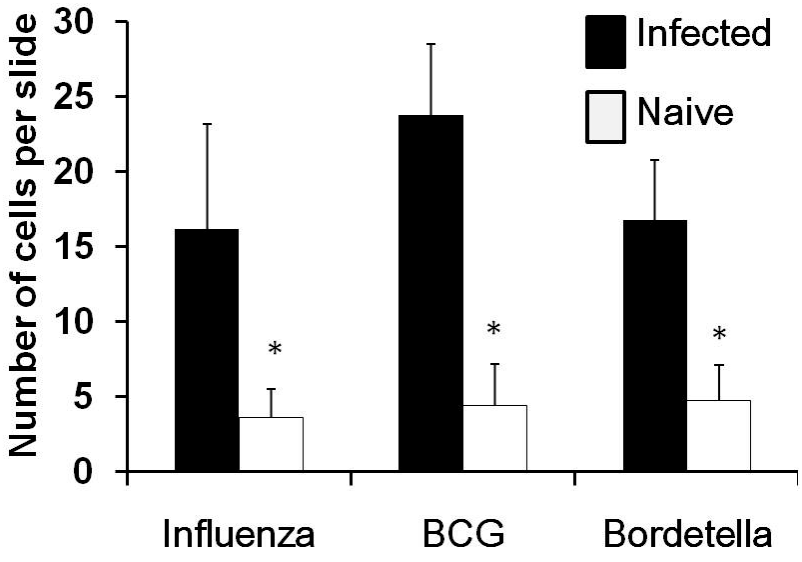


Figure 3.2.2 (C) BALB/c mice were infected with 10^6 CFU of B. pertussis and femur bones were harvested 3 days later. Femur bones were immunohistochemically stained for CD137 (brown) using a polyclonal goat anti-CD137 antibody. Goat IgG was used as an isotype control. The slides were counterstained with hematoxylin (blue). (D) Quantification of number of cells stained positive for CD137 in each slide. Magnification: 10x. Data were representative of two independent experiments.

3.2.3 CD137^{+} T cells are expanded in bone marrow during infection

Expression of CD137 can be found on a wide range of cells, including T cells, NK cells, epithelial cells and DCs. Due to the potential role of T cells in myelopoiesis in the bone marrow, it is hypothesized that T cell is the major cell population that express CD137. In order to demonstrate the wider significance of CD137 expression during infections and to characterize the CD137-expressing cells as T cells in the bone marrow, WT and CD137$^{-/-}$ mice were infected with E. coli or administered LPS, a component of the E. coli cell wall. Both bacteria and LPS induced lethargy and acute inflammation in the animals. Mice were euthanized 48 hr after administration of bacteria and LPS and bone marrow cells were harvested for flow cytometry. T cells were identified among bone marrow cells based on size, granularity and CD3 expression, and were further subcategorized into helper and cytotoxic T cells based on CD4 and CD8 expression, respectively. CD137$^{-/-}$ mice served as background control of CD137 (**Figure 3.2.3A**).

Among the bone marrow cells from naïve WT mice no CD137-expressing CD8^{+} T cells and minimal numbers of CD4^{+} T cells could be detected. In contrast, injection of LPS or infection by E. coli increased the percentages of CD137^{+}CD4^{+} T cells significantly by 8.5 fold and 10 fold, respectively (**Figure 3.2.3BC**). CD137^{+} CD8^{+} T cells also became detectable but their percentage was relatively low in

comparison to those of $CD4^+$ T cells (**Figure 3.2.3BC**). The absolute number of $CD137^+$ $CD4^+$ and $CD8^+$ T cells also increased correspondingly (**Figure 3.2.3D**).

About two thirds of the CD137-expressing cells in the bone marrow expressed CD3, identifying them as T cells (**Fig. 3.2.3 E**). These data showed that the majority of the $CD137^+$ cells which were present in the bone marrow during inflammatory conditions were T cells, predominantly $CD4^+$ T cells.

$CD3^+CD137^+$ cells were also sorted from mice with LPS injection together with $CD3^+CD137^-$ and $CD3^-CD137^+$ cells (**Fig. 3.2.3 F**). Comparison of the cell morphology revealed that $CD3^+CD137^+$ cells had a round morphology that was similar to the $CD3^+CD137^-$ cells, of which the majority was T cells. In the $CD3^-$ $CD137^+$ cells, however, cells with macrophage-like morphology were observed.

A

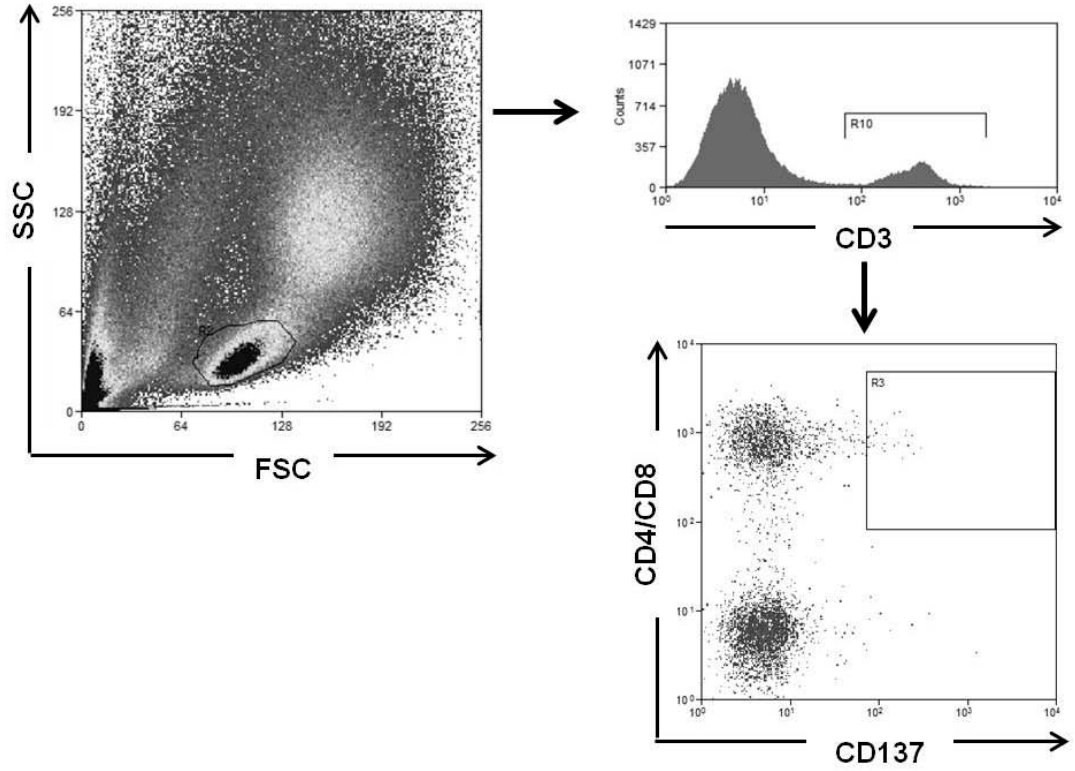


Figure 3.2.3 Identification of the CD137-expressing bone marrow cells (A) Gating strategy: Lymphocytes among total bone marrow cells were gated based on forward scatter (FSC) and side scatter (SSC). T cells were identified by CD3 expression, and were further subdivided by CD4 and CD8 staining. 100000 $CD3^+$ T cells were acquired for each sample.

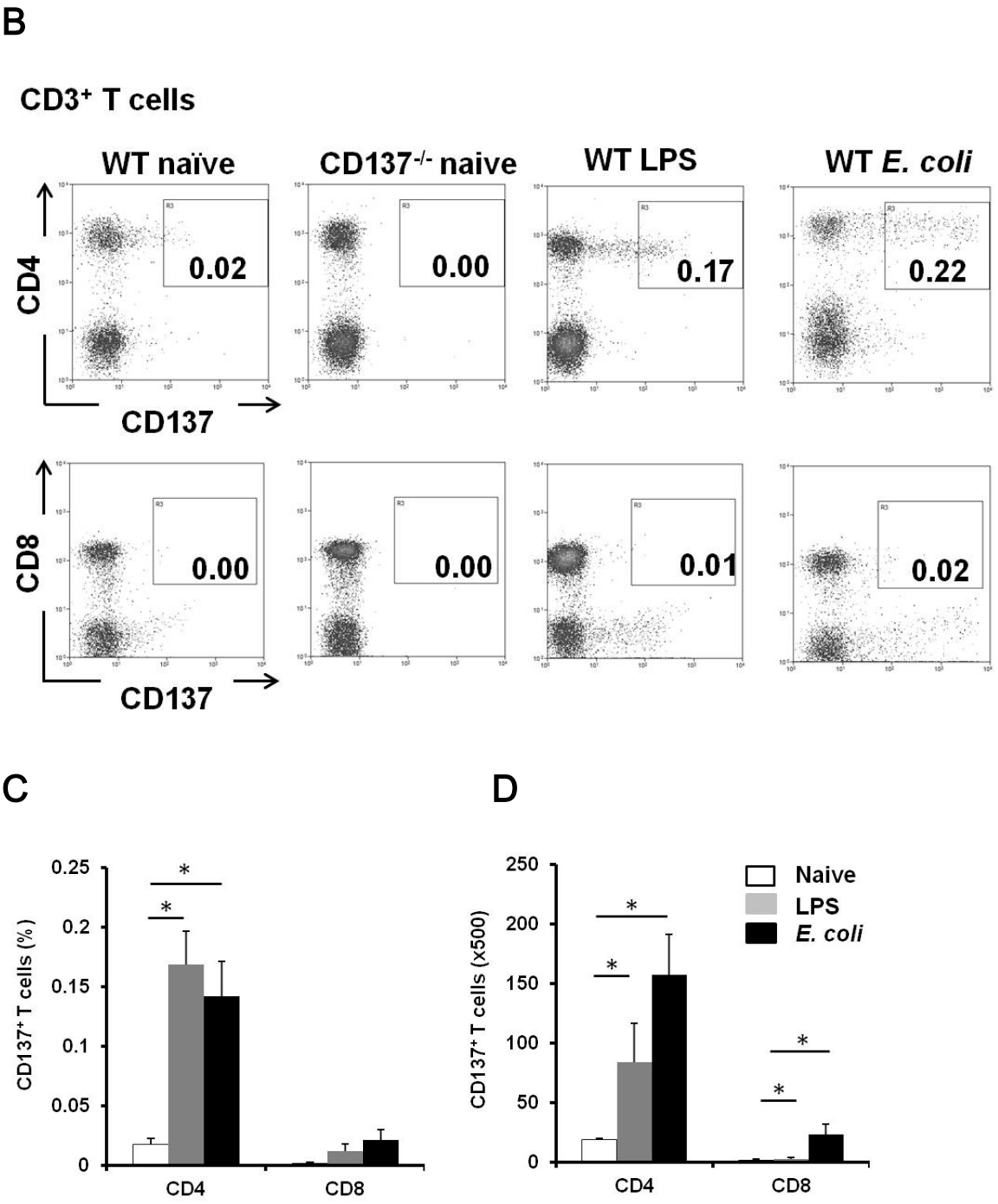
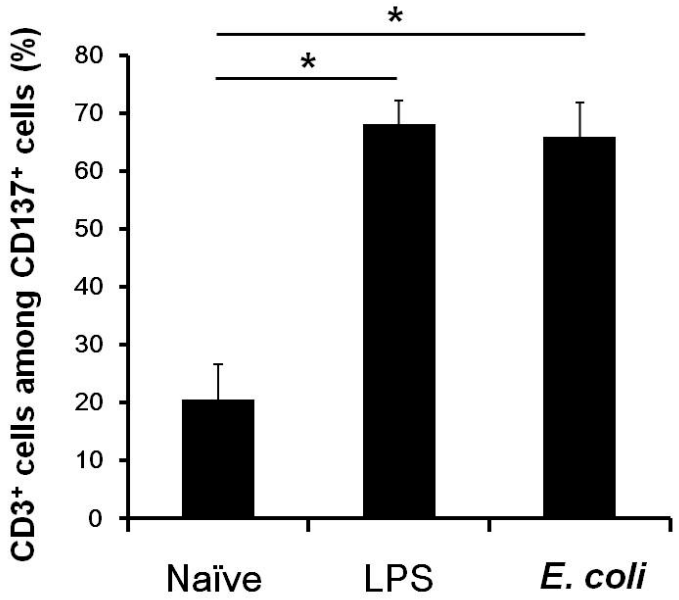


Figure 3.2.3 Identification of the CD137-expressing bone marrow cells (B) C57BL/6 WT mice were injected with 10^6 CFU E. coli or 100 µg LPS, and bone marrow cells were isolated 48 hr later. The cells were stained for CD3, CD4, CD8 and CD137. Bone marrow cells of naïve mice were used as controls. Numbers in each square represent percentages of $CD137^+$ T cells among the total bone marrow cells. (C, D) Quantification of percentages (C) and absolute numbers (D) of $CD4^+$ and $CD8^+$ T cells among $CD137^+$ T cells and $CD137^-$ T cells. Depicted are means ± SD of 3 - 4 mice. Data were representative of two independent experiments.

E



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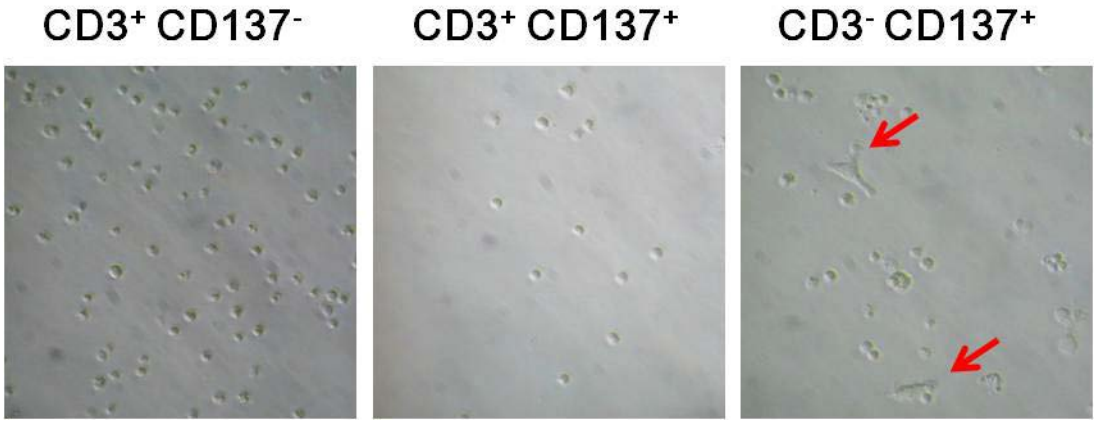


Figure 3.2.3 Identification of the CD137-expressing bone marrow cells (E) Percentage of $CD3^+$ T cells among $CD137^+$ cells in the bone marrow. (F) $CD3^+CD137^-$, $CD3^+CD137^-$, $CD3^+CD137^-$ cells were sorted from mice with LPS injection. Cells were cultured in RPMI with 10% FBS. Morphology was recorded on third day of culture. Arrow: Cells with macrophage-like morphology. Data were representative of two independent experiments.

3.2.4 In vitro activated T cells that express CD137 can home to bone marrow and other lymphoid organs

Since the majority of the CD137-expressing bone marrow cells that increased in numbers during infection are T cells, it is hypothesized that they migrate to the bone marrow from the site of infection or inflammation or from the draining lymph nodes. Therefore, the migratory ability of activated, CD137-expressing T cells from a site of inflammation to the bone marrow was investigated.

Splenic T cells were isolated from WT and $CD137^{-/-}$ mice and activated with anti-CD3 and anti-CD28 antibodies for 48 h. More than 30% of the activated WT T cells expressed CD137 (**Figure 3.2.4A**). The cells were then labeled with 5 μM CFSE and injected i.v. into WT mice. Bone marrow cells, splenocytes and draining lymph node cells were analyzed 24 hr after the injection. WT naïve mice were used as a negative control. Before injection the expression of CD137 as well as the activation markers CD44, CD62L and CD69 were examined (**Figure 3.2.4B**). These markers are known to be upregulated in activated T cells and essential for T cells migration. CD62L, also known as L-selectin, was slightly upregulated in $CD137^{-/-}$ activated T cells compared to WT (43.7% vs 31.5%). However, no difference was found in the expression levels of CD44 and CD69 between activated WT and $CD137^{-/-}$ T cells.

Migration of T cells to lymphoid organ was done by detecting $CFSE^+$ cells using flow cytometry (**Figure 3.2.4C**). Data showed that WT and $CD137^{-/-}$ activated T cells were able to migrate to the bone marrow as well as to secondary lymphoid organs such as spleen and lymph nodes. The efficiency of T cell migration was significantly higher to the spleen than to the bone marrow regardless of the origin of the T cells as the absolute cell number of $CFSE^+$ T cells in the spleen was almost 6 fold higher than that in the bone marrow for both activated WT and $CD137^{-/-}$ T cells. The number of activated T cells homing to the bone marrow was similar for the two strains both in percentages and absolute cell numbers (**Figure 3.2.4DE**). Unexpectedly, $CD137^{-/-}$ T cells had a higher efficacy of homing to the spleen than WT T cells.

A

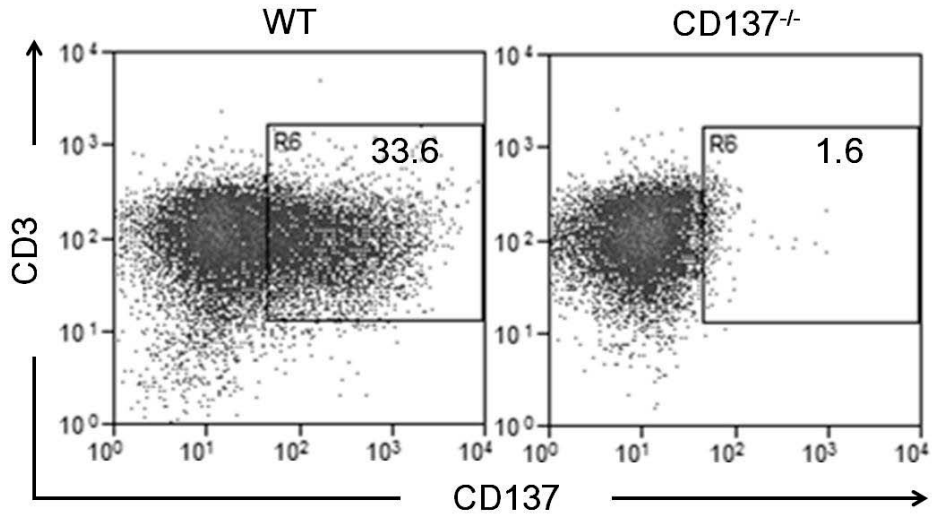
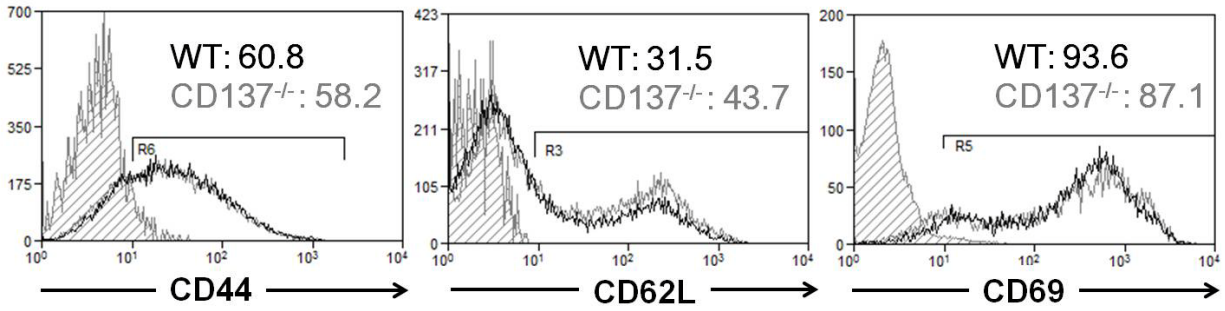


Figure 3.2.4 Migration of activated, CD137-expressing T cells to the bone marrow. Splenic T cells were isolated from WT and $CD137^{-/-}$ mice, activated by anti-CD3 and anti-CD28 antibodies for 48 hr, and labeled with CFSE. (A) Expression of CD137 on activated WT T cells. T cells from $CD137^{-/-}$ mice served as a background control.

B



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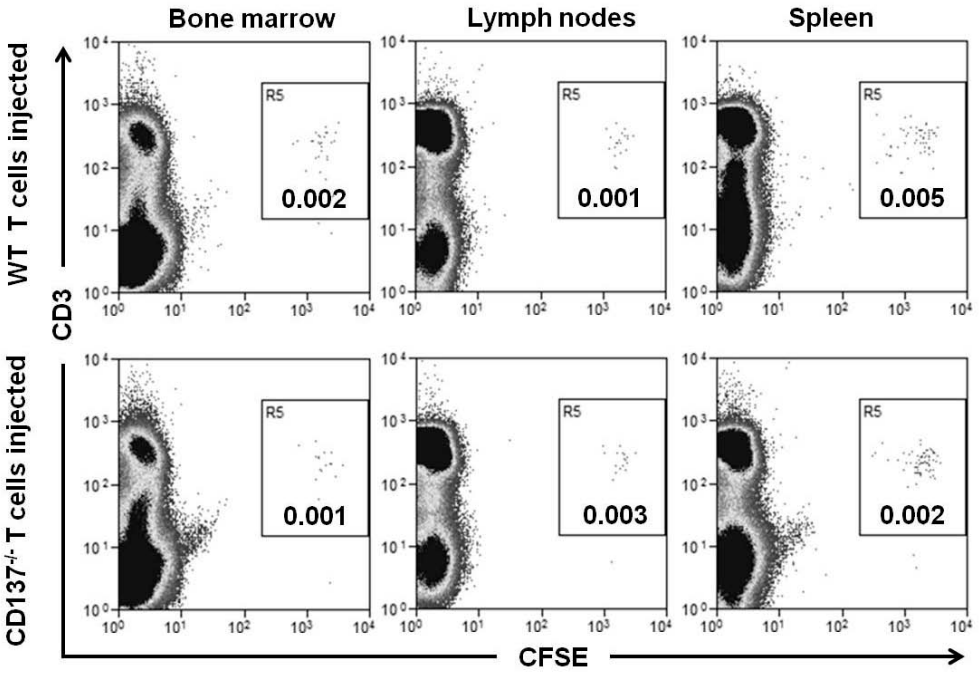


Figure 3.2.4 (B) Expression of homing markers was determined by flow cytometry. Isotype: hatched. WT: dark line. $CD137^{-/-}$: grey line. Numbers indicate percentages of positive cells relatively to isotype control. Data were representative of two independent experiments. (C) Activated WT or $CD137^{-/-}$ T cells were injected i.v. into WT mice. 24 hr later mice were euthanized and the number of $CFSE^{+}$, $CD3^{+}$ cells that migrated to the bone marrow, spleen and lymph nodes were analyzed by flow cytometry. 100000 $CD3^{+}$ T cells were acquired for each sample.Numbers in graph insets represent percentages of $CFSE^{+}$, $CD3^{+}$ cells among total lymphocytes. Each treatment group consisted of 5 mice.

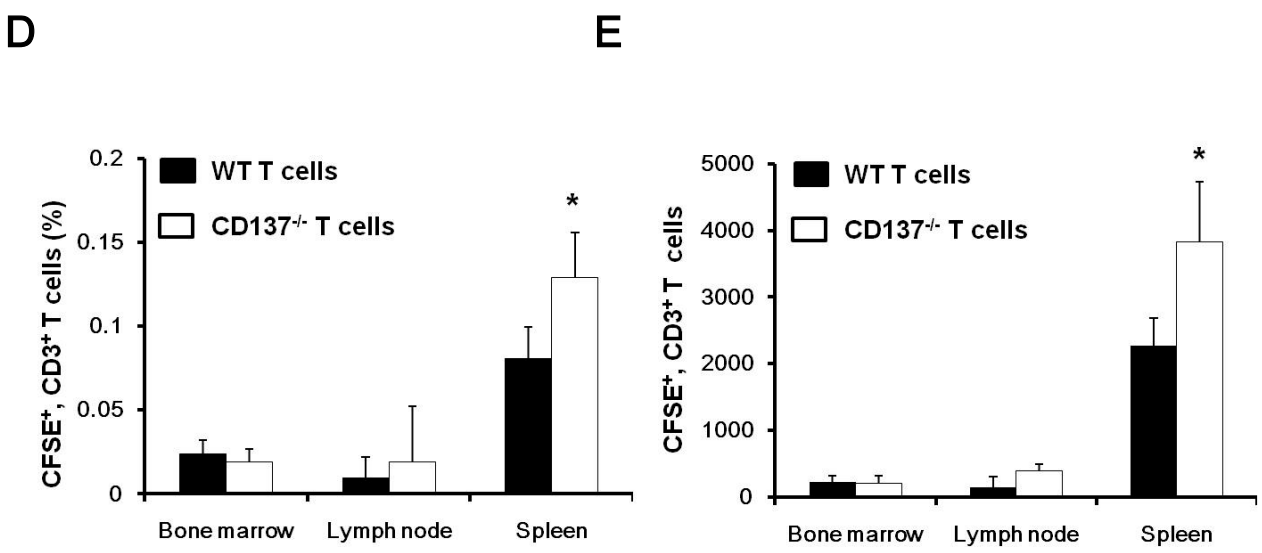


Figure 3.2.4 (D) Percentages and (E) absolute numbers of $CFSE^+$, $CD3^+$ cells that have migrated to bone marrow, lymph node and spleen. *$p<0.05$. Statistical analysis was done by two-tailed unpaired Student's *t*-test. Data were representative of three independent experiments.

3.2.5 Activated $CD4^+$ T cells induce bone marrow cells proliferation through CD137L reverse signaling

Having demonstrated that activated, $CD137^+$ T cells can migrate from peripheral tissue to the bone marrow the next aim was to test whether the $CD137^+$ T cells can contribute to myelopoiesis. The major focus was on $CD4^+$ T cells since they rather than $CD8^+$ T cells constituted the majority of $CD137^+$ T cells in the bone marrow of mice during peritonitis (**Figure 3.2.2**).

$CD4^+$ T cells were isolated from splenocytes of WT and $CD137^{-/-}$ mice by magnetic cell sorting, and were activated with 5 µg/ml of immobilized anti-CD3 and

anti-CD28 antibodies. 48 hr later WT bone marrow cells which have been labeled with CFSE were added to the activated $CD4^+$ T cells at a ratio of 2:1. After 6 days of coculture the proliferation of bone marrow cells was determined based on CFSE dilution. The CFSE MFI of bone marrow cells cocultured with WT or $CD137^{-/-}$ $CD4^+$ T cells was 267.3 and 775.7, respectively, demonstrating that WT $CD4^+$ T cells induced a much stronger proliferation of bone marrow cells than $CD137^{-/-}$ $CD4^+$ T cells (**Figure 3.2.5 AB**). This increased proliferation was accompanied by an increase in the percentage of $CD11b^+$ bone marrow cells (92.8 ± 1.8% versus 67.8 ± 16. 7%) in the cocultures with WT $CD4^+$ T cells (**Figure 3.2.5 CD**). These data indicated that CD137 expressed on $CD4^+$ T cells induced bone marrow cell proliferation and enhanced myeloid differentiation.

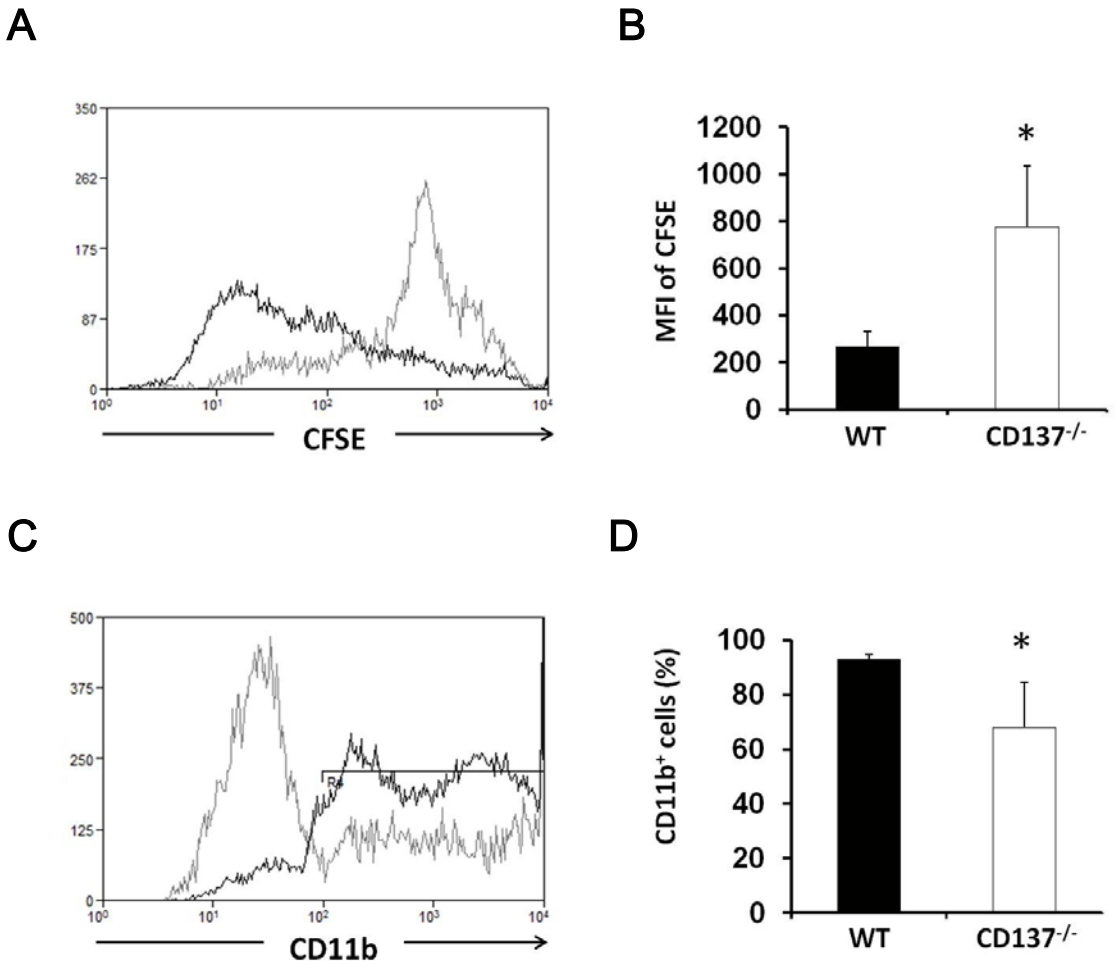


Figure 3.2.5 CD137-experssing CD4$^+$ T cells promote myelopoiesis in vitro. CD137-expressing CD4$^+$ T cells promote myelopoiesis in vitro. WT and CD137$^{-/-}$ CD4$^+$ T cells were isolated from spleens and were activated for 48 h with 5 µg/ml α-CD3/CD28 antibodies. WT bone marrow cells were isolated and labeled with CFSE, and cocultured with activated CD4$^+$ T cells at a ratio of 1:2. After 6 days of coculture, cells were harvested and CD11b expression was quantified by flow cytometry. Equal numbers of CFSE$^+$ cells were acquired for each sample. (A) CFSE dilution of bone marrow cells. The dark and grey lines represent the dilution of CFSE of bone marrow cells cocultured with WT or CD137$^{-/-}$ CD4$^+$ T cells, respectively. (B) Quantification of (A) from quadruplicate measurements. Depicted are means of mean fluorescent intensities (MFI) ± SD. (C) Expression of CD11b on CFSE$^+$ bone marrow cells. The dark and grey lines represent CD11b staining of bone marrow cells cocultured with WT or CD137$^{-/-}$ CD4$^+$ T cells, respectively. (D) Quantification of (C) from quadruplicate measurements. Depicted are means of percentages of CD11b$^+$ cells ± SD. Data were representative of two independent experiments.

3.2.6 Activated $CD4^+$ T cells induce Lin^- progenitor cell proliferation through CD137L reverse signaling

Bone marrow cells include both Lin^- progenitor cells and monocytes, both of which have been demonstrated to proliferate and differentiate in response to recombinant CD137 stimulation. Lin^- progenitor cells are of particular interest because of their ability to give rise to downstream myeloid cells.

To examine the response of Lin^-progenitor cells to T cell stimulation with or without the interaction of CD137 and CD137L, Lin^- progenitor cells were isolated from bone marrow cells of WT and $CD137L^{-/-}$ mice by negative selection. The cells were CFSE labeled before being cocultured with activated WT and $CD137^{-/-}$ T cells. Cells were stained for CD4, CD11b and Gr-1. Progenitor cells and activated T cells were separated by CD4 expression and CFSE intensity (**Figure 3.2.5 A**)

Similar to the results observed using total bone marrow cells, when CD137L was absent from the progenitor cells, cells displayed reduced proliferation as shown by MFI of CFSE dilution (328 vs 476) (**Figure 3.2.5 B**). On the other hand WT T cells induced a higher degree of cell proliferation in progenitor cells than $CD137^{-/-}$ T cells (328 vs 413) (**Figure 3.2.5 C**), suggesting that the presence of CD137 on the

stimulator cells or the presence of ligand on the responder cell are essential for CD137L-mediated myelopoiesis.

Costaining of CD11b or Gr-1 together with CFSE labeling of the progenitor showed that although both WT and CD137L$^{-/-}$ progenitor could proliferate when cocultured with activated T cells, CD137L reverse signaling clearly favored myeloid differentiation as more proliferating WT progenitor cells belonged to the CD11b and Gr-1 compartment compared to CD137L$^{-/-}$ progenitor cells (**Figure 3.2.5 EF**). CD137L$^{-/-}$ progenitor cells cocultured with CD137$^{-/-}$ T cells had the lowest level of myeloid differentiation of all groups and only one third of the CD137L$^{-/-}$ progenitor cells cocultured with WT T cell expressed CD11b (18.0% vs 46.0%, **Figure 3.2.5 F**).

In addition to surface bound CD137, myeloid differentiating factors such as GM-CSF, M-CSF and G-CSF may synergize to reinforce myelopoiesis. The levels of these three cytokines in the supernatants of the coculture were tested. Surprisingly, cocultures with CD137$^{-/-}$ activated T cells showed a significantly higher amount of GM-CSF and G-CSF than cocultures with WT activated T cells in spite of the reduced myelopoiesis. M-CSF was not detectable in all coculture groups (**Figure 3.2.5 G**)

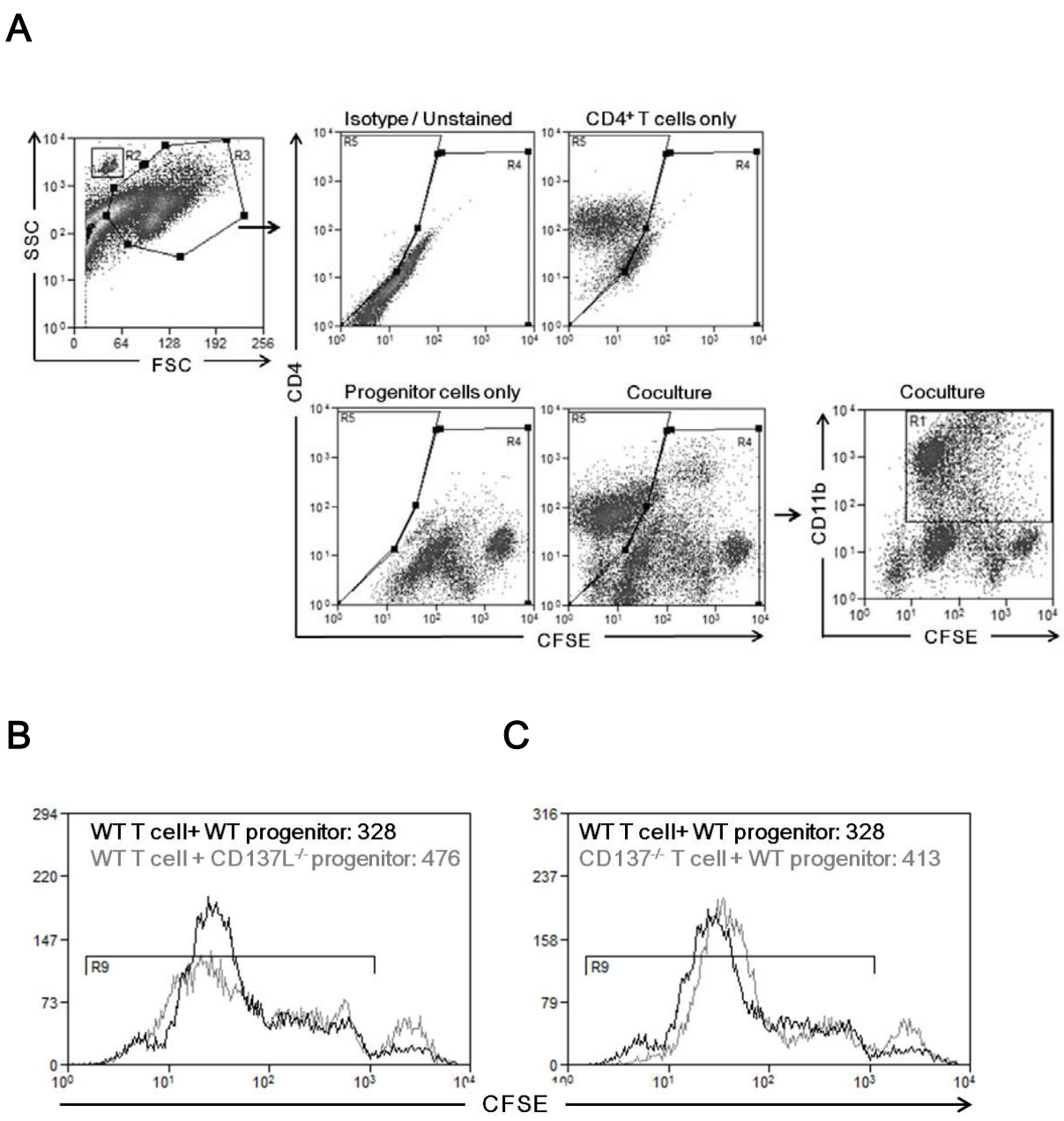
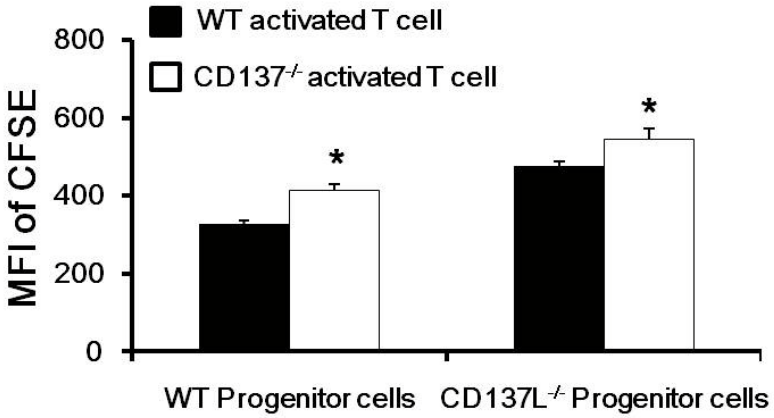


Figure 3.2.6 CD137-experssing CD4$^+$ T cells promote myeloid lineage proliferation and differentiation of Lin$^-$ progenitor cells in vitro. Lin$^-$ progenitor cells were isolated from WT and CD137L$^{-/-}$ mice, labeled with CFSE, and were cocultured with activated WT or CD137$^{-/-}$ T cells. On day 6, cells were harvested and stained for CD4, CD11b and Gr-1. Equal numbers of CFSE$^+$ cells were acquired for each sample. (A) Cells were first gated on forward and side scatter. CD4$^+$ T cells were gated out based on low CFSE fluorescence and high CD4 expression. Progenitor cells were gated based on high CFSE intensity. (B,C) Proliferation of WT (dark lines) or CD137L$^{-/-}$ (grey lines) progenitor cells cocultured with WT or CD137$^{-/-}$ activated T cells. Numbers in the histograms represent MFI of the respective populations.

D



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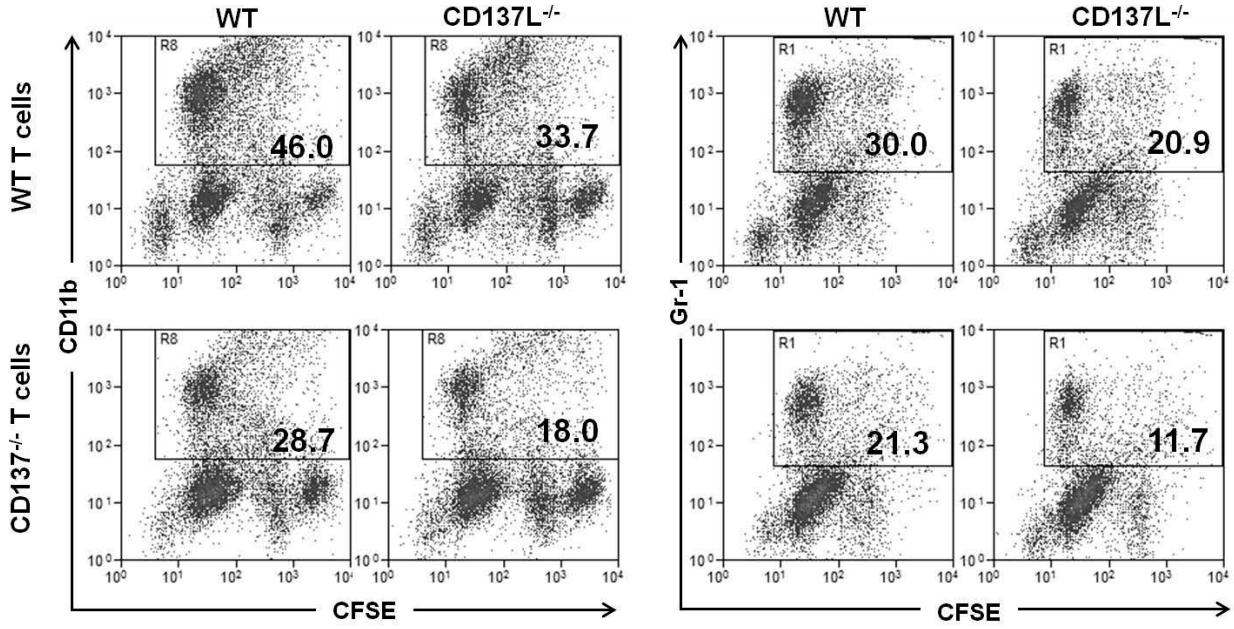
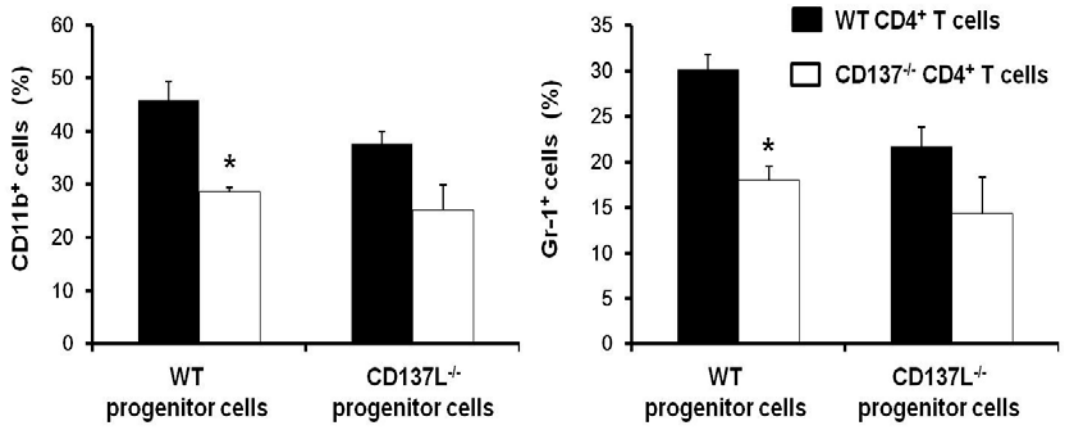


Figure 3.2.6 (D) Means ± SD of 5 repeats of (B and C). (E) Expression of CD11b and Gr-1 on CFSE-labeled WT or CD137L$^{-/-}$ progenitor cells that had been cocultured with WT or CD137$^{-/-}$ activated CD4^{+} T cells. Numbers represent percentages of CD11b^{+} or Gr-1^{+} cells among total CFSE^{+} progenitor cells.

F



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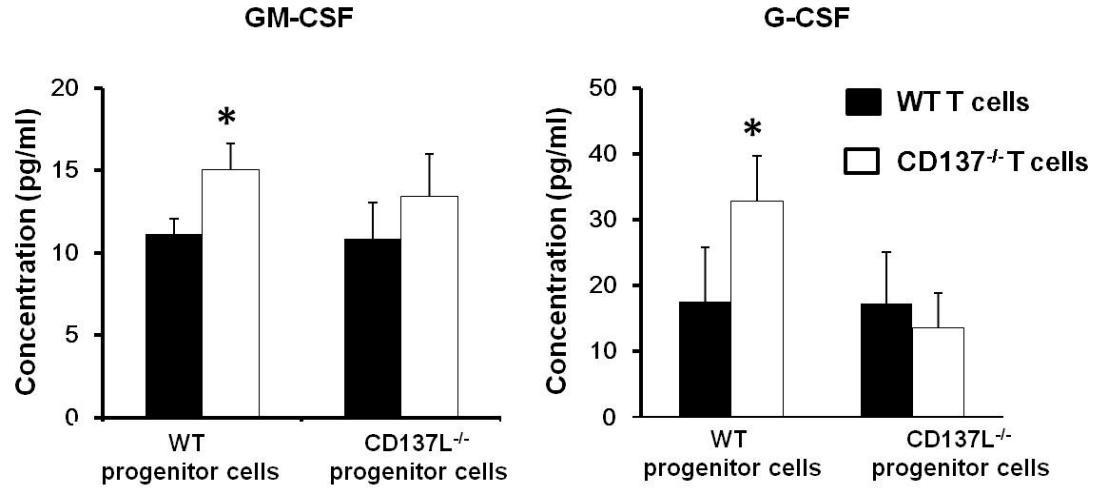


Figure 3.2.6 (F) Quantification of percentages of $CD11b^+$ and $Gr\text{-}1^+$ cells from (E). (G) GM-CSF and G-CSF concentrations in supernatants of cocultures. Depicted are means ± SD of 5 measurements. * $p<0.05$. Statistical analysis was done by two-tailed unpaired Student's *t*-test. Data are representative of two independent experiments.

3.2.7 Activated WT and $CD137^{-/-}$ T cells do not differ in GM-CSF production

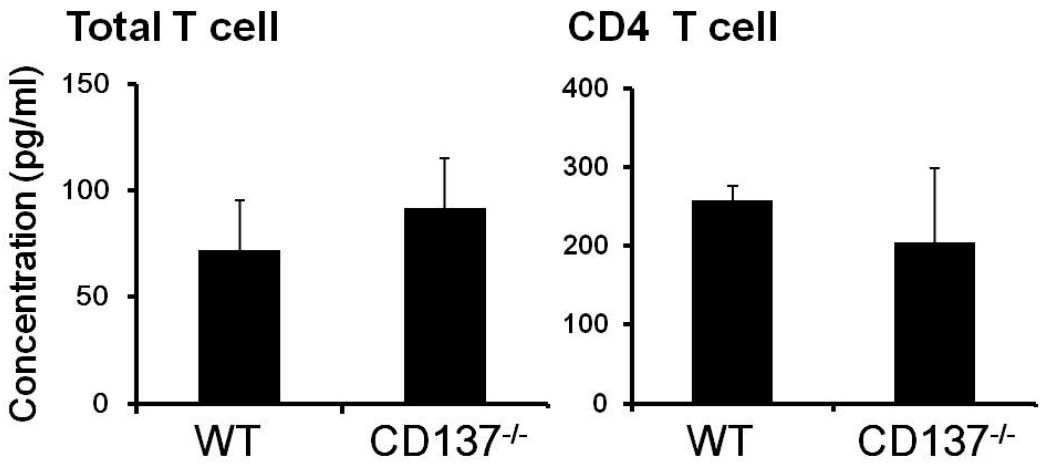
It is surprising that $CD137^{-/-}$ T cells actually secrete more GM-CSF and G-CSF into the supernatant than WT T cells when cocultured with progenitor cells. The

differences can be due to two causes: 1. the hyperproliferation of $CD137^{-/-}$ T cells; and/or 2. the absence of CD137 on T cells upregulates of GM-CSF. To investigate whether CD137 inhibits GM-CSF production in T cell, supernatants of WT and $CD137^{-/-}$ T cells were tested for GM-CSF levels. GM-CSF production by either total T cells or $CD4^{+}$ T cell after activation by anti-CD3 and anti-CD28 antibodies was not affected by the presence of CD137 (**Figure 3.2.7 A**). M-CSF was not detectable in all conditions.

Although expression of CD137 in vitro does not give an advantage to the T cells in regards to GM-CSF production, it may enhance the levels of GM-CSF in vivo. $CD3^{+}CD137^{-}$ and $CD3^{+}CD137^{+}$ T cells sorted from mice injected with LPS were cultured for 3 days before harvest of the supernatants. There was no significant difference between the two T cell populations in GM-CSF production, suggesting that in vivo CD137 did not enhance levels of GM-CSF(**Figure 3.2.7 B**).

The data suggested that CD137 did not influence the production of GM-CSF by T cells. The difference observed in the supernatants of cocultures with progenitor cells was probably a result of hyperproliferating $CD137^{-/-}$ T cells.

A



B

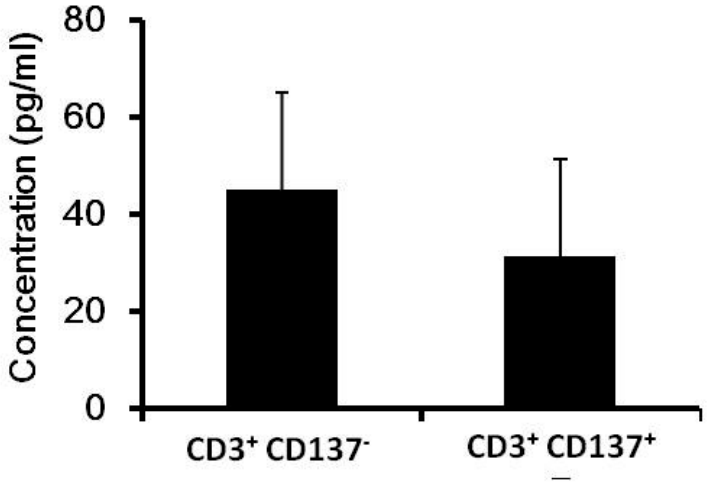


Figure 3.2.7 Expression of CD137 does not affect levels of GM-CSF expression. (A) Levels of GM-CSF were measured in activated WT and CD137$^{-/-}$ total or CD4^{+} T cells by ELISA. Each sample was done in triplicates. (B) Levels of GM-CSF were measured in supernatants of sorted cells from WT mice after receiving LPS injection. Data were representative of two independent experiments.

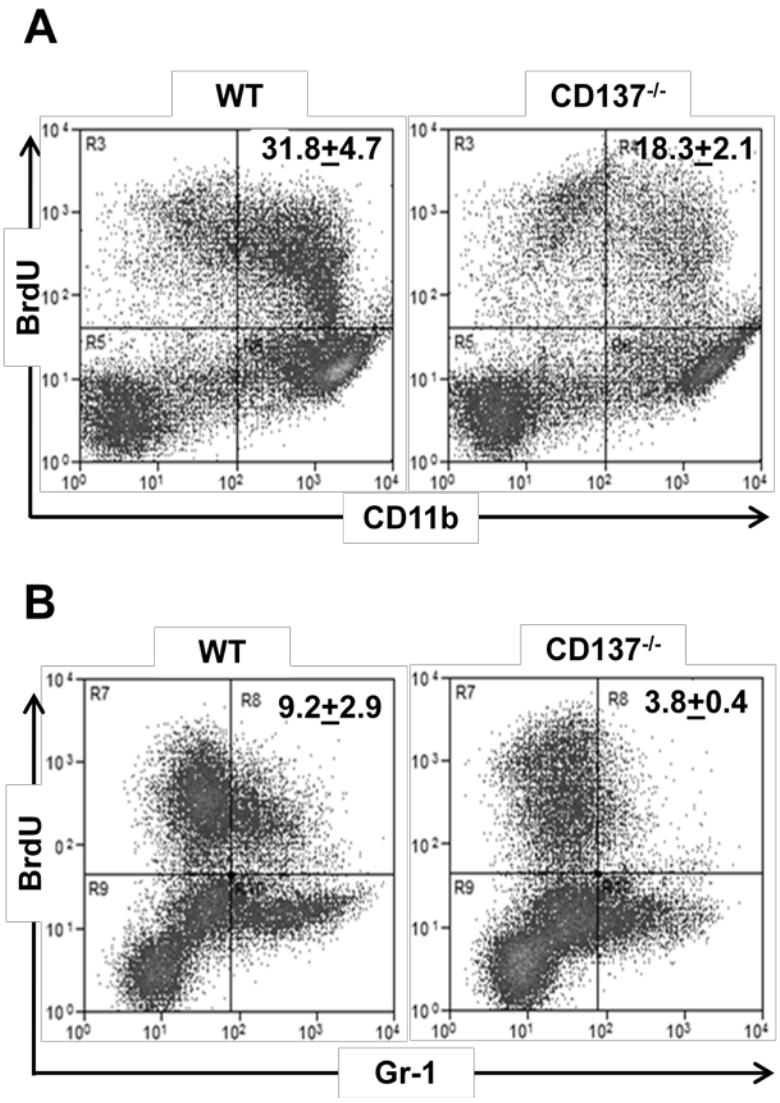
3.2.8 CD137L reverse signaling enhances primary myelopoiesis during acute peritonitis

So far it has been shown that in vitro $CD137^+$ $CD4^+$ T cells can directly induce myelopoiesis in Lin^- progenitor cells. However, the effect has yet to be demonstrated in vivo. A model of acute peritonitis using E.coli was employed in order to determine whether under inflammatory situation CD137L reverse signaling can induce myelopoiesis in vivo too. To ensure that any increase in myeloid cell number was due to local proliferation but not trafficking of cells in and out of bone marrow compartment, the newly emerging cells were labeled by BrdU. 10^6 CFU E. coli were injected i.p. into WT and $CD137^{-/-}$ mice. After 48 hr when acute peritonitis had developed the mice were sacrificed but 6 hr prior to euthanization 1 mg BrdU per mouse was injected.

As in the case of infection with Influenza, B. pertussis and BCG (**Figure. 3.2.1 ABC**) infection with E. coli also led to an increase in the number of CD137-expressing cells in the bone marrow (**Figure 3.2.2 B**). The percentages of proliferating (i.e. $BrdU^+$) $CD11b^+$ /$Gr\text{-}1^+$ cells (**Figure 3.2.8 ABC**) were significantly enhanced in the bone marrow of WT compared to $CD137^{-/-}$ mice. Also the absolute numbers of proliferating $CD11b^+$ and $Gr\text{-}1^+$ cells in the bone marrow were significantly higher in the WT than in the $CD137^{-/-}$ mice (**Figure 3.2.8 D**). This increased proliferation of myeloid cells resulted in an increased percentage (**Figure**

3.2.8 E) and total number (**Figure 3.2.8 F**) of $CD11b^+$ and $Gr\text{-}1^+$ cells in the bone marrow of WT mice.

Extramedullary myelopoiesis in the spleen was also examined. Both the percentage and the number of proliferating $CD11b^+$ /$Gr\text{-}1^+$ cells in the spleen were much lower than that of bone marrow. The absence of CD137 did not influence extramedullary myelopoiesis as no difference was detected between WT and $CD137^{-/-}$ mice with acute peritonitis (**Figure 3.2.8 G-L**)


A
WT
CD137-/-
31.8±4.7
18.3±2.1
R3
R4
R5
R6
BrdU
CD11b
B
WT
CD137-/-
9.2±2.9
3.8±0.4
R7
R8
R9
R10
BrdU
Gr-1

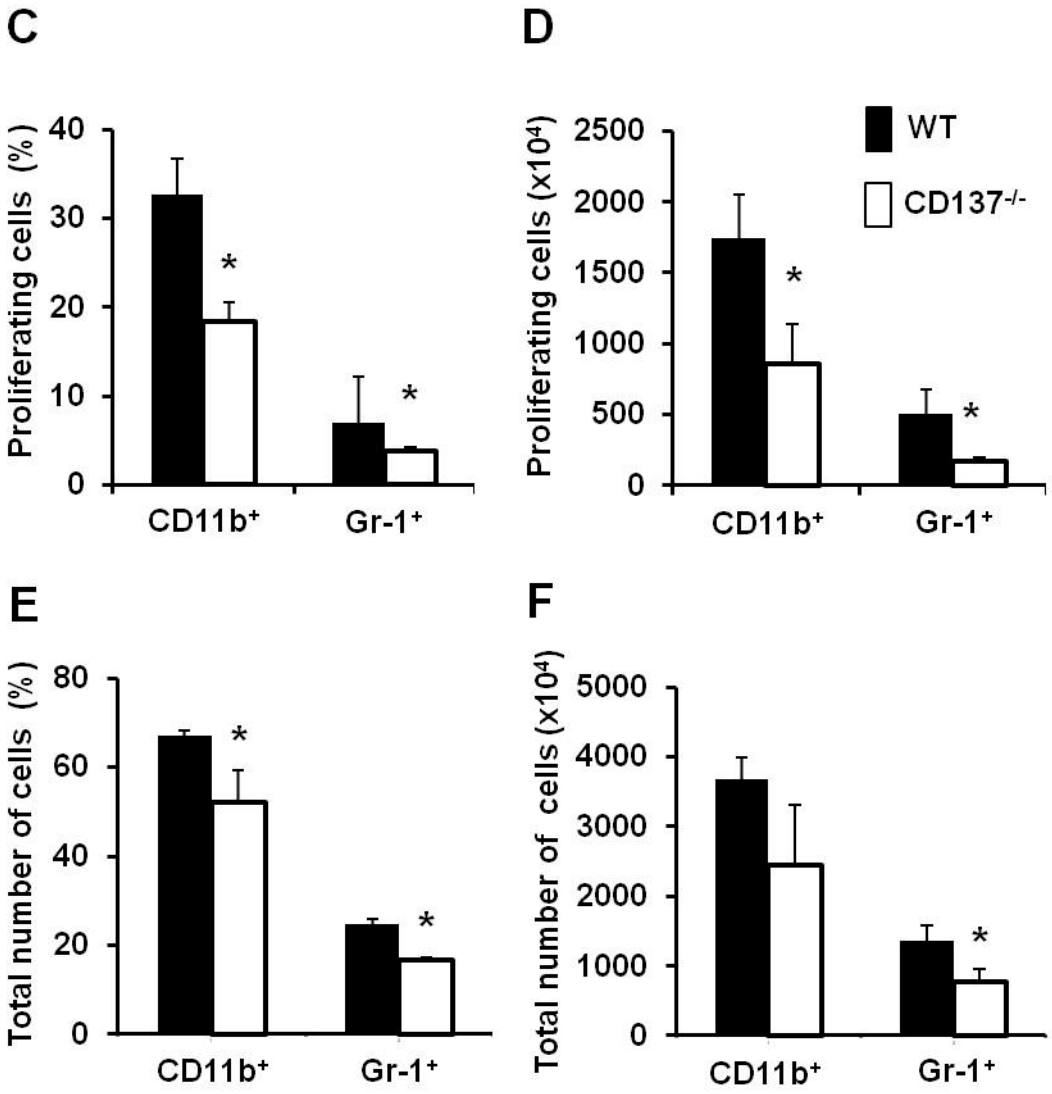
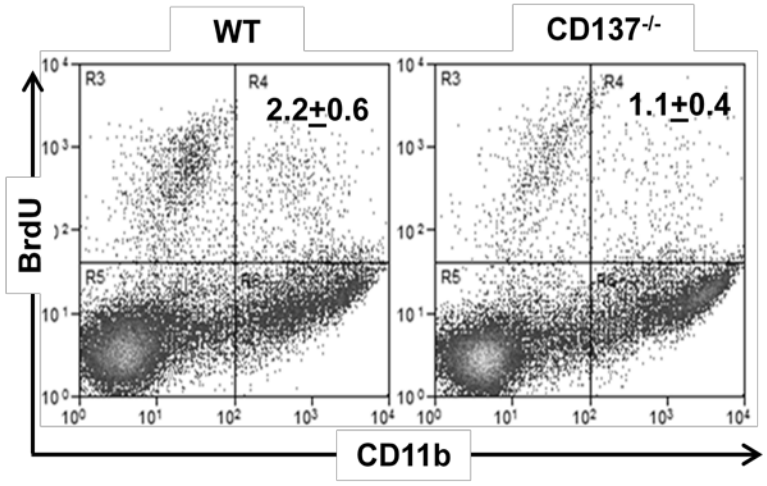
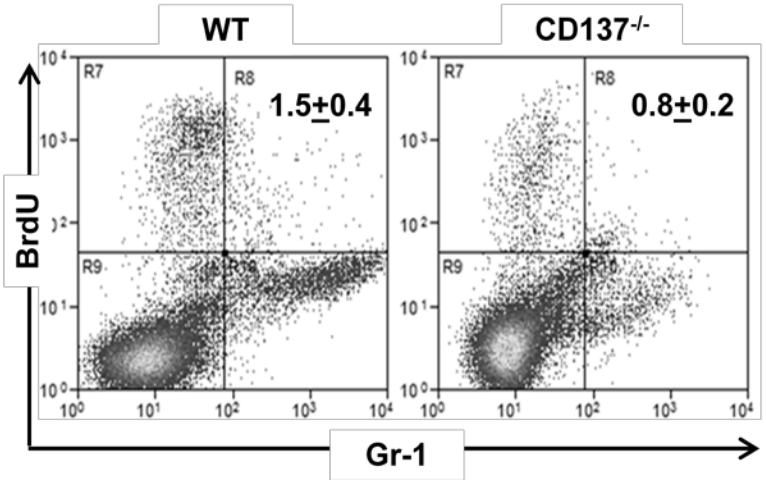


Figure 3.2.8 CD137L reverse signaling enhances primary myelopoiesis during acute peritonitis. C57BL/6 WT and CD137$^{-/-}$ mice were injected i.p. with 10^6 CFU E. coli. Mice were sacrificed after 48 hr. 6 hr prior to the euthanization, 1 mg BrdU was injected i.p. per animal to label proliferating bone marrow cells. Equal numbers of bone marrow cells were acquired for each sample. (A-F) Bone marrow cells were stained for CD11b and BrdU (A), or for Gr-1 and BrdU (B) and analyzed by flow cytometry. Counting beads (Invitrogen) were added to determine absolute cell numbers. Three mice per strain were analyzed. Percentages (C) and absolute numbers (D) of proliferating CD11b$^+$, BrdU$^+$ and Gr-1$^+$, BrdU$^+$ cells in the bone marrow. Percentages (E) and absolute numbers (F) of total CD11b$^+$ and Gr-1$^+$ cells in the bone marrow

G


WT
CD137-/-
R3
R4
2.2±0.6
R5
R6
1.1±0.4
BrdU
CD11b

H


WT
CD137-/-
R7
R8
1.5±0.4
R9
R10
0.8±0.2
BrdU
Gr-1

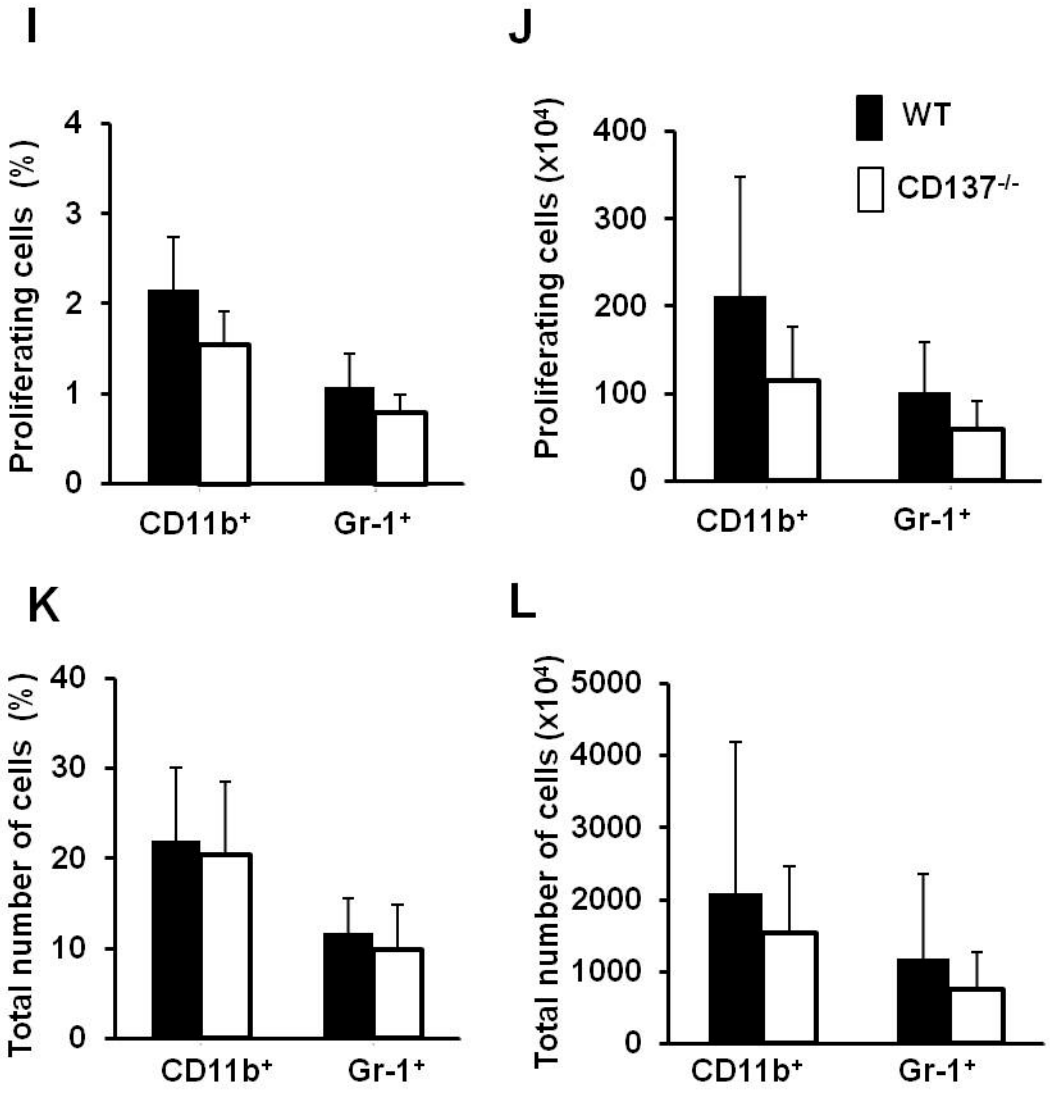


Figure 3.2.8 (G-L) Splenocytes were stained for CD11b and BrdU (G), or for Gr-1 and BrdU (H). Percentages (I) and absolute numbers (J) of proliferating $CD11b^+$, $BrdU^+$ and $Gr\text{-}1^+$, $BrdU^+$ cells in the spleen. Equal numbers of splenocytes were acquired for each sample. Percentages (K) and absolute numbers (L) of total $CD11b^+$, $BrdU^+$ and $Gr\text{-}1^+$, $BrdU^+$ cells in the spleen. Data were representative of three independent experiments. * $p<0.05$.

3.3 CD137L reverse signaling maintains myelopoiesis during aging

Having demonstrated that CD137L reverse signaling is essential for myelopoiesis during acute inflammation, the next question asked is whether CD137L plays a role in promoting myelopoiesis during chronic inflammation, for example, aging. Hematopoiesis is strongly skewed towards myelopoiesis during aging while lymphopoiesis is much reduced. Identifying CD137L as a contributing factor in immuneaging will help to devise novel therapeutic tool to intervene with age-related hematopoietic disease.

3.3.1 Numbers of myeloid cells are increased in WT during aging, but not in CD137$^{-/-}$ and CD137L$^{-/-}$ mice

Two major proliferating myeloid populations in the bone marrow that are steadily derived from progenitor cells are granulocytes and monocytes. To better determine the level of myelopoiesis, granulocytes and monocytes were categorized by the differential expression of Ly6G and Ly6C: granulocytes as CD11b^{+}Ly6G^{+}Ly6C^{+} and monocytes as CD11b^{+}Ly6G^{-}Ly6C^{+}. Gender-matched 3-month and 12-month WT, CD137$^{-/-}$ and CD137L$^{-/-}$ mice were sacrificed and bone marrow cells were stained for CD11b, Ly6G and Ly6C. Granulocytes and monocytes were grouped based on the expression of Ly6G and Ly6C (**Figure 3.3.1A**).

Percentages of granulocytes and monocytes in 3-month mice did not differ significantly among the 3 strains. As the animals aged, percentages of granulocytes of 12-month WT mice were significantly increased compared to 3-month mice from 27.3% to 34.7%, corresponding to previous studies that aging led to an increase of myeloid cells (Linton and Dorshkind 2004, Gruver et al. 2007)(**Figure 3.3.1B**). However, the percentages of granulocytes of 12-month CD137$^{-/-}$ and CD137L$^{-/-}$ mice remained unchanged. On the other hand percentages of monocytes were not affected by aging in any of the three strains (**Figure 3.3.1B**).

Change in percentages could be caused by altered frequencies of other cell populations such as B cells in the bone marrow. To better determine the change of myeloid populations, absolute cell numbers of granulocytes and monocytes in the bone marrow were calculated. Similar to the change of percentages, a significant increase of 1.5 fold in the number of granulocytes was detected in 12-month WT mice compared to the 3-month control. However, the numbers of granulocytes again remained unchanged in both 12-month CD137$^{-/-}$ and CD137L$^{-/-}$ mice. While the percentage of monocytes remained similar in the aged animals across the 3 strains, a significant increase was observed in the absolute cell number of monocytes where WT mice have almost twice the number of cells as the animal age while CD137$^{-/-}$ and CD137L$^{-/-}$ mice have unchanged numbers of cells (**Figure 3.3.1C**).

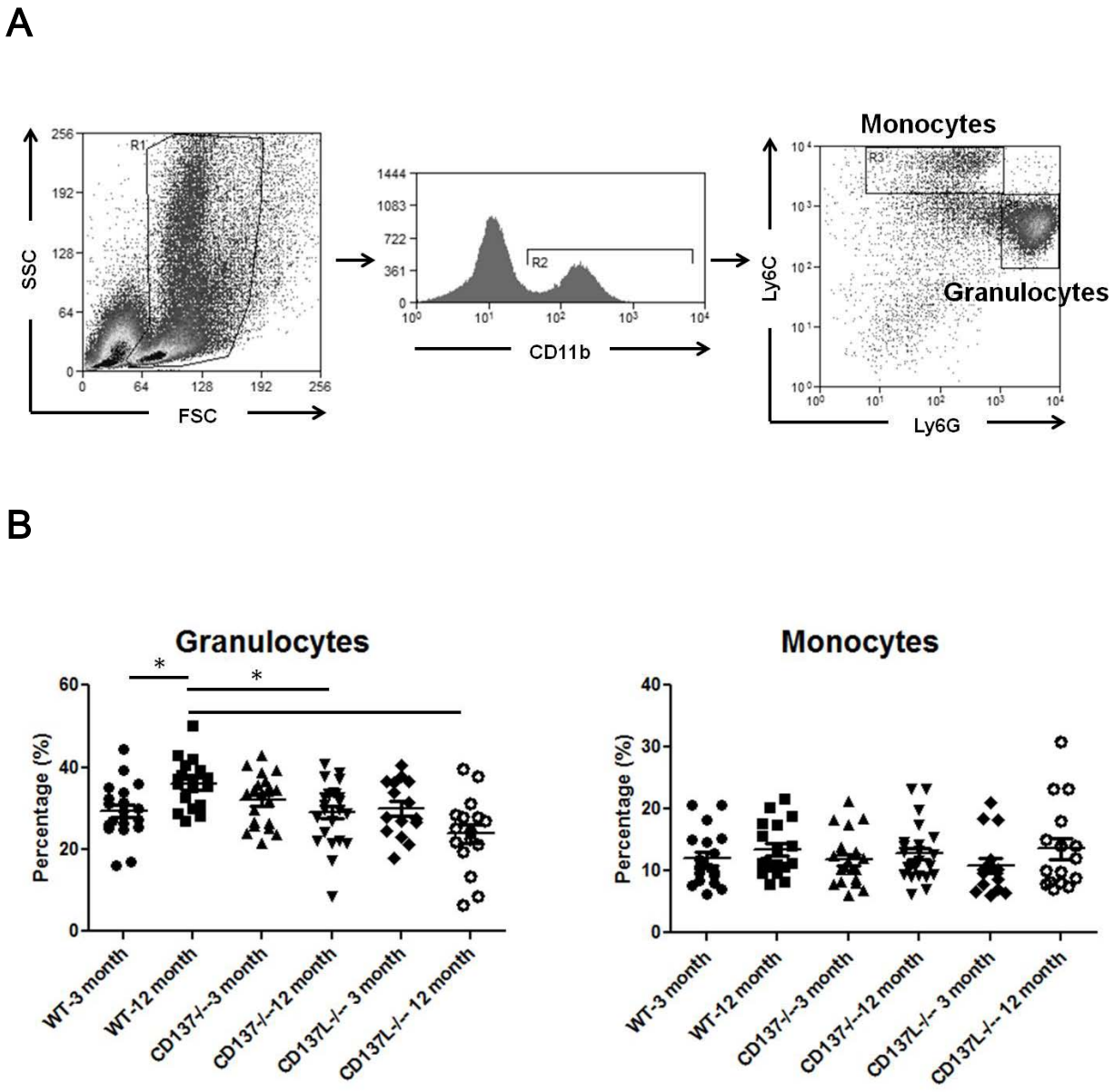


Figure 3.3.1 Aged CD137$^{-/-}$ and CD137L$^{-/-}$ mice have reduced myelopoiesis in the bone marrow compared to WT. 3-month and 12-month WT, CD137$^{-/-}$ and CD137L$^{-/-}$ mice were euthanized and bone marrow cells were stained for CD11b, Ly6G and Ly6C. Equal numbers of bone marrow cells were acquired for each sample. (A) Gating strategy of granulocytes (CD11b^{+}Ly6G^{+}Ly6C^{+}) and monocytes (CD11b^{+}Ly6G^{-}Ly6C^{+}) in the bone marrow. (B). Percentages of granulocytes and monocytes in the bone marrow.

C

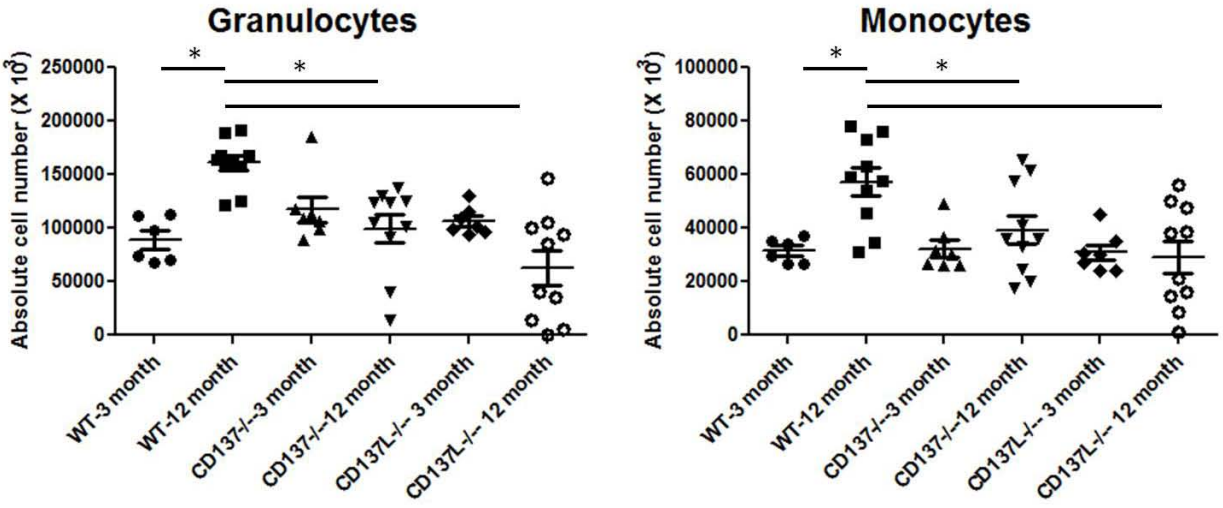


Figure 3.3.1 (C) Absolute cell numbers of granulocytes and monocytes in the bone marrow. Each group consisted of at least 5 mice. Data were representative of two independent experiments. *p<0.05.

3.3.2 $CD4^+$ T cells are increased in bone marrow of aged mice.

Compared to acute peritonitis where mice experience a rapid induction of inflammation, aging is a chronic inflammatory process induced by environmental factors (Wang and Wagers 2011, Woolthuis et al. 2011). It is wondered whether during this relatively slow inflammatory process $CD137^+$ T cells will be also increased in the bone marrow similarly as during infection.

Bone marrow cells were isolated from 3-month and 12-month WT, $CD137^{-/-}$ and $CD137L^{-/-}$ mice and stained for CD3, CD4, CD8 and CD137. $CD137^{-/-}$ mice

served as a negative control for the background staining of CD137 (**Figure 3.3.2ABC**).

As shown previously, there were very few $CD137^+$ T cells in the bone marrows at the steady state in young mice. As the WT mice aged, surprisingly, there was no significant increase of $CD137^+$ T cells either in absolute cell numbers for both $CD4^+$ and $CD8^+$ T cells in WT mice. However, a significant increase in the absolute cell number of $CD137^+$ $CD4^+$ T cells was noted in 12-month $CD137L^{-/-}$ mice in spite of the reduced myelopoiesis. The number of $CD137^+$ $CD4^+$ T cells was 6 times higher in 12-months $CD137L^{-/-}$ mice than in WT mice and in their younger controls (**Figure 3.3.2B**).

It was also wondered whether the $CD137^-$ T cells populations were altered during aging because a general increase of polyfunctional memory T cells have been reported to accumulate in human bone marrow (Herndler-Brandstetter et al. 2011, Herndler-Brandstetter et al. 2012). A general increase in $CD137^-$ $CD4^+$ T cells was observed in all three strains of mice with the number of cells doubling over 12 months. However the absence of either CD137 or CD137L did not affect the number of $CD137^-$ $CD4^+$ T cells (**Figure 3.3.2F**)..

No significant change was observed in $CD8^+$ T cells, regardless of expression of CD137 (**Figure 3.3.2G**).

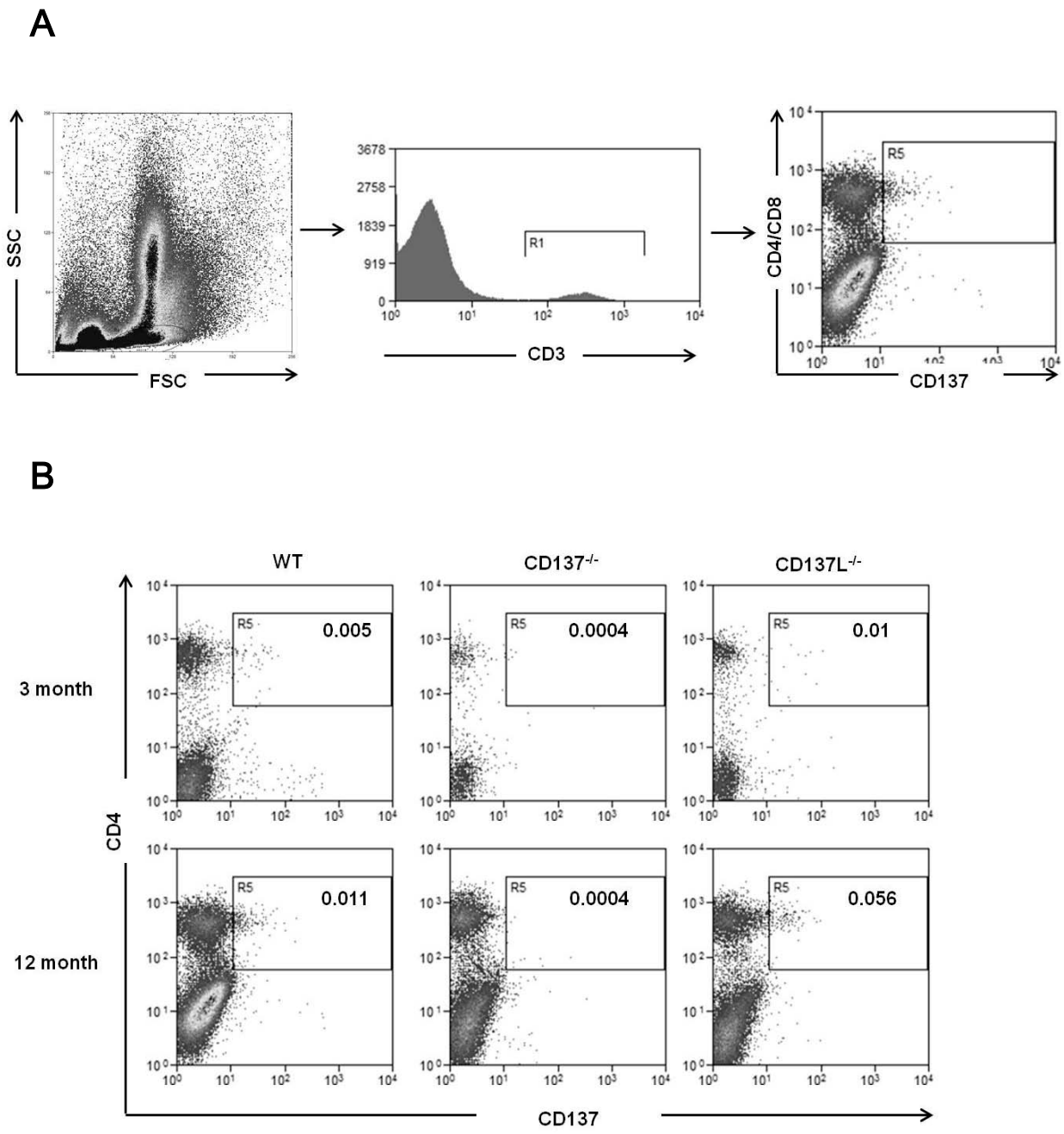


Figure 3.3.2 Increased number of $CD4^+$ T cells in aged mice. Bone marrow cells were isolated from 3-months and 12-months old WT, $CD137^{-/-}$ and $CD137L^{-/-}$ mice and stained for CD3, CD137, CD4 and CD8. Absolute number of cells were determined by addition of CountBright beads (Invitrogen). (A) Gating strategy of $CD137^+$ T cells. (B) Illustration of $CD137^+$ $CD4^+$ T cells in 3-month and 12-month WT, $CD137^{-/-}$ and $CD137L^{-/-}$ mice.

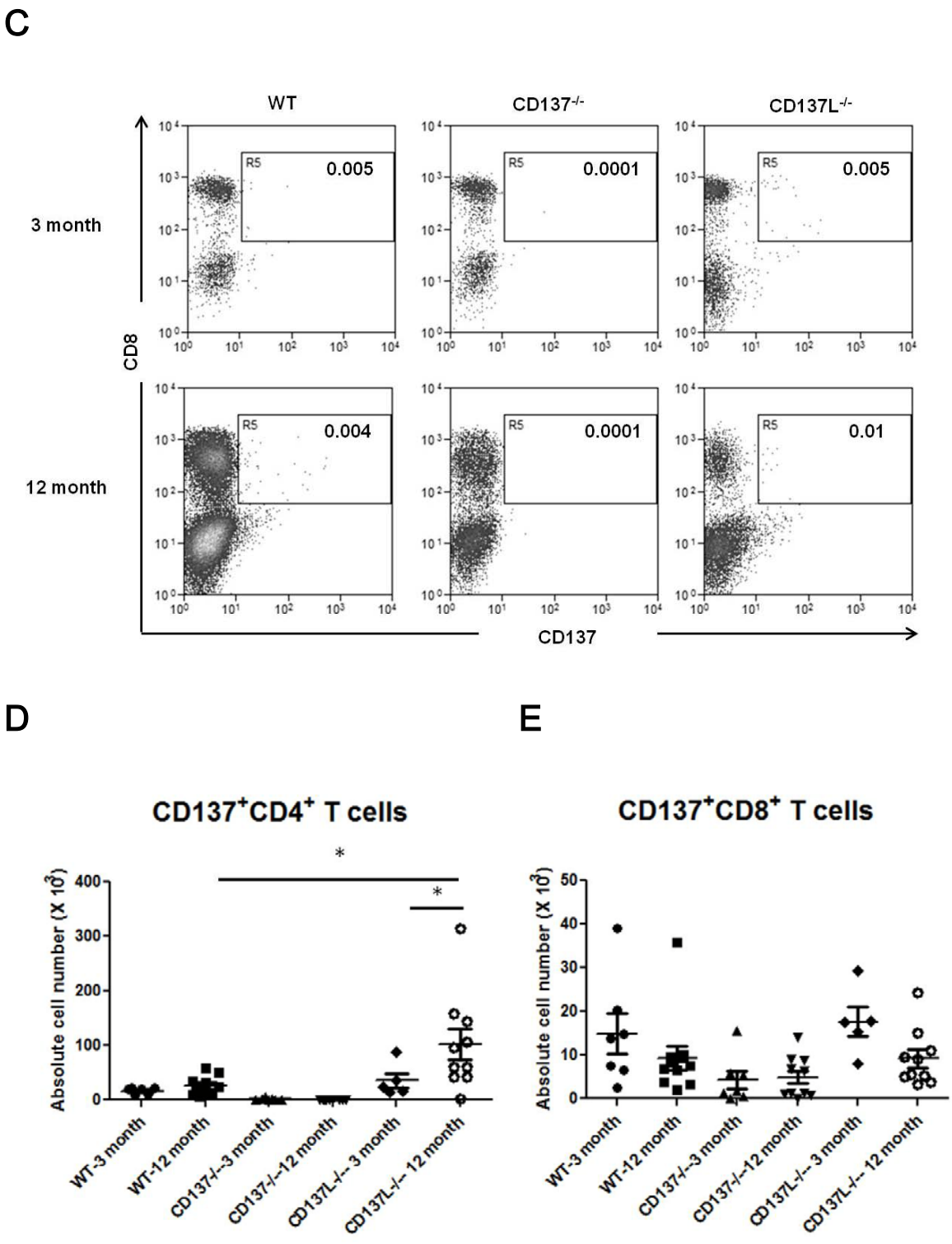


Figure 3.3.2 (C) CD137^{+} CD8^{+} T cells in 3-month and 12-month mice of WT, CD137$^{-/-}$, CD137L$^{-/-}$ mice. (D, E) Absolute cell number of CD137^{+} T cells in 3-months and 12-months old mice.

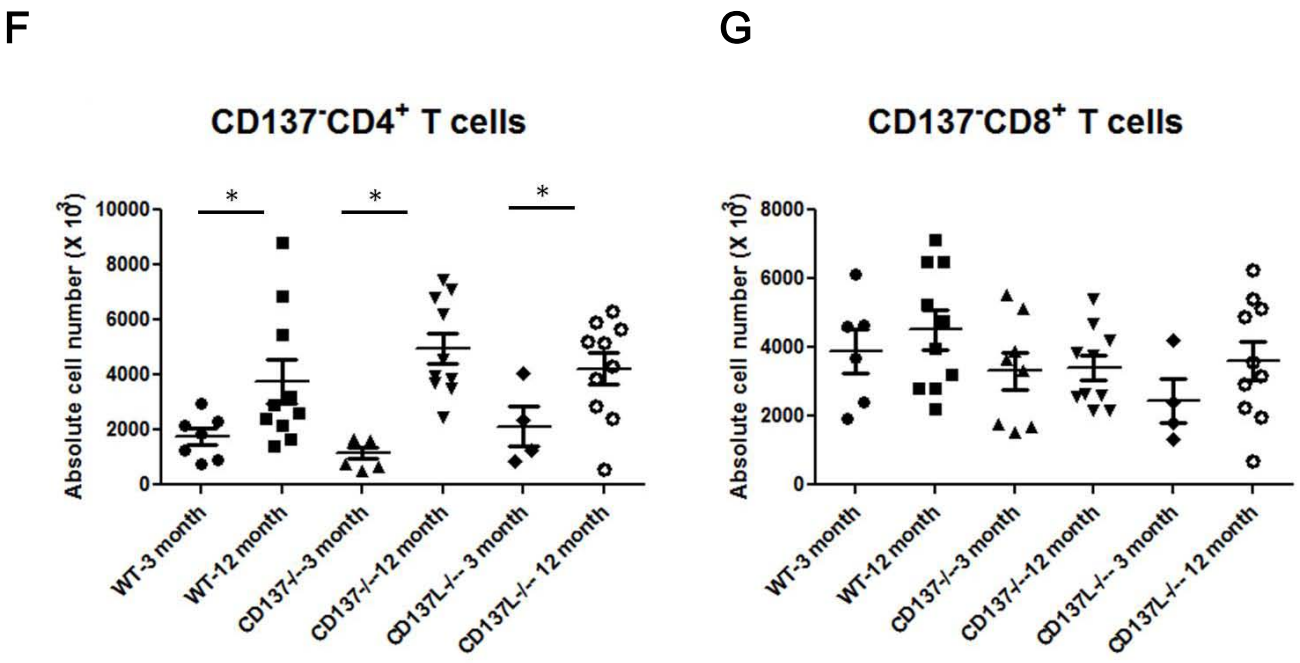


Figure 3.3.2 (F, G) Absolute cell numbers of CD137$^-$ CD4$^+$/CD8$^+$T cells. Each group consisted of at least 5 mice. *p<0.05. Data were represenatative of two independent experiments.

3.3.3 Increased numbers of myeloid progenitor cells in the absence of CD137

The major cell populations that may give rise to mature differentiated granulocytes and monocytes in the bone marrow in response to environmental stimuli are the HSCs and myeloid progenitor cells. To investigate whether these two populations are affected by the absence of CD137L reverse signaling during aging, their numbers were examined in 3-months and 12-months WT, CD137$^{-/-}$ and CD137L$^{-/-}$ mice.

Bone marrow cells isolated from the 3-months and 12-months WT, $CD137^{-/-}$, $CD137L^{-/-}$ mice were stained for lineage marker (CD3, CD19, B220, Ter-119, CD11b, Gr-1, CD11c, F4/80), Sca-1, CD117 (c-kit) and IL-7R. For the simplicity, hematopoietic stem cells were termed KSL. Categorization of KSL and myeloid progenitor cells were based on the expression of Sca-1 and CD117 (**Figure 3.3.3A**)

The data showed that KSL accumulated during the aging process in both $CD137^{-/-}$ and $CD137L^{-/-}$ mice, with significantly higher number than 12-months WT mice. On the other hand, surprisingly the number of KSL in12-months WT mice was unaltered compared to 3-months WT mice (**Figure 3.3.3 B**).

Similarly the downstream myeloid progenitor cells showed a significant increase in absolute cell numbers in the 12-months $CD137^{-/-}$ and $CD137L^{-/-}$ mice. The increment is particularly evident in $CD137L^{-/-}$ mice where a 2 fold difference was observed between 12-months WT and $CD137L^{-/-}$ mice (**Figure 3.3.3 C**).

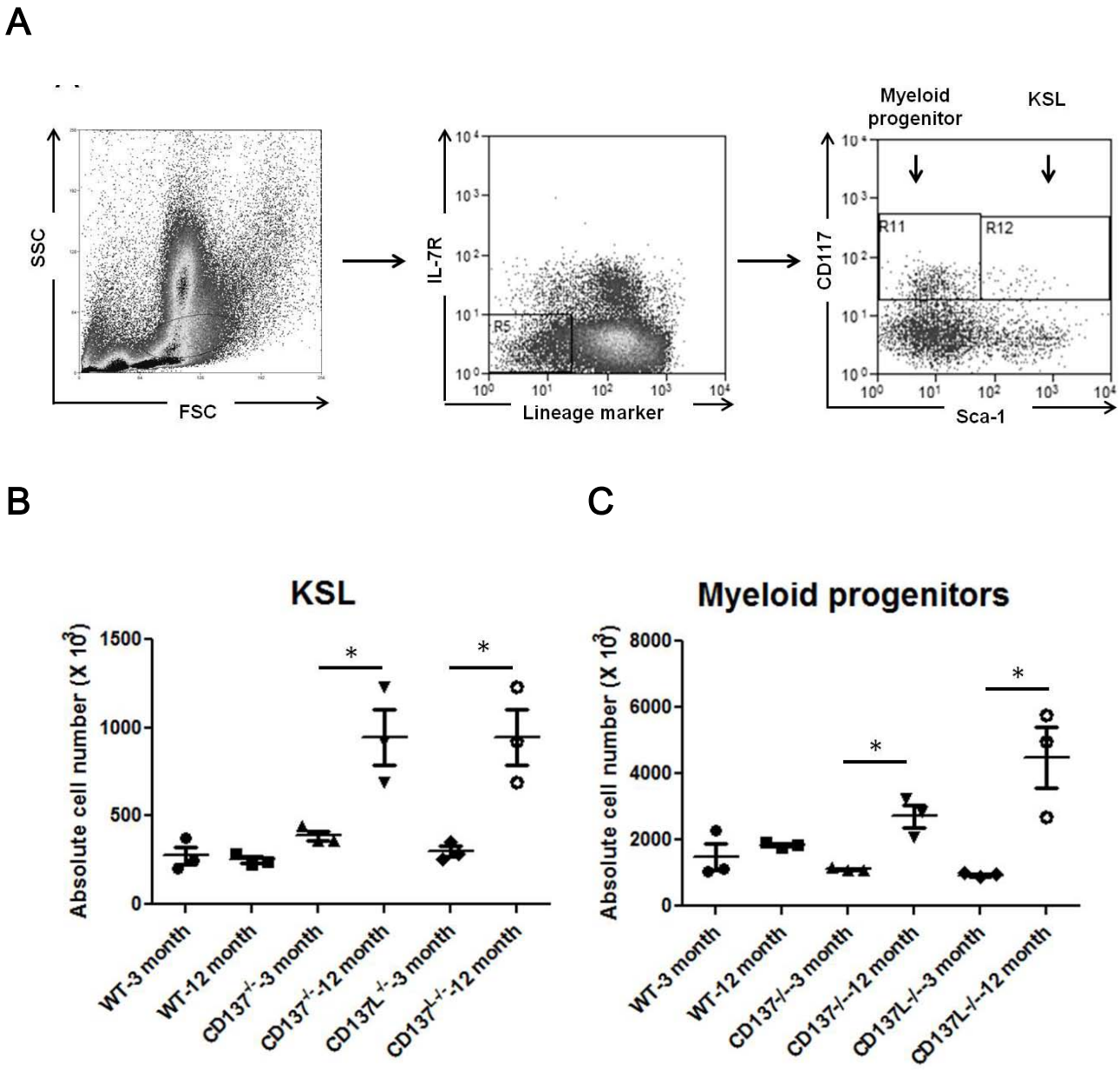


Figure 3.3.3 Increased number of myeloid progenitor cells in aged CD137$^{-/-}$ and CD137L$^{-/-}$ mice. Bone marrow cells were isolated from 3-months and 12-months old WT, CD137$^{-/-}$ and CD137L$^{-/-}$ mice and stained for the lineage marker, IL-7R, CD117 and Sca-1. Cells were first gated on progenitor cell region based on forward and side scatter. Then Lin^{-}IL-7R^{-} cells were gated out for analysis of myeloid progenitor cells (CD117^{+}Sca-1^{-}) and KSL (CD117^{+}Sca-1^{+}). Absolute numbers of cells were counted by addition of CountBright beads (Invitrogen). (A) Gating strategy of KSL and myeloid progenitor cells. (B) Absolute cell number of KSL and (C) myeloid progenitor cells in 3-months and 12-months mice. Each group consisted of 3 mice. Data were representative of two independent experiments. *P<0.05.

3.3.4 Increasing colony forming units of myeloid lineage of 12-month CD137$^{-/-}$ and CD137L$^{-/-}$ mice

To further confirm that the absence of CD137 or CD137L leads to increasing numbers of myeloid progenitor cells, colony forming unit assays were performed to assess the number of colony forming unit (CFU) of myeloid lineage in the bone marrow of 3-months and 12-months mice.

Three types of colonies, CFU-Granulocytes (CFU-G), CFU-Macrophage (CFU-M), CFU-Granulocyte/Macrophage (CFU-GM) were detected in Methocult3434 (Stemcell Technologies). Types of colonies were determined based on the morphology according to manufacturer's instruction (**Figure 3.3.4 A**).

The most abundant type of CFU detected was the CFU-GM which contained progenitor cells with potential for granulocytes and monocytes. In the 3-month age group, CD137$^{-/-}$ and CD137L$^{-/-}$ mice had small but significant increase in the number of CFU-GM than WT, corresponding to data previously reported (Kwon, 2002). As the mice aged CFU-GM of CD137$^{-/-}$ and CD137L$^{-/-}$ mice continue to increase compared to WT. Again 12-months CD137L$^{-/-}$ mice have the highest number of CFU-GM among all three strains, with the number increased two fold. 12-months CD137$^{-/-}$

mice also had a significant increase but to a lesser degree. WT mice had the lowest increase in the number of CFU-GM (**Figure 3.3.4 B**).

CFU-G and CFU-M represent progenitor cells with even more restricted differentiation potential that can only develop into granulocytes or monocytes. WT and CD137L$^{-/-}$ mice showed significant increases in CFU-G as the animals aged. A similar pattern was observed in the CD137$^{-/-}$ mice although no statistical significance was detected. No clear pattern of change was observed for CFU-M in all three strains regardless of the age.

A

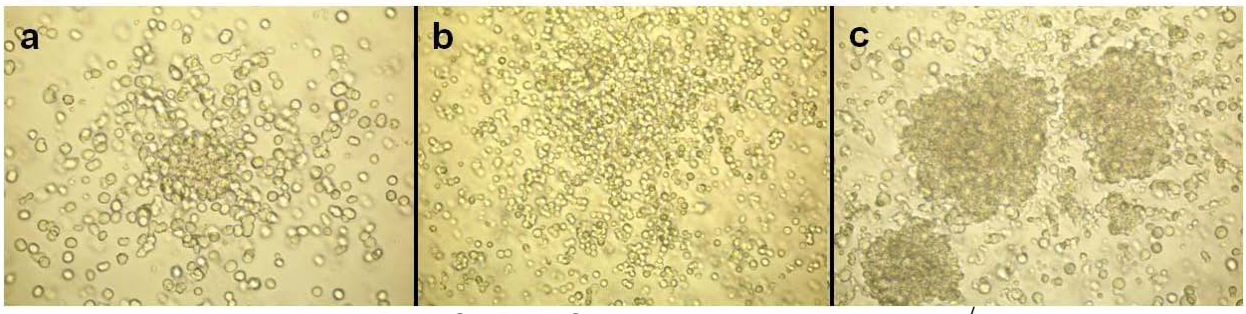


Figure 3.3.4 Increased number of colony forming units in aged CD137$^{-/-}$ and CD137L$^{-/-}$ mice. Colony forming units (CFU) were detected on the frequency of 0.1 million of total bone marrow cells. (A) Morphology of CFU-(a)Macrophage/(b)Granulocyte/(c)Granulocyte-Macrophage. Magnification: 20x.

B

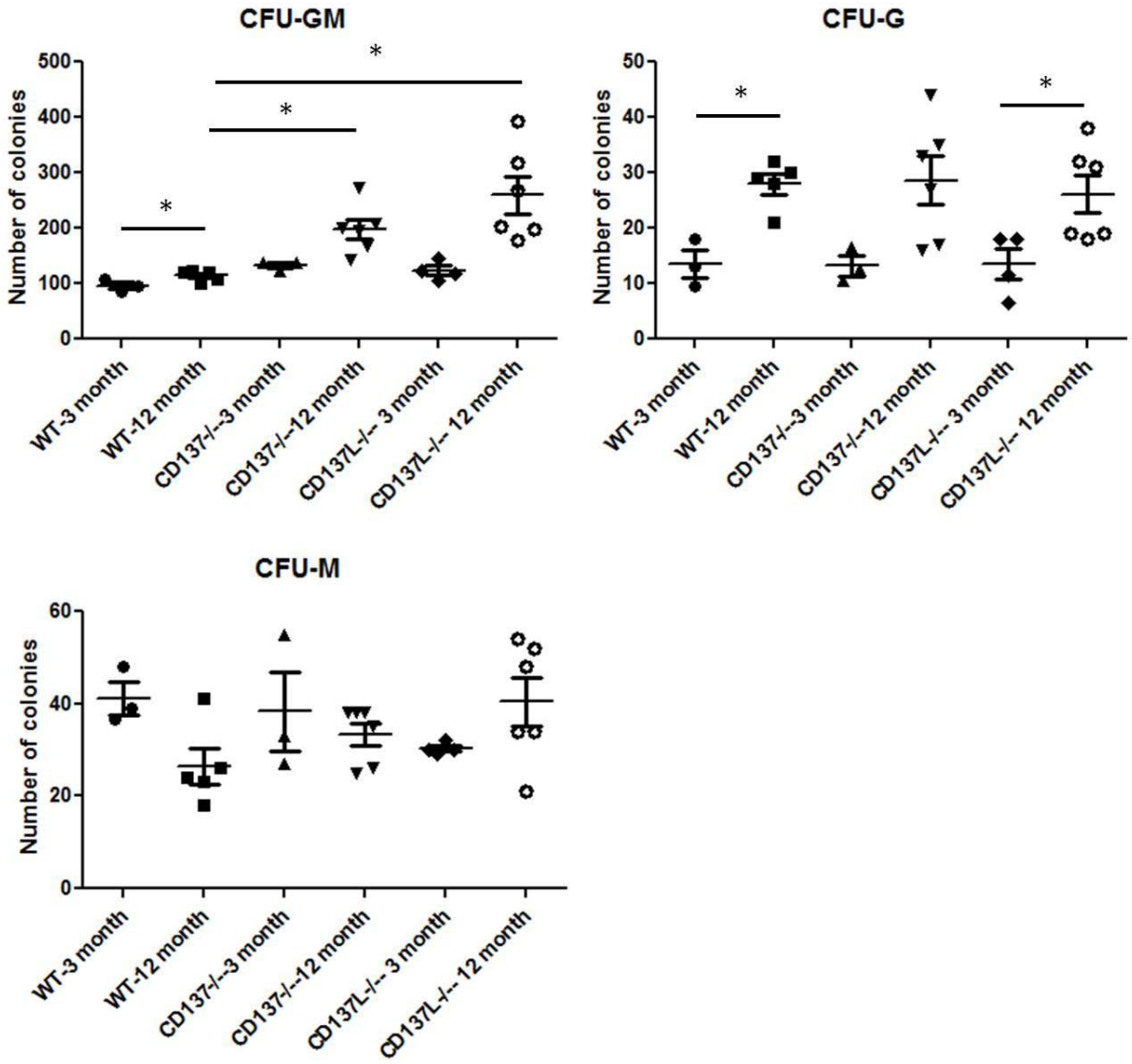


Figure 3.3.4 (B) Number of CFU per 0.1 million cells. Each group consisted of at least 3 mice. Data were representative of two independent experiments. *$p<0.05$.

3.3.5 CD137$^+$ CD4$^+$ T cells enhance myeloid cell differentiation of aged Lin$^-$ progenitor cells

Previously it has been shown that CD137$^+$ CD4$^+$ T cells can induce differentiation of Lin$^-$ progenitor cells from young mice. It is asked whether CD137L reverse signaling is also essential for the aged progenitor cells to differentiate to myeloid lineage.

Lin$^-$ progenitor cells were isolated from bone marrow cells of 12-months WT and CD137L$^{-/-}$ mice. The progenitor cells were labeled with CFSE before being cocultured with activated WT CD4$^+$ T cells for 6 days. To better separate the two populations, T cells were further stained for CD4. Percentages and absolute cell numbers of CD11b$^+$, Ly6G$^+$ and Ly6C$^+$ cells were analyzed in the CFSE$^+$CD4$^-$ population (**Figure 3.3.5A**).

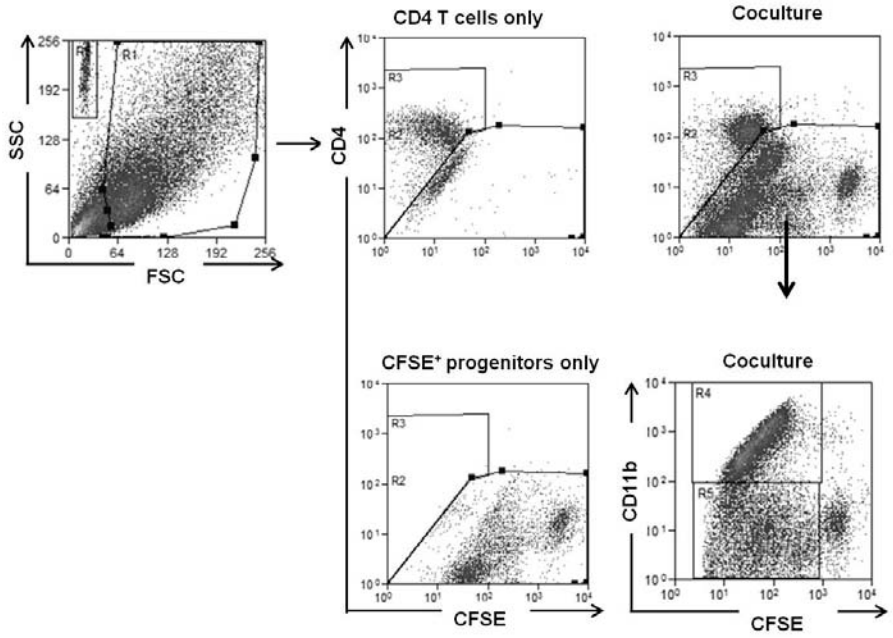
When cocultured with activated CD4$^+$ T cells, 60% of WT progenitor cells expressed CD11b$^+$ cells in the CFSE$^+$ population while only 35% of CD137L$^{-/-}$ progenitor cells became positive for CD11b (**Figure 3.3.5B**). With a change close to two fold WT progenitor cells clearly had a higher propensity of differentiating to myeloid cells than CD137L$^{-/-}$ progenitor cells. The two other myeloid markers, Ly6G

and Ly6C, were also expressed by higher percentages of WT progenitor cells than $CD137L^{-/-}$ progenitor cells.

To determine that the observed change in frequency of myeloid cells was not due to apoptosis of other cell populations, the absolute number of $CD11b^{+}$, $Ly6G^{+}$ and $Ly6C^{+}$ cells were calculated. Similar differences was also detected in terms of absolute cell numbers where WT progenitor cells had greater numbers of $CD11b^{+}$, $Ly6G^{+}$ and $Ly6C^{+}$ cells than $CD137L^{-/-}$ progenitor cells (**Figure 3.3.5C**).

Costaining of CFSE and myeloid markers showed that although WT and $CD137L^{-/-}$ progenitor cells can equally proliferate in response to activated T cells, WT progenitor cells clearly have a preference of differentiating to the myeloid lineage compared to $CD137L^{-/-}$ progenitor cells (**Figure 3.3.5D**)

A



B

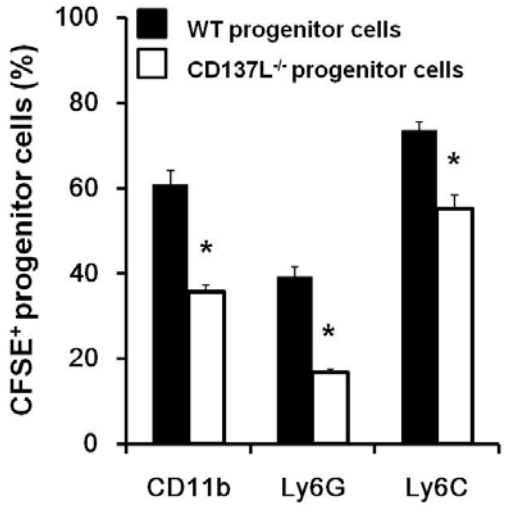
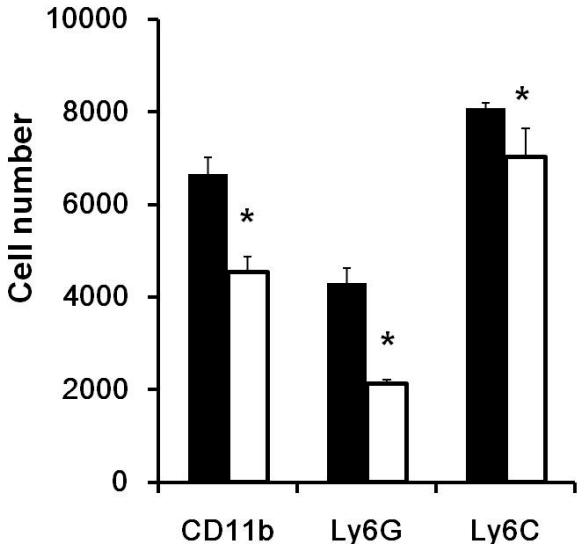


Figure 3.3.5 Increased differentiation and proliferation of myeloid cells of aged WT progenitor cells. Lineage$^-$ progenitor cells labeled with CFSE were isolated from 12-months WT and CD137L$^{-/-}$ mice and cocultured with activated WT CD4$^+$ T cells at a ratio of 1:2 for 6 days. Cells were then harvested and stained for CD4, CD11b, Ly6G and Ly6C. Countbright beads were added to calculate the absolute cell numbers. Equal numbers of CFSE$^+$ cells were acquired for each sample. (A) Gating strategy of identifying CD4$^+$ T cells and CFSE labeled progenitor cells. (B) Percentages of CD11b$^+$, Ly6G$^+$ and Ly6C$^+$ in the CFSE$^+$ population.

C



D

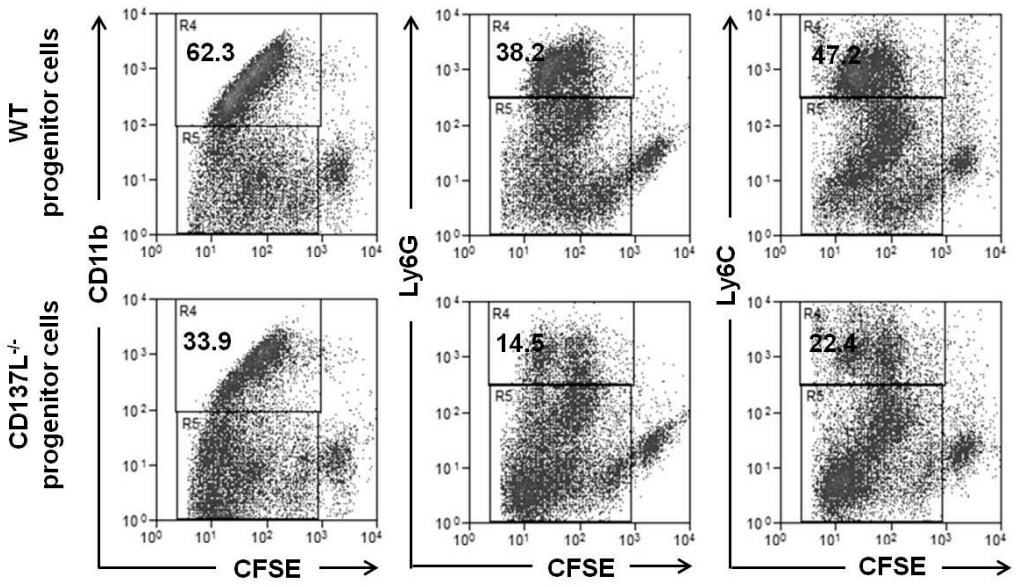


Figure 3.3.5 (C) Absolute numbers of $CD11b^+$, $Ly6G^+$ and $Ly6C^+$ in $CFSE^+$ population. (D) CFSE dilution of $CD11b^+$, $Ly6G^+$ and $Ly6C^+$ cells. Quadruplicates were performed for each treatment group. Data were representative of two independent experiments. *$p<0.05$.

Chapter 4 Discussion

In the last section, the role of CD137L reverse signaling in promoting myelopoiesis has been demonstrated in acute and chronic inflammation models. This section will focus on discussing the potential mechanism underlying the CD137L-mediated myelopoiesis and attempt to resolve conflicts of data reported from various research groups.

4.1 Species difference of CD137L reverse signaling between human and murine monocytes

4.1.1 Can CD137L induce DC differentiation in murine monocytes?

It was recently reported that CD137L signaling induces differentiation of peripheral blood human monocytes to inflammatory DCs, and that these CD137L-DCs were more potent than classical DCs generated by GM-CSF + IL-4 and matured by LPS + IFN-γ (Kwajah and Schwarz 2010). The CD137L-DCs were particularly efficient in inducing cytotoxic $CD8^+$ T cell expansion (Kwajah and Schwarz 2010). Similar findings were reported by another group showing that CD137L reverse signaling synergized with IL-4 to induce DC differentiation from human monocytes (Ju, Ju et al. 2009). The above data became the driving force for developing murine CD137L-DCs to investigate their T cell stimulatory effect in vivo. DCs have been the long-time tool for immunotherapy and it has been shown that successful rejection of

tumors largely depends on effective antigen presentation by DCs (Diamond, Kinder et al. 2011, Fuertes, Kacha et al. 2011, Palucka and Banchereau 2012). DCs with a higher potency would be expected to enhance the efficacy of immunotherapy. One major advantage of CD137L-DC over classic DC is that only one recombinant protein is required for the ex vivo expansion which reduces variables introduced. The generation of the murine equivalent of the human CD137L-DCs promises to allow characterization of CD137L-DCs in murine models and thereby obtaining more relevant in vivo data on the potency of CD137L-DCs.

Based on the reports with human CD137L-DCs it was hypothesized that CD137L reverse signaling should be able to induce differentiation of murine monocytes to inflammatory DCs. Indeed, the phenotypical response, i.e. increased attachment, morphological changes and induction of proliferation are identical between monocytes from the two species. But surprisingly, CD137 treatment fails to generate inflammatory DCs from murine monocytes as marked by the absence of costimulatory molecules and MHC-II, low levels of IL-12p70 and the inability to stimulate allogenic T cell proliferation.

In the human cells, CD137L-DC does not require further maturation by LPS (Kwajah and Schwarz 2010). In the murine cells, however, addition of LPS as

maturation factor does not convert the monocytes to inflammatory DCs but rather enhances their IL-10 secretion which inhibits allogenic T cell proliferation. Therefore, it has to be concluded that a species difference exists between murine and human monocytes in their differentiation response to CD137L reverse signaling. While human monocytes differentiate to inflammatory DCs murine monocytes do not. It is at present entirely unknown what the biological reason for this species difference may be.

This species difference becomes also evident in the effects of CD137L reverse signaling on immature classical DCs generated by GM-CSF + IL-4. Exposure of human immature, classical DCs to CD137 protein or CD137-expressing cells induces maturation as evidenced by a higher expression of costimulatory molecules and IL-12p70, stronger migratory activity and a higher T cell stimulatory capacity (Kim, Li et al. 2002, Lippert, Zachmann et al. 2008). In contrast, CD137L reverse signaling does not induce costimulatory molecule and IL-12p70 expression in murine immature DCs (Tang, Jiang et al. 2011). Most importantly, the CD137-treated murine DC does not exhibit a higher T cell stimulatory ability than Fc-treated murine DC.

4.1.2 What cell types are the CD137-treated monocytes?

If CD137L reverse signaling cannot induce DC differentiation from monocytes, then what identity have the cells adopted?

During an inflammatory state after monocytes exit the circulation they may become either macrophages or DCs based depending on the environmental cues (Geissmann, Manz et al. 2010). Therefore, it is quite likely that the CD137-treated monocytes differentiate to macrophages. Indeed, the enhanced phagocytic activity and the absence of allogeneic T cell stimulation strongly suggest that the CD137-treated monocytes have characteristics of macrophages. By comparing the CD137-treated monocytes to BMM generated by M-CSF, a highly similar cytokine profile is found between the two cells types, most notably the low IL-12p70/IL10 ratio. The induction of IL-10 production is further augmented by the addition of LPS.

The data corresponds well to previous studies by Shin in 2007 (Shin, Lee et al. 2007). It was shown that immobilization of CD137 protein induced IL-10 production from peritoneal macrophage. When mice received LPS injection, the influx of cells to peritoneal cavity is enhanced in $CD137^{-/-}$ mice because of impaired IL-10 production. Together with Shin's data, interaction of CD137 and CD137L is crucial for IL-10 production in differentiated macrophage, particularly when synergized with LPS.

Crosslinking of CD137L on the murine monocytes also releases large amounts of MCP-1 which is a key chemokine for monocytes trafficking during infection (Huffnagle, Strieter et al. 1995, McCord, Burgess et al. 2005, Deshmane, Kremlev et al. 2009). The high level of MCP-1 suggests an activated state of the CD137-treated monocytes and may promote recruitment of phagocytes in vivo for pathogen clearance.

4.1.3 Is the macrophage differentiation signal truly through CD137L?

When it is found that CD137L reverse signaling induces macrophage differentiation, a question arises whether the effect observed is truly the result of CD137L reverse signaling or due to binding of CD137 to other surface proteins such as other members of TNF receptor family that may share certain degrees of structural homology. Although it has been demonstrated by in vitro competitive assays that, unlike some of the TNF receptor super family members that are able to bind to multiple ligands, CD137 and CD137L are the sole receptor/ligand partner found so far (Bossen, Ingold et al. 2006), the possibility that CD137 may have hitherto unrecognized ligand outside the TNF family cannot be completely excluded.

Moreover monocytes can spontaneously differentiate to macrophages when in contact with ECM in vivo as well as when seeded on tissue culture dishes in vitro

(Jacob, Shastry et al. 2002, Sudhakaran, Radhika et al. 2007). Is it possible that the observed results are merely a combination of random binding of CD137 to surface protein and Fc fragment binding to Fc receptor on monocytes?

By using two other members of the TNF receptor super family, OX40 and TNFRI, the above possibilities are excluded because each of the individual receptor, CD137, OX40 and TNFR1 induce very unique morphological changes and cytokine profiles, suggesting that each receptor mediates the signal through its own ligand. Although it is not as effective in inducing cytokine production as TNFRI, CD137L reverse signaling is particularly effective in inducing attachment, survival and morphological changes compared to the two other members. Most importantly, all three recombinant proteins differ significantly from the Fc protein, clearly indicating that the observed attachment, morphological change and cytokine production are not merely mediated by random binding to another surface protein or attachment to the culture plate.

4.1.4 Underlying mechanism of species differences?

The striking species differences in response to CD137L reverse signaling between murine and human monocytes begs the question what the underlying mechanism can be. This species difference may have its molecular basis in the low conservation of the human and murine CD137 ligand. While the amino acid sequence identity between other human and murine members of the TNF family ranges between 70 and 80%, it is only 36% for CD137L. Therefore, it seems plausible that different signaling pathways and accordingly different biological effects are initiated by human and murine CD137L reverse signaling in the monocytes. Nevertheless, this species difference is unexpected since CD137L reverse signaling has the same effect on human and murine hematopoietic progenitor cells and induces macrophage differentiation in both species (Jiang 2008, Jiang, Yue et al. 2008). One possible explanation is that as the human and murine progenitor cells gradually lose plasticity of lineage potential, they develop distinct intrinsic changes, for example, a different epigenetic regulation and surface marker profile which leads to a different response to CD137L reverse signaling. Screening of gene regulation and surface marker profiles in both cell populations may help to answer this question.

4.1.5 Other Concerns on species differences in CD137/CD137L biology

It was not the first time that species differences between man and mouse were described for the CD137/CD137L system. While CD137 signaling delivers potent costimulatory signals to both human and murine T cells it affects NK cells differently in the two species. Crosslinking of CD137 by CD137L expressed on AML cell lines enhances the activity of murine NK cells while it inhibits the cytotoxicity and IFN-gamma release of human NK cells (Baessler, Charton et al. 2010). Combining the evidence from the above studies, one can foresee that the species differences between human and murine CD137/CD137L bidirectional signaling systems can significantly impact the interpretation of experimental data and eventually clinical trials. Agonistic CD137 antibodies have been viewed as a potent therapeutic tool because administration of the antibodies can reject established melanoma in mice (Melero, Shuford et al. 1997). However, it is possible that a large proportion of the therapeutic effect comes from the activating effect on murine NK cells (Baessler, Charton et al. 2010). Due to the inhibitory effect of CD137 on human NK cells, clinicians may face disappointing outcomes when administering the antibodies to human patients. On the other hand, while CD137L fails to generate DCs from murine monocytes, its ability to induce inflammatory DC from human monocytes should not be neglected, and it is possible to test the tumoricidal effect of CD137L-DC in humanized mice.

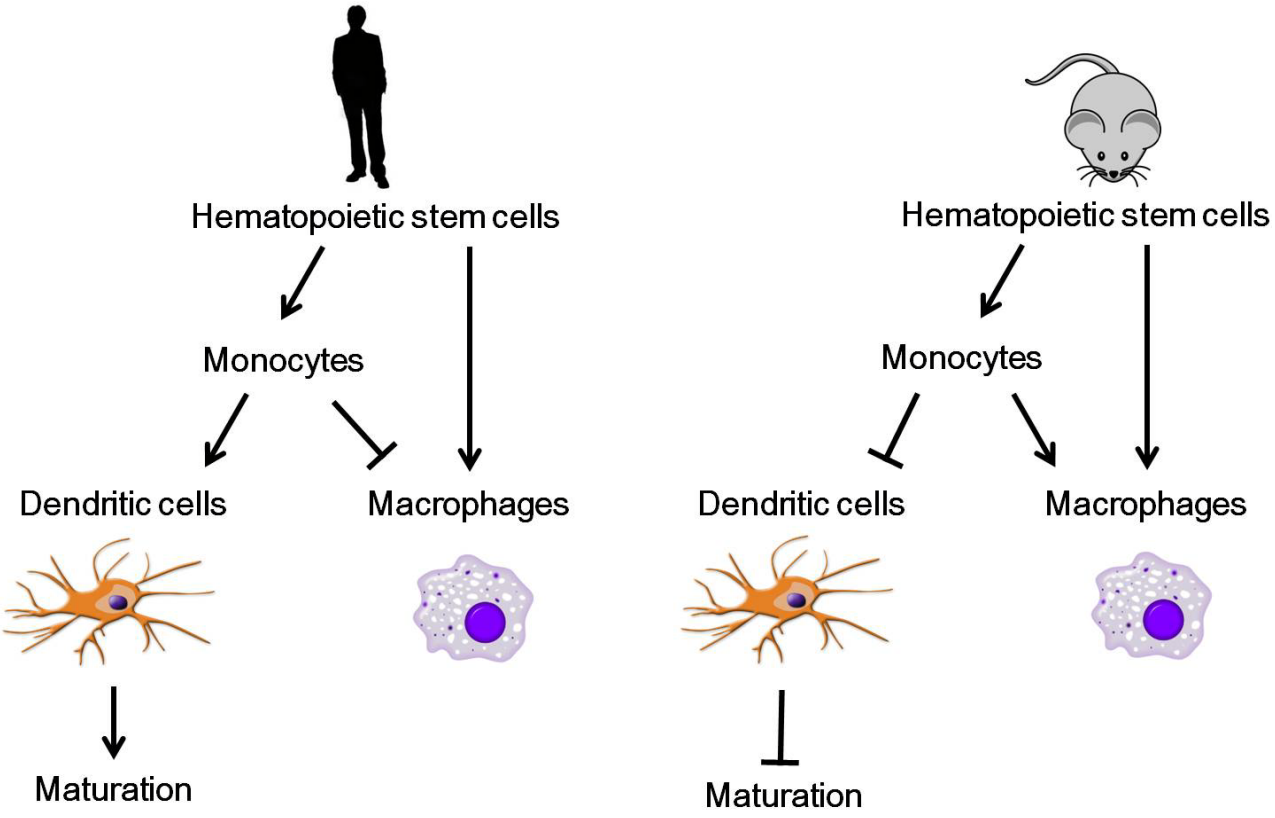


Figure 4.1 Species difference between human and murine cells in response to CD137L stimulation in hematopoietic cells at different stages. Left panel: Differentiation pathway of human cells in in response to stimulation by CD137 protein. Right panel: Differentiation pathway of murine cells in in response to stimulation by CD137 protein. Arrow: direction of differentiation.

4.2 The role of CD137L reverse signaling in myelopoiesis during infection

Although CD137L reverse signaling shows a considerable species difference in the human and murine cells, the response of early progenitor cells is similar and CD137L remains a potent myeloid growth factor. It is particularly important to understand the effect of CD137L at the progenitor cells level because it is the one of the major compartments affected during many disease states. For instance, the origin of AML is caused by a differentiation arrest at the progenitor stage and malignant cells retain the proliferative property without further differentiation which usually leads to reduced mitotic potential (Catenacci and Schiller 2005, Licciulli, Cambiaghi et al. 2010, Alberich-Jorda, Wouters et al. 2012, Sexauer, Perl et al. 2012).

Moreover during emergency myelopoiesis it is the progenitor compartment that responds to the proinflammatory stimulus and produces more cells to replace the loss in the periphery (Scumpia, Kelly-Scumpia et al. 2010, MacNamara, Oduro et al. 2011). Hence, understanding the influence of CD137L during infection helps to elucidate the underlying mechanism of defective innate immunity such as neutropenia at the progenitor level.

4.2.1 What is the source of CD137 during infection?

Due to its inducible property, the level of CD137 at steady state is extremely low. If CD137L is to induce signaling into progenitor cells, the level of CD137 has to increase in the first place. Indeed during infection by various pathogens, including virus and extra- and intracellular bacteria, a significant increase of $CD137^+$ cells is observed in the bone marrow compared to naïve mice by IHC. Further analysis by flow cytometry identified the cells as mainly T cells, with more than 60% of the cells expressing CD3. It is surprising, though, that the majority of the $CD137^+$ T cells are actually $CD4^+$ instead of $CD8^+$ T cells because CD137 has been known to preferentially propagate $CD8^+$ T cell population over $CD4^+$ T cells and promote Th1 response during virus and mycobacterium infection (Shuford, Klussman et al. 1997, Lee, Park et al. 2002).

In the context of myelopoiesis, however, the preference of $CD4^+$ T cells over $CD8^+$ T cells can be explained by their distinct roles in hematopoiesis. It has been reported that it was $CD4^+$ T cells but not $CD8^+$ T cells that are essential to maintain normal myelopoiesis in bone marrow because reconstitution of the latter ones cannot rescue the defect myelopoiesis in athymic mice ( ). Even in the spleen, the major secondary lymphoid organ where extramedullary myelopoiesis occurs, $CD4^+$ effector T cells were found to be responsible for myeloid cell development (Broxmeyer, Sehra et al. 2007, Lee, Wang et al. 2009). Moreover, it

was reported that $CD4^+$ T cells instead of $CD8^+$ T cells are the major sources of IFN-gamma which is essential for HSCs activation and proliferation in the bone marrow during intracellular bacterial infection. Therefore, even though CD137 may preferentially activate CD8 T cell in the periphery, during infection, it is the $CD4^+$ T cells that have a higher expression of CD137 in the bone marrow to facilitate myelopoiesis.

Can the $CD137^+$ cells be myeloid cells such as DC that constitutively express CD137? Indeed CD137 has been shown to be expressed on both myeloid DC and activated bone marrow macrophages, and both cell types can reside in the bone marrow (Futagawa, Akiba et al. 2002, Saito, Ohara et al. 2004, Choi, Kim et al. 2009). Analysis of the myeloid marker CD11b on the $CD137^+$ cells show that indeed about 30% of the $CD3^-CD137^+$ cells are myeloid cells. However, whether the $CD137^+$ myeloid cells contribute to the myelopoiesis during infection remains largely unknown. Based on the fact that the majority of the $CD137^+$ cells that increase during infections are T cells, it is suggested that T cells are the major contributors of CD137 to the bone marrow compartment. One may wonder what the rest of the $CD3^-CD137^+$ cells are. Under the microscope, aside from the few lamellipodia forming, myeloid-like cells, the majority of the $CD3^-CD137^+$ cells appear round and are probably of lymphoid origin. One speculation is that the cells are NK cells. Their identities require

further investigation and how much they may contribute to the CD137-mediated myelopoiesis remains a topic for future investigation.

4.2.2 Where do the CD137$^+$ T cells come from?

The next question is the origin of CD137$^+$ T cells. T cells are uncommon in bone marrow and usually comprise of only 1 to 3% of total cells during the steady state (Slifka, Whitmire et al. 1997, Herndler-Brandstetter, Landgraf et al. 2012). Then where do all the activated T cells expressing CD137 come from during infection?

The first explanation is that activated T cells traffic from the periphery to the bone marrow, functioning as messenger to deliver the stimulating signal for myelopoiesis. This hypothesis is difficult to test directly because there is no means to label activated T cells in the periphery in order to distinguish them from the bone marrow residing T cells. Instead the hypothesis is tested indirectly by injecting CFSE labeled activated T cells to naïve mice i.v., mimicking a situation where T cells become activated in the periphery and traffick to other lymphoid tissue. The pattern of migration suggests that the activated T cells are able to migrate to bone marrow. Although the experimental set up does not completely recapitulate the in vivo situation, it was shown by other groups that during LCMV infection, T cells enter bone marrow and enhance myelopoiesis to combat the infection (de Bruin 2012).

Therefore, it is possible that during infection T cells become primed and activated in periphery such as lymph nodes and that some of these activated T cells migrate to the bone marrow.

It should be noted, however, that the expression of CD137 does not convey an advantage on T cell migration as no increase in the expression of homing markers was observed between WT and $CD137^{-/-}$ activated T cells. One of the markers, CD62L, which is known to mediate the adhesion of leukocytes on blood vessels, was even slightly upregulated on $CD137^{-/-}$ T cells. The similar level of homing markers results in similar numbers of WT and $CD137^{-/-}$ T cells migrating to the bone marrow. In the spleen, $CD137^{-/-}$ T cells have surprisingly higher rates of migration than WT T cells, probably due to the higher CD62L expression that helps the extravasation of T cells in the vessel-rich spleen. How much the migratory efficacy contributes to the eventual output of myeloid cells is unclear. However, the number of cells may play a minor role because even with a higher influx of cells, $CD137^{-/-}$ T cells cannot crosslink CD137L in progenitor cells and mediate myelopoiesis.

A second explanation is that resident T cells in the bone marrow become activated by dissemination of pathogen or cytokine to the bone marrow and enhance expression of CD137. The likelihood for this is low because in all the infection

models that have been tested, none of the pathogens is known to disseminate to the bone marrow. Induction of CD137 on T cells also requires ligation of TCR (Schwarz, Tuckwell et al. 1993, Schwarz, Valbracht et al. 1995). Therefore, even if the expression of CD137 on T cells is through systemic increase in proinflammatory cytokines, it cannot directly act on T cells. However, it cannot be excluded that an inflammation-induced cytokine storm causes a systemic reaction and activates myeloid cells in the bone marrow which in turn induce CD137 expression on T cells.

4.2.3 What is the cell type responding to activated T cells during infection?

Bone marrow is composed of a mixture of cell populations including progenitor cells, B cells, monocytes, granulocytes and macrophages. Among all the cell populations, progenitor cells and monocytes are most likely the responding population to $CD137^{+}$ T cells because both cell types have been shown previously to proliferate in vitro in response to recombinant CD137 protein (Jiang 2008, Jiang, Yue et al. 2008, Tang, Jiang et al. 2011). When cocultures of activated T cells were performed with either Lin^{-} progenitor cells or total bone marrow cells, it was found both populations were able to proliferate, though at different rates. While WT T cells clearly promote more myeloid differentiation and proliferation of both cell populations compared to $CD137^{-/-}$ T cells, total bone marrow cells have a higher proliferation rate and higher myeloid differentiation than Lin^{-} progenitor cells. Does

that mean progenitor cells have a weaker response to CD137 stimulation in myelopoiesis than total bone marrow cells?

The data appear unexpected at first glance, but it is probably due to the mixed population present in total bone marrow cells. The majority of the cells that respond to CD137 treatment are monocytes, with the rest consisting of a small number of progenitor cells. Granulocytes and B cells probably undergo apoptosis due to insufficient growth support. While monocytes can only commit to the fate of macrophages or DCs, Lin^- progenitor cells have a much wider choice. The differentiating efficacy of CD137 is not 100%, and part of the Lin^- progenitor population may escape the influence of CD137 and commit to other lineages. Therefore, in the case of total bone marrow, the majority of the responding cells can only become or maintain myeloid proliferation or differentiation. On the other hand, when the responding cells only consist of progenitor cells, part of the population retains the ability to differentiate to lineages other than myeloid cells, resulting in a lower percentage of mature myeloid cells.

4.2.4 Why is extramedullary myelopoiesis not affected by CD137L reverse signaling?

When assessing the level of myelopoiesis in the bone marrow of infected mice, a higher number of $BrdU^+$ myeloid cells were found in the bone marrow, suggesting that the observed enhanced myelopoiesis was not due to mobilization of granulocytes and monocytes from the bone marrow compartment to the periphery. Rather it was due to local proliferation as the cells actively incorporate BrdU, a nucleotide analog. It is interesting that an increase of proliferation was only observed in the bone marrow but not in the spleen. Does that mean that CD137L reverse signaling has no effect on extramedullar myelopoieisis during infection? The explanation may lie in the cell composition in the spleen. Unlike in the bone marrow where the majorities of the cells are immature and retain the potential for proliferation and differentiation, the spleen consists mainly of terminally differentiated myeloid cells with limiting ability to proliferate. Although HSCs reside in the spleen during steady state, and are reported to migrate from bone marrow to spleen during infection or tumor growth, their frequency in spleen is much lower than that in the bone marrow, and the splenic HSCs have a lower proliferation rate and longer cell cycle (Morita, Iseki et al. 2011). Therefore, it may not be surprising that the number of proliferating myeloid cells is relatively lower than that of bone marrow. The low degree of cell proliferation may also masks any difference between WT and $CD137^{-/-}$ mice.

In addition, previously it was shown that $CD137^{-/-}$ T cells can mobilize to the spleen more efficiently than WT T cells. In spite of their possible higher migratory property WT and $CD137^{-/-}$ mice do not differ in extremedullar myelopoiesis during infection, again suggesting that the pivotal factor of myelopoiesis lies in the expression of CD137 instead in the number of T cells that have trafficked to the lymphoid organs.

4.2.5 Is CD137L reverse signaling the sole mechanism of the observed myelopoiesis

Besides surface-bound CD137, activated T cells secrete numerous cytokines including GM-CSF, G-CSF, IL-3 and M-CSF. Many of these cytokines can promote myelopoiesis. Therefore, one may ask the question whether CD137L reverse signaling is the only mechanism responsible of the myelopoiesis, both in vitro and in vivo? The level of myeloid growth factors in the coculture of Lin^- progenitor cells and activated $CD4^+$ T cells has been tested for GM-CSF, G-CSF and M-CSF. Surprisingly, the levels of both GM-CSF and G-CSF were higher in cocultures of $CD137^{-/-}$ than WT T cells with Lin^- progenitor cells, even though the Lin^- progenitor cells in this coculture had reduced proliferation and myeloid differentiation. If GM-CSF and G-CSF contribute to the myelopoiesis, one would have expected that Lin^- progenitor cells in the coculture with $CD137^{-/-}$ T cells have a comparable or even a higher degree of

myeloid differentiation. Instead, the data rather proved that the influence of CD137L reverse signaling on Lin-progenitor cells surpasses that of GM-CSF and G-CSF.

Why $CD137^{-/-}$ T cells produce more GM-CSF and G-CSF remains unclear. ELISA data of supernatants from activated WT and $CD137^{-/-}$ T cells showed that when activated by anti-CD3 and anti-CD28 antibodies T cells from the two strains produce similar levels of GM-CSF. However when cocultured with progenitor cells $CD137^{-/-}$ T cells have a higher production, suggesting an involvement of CD137L. The data actually fit to previous findings that splenocytes but not purified T cells from $CD137^{-/-}$ mice hyperproliferate when activated (Kwon 2002). The reason that total splenocytes instead of purified T cells indicates that CD137L on other cell types must crosslink the CD137 on T cells to prevent hyperproliferation. In the coculture system the Lin^{-} progenitor cells and monocytes express CD137L and therefore, crosslink CD137 on WT T cells to prevent hyperproliferation while in the activated $CD137^{-/-}$ T cells such a braking system is absent. The higher number of T cells may result in a larger amount of GM-CSF and G-CSF in the supernatants.

The in vivo situation is far more complicated than the in vitro system as other immune and non-immune cell types are involved in the process as well. CD137 has been shown to be expressed on endothelial cells stimulated by TNF-alpha, LPS and

IL-1 beta. Crosslinking of CD137 causes production of the chemokine CCL21, leading to leukocytes infiltration and cytokine storm including IFN-gamma and MCP-1 (Jeon, Choi et al. 2010, Teijeira, Palazon et al. 2012). As previously discussed, many of the proinflammatory cytokines such as IFN-gamma, IFN-beta and IL-6 favor myelopoiesis. Therefore, the possibilities that these cytokines play certain roles in the CD137L-mediated myelopoiesis cannot be completely excluded.

Last but not the least CD137L may exert its influence on myeloid differentiation by upregulating receptors for cytokines, for instance, GM-CSFR, M-CSFR and FLT-3. Currently no reports have touched on this subject although CD137L reverse signaling on human monocytes has been shown to induce M-CSF which acts in an autocrine manner to promote monocytes proliferation and survival (Langstein, Michel et al. 1999, Langstein and Schwarz 1999).

4.2.6 Is CD137L reverse signaling absolutely dependent on CD137 crosslinking?

Although studies have shown that so far CD137 is the only recognized receptor for CD137L (Bossen, Ingold et al. 2006), evidence has emerged that on occasions CD137L can signal without the presence of CD137. A study employing yeast-two-hybrid screen found that CD137L can associate with TLR-4 and induce signal on macrophages (Kang, Kim et al. 2007). The formation of this signaling

complex is completely independent of CD137. Moreover, the CD137L-TLR-4 complex is required for a sustained TNF-alpha production in macrophages. Even though in the absence of this complex, TNF-alpha can be produced in large quantities, the production returns quickly to base level without CD137L. Another study by Moh showed that in human THP-1 cell line CD137L binds to TNFRI (Moh, Lorenzini et al. 2013). Different from the previous case, association of CD137L and TNFRI requires crosslinking of CD137.

Whether there are other surface proteins that CD137L may bind to is still to be elucidated. However, based on current knowledge, it can be speculated that the association of CD137L and other surface proteins may have a significant impact on the cell population it is expressed on. In the case of myelopoiesis, TLR-4 has been reported to be expressed on HSCs. It is possible that TLR-4 expressed on HSCs can associate with CD137L and hence mediate reverse signaling. Based on the in vivo model of acute peritonitis, the data suggest that in the case of infection-induced myelopoiesis, CD137-CD137L interaction is necessary for the action because in $CD137^{-/-}$ mice where the receptor is missing, mice have fewer proliferating cells than their WT counterparts. Besides, even though CD137L has been proven to associate with TLR-4 and the interaction of the two molecules is required for a sustained TNF-alpha production, the signaling pathway my differ from the one initiated by CD137 crosslinking. It cannot be excluded that the two signaling pathways overlap in certain

functions. The association of TLR-4 and CD137L may even augment the response of HSC to CD137 stimulation. A double-knockout mouse strain without both TLR-4 and CD137L can answer the question.

4.2.7 What is the significance of the biphasic role of CD137L reverse signaling in myelopoiesis?

The data on CD137L reverse signaling during infection indicate a biphasic role of the molecule in myelopoiesis: inhibitory during steady state but proliferative during inflammation. Such a phenomenon is not limited to CD137L. It was also reported that IFN-gamma limits myeloid lineage colony formation when added in vitro but is essential for monocytes development during LCMV infection (MacNamara, Oduro et al. 2011, de Bruin 2012). Similarly, IFN-beta also inhibits myelopoiesis in vitro but promotes myeloid cells development in vivo (Deonarain, Verma et al. 2003, Wilkison, Gauss et al. 2012). The complete opposite effects of these inflammatory molecules including CD137L are possibly regulatory mechanisms of the immune system: the low constitutive level of the molecule present in the primary hematopoietic compartment during steady state is to prevent excess myeloid cells output that may produce undesirable inflammation and tissue damage. It is also to prevent a compromising lymphopoieisis when the hematopoieisis skews to myelopoiesis. The switch from inhibition to promotion occurs when large amounts of these inflammatory cytokines are introduced by pathogen invasion. Under such

emergency situations the proinflammatory molecules function as an alarm signal by increasing concentrations or expression levels in the bone marrow compartment and drive the hematopoiesis into an emgergency myelopoiesis.

It should be noted that the CD137L reverse signaling mediated myelopoiesis may not be limited to a particular infection model as similar increases of $CD137^+$ T cells are observed in the bone marrow of mice infected with E. coli or receiving LPS injection. While E. coli is a natural pathogen, LPS is only a bacterial cell wall component that induces systemic inflammation. It is more likely that the enhanced expression of CD137 is a general response of inflammation regardless of the nature of the cause. It is even possible that CD137L reverse signaling may contribute to the production of inflammatory myeloid cells in autoimmune diseases such as EAE and rheumatoid arthritis.

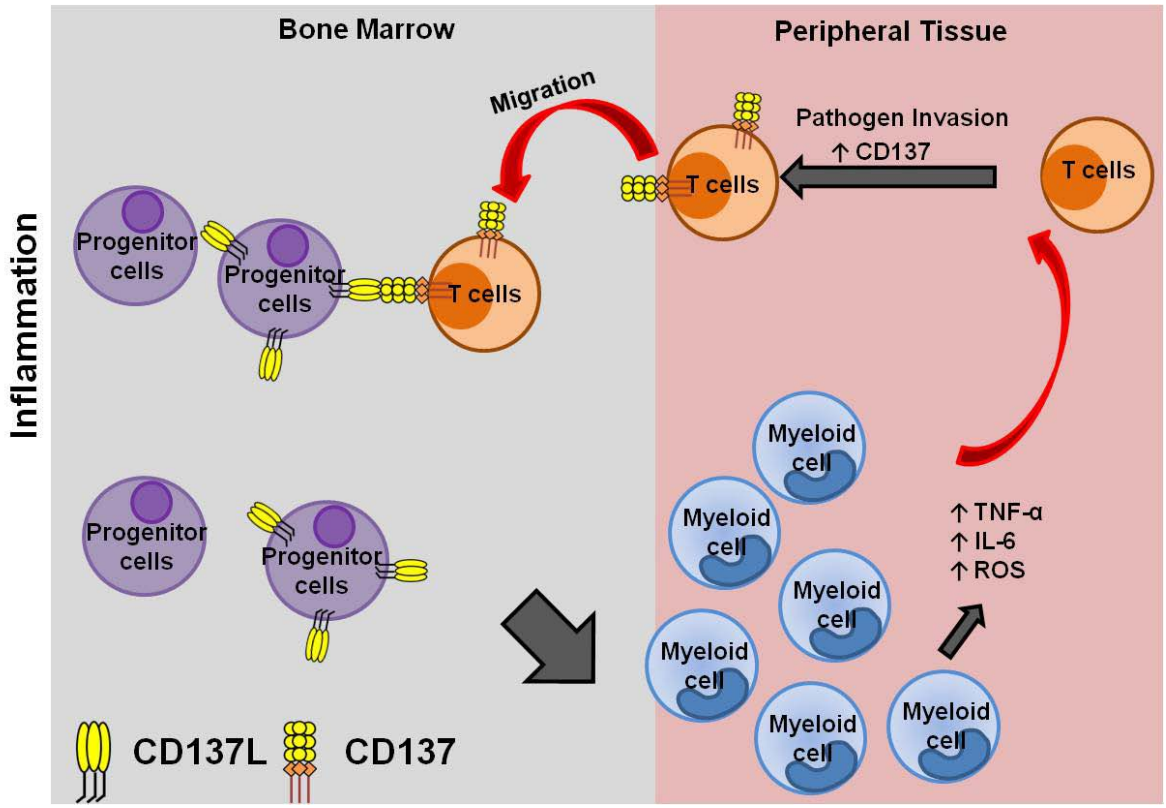


Figure 4.2 Model CD137L reverse signaling induces myelopoiesis during infection. Infection by pathogen induces T cell activation and CD137 expression. The activated T cells migrate to the bone marrow and crosslink CD137L on HSCs to promote myelopoiesis. The increased numbers of myeloid cells exit the bone marrow and eliminate the pathogens.

4.3 Role of CD137L reverse signaling in age-related myelopoiesis

The previous section demonstrates that the CD137/CD137L system plays a dichotomic role, inhibiting myelopoiesis during steady state conditions and inducing myelopoiesis during inflammatory conditions. It also identifies a novel mechanism of infection-induced myelopoiesis by demonstrating that the interaction of CD137 on activated $CD4^+$ T cells and CD137L on HSCs induces myelopoiesis during infections.

Age-related disruption in hematopoiesis is a well-known phenomenon and has been linked to development of various types of disease including the increasing occurrence of infection and tumor incidents (Gruver, Hudson et al. 2007, Hakim and Gress 2007). Understanding the underlying mechanisms that skew hematopoiesis towards myelopoiesis will facilitate in implanting new therapeutic tools for intervening the process.

The role of proinflammatory molecules in driving myelopoiesis has been extensively investigated in the last decade. Cytokines including IFN-γ and TNF-α have been reported to be essential for myeloid cell development (MacNamara, Oduro et al. 2011, de Bruin 2012, Oduro, Liu et al. 2012). It is considered a positive feedback mechanism to the hematopoietic system to increase numbers of myeloid cells which are the major forces in combating infection. In the context of aging,

however, these proinflammatory cytokines may be the promoting factors for bias towards myelopoiesis.

Based on the role of CD137L reverse signaling on myelopoiesis during acute inflammation mediated by E. coli infection, this section discusses its effect on aging where mice experience low degree of chronic inflammation.

4.3.1 Is CD137L a driving force of myelopoiesis during aging?

The next question to address is the role of CD137L reverse signaling in myelopoiesis during aging. Previously it has been reported that CD137L reverse signaling enhances myelopoiesis during inflammation. Since aging is also an inflammatory process it is asked whether CD137L is essential for myelopoiesis during this process. The major newly differentiated myeloid populations derived from progenitor cells are mainly granulocytes and monocytes. The data showed that in the absence of CD137L or CD137, mice of 12-months of age do not experience a similar increase in the numbers of granulocytes and monocytes as WT mice, strongly suggesting that the myeloid skewing process in the $CD137^{-/-}$ and $CD137L^{-/-}$ mice is stalled. This phenomenon is similar to the published data where CD137L reverse signaling enhances myelopoiesis during inflammation (Tang, Jiang et al. 2013). Both $CD137^{-/-}$ and $CD137L^{-/-}$ mice showing reduced myelopoiesis in aged animals strongly

suggests that the age-related myelopoiesis depends on the interaction of CD137 and CD137L.

4.3.2 What is the role of $CD137^+$ T cells in age-related myelopoiesis?

Contrasting to the findings in the infection models where $CD137^+$ $CD4^+$ T cells accumulate during the inflammation, no increased number of $CD137^+$ $CD4^+$ T cells in the 12-months WT mice was observed compared to their 3-months counterparts. This is probably due to the different degrees of inflammation the mice experience. During acute peritonitis mice develop symptoms of inflammation within 48 hr while in the aging model mice have only low degree of chronic inflammation throughout 12 months. Therefore, possibly the low numbers of $CD137^+$ $CD4^+$ cells are sufficient to maintain the myelopoiesis while the fast expansion of $CD137^+$ $CD4^+$ T cells is only required during emergency myelopoiesis where a quick response is needed for pathogen clearance.

It is interesting that a significant increase of $CD137^+$ $CD4^+$ T cells in 12-months $CD137L^{-/-}$ mice is observed in spite of the lower myelopoiesis in the bone marrow. One potential explanation could be the reciprocal relationship between expression levels of CD137 and CD137L, where a decrease of CD137L expression leads to an increase in CD137 expression, and vice versa (Wang, Lin, Ho et al., 2013;

Wang et al., 2009). An alternative explanation may be due to an unclosed feedback loop. Since $CD137^+$ $CD4^+$ T cells function as stimulators of myelopoiesis, and since a positive feedback signal in the form of more myeloid cells does not occur in the absence of CD137L reverse signaling, the $CD137^+$ $CD4^+$ T cells continue to home to the bone marrow.

The phenotype of the $CD137^+$ $CD4^+$ T cells may differ from the ones observed during infection. While the $CD137^+$ T cells whose numbers increase during infections are probably effector T cells primed in the periphery, the accumulated T cells in aged animals are possibly memory T cells that have encountered antigen in the periphery and homed to the bone marrow during the aging process. Previous studies on T cells in bone marrow of aged adults indicated that $CD4^+$ and $CD8^+$ T cells in the bone marrow are mainly antibacterial and anti tumor memory T cells (Herndler-Brandstetter, Landgraf et al. 2011). This speculation needs to be validated by further experiments that examine the surface marker profile of the T cells in the bone marrow of mice with infection and of aged mice.

A general increase of $CD137^-CD4^+$ T cells is observed in the bone marrow of aged animals in all three strains of mice. This accumulation is independent of the expression of CD137 or CD137L. It seems unlikely that this population plays a major role in the age-related myelopoiesis because all three strains of mice have similar

numbers of $CD137^{-}CD4^{+}$ T cells. However, it cannot be excluded that inherent changes have been induced in the T cells across the strains. Despite their similar numbers, it is unknown whether the T cells from $CD137^{-/-}$ and $CD137L^{-/-}$ aged mice actually have reduced cytokine production or a different surface marker expression profile that may eventually affect their function in the age-related myelopoiesis. Previous findings have shown that in spite of their hyperproliferation, the cytokine production of $CD137^{-/-}$ T cells is clearly impaired when activated through TCR (Kwon 2002). Recently it was reported that during infection $CD4^{+}$ T cells instead of $CD8^{+}$ T cells are the major source of IFN-gamma which is required for HSCs activation and proliferation during intracellular bacterial infection. Therefore, it is possible that the $CD4^{+}$ T cells in the bone marrow of the knock out strains are dysfunction in terms of cytokine production and have a smaller impact on myelopoiesis than WT $CD4^{+}$ T cells. To better compare the functions of bone marrow residing T cells in myelopoiesis, it will be useful to sort out the $CD4^{+}$ and $CD8^{+}$ T cells from bone marrow of aged WT, $CD137^{-/-}$ and $CD137L^{-/-}$ mice and compare them to their young counterparts.

4.3.3 Is CD137L necessary for transition from progenitor cells to mature cells?

Because the myelopoiesis level of $CD137L^{-/-}$ mice is much lower than of WT in spite of the increased numbers of $CD137^{+}$ and $CD137^{-}$ $CD4^{+}$ T cells, it is suspected that the recipient of the signal, mainly the myeloid progenitor cells, may experience a

block while transiting from the progenitor stage to the more differentiated granulocytes and monocytes. Proinflammatory cytokines are known to shift the myeloid progenitor cells to downstream granulocytes and monocytes during inflammation (MacNamara, Oduro et al. 2011). It was found that indeed numbers of both KSL and myeloid progenitor cells were significantly increased by the aging process in $CD137^{-/-}$ and $CD137L^{-/-}$ mice but remained unchanged in the WT mice, indicating an accumulation of undifferentiated hematopoietic cells in the two knock-out strains. The data of colony forming assays further confirmed that the two knock-out strains, particularly the $CD137L^{-/-}$ mice, had higher numbers of myeloid colony forming units during aging. The data strongly suggest that the absence of CD137L reverse signaling causes a differentiation block in the progenitor cells, and hence arrests them at the less differentiated stage, leading to lower numbers of mature granulocytes and monocytes in the bone marrow.

The direct link between activated $CD4^+$ T cells and myeloid differentiation of progenitor cells is confirmed by coculture of activated WT $CD4^+$ T cells with WT or $CD137L^{-/-}$ $Lineage^-$ progenitor cells where the lack of CD137L reverse signaling strongly diverts the progenitor cells away from the myeloid lineage as shown by a lower numbers of $CD11b^+$, $Ly6G^+$ and $Ly6C^+$ cells.

Notably other groups have reported that during steady stage $CD137^{-/-}$ and $CD137L^{-/-}$ progenitor cells have a higher myeloid differentiation potential and turnover rate in naïve young mice (Kwon 2002, Lee, Park et al. 2008). These data are not necessarily contradictory to the observation in the aging model. CD137L reverse signaling at steady stage may serve as a regulator of myelopoiesis to prevent overproduction of myeloid cells. Although the absence of CD137L reverse signaling may subject the myeloid progenitor cells to a higher turnover rate , under inflammation where CD137L is required for terminal differentiation to granulocytes and monocytes, the progenitor cells cannot exit and therefore, continue to accumulate in the bone marrow. Such seemingly conflicting role of CD137L was also reported in other studies where activation of CD137L on B cells led to proliferation in vitro (Pollok, Kim et al. 1994, Pauly, Broll et al. 2002) while in vivo CD137L regulates hyperproliferation of germinal centre B cells, evidenced by the fact that aged $CD137L^{-/-}$ mice tend to develop germinal B cell lymphoma (Middendorp, Xiao et al. 2009).

4.3.4 Implication of CD137L-mediated myelopoiesis during aging

It is particular important to understand the mechanism underlying biased myelopoiesis during aging because this is one of the driving forces that cause an imbalance in the hematopoietic system. As previously mentioned, researchers have been experimenting to rejuvenate the lymphopoiesis in aged mice by depleting

peripheral B cells (Keren, Naor et al. 2011). With the understanding of the role of CD137L reverse signaling in aged-related myelopoiesis, there may be additional approaches to reprogram the aging hematopoietic system. Neutralizing antibodies targeting CD137L on HSCs can be developed to block the interaction between progenitor cells and T cells. In a future study, it would be interesting to see whether aged $CD137^{-/-}$ and $CD137L^{-/-}$ mice have enhanced vaccine efficacy or lower tumor development than WT mice. On the other hand, due to their higher number of innate myeloid cells, aged WT mice may have an advantage over the two knock out strains in acute bacterial infections as they can clear the pathogen in a faster manner.

Is the myelopoiesis driving effect of CD137L reverse signaling absolutely deleterious? Not necessarily. Without proceeding to terminal differentiation of granulocytes and monocytes the progenitor cells become arrested at the immature stage and hence prone to the development of malignant leukemia such as AML. The development of leukemic cells usually starts with genetic mutations in a few clones which escape immune surveillance and continue to expand. In the case of CD137/CD137L system, besides the inability to promote myelopoiesis, $CD137^{-/-}$ and $CD137L^{-/-}$ mice may also be inferior to the WT counterparts in eliminating leukemic clones. In the future it would be interesting to know whether aged $CD137^{-/-}$ and $CD137L^{-/-}$ mice have more genetic mutations or DNA damage than aged WT progenitor cells.

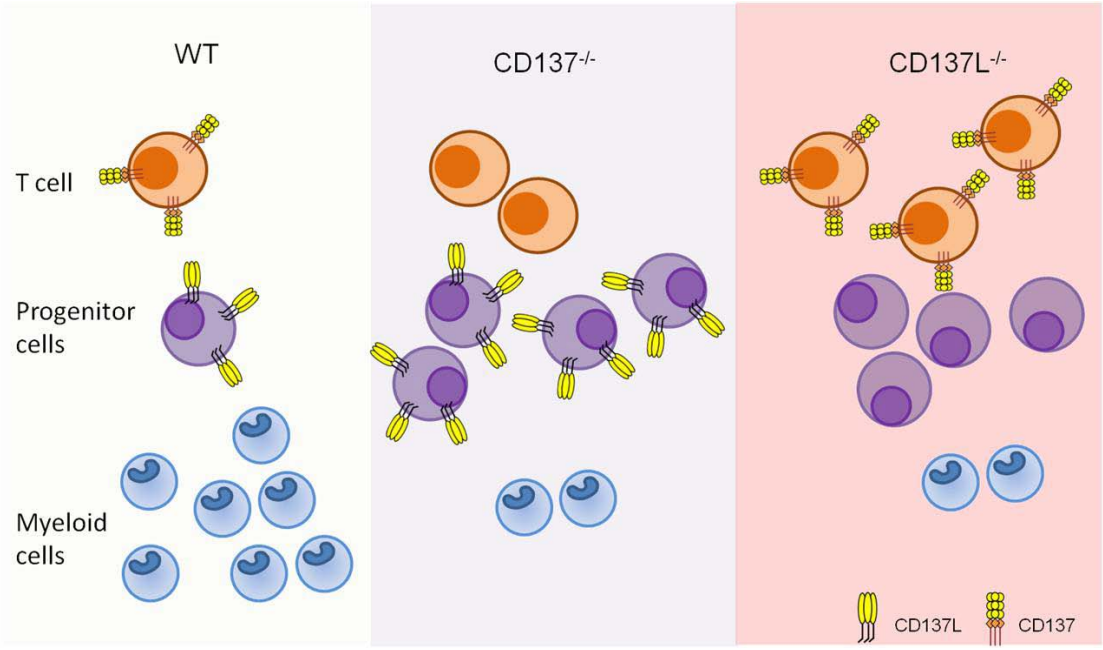


Figure 4.3.2 Model of CD137L-mediated myelopoiesis in aging animals. $CD137^{+}CD4^{+}$ T cells in bone marrow of aged animals continuously promote differentiation of hematopoietic progenitor cells to mature myeloid cells. In the absence of CD137, progenitor cells accumulate at the undifferentiated stage and fail to progress to the downstream myeloid cells. When CD137L is absent from the progenitor cells, $CD137^{+}CD4^{+}$ T cells keep homing to the bone marrow due to the missing increase of myeloid cells in the periphery. The progenitor cells are arrested in the undifferentiated stage due to the lack of CD137L reverse signaling.

Chapter 5 Conclusion

In this project, the role of CD137L reverse signaling in myeloid cells has been investigated in the context of acute and chronic inflammation.

Firstly this project addresses the issue of the species difference of CD137L reverse signaling between human and murine monocytes. Although the inability of CD137L reverse signaling to induce murine DC differentiation limits the usage of murine model for testing the tumoricidal effect of CD137L-DC in vivo, it brings up the awareness of potential implication of exploiting CD137/CD137L system in clinical usage.

Furthermore the findings in both acute and chronic inflammation in the context of infection and aging respectively show that CD137L reverse signaling is a driving force for myelopoiesis, mainly mediated by $CD137^+$ $CD4^+$ T cells. In the future manipulation of the CD137L reverse signaling pathway may provide insight of therapeutic tools for dysregulated hematopoiesis. The data also resolve long lasting conflict on the role of CD137L reverse signaling in myelopoiesis during steady and inflammatory state.

Reference

Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. (2007). "Local self-renewal can sustain CNS microglia maintenance and function throughout adult life." Nat Neurosci **10**(12): 1538-1543.

Alberich-Jordà M, Wouters B, Balastik M, Shapiro-Koss C, Zhang H, Di Ruscio A, Radomska HS, Ebralidze AK, Amabile G, Ye M, Zhang J, Lowers I, Avellino R, Melnick A, Figueroa ME, Valk PJ, Delwel R, Tenen DG. (2012). "C/EBPgamma deregulation results in differentiation arrest in acute myeloid leukemia." J Clin Invest **122**(12): 4490-4504.

Baessler T, Charton JE, Schmiedel BJ, Grünebach F, Krusch M, Wacker A, Rammensee HG, Salih HR. (2010). "CD137 ligand mediates opposite effects in human and mouse NK cells and impairs NK-cell reactivity against human acute myeloid leukemia cells." Blood **115**(15): 3058-3069.

Blanco P, Palucka AK, Pascual V, Banchereau J. (2008) "Dendritic cells and cytokines in human inflammatory and autoimmune diseases." Cytokine Growth Factor Rev. **19**(1):41-52.

Boettcher S, Ziegler P, Schmid MA, Takizawa H, van Rooijen N, Kopf M, Heikenwalder M, Manz MG.(2012) "LPS-induced emergency myelopoiesis depends on TLR4-expressing nonhematopoietic cells." J Immunol. **188**(12):5824-8.

Bosschaerts T, Guilliams M, Stijlemans B, Morias Y, Engel D, Tacke F, Hérin M, De Baetselier P, Beschin A. (2010). "Tip-DC development during parasitic infection is regulated by IL-10 and requires CCL2/CCR2, IFN-gamma and MyD88 signaling." PLoS Pathog **6**(8): e1001045.

Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, Ambrose C, Tschopp J, Schneider P. (2006). "Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human." J Biol Chem **281**(20): 13964-13971.

Broxmeyer HE, Sehra S, Cooper S, Toney LM, Kusam S, Aloor JJ, Marchal CC, Dinauer MC, Dent AL. (2007). "Aberrant regulation of hematopoiesis by T cells in BAZF-deficient mice." Mol Cell Biol **27**(15): 5275-5285.

Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, Hedrick CC, Cook HT, Diebold S, Geissmann F. (2013) "Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal." Cell. **153**(2):362-75

Catenacci, D. V, G. J. Schiller (2005). "Myelodysplasic syndromes: a comprehensive review." Blood Rev **19**(6): 301-319.

Choi BK, Kim YH, Kwon PM, Lee SC, Kang SW, Kim MS, Lee MJ, Kwon BS. (2009). "4-1BB functions as a survival factor in dendritic cells." J Immunol **182**(7): 4107-4115.

Chong SZ, Wong KL, Lin G, Yang CM, Wong SC, Angeli V, Macary PA, Kemeny DM. (2011). "Human CD8(+) T cells drive Th1 responses through the differentiation of TNF/iNOS-producing dendritic cells." Eur J Immunol **41**(6): 1639-1651.

de Bruin AM, Libregts SF, Valkhof M, Boon L, Touw IP, Nolte MA. (2012). "IFNγ induces monopoiesis and inhibits neutrophil development during inflammation." Blood **119**(6): 1543-1554.

Deonarain R, Verma A, Porter AC, Gewert DR, Platanias LC, Fish EN. (2003). "Critical roles for IFN-beta in lymphoid development, myelopoiesis, and tumor development: links to tumor necrosis factor alpha." Proc Natl Acad Sci U S A **100**(23): 13453-13458.

Deshmane SL, Kremlev S, Amini S, Sawaya BE. (2009). "Monocyte chemoattractant protein-1 (MCP-1): an overview." J Interferon Cytokine Res **29**(6): 313-326.

Di Rosa F, Pabst R. (2005). "The bone marrow: a nest for migratory memory T cells." Trends Immunol **26**(7): 360-366.

Diamond MS, Kinder M, Matsushita H, Mashayekhi M, Dunn GP, Archambault JM, Lee H, Arthur CD, White JM, Kalinke U, Murphy KM, Schreiber RD. (2011). "Type I interferon is selectively required by dendritic cells for immune rejection of tumors." J Exp Med **208**(10): 1989-2003.

Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR, Cumano A, Geissmann F. (2006). "A clonogenic bone marrow progenitor specific for macrophages and dendritic cells." Science **311**(5757): 83-87.

Fuertes MB, Kacha AK, Kline J, Woo SR, Kranz DM, Murphy KM, Gajewski TF. (2011). "Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8 dendritic cells." J Exp Med **208**(10): 2005-2016.

Futagawa T, Akiba H, Kodama T, Takeda K, Hosoda Y, Yagita H, Okumura K. (2002). "Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells." Int Immunol **14**(3): 275-286.

Geissmann F, Jung S, Littman DR. (2003). "Blood monocytes consist of two principal subsets with distinct migratory properties." Immunity **19**(1): 71-82.

Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. (2010) Development of monocytes, macrophages, and dendritic cells." Science **327**(5966): 656-661.

Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M. (2010) “Fate mapping analysis reveals that adult microglia derive from primitive macrophages.” Science. **330**(6005):841-5.

Gruver AL, Hudson LL, Sempowski GD. (2007). "Immunosenescence of ageing." J Pathol **211**(2): 144-156.

Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, Becker CD, See P, Price J,
Lucas D, Greter M, Mortha A, Boyer SW, Forsberg EC, Tanaka M, van Rooijen N, García Sastre A, Stanley ER, Ginhoux F, Frenette PS, Merad M. (2013) " Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes.” Immunity. **38**(4):792-804.

Hakim FT, Gress RE. (2007). "Immunosenescence: deficits in adaptive immunity in the elderly." Tissue Antigens **70**(3): 179-189.

Herndler-Brandstetter D, Landgraf K, Jenewein B, Tzankov A, Brunauer R, Brunner S, Parson W, Kloss F, Gassner R, Lepperdinger G, Grubeck-Loebenstein B. (2011). "Human bone marrow hosts polyfunctional memory CD4+ and CD8+ T cells with close contact to IL-15-producing cells." J Immunol **186**(12): 6965-6971.

Herndler-Brandstetter D, Landgraf K, Tzankov A, Jenewein B, Brunauer R, Laschober GT, Parson W, Kloss F, Gassner R, Lepperdinger G, Grubeck-Loebenstein B. (2012). "The impact of aging on memory T cell phenotype and function in the human bone marrow." J Leukoc Biol **91**(2): 197-205.

Hettinger J, Richards DM, Hansson J, Barra MM, Joschko AC, Krijgsveld J, Feuerer M. (2013) Origin of monocytes and macrophages in a committed progenitor. Nat Immunol. 14(8):821-30.

Hou W, Gibbs JS, Lu X, Brooke CB, Roy D, Modlin RL, Bennink JR, Yewdell JW. (2010)

"Viral infection triggers rapid differentiation of human blood monocytes into dendritic cells." Blood. **119**(13):3128-31.

Huffnagle GB, Strieter RM, Standiford TJ, McDonald RA, Burdick MD, Kunkel SL, Toews GB. (1995). "The role of monocytes chemotactic protein-1 (MCP-1) in the recruitment of monocytes and CD4+ T cells during a pulmonary Cryptococcus neoformans infection." J Immunol **155**(10): 4790-4797.

Jakubzick C, Gautier EL, Gibbings SL, Sojka DK, Schlitzer A, Johnson TE, Ivanov S, DuanQ, Bala S, Condon T, van Rooijen N, Grainger JR, Belkaid Y, Ma'ayan A, Riches DW, Yokoyama WM, Ginhoux F, Henson PM, Randolph GJ. Immunity. (2013) "Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes."Immunity **39**(3):599-610.

Jacob SS, Shastry P, Sudhakaran PR. (2002). "Monocytes-macrophage differentiation in vitro: modulation by extracellular matrix protein substratum." Mol Cell Biochem **233**(1-2): 9-17.

Jeon HJ, Choi JH, Jung IH, Park JG, Lee MR, Lee MN, Kim B, Yoo JY, Jeong SJ, Kim DY, Park JE, Park HY, Kwack K, Choi BK, Kwon BS, Oh GT. (2010). "CD137 (4-1BB) deficiency reduces atherosclerosis in hyperlipidemic mice." Circulation **121**(9): 1124-1133.

Jiang D, Chen Y, Schwarz H. (2008). "CD137 induces proliferation of murine hematopoietic progenitor cells and differentiation to macrophages." The Journal of immunology (1950) **181**(6): 3923-3932.

Jiang D, Yue PS, Drenkard D, Schwarz H. (2008). "Induction of proliferation and monocytic differentiation of human CD34+ cells by CD137 ligand signaling." STEM CELLS **26**(9): 2372-2381.

Ju S, Ju S, Ge Y, Qiu H, Lu B, Qiu Y, Fu J, Liu G, Wang Q, Hu Y, Shu Y, Zhang X. (2009). "A novel approach to induce human DCs from monocytes by triggering 4-1BBL reverse signaling." Int Immunol **21**(10): 1135-1144.

Kang YJ, Kim SO, Shimada S, Otsuka M, Seit-Nebi A, Kwon BS, Watts TH, Han J. (2007). "Cell surface 4-1BBL mediates sequential signaling pathways 'downstream' of TLR and is required for sustained TNF production in macrophages." Nat Immunol **8**(6): 601-609.

Kaufman CL, Colson YL, Wren SM, Watkins S, Simmons RL, Ildstad ST. (1994). "Phenotypic characterization of a novel bone marrow-derived cell that facilitates engraftment of allogeneic bone marrow stem cells." Blood **84**(8): 2436-2446.

Keren Z, Naor S, Nussbaum S, Golan K, Itkin T, Sasaki Y, Schmidt-Supprian M, Lapidot T, Melamed D. (2011). "B-cell depletion reactivates B lymphopoiesis in the BM and rejuvenates the B lineage in aging." Blood **117**(11): 3104-3112.

Kim YJ, Li G, Broxmeyer HE. (2002). "4-1BB ligand stimulation enhances myeloid dendritic cell maturation from human umbilical cord blood CD34+ progenitor cells." J Hematother Stem Cell Res **11**(6): 895-903.

King IL, Dickendesher TL, Segal BM. (2009). "Circulating Ly-6C+ myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease." Blood **113**(14): 3190-3197.

Krutzik SR, Tan B, Li H, Ochoa MT, Liu PT, Sharfstein SE, Graeber TG, Sieling PA, Liu YJ, Rea TH, Bloom BR, Modlin RL.(2005) "TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells." Nat Med. **11**(6):653-60.

Kwajah M M S, Schwarz H. (2010). "CD137 ligand signaling induces human monocytes to dendritic cell differentiation." Eur J Immunol **40**(7): 1938-1949.

Kwon BS, Hurtado JC, Lee ZH, Kwack KB, Seo SK, Choi BK, Koller BH, Wolisi G, Broxmeyer HE, Vinay DS. (2002). "Immune responses in 4-1BB (CD137)-deficient mice." The Journal of immunology (1950) **168**(11): 5483-5490.

Langstein J, Michel J, Schwarz H. (1999). "CD137 induces proliferation and endomitosis in monocytes." Blood **94**(9): 3161-3168.

Langstein J, Schwarz H. (1999). "Identification of CD137 as a potent monocytes survival factor." J Leukoc Biol **65**(6): 829-833.

Lee HW, Park SJ, Choi BK, Kim HH, Nam KO, Kwon BS. (2002). "4-1BB promotes the survival of CD8+ T lymphocytes by increasing expression of Bcl-xL and Bfl-1." J Immunol **169**(9): 4882-4888.

Lee JH, Wang C, Kim CH. (2009). "FoxP3+ regulatory T cells restrain splenic extramedullary myelopoiesis via suppression of hemopoietic cytokine-producing T cells." J Immunol **183**(10): 6377-6386.

L Lee SW, Park Y, So T, Kwon BS, Cheroutre H, Mittler RS, Croft M. (2008). "Identification of regulatory functions for 4-1BB and 4-1BBL in myelopoiesis and the development of dendritic cells." Nat Immunol **9**(8): 917-926.

León B, López-Bravo M, Ardavín C. (2007). "Monocytes-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania." Immunity **26**(4): 519-531.

Licciulli S, Cambiaghi V, Scafetta G, Gruszka AM, Alcalay M. (2010). "Pirin downregulation is a feature of AML and leads to impairment of terminal myeloid differentiation." Leukemia **24**(2): 429-437.

Linton PJ, Dorshkind K. (2004). "Age-related changes in lymphocyte development and function." Nat Immunol **5**(2): 133-139.

Lippert U, Zachmann K, Ferrari DM, Schwarz H, Brunner E, Mahbub-Ul Latif AH, Neumann C, Soruri A. (2008). "CD137 ligand reverse signaling has multiple functions in human dendritic cells during an adaptive immune response." Eur J Immunol **38**(4): 1024-1032.

Liu K, Nussenzweig MC. (2010). "Origin and development of dendritic cells." Immunol Rev **234**(1): 45-54.

MacNamara KC, Oduro K, Martin O, Jones DD, McLaughlin M, Choi K, Borjesson DL, Winslow GM. (2011). "Infection-induced myelopoiesis during intracellular bacterial infection is critically dependent upon IFN-gamma signaling." J Immunol **186**(2): 1032-1043.

Mazo IB, Honczarenko M, Leung H, Cavanagh LL, Bonasio R, Weninger W, Engelke K, Xia L, McEver RP, Koni PA, Silberstein LE, von Andrian UH. (2005). "Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells." Immunity **22**(2): 259-270.

McCord AM, Burgess AW, Whaley MJ, Anderson BE. (2005). "Interaction of Bartonella henselae with endothelial cells promotes monocytes/macrophage chemoattractant protein 1 gene expression and protein production and triggers monocytes migration." Infect Immun **73**(9): 5735-5742.

Melero I, Shuford WW, Newby SA, Aruffo A, Ledbetter JA, Hellström KE, Mittler RS, Chen L. (1997). "Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors." Nat Med **3**(6): 682-685.

Middendorp S, Xiao Y, Song JY, Peperzak V, Krijger PH, Jacobs H, Borst J. (2009). "Mice deficient for CD137 ligand are predisposed to develop germinal center-derived B-cell lymphoma." Blood **114**(11): 2280-2289.

Mildner A, Schmidt H, Nitsche M, Merkler D, Hanisch UK, Mack M, Heikenwalder M, Brück W, Priller J, Prinz M. (2007). "Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions." Nat Neurosci **10**(12): 1544-1553.

Moh MC, Lorenzini PA, Gullo C, Schwarz H. (2013). "Tumor necrosis factor receptor 1 associates with CD137 ligand and mediates its reverse signaling." FASEB J.

Morita Y, Iseki A, Okamura S, Suzuki S, Nakauchi H, Ema H. (2011). "Functional characterization of hematopoietic stem cells in the spleen." Exp Hematol **39**(3): 351-359 e353.

O'Doherty U, Peng M, Gezelter S, Swiggard WJ, Betjes M, Bhardwaj N, Steinman RM. (1994) “Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature.” Immunology.**82**(3):487-93.

Oduro KA Jr, Liu F, Tan Q, Kim CK, Lubman O, Fremont D, Mills JC, Choi K. (2012). "Myeloid skewing in murine autoimmune arthritis occurs in hematopoietic stem and primitive progenitor cells." Blood **120**(11): 2203-2213.

Palucka K, Banchereau J. (2012). "Cancer immunotherapy via dendritic cells." Nat Rev Cancer **12**(4): 265-277.

Pauly S, Broll K, Wittmann M, Giegerich G, Schwarz H. (2002). "CD137 is expressed by follicular dendritic cells and costimulates B lymphocyte activation in germinal centers." J Leukoc Biol **72**(1): 35-42.

Pollok KE, Kim YJ, Hurtado J, Zhou Z, Kim KK, Kwon BS. (1994). "4-1BB T-cell antigen binds to mature B cells and macrophages, and costimulates anti-mu-primed splenic B cells." Eur J Immunol **24**(2): 367-374.

Price PW, Cerny J. (1999). "Characterization of CD4+ T cells in mouse bone marrow. I. Increased activated/memory phenotype and altered TCR Vbeta repertoire." Eur J Immunol **29**(3): 1051-1056.

Randolph GJ, Beaulieu S, Lebecque S, Steinman RM, Muller WA. (1998). "Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking." Science **282**(5388): 480-483.

Rivollier A, He J, Kole A, Valatas V, Kelsall BL. (2012). "Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon." J Exp Med **209**(1): 139-155.

Saito K, Ohara N, Hotokezaka H, Fukumoto S, Yuasa K, Naito M, Fujiwara T, Nakayama K. (2004). "Infection-induced up-regulation of the costimulatory molecule 4-1BB in osteoblastic cells and its inhibitory effect on M-CSF/RANKL-induced in vitro osteoclastogenesis." J Biol Chem **279**(14): 13555-13563.

Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, Prinz M, Wu B, Jacobsen SE, Pollard JW, Frampton J, Liu KJ, Geissmann F. (2012) "A lineage of myeloid cells independent of Myb and hematopoietic stem cells." Science. 2012 **336**(6077):86-90

Schwarz H, Tuckwell J, Lotz M. (1993). "A receptor induced by lymphocyte activation (ILA): a new member of the human nerve-growth-factor/tumor-necrosis-factor receptor family." Gene **134**(2): 295-298.

Schwarz H, Valbracht J, Tuckwell J, von Kempis J, Lotz M.. (1995). "ILA, the human 4-1BB homologue, is inducible in lymphoid and other cell lineages." Blood **85**(4): 1043-1052.

Scumpia PO, Kelly-Scumpia KM, Delano MJ, Weinstein JS, Cuenca AG, Al-Quran S, Bovio I, Akira S, Kumagai Y, Moldawer LL. (2010). "Cutting edge: bacterial infection induces

hematopoietic stem and progenitor cell expansion in the absence of TLR signaling." J Immunol **184**(5): 2247-2251.

Senthilkumar R, Lee HW. (2009). "CD137L- and RANKL-mediated reverse signals inhibit osteoclastogenesis and T lymphocyte proliferation." Immunobiology **214**(2): 153-161.

Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. (2003). "TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection." Immunity **19**(1): 59-70.

Sexauer A, Perl A, Yang X, Borowitz M, Gocke C, Rajkhowa T, Thiede C, Frattini M, Nybakken GE, Pratz K, Karp J, Smith BD, Levis M. (2012). "Terminal myeloid differentiation in vivo is induced by FLT3 inhibition in FLT3/ITD AML." Blood **120**(20): 4205-4214.

Shin HH, Lee JE, Choi HS. (2007). "Absence of 4-1BB increases cell influx into the peritoneal cavity in response to LPS stimulation by decreasing macrophage IL-10 levels." FEBS Lett **581**(22): 4355-4360.

Shuford WW, Klussman K, Tritchler DD, Loo DT, Chalupny J, Siadak AW, Brown TJ, Emswiler J, Raecho H, Larsen CP, Pearson TC, Ledbetter JA, Aruffo A, Mittler RS. (1997). "4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses." J Exp Med **186**(1): 47-55.

Slifka MK, Whitmire JK, Ahmed R. (1997). "Bone marrow contains virus-specific cytotoxic T lymphocytes." Blood **90**(5): 2103-2108.

Sudhakaran PR, Radhika A, Jacob SS. (2007). "Monocytes macrophage differentiation in vitro: Fibronectin-dependent upregulation of certain macrophage-specific activities." Glycoconj J **24**(1): 49-55.

Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, Garin A, Liu J, Mack M, van Rooijen N, Lira SA, Habenicht AJ, Randolph GJ.. (2007). "Monocytes subsets

differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques." J Clin Invest **117**(1): 185-194.

Tang Q, Jiang D, Alonso S, Pant A, Martínez Gómez JM, Kemeny DM, Chen L, Schwarz H. (2013). "CD137 ligand signaling enhances myelopoiesis during infections." Eur J Immunol. **43**(6):1555-67

Tang Q, Jiang D, Shao Z, Martínez Gómez JM, Schwarz H. (2011). "Species difference of CD137 ligand signaling in human and murine monocytes." PLoS One **6**(1): e16129.

Teijeira Á, Palazón A, Garasa S, Marré D, Aubá C, Rogel A, Murillo O, Martínez-Forero I, Lang F, Melero I, Rouzaut A. (2012). "CD137 on inflamed lymphatic endothelial cells enhances CCL21-guided migration of dendritic cells." FASEB J **26**(8): 3380-3392.

Wang LD, Wagers AJ. (2011). "Dynamic niches in the origination and differentiation of haematopoietic stem cells." Nat Rev Mol Cell Biol **12**(10): 643-655.

Wilkison M, Gauss K, Ran Y, Searles S, Taylor D, Meissner N. (2012). "Type 1 interferons suppress accelerated osteoclastogenesis and prevent loss of bone mass during systemic inflammatory responses to Pneumocystis lung infection." Am J Pathol **181**(1): 151-162.

Woolthuis CM, de Haan G, Huls G. (2011). "Aging of hematopoietic stem cells: Intrinsic changes or micro-environmental effects?" Curr Opin Immunol **23**(4): 512-517.

Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, Strauss-Ayali D, Viukov S, Guilliams M, Misharin A, Hume DA, Perlman H, Malissen B, Zelzer E, Jung S. (2013) "Fate mapping reveals origins and dynamics of monocytes and tissue macrophages underhomeostasis."Immunity. **38**(1):79-9.

Yang J, Park OJ, Lee YJ, Jung HM, Woo KM, Choi Y. (2008). "The 4-1BB ligand and 4-1BB expressed on osteoclast precursors enhance RANKL-induced osteoclastogenesis via bi-directional signaling." Eur J Immunol **38**(6): 1598-1609.

Yurkovetsky ZR, Shurin GV, Barry DA, Schuh AC, Shurin MR, Robbins PD. (2006). "Comparative analysis of antitumor activity of CD40L, RANKL, and 4-1BBL in vivo following intratumoral administration of viral vectors or transduced dendritic cells." J Gene Med **8**(2): 129-137.

Zhu G, Flies DB, Tamada K, Sun Y, Rodriguez M, Fu YX, Chen L. (2001). "Progressive depletion of peripheral B lymphocytes in 4-1BB (CD137) ligand/I-Ealpha)-transgenic mice." J Immunol **167**(5): 2671-2676.

Appendices

Appendix I Buffer and solutions

PBS (Working concentration, 1x)

8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1L H_2O, ph7.4

FACS buffer

0.5% FBS, 0.02%NaN_3 in PBS, Ph7.4

MACS buffer

0.5% FBS, 2mM EDTA in PBS, Ph7.4

RBC lysis buffer

0.15M NH_4Cl, 10mM $KHCO_3$, 0.1Mm Na_2EDTA in H_2O, Ph 7.4

ELISA wash buffer (PBST)

0.05% Tween-20 in PBS

Appendix II Publications

1. Species difference of CD137 ligand signaling in human and murine monocytes. Plos One, 2011 Jan 14;6(1):e16129. doi:10.1371/journal.pone.0016129

Tang Q, Jiang D, Shao Z, Martínez Gómez JM, Schwarz H.

2. Involvement of the cytokine receptor CD137 in murine hematopoiesis.

Advances in Experimental Medicine and Biology, 2011;691:375-82. doi: 10.1007/978-1-4419-6612-4_38.

Jiang D, Tang Q, Schwarz H.

3. CD137 ligand signaling enhances myelopoiesis during infections

European Journal of Immunology, 2013 Mar 20. doi: 10.1002/eji.201243071

Tang Q, Dongsheng Jiang, Alonso S, Pant A, Martínez Gómez JM, Kemeny DM, Chen L, and Schwarz H.

4. CD137L signaling maintains myelopoiesis during aging (Manuscript in preparation)

Tang Q, Koh L, Jiang D, Schwarz H

Appendix III Selected oral presentation and poster

1.Influence of CD137L signalling in myelopoiesis during infection, IFRec-SIgN Winter School, Japan, 2010

2. Influence of CD137L signalling in myelopoiesis during acute inflammation, Model of Physiology and Disease, Singapore, 2012

3. Influence of CD137L signalling in myelopoiesis during acute and chronic inflammation, 5th Annual National University of Singapore Graduate School of Integrative Science and Engineering Symposium, Singapore, 2013

4. Influence of CD137L signalling in myelopoiesis during acute and chronic inflammation, International Congress of Immunology, Italy, 2013