AUGMENTATION AND DIFFERENTIATION OF HEMATOPOIETIC PROGENITOR CELLS BY CD137

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A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

YONG LOO LIN SCHOOL OF MEDICINE

NATIONAL UNIVERSITY OF SINGAPORE

2009

ACKNOWLEDGEMENTS

I would first like to express my heartfelt gratitude to my supervisor, A/P Herbert Schwarz, for his invaluable guidance throughout the course of this project. I truly appreciate his unreserved encouragement and support, irradiative advice and critiques, and remarkable patience.

Special thanks to the following people for their help with my work: Dr. Sylvie Alonso and Dr. Seah Geok Teng for their initiative discussions and suggestions; Ariel and Poh Cheng for guiding me when I first joined the lab; Sun Feng and Doddy for helping me with the immunohistochemistry and radioactive work; Richard for the influenza A infection in C57BL/6 mice; Aakansha for the *Bordetella pertussis* infection in BALB/c mice; and Isabel and Eunice for teaching and helping me during the first year of my study.

I am also grateful to all the current and previous members in my lab, including Shao Zhe, Jane, Shaqireen, Elaine, Liang Kai, Sharon, Diana, Jeanette, Dr. Yan, Dipanjan, Wen Tong, Kok Leng, Shi Hao, Mira, and Qianqiao. With whom I have shared four cherished years in such a comfortable environment. This wonderful experience is a priceless treasure in my life.

I also would like to express my appreciation to the General Offices of Department of

Physiology and Immunology Program for their generous support.

Last but not least, I would like to thank my parents for their constant love and support.

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ABSTRACT

CD137 is a member of the TNF receptor family, and is involved in the regulation of activation, proliferation, differentiation and cell death of leukocytes. Bidirectional signaling exists for the CD137 receptor / ligand system as CD137 ligand which is expressed as a transmembrane protein, can also transduce signals into the cells it is expressed on. In this study we have identified the expression of CD137 and CD137 ligand on small subsets of bone marrow cells. Activation of hematopoietic progenitor cells – $CD34^+$ cells in the human system and Lin^-, $CD117^+$ cells in the murine system – through CD137 ligand induces activation, prolongation of survival, proliferation and colony formation. Concomitantly to proliferation, the cells differentiate to colony forming units granulocyte macrophage (CFU-GM), and then to monocytes and macrophages but not to granulocytes or dendritic cells. Hematopoietic progenitor cells differentiated in the presence of CD137 protein display enhanced phagocytic activity, secrete high levels of IL-10 but no IL-12 in response to LPS, and can partially suppress T cell activation and proliferation in an allogeneic mixed lymphocyte reaction. CD137 and G-CSF cooperatively induce survival and proliferation of murine bone marrow cells, while they compete for inducing monocytic and granulocytic differentiation, respectively. The number of $CD137^+$ cells in the bone marrow is positively regulated during infection and inflammation. These data uncover a novel function of CD137 and CD137 ligand by showing their participation in hematopoiesis, in particular, myelopoiesis and monocytosis.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cells
BM	Bone marrow
bp	Base pair
CBA	Cytokine beads array
CD137L	CD137 ligand
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
CFU-G	Colony forming unit-granulocyte
CFU-GM	Colony forming unit-granulocyte/macrophage
CFU-M	Colony forming unit-macrophage
CPM	Count per minute
CMP	Common myeloid progenitors
DC	Dendritic cells
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ES	Embryonic stem
FACS	Fluorescence activated cell sorting
Fc	Fc portion of an antibody
FITC	Fluorescein isothiocyanate
Flt3L	Fms-like tyrosine kinase 3 (Flt3) ligand
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Granulocyte macrophage progenitors
HPC	Hematopoietic progenitor cells
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
HSPC	Hematopoietic stem / progenitor cells
ICC	Immunocytochemistry
IFN	Interferon
IHC	Immunohistochemstry
IL	Interleukin
ILA	Induced by lymphocyte activation
i.p.	Intra-peritoneal
IRB	Institutional review board
i.v.	Intra-venous
KC	Keratinocyte cytokine
KO	Knockout
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccarides
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting

mCD137-Fc	Murine CD137-Fc
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Monocyte colony stimulating factor
MDSC	Myeloid derived suppressor cells
MoDC	Monocyte derived dendritic cells
MFI	Mean fluorescence intensity
MLR	Mixed lymphocyte reaction
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	PBS + 0.05% Tween-20
PE	Phycoerythrin
PFA	Paraformaldehyde
RBC	Red blood cells
RT-PCR	Reverse transcription – polymerase chain reaction
SCF	Stem cell factor
SD	Standard deviation
SE	Standard error
TAE	Tris-acetate-EDTA
TAM	Tumor associated macrophages
TECK	Thymus-expressed chemokine
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TPO	Thrombopoietin
WT	Wide type

CHAPTER 1 INTRODUCTION

1.1 Hematopoietic stem and progenitor cells (HSPC)

Stem cells hold great promise for regenerative medicine and the study of cell and tissue differentiation. Hematopoietic stem and progenitor cells (HSPC) are to date the best-studied stem cell population, and are already used for several clinical applications.

First, HSPCs are an indispensable source of replenishment of blood and immune cells. All adult hematopoietic lineages are derived from very small numbers of self-replicating hematopoietic stem cells (HSC). This breathtaking ability to reconstitute the hematopoietic system makes transplantation of HSPCs after ablative chemotherapy the gold standard of care for treatment of leukaemia and lymphoma nowadays. These chemotherapy and radiation therapies inevitably destroy actively dividing healthy cells of the hematopoietic system along with the cancer cells. Patients thus often become immune deficient and highly susceptible to opportunistic infections which may even be fatal. The traditional treatment regiment involves the use of granulocyte colony-stimulating factor (G-CSF / Neupogen), but the short half life of this factor demands for daily injections. Furthermore, this treatment is specific for the augmentation of granulocytes. HSPCs with their multipotential seem to offer a better alternative solution for shortening the period of profound pancytopenia following chemotherapy or chemo-radiotherapy. This may in turn lessen the chances

of opportunistic infections and improve the patients' quality of life. Engraftment of HSPCs to cancer patients following chemotherapy also means that patients can be treated more aggressively, thereby increasing the potential for complete remission (Elias, 1995). Therefore, in the recent decade, transplantation of HSPCs have been more frequently used in autologous and allogeneic settings to restore the immune functions (Corringham and Ho, 1995; Sorrentino, 2004; Heike *et al.*, 2002).

More recently, there are emerging clinical applications of HSPCs for other malignant and non-malignant diseases. For example, HSPCs are capable of trans-differentiation to non-hematopoietic tissues to replace damaged or lost tissues (Orlic *et al.*, 2001; Bailey *et al.*, 2004). Recent cardiac clinical trials of introducing autologous hematopoietic progenitor cells during coronary artery bypass grafting demonstrated that patients had significantly improved circulation into infarct area and cardiac functions (Stamm *et al.*, 2003). Clinical applications for hematopoietic progenitor cells are therefore no longer restricted to hematopoietic and immune reconstitution.

This enormous clinical potential of HSPCs is, however, limited by their low availability. It is therefore of great importance to find ways to effectively and efficiently amplify the numbers of these cells. Hematopoietic growth factors and cocktails of such factors are an essential part of the *ex vivo* or *in vivo* amplification protocols of HSPCs. Unfortunately, the effective expansion of HSPCs has yet to be attained in spite of over 20 years of research in animal models and human clinical

trials (Devine *et al.*, 2003).

1.2 Hematopoiesis and myeloid cells

1.2.1 Overview of hematopoiesis

Hematopoiesis is a complex and tightly regulated process, and is essential for the homeostasis of tissue oxygenation and immune functions. Deepening our understanding of the regulation and mechanisms of hematopoiesis is expected to continue to offer new and effective therapies.

Hematopoiesis (Fig. 1.1) starts with HSCs, cells that can renew themselves and can differentiate to a variety of specialized cells. HSCs can be further divided into three groups, long term (LT)-HSC, short term (ST)-HSC and multi-potential progenitor (MPP). LT-HSCs are very rare and usually in a quiescent state, while MPPs are in a more active state. The MPPs commit to common myeloid progenitors (CMP) in the presence of stem cell factor (SCF) and thrombopoietin (TPO), or to common lymphoid progenitors (CLP) in the presence of IL-7. (Robb, 2007).

CMPs further differentiate to megakaryocyte erythroid progenitors (MEP) or granulocyte-macrophage progenitors (GMP). MEPs give rise to megakaryocytes (platelets) under the influence of TPO, or erythrocytes (red blood cells) under the influence of erythropoietin (EPO) (Robb, 2007). GMPs have potential to give rise to

monocytes, which further differentiate to macrophages or myeloid DCs in the peripheral tissues depending on the microenvironment; or to granulocytes, including neutrophils, eosinophils and basophils. This process is called myelopoiesis, and colony stimulating factors, such as GM-CSF, G-CSF and M-CSF, are crucial regulators of myelopoiesis (Fig. 1.1).

CLPs undergo lymphopoiesis and give rise to B cells, T cells and NK cells, depending on the presence of various interleukins as shown in Fig. 1.1. (Robb, 2007).

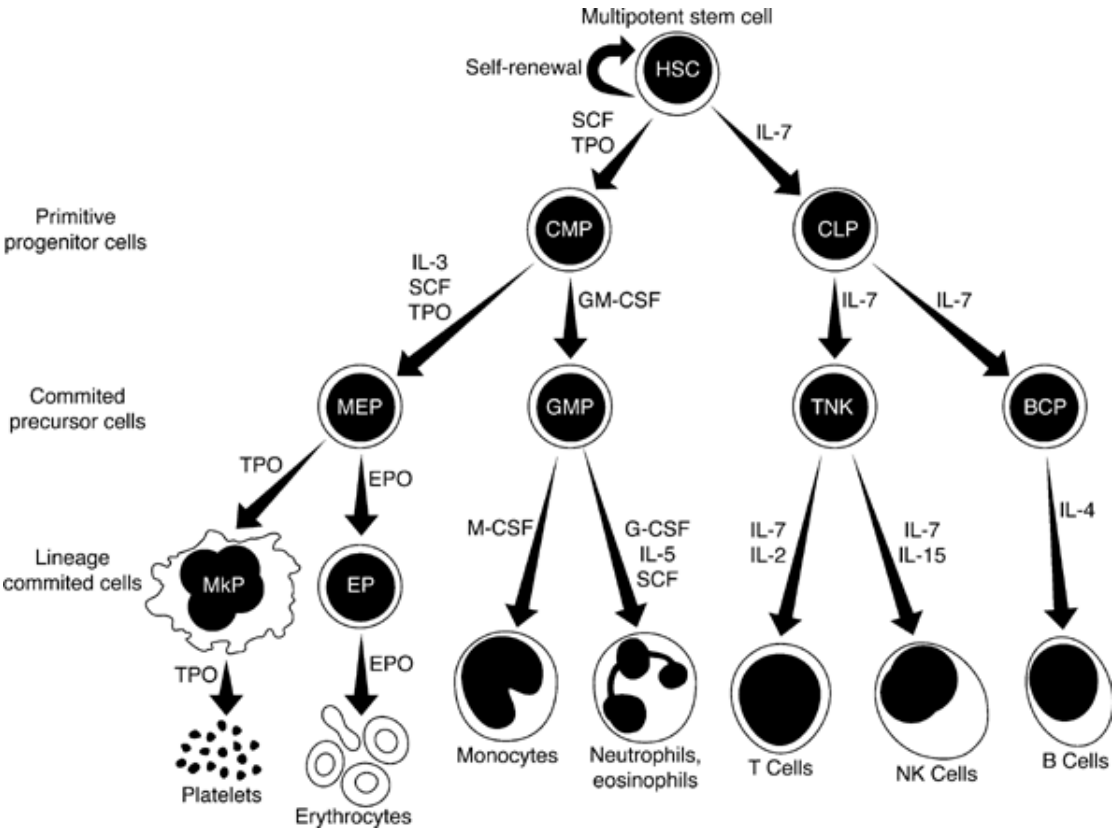


Figure 1.1. Overview of hematopoiesis and the role of cytokines *in vivo*. HSC, hematopoietic stem cells; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte erythroid progenitor; GMP, granulocyte-macrophage progenitor; MkP, megakaryocyte progenitor; EP, erythroid

progenitor; TNK, T-cell natural killer cell progenitor; BCP, B-cell progenitor. IL, interleukin; SCF, stem cell factor; TPO, thrombopoietin; EPO, erythropoietin; GM-CSF, granulocyte-macrophage-colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; M-CSF, macrophage-colony stimulating factor. (Robb, 2007).

1.2.2 Monocytes / Macrophages

Macrophages have long been considered to be important immune effector cells. 100 years ago (1908), Elie Metchnikoff who won the Nobel Prize for his description of phagocytosis, proposed that the key to immunity was to "stimulate the phagocytes" (Gordon, 2008). Macrophages are present in virtually all tissues. During monocyte development, GMPs sequentially give rise to monoblasts, pro-monocytes and finally monocytes, which are released from the bone marrow into the bloodstream. Monocytes migrate from the blood into tissues to replenish long-lived tissue-specific macrophages of the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum (Gordon and Taylor, 2005).

In innate immunity, resident macrophages provide immediate defense against foreign pathogens and coordinate leukocyte infiltration (Martinez *et al.*, 2008). Macrophages contribute to the balance between antigen availability and clearance through phagocytosis and subsequent degradation of apoptotic cells, microbes and possibly neoplastic cells (Gordon, 2003). In adaptive immunity, macrophages collaborate with T and B cells, through both cell-to-cell interactions and fluid phase-mediated

mechanisms, based on the release of cytokines, chemokines, enzymes, arachidonic acid metabolites, and reactive radicals (Gordon, 2003; Martinez *et al.*, 2008).

Macrophages display remarkable plasticity and can change their physiology in response to environmental cues. These changes can give rise to different populations of cells with distinct functions. Now, it is commonly accepted that activated macrophages can be classified into two main groups (Fig. 1.2 A): classically activated macrophages (or M1), whose prototypical activating stimuli are IFN-γ and LPS; and alternatively activated macrophages (or M2), further subdivided into M2a (after exposure to IL-4 or IL-13), M2b (immune complexes in combination with IL-1β or LPS) and M2c (IL-10, TGF-β or glucocorticoids) (Martinez et al, 2008). M1 macrophages exhibits potent microbicidal properties and promote strong IL-12-mediated Th1 responses, whilst M2 macrophages support Th2-associated effector functions. Beyond infection, M2-polarized macrophages play a role in the resolution of inflammation through high endocytic clearance capacities and trophic factor synthesis, accompanied by reduced pro-inflammatory cytokine secretion (Martinez et al, 2008). In the tumor microenvironment, M2 macrophages could be redirected to tumor-associated macrophages (TAM), which are believed to facilitate tumor growth, angiogenesis and metastasis by secreting various growth factors and suppressing the anti-tumor immune responses (Bingle *et al.*, 2002; Lin and Pollard, 2004).

More recently, a new grouping of macrophage populations has been suggested, based on the three different homeostatic activities – host defense, would healing and immune regulation (Fig. 1.2 B) (Mosser and Edwards, 2008). It is proposed that similarly to primary colors these three basic macrophage populations can blend into various other "shades" of activation. Compared to the linear polar classification of M1 / M2, this classification better illustrates how macrophages can evolve to exhibit characteristics that are shared by more than one macrophage population. The concept of "regulatory macrophages" is raised in this new classification. Regulatory macrophages, which are anti-inflammatory, can arise following innate or adaptive immune responses. This population of macrophages produces high levels of the immunosuppressive cytokine IL-10, and also downregulates IL-12 production (Gerber and Mosser, 2001). Therefore, the ratio of IL-10 to IL-12 could be used to define regulatory macrophages. Unlike wound-healing macrophages, these regulatory macrophages do not contribute to the production of extracellular matrix, and many of these regulatory cells express high levels of costimulatory molecules (CD80 and CD86) and therefore can present antigens to T cells (Edwards *et al.*, 2006). So, there are clear functional, as well as biochemical differences between regulatory and wound-healing macrophages.

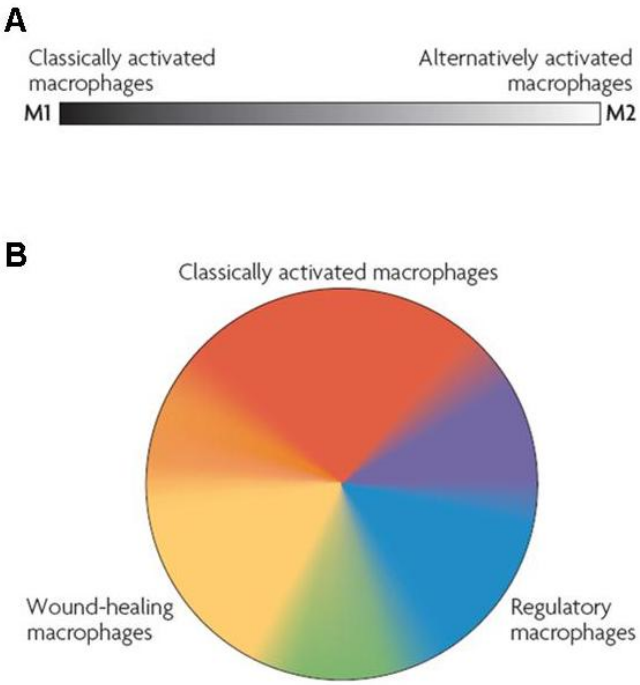


Figure 1.2. Classification of macrophage populations. (A) A monochromatic depiction of the nomenclature showing the linear scale of the two macrophage designations, M1 and M2. **(B)** The three populations of macrophages that are classified based on the three different homeostatic activities – classically activated macrophages, would-healing macrophages, and regulatory macrophages. (Mosser and Edwards, 2008).

1.2.3 Myeloid dendritic cells

Dendritic cells (DCs), the so-called "conductor of the immune orchestra", are professional antigen presenting cells (APCs) endowed with the unique capacity to activate naive T cells. DCs also have important effector functions during innate immune responses, such as pathogen recognition and cytokine production. Therefore, DCs are a bridge between innate and adaptive immunity.

Similar as monocytes and macrophages, bone marrow derived myeloid DCs (also called conventional DCs) consist of a complex and heterogeneous population of cells

with distinctive stages of cell development, activation and maturation. Various subsets endowed with specific pathogen recognition mechanisms, locations, phenotypes, and functions have been described. DCs can be immunogenic or tolerogenic (Steinman *et al.*, 2003; Morelli and Thomson, 2007; Rossi and Young, 2005). The immunogenic DCs, in the adaptive immunity, influence the type of subsequent immune response by secreting specific cytokines. For example, IL-12 produced by DCs favors a Th1 response that is highly effective against viral infections and neoplastic cells; while IL-4 production drives T cell polarization towards a Th2 response that is more advantageous during infections by extracellular bacteria (Steinman, 2003). Differing from their immunogenic counterparts, tolerogenic DCs can sufficiently suppress the activities of effector T cells and induce regulatory T cells (Menges *et al.*, 2002). Thus, they are important in keeping the balance of the immune system by preventing harmful autoimmune responses and excessive inflammation and tissue damage during anti-pathogen immune responses. In spite of their regulatory roles, however, the tolerogenic DCs sometimes can render the immune system unresponsive to infections or malignancies and hence cause pathogenesis.

1.2.4 Granulocytes

Granulocytes are essential cells of the innate immune system. As neutrophil and eosinophil granulocytes they form the first defense line against bacteria and multicellular parasites, respectively. Through release of cytotoxic and inflammatory mediators granulocytes participate in the elimination of pathogens, recruitment of

additional immune cells and perpetuation of the inflammatory reaction (Hogan *et al.*, 1988). The activity of granulocytes is partly regulated via their life span which is short under normal conditions. Neutrophils, which constitute about 95% of all granulocytes, have a half life of a just few hours in circulation. At sites of inflammation proinflammatory cytokines such as G-CSF, GM-CSF, TNF and IFN-γ extend the life span of granulocytes by preventing apoptosis (Simon *et al.*, 1997; Savill, 1997). Numbers of granulocytes can also be increased by enhancing the proliferation rate of hematopoietic progenitor cells and their differentiation rate to granulocytes. G-CSF is the single most important factor for inducing the generation of new granulocytes from bone marrow. G-CSF is also used to treat neutropenia induced by cancer chemo or radiation therapy (Moore, 1990).

1.2.5 Myeloid derived suppressor cells

The myeloid derived suppressor cells (MDSC) are a group of myeloid cells comprised of precursors of macrophages, granulocytes, dendritic cells and myeloid cells at earlier stages of differentiation (Talmadge, 2007). The accumulation of MDSC in lymphoid organs, tumor masses and peripheral blood has been observed in tumor-bearing individuals and is often associated with large tumor burdens. In mice these cells are broadly defined as $CD11b^{+}Gr\text{-}1^{+}$ cells. They have a very rapid turnover and accumulate in large numbers in lymphoid tissues of tumor-bearing mice as well as in mice with infectious diseases, sepsis, and trauma (Delano *et al.*, 2007; MacDonald *et al.*, 2005; Youn *et al.*, 2008). MDSC have also been described in cancer patients. In

a recent study circulating MDSC were found to be significantly increased in cancer patients of all stages relative to healthy volunteers. A significant correlation between circulating MDSC and clinical cancer stage was also observed. Among stage IV patients, those with extensive metastatic tumor burden had the highest percentage and absolute number of MDSC (Diaz-Montero *et al.*, 2009). The main feature of these cells is their ability to suppress T cell responses in Ag-specific or nonspecific manners depending on the condition of T cell activation (Youn et al., 2008; Movahedi *et al.*, 2008). MDSCs can suppress T cell sensitization in tumor-draining lymph nodes *in vivo* (Watanabe *et al.*, 2008). Furthermore, it is reported that MDSC quickly differentiate to tumor-associated macrophages (TAM) (Serafini and Bronte, 2007; Mantovani *et al.*, 2009). Sinha and colleagues have demonstrated that cross-talk between MDSC and macrophages further subverts tumor immunity by increasing MDSC production of IL-10, and by decreasing macrophage production of IL-12 (Sinha *et al.*, 2007). Therefore, MDSCs are now considered as one of the major factors responsible for tumor associated immune defects and are an attractive target for therapeutic intervention.

1.3 CD137

1.3.1. Expression of CD137

CD137 (TNFRSF9 / 4-1BB / ILA), is a member of the tumor necrosis factor (TNF) receptor family, and it has originally been identified as a potent T cell costimulatory

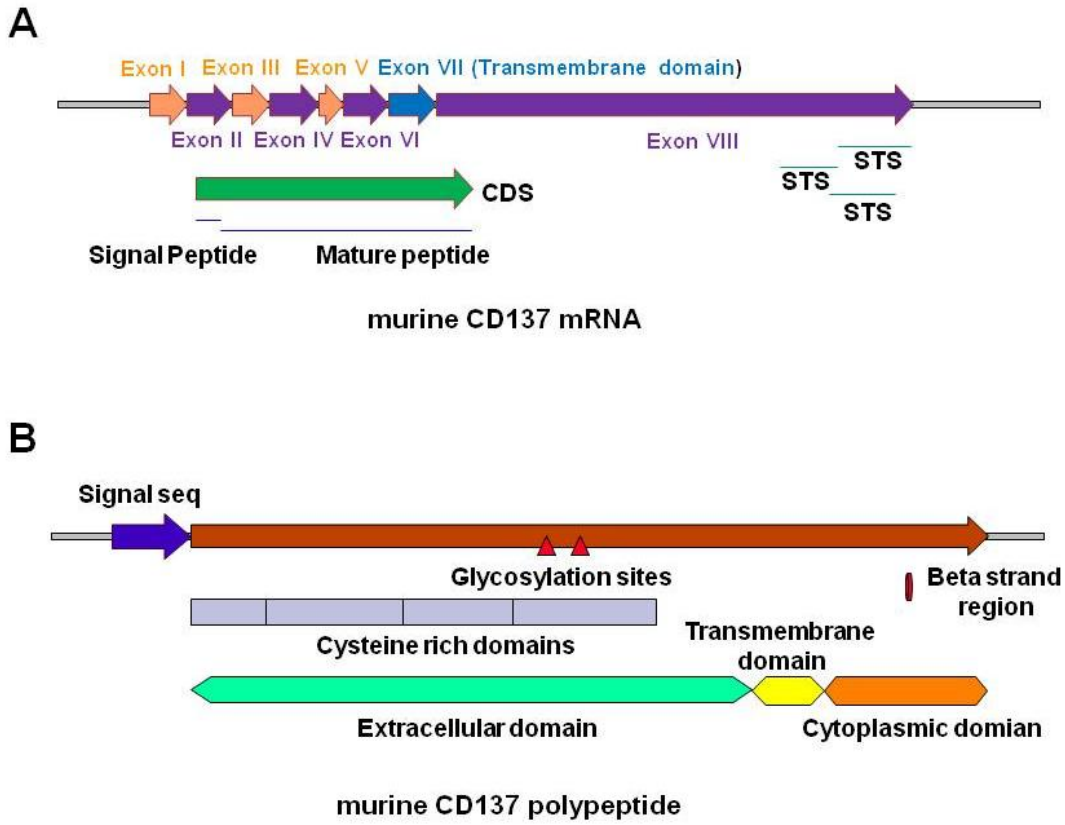
molecule (Kwon *et al.*, 1989; Schwarz *et al.*, 1993). It is a type I transmembrane glycoprotein expressed by activated T cells (Schwarz *et al.*, 1995, 1996), activated NK cells (Melero *et al.*, 1998), monocytes (Kienzle *et al.*, 2000), follicular dendritic cells (FDCs) in germinal centres (Pauly *et al.*, 2002), and a small fraction of neutrophils (Simon, 2001). Its expression on primary immune cells is strictly activation-dependent and transient (Schwarz *et al.*, 1995), and is involved in the regulation of multiple and diverse types of immune responses (Watts, 2005).

Besides immune cells, primary articular chondrocytes express CD137 after stimulation by proinflammatory factors (von Kempis *et al.*, 1997). The walls of blood vessels at sites of inflammation (Drenkard *et al.*, 2007) and in certain cancer cells (Lisignoli *et al.*, 1998; Ringel *et al.*, 2001).

1.3.2 Structure of CD137

The murine *CD137* gene is located on chromosome 4 (Kwon *et al.*, 1989). The human *CD137* gene has been mapped to chromosome band 1p36, where the genes of four other members of the TNFRSF, *TNFRII, CD30, OX40* and *TRAMP/Apo3,* are also found (Schwarz *et al.*, 1993). Both human and murine CD137 are made up of eight exons. Exon VII encodes the transmembrane domain (Fig. 1.3 A, C). A smaller mRNA isoform lacking this exon is generated by differential splicing, and leads to the production of soluble CD137 (sCD137) (Setareh *et al.*, 1995; Michel *et al.*, 1998).

Murine and human CD137 proteins (Fig. 1.3 B, D) are 60% homologous and have similar topological structures. CD137 protein is comprised of a signal peptide, an extracellular domain, a transmembrane domain, and a cytoplasmic domain, which is necessary for signal transduction into the cell. Within the extracellular region lie four cysteine-rich domains (CRDs), which are characteristic for TNFR superfamily members. In the cytoplasmic domain, five regions are conserved between mouse and man, indicating that they might be important for the function of CD137. All the structural features of human CD137 protein are listed in Table 1.



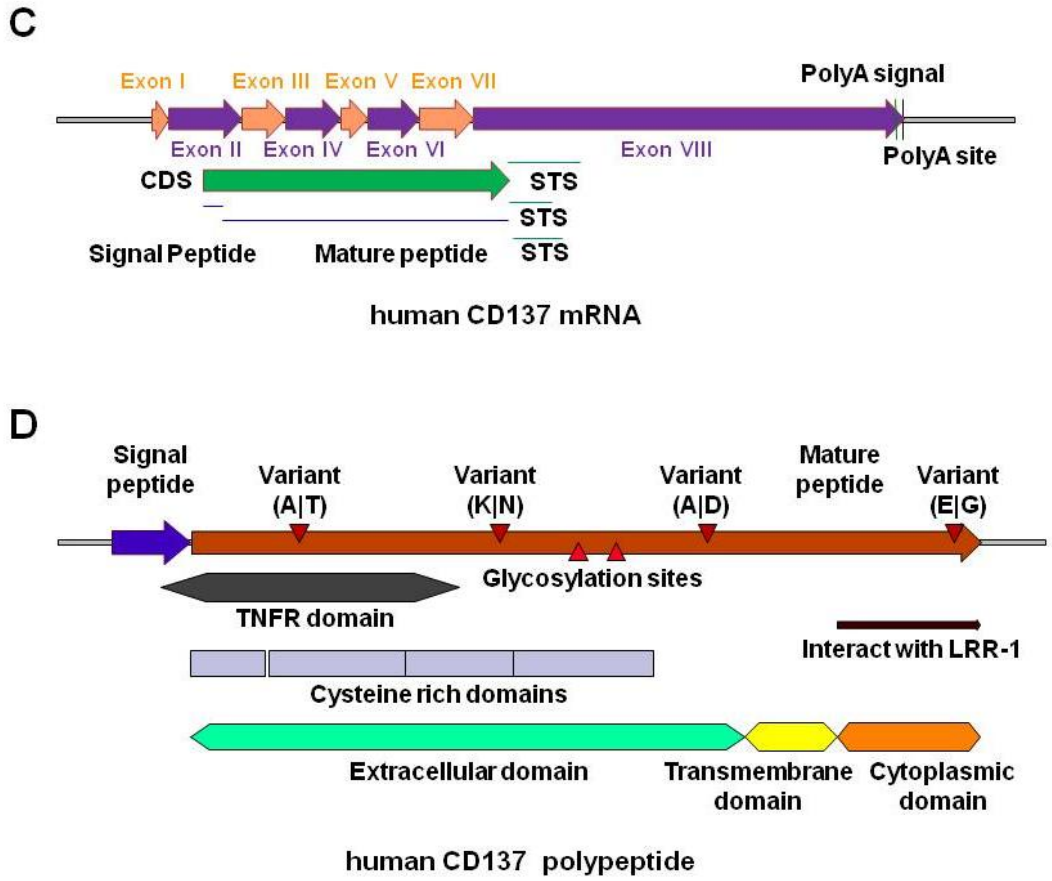


Figure 1.3. Schematic diagram of the structure of murine and human CD137 mRNA and protein. (A) murine CD137 mRNA, accession number: NM_011612; **(B)** murine CD137 protein, accession number: P20334; **(C)** human CD137 mRNA, accession number: NM_001561; and **(D)** human CD137 protein, accession number: Q07011. STS, sequence-tagged site; CDS, coding sequence. All the information of sequences and primary structures is from NCBI Entrez Nucleotide and Entrez Protein databases (As in May 2009).

Table 1. Structure features of human CD137 protein.

Position (amino acid)	Structure feature
1-23	signal peptide
24-255	mature peptide
19-106	TNFR domain
24-186	extracellular domain
187-213	transmembrane domain
214-255	cytoplasmic domain, interaction with LRR-1
24-45	cysteine rich domain
47-86	cysteine rich domain
87-118	cysteine rich domain
119-159	cysteine rich domain

29-36, 43-47, 79-81	protein binding site
19, 29, 33, 35, 44, 47, 57	parallel homodimerization interface
53, 63, 72, 94, 102	antiparallel homodimerization interaface
56-57, 62,64	50's loop TNF binding site what is that?
94, 102-106	90's loop TNF binding site
138, 149	glycosylation site
56	variant (A → T)
115	variant (K → N)
176	variant (A → D)
250	variant (E → G)
28, 37	disulfide bond
31, 45	disulfide bond
48, 62	disulfide bond
65, 78	disulfide bond
68, 86	disulfide bond
88, 94	disulfide bond
99, 106	disulfide bond
102, 117	disulfide bond
121, 133	disulfide bond
139, 158	disulfide bond

LRR-1: Leucine rich repeat protein.

1.3.3 Biological functions of CD137

High levels of CD137 can be found on activated T cells, and crosslinking of CD137 delivers potent costimulatory signals to T cells. Recombinant CD137 ligand or agonistic anti-CD137 antibodies enhance both T cell and NK cell activities, such as vigorous proliferation and IFN-γ secretion (Melero *et al.*, 1997, 1998; Wilcox *et al.*, 2002). These enhaced immune responses lead to induce complete or partial regression of established tumors in various mouse models (Sica and Chen, 2000; Croft, 2003; Watts, 2005; Zhu *et al.*, 2009). Humanized anti-CD137 antibodies are currently undergoing phase I clinical trials for cancer immunotherapy (Hong *et al.*, 2000; Son *et al.*, 2004).

The CD137 signal to T cells is able to substitute the CD28 costimulatory signal, and CD137 agonists have been proven to be effective immune stimulators, leading to elimination of mastocytoma, sarcoma, colon carcinoma and melanoma in mice (Croft, 2003; Sica *et al.*, 2000; Watts, 2005).

Stimulation of CD137 also enhances protective immune responses against pathogen infections. Agonistic anti-CD137 antibodies have been shown to enhance the efficacy of vaccines against influenza and poxvirus (Halstead *et al.*, 2002; Munks *et al.*, 2004). Inclusion of a CD137 ligand-expressing vector in a vaccine, or engineered expression of CD137 ligand on monocytes enhanced their ability to induce anti-HIV responses (Harrison *et al.*, 2006; Wang *et al.*, 2007). Therefore, the induction of CD137 expression on immune cells is part of the anti-pathogen immune response.

Interestingly, CD137 agonists can also dampen immune responses and ameliorate autoimmune diseases under certain conditions (Mittler, 2004; Myers *et al.*, 2005). The underlying mechanism is still not elucidated.

A few studies have investigated the role of CD137 in granulocytes. Neutrophils from human and mouse are found to constitutively express CD137 (Simon, 2001; Lee *et al.*, 2005). In human, CD137 expression on eosinophils could be observed in patients suffering from IgE-mediated allergic responses, but not in normal subjects or those patients suffering from non-IgE-mediated eosinophilic disorders (Heinisch *et al.*,

2001). In both neutrophils and eosinophils, CD137 stimulation promotes apoptosis in these cells, even in the presence of the survival factors GM-CSF and / or IL-5 (Heinisch *et al.*, 2000). In mouse, however, ligation of CD137 on neutrophils by anti-CD137 antibody was reported to induce intracellular Ca^{2+} influx, and to enhance the reactive oxygen species generation and phagocytic activities, which was very important for the host defense against intracellular pathogens such as *Listeria monocytogenes* (Lee *et al.*, 2005). Nishimoto *et al.* found CD137 expression is inducible in mast cells upon the stimulation through the high-affinity receptor for IgE (FcεRI). Agonistic anti-CD137 antibodies can enhance FcεRI-induced cytokine production and secretion from mast cells (Nishimoto *et al.*, 2005). Despite the discrepancy, the consensus is that CD137 stimulation seems to play an important role in regulating granulocyte function during the initiation and resolution of an inflammatory response.

1.3.4 Diseases associated with CD137

CD137 is expressed as a neoantigen by several types of tumor cells, such as in Reed-Sternberg cells in Hodgkin's lymphoma (personal communication, Schwarz H), osteosarcoma (Lisignoli *et al.*, 1998), rhabdomyosarcoma (personal communication, Schwarz H) and pancreatic cancer (Ringel *et al.*, 2001). Elevated sCD137 levels have been reported in sera of patients with chronic lymphocytic leukemia (CLL) (Furtner *et al.*, 2005).

CD137 in both membrane-bound form and soluble form is also reported to be involved in or associated with some autoimmune diseases, such as herpetic stromal keratitis (HSK) (Seo *et al.*, 2003), rheumatoid arthritis (RA) (Michel *et al.*, 1998; Seo *et al.*, 2004), multiple sclerosis (MS) (Sharief, 2002; Jung *et al.*, 2004), systemic lupus erythematosus (SLE) and Behcet's disease (Shao *et al.*, 2008; Jung *et al.*, 2004).

1.4 CD137 ligand

1.4.1 Expression of CD137 ligand

CD137 ligand (CD137L / TNFSF9 / 4-1BBL), also known as CD137 counter-receptor, is a type II membrane protein of the TNF superfamily (Goodwin *et al.*, 1993; Alderson *et al.*, 1994). CD137L is expressed mainly on APCs, including B cells, DCs and monocytes/macrophages. Human and murine transformed B cells express CD137L protein constitutively while activation may be required for primary B cells (Pollok *et al.*, 1994; Zhou *et al.*, 1995; DeBenedette *et al.*, 1997; Palma *et al.*, 2004). CD137L is also expressed constitutively on peripheral monocytes and monocyte/macrophage cell lines (Pollok *et al.*, 1994; Futagawa *et al.*, 2002; Ju *et al.*, 2003; Laderach *et al.*, 2003). In DCs, CD137L is expressed at low levels in both the murine and human system. However, it can be enhanced by proinflammatory stimuli, including IL-1, CD40 ligand, LPS and double stranded RNA (Futagawa *et al.*, 2002; Laderach *et al.*, 2003; Kim *et al.*, 2002; Lee *et al.*, 2003). Progress in understanding the CD137L biology has not gained much momentum as that of its counterpart,

CD137.

1.4.2 Structure of CD137L

The gene for CD137L is located on chromosome 17 in mouse (Goodwin *et al.*, 1993) and on chromosome 19p13.3 in man (Alderson *et al.*, 1994), (Fig. 1.4 A, C). The CD137 protein has a TNF domain, whose tertiary structure is very similar to that of TNF and lymphotoxin (LT)-α (Fig. 1.4 B, D). Like other members of the TNF superfamily, it is present in a trimeric or oligometric form on cell surfaces (Rabu *et al.*, 2005).

Sequence analysis of the human CD137L protein has revealed only 36% aminoc acid homology to the mouse CD137L, compared to 70–80% of homology between human and murine ligands reported for other members of the TNF family (Alderson *et al.*, 1994). In addition, there is almost no homology of human CD137L to other members of the human TNF cytokine family (Smith *et al.*, 1994). Chalupny *et al.* reported that, besides CD137, a murine CD137-Fc fusion protein could bind to various extracellular matrix proteins, including fibronectin, vitronectin, laminin, and collagen VI (Chalupny *et al.*, 1992). All this information suggests that an alternative ligand may exist.

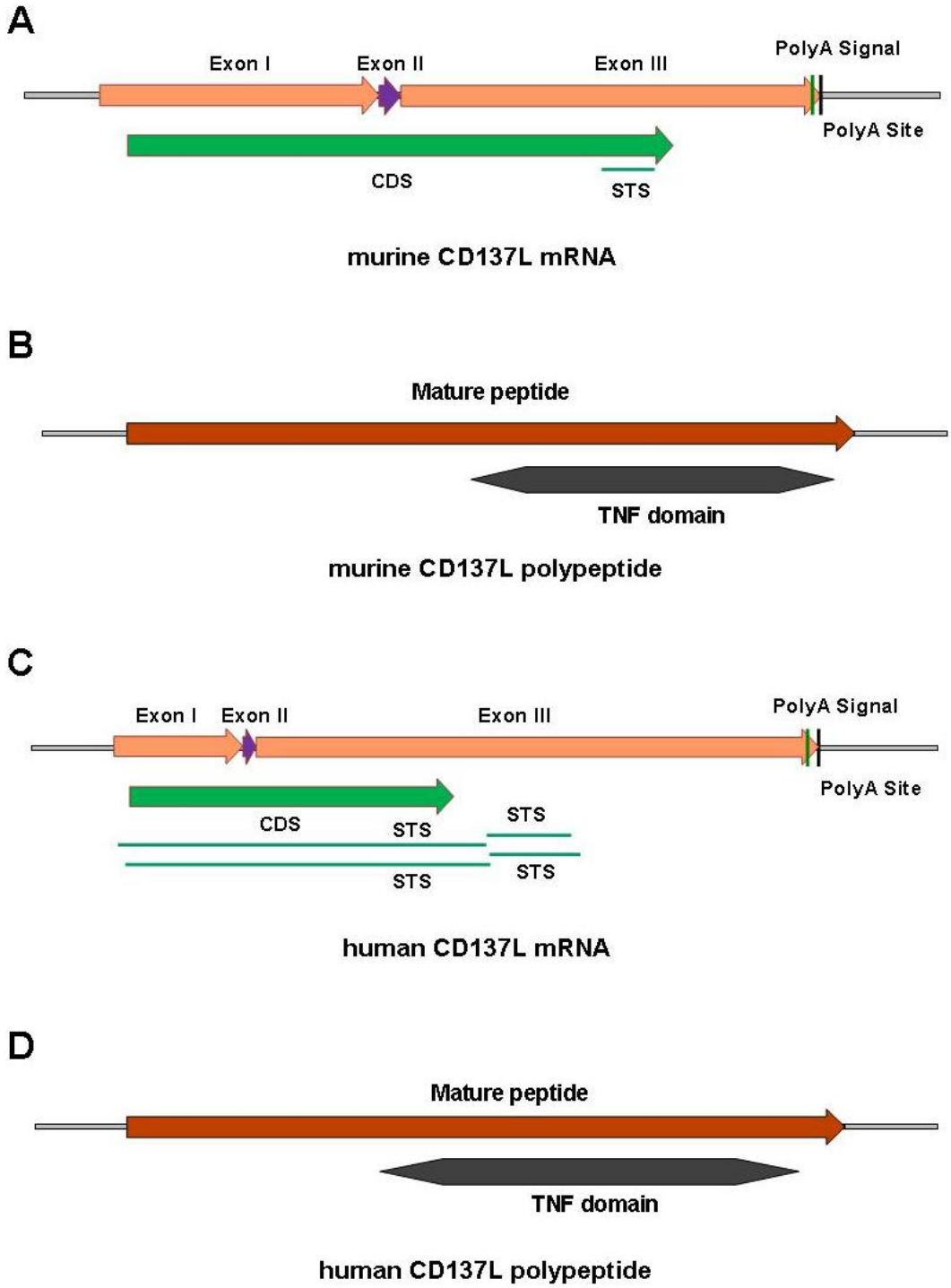


Figure 1.4. Schematic diagram of the structure of murine and human CD137L mRNA and protein. (A) murine CD137L mRNA, accession number: NM_009404; **(B)** murine CD137L protein, accession number: NP_033430; **(C)** human CD137L mRNA, and accession number: NM_003811; **(D)** human CD137L protein, accession number: NP_003802. STS, sequence-tagged site; CDS, coding sequence. All the information of sequences and primary structures is from NCBI Entrez Nucleotide and Entrez Protein databases (As in May 2009).

1.4.3 Bidirectional signaling of CD137 receptor / ligand system

The CD137 receptor / ligand system has the ability to signal bidirectionally. CD137L is expressed as a type II transmembrane protein and it too can transduce signals into the cells it is expressed on, a process referred to as reverse signaling or bidirectional signal transduction (Schwarz, 2005). The ability of reverse signaling is common among other members of TNFR and TNF families (Eissner *et al.*, 2004). During APC-T cell interactions, the signals through CD137L are activating or costimulatory for APCs. Together with CD137, which can deliver costimulatory signals to T cells, the bidirectional signals escalate the activation process for both APCs and T cells and therefore, form a potent proinflammatory system (Fig. 1.5).

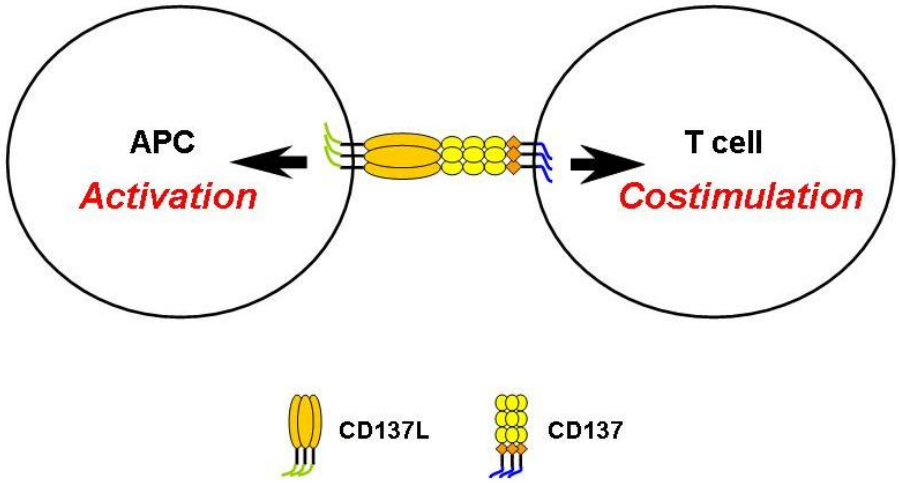


Figure 1.5. Schematic diagram of bidirectional signal transduction of the CD137 receptor / ligand system. (Schwarz, 2005).

1.4.4 Biological functions of reverse signaling through CD137L

The biological functions of reverse signaling through CD137L in hematopoietic cells are summarized in Table 2.

1.4.4.1 Reverse signaling through CD137L in monocytes

Reverse signaling through the CD137L in monocytes has been well studied. Crosslinking of the CD137L on peripheral monocytes induces a specific signaling cascade involving protein tyrosine kinases (PTK), p38 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)-1/2, phosphoinositide -3-kinase (PI3K) and protein kinase A (PKA) (Sollner *et al.*, 2007). Signaling through CD137L leads to activation of the monocytes with induction of adherence, morphological changes and enhanced expression of proinflammatory cytokines (TNF, IL-6, IL-8 and IL-12) and activation markers (ICAM-1). Concurrently, it inhibits the expression of anti-inflammatory cytokines (IL-10) and differentiation markers (FcyRIII) in monocytes (Langstein *et al.*, 1998, 2000; Laderach *et al.*, 2003). In addition, it significantly prolongs monocyte survival via induction of M-CSF (Langstein *et al.*, 1999b), and it induces a profound proliferation and endomitosis of human peripheral monocytes (Langstein *et al.*, 1999a). The potency of CD137L in stimulating proliferation of monocytes is profoundly higher than that of other monocyte growth factors such as M-CSF or GM-CSF (Langstein *et al.*, 1999a; Ju *et al.*, 2003). Recently, the activating effects of CD137L on monocytes have been confirmed in vivo. Transgenic mice overexpressing CD137 ligand on APC develop a threefold increased number of macrophages (Zhu *et al.*, 2001). CD137-deficient mice on the other hand have an increased number of myeloid progenitor cells in the peripheral blood, bone marrow, and spleen (Kwon *et al.*, 2002). It seems these cells cannot fully mature to monocytes, macrophages, and possibly dendritic cells due to

the lack of CD137L signaling.

CD137L provides potent migration signals to peripheral monocytes (Drenkard *et al.*, 2007). Since the expression of CD137 is strictly activation-dependent, monocytes would encounter CD137 in tissues where activated immune cells or endothelial cells are present (Schwarz *et al.*, 1995; Drenkard *et al.*, 2007; Broll *et al.*, 2001). Therefore, reverse signaling by CD137L into monocytes is expected to induce proliferation, activation, and migration, i.e. amplifying and regulating an ongoing immune response.

1.4.4.2 Reverse signaling through CD137L in DCs

DCs are professional and potent APCs in the initiation of an immune response and adaptive immunity. CD137L is expressed at low levels on murine and human DCs, which were derived *in vitro* from monocytes or hematopoietic progenitor cells (Laderach *et al.*, 2003; Kim *et al.*, 2002; Futagawa *et al.*, 2002). Crosslinking of CD137L on DCs enhances the expression of CD11c, CD80, CD86, MHC class II, and CCR7, and induces cellular adherence and the release of IL-6, IL-12, and TNF (Kim *et al.*, 2002; Futagawa *et al.*, 2002; Laderach *et al.*, 2003; Lippert *et al.*, 2008). CD137L-activated DCs are able to induce the proliferation of autologous T cells in antigen-specific manner, the release of IL-12p70 and IFN-γ, and the differentiation to potent Th1 effectors (Lippert *et al.*, 2008). This implies that CD137L signaling induces the maturation, activation and migration of immature DCs. The

cross-linking of CD137L and CD137 allows simultaneous costimulation of T cells and DCs when they interact, thereby endowing the CD137 receptor / ligand system with potent proinflammatory activities.

Interestingly, Wilcox *et al.* also reported that freshly isolated murine splenic DCs and bone marrow-derived DCs express CD137 on the cell surface and in soluble form. Triggering CD137 increased the secretion of IL-6 and IL-12 from DCs and enhanced the ability of DCs to stimulate T cell proliferation *in vitro* (Wilcox *et al.*, 2002). This implies that DCs can express both CD137L and CD137 on their surface, and both signals are activating. However, it is not clear whether both signaling can occur at the same time and whether there is a positive feedback loop in the DCs that express both CD137L and CD137.

The physiological function of CD137L signal in DCs is an induction or enhancement of their antigen-presenting capacity, and consequently an initiation or enhancement of immune response. However, it is still controversial whether CD137L signal alone is sufficient to induce DC activation or whether it only works in combination with additional signals such as CD40L. In addition, it is still unclear whether CD137L alone can induce DC differentiation directly from monocytes or hematopoietic progenitors.

1.4.4.3 Reverse signaling through CD137L in B cells

Human and murine transformed B cells express CD137L protein constitutively while activation may be required for primary B cells (Pauly *et al.*, 2002; Pollok *et al.*, 1994). Unlike in the case of monocytes and DCs, signals through CD137L do not initiate activation of B cells. Rather, they enhance proliferation and immunoglobulin synthesis of preactivated B cells (Pauly *et al.*, 2002; Pollok *et al.*, 1994). In contrast to these *in vitro* data, constitutive expression of CD137L on APC in transgenic mice causes a gradual elimination of peripheral B cells (Zhu *et al.*, 2001). This could indicate that initially the signals through CD137L are activating for B cells, while prolonged CD137L signals may be deleterious for B cells, possibly due to the activation induced cell death. Indeed, it has been shown that crosslinking of CD137 on monocytes causes them to induce apoptosis in B cells (Kienzle *et al.*, 2000). The findings of CD137 expression on follicular DCs in germinal centers support CD137L-mediated B cell activation. The expression of CD137 on follicular DCs provide costimulatory and survival signals to those B cells in the germinal centers, which have rearranged their immunoglobulin genes resulting in a high affinity binding to the antigen (Pauly *et al*., 2002; Lindstedt *et al.*, 2003). This implicates that the CD137 / CD137L system plays a role in B cells affinity maturation.

1.4.4.4 Reverse signaling through CD137L in bone marrow cells

The expression of CD137L and CD137 in the bone marrow (Saito *et al.*, 2004; Jiang *et al.*, 2008a, 2008b; Lee *et al.*, 2008) suggests CD137L signaling takes place not only in differentiated hematopoietic cells but also in bone marrow progenitor cells.

Proliferation of murine bone marrow cells treated with M-CSF is enhanced when cells are exposed to immobilized CD137 protein (Saito *et al.*, 2004). CD137L signals also inhibit RANK ligand-induced differentiation of bone marrow cells towards osteoclasts (Saito *et al.*, 2004; Shin *et al.*, 2006a, 2006b). These data indicate that CD137L signals direct differentiation of bone marrow cells away from osteoclast and towards the myeloid lineage. However, total bone marrow cells are unable to provide a clean system to study the effects of CD137 ligand signaling in HSPCs, because bone marrow cells are a mixture of various cell populations, including those differentiated APCs mentioned above.

1.4.4.5 Reverse signaling through CD137L in T cells

Reverse signaling also takes place in T cells, and contrary to the situation in APCs, the CD137L signal has been shown to inhibit proliferation and to induce apoptosis in human T cells (Schwarz *et al.*, 1996; Ju *et al.*, 2003; Michel *et al.*, 1999). A recent study however, found that CD137L signaling in murine T cells induces IFN-γ release, contributing to immune deviation and an inhibition of Th2-mediated allergic lung inflammation (Polte *et al.*, 2007).

In conclusion, except in T cells, the reverse signaling through CD137L into APCs and probably their progenitors leads to activation, maturation and even proliferation. This leads to the initiation or amplification of immune responses. Together with the

costimulatory signal through CD137 into T cells, the bidirectional signaling of the CD137 receptor / ligand system allows a more coordinated immune response and its fine-tuning.

To date, very limited data are available that support a role of CD137L signaling in hematopoiesis. In fact, some other members of the TNF family have already been shown to regulate survival and expansion of hematopoietic precursor cells. According to Greil and colleagues, CD95 and TNF related apoptosis inducing ligand (TRAIL) are of critical importance for lymphopoiesis and myelopoiesis (Greil *et al.*, 2003). TNF can induce or inhibit hematopoiesis, depending on its dose and its interaction with other hematopoietic growth factors (Jacobsen *et al.*, 1994).

Table 2. Biological functions of reverse signaling through CD137L in hematopoietic cells.

Cell type	Species	Activity	System	References
Monocytes	Human	Induction of TNF, IL-6, IL-8, IL-12, ICAM, and adherence. Inhibition of FcγRIII and IL-10	*In vitro*	Langstein *et al.*, 1998, 2000; Laderach *et al.*, 2003
	Human	Induction of M-CSF and prolongation of survival	*In vitro*	Langstein *et al.*, 1999b
	Human	Induction of proliferation and endomitosis	*In vitro*	Langstein *et al.*, 1999a; Ju *et al.*, 2003
	Human	Enhanced migration and extravasation.	*In vivo*	Drenkard *et al.*, 2007
	Mouse	Amplification of cell numbers	CD137L-trg mice	Zhu *et al.*, 2001
Macrophages	Mouse	Downstream of TLR, sustained TNF production	CD137L-trg mice	Kang *et al.*, 2007
Dendritic cells	Human	Maturation, induction of CD11c, CD86, CD137L, MHC-II, IL-12, and cellular adherence	*In vitro*	Laderach *et al.*, 2003; Kim *et al.*, 2002
	Mouse	Induction of IL-6, IL-12, CD80 and CD86	*In vitro*	Futagawa *et al.*, 2002
	Human	Maturation by induction of TNF-α, migration by induction of CCR7, induce T cell proliferation and release of IL-12p70 and IFN-γ	*In vitro*	Lippert *et al.*, 2008
B cells	Human	Costimulation of proliferation and Ig secretion	*In vitro*	Pauly *et al.*, 2002
	Mouse	Costimuation of proliferation	*In vitro*	Pollok *et al.*, 1994
	Mouse	Elimination of peripheral B cells (after 3 months)	CD137L-trg mice	Zhu *et al.*, 2001
Bone marrow cells	Mouse	Induction of proliferation	*In vitro*	Saito *et al.*, 2004
	Mouse	Inhibition of osteoclast differentiation	*In vitro*	Saito *et al.*, 2004; Shin *et al.*, 2006a
	Mouse	Enhanced osreoclastogenesis caused by reduced IL-10	CD137-def mice	Shin *et al.*, 2006b
T cells	Human	Inhibition of proliferation and induction of apoptosis	*In vitro*	Schwarz *et al.*, 1996; Ju *et al.*, 2003

IL: interleukin, ICAM: intercellular adhesion molecule, FCγR: Fc receptor for IgG, M-CSF: macrophage-colony stimulating factor, CD137L-trg: CD137 ligand-transgenic, MHC: major histocompatibility complex, CD137-def: CD137-deficient. Modified from Schwarz, 2005.

1.5 Scope and objectives of the study

The data of reverse signaling through CD137L in hematopoietic cells described above imply a role of CD137L signaling in hematopoiesis. In addition, preliminary evidence has shown that immobilized recombinant CD137 protein can induce proliferation of human and murine HSPCs. However, this activity of CD137L signaling has not been experimentally proven. This study aims to look at the involvement of CD137L signaling in the proliferation and differentiation of HSPCs in both the murine and the human system.

The specific objectives of this study are,

1. Investigate the effects of CD137 on HSPC proliferation and differentiation in both the murine and the human system;
2. Investigate the lineage and the functions of HSPCs differentiated by CD137;
3. Explore potential synergistic or additive effects of CD137 with other hematopoietic growth factors on HSPC proliferation and differentiation;
4. Identify the CD137L- and CD137-expressing cells in the bone marrow;
5. Investigate the regulation of CD137 expression in the bone marrow.

The data obtained are expected (1) to increase our knowledge of the CD137 / CD137L biology and CD137L signaling in hematopoiesis, (2) to lay the basis for *ex vivo* or *in vivo* expansion of HSPCs with CD137, and (3) to explore the potential to use CD137 as a candidate for future clinical applications in terms of immunotherapy.

CHAPTER 2 MATERIALS AND METHODS

2.1 Preparations of animal and human samples

2.1.1 Mice

BALB/c or C57/BL6 wide type and CD137-deficient mice between 16 and 20 weeks of age were used as a source of bone marrow cells or splenocytes. 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.

2.1.2 Preparation of murine bone marrow cells and splenocytes

BALB/c or C57/BL6 mice were sacrificed 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 complete medium (RPMI1640 (Sigma), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin).

Spleens were aseptically removed from the abdominal cavity and minced through a 40-μm nylon cell strainer (BD Falcon) with a 5-ml syringe core in 10 ml of PBS. Red

blood cells were depleted with Tris-NH_4Cl lysis buffer (0.144 M NH_4Cl, 0.017 M Tris-HCl). Splenocytes were washed with PBS containing 2 mM EDTA and resuspended in complete medium.

2.1.3 Isolation of Lin^-, $CD117^+$ and $Gr\text{-}1^+$ cells from mouse bone marrow cells

The Lin^-, $CD117^+$ progenitor cells were isolated by immunomagnetic separation (MACS) using the mouse lineage cell depletion kit and mouse CD117 selection kit (Miltenyi) following the manufacturer's instructions. Briefly, the fresh bone marrow cells were labeled with a cocktail of biotinylated antibodies against a panel of antigens (CD5, CD45R (B220), CD11b, anti-Ly-6G (Gr-1), 7-4, and Ter119 antibodies) expressed on mature cells, followed by anti-biotin microbeads. The cell suspension was passed through a LD column in a strong magnetic field, and the lineage negative (Lin^-) cells were collected in the effluent. These Lin^- cells were then positively selected for CD117 expression using CD117 microbeads. The purity of Lin^-, $CD117^+$ cells was >90% as determined by flow cytometry using a FITC-conjugated anti-CD117 antibody and an APC-conjugated anti-biotin antibody (Miltenyi).

The $Gr\text{-}1^+$ cells were also isolated by MACS. Briefly, the fresh bone marrow cells were treated with mouse FcR blocker (Miltenyi Biotec) and labeled with biotinylated anti-mouse Gr-1 antibody (Biolegend) followed by anti-biotin microbeads (Miltenyi Biotec). The cell suspension was passed through a LS column (Miltenyi Biotec) in a strong magnetic field. The $Gr\text{-}1^+$ cells were retained in the column. After three washes,

the column was removed from the magnetic field and the Gr-1$^+$ cells were flushed out. The purity of the Gr-1$^+$ cells was >95% as determined by flow cytometry using a PE-conjugated anti-Gr-1 antibody (eBioscience).

2.1.4 Isolation of pan T cells from mouse splenocytes

Splenic T cells were isolated by MACS using the mouse Pan T Cell isolation kit (Miltenyi). 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. The purity of the T cells was >90% as determined by flow cytometry using a PE-conjugated anti-CD3 antibody (eBioscience).

2.1.5 Isolation of CD34$^+$ cells and monocytes from human cord blood

Human umbilical cord blood was obtained from the Singapore Cord Blood Bank and the protocols were approved by IRB (Yong Loo Lin School of Medicine, National University of Singapore). CD34$^+$ cells were isolated from cord blood by immunomagnetic selection (MACS) using the Direct CD34 Progenitor Kit (Miltenyi Biotech). Briefly, the layer of mononuclear cells was obtained from cord blood by Ficoll-Hypaque density (1.077g/dl) (Sigma) gradient centrifugation. These

mononuclear cells were labeled with microbeads which were conjugated to the monoclonal mouse anti-human CD34 antibody, QBEND/10, in the presence of FcR blocking reagent (Miltenyi) to prevent non-specific binding. The magnetic labeled cells were passed through a LS column in a strong magnetic field. The column was then removed from the magnetic field and the retained $CD34^+$ cells were flushed out. The purity of $CD34^+$ cells was >90% as determined by flow cytometry using FITC-conjugated mouse anti-human CD34 monoclonal antibody, AC136 (Miltenyi). The purified $CD34^+$ cells were resuspended in RPMI1640 culture medium (Sigma) supplemented with 10% FBS, 50 μg/ml streptomycin and 50 IU/ml penicillin, or in serum-free expansion medium (SFEM) from StemCell Technologies (Vancouver, British Columbia, Canada).

Monocytes were purified from umbilical cord blood with the monocyte isolation kit II (Miltenyi). The purity was higher than 95% as determined by flow cytometric analysis after CD14 staining.

2.1.6 Intra-peritoneal (i.p.) injection of LPS

8 – 10 weeks old healthy C57/BL6 mice, of around 20 g of weight were divided into three groups (2 mice per group). Dosing solutions of LPS from *E. coli* 0111:B4 (Sigma) were prepared in PBS. Mice were anesthetized with xylazine (5 mg/kg), and then dosed with 100 μL of 1 mg/ml LPS (high dose = 5 mg/kg = 100 μg/mouse) or 0.2 mg/ml LPS (low dose = 1 mg/kg = 20 μg/mouse) or PBS by intraperitoneal (i.p.)

injection. Mice were sacrificed after 24 h. Femur bones were collected, and EDTA decalcified, formalin fixed, paraffin embedded bone sections were used for IHC staining.

2.1.7 Adoptive transfer of T cells

T cells were isolated from spleens of C57/BL6 wide type (WT) or CD137-deficient (CD137-KO) mice by MACS using the Pan T cell isolation kit (Miltenyi). The cells were seeded at a density of $2 – 3 \times 10^6$ per ml per well into 12-well plates that were precoated with 5 μg/ml of anti-mouse CD3 mAb (Clone 17A2) and 5 μg/ml of anti-mouse CD28 mAb (Clone 37.51) or their isotypes, rat IgG2b and Syrian hamster IgG, respectively, both at 5 μg/ml. Cells were harvested after culturing for 48 h, and labeled with CFSE (Invitrogen) at a final concentration of 5 μM. Cells were incubated for 5 min in 2 mM EDTA in PBS to dissociate any aggregates. After washing twice, the CSFE labeled isotype Abs treated or anti-CD3 and anti-CD28 treated T cells from WT or CD137-KO C57/BL6 mice were resuspended in serum-free RPMI medium at 2×10^7/ml. 2×10^6 cells in a volume of 100 μL or 100 μL of serum-free RPMI medium were aseptically i.v. injected into the lateral tail veins of 5 healthy C57/BL6 mice. Mice were sacrificed after 24 h. Bone marrow cells, splenocytes and peripheral blood mononuclear cells were collected and analyzed for CFSE fluorescence by flow cytometry.

2.2 Molecular and biochemical techniques

2.2.1 Recombinant proteins and chemicals

Recombinant human CD137-Fc protein, comprising the extracellular domain of human CD137 and Fc portion of human IgG1 (Fig. 2.1), was purified from supernatants of stable transfected CHO cells by protein G sepharose, as described previously (Schwarz, *et al* 1996). The endotoxin concentration in the CD137-Fc protein is 55 I.U./mg. Recombinant murine CD137-Fc protein, comprising the extracellular domain of murine CD137 and Fc portion of human IgG1, was purchased from R&D systems (Minneapolis, MN USA). Human IgG1 Fc protein was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY, USA). Recombinant murine GM-CSF, IL-4, M-CSF, G-CSF, Flt3L, LIF, SCF and recombinant human GM-CSF, IL-4, M-CSF were purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant human G-CSF and LPS (*E. coli* 0111:B4) were obtained from Sigma (St. Louis, MO, USA).

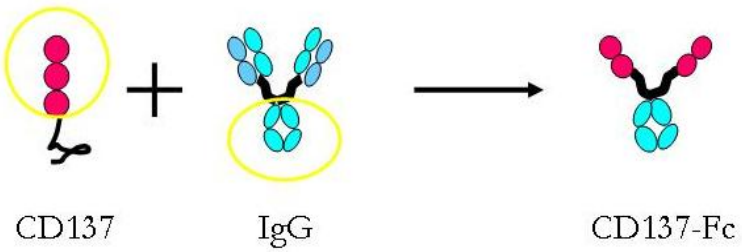


Figure 2.1. Schematic diagram of the structure of recombinant CD137-Fc protein.

2.2.2 RT-PCR

Total RNA from murine bone marrow cells was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed in a Mastercycler® gradient thermal cycler (Eppendorf, Hamburg, Germany). cDNA was reverse transcribed from total RNA using the RevertAid^{TM} first strand cDNA synthesis kit (Fermentas, Hanover, MD, USA). Amplification of the murine *GAPDH* gene or human *Cyclophilin* gene was used to verify equal amounts of cDNA in the murine or human cDNA samples, respectively. The target genes, primer sequences, annealing temperatures, cycle numbers, and the length of PCR products are listed in Appendix III. The cycling parameters for a PCR amplification usually included 1 cycle at 95℃ for 5 min, followed by 25 - 40 cycles at 95℃ for 30 sec, indicated annealing temperature for 30 sec and 72℃ for appropriate time based on 1 min for every 1000 bp, and a final extension at 72℃ for 10 min.

2.3 Cell biology techniques

2.3.1 Coating of recombinant proteins and antibodies

In the experiments that require precoating of recombinant proteins or antibodies, tissue culture plates were incubated with coating solution at 4℃ overnight, and the wells were washed with PBS before cells were seeded (Fig. 2.2). The final concentrations of coating solutions were: 10 μg/ml (Langstein *et al.*, 1998, 1999a,

1999b, 2000; unless otherwise stated) for human or mouse CD137-Fc protein and their control protein, Fc of human IgG1 in PBS; 10 μg/ml for anti-human CD137L (clone 5F4 and C65-485) and their isotype control mouse IgG1 (clone MOPC-21) in PBS; 5 μg/ml for anti-mouse CD3 (clone 17A2), anti-mouse CD28 (clone 37.51) and their isotype control rat IgG2b (clone RTK4530), Syrian hamster IgG (clone SHG-1) in PBS. The volumes of coating solutions per well for different tissue culture plates were: 100 μL for 96-well plates, 300 μL for 24-well plates, 500 μL for 12-well plates and 1 ml for 6-well plates (unless otherwise stated). When the recombinant proteins or antibodies were added as soluble, the plate wells were precoated with 3% BSA at 4ºC overnight.

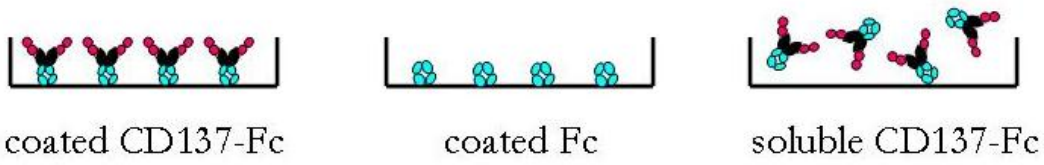


Figure 2.2. Schematic diagram of coating proteins or antibodies onto the tissue culture plates.

2.3.2 Cell count

2.3.2.1 Manual cell count with Trypan blue

Cells were harvested after incubation with 10 mM EDTA for 10 min. Cells were centrifuged and resuspended in PBS. The numbers of viable cells were assessed by 0.4% Trypan blue (Sigma) staining and counted using a haemocytometer (Neubauer).

2.3.2.2 Differential Cell Count

For cytological examination, cytospin preparations were prepared using a Cytospin (Thermo Shandon, Pittsburgh, PA), fixed, and stained in a modified Wright stain (Bao, *et al* 2007). Differential cell count was then performed on at least 500 cells in each cytospin slide.

2.3.2.3 Cell count by FACS with counting beads

Cell samples were stained with 0.5 μg/ml 7-AAD for 15 min at room temperature in the dark. After washing for three times, cells were resuspended in 450 μl PBS. 50 μl of Sphero AccuCount Blank Particles (about 50,000 beads per 50 μl) (ACBP-100-10, Spherotech, IL, USA) were added to the suspension and mixed well. Samples were analyzed by flow cytometry with CyAn™ ADP Analyzer (Beckman Coulter, CA, USA). 7-AAD negative cells were gated as live cells. The population of beads and target cells were gated separately on the forward/side scatter plot based on different sizes. The number of cells in each sample was calculated by the following formula.

$$\text{No. of cells in each sample} = \frac{\text{No. of events of test sample}}{\text{No. of events of beads}} \times \text{No. of beads (50,000)}$$

2.3.3 Flow cytometry analysis and antibodies

Aliquots of cultured cells (2 – 3 × 10^5 cells) were stained with respective fluorochrome conjugated antibodies in PBS containing 0.5% BSA and 0.1% sodium azide (FACS buffer) together with mouse or human FcR blocker (Miltenyi Biotech, Bergisch Gladbach, Germany) for 1 h at 4℃ 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 at 4ºC for 1 h. 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. Nonspecific staining was controlled by isotype-matched antibodies. All the antibodies used in the study were listed in Appendix II.

2.3.4 CFSE labeling

Fresh bone marrow cells at a concentration of 10^6 cells/ml were labeled with 5 μM Carboxyfluorescein diacetate, succinimidyl ester (CFSE) using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, CA) according to the manufacturer's protocol. Labeled cells were cultured in plates precoated with 10 μg/ml Fc or CD137-Fc. The cells were harvested on day 7 and 14, and analyzed by flow cytometry.

2.3.5 Proliferation assay

Cell proliferation was determined by ^{3}H-thymidine incorporation. Cells were pulsed with 0.5 μCi of ^{3}H-thymidine (PerkinElmer, Boston, MA) for the last 24 h 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, CT).

For immobilization of CD137L agonists on beads, protein A-coupled polystyrene

beads (Spherotech, IL, USA) were incubated with CD137-Fc, Fc, anti-CD137L antibody (clone 5F4) or isotype control mouse IgG1 (clone MOPC-21) at a ratio of 50 μg beads (~ 1.3×10^6 beads) to 1.5 μg protein or antibody for 1 h at 4 ℃. The coated beads were washed three times with PBS before seeded into round-bottom 96-well plates (Nunc) at 50 μg beads per well.

2.3.6 Colony-forming assay

2.3.6.1 Colony-forming assay for mouse bone marrow and Lin$^-$, CD117$^+$ cells

Freshly isolated bone marrow cells at density of 5×10^4 cells/ml or Lin$^-$, CD117$^+$ cells at density of 10^4 cells/ml were cultured in 35 mm dishes in triplicates in 1 ml of semi-solid methylcellulose based medium (MethoCult® SF^{BIT} M3236, StemCell Technologies) supplemented with 10% bovine calf serum. Dishes were cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2. Colonies were counted under the microscope at day 14 for bone marrow cells, and day 7 for Lin$^-$, CD117$^+$ cells. The number of colonies consisting of more than 50 cells was counted, and the average numbers of colonies and standard deviations were calculated from triplicate plates. The types of colonies were identified based on their morphological characteristics (Yoon *et al.* 2006, Coutinho *et al.* 2003).

2.3.6.2 Colony-forming assay for human CD34$^+$ cells

CD34$^+$ cells were cultured in 35 mm dishes in triplicates at a density of 2×10^3 cells/ml in 1 ml of semi-solid methylcellulose based medium (StemCell Technology)

supplemented with 10% bovine calf serum (BCS). Dishes were cultured at 37℃ in a humidified atmosphere containing 5% CO_2. Colonies were counted under the microscope 14 days after culture. The number of colonies consisting of more than 30 cells was counted, and the average number of colonies and standard deviations were calculated from triplicates.

2.3.7 Apoptosis assay

Cells were labeled with Annexin V-FITC and 7-AAD (BD Bioescience) following the the manufacturer's instructions. The percentage of apoptotic cells was determined by flow cytometry.

2.3.8 Phagocytosis assay

2.3.8.1 Phagocytosis assay for mouse bone marrow cells and Lin^-, $CD117^+$ cells

2×10^6 bone marrow cells or 5×10^5 Lin^-, $CD117^+$ cells per well were cultured in plates that had been precoated with 10 μg/ml of Fc or CD137-Fc for 7 days. Subsequently, 1 μm yellow-green fluorescent carboxylate-modified microspheres (FluoSpheres, Molecular Probes) were added at a ratio of 50 beads per cell. The plates were incubated for 1 h at 37℃ in the dark. Cells that had undergone the same treatment but without addition of beads served as negative controls. After 1 h incubation, 1 ml of cold PBS was added into each well to stop phagocytosis. Cells were washed and trypsin-EDTA was added to dislodge surface adherent beads. Cells were washed again and resuspended in FACS buffer for analysis by flow cytometry.

2.3.8.2 Phagocytosis assay for human cord blood CD34$^+$ cells and monocytes

2.5 × 10^5 CD34$^+$ cells or 5 × 10^5 cord blood monocytes per well were cultured in plates that had been precoated with 10 μg/ml of Fc or CD137-Fc. Monocytes were cultured for 3 days and CD34$^+$ cells were cultured for 10 days. Subsequently, 1 μm yellow-green fluorescent carboxylate-modified microspheres were added and then analyzed by flow cytometry, same as described above.

2.3.9 Allogeneic mixed lymphocyte reaction

2.3.9.1 Allogeneic MLR for mouse Lin$^-$, CD117$^+$ cells

Bone marrow cells were isolated from femurs of 12-week old BALB/c mice. 2 × 10^6 bone marrow cells or 3 × 10^5 Lin$^-$, CD117$^+$ cells per well were cultured in 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 DCs. Maturation of DCs was induced on day 6 by addition of 1 μg/ml LPS (Sigma). Cells were treated with 50 μg/ml mitomycin C at 37℃ for 2 h to disable the proliferation on day 7. Subsequently, cells were harvested by incubation with 10 mM EDTA at room temperature for 10 min and washed twice with PBS, and served as stimulator cells. T cells were isolated from splenocytes of 8-week old C57/BL6 mice by magnetic selection using the mouse Pan T cell isolation kit (Miltenyi) to a purity of >90%, as analyzed by flow cytometry for mouse CD3. 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 18 h. The rate of T cell

proliferation was determined with a scintillation counter (Packard).

2.3.9.2 Allogeneic MLR for human cord blood CD34$^+$ cells and monocytes

2.5 × 10^5 $CD34^+$ cells or 5 × 10^5 cord blood monocytes per well were cultured in plates that had been precoated with 10 μg/ml of Fc or CD137-Fc for 7 days. In parallel, cells were treated with 100 ng/ml GM-CSF + 25 ng/ml IL-4 (Peprotech) to generate immature DCs. Maturation of DCs was induced on day 6 by addition of 1 μg/ml LPS (Sigma). Cells were treated with 50 μg/ml mitomycin C at 37℃ for 2 h to disable the proliferation on day 7. Subsequently, cells were harvested by incubation with 10 mM EDTA at room temperature for 10 min and washed twice with PBS, and served as stimulator cells. $CD3^+$ T cells were isolated from another unit of buffy coat by magnetic selection using the human CD3 MicroBeads (Miltenyi) to a purity of >90%, as analyzed by flow cytometry for human CD3. 10^5 $CD3^+$ 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 18 h. The rate of T cell proliferation was determined with a scintillation counter (Packard).

2.3.9.3 MLR for suppressor cell function

Bone marrow cells and Lin^-, $CD117^+$ cells were isolated from BALB/c mice as described above. Bone marrow derived DCs were generated by culturing bone marrow cells with 100 ng/ml GM-CSF and 25 ng/ml IL-4 for 7 days with 1 μg/ml LPS for the last 24 h. Lin^-, $CD117^+$ cells were cultured in 12-well plates that were

precoated with 10 μg/ml CD137-Fc or Fc proteins for 7 days at a density of 5×10^5 cells per well. Cells and supernatants were harvested on day 7. Supernatants were used for ELISA. Bone marrow derived DCs were used as stimulator cells, and CD137-Fc or Fc treated cells were used as suppressor cells. T cells were isolated from C57/BL6 mice spleen by MACS using the Pan T cell isolation kit (Miltenyi). T cells, stimulator cells (bone marrow derived DCs) and suppressor cells were cocultured in round-bottom 96-well plate at indicated ratios, with a constant T cell number of 10^5 per well, in a total volume of 200 μL complete medium. Single cell types of T cells or stimulator cells or suppressor cells were used as negative controls. Cells were cocultured for 4 days and pulsed with 0.5 μCi ^{3}H-thymidine per well for the last 18 h. The rate of T cell proliferation was determined with a scintillation counter (Packard).

2.3.10 ELISA

The concentrations of murine IL-10, IL-12p70, G-CSF, M-CSF, GM-CSF, IL-3, IL-6, and SCF in cell supernatants were determined by mouse IL-10 ELISA MAX set (BioLegend), mouse IL-12p70 DuoSet ELISA kit (R&D systems) and mouse G-CSF, M-CSF, GM-CSF, IL-3, IL-6, SCF ELISA Development kits (Peprotech), respectively. The concentrations of human IL-10, IL-12p70, IL-6 and IL-8 in cell supernatants were determined by human IL-10 ELISA MAX set (BioLegend), human IL-12p70, IL-6, IL-8 DueSet ELISA kits (R&D systems) according to the manufacturer's instructions. All measurements were done in triplicates.

2.3.11 Transfection

MCF-7 cells were plated in 6-well plates at a density of 2×10^5 cells/well, and cultured to 80% confluency. The cells were transiently transfected with 10 μg of human or murine CD137 ligand expression vector or the empty vector pcDNA3.1 (Invitrogen), together with 10 μl of Lipofectamine^{TM} 2000 (Invitrogen) in serum-free medium. The culture medium was changed to complete medium after 4 h of transfection. Cells were harvested after 48 h of transfection using 10 mM EDTA in PBS for flow cytometric analysis.

2.3.12 Cytokine antibody array

Mouse bone marrow cells were cultured for 14 days in plates that were precoated with 10 μg/ml CD137-Fc or Fc. Supernatants were harvested on day 7 and 14, and analyzed with using the RayBio Mouse Cytokine Antibody Array III (RayBiotech, GA, USA) according to the manufacturer's instructions. Briefly, cytokine array membranes were blocked in blocking buffer for 30 min and then incubated with 1 ml of samples at room temperature for 2 h. After five times washing, membranes were incubated at room temperature with biotinylated primary antibodies for 2 h and then with streptavidin-HRP for 1 h. Membranes were washed thoroughly for 5 min in the dark before imaging. Membranes were exposed to X-ray film (Kodak X-OMAT AR film) within 30 min of exposure to the substrate. Signal intensities were scanned with the Typhoon 9400 blot imager (Amersham Biosciences, CA, USA) and quantified with ImageQuant TL v2005 (Amersham). Data were analyzed by RAYBIO analysis tool

(RayBiotech). The signal intensities on different membranes were normalized based on six positive control spots on each membrane.

2.3.13 Cytometric bead array (CBA)

Tissue culture plates were precoated with 10 μg/ml CD137-Fc or Fc protein at 4 ºC overnight. 2×10^6 BALB/c bone marrow cells were cultured in 6-well plates for 7 days or 5×10^5 human cord blood $CD34^+$ cells were cultured in 12-well plates for 10 days. Supernatants were collected and concurrently assayed for IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 using the BD™ Cytometric Bead Array (CBA) mouse inflammation kit; or IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 using the human inflammation kit (BD Biosciences), respectively, according to the manufacturer's protocol.

Six bead populations with distinct fluorescence intensities had been coated with capture antibodies specific for the above mentioned cytokine proteins and were detected on the FL3 channel of the Calibure flow cytometer (Becton Dickinson, Mountain View, CA). The antibody-coated bead mixture was then tagged to a PE-conjugated detection-antibody system and the fluorescence signals were read on the FL2 channel. Standard curves for each cytokine were plotted and the unknown values for each of the test samples were calculated using the CBA software. Results are represented as mean fluorescence intensity obtained on the FL2 channel.

2.3.14 Microscopy and photography

Morphological changes of cells were documented by using a Zeiss Axiovert 40 inverted microscope (Zeiss, Göttingen, Germany) and Canon PowerShot G6 digital camera. Immunofluorescence was taken under Nikon Eclipse TE2000-U Fluorescence Microscope.

2.4 Staining procedures

2.4.1 Immunocytochemistry

$2 – 5 \times 10^6$ cells were smeared onto the TBS-coated glass slides. The air-dried slides were fixed with ice cold absolute methanol. After blocking with 3% skim milk for 30 min and 3% H_2O_2 for 10 min, the slides were incubated with 1 µg/ml biotinylated primary antibodies for 1 h and subsequently with 1 µg/ml streptavidin-peroxidase polymer (Sigma) for 30 min. After three washes, the slides were incubated with liquid DAB^+ substrate (Dako, Denmark) for 5 - 10 min in the dark. Then the slides were counterstained with hematoxylin (Sigma), and mounted in DPX mounting media (Sigma). All incubations were done at room temperature.

2.4.2 Immunohistochemistry

Formalin fixed paraffin embedded human bone marrow tissue slides were purchased from Spring Bioscience (Fremont, CA, USA). After deparaffinization in Xylene, and

hydration in a graded series of alcohol, the slides were pretreated with citrate buffer (Dako) in a pressure cooker at 109 ℃ for 15 min 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. 2 µg/ml of anti-CD137 (clone BBK-2, Bioscource) or an isotype control antibody (MOPC21, Sigma) in TBS were used as primary antibodies and hybridized overnight. The secondary HRP-conjugated anti-mouse antibody (Dako) was added for 30 min, followed by DAB^+ substrate (Dako). 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 and mounted.

2.4.3 Esterase stain

2 - 5 × 10^6 cells were smeared onto glass slides. Esterase staining was performed successively with the α-Naphyl Acetate Esterase kit (Sigma) for non-specific esterase and the Naphthol AS-D Chloroacetate Esterase kit (Sigma) for specific esterase following the manufacturer's instructions.

2.5 Statistics

Data are expressed as means ± SD for replicates in an independent experiment, or mean ± SE for repeats of several independent experiments. Unless otherwise stated, two tailed unpaired Student's t-tests were used to determine statistical significance.

CHAPTER 3 RESULTS

Since CD137 agonists (recombinant CD137 protein and anti-CD137L antibodies) have been shown to induce proliferation and endomitosis in peripheral monocytes, a cell population which was believed not to be able to proliferate (Langstein *et al.*, 1999a; Ju *et al.*, 2003) we hypothesized that they may also be able to induce proliferation in the precursor of monocytes, the hematopoietic progenitor cells. We started our study in the human system with umbilical cord blood $CD34^+$ cells.

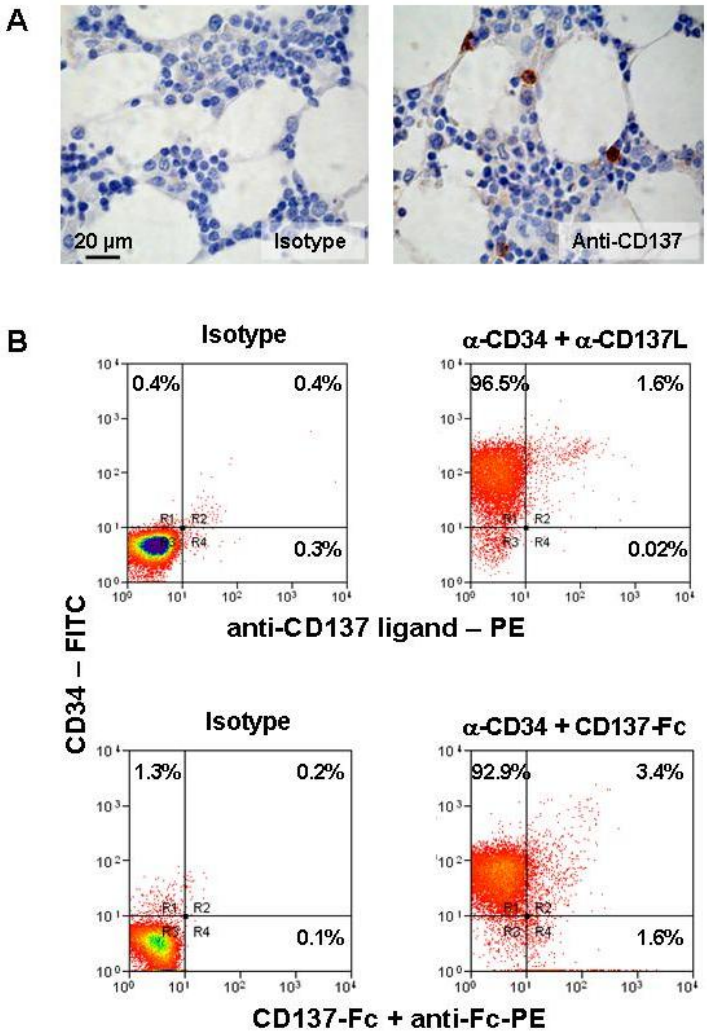
3.1 Induction of proliferation and monocytic differentiation of human $CD34^+$ cells by CD137L signaling

3.1.1 CD137 and its ligand are expressed in the bone marrow

CD137 is expressed on few but distinct cells in the human bone marrow as detected immunohistochemically (Fig. 3.1 A). CD137L is expressed on a small subset of hematopoietic progenitor cells. 1.9% and 3.2% of freshly isolated $CD34^+$ cells stained positive with an anti-CD137L antibody and a recombinant CD137 protein, respectively (Fig. 3.1 B). Confirming data on CD137L protein expression we detected CD137L mRNA in $CD34^+$ cells by RT-PCR (Fig. 3.1 C).

Expression of both, CD137 and CD137L by bone marrow cells suggested a function

for this receptor/ligand pair in the bone marrow. Since CD137L crosslinking has been shown to induce proliferation of peripheral monocytes and maturation of immature DCs we suspected similar functions for the CD137L-expressing hematopoietic progenitor cells.



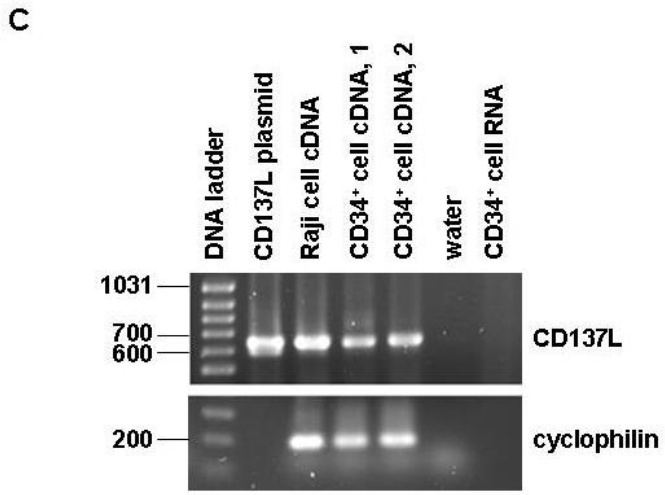


Figure 3.1. CD137 and CD137L expression. (A) Immunohistochemical detection of CD137 expression (brown stain) in human bone marrow. CD137 was detected by staining with anti-CD137 antibody (clone BBK-2); and mouse IgG (clone MOPC-21) was used as an isotype control. Magnification ×630. **(B)** Freshly isolated $CD34^+$ cells (2×10^5 cells) were stained with PE-labeled anti-CD137L antibody, clone 5F4 (1:10), or 1.2 μg of CD137-Fc protein followed by anti-human IgG1-PE. Mouse IgG1 and Fc protein were used as isotype controls for 5F4 and CD137-Fc, respectively. CD34 was detected by an FITC-labeled anti-CD34 antibody, clone AC136 (1:10). Cells were analyzed by flow cytometry, and the resulting dot blot analyses are depicted. Percentages of cells are stated in each quadrant. **(C)** Expression of CD137L mRNA was determined by RT-PCR using cDNAs from purified $CD34^+$ cells of two donors. Cloned human CD137L cDNA (CD137L plasmid) and cDNA from Raji cells were used as positive controls. Reactions with no template (water) or RNA from $CD34^+$ cells that was not reverse-transcribed served as negative controls. Comparable amounts of cDNA were verified by amplification of cyclophilin. GeneRuler DNA ladder (Fermentas) was used as a size standard. PCR products were analyzed on a 1% TAE agarose gel. These experiments were performed three times with same results.

3.1.2 CD137 induces morphological changes of $CD34^+$ cells

Hematopoietic progenitor cells were isolated from umbilical cord blood via their CD34 expression. The purity of enriched $CD34^+$ cells was higher than 90%. The cells were cultured in tissue culture plates that were coated with a fusion protein consisting of the extracellular domain of CD137 and the constant domain (Fc) of IgG1

(CD137-Fc) or an Fc control protein.

CD137-Fc protein induced attachment and spreading of subsets of $CD34^+$ cells whereas other cells appeared to attach only lightly or not at all. Cells in the control wells resembled this latter group as they too remained round and did not attach. Attachment and spreading in response to CD137 protein was accompanied by morphological changes of the cells, which became apparent on the days 5 – 7, and were the most prominent on day 14. Besides a general increase in cell size, the following three types of morphological changes were observed: (1) Elongation to spindle-shaped cells, formation of (2) lamelopodia and filopodia, and (3) dendrites (Fig. 3.2).

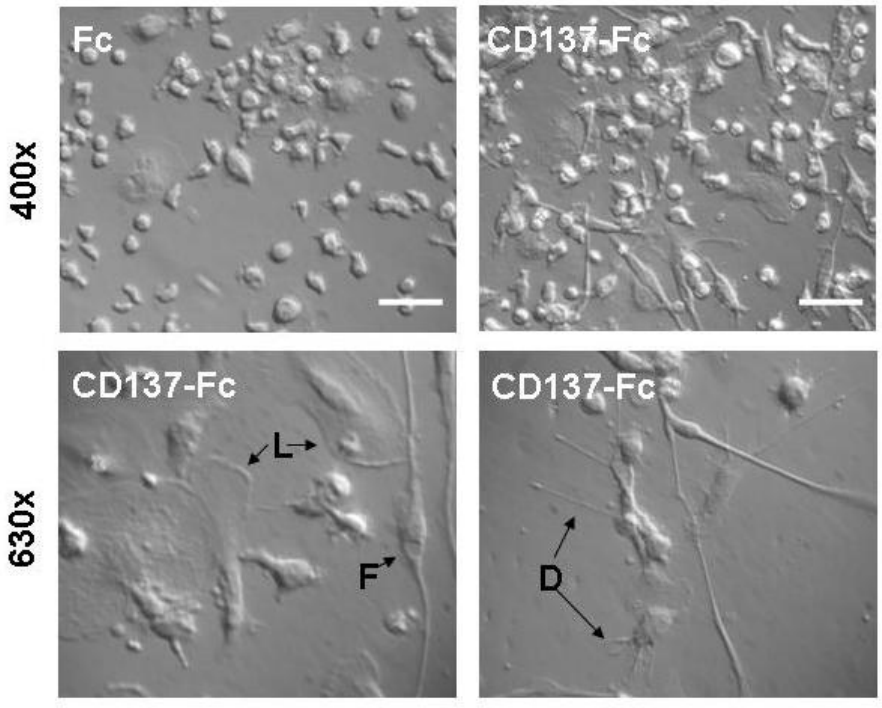


Figure 3.2. CD137 induces morphological changes of $CD34^+$ cells. Human $CD34^+$

cells at a density of 5×10^5 /ml were cultured in RPMI, 10% FBS (complete medium) in wells coated with 10 μg/ml Fc or CD137-Fc protein. Photographs were taken on day 14 at room temperature at magnifications of 400× and 630×. Scale bars: 25 μm. L: Lamellopodia. F: Filopodia. D: Dendrites.

3.1.3 CD137 induces proliferation of CD34$^+$ cells

We noticed that besides morphological changes CD137-Fc protein also enhanced the number of viable cells. For quantification, 2.5×10^4 CD34$^+$ cells were cultured on Fc- or CD137-Fc-coated plates and numbers of live cells were counted on days 3, 7, 10 and 14. More than half of the cells died during the first 3 days. CD137-Fc induced an amplification of live cell numbers that peaked at day 14 at around 4×10^4 cells. At that time point CD137-Fc had increased cell numbers 4-fold over the Fc control and above the original cell number plated (Fig. 3.3 A). The cell number did not increase further after day 14, likely due to exhaustion of nutrients in the culture (not shown). The increase in cell number was accompanied by cellular proliferation in response to CD137-Fc protein as measured by ^{3}H-thymidine incorporation (Fig. 3.3 B, C). The time course curve of cell numbers showed a delayed kinetics compared to that of cell proliferation which peaked at day 10. CD137-Fc added as soluble protein could not induce proliferation of CD34$^+$ cells (Fig. 3.3 B). This demonstrates that CD137-Fc works by crosslinking CD137L on a subset of CD34$^+$ cells, and excludes that the observed effects are due to potential contaminants in the protein preparation.

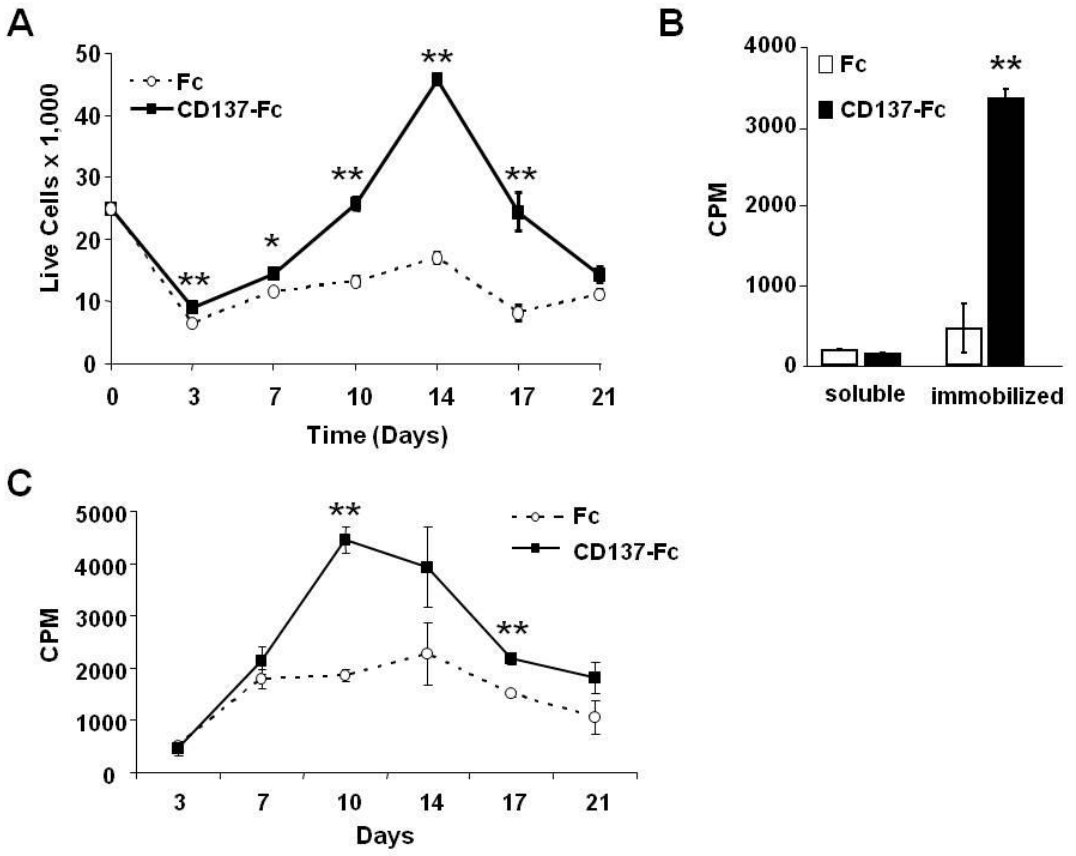


Figure 3.3. CD137 maintains survival and induces proliferation of CD34$^+$ cells. Human CD34$^+$ cells at a density of 2.5 × 10^5 cells/ml were cultured in complete medium in wells coated with 10 μg/ml Fc or CD137-Fc protein or the proteins were added soluble. **(A)** Live cells were counted after trypan blue staining on days 3, 7, 10, 14, 17, and 21. Depicted are means ± SD of four representative fields of each of duplicate cultures. **(B)** Proliferation was determined on day 10 after cells had been labeled for the last 24 h with 0.5 μCi of ^{3}H-thymidine. **(C)** Proliferation was determined on day 3, 7, 10, 14, 17, 21 after cells had been labeled for the last 24 h with 0.5 μCi of ^{3}H-thymidine. Depicted are means ± SD of triplicate measurements. *, $p < 0.05$; **, $p < 0.01$. This experiment was performed three times with similar results.

In order to verify that the observed effects of CD137-Fc protein on CD34$^+$ cells are indeed due to CD137L crosslinking we performed several control experiments. Immobilization of CD137-Fc was prevented by prior coating of the culture dishes

with BSA. Immobilized but not soluble CD137-Fc protein induced morphological changes (Fig. 3.2; personal communication by Yue P. S. and Schwarz H.), and proliferation (Fig. 3.3 B). In addition, two monoclonal antibodies specific for CD137L induced identical morphologies as CD137-Fc protein (not shown), and enhanced proliferation of $CD34^+$ cells (Fig. 3.4 A). Further, CD137-Fc and anti-CD137L antibody (clone 5F4), but not the Fc or MOPC-21 isotype controls, immobilized on protein-A coated polystyrene beads enhanced proliferation of $CD34^+$ cells (Fig. 3.4 B), similar as they did when immobilized on the culture plates. This rules out the possibility that the Fc portion of the fusion protein provides synergistic effect to CD137L signaling.

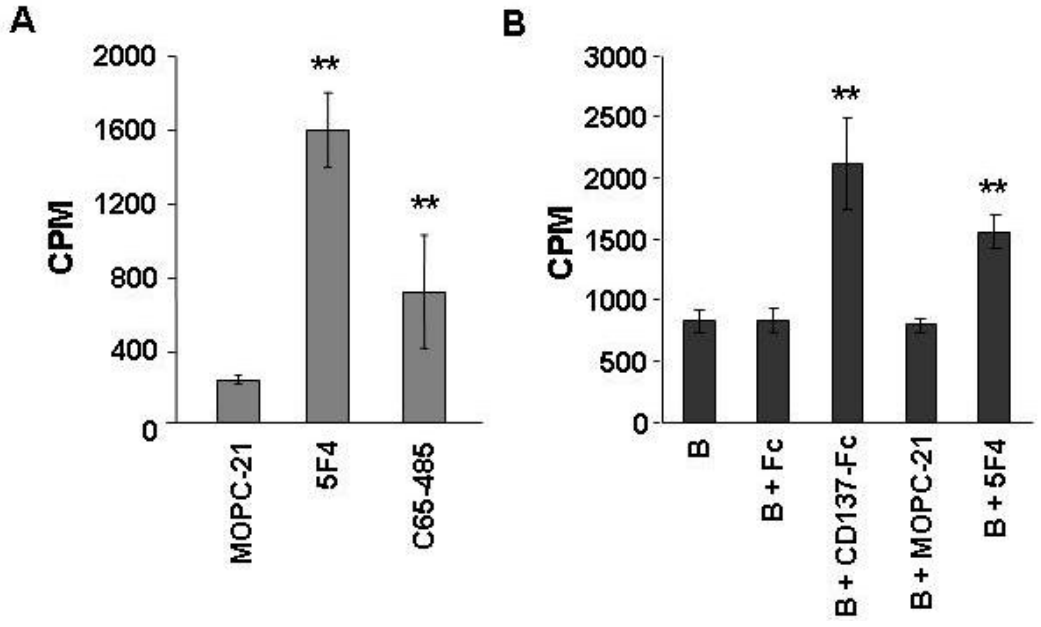


Figure 3.4. CD137L agonists immobilized on beads induce proliferation of $CD34^+$ cells. Human $CD34^+$ cells at a density of 2×10^5/ml were cultured in complete medium. Proliferation was determined on day 10 after cells had been labeled for the last 24 h with 0.5 μCi of ^{3}H-thymidine. **(A)** Cells were treated with 10 μg/ml immobilized monoclonal anti-CD137L antibodies (clone 5F4 or C54-485) or isotype control (mouse IgG1, clone MOPC-21). **(B)** Cells were cocultured with 50 μg of

protein-A coated polystyrene beads (labeled as B on x-axis) conjugated with 1.5 μg of Fc, CD137-Fc, MOPC-21, or 5F4 in round-bottom 96-well plates. Depicted are means ± SD of triplicate measurements. **, $p < 0.01$. This experiment was performed three times with similar results.

3.1.4 CD137 induces colony formation of CD34$^+$ cells

CD137L crosslinking also induced colony formation by hematopoietic progenitor cells. 2×10^3 CD34$^+$ cells were seeded in Fc- or CD137-Fc-coated 35 mm dishes with methylcellulose medium supplemented with 10% serum. No other growth factors were added. Due to the high viscosity of methylcellulose medium only a fraction of the plated CD34$^+$ cells are expected to come in contact with the plate-bound Fc or CD137-Fc protein. By day 20, 3 - 4 colonies had emerged in the Fc-coated control dishes, but more than 40 colonies had formed in response to CD137-Fc. While Fc-induced colonies were comparatively small containing less than 50 cells, CD137 protein had induced larger colonies, with some containing up to 500 cells (Fig. 3.5). The colonies formed in response to CD137L crosslinking were of the CFU-GM type (Eaves *et al.* 1995), implying that CD137L signaling may induce myeloid differentiation.

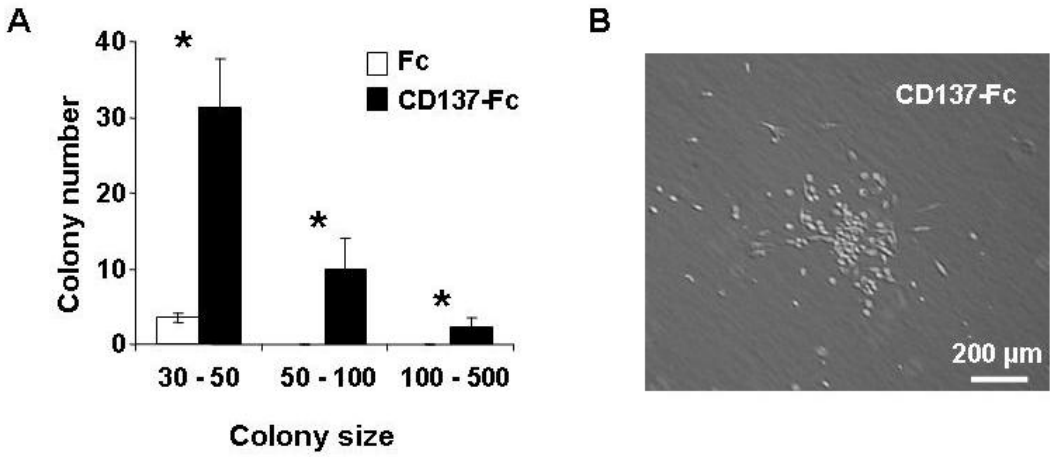


Figure 3.5. CD137L signaling promotes colony formation. 2×10^3 $CD34^+$ cells were cultured in 1 ml methylcellulose medium, 10% BCS, on 35 mm dishes which were coated with 10 µg/ml Fc or CD137-Fc protein. **(A)** At day 14, the numbers and sizes of the colonies were counted. Depicted are means ± SD of triplicate measurements. * $p<0.05$. **(B)** Photographs of colonies in CD137-Fc-coated dishes were taken at day 14. This experiment was performed three times with comparable results.

3.1.5 CD137 ligand signaling induces differentiation towards the myeloid lineage

Generally, growth factors which induce proliferation of hematopoietic progenitor cells also induce differentiation to a specific lineage. We therefore investigated whether CD137L signaling also influences differentiation using flow cytometry.

Differentiation is characterized by a decreased expression of the CD34 antigen (Fackler *et al.* 1995). Among freshly isolated cells, more than 90% stained positive for CD34 by flow cytometry. However, after culturing the cells for 10 days on Fc- or CD137-Fc-coated dishes the expression of CD34 had decreased to around 12%, indicating that differentiation of the cells had occurred (Fig. 3.6).

We analyzed expression of lineage-associated cell surface markers to determine to which lineage CD137 agonists are driving differentiation of the $CD34^+$ cells. Antibodies to CD3, CD19, CD41 and CD235a were used to identify T cells, B cells, megakaryocytic and erythroid cells, respectively. CD11b and CD14 served as markers for the myeloid lineage and monocytes/macrophages, respectively. No influence of CD137-Fc on T and B cell survival or differentiation could be detected. T cells and B cells which each made up close to 5% of freshly isolated $CD34^+$ cells were strongly reduced or had completely disappeared at day 10 (Fig. 3.6 A, B).

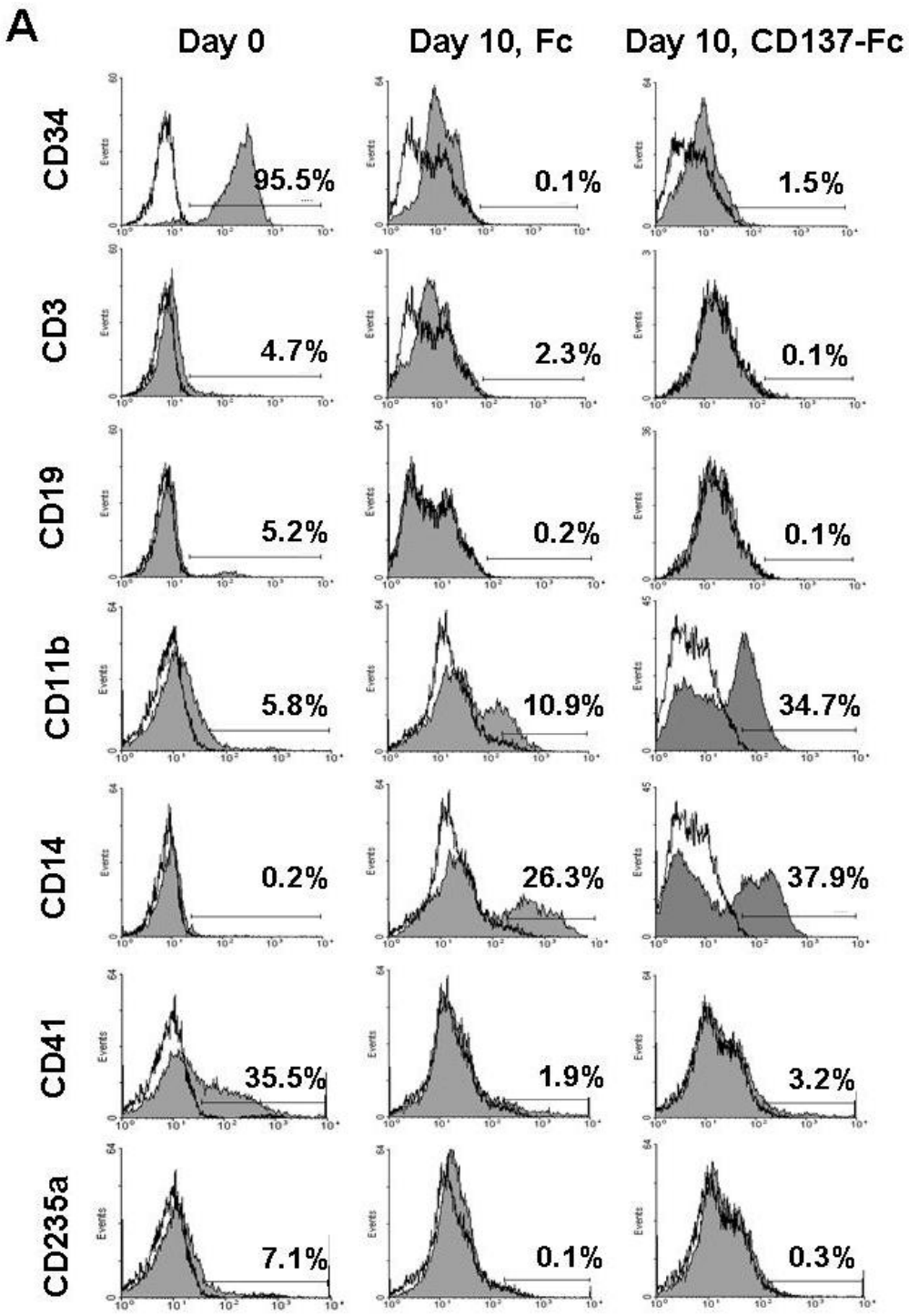
Similarly, CD137-Fc had no influence on the megakaryocytic and erythroid lineages. Around 60% of the freshly isolated $CD34^+$ cells expressed CD41. After the 10 day culture, the percentage of $CD41^+$ cells was reduced to 5 – 10%. The percentage of $CD235a^+$ cells had decreased from around 16% to 1 – 2% by day 10 (Fig. 3.6 A, B). There were no significant differences in CD3, CD19, CD41 and CD235 expression for the cells cultured on Fc- or CD137-Fc-coated plates.

CD137-Fc however, promoted differentiation to myeloid cells. Among freshly isolated $CD34^+$ cells $5.7 \pm 2.7\%$ expressed CD11b. After the cells were cultured for 10 days, CD11b expression had increased significantly, to $16.2 \pm 3.4\%$ and $29.3 \pm 3.8\%$ $CD11b^+$ cells for Fc- and CD137-Fc-treated cells, respectively (Fig. 3.6 A, B). More pronounced was the effect of CD137-Fc on the absolute number of live $CD11b^+$ cells. Those had tripled from $(57 \pm 27) \times 10^3$ to $(168.5 \pm 35.4) \times 10^3$ in the Fc control conditions

but had increased 18-fold to $(946.4 \pm 122.7) \times 10^3$ in the CD137-Fc conditions (Fig. 3.6 A, B).

The monocytic marker CD14 was expressed on $3.3 \pm 1.2\%$ of the freshly isolated $CD34^+$ cells. After the 10 day culture, the percentage of Fc-treated cells which stained positive for CD14 had increased to $18.6 \pm 3.5\%$ while that for CD137-Fc-treated cells had increased to $30.4 \pm 3.9\%$. The absolute number of $CD14^+$ cells had increased from $(33 \pm 12) \times 10^3$ to $(193.4 \pm 36.4) \times 10^3$ in the Fc controls, and to $(991.9 \pm 126) \times 10^3$ in the CD137-Fc conditions (Fig. 3.6 A, B).

These data indicate that CD137L signaling drives differentiation of $CD34^+$ cells to the myeloid lineage, especially to monocytes/macrophages, but has no effect on the lymphoid, megakaryotic and erythroid lineage.


A
Day 0
Day 10, Fc
Day 10, CD137-Fc
CD34
95.5%
0.1%
1.5%
CD3
4.7%
2.3%
0.1%
CD19
5.2%
0.2%
0.1%
CD11b
5.8%
10.9%
34.7%
CD14
0.2%
26.3%
37.9%
CD41
35.5%
1.9%
3.2%
CD235a
7.1%
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0.3%

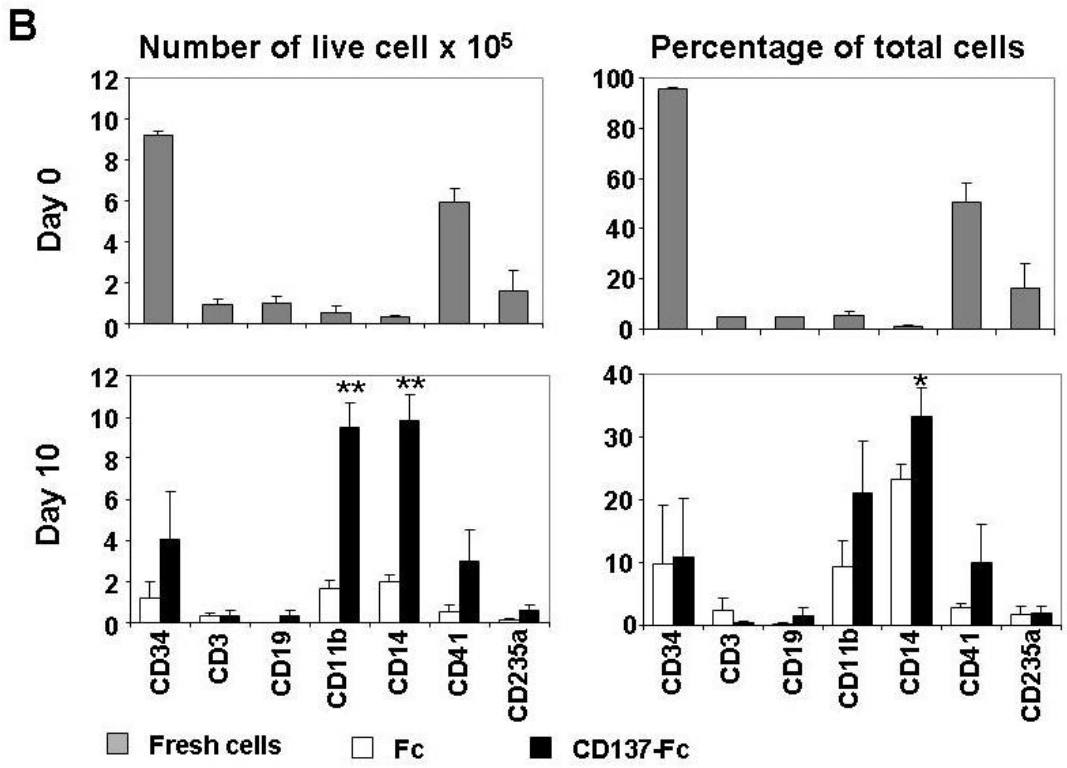


Figure 3.6. Flow cytometric analysis of CD137-induced differentiation of CD34$^+$ cells. Freshly isolated CD34$^+$ cells or CD34$^+$ cells which had been cultured on dishes coated with 10 μg/ml Fc or CD137-Fc protein for 10 days. **(A)** Cells were stained for CD34, CD11b, CD3, CD19, CD14, CD41 and CD235a expression and analysed by flow cytometry. Black line: Isotype. Gray, filled curve: Marker-specific antibody. **(B)** Depicted are means ± SE for absolute numbers of live cells (left column) and percentages (right column) of data in (A) for indicated subpopulations at days 0 and 10 of eight independent experiments.

3.1.6 CD137 induces differentiation to macrophages

Previous experiments show CD137L crosslinking by recombinant CD137 protein on CD34$^+$ cells and subsequent CD137L signaling in CD34$^+$ cells causes (1) activation resulting in attachment and morphological changes, (2) survival and/or proliferation, and (3) induces differentiation of CD34$^+$ cells to monocytic cells. Monocytic cells include monocytes, macrophages, and myeloid DCs. Circulating monocytes can

differentiate to DCs or macrophages in the peripheral tissues upon stimulation. The next question we want to address is whether the monocytic cells differentiated by CD137 are DCs or macrophages.

First, we started with cell morphology since DCs and macrophages adopt different morphologies. When comparing CD137-differentiated $CD34^+$ cells to cells exposed to M-CSF or GM-CSF + IL-4, classical macrophage or DC differentiation factors, respectively, we noticed the presence of elongated or ovoid macrophages, and of smaller, round, detached cells with DCs morphology, implying that CD137 may support macrophage as well as DC differentiation (Fig. 3.7).

For reference we included cord blood monocytes from the same donor. CD137-Fc-treated monocytes resembled more macrophages than DCs but contained more cells than M-CSF-treated monocytes. This observation is in line with the known activities of CD137 as a monocyte survival and growth factor (Langstein *et al.* 1999a, b). Further, $CD34^+$ cells and monocytes exposed to CD137 differed profoundly in morphology indicating that it were progenitor cells that responded to CD137. It excludes the possibility that the observed effects are due to contaminating monocytes (1 – 2% $CD34^-CD137L^+$ cells, Fig. 3.1 B) in $CD34^+$ cells.

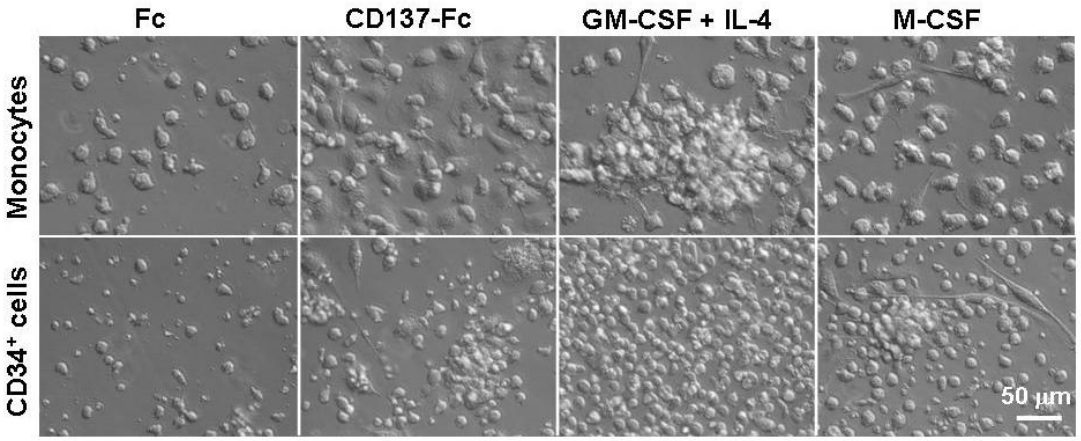


Figure 3.7. Morphological comparison of CD137 differentiated cells, DCs and macrophages. Human $CD34^+$ cells or cord blood monocytes were cultured for 10 days in complete medium in wells coated with 10 μg/ml Fc or CD137-Fc protein. 100 ng/ml of GM-CSF + 25 ng/ml of IL-4 and 50 ng/ml of M-CSF were used as controls for DCs differentiation and macrophage differentiation, respectively. Photographs were taken on day 10 at room temperature at magnifications of 400×. Scale bars: 50 μm.

Next, gene expression analysis was carried in order to gain a more detailed understanding of CD137-induced differentiation of hematopoietic progenitor cells. We checked the expression of DC- and macrophage-specific genes. It is reported that during differentiation of human monocytes to macrophages or DCs, *FcγR1A* and *TCF7L2* are macrophage-specific, and *WNT5A*, *FceR1A*, *Fibronectin*, *SOCS1*, and *TGG-α* are DC-specific (Lehtonen *et al.* 2007). The expression profile of these genes was analyzed and compared in the cultures of CD137-Fc- and Fc-treated $CD34^+$ cells and cord blood monocytes by RT-PCR. The expression of *FcγR1A* and *TCF7L2* were higher in M-CSF-treated $CD34^+$ cells and monocytes than in GM-CSF + IL-4-treated monocytes, indicating that they are indeed macrophage-specific genes. CD137-Fc-treated cells showed higher expression of these two genes than Fc-treated

counterparts, and the expression levels were similar as in M-CSF-treated cells (Fig. 3.8). This suggested that CD137-treated $CD34^+$ cells and monocytes were in the process of macrophage differentiation. *SOCS1* was expressed in GM-CSF + IL-4-treated cells but not in M-CSF-treated cells, indicating it was a DC-specific gene. CD137-Fc-treated $CD34^+$ cells expressed no or much less *SOCS1* than Fc-treated cells on day 7 and 13 (Fig. 3.8). This suggested that CD137 drives differentiation away from DCs. The rest of the DC-specific genes failed to give a clear result. From the signature gene expression profile, the CD137-differentiated cells were likely to be macrophages.

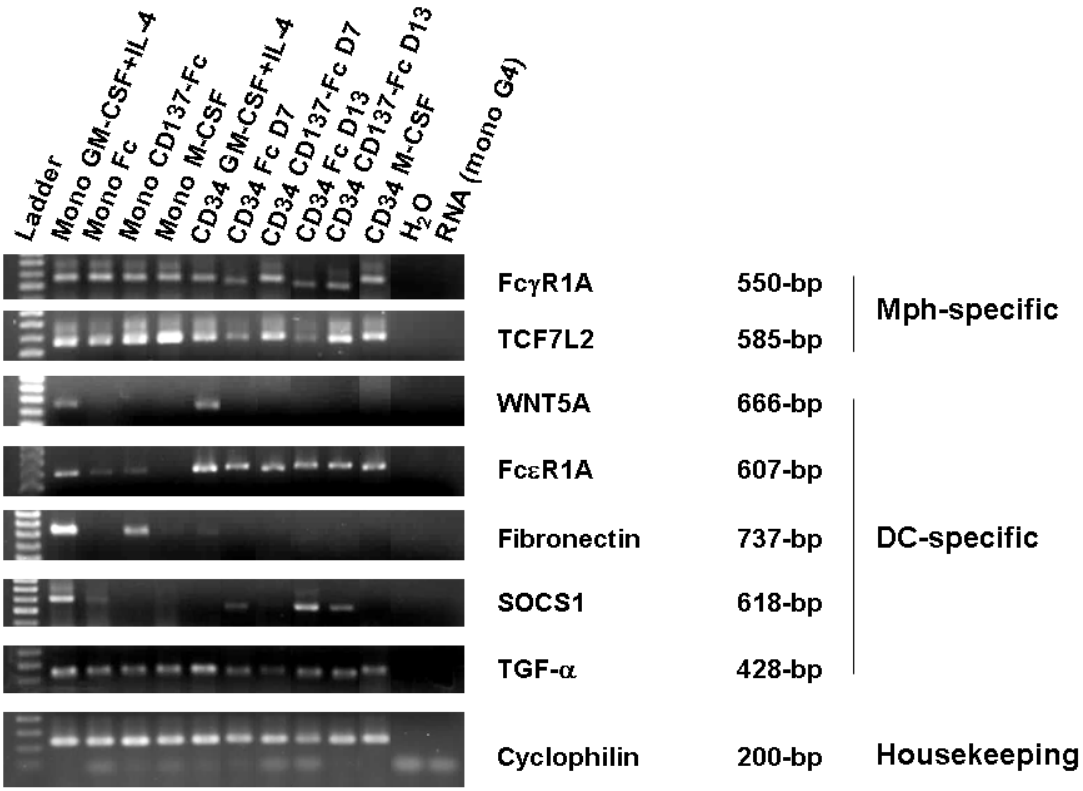


Figure 3.8. RT-PCR of macrophage- and DC-specific genes. $CD34^+$ cells and monocytes isolated from human cord blood were cultured in complete medium in wells coated with 10 μg/ml Fc or CD137-Fc protein. 100 ng/ml of GM-CSF + 25 ng/ml of IL-4 and 50 ng/ml of M-CSF were used as controls for DC and macrophage

differentiation, respectively. Total RNA was extracted and converted to cDNA from these cultures on day 7 and 13. Expression of *FcγR1A*, *TCF7L2*, *WNT5A*, *FceR1A*, *Fibronectin*, *SOCS1* and *TGG-α* mRNA were determined by RT-PCR. Reactions with no template (water) or RNA from GM-CSF + IL-4-treated monocytes that was not reverse-transcribed served as negative controls. Comparable amounts of cDNA were verified by amplification of cyclophilin. GeneRuler DNA ladder (Fermentas) was used as a size standard. PCR products were analyzed on a 1% TAE agarose gel. These experiments were performed twice with similar results. The numbers indicate the lengths of the PCR products for the respective genes.

In the next step, we analyzed CD137-induced changes in cell surface marker expression to assess DC and macrophage differentiation phenotypically. CD1a and CD83 were not detectable or expressed at low levels, respectively, on CD137-differentiated $CD34^+$ cells (not shown). CD209 (DC-SIGN) was neither expressed on CD137-Fc-treated $CD34^+$ cells nor monocytes, but was expressed slightly by GM-CSF + IL-4-treated $CD34^+$ cells (9% positive), and strongly by GM-CSF + IL-4-treated monocytes (95% positive), (Fig. 3.9). HLA-DR levels were high (>75% positive) but not affected by CD137L signaling on $CD34^+$ cells, and interestingly, were reduced on cord blood monocytes from the same donor (85% vs. 53.8%), (Fig. 3.9). CD14 expression was increased by exposure of $CD34^+$ cells to CD137 protein (31% vs. 43%), but was lower than in monocytes (around 80%), (Fig. 3.9). These data from the analysis of cell surface marker expression were more compatible with macrophage than DC differentiation.

Though expression of cell surface markers could indicate cellular differentiation, a more reliable test is a functional evaluation. We tested the phagocytic capacity of

$CD34^+$ cells that had been cultured for 10 days on CD137-Fc or Fc protein. Stimulation by CD137 protein more than doubled the percentage of cells phagocytosing flourescent latex beads (8.1% vs 18.6%). In contrast, in cord blood monocytes CD137 reduced the number of phagocytic cells to less than half (38.3% vs 18%), (Fig. 3.10).

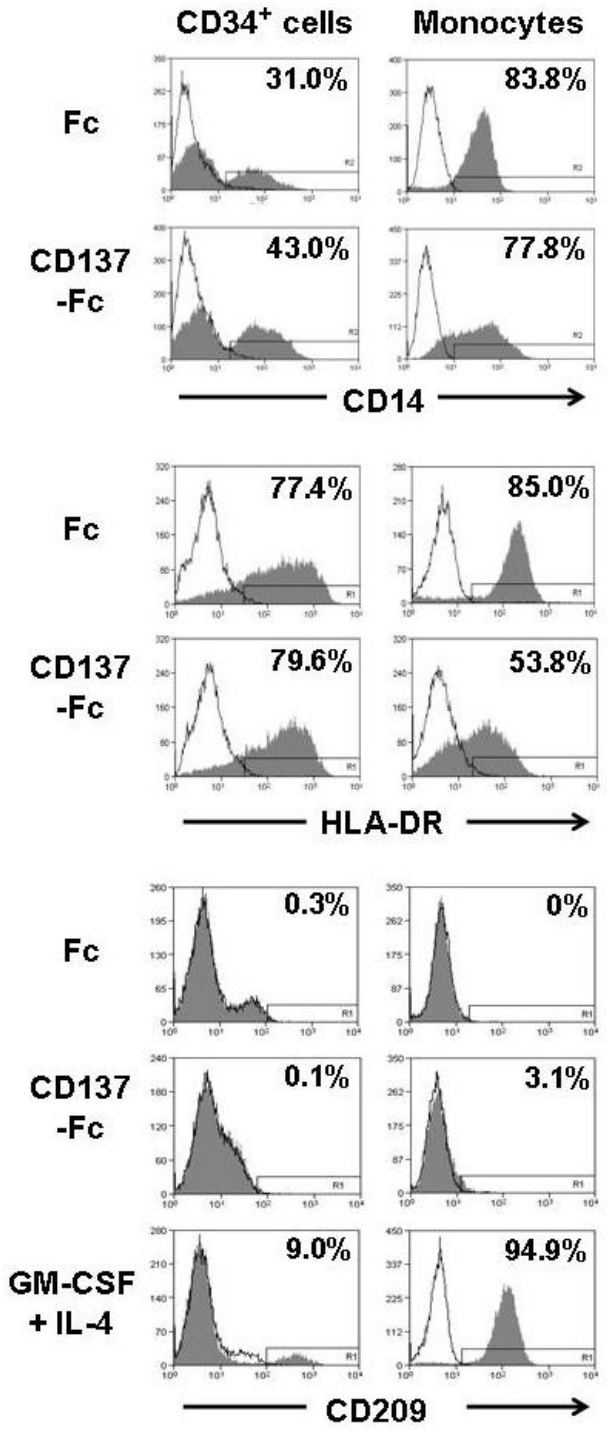


Figure 3.9 Flow cytometric analysis of DC / Macrophage cell surface marker expression on CD137-treated cells. Human $CD34^+$ cells or cord blood monocytes were cultured in complete medium in wells coated with 10 μg/ml Fc or CD137-Fc protein. 100 ng/ml of GM-CSF + 25 ng/ml of IL-4 and 50 ng/ml of M-CSF were used as controls for DCs differentiation and macrophage differentiation, respectively. Cells were harvested on day 10 and stained for CD14, HLA-DR and CD209 expression and analysed by flow cytometry. Black line: Isotype. Gray, filled curve: Marker-specific antibody. This data is representative of three independent experiments.

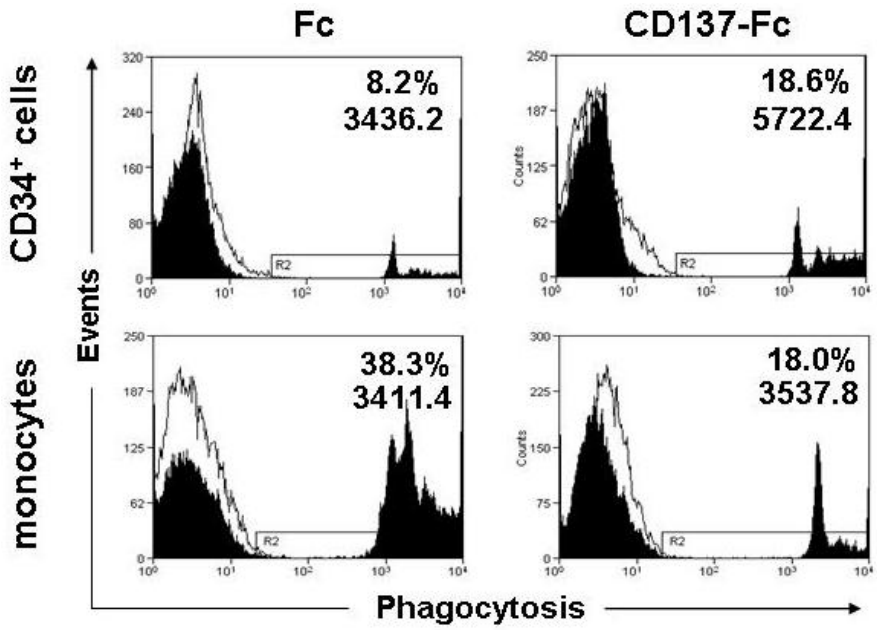


Figure 3.10 Phagocytic function analysis. Human CD34$^+$ cells were cultured for 10 days and cord blood monocytes were cultured for 3 days in complete medium in wells coated with 10 μg/ml Fc or CD137-Fc protein. Phagocytosis was measured by a 1 h pulse with fluorescent latex beads (black curves) at 50 beads per cell, followed by flow cytometry. White lines: Control without beads. This data is representative of three independent experiments. The percentage of beads containing cells and mean fluorescence intensity (MFI) were shown in the upper right corner for each sample.

In addition, we analyzed secretion of cytokines associated with DC and macrophage differentiation. CD137-Fc differentiated CD34$^+$ cells produced low levels of IL-10, and secretion of this cytokine was strongly induced by LPS. The same pattern was found in CD34$^+$ cells differentiated with the Fc control protein, or in immature DCs, differentiated with GM-CSF + IL-4. In cord blood monocytes from the same donor CD137 protein as well as GM-CSF + IL4 induced moderate IL-10 secretion while high levels were obtained after LPS stimulation (Fig. 3.11 A). IL-12 was secreted in moderate and high amounts by GM-CSF + IL-4 treated monocytes and CD34$^+$ cells, respectively, upon LPS maturation. CD137-Fc treatment could only give rise to low

levels of IL-12 in monocytes but not in $CD34^+$ cells after LPS maturation. Without LPS treatment CD137L signaling had actually slightly reduced basal IL-12 levels in monocytes and $CD34^+$ cells (Fig. 3.11 B).

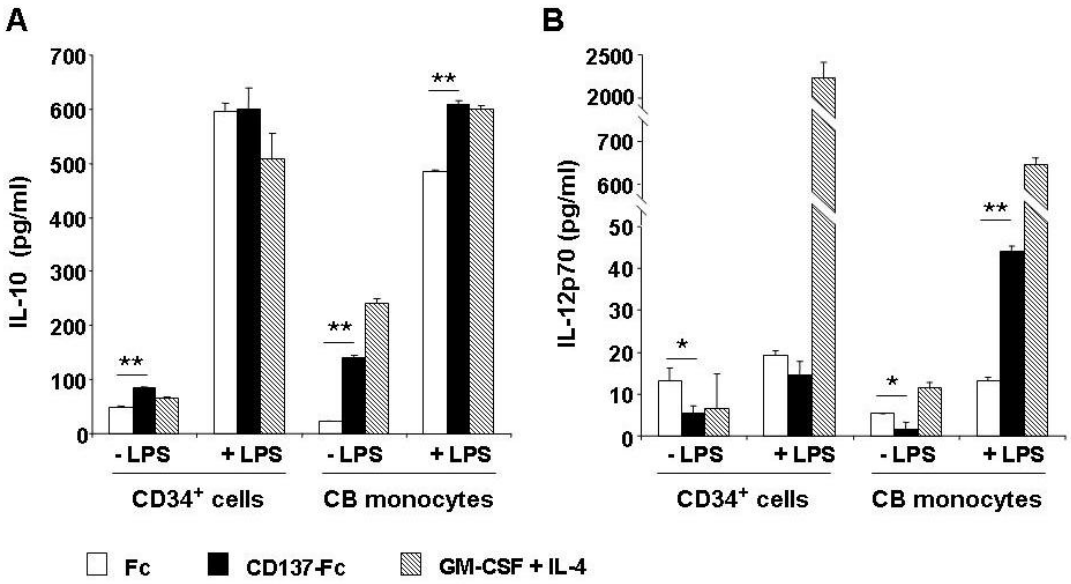


Figure 3.11. IL-10 and IL-12p70 ELISA. Human $CD34^+$ cells or cord blood monocytes were cultured for 10 days in RPMI, 10% FBS in wells coated with 10 μg/ml Fc or CD137-Fc protein. 100 ng/ml of GM-CSF + 25 ng/ml of IL-4 were used as controls for DC differentiation. LPS was added for the last 24 h at 1 μg/ml. IL-10 **(A)** and IL-12p70 **(B)** in cell culture supernatants were measured by ELISA. Depicted are means ± SD of triplicate measurements. * $p<0.05$; ** $p<0.01$. These experiments were repeated three times with similar results.

The ability to activate and induce clonal expansion of T cells is one of the most important signatures and functions of DCs. We tested this by using CD137-Fc-treated $CD34^+$ cells and cord blood monocytes as stimulator cells in an allogeneic mixed lymphocyte reaction (MLR). Compare with Fc-treated monocytes, CD137-Fc-treated ones induced T cell proliferation (Fig. 3.12); however, CD137-Fc-treated $CD34^+$ cells did not enhance T cell proliferation in the presence or absence of LPS (Fig. 3.12).

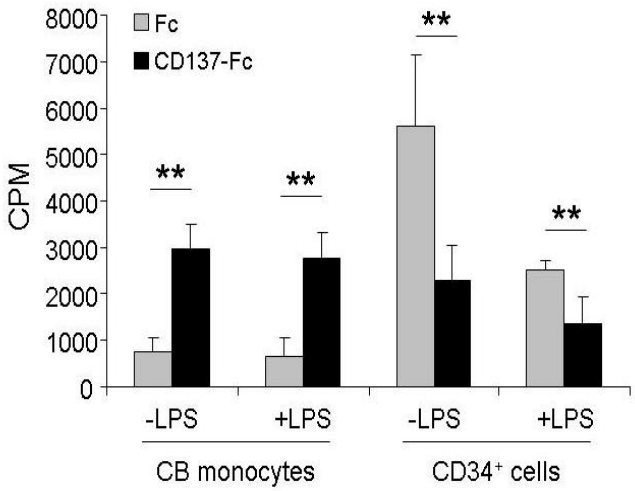


Figure 3.12. MLR. Human $CD34^+$ cells or cord blood monocytes were cultured for 10 days in RPMI, 10% FBS in wells coated with 10 μg/ml Fc or CD137-Fc protein. After mitomycin C treatment, the cells were used as stimulators in an allogeneic MLR with T cells from another buffy coat PBMC at a stimulator:responder cell ratio of 1:10. T cell proliferation was measured ^{3}H-thymidine incorporation. Depicted are means ± SD of triplicate measurements. **, $p<0.01$. This experiment was performed three times with similar results.

Taken together, the absence of DC markers (*SOCS1*, CD1a, CD83, CD209, IL-12p70, and inability to induce allogeneic T cell proliferation), expression of macrophage markers (IL-10 and enhanced phagocytosis) argue that CD137L signaling induces macrophage rather than DC differentiation in hematopoietic progenitor cells.

3.1.7 Maintenance of cell survival and induction of cell proliferation on $CD34^+$ cells by CD137 were partially dependent on IL-8 and cell density

Next, we wanted to investigate the underlying mechanism of CD137-induced

proliferation and differentiation of hematopoietic progenitor cells (HPC). We noticed that immobilized CD137L agonists-treated $CD34^+$ cells had significantly elevated IL-8 secretion in the presence (Fig. 3.13 A) or absence of serum (Fig. 3.13 B), compared with the cells treated with Fc or MOPC-21 controls or soluble CD137-Fc (Fig. 3.13).

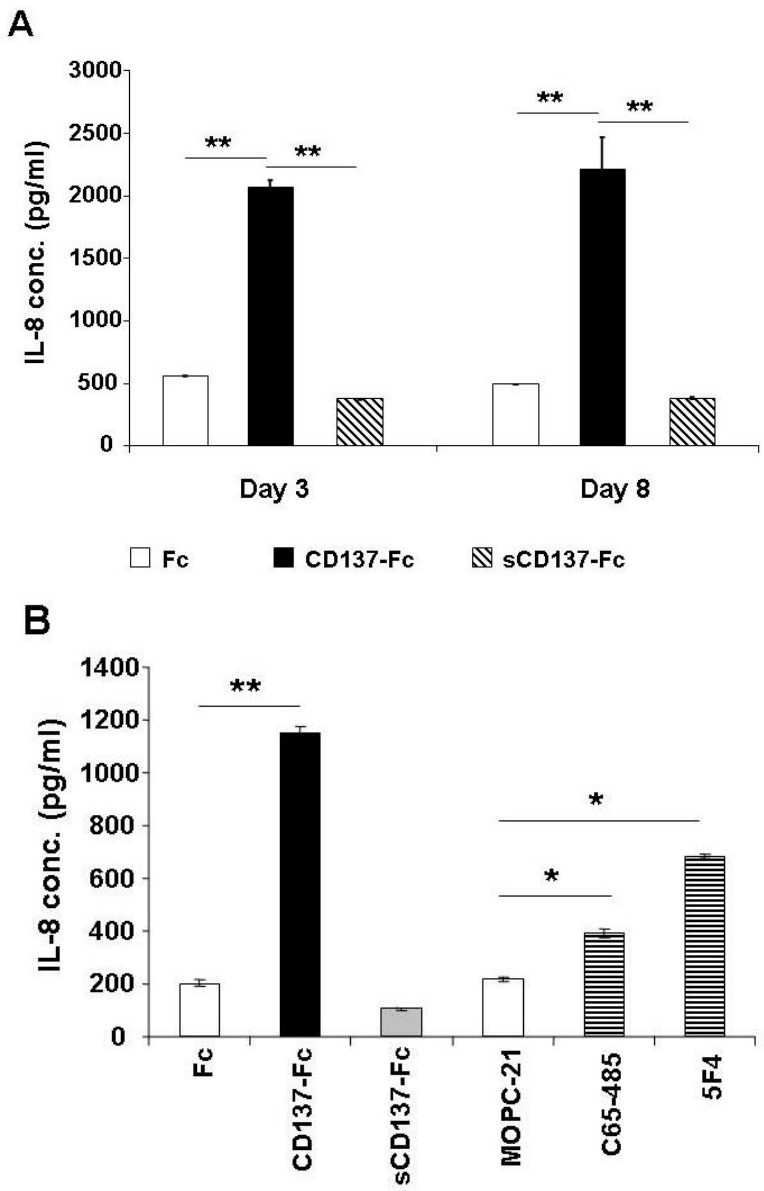


Figure 3.13. IL-8 ELISA. 2×10^5 of human $CD34^+$ cells at density of 4×10^5 / ml were cultured in complete medium for 8 days **(A)** or serum free medium for 10 days **(B)** in wells coated with 10 μg/ml Fc or CD137-Fc protein, 10 μg/ml MOPC-21 or

anti-CD137L mAb (clone C65-485 and 5F4), or CD137-Fc was added soluble. Supernatants were collected at day 3, 8 and 10, respectively. The concentration of IL-8 in the supernatants was measured by ELISA. Depicted are means ± SD of triplicate measurements. *, $p<0.05$, **, $p<0.01$. These experiments were repeated three times with similar results.

Since IL-8 is associated with activation and proliferation of cells such as monocytes / macrophages (Milara *et al.* 2009), endometriotic stromal cells (Takemura *et al.* 2007), epithelial cells (Li *et al.* 2003; Joh *et al.* 2005), and carcinoma cells (Itoh *et al.* 2005), it may also play a role in CD137-induced HPC activation and proliferation. We tested this possibility by adding neutralizing anti-human IL-8 antibody or the isotype control antibody (MOPC-21) into the culture. 0.5 μg/ml and 5 μg/ml of anti-IL-8 neutralizing antibody but not the isotype control could significantly reduce the viable cell numbers of CD137-Fc-exposed $CD34^+$ cells (Fig. 3.14). However, the cultures with anti-IL-8 neutralizing antibody still had statistically significant more viable cells than the cells treated with Fc protein (Fig. 3.14). Therefore, blocking IL-8 could partially abolish the effects of CD137L reserves signaling in $CD34^+$ cells. It suggested that CD137L agonists induced survival and proliferation of $CD34^+$ cells were partially dependent on autocrine or paracrine of IL-8.

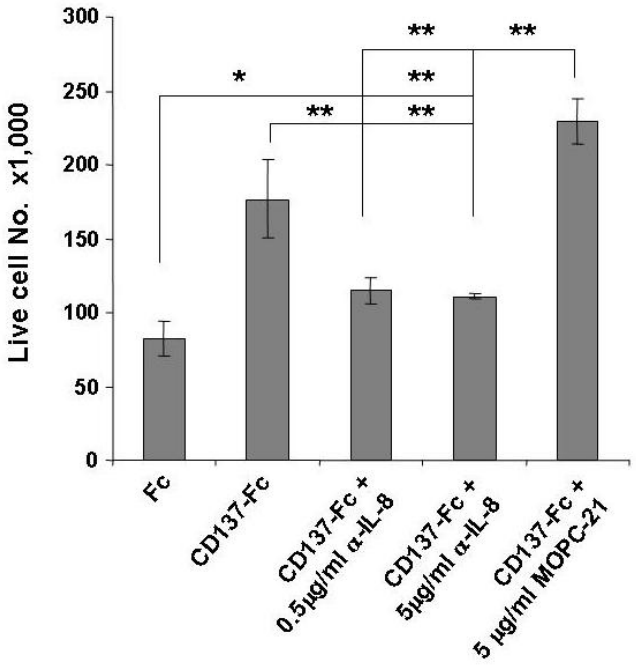


Figure 3.14. CD137-induced survival and proliferation of CD34$^+$ cells are partially dependent on IL-8. Human CD34$^+$ cells at a density of 5 × 10^5/ml were cultured in complete medium in wells coated with 10 μg/ml Fc or CD137-Fc protein. Anti-human IL-8 neutralizing antibody at 0.5 μg/ml and 5 μg/ml or isotype control antibody (MOPC-21) at 5 μg/ml were added into the respective wells. The cells were harvested on day 10 and the numbers of viable cells were determined by cell counting after trypan blue staining. Depicted are means ± SD of four representative fields of each of duplicate cultures. *, $p<0.05$; **, $p<0.01$. This experiment was performed twice with the same result.

During the culture, we also noticed that the initial cell density affected the magnitude of the effects on CD34$^+$ cells induced by CD137L agonists. A proliferation assay of CD137-Fc-treated CD34$^+$ cells was set up with different initial seeding densities. The cell proliferation increased exponentially with the increasing initial cell density (Fig. 3.15). Hence, the initial cell density plays a role in CD137L agonist-induced proliferation of CD34$^+$ cells. This suggests that some cytokines (e.g. IL-8) with autocrine or paracrine properties or cell-cell interactions are crucial in the CD137L reverse signaling pathway in HPC.

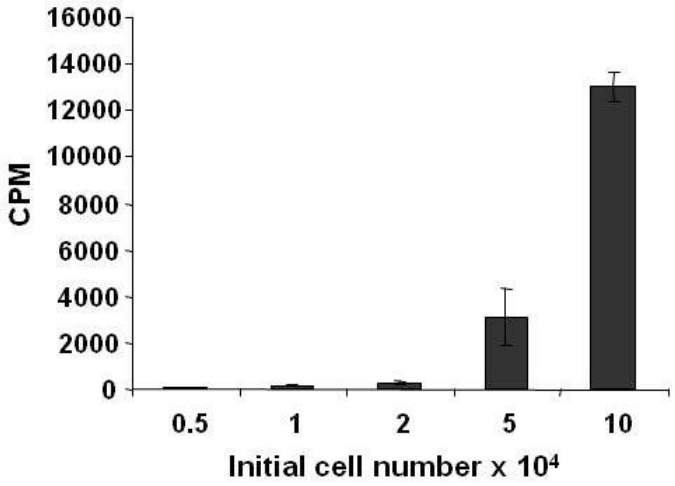


Figure 3.15. CD137-induced proliferation of CD34$^+$ cells increases exponentially with the increasing initial cell density. Human CD34$^+$ cells were cultured in complete medium in wells coated with 10 μg/ml CD137-Fc at 0.5 × 10^4, 1 × 10^4, 2 ×10^4, 5 × 10^4, 10 × 10^4 per well, respectively. Proliferation was determined on day 8 after cells had been labeled for the last 24 h with 0.5 μCi of ^{3}H-thymidine. Depicted are means ± SD of triplicate measurements. This experiment was performed twice with similar results.

3.1.8 CD137 is unable to induce the proliferation of DCs

Previous studies have demonstrated that crosslinking of CD137L induces proliferation and activation of peripheral monocytes (Langstein *et al.*, 1999a; Langstein *et al.*, 2000). It can also induce the maturation of the immature DCs and enhance the DCs activity. (Wilcox *et al.*, 2002; Kim *et al.*, 2002; Laderach *et al.*, 2003; Lippert *et al.* 2008). We therefore investigated whether CD137L signaling also induces the proliferation of DCs.

Monocytes were isolated from fresh peripheral blood (Fig. 3.16), buffy coat (not shown) or cord blood (not shown) and were cultured with GM-CSF + IL-4 for 7 days

to induce the differentiation of immature monocyte derived DCs (MoDCs). The maturation was pulsed by LPS or LPS + IFN-γ. Morphologically, the cells were semi-adherent and tended to form aggregates, and the cells treated with LPS + IFN-γ extended long dendrites (Fig. 3.16 A). Phenotypically, they all expressed high levels of DC markers DC-SIGN (CD209) and HLA-DR. The costimulatory molecules CD80 and CD86, the DC activation marker CD83 were upregulated, especially in LPS + IFN-γ treated cells (Fig. 3.16 B). Therefore, these cells were indeed immature and mature MoDCs.

These cells were then transferred to wells that had been coated with CD137-Fc or Fc in the presence or absence of conditioned medium from the DCs culture. There was no proliferation detectable in these cells by day 3 (not shown) and 6 (Fig. 3.16 C), except that the immature MoDCs in fresh medium had a basal level of DNA synthesis with no difference in CD137-Fc or Fc treatment. Therefore, no evidence could be obtained for CD137L signaling inducing the proliferation of the terminally differentiated human MoDCs.

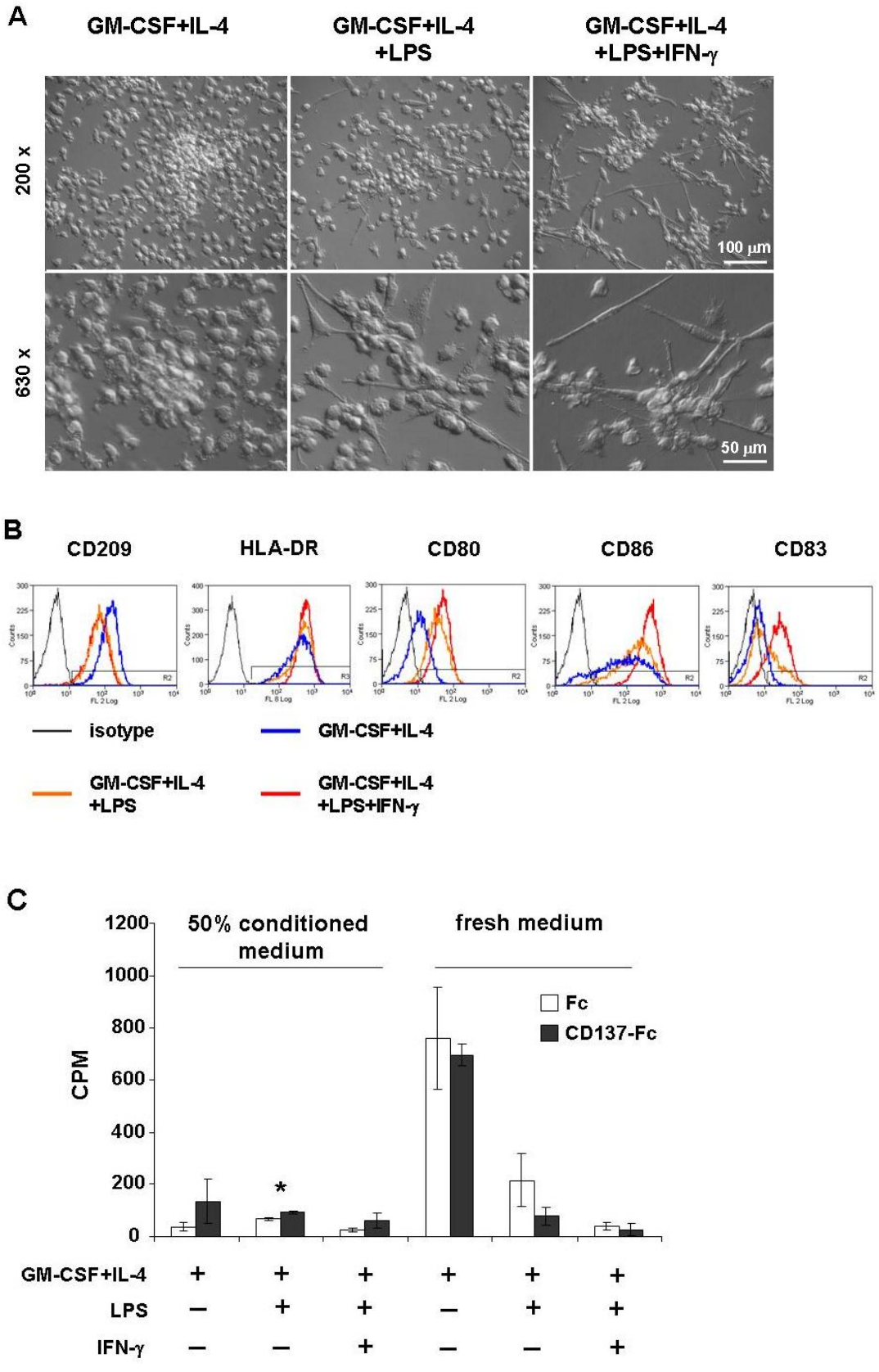


Figure 3.16. CD137 is unable to induce proliferation of human MoDCs. Monocytes purified from fresh peripheral blood at the density of 1 × 10^6 /ml were

cultured for 7 days in complete medium supplemented with 100 ng/ml of GM-CSF + 25 ng/ml of IL-4. 1 μg/ml LPS and 100 ng/ml IFN-γ were added in the corresponding wells for the last 24 h. **(A)** Photographs were taken on day 7 at room temperature at magnifications of 200× (scale bars: 100 μm) and 630× (scale bars: 50 μm). **(B)** Cells were harvested and stained for CD209, HLA-DR, CD80, CD86 and CD83 expression, and analyzed by flow cytometry. Black line: Isotype; blue line: GM-CSF + IL-4; orange line: GM-CSF + IL-4 + LPS; red line: GM-CSF + IL-4 + LPS + IFN-γ. **(C)** Culture supernatants were harvested as conditioned medium (CM) together with the cells. The cells were transferred to wells that had been precoated with 10 μg/ml Fc or CD137-Fc protein with 5×10^4 cells per well in fresh medium or complete medium that contained 50% CM. Cells were cultured for another 6 days and proliferation was determined after cells had been labeled for the last 24 h with 0.5 μCi of ^{3}H-thymidine. Depicted are means ± SD of triplicate measurements. These data are representatives of three independent experiments.

3.2 CD137 induces proliferation of murine hematopoietic progenitor cells and differentiation to macrophages

3.2.1 CD137 ligand signaling regulates survival and proliferation of murine bone marrow cells

In the experiments described in the previous section, our hypothesis that CD137 agonists are able to induce proliferation and differentiation of HSPCs proved to be true in the human system. We wanted to confirm these activities of CD137 agonists also in the murine system in order to use murine animal models.

Bone marrow cells were isolated from the femur bones of BALB/c mice. The cells were cultured on tissue culture plates that had been coated with CD137-Fc. Fc protein was used as a negative control. Coating was performed overnight at 4℃ and plates were washed with PBS afterwards. Cell numbers were determined by counting using a haemocytometer and viability was determined by trypan blue staining twice a week. 2×10^6 bone marrow cells were plated per condition and about 90% of the cells died during the first three days. But from day three onwards the number of live cells started to increase in the wells which had been coated with CD137-Fc protein until day 10 after which the live cell number declined again. By that time all cells in the other conditions had died. This and the decline of cell numbers in the CD137-Fc-treated wells after day 10 may be due to exhaustion of nutrients.

But more live cells were present in the CD137-Fc-coated wells compared to Fc-coated wells or untreated wells (PBS) throughout the experiment. Even at day 21 when the experiment was terminated CD137-treated conditions contained $>10^5$ live cells. G-CSF which had been added daily to the cells until day 21 resulted in a less rapid decline in cell numbers during the first seven days, but had no effect afterwards (Fig. 3.17 A). Enhancement of cell numbers by CD137 protein was dose-dependent. Saturation was reached at a coating concentration of 20 μg/ml CD137-Fc but a significant enhancement in live cell numbers could already be observed at 1.2 μg/ml (Fig. 3.17 B).

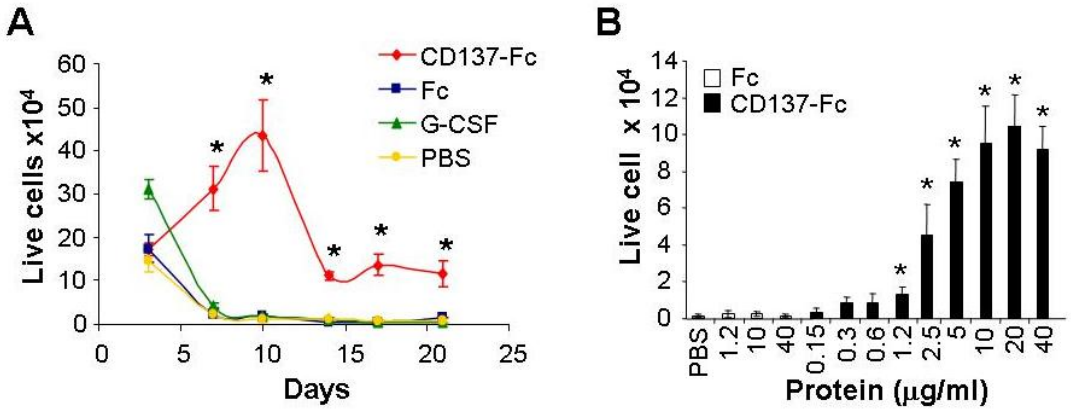


Figure 3.17. CD137L crosslinking increases cell numbers of murine bone marrow cells. Murine bone marrow cells at a density of 10^6 cells/ml were cultured on plates coated with indicated concentrations of Fc or CD137-Fc protein or on uncoated plates (PBS). **(A)** Time course. 2×10^6 cells were plated, and the number of live cells was determined by trypan blue staining and counting at indicated time points. PBS: yellow ovals; Fc (10 μg/ml): blue squares; CD137-Fc (10 μg/ml): red diamonds; G-CSF (0.1 ng/ml daily): green triangles. Depicted are means ± SD of 4 countings. **(B)** Dose response. Experimental conditions as in A, but the number of live cells in B was determined at day 12. *, $p<0.05$. The experiments were performed at least 3 times with similar results.

The increase in numbers of living cells by CD137-Fc could be due to survival,

proliferation or a combination of both. We determined therefore the rate of CD137-induced proliferation by measuring DNA synthesis and cell division using ^{3}H-thymidine and CFSE labeling, respectively. Concentrations of Fc and CD137-Fc protein ranging from 0.15 to 40 μg/ml were used to coat plates before 10^{5} bone marrow cells were added for 7 days. CD137-Fc induced a significant proliferation of bone marrow cells in both complete medium (Fig. 3.18 A) and serum free medium (Fig. 3.18 B) at concentrations as low as 1.2 μg/ml and reached its maximum activity at 20 μg/ml. Proliferation of cells in the Fc-coated wells was not different from that of cells in uncoated wells (PBS), (Fig. 3.18). Both, the number of live cells and the ^{3}H-thymidine incorporation rate exhibited an identical dose-dependency.

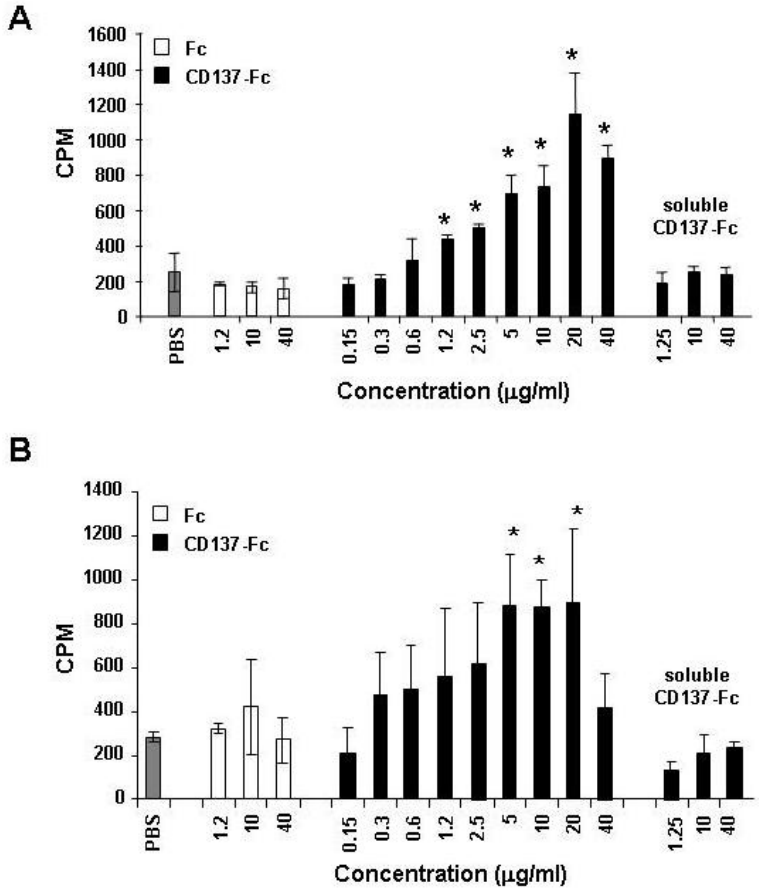


Figure 3.18. CD137L crosslinking induces proliferation of bone marrow cells. 10^5 bone marrow cells per well at the density of 10^6 cells/ml were cultured either in complete medium **(A)** or serum free medium **(B)** on plates coated with indicated concentrations of Fc or CD137-Fc protein or on uncoated plates (PBS) or indicated concentrations of soluble CD137-Fc protein. Cells were cultured for 7 days and labeled for the last 24 h with 0.5 μCi ^{3}H-thymidine. The rate of proliferation was determined as ^{3}H-thymidine incorporation with a scintillation counter. Depicted are means ± SD of triplicate measurements. *, $p<0.05$. The experiments were performed 3 times with similar results.

CD137-Fc needs to be immobilized onto the tissue culture plates in order to induce proliferation and survival of bone marrow cells. CD137-Fc in its soluble form is not active. When CD137-Fc was added to the cells as a soluble protein and its immobilization to the tissue culture plates was prevented by precoating the plates with serum, no increase in cell numbers was observed (Fig. 3.18). This demonstrates that CD137-Fc works by crosslinking CD137L on bone marrow cells or a subset of these cells, and excludes that the observed effects are due to potential contaminants in the protein preparation.

The subset of bone marrow cells that is considered to be most enriched for hematopoietic progenitor cells is negative for lineage-specific markers (Lin^-) and positive for CD117 (c-kit), the receptor for stem cell factor. Lin^-, $CD117^+$ cells constitute only 0.5 - 1% of bone marrow cells. CD137-Fc increased proliferation of the Lin^-, $CD117^+$ cells to the same extend (about five fold) than that of total bone marrow cells, and soluble CD137-Fc was inactive (Fig. 3.19). But total cell proliferation was about 10-fold higher in Lin^-, $CD117^+$ cells than in unfractionated

bone marrow cells.

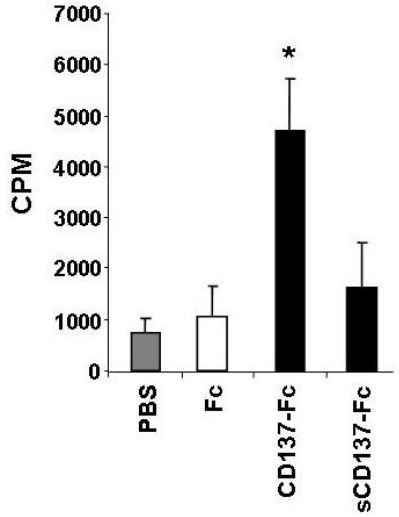


Figure 3.19. CD137L crosslinking induces proliferation of Lin$^-$, CD117$^+$ cells. 5 × 10^4 Lin$^-$,CD117$^+$ cells per well were cultured on plates coated with 10 μg/ml Fc or CD137-Fc protein or on uncoated plates (PBS) or 10 μg/ml soluble CD137-Fc protein. Cells were cultured for 7 days and labeled for the last 24 h with 0.5 μCi ^{3}H-thymidine. The rate of proliferation was determined as ^{3}H-thymidine incorporation with a scintillation counter. Depicted are means ± SD of triplicate measurements. *, $p<0.01$. The experiments were performed 3 times with similar results.

36 × 10^6 bone marrow cells were labeled with 5 μM CFSE. Labeling efficacy was verified by flow cytometry and the area of CFSE-negative cells was defined as M1 (Fig. 3.20). Only 0.9 % of the CFSE-labeled cells were in M1. The CFSE-labeled cells were cultured for 14 days on immobilized CD137-Fc or Fc protein, or treated daily with 0.1 ng/ml of G-CSF. Flourescence of CSFE-labeled bone marrow cells which were cultured in the presence of Fc control protein had diminished by day 14, indicating some cell division had occurred but only 0.9% of the cells were in M1 which was the same percentage as for freshly labeled cells (Fig. 3.20). In contrast,

CD137-Fc induced strong proliferation causing 44% of the cells to shift into the M1 region. G-CSF also induced cell division, and 9.1% of the G-CSF-treated cells were in M1 (Fig. 3.20). This data was adopted from my colleague Chen Yifeng to solidify the proliferation of bone marrow cells induced by immobilized CD137-Fc protein (Jiang *et al.*, 2008a).

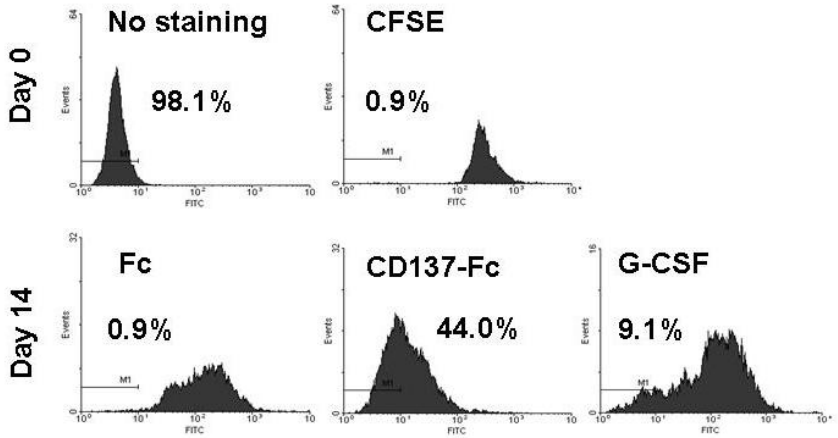
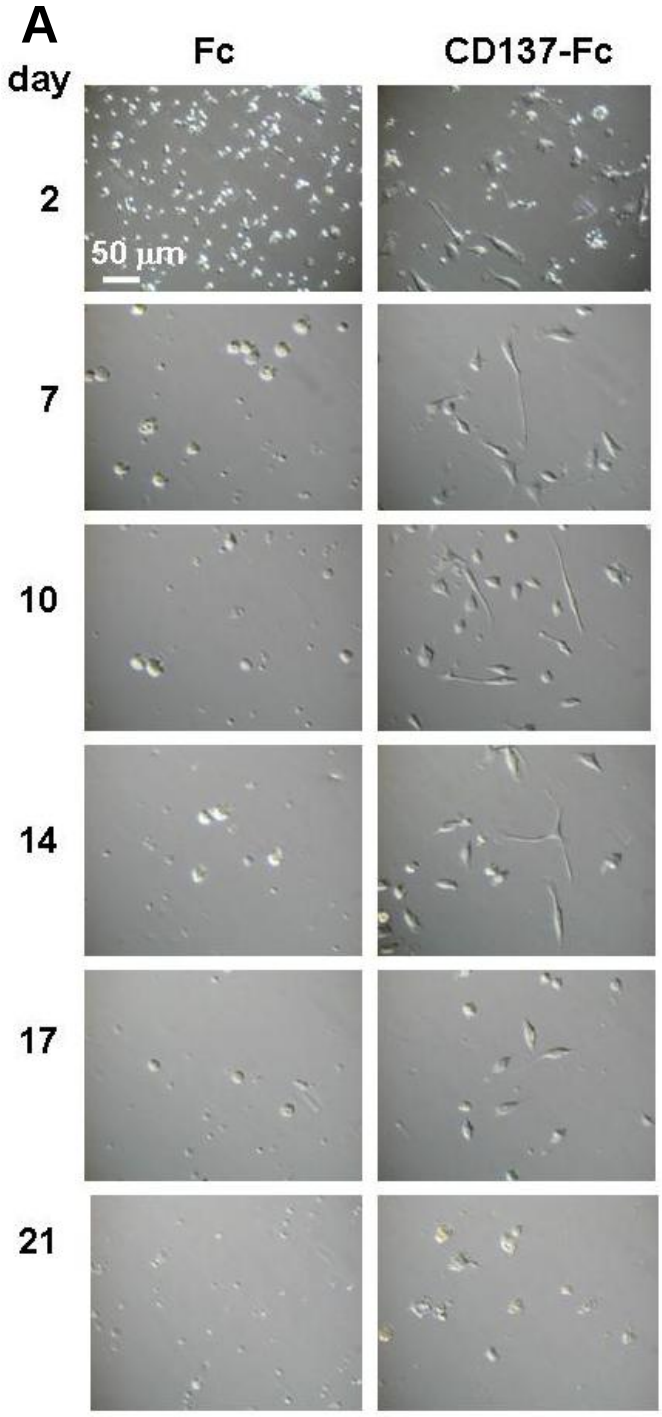


Figure 3.20. Tracking of cell division. Freshly isolated bone marrow cells were either not treated or stained with 5 μM CFSE and were immediately (day 0) analyzed by flow cytometry. CFSE-stained bone marrow cells were incubated for 14 days on immobilized Fc or CD137-Fc protein, or were treated daily for 14 days with 0.1 ng/ml of G-CSF and analyzed by flow cytometry for CFSE flourescence. Depicted are histograms with percentages of CFSE-negative cells (gated in M1). This experiment was performed twice by Chen Y. with similar results. (Jiang *et al.*, 2008a).

3.2.2 CD137 ligand signaling changes the morphology of murine bone marrow cells and Lin$^-$, CD117$^+$ cells

Concomitant with survival and proliferation CD137-Fc protein induced adherence and morphological changes. These morphological changes were of the same type in the subpopulation of whole bone marrow cells which survived until day 7 (Fig. 3.21 A) as

in the Lin^-, $CD117^+$ cells (Fig. 3.21 B). However, the magnitude of the response was greater in Lin^-, $CD117^+$ cells, as a higher percentage of the Lin^-, $CD117^+$ cells responded to the CD137L crosslinking. Many more cells attached to CD137-Fc-coated plates than to the Fc-coated or untreated control plates (Fig. 3.21 B). During the first three days CD137-Fc induced spreading in the attached cells, and some grew a tail-like extension on one end and lamellopodia on the opposite end. By day seven, the tails had grown longer, sometimes reaching five times the length of the cell body, while the lamellopodia were changing to filopodia. Some cells had several branches and displayed star-like shapes. From day 10 - 17, some cells adopted round and flat ovoid shapes. Several morphological characteristics described above coexisted in the culture. From day 21 onwards, filopodia disappeared and tails shortened, the cells adopted spindle-like shapes (Fig. 3.21 B). The appearance of the tail-like extensions and the lamellopodia and filopodia coincided with the phase of cell growth. They disappeared after day 21 when the cells stopped proliferating and the cell numbers started to decline. Soluble CD137-Fc protein (sCD137-Fc) did not induce such morphological changes (Fig. 3.21 B).


A
Fc
CD137-Fc
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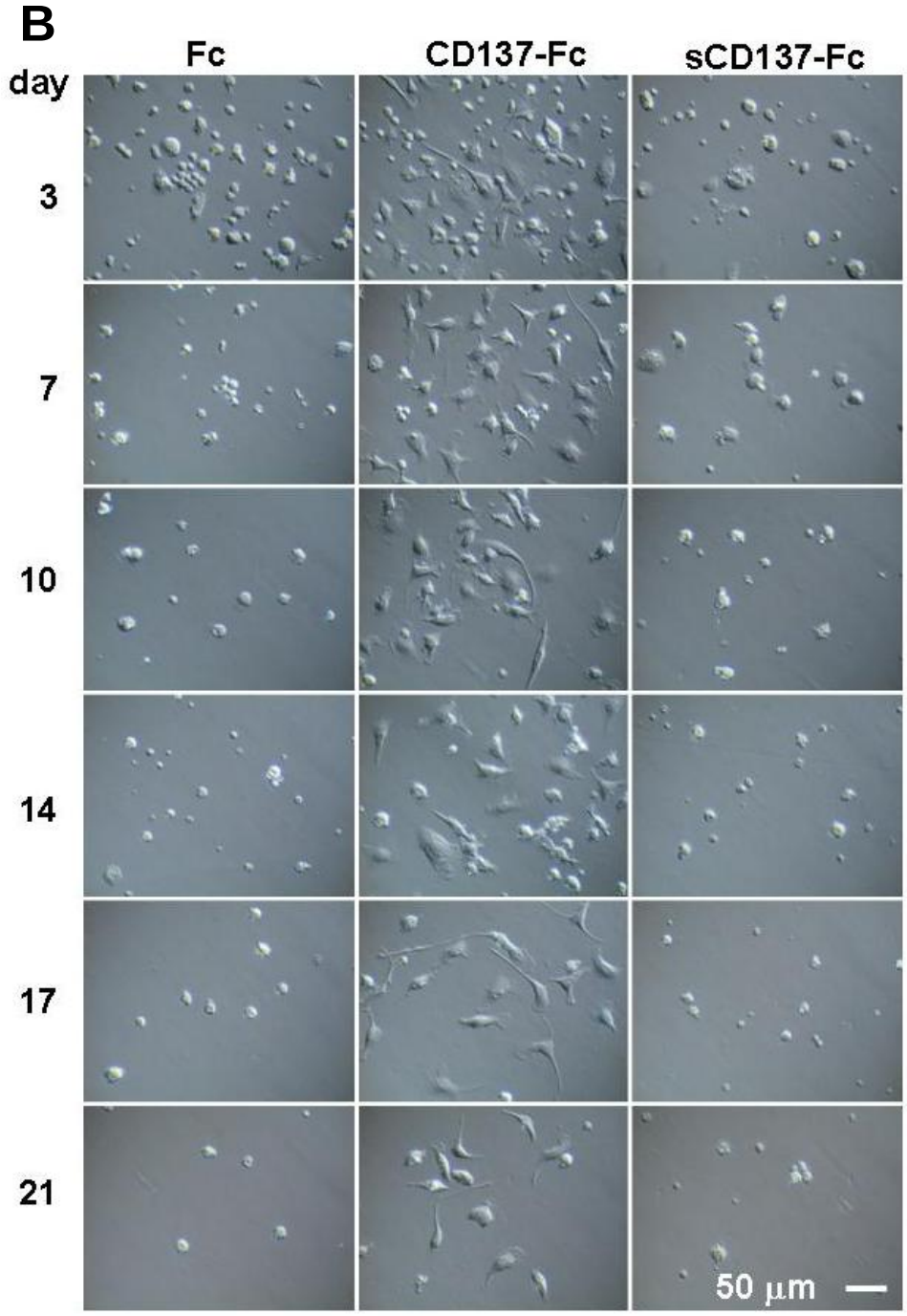


Figure 3.21. Morphological changes induced by CD137 protein. (A) 2×10^6 murine bone marrow cells at a density of 10^6 cells/ml, or **(B)** 5×10^5 Lin^-, $CD117^+$ cells at a density of 5×10^5 cells/ml were cultured on plates coated with 10 μg/ml of Fc or CD137-Fc protein. Photographs were taken at indicated times at a magnification of 400×. Scale bar is 50 μm. This experiment was performed three times with identical results.

3.2.3 CD137 ligand signaling induces colony formation in hematopoietic progenitor cells

Proliferation induced by CD137L stimulation was also confirmed by colony assays. Bone marrow cells were cultured for 14 days in methylcellulose medium containing 10% bovine calf serum on tissue culture plates with immobilized CD137-Fc or Fc protein. There were in average only 3 ± 1 colonies in the Fc-coated dishes compared to 40 ± 10 in the CD137-Fc-coated dishes. In addition, colonies on Fc protein contained fewer than 100 cells whereas colonies on CD137-Fc protein were composed of up to 5,000 cells (Fig. 3.22).

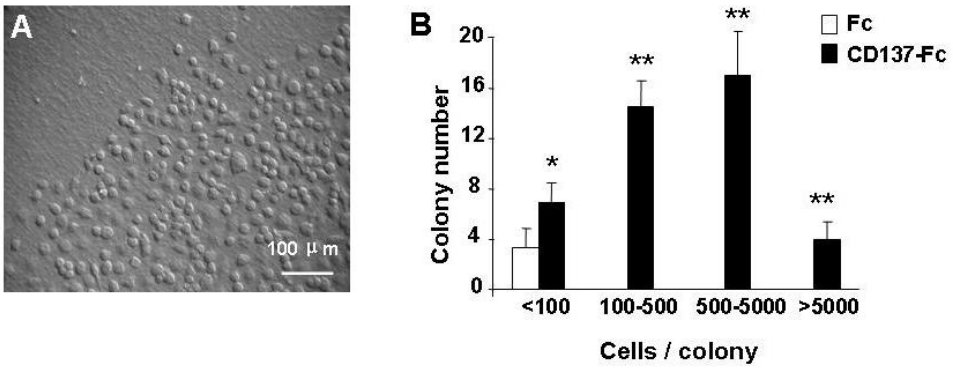


Figure 3.22. Colony formation from bone marrow cells in response to CD137. 5×10^4 bone marrow were cultured in methylcellulose medium containing 10% bovine calf serum on 35 mm dishes which were coated with 10 µg/ml Fc or CD137-Fc protein. **(A)** A photograph of the edge of a large colony grown on CD137-Fc-coated plates were taken at day 14 at a magnification of 200×. **(B)** Numbers and seizes of the colonies were counted on day 14. *, $p<0.05$; **, $p<0.01$. The cell numbers in the colonies larger than 50 were determined by counting representative sections of the colony. Depicted are means ± SD of triplicate measurements. This experiment was performed 3 times with similar results.

The Lin^-, $CD117^+$ cells formed colonies at a much higher rate than bone marrow cells. Since Lin^-, $CD117^+$ cell colonies had grown to confluency by day 14 they had to be

assessed already at day 7 (Fig. 3.23).

CD137-induced similar looking colonies in total bone marrow cells and lin^-, $CD117^+$ cells, implying that it were the lin^-, $CD117^+$ cells among the bone marrow cells that responded to CD137. Most colonies (80.8±1.9%) were colorless with dark, dense cores or consisted of granular or foamy cells. Based on these morphological characteristics they were identified as colony forming unit granulocyte-macrophage (CFU-GM), (Yoon *et al.*, 2006, Coutinho *et al.*, 1993). CFU macrophage (CFU-M), recognizable by their larger and individually identifiable cells were also induced by CD137 and constituted 16.9±1.4% of the colonies. Very few (2.3±0.5%) CFU granulocyte (CFU-G), (smaller and more tightly packed cells) could be identified (Fig. 3.23 B-D). No additional growth factors besides CD137 were present in this assay to support colony formation.

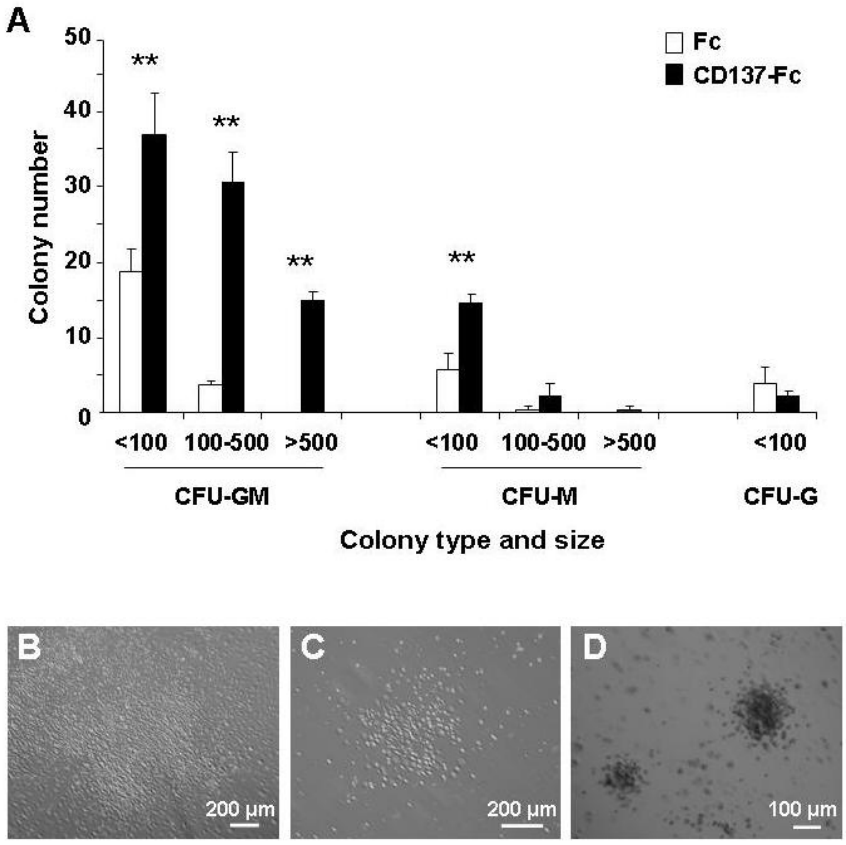


Figure 3.23. Colony formation from Lin$^-$, CD117$^+$ cells in response to CD137. 10^4 Lin$^-$, CD117$^+$ cells were cultured in methylcellulose medium containing 10% bovine calf serum on 35 mm dishes which were coated with 10 µg/ml Fc or CD137-Fc protein. Colonies formed by Lin$^-$, CD117$^+$ cells on day 8. **(A)** Quantitative evaluation. Depicted are means ± SD of triplicate measurements. **(B) – (D)** Photographs of CFU-GM, CFU-M and CFU-G, respectively, at a magnification of 50×. **, $p<0.01$. The cell numbers in the colonies larger than 50 were determined by counting representative sections of the colony. This experiment was performed twice with similar results.

Since CD137L signaling induced formation of myeloid colonies and of cells with myeloid morphology it was possible that CD137 protein exerted its activities through induction of GM-CSF. However, a neutralizing anti-GM-CSF antibody had no influence on the appearance (Fig. 3.24 A), proliferation rate (Fig. 3.24 B), nor on the type, number and size of colonies of Lin$^-$, CD117$^+$ cells (Fig. 3.24 C).

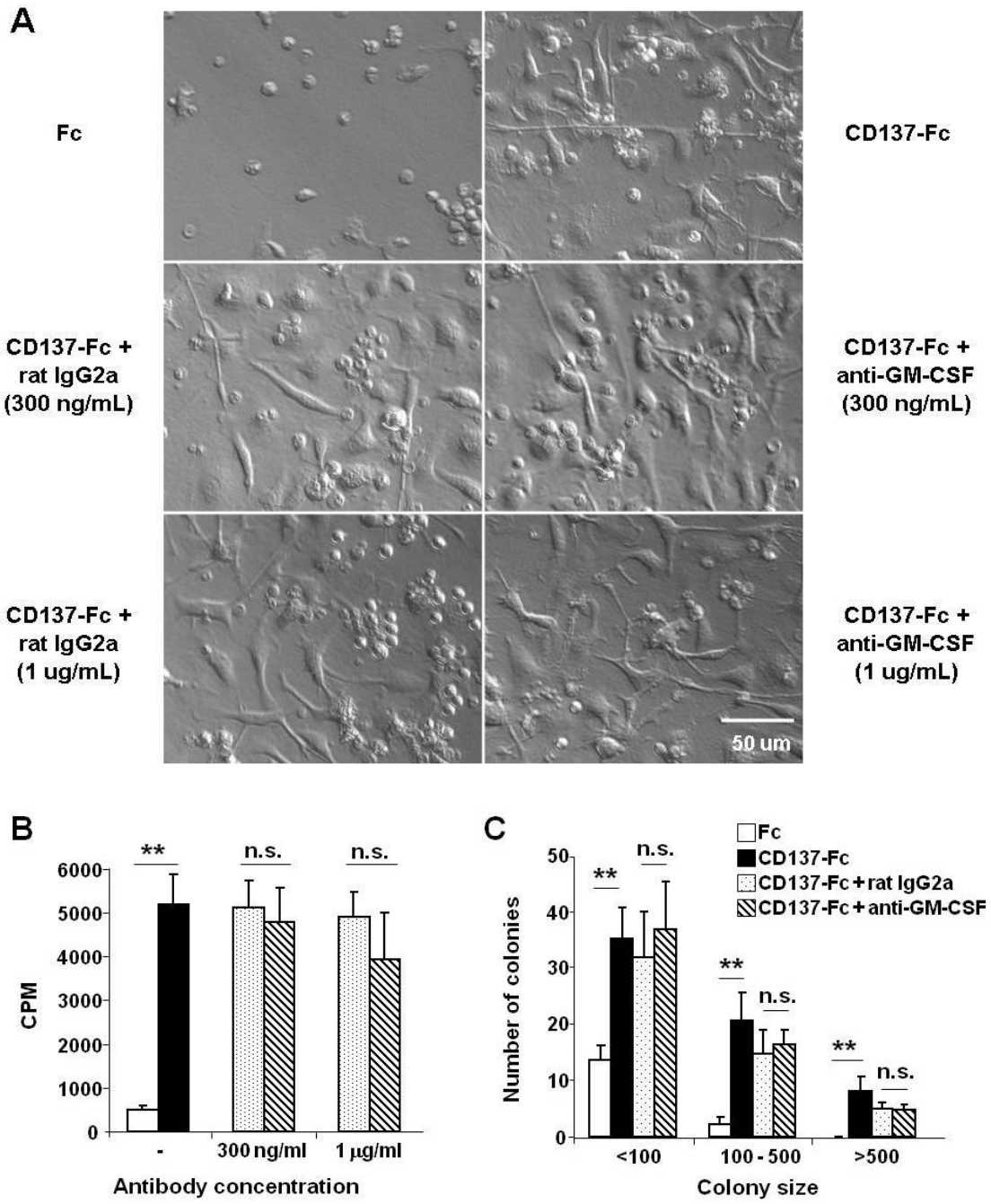


Figure 3.24. Effect of neutralizing anti-GM-CSF antibody on CD137-induced (A) morphological changes, (B) proliferation, and (C) colony formation. Rat IgG2a: isotype control. Scale bar in (A) is 50 μm. Concentration of antibodies in **(C)** is 300 ng/ml. **, $p<0.01$; n.s.: not significant. The cell numbers in the colonies larger than 50 were determined by counting representative sections of the colony. Depicted in (B) and (C) are means ± SD of triplicate measurements. This experiment was performed three times with similar results.

3.2.4 Expression of CD137 and CD137 ligand on bone marrow cells

The above described proliferative response of bone marrow cells to CD137L agonists implied CD137L expression by bone marrow cells. Further, if the CD137L induced proliferation were to occur in the bone marrow then CD137 should also be expressed there.

Indeed, CD137L is expressed constitutively by a subset of murine bone marrow cells. By immunocytochemical staining with a polyclonal or a monoclonal anti-CD137L antibody 8.9±3.8% or 6.8±0.7%, respectively, of freshly isolated and untreated bone marrow cells were found to express CD137L (Fig. 3.25 A). Also, the expression of CD137 on few (4.2±0.4%) but distinct cells in the murine bone marrow was detected immunocytochemically (Fig. 3.25 B). Expression of CD137L and CD137 mRNAs could also be verified by RT-PCR (Fig. 3.25 C, D). Bone marrow cells from healthy naïve C57/BL6 mice express very little CD137, but the expression elevated significantly after nasal infection of *Bordetella pertussis* bacteria (Fig. 3.25 D). The details of regulation of CD137 expression in the bone marrow upon infection will be discussed in Section 3.5.

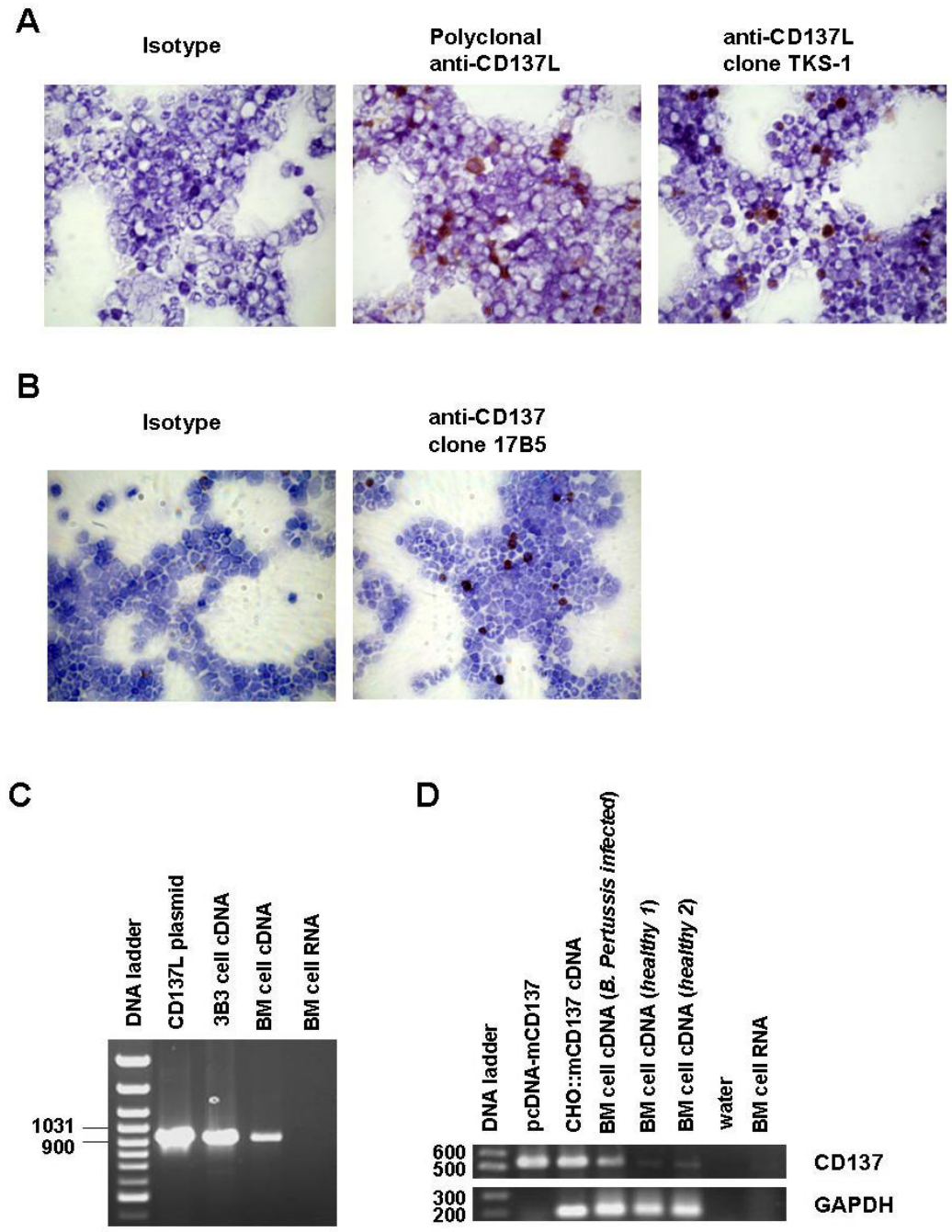


Figure 3.25. Expression of CD137 and CD137L in the bone marrow. Fresh bone marrow cell smears were fixed and immunocytochemically stained (brown). **(A)** CD137L expression was detected with a polyclonal and a monoclonal anti-CD137L antibody (clone TKS-1). **(B)** CD137 expression was detected by staining with anti-CD137 antibody (clone 17B5). Magnification: 200×. Expression of **(C)** CD137L and **(D)** CD137 mRNA was determined by RT-PCR using cDNA from bone marrow cells as a template. Cloned CD137L cDNA (CD137L plasmid) and cDNA from 3B3 cells were used as positive controls for CD137L. Cloned CD137 (pcDNA-mCD137) and cDNA from mCD137 transfected CHO cells (CHO::mCD137) were used as

positive controls for CD137. Reactions with no template (water) or RNA form bone marrow cells which was not reverse transcribed served as a negative control. Comparable amounts of cDNA were verified by amplification of GAPDH. GeneRuler DNA ladder (Fermentas) was used as a marker. PCR products were analyzed on a 1% TAE agarose gel and stained by ethidium bromide. These experiments were performed 3 times with similar results.

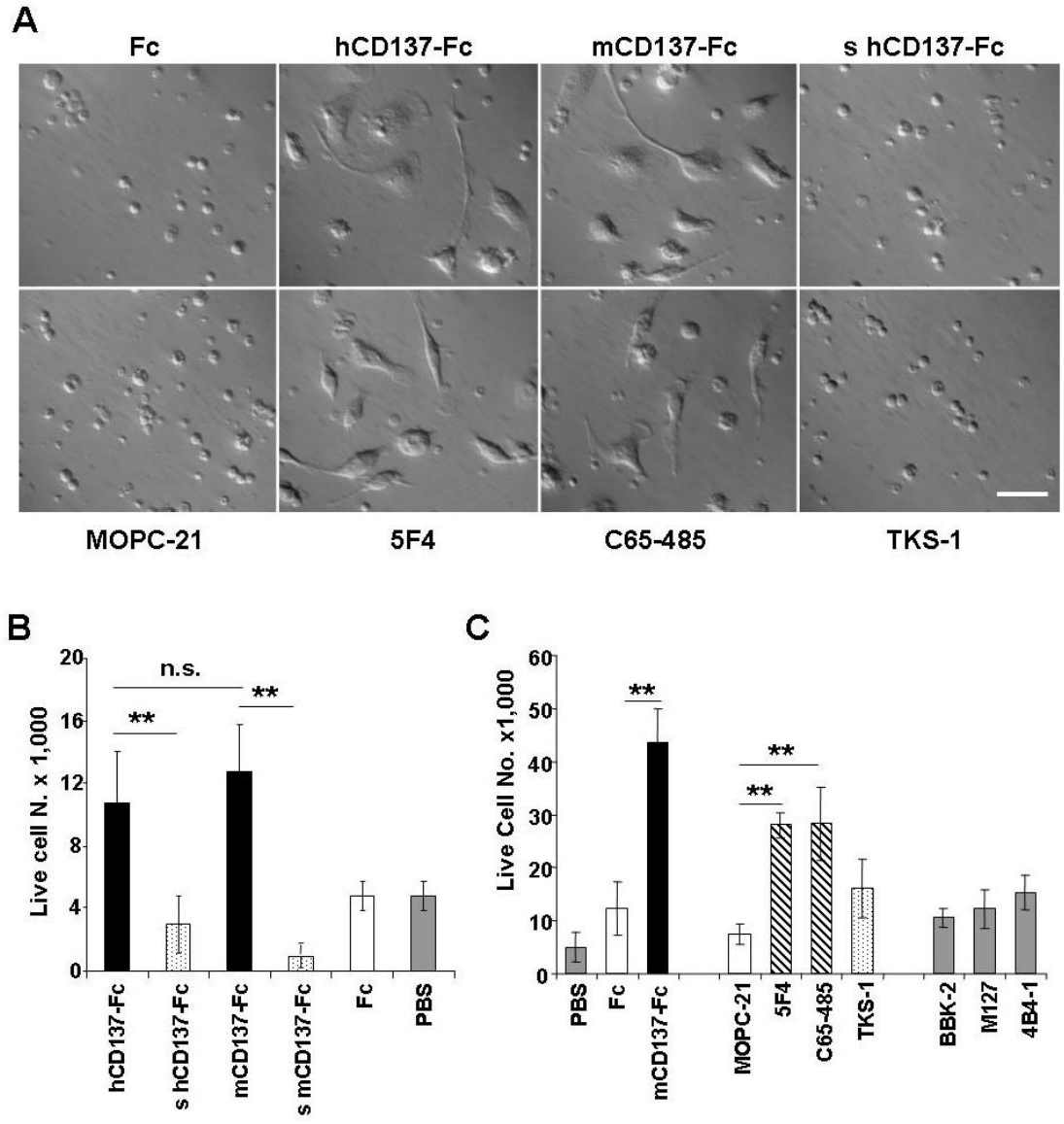
3.2.5 Murine CD137 elicits same effects as human CD137 on murine bone marrow cells

We have detected the expression of both murine CD137L and CD137 on bone marrow cells; however, the CD137-Fc recombinant protein we used here contains the extracellular domain of human CD137. Therefore, we wanted to determine whether murine CD137 was functionally equivalent to human CD137 on nurine bone marrow cells.

Murine bone marrow cells adopted the same morphological changes in response to murine CD137-Fc as was observed with human CD137-Fc protein (Fig. 3.26 A). Both proteins induced a similar increase in viable cell numbers (Fig. 3.26 B) and proliferation (Fig. 3.26 D, compared with Fig. 3.18 A); and both proteins were inactive when they were added as soluble proteins (Fig. 3.26 B). Interestingly, anti-human CD137L mAb (clone 5F4 and C65-485) could induce similar morphological changes and a similar increase in viable cell number too (Fig. 3.26 A, C), while their isotype control (clone MOPC-21) and the antagonistic anti-mouse CD137L mAb (clone TKS-1) could not. Moreover, anti-human CD137 mAbs (clone

BBK-2, M127, and 4B4-1) had no such activities (Fig. 3.26 C and not shown) further proving that the signal was transduced through CD137L.

Further more, the human or murine CD137 expressed on CHO cells could also induce the proliferation of cocultured bone marrow cells with similar extend (Fig. 3.26 E). Again, it proved functional equivalence between human and murine CD137, and it also indicated that the Fc portion of the fusion protein was not required.



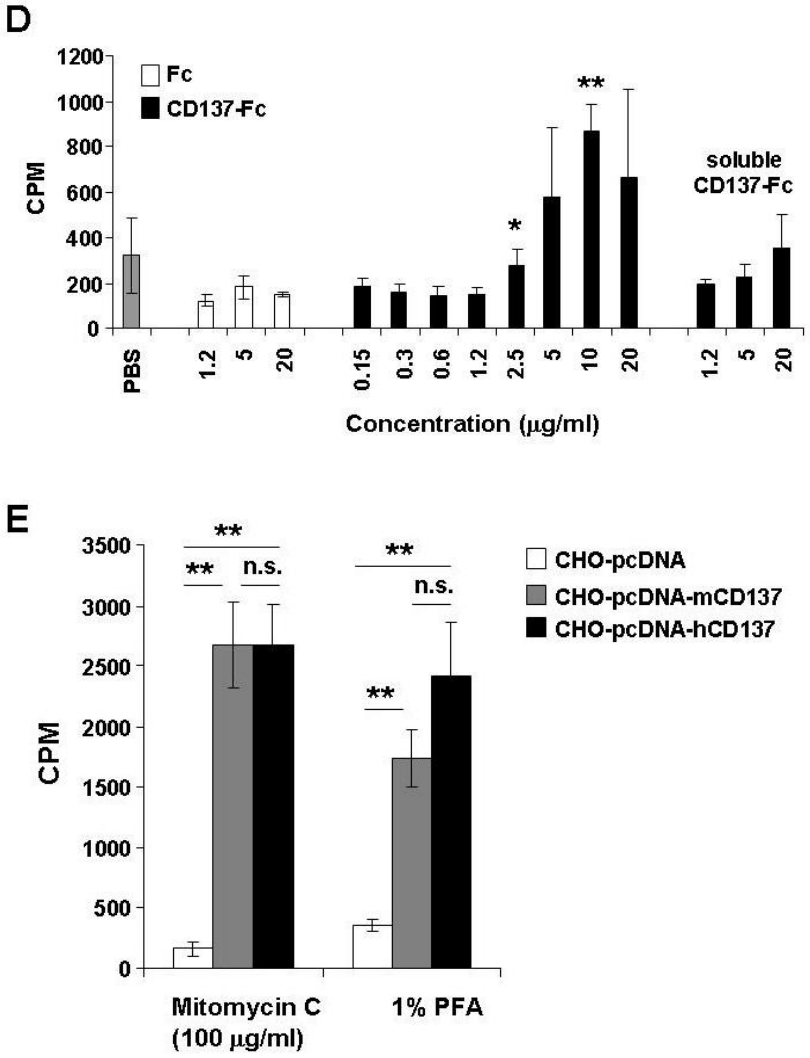


Figure 3.26. Functional equivalence of human and murine CD137. (A) 2×10^6 murine bone marrow cells were cultured on uncoated plates (PBS) or on plates coated with 10 μg/ml Fc or human CD137-Fc or murine CD137-Fc protein; anti-human CD137L mAbs (clone 5F4 and C65-485) or their isotype control (clone MOPC-21) or antagonistic anti-mouse CD137L mAb (clone TKS-1) or anti-human CD137 mAbs (clone BBK-2, M127, and 4B4-1). In addition, human or murine CD137-Fc proteins were added as soluble proteins. Photographs were taken at day 14. Scale bar = 25 μm, magnification = 630×. **(B)** 3×10^5 or **(C)** 2×10^6 bone marrow cells were undergone same treatment in (A) and viable cell numbers were counted after trypan blue staining on day 7. **(D)** 1×10^5 bone marrow cells at density of 1×10^6 /ml were cultured for 7 days on uncoated plates (PBS) or on plates coated with indicated concentration of murine CD137-Fc or Fc, or with indicated concentration of soluble murine CD137-Fc. **(E)** CHO cells stably transfected with murine CD137 (CHO-pcDNA-mCD137) or human CD137 (CHO-pcDNA-hCD137) on pcDNA3.1 vector or empty vector (CHO-pcDNA) were arrested by treating overnight with 100 μg/ml Mitomycin C or fixed overnight with 1% paraformaldehyde (PFA). 1×10^5 bone marrow cells at density

of 1×10^6 /ml were cocultured with arrested or fixed CHO cells for 7 days. For proliferation assessment, cells were labeled for the last 24 h with 0.5 μCi ^{3}H-thymidine, and the rate of proliferation was determined with a scintillation counter (Packard). Depicted are means ± SD of triplicate measurements. *, $p<0.05$; **, $p<0.01$; n.s., not significant. These experiments were performed three times with similar results.

3.2.6 CD137 ligand signaling induces differentiation to monocytic cells

Induction of colony formation, especially of the CFU-GM type had already implied that CD137L signaling induces differentiation of bone marrow cells as well as of Lin^-, $CD117^+$ cells to the myeloid lineage. We confirmed this by analyzing cell marker expression by flow cytometry.

Among freshly isolated bone marrow cells the most prominent subpopulations were $Gr\text{-}1^+$ granulocytes (46.7%), and $CD11b^+$ myeloid cells (36.8%). However, most of the myeloid cells seemed to be immature since CD14 and F4/80, markers for monocytes and macrophages, were present only on 0.1% and 9.2% of the cells, respectively. CD11c a marker for DCs was found only on 2.6% of the cells. 1.2% and 11.6% of the cells were T and B cells, respectively, as determined via CD3 and CD19 expression (Fig. 3.27 A, B).

At day seven, only 12 % of the CD137-Fc-treated cells and 3.2% of the Fc-treated cells had survived. T and B cells and granulocytes had disappeared (0.4%, 0.6% and 3.2%, respectively), indicating that lymphocyte and granulocyte survival and/or differentiation are not supported under these conditions. Among the CD137-Fc-treated

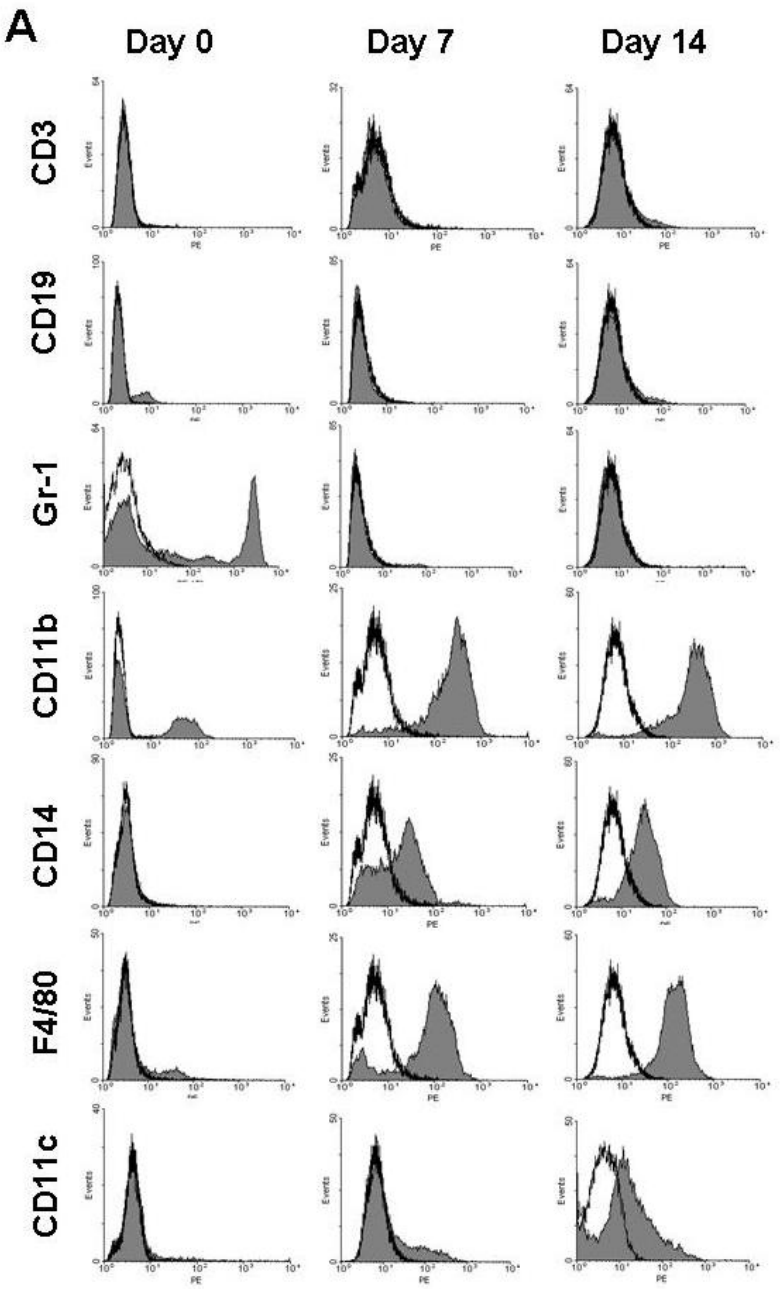
cells, the percentages of cells positive for CD11b, CD14, and F4/80 had increased to 91.4%, 43.9% and 82.2%, respectively. However, since the total cell number had dropped to 12% by day 7 this increase in percentage did not translate into an increase in total cell numbers for $CD11b^+$ and $F4/80^+$ cells. The number of myeloid cells ($CD11b^+$) actually decreased while the $F4/80^+$ population remained at the same level. The only cell population that was increased in relative proportion (>400-fold) and absolute numbers (50-fold) were the $CD14^+$ cells. Among the Fc-treated cells, the percentages of cells positive for CD11b, CD14, F4/80 had also increased (79.8%, 27.7% and 67.7%, respectively) compared to fresh bone marrow cells, but to a lesser degree compared to CD137-Fc-treated cells. Also, there was only an increase in percentage, not in absolute cell numbers for any of these cell populations (Fig. 3.27 A, B*)*.

At day 14 the absolute cell numbers had further decreased to 9.3% and 0.4% in the CD137-Fc and Fc conditions, respectively. Among the CD137-Fc-treated cells the $F4/80^+$ population was unchanged compared to day 7, the $CD11b^+$ population had slightly declined in percentage and absolute numbers, while the $CD14^+$ population had further increased in percentage and absolute number. Among the Fc-treated cells the $CD11b^+$, $CD14^+$, $F4/80^+$ populations were lower in percentage and absolute numbers (Fig. 3.27 A, B).

The absolute number of DCs as detected by CD11c was the same in fresh bone

marrow cells and CD137-Fc-treated cells throughout the experiment but their percentage in the overall population had increased from 2.6% to 21.8%. The Fc control protein was not able to support DCs survival or differentiation (Fig. 3.27 A, B).

These findings were confirmed by immunofluorescent staining. CD137-Fc-treated bone marrow cells contained more $CD11b^+$, $CD14^+$, $F4/80^+$, and $CD11c^+$ cells than the Fc treated bone marrow cells (Fig. 3.27 C).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Day 0
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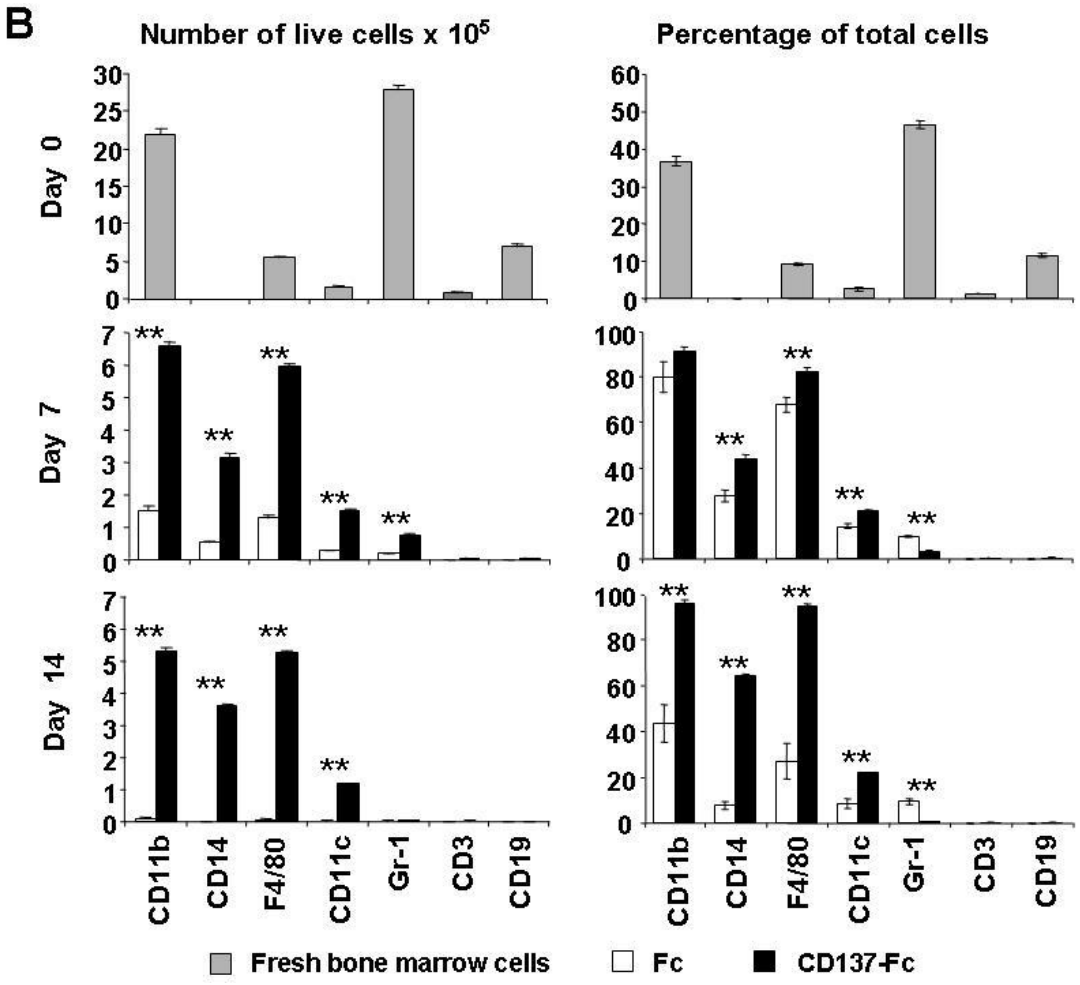
Day 14
CD3
CD19
Gr-1
CD11b
CD14
F4/80
CD11c
Events
PE


B
Number of live cells x 10^5
Percentage of total cells
Day 0
Day 7
Day 14
CD11b
CD14
F4/80
CD11c
Gr-1
CD3
CD19
Fresh bone marrow cells
Fc
CD137-Fc

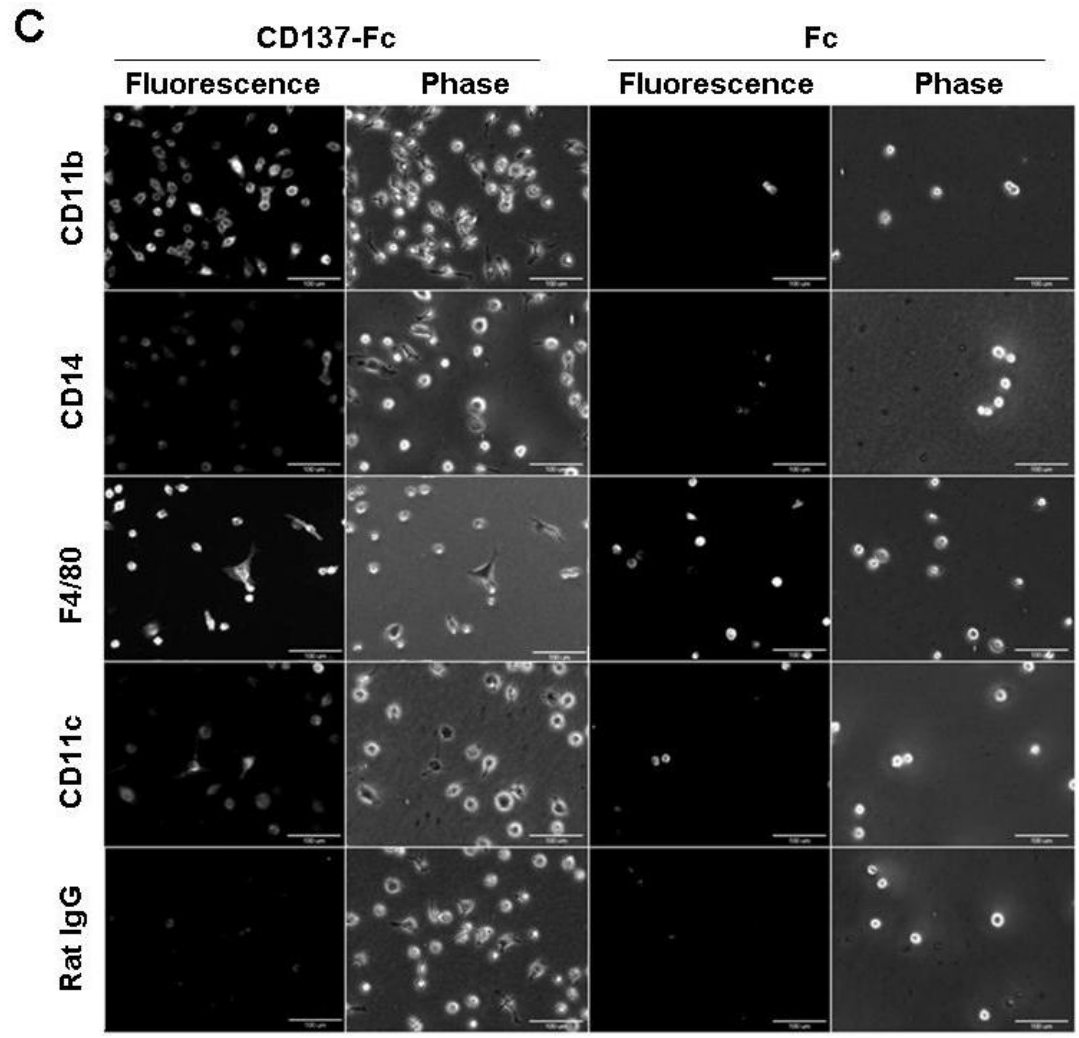


Figure 3.27. CD137L signaling induces differentiation of bone marrow cells to monocytic cells. (A) Bone marrow cells were analyzed immediately after isolation (day 0) for cell surface marker expression by flow cytometry, or 6×10^6 bone marrow cells were cultured in T25 flasks coated with 1 ml of 10 μg/ml of Fc or CD137-Fc protein and analyzed on days 7 and 14. Black line: Isotype. Gray, filled curve: Marker-specific antibody. **(B)** Depicted are absolute numbers of live cells (left column) and percentages (right column) of data in (A) for indicated subpopulations at days 0, 7 and 14. This experiment was performed three times with identical results. **(C)** 1×10^6 bone marrow cells were cultured in 6-well plate coated with 1 ml of 10 μg/ml of Fc or CD137-Fc protein. On day 7 supernatants were removed and cells were stained with PE-conjugated antibodies. Photos were taken under light phase contrast or fluorescence microscope. Scale bar = 100 μm.

Since the total bone marrow cells contain mature myeloid cells, such as monocytes and macrophages, which are known to respond to CD137L signals with survival and proliferation (Langstein *et al.* 1999a, 1999b, Ju *et al.* 2003), it is possible that these mature cells had influenced the differentiation results we had obtained. Hence, we further investigated CD137-induced differentiation using Lin^-, $CD117^+$ cells.

The Lin^-, $CD117^+$ cells were entirely depleted of mature cells assuring that any mature cells present after CD137L stimulation would necessarily have to have differentiated from hematopoietic progenitor cells (Fig 3.28 A). Lin^-, $CD117^+$ cells cultured for 7 days in the presence of immobilized CD137-Fc protein contained around 30 - 40% of $CD11b^+$, $CD14^+$, $F4/80^+$ or $CD11c^+$ cells while expression of these markers on the Fc control cells was negligible (Fig 3.28 B). The numbers of live cells expressing these myeloid markers were enhanced accordingly by CD137L signaling (Fig 3.28 C).

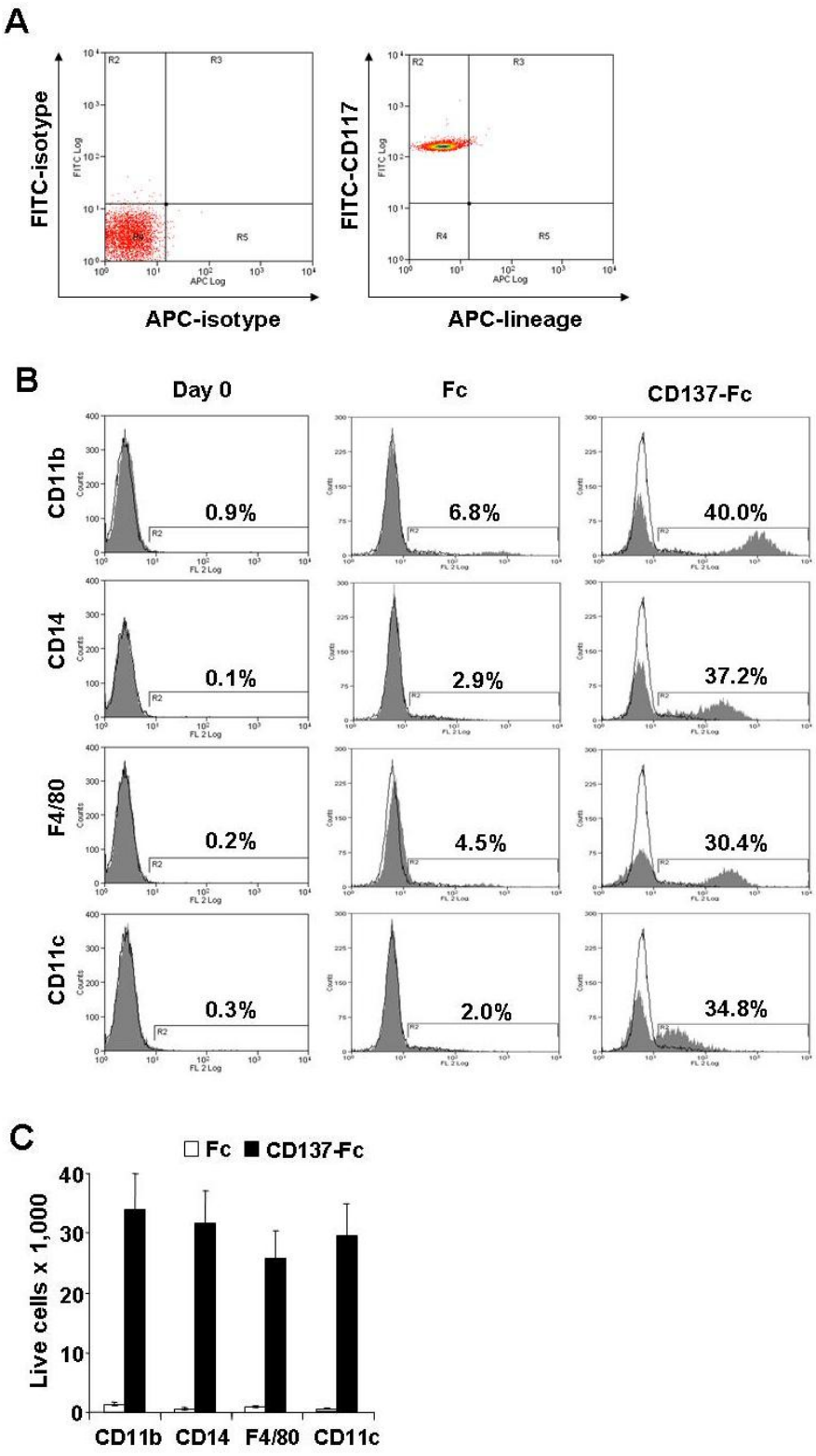


Figure 3.28. CD137L signaling induces differentiation of Lin$^-$, CD117$^+$ cells to monocytic cells. (A) Lin$^-$, CD117$^+$ cells were analyzed immediately after isolation for expression of mature cell markers (CD5, CD45R (B220), CD11b, anti-Ly-6G (Gr-1), 7-4, and Ter119 antibodies) by flow cytometry. **(B)** Lin$^-$, CD117$^+$ cells at a density of 5×10^5 cells/ml were cultured on plates coated with 10 μg/ml of Fc or CD137-Fc

protein, and expression of cell surface marker was analyzed by flow cytometry on day 7. Black line: Isotype. Gray, filled curve: Marker-specific antibody. **(C)** Depicted are absolute numbers of live cells of data in (B). This experiment was performed 3 times with similar results.

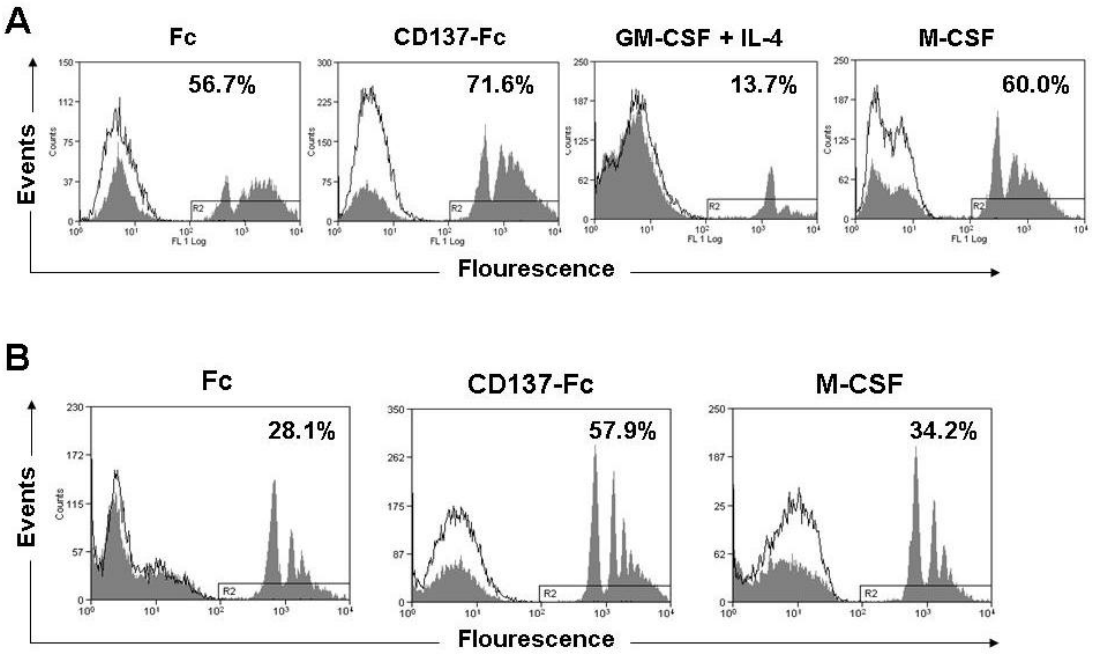
The identical nature of CD137 effects on total bone marrow cells and Lin^-, $CD117^+$ cells, and the fact that the Lin^-, $CD117^+$ cells respond more strongly than the total bone marrow cells suggest that the small subpopulation in the bone marrow cells which responded to CD137-Fc protein is largely contained within the Lin^-, $CD117^+$ cells.

3.2.7. CD137 ligand signaling induces macrophage differentiation

The comparable numbers of cells expressing macrophage markers (CD14, F4/80) and DC markers (CD11c) which CD137L signals had induced in both bone marrow cells and Lin^-, $CD117^+$ cells were compatible with macrophage and DC differentiation. In order to clarify the nature of cells that had been derived from hematopoietic progenitor cells after differentiation with CD137 protein, we assessed their biological functionalities, including the phagocytotic ability, the ability to induce allogeneic T cell proliferation, and the cytokine profile.

Bone marrow cells that were exposed for seven days to CD137-Fc exhibited an enhanced phagocytosis compared to Fc-treated cells (71.6% vs. 56.7%), (Fig. 3.29 A).

Bone marrow cells that had been treated with M-CSF as a positive control for inducing macrophage differentiation, displayed a similar phagocytic activity as Fc-treated control cells (60.0% vs. 56.7%) but surprisingly were not as active as CD137-Fc-treated cells (60.0% vs. 71.6%). The cells that had been treated with GM-CSF + IL-4 as a positive control for inducing DC differentiation, displayed a lowest phagocytotic activity (13.7%) as expected. Similarly, Lin^-, $CD117^+$ cells that were exposed for seven days to CD137-Fc exhibited an enhanced phagocytosis compared to Fc-treated cells (57.9% vs. 28.1%), (Fig. 3.29 B). This increased phagocytic activity suggested a macrophage differentiation. In addition, almost all the phagocytotic CD137-treated bone marrow cells were $F4/80^+$ (99.1%), further confirming macrophage differentiation. Interestingly, 30.7% of the phagocytic cells also expressed CD11c (Fig. 3.29 C).



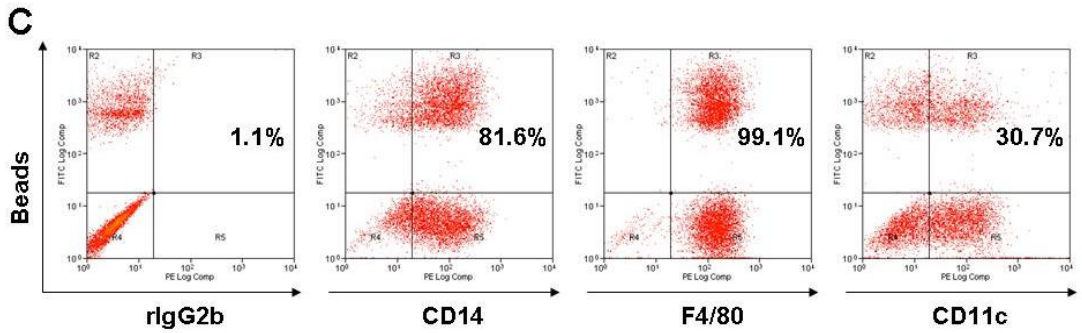


Figure 3.29. Phagocytosis assay. (A) 2×10^6 bone marrow cells or **(B)** 5×10^5 Lin^-, $CD117^+$ cells were cultured for 7 days in complete medium in wells coated with 10 μg/ml Fc or CD137-Fc protein. Or the cells were treated with 100 ng/ml GM-CSF + 25 ng/ml IL-4, or 50 ng/ml M-CSF as the controls for DC and macrophage differentiation, respectively. Phagocytosis was measured by a 1 h pulse with fluorescent latex beads (gray shade) at 50 beads per cell, followed by flow cytometry. White lines: Control without beads. **(C)** After being pulsed with fluorescent beads, the CD137-Fc-treated bone marrow cells in (A) were harvested and stained with PE-conjugated antibodies for CD14, F4/80, CD11c or their isotype control rat IgG2b, and were analyzed by flow cytometry. These data are representative of three independent experiments.

Since only about 70% of bone marrow cells and 60% of Lin^-, $CD117^+$ cells that had been differentiated with CD137-Fc were phagocytically active, and since CD137-Fc treatment had induced expression of CD11c, it was possible that some of the CD137-Fc-treated bone marrow cells and Lin^-, $CD117^+$ cells had differentiated to DCs. We tested this possibility by using CD137-Fc-treated cells as stimulator cells in an allogeneic MLR. CD137-Fc-treated bone marrow cells (Fig. 3.30 A) and Lin^-, $CD117^+$ cells (Fig. 3.30 B) did not enhance T cell proliferation and neither did the Fc control protein nor M-CSF (Fig. 3.30 B). However, cells that had been treated with GM-CSF + IL-4, standard conditions for DCs generation, induced significant T cell proliferation (Fig. 3.30 A, B).

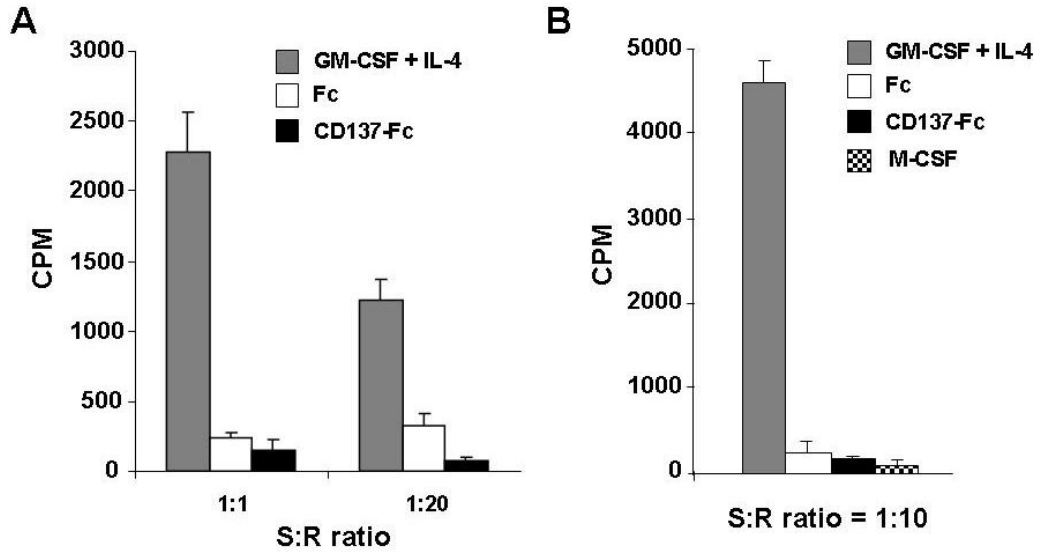


Figure 3.30. Allogeneic MLR. (A) BALB/c bone marrow cells or **(B)** Lin^-, $CD117^+$ cells were cultured for 7 days in wells coated with 10 μg/ml Fc or CD137-Fc protein, or in wells containing 50 ng/ml M-CSF or 100 ng/ml GM-CSF + 25 ng/ml IL-4. After mitomycin C treatment, the cells were used as stimulators in an allogeneic MLR with T cells from C57/BL6 mice at the indicated stimulator:responder (S:R) cell ratios. T cell proliferation was measured by ^{3}H-thymidine incorporation. Depicted are means ± SD of triplicate measurements. These experiments were repeated three times with similar results.

DCs also differ from macrophages in their ability to secrete IL-12 (Smith *et al.*, 1998). Fc-, CD137-Fc- or M-CSF-treated Lin^-, $CD117^+$ cells secreted large amounts of IL-10 upon LPS stimulation but little IL-12 (Fig. 3.31). GM-CSF + IL-4-treated cells had the reverse pattern with high levels of IL-12 and low levels of IL-10. CD137L signals enhanced IL-10 and reduced IL-12 slightly but significantly (Fig.3.31).

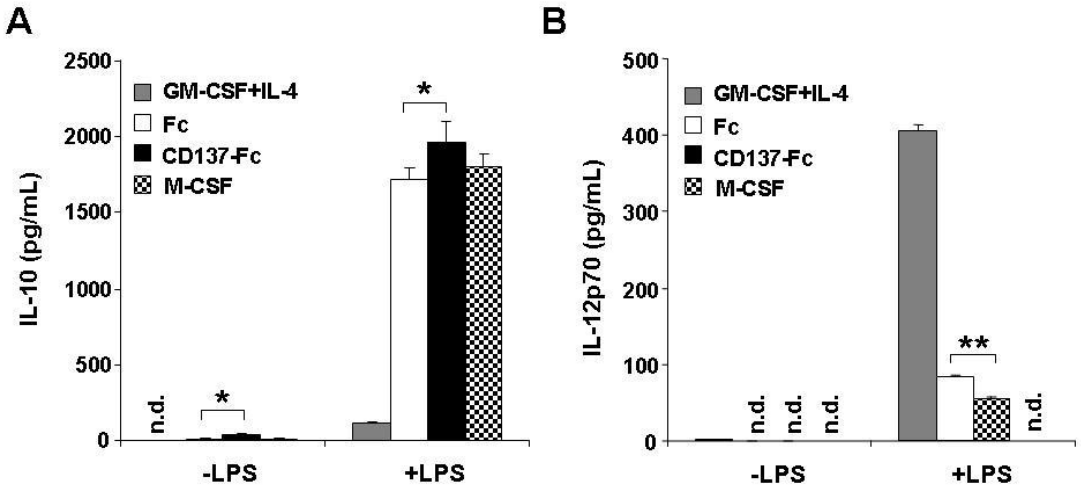


Figure 3.31. ELISA of IL-10 and IL-12p70. Lin$^-$, CD117$^+$ cells were cultured for 7 days in wells coated with 10 μg/ml Fc or CD137-Fc protein, or in wells containing 50 ng/ml M-CSF or 100 ng/ml GM-CSF + 25 ng/ml IL-4. LPS was added for the last 24 h at 1 μg/ml. Supernatants were harvested on day 7. **(A)** IL-10 and **(B)** IL-12p70 were measured by ELISA. Depicted are means ± SD of triplicate measurements. *, $p<0.05$; **, $p<0.01$. n.d.: Not detectable. This experiment was repeated three times with similar results.

Besides this IL-10^{hi}IL-12^{lo} profile, CD137-Fc-treated bone marrow cells and Lin$^-$, CD117$^+$ cells also secreted significantly higher levels of macrophage associated cytokines compare to the Fc treated counterparts, such as MCP-1, TNF, M-CSF, KC, MCP-5, and TECK, detected by cytokine bead array (BD Biosciences) (Fig. 3.32 A) and by cytokine antibody array (RayBiotech) (Fig. 3.32 B; Table 3). The bone marrow cells released cytokines with fold change ≥ 2 detected by cytokine antibody array are listed in Table 3.

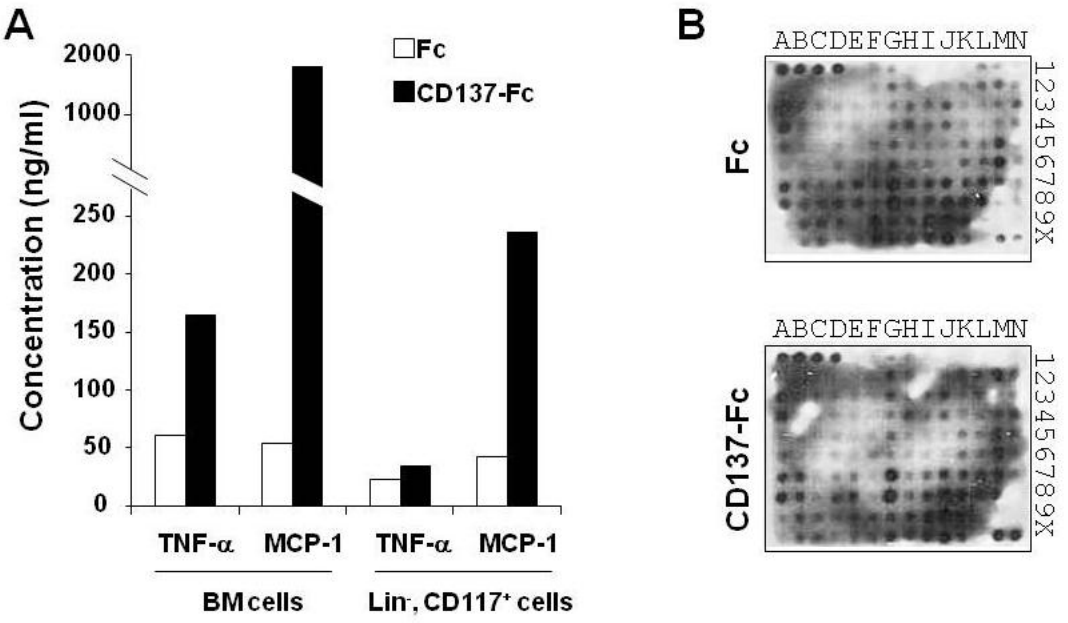


Figure 3.32. Cytokine profile. 2×10^6 bone marrow cells or 5×10^4 Lin^-, $CD117^+$ cells were cultured in plate that had been coated with 10 mg/ml Fc or CD137-Fc. Supernatants were harvested on day 7. **(A)** Cytokine levels in supernatants from bone marrow cells and Lin^-, $CD117^+$ cells were analyzed by cytokine bead array for murine TNF and MCP-1 (IL-12p70, IFN-γ, IL-10, and IL-6 were lower than detection limit). **(B)** Cytokine levels in supernatants from bone marrow cells were analyzed by cytokine antibody array. Membranes were exposed to X-ray film, and signal intensities were scanned and quantified with ImageQuant TL v2005 (Amersham). The cytokines of the corresponding dots on the membrane were listed in Appendix IV. The cytokines with fold change ≥ 2 were listed in Table 3.

Table 3. Differential cytokine profiles between CD137-Fc- and Fc-treated bone marrow cells on day 7 detected by cytokine antibody array.

Cytokines	Signal (Fc)	Signal (CD137-Fc)	Fold change (CD137-Fc / Fc)
sTNF-RII	64.47	647.93	**10.1**
sTNF-RI	102.82	867.56	**8.4**
TNF	117.82	621.51	**5.3**
TPO	351.92	1,068.38	**3.0**
TECK	116.81	306.46	**2.6**
MCP-1	469.40	1,130.27	**2.4**
KC	115.11	268.95	**2.3**
M-CSF	362.79	748.09	**2.1**
MCP-5	261.16	511.29	**2.0**

IL-13	194.90	396.87	**2.0**
SCF	216.56	48.91	**–4.4**

TPO, thrombopoietin; TECK, thymus-expressed chemokine; MCP-1, monocyte chemoattractant protein-1; KC, keratinocyte cytokine; SCF, stem cell factor.

In summary, the high levels secretion of macrophage associated cytokines and the IL-10^{hi}IL-12^{lo} profile of CD137-Fc-treated bone marrow cells and Lin^-, $CD117^+$ cells are in agreement with the enhancement of phagocytosis and the inability to induce T cell proliferation in an allogeneic MLR, and demonstrate that CD137L signaling induces differentiation to macrophages but not to DCs.

3.2.8. Macrophages induced by CD137L signaling are immunosuppressive

Based on the IL-10^{hi}IL-12^{lo} profile and inability to simulate T cells in an allogenic MLR in previous experiments, it hinted that the cells induced by CD137 may be suppressive instead of inflammatory. To test the potential suppressive properties of CD137-treated cells, and to identify the cells they influence, we cocultured GM-CSF + IL4 generated bone marrow derived DCs from BALB/c, splenic T cells from C57/BL6, and CD137-Fc-treated Lin^-, $CD117^+$ cells from BALB/c. The MLR showed that mature bone marrow derived DCs could induce robust T cell proliferation (Fig. 3.33). However, the T cell proliferation was partially suppressed by addition of CD137-Fc-treated Lin^-, $CD117^+$ cells. This suppression was dose -dependent, as less CD137-Fc-treated cells resulted less suppression. The T cell proliferation was restored

when the ratio of CD137-Fc-treated cells : DCs : T dropped to 0.05 : 1 : 5 (i.e. 10^3 CD137-Fc-treated cells per 2×10^4 DCs and 10^5 T cells) (Fig. 3.33). The paraformaldehyde fixed CHO in the DCs and T cells coculture failed to elicit the similar suppression on T cell proliferation (Fig. 3.33) indicated that the suppressive effect of CD137-Fc-treated cells was specific and not due to the spatial blockage of interaction between DCs and T cells. Therefore, the *in vitro* differentiated macrophages induced by CD137L signaling were immunosuppressive.

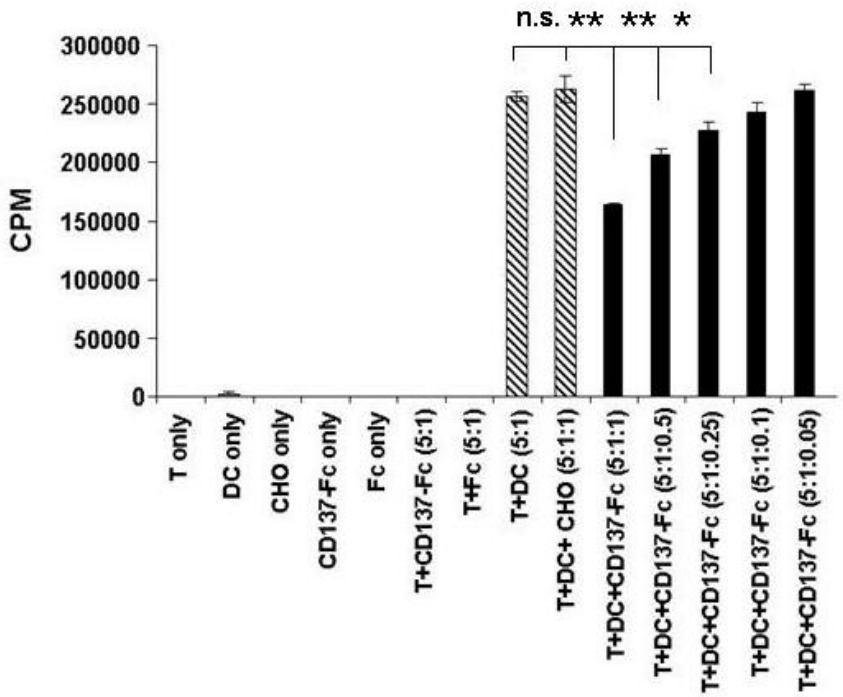


Figure 3.33. CD137-induced Lin$^-$, CD117$^+$ macrophages partially suppress T cell proliferation in an allogeneic MLR. Lin$^-$, CD117$^+$ cells were isolated from BALB/c bone marrow cells by MACS using the lineage depletion kit followed by CD117 selection kit (Miltentyi). Bone marrow derived DCs were generated by culturing fresh BALB/c bone marrow cells with 100 ng/ml GM-CSF and 25 ng/ml IL-4 for 7 days with 1 μg/ml LPS for the last 24 h. Lin$^-$, CD117$^+$ cells were cultured in 12-well plates that were precoated with 10 μg/ml CD137-Fc proteins for 7 days at a density of 5×10^5 cells per well. T cells were isolated from C57/BL6 mice spleen by MACS using the Pan T cell isolation kit (Miltenyi). Bone marrow derived DCs were used as effector cells, and and T cells were used as target cells. CD137-Fc-treated cells were co-cultured with DCs and T cells in round-bottom 96-well at various ratios as

indicated, with a constant T cell number of 10^5 per well, in a total volume of 200 μL complete medium. 2% paraformaldehyde fixed CHO cells were used as negative control for CD137-Fc-treated cells in coculture. Single cell types of T cells or stimulator cells or CD137-Fc-treated cells were used as negative controls for proliferation. Cells were cocultured for 4 days and pulsed with 0.5 μCi ^{3}H-thymidine per well for the last 18 h. The rate of T cell proliferation was determined with a scintillation counter (Packard). Depicted are means ± SD of triplicate measurements. DC, GM-CSF + IL-4 + LPS treated BM-derived DC; CHO, 2% paraformaldehyde fixed Chinese hamster ovary cells; CD137-Fc, CD137-Fc treated Lin^-, $CD117^+$ cells; *, $p<0.05$; **, $p<0.01$; n.s., not significant. This experiment was performed three times with comparable results.

3.2.9. CD137 does not induce proliferation of murine embryonic stem cells

Since CD137 can induce proliferation and differentiation of hematopoietic stem / progenitor cells (HSPC), we wanted to investigate whether CD137 has similar effects on the more primitive pluripotent embryonic stem (ES) cells.

Mouse ES cells AB2.2 (a gift from Dr. Wang Nai Di, Dept. of Physiology, NUS) were cultured in DMEM supplemented with 15% FBS, 2 mM L-Glutamine, 1% Penicillin-Streptomycin, 100 μM β-mercaptoethanol and 1000 U/ml LIF (ESC culture medium) for 4 passages. Cells from each passage were seeded into wells that were precoated with CD137-Fc or murine CD137-Fc (mCD137) or Fc proteins, or CD137-Fc was added as a soluble protein. On day 2, embryoid bodies were formed in each condition. There were no significant differences in proliferation among the different treatment groups in any passage (Fig. 3.34). Hence, CD137 may have no influence on ES cell proliferation.

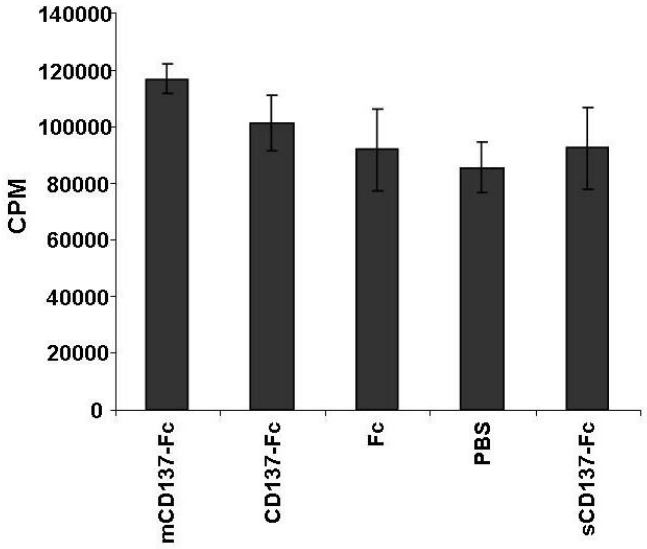


Figure 3.34. CD137 does not induce proliferation of mouse ES cells. AB2.2 ES cells were cultured for four passages and then seeded into wells coated with 10 μg/ml Fc or CD137-Fc or mCD137-Fc protein or in uncoated wells (PBS) or 10 μg/ml soluble CD137-Fc protein were added. Cells were cultured for 3 days and labeled for the last 24 h with 0.5 μCi ^{3}H-thymidine. The rate of proliferation was determined as ^{3}H-thymidine incorporation with a scintillation counter. Depicted are means ± SD of triplicate measurements. The experiments were performed 4 times for AB2.2 cells harvested in each passage 1-4 with similar results.

3.3 G-CSF and CD137 cooperatively induce proliferation of bone marrow cells but antagonize each other in promoting granulocytic or monocytic differentiation

3.3.1 CD137 ligand signaling in bone marrow cells leads to an increase in myeloid cell numbers except granulocyte numbers

As showed in Section 3.2.6, CD137L signaling can induce the colony formation of CFU-GM type. This implied that CD137L signaling could induce the differentiation of HSPC to myeloid cells, which are mainly comprised of two major groups, the monocytic cells and the granulocytic cells. After treating bone marrow cells with CD137-Fc for 7 days, the percentages of $CD14^+$ and $F4/80^+$ cells, markers for monocytes and macrophages, were increased from 0.1% and 9.2% to 43.9% and 82.2%, respectively, and higher than in the Fc treated cultures (27.7% and 67.7%, respectively). $Gr\text{-}1^+$ cells, a pan marker for bone marrow granulocytes dropped from 46.7% to 3.2%, and even lower in the Fc treated cells (9.9%) (Fig. 3.27 A, B in Section 3.2.6). A similar pattern was seen on day 14. $CD14^+$ and $F4/80^+$ cells were increased to 64.2% and 94.6%, respectively, in CD137-Fc-treated bone marrow cells, accompanied by a substantial increase in absolute cell numbers. In contrast, the $Gr\text{-}1^+$ cells were decreased to 0.7% (Fig. 3.27 A, B in Section 3.2.6).

3.3.2 CD137 does not induce apoptosis of bone marrow granulocytes

CD137 protein has been shown to induce T cell death by apoptosis and crosslinking

of CD137 on granulocytes can also induce apoptosis (Schwarz *et al.* 1996, Michel *et al.* 1999, Heinisch *et al.* 2000, Heinisch *et al.* 2001). Since a possible explanation for the decrease in the percentage of granulocytes could be CD137-induced granulocyte apoptosis, we tested the effects of CD137 on granulocyte survival and death. Cell death in granulocytes among bone marrow cells (Fig. 3.35 A) or in purified granulocyte populations (Fig. 3.35 B, C) were not different between Fc and CD137-Fc conditions ($p = 0.48$ in Fig. 3.35 A; $p = 0.45$ in Fig. 3.35 B), evaluated by Annexin v / 7-AAD apoptosis assay. This was confirmed by ethidium bromide and acridine orange staining. Cell death (yellow or red fluorescent) could be found in both Fc and CD137-Fc-treated Gr-1^+ bone marrow cells (Fig. 3.36 A). Although the latter one had a slightly higher death rate, the differences were statistically not significant (Fig. 3.36 B). The cell blebbing and chromatin condensation (Fig. 3.36 C) and the externalization of phosphatidylserine (PS) to the outer membrane (Annexin V staining in Fig. 3.35) indicated that the cells had undergone apoptosis, which may due to the short half-life of neutrophils, the main component of granulocytes.

Since CD137 does not influence granulocyte survival, the reduction of granulocytes in the bone marrow cell population must be due to a suppression of granulocytic differentiation of progenitor cells by CD137.

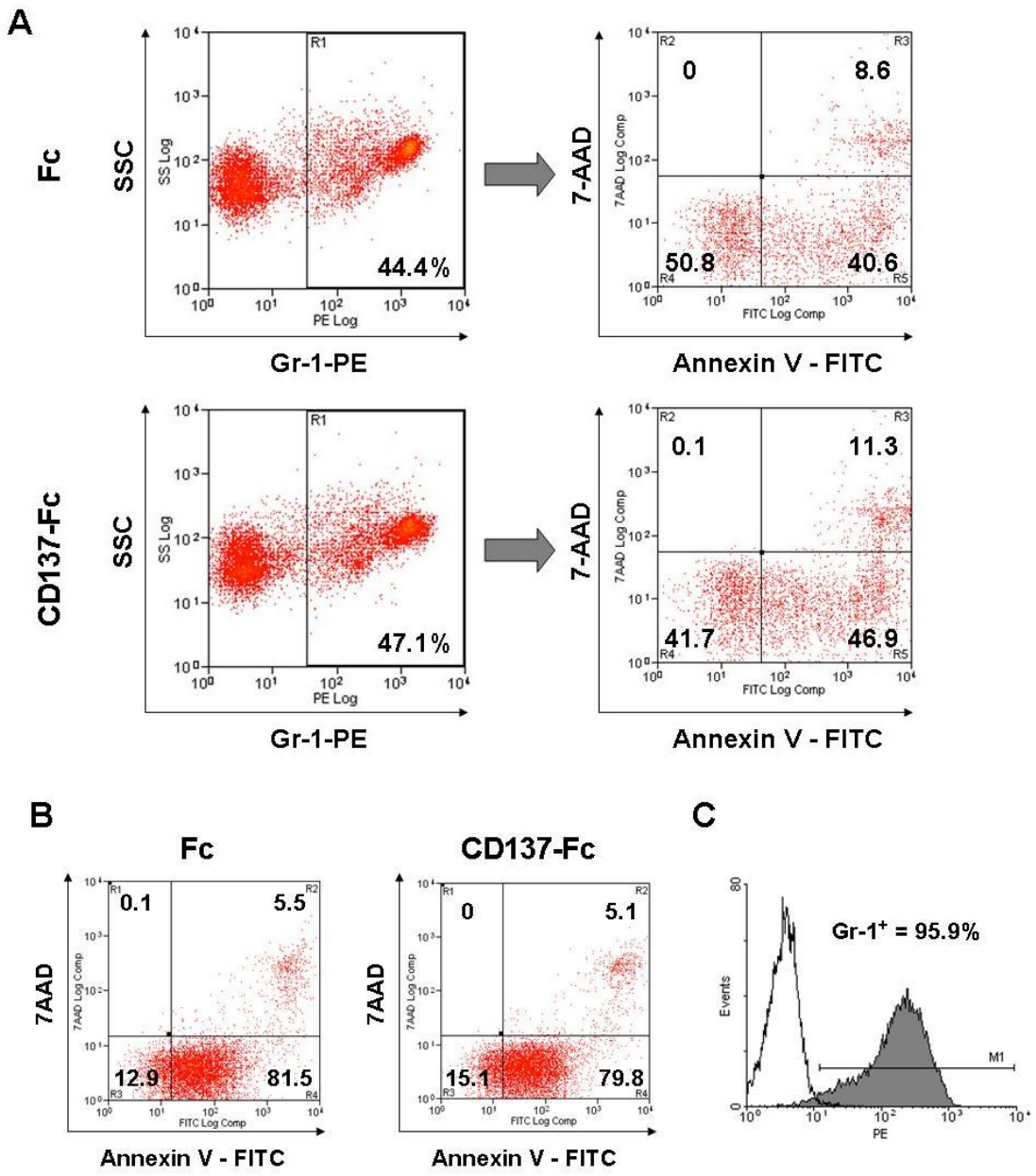


Figure 3.35. CD137 does not induce apoptosis of bone marrow granulocytes. 2×10^6 **(A)** BALB/c bone marrow cells or **(B)** MACS purified Gr-1^+ cells at a density of 10^6 cells/ml were cultured on plates coated with 10 µg/ml Fc or CD137-Fc protein for 4 h. Cells were stained with Gr-1-PE, Annexin V-FITC and 7-AAD and analyzed by flow cytometry. Depicted are Annexin V and 7-AAD analysis on Gr-1^+ cells. **(C)** Gr-1^+ cells were purified from BALB/c bone marrow cells by MACS using biotin conjugated anti-Gr-1 antibody and anti-biotin microbeads. Depicted histogram shows the purity of enriched cells after MACS separation. Black line: Isotype (rat IgG2b). Gray, filled curve: anti-Gr-1 antibody. These experiments were performed three times with similar results.

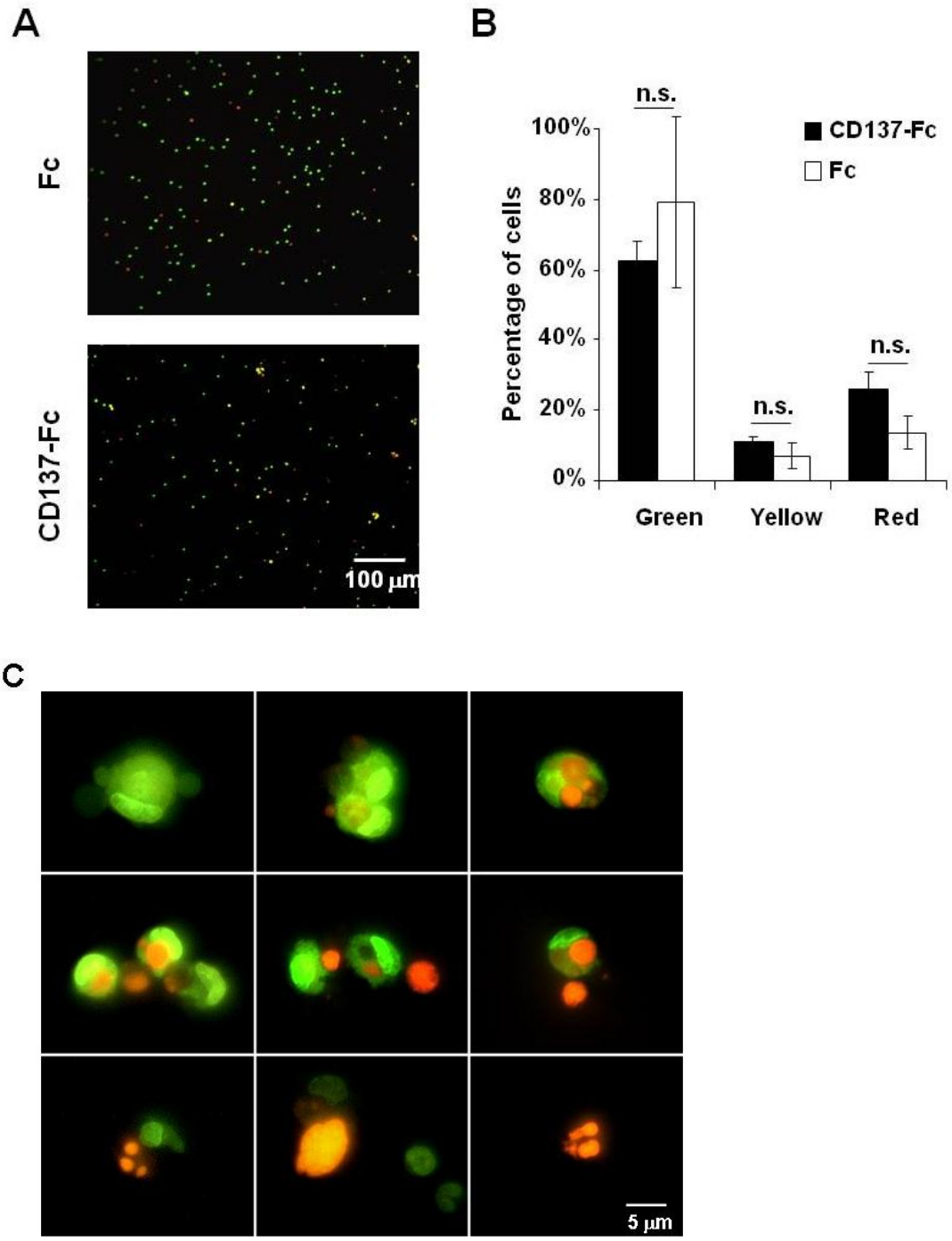


Figure 3.36. Ethidium bromide and acridine orange staining. 2×10^6 Gr-1^+ cells at a density of 10^6 cells/ml were cultured on plates coated with 10 µg/ml Fc or CD137-Fc protein for 40 h. Cells were harvested and stained with 10 µg/ml Ethidium Bromide and 3 µg/ml Acridine Orange, and examined and counted under fluorescent microscope. **(A)** Representative photos for Fc and CD137-Fc-treated Gr-1^+ cells after staining. Green cells, live; Red cells, late apoptosis or secondary necrosis; yellow, early apoptosis. Magnification: 50×; scale bar: 100 µm. **(B)** Depicted are the cell counts of the different fluorescent colors as mean ± SD of 10 fields. n.s.: not significant. **(C)** Photos were taken at magnification of 400×, scale bar: 5 µm.

3.3.3 G-CSF and CD137 cooperatively induce survival and proliferation and morphological changes of bone marrow cells

Since CD137 supports monocyte but not granulocyte survival and/or differentiation we investigated the interaction of CD137 with G-CSF, the classical granulocyte growth and differentiation factor, in regulating bone marrow cell survival, proliferation and differentiation.

Bone marrow cells were treated with immobilized CD137-Fc or G-CSF, which was added daily at a concentration of 1 ng/ml, or with both. Cells treated with Fc or Fc + G-CSF were used as controls. Both CD137-Fc and G-CSF increased cell proliferation (Fig. 3.37 A) and numbers (Fig. 3.37 B), with CD137 being more potent. Comparing with CD137-Fc or G-CSF alone, the combination of CD137-Fc and G-CSF further increased cell proliferation and cell numbers (Fig 3.37).

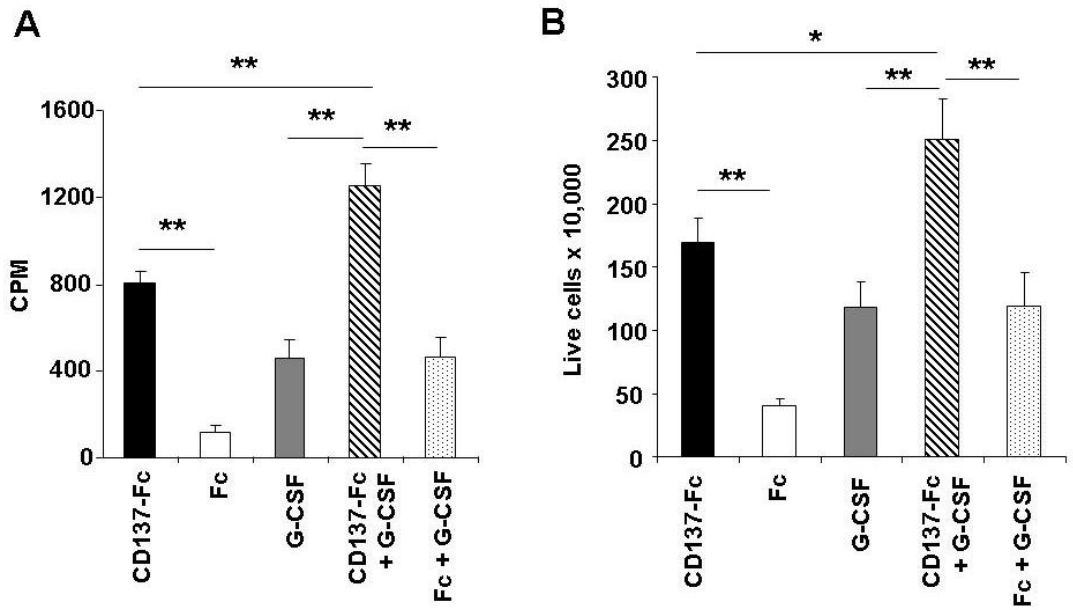


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Figure 3.37. G-CSF and CD137 synergizes to induce proliferation and survival of bone marrow cells. 2×10^6 murine bone marrow cells at a density of 10^6 cells/ml were cultured for 7 days with respective treatment. 10 μg/ml of Fc or CD137-Fc protein were pre-coated on the flask, 1 ng/ml of G-CSF was added daily in respective wells. **(A)** Cells were labeled for the last 24 h with 0.5 μCi ^{3}H-thymidine, and the rate of proliferation was determined with a scintillation counter (Packard, Meriden, CT). Depicted are means ± SD of triplicate measurements. **(B)** 10^5 bone marrow cells were treated as in (A). The cells were harvested on day 7, and cell counts were determined by trypan blue staining of 4 representative microscopic fields. *, $p<0.05$; **, $p<0.01$. Depicted are means and standard errors of 7 independent experiments.

G-CSF and CD137 had more than additive effects on cell morphology. Treatment of bone marrow cells with CD137 protein resulted in a population of adherent and spread-out cells with few cells remaining in suspension. G-CSF treatment resulted in the opposite phenotype. However, the combination of G-CSF + CD137-Fc resulted in an enhanced cell adherence and spreading of the cells, yet more cells remained in suspension compared to CD137 alone (Fig. 3.38).

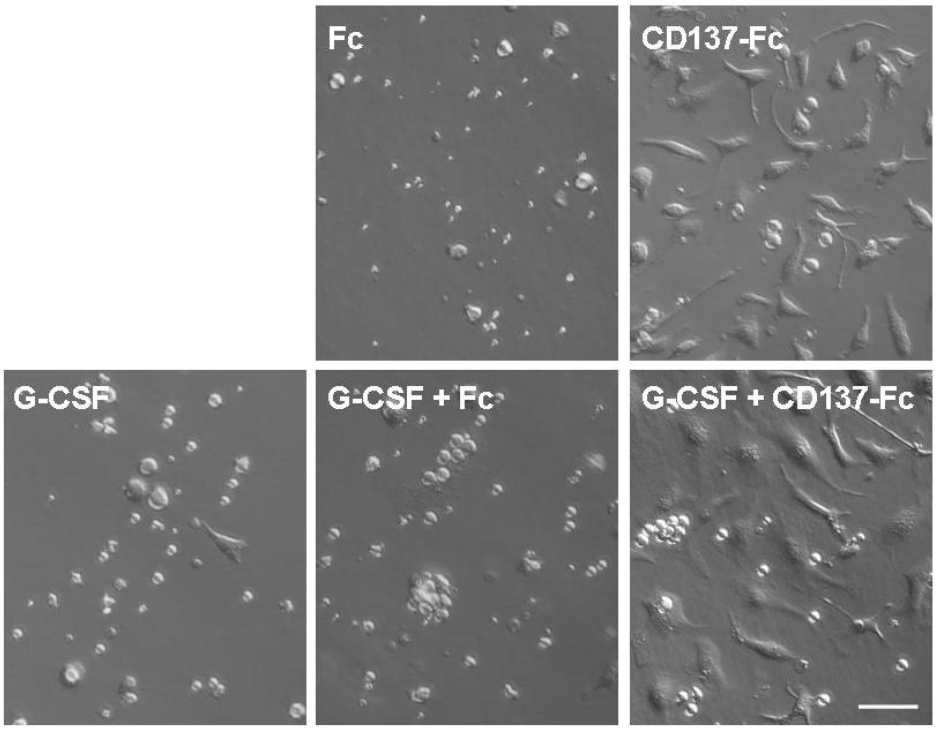


Figure 3.38. Cell morphology. 2×10^6 BALB/c bone marrow cells were cultured in wells that had been precoated with 10 μg/ml CD137-Fc or Fc, in the presence or absence of daily added 1 ng/ml G-CSF. Photographs were taken on day 7 at a magnification of 200×. Scale bar: 50 μm.

Adherence is a characteristic of monocytic cells while granulocytes are non-adherent cells. The observed morphologies are therefore in line with the expected effects of CD137 and G-CSF. The presence of adherent as well as suspension cells in conditions that were treated with G-CSF + CD137 indicated that none of the factors exerted a dominant influence over the differentiation of bone marrow cells.

3.3.4 CD137 and G-CSF antagonize each other in inducing differentiation of bone marrow cells

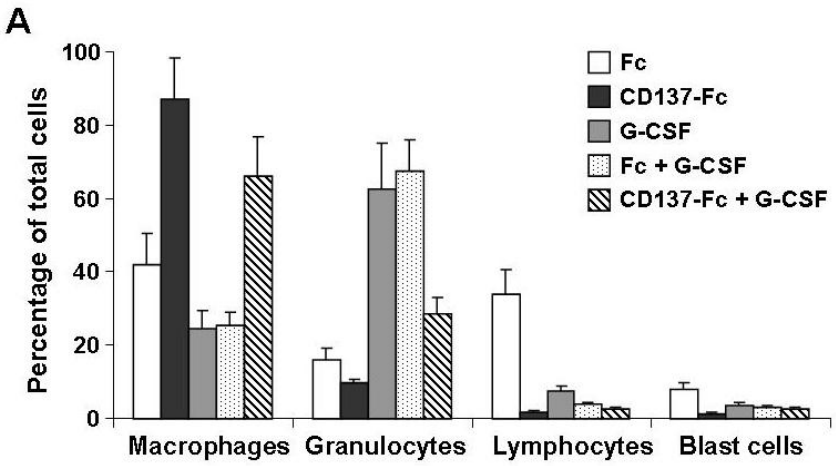
Granulocytic versus monocytic differentiation of bone marrow cells was investigated by differential cell counting. Data in Figure 3.28 had implied that CD137 inhibits granulocyte differentiation. This could indeed be verified since treatment of bone marrow cells with CD137-Fc for 7 days resulted in a decrease in granulocytes (9.6 vs 16.1 %), and an concomitant increase of macrophages (87.0 vs 41.9 %) as determined by differential cell counting (Fig. 3.39 A). As expected, the treatment of bone marrow cells with G-CSF increased granulocytes numbers. However, the combination of CD137 and G-CSF resulted in a smaller increase of macrophages than with CD137 alone (66.2 vs 87.0 %), and in a lesser increase in granulocytes than with G-CSF

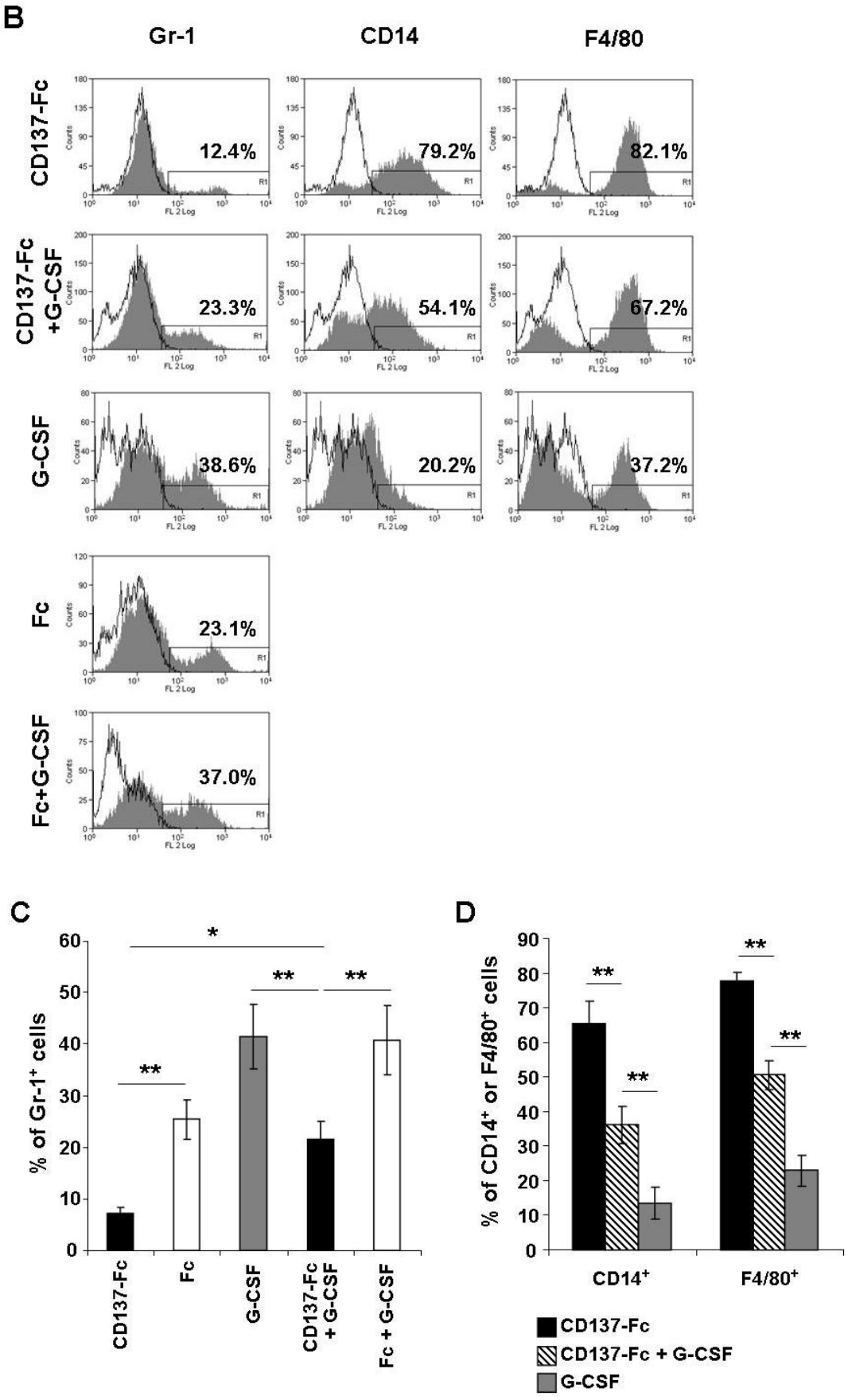
alone (28.6 vs 62.4 %), implying that CD137 and G-CSF inhibit each other in inducing granulocytic vs monocytic differentiation (Fig. 3.39 A).

The antagonistic activities of G-CSF and CD137 in myelopoiesis of bone marrow cells could be confirmed by flow cytometry via Gr-1 and CD14 and F/4/80 expression, markers for granulocytes and monocytes/macrophages, respectively (Fig. 3.39 B). Bone marrow cells treated for 7 days with CD137-Fc contained a significantly lower percentage of Gr-1^+ cells than the Fc control condition (7.2 ± 1.2% vs. 25.4 ± 3.9%), (Fig. 3.39 C). In bone marrow cells treated with G-CSF 41.5 ± 6.2% of the cells were Gr-1^+, demonstrating the expected induction of granulocyte differentiation by G-CSF. The presence of CD137 however prevented this G-CSF-induced granulocyte differentiation and the percentage of Gr-1^+ cells in the cultures treated with G-CSF + CD137 was 21.5 ± 3.6%. The Fc control protein had no influence on the percentage of Gr-1^+ cells (40.7 ± 6.6%) (Fig. 3.39 C). Reciprocally, G-CSF also interfered with CD137-induced monocytic cell differentiation. Of CD137-Fc-treated bone marrow cells 65.4 ± 5.4% and 77.9 ± 2.4% expressed CD14 and F4/80, respectively, and these number dropped to 36.1 ± 5.3% and 50.6 ± 4.2%, respectively, when G-CSF was added (Fig. 3.39 D).

The antagonism of G-CSF to CD137-induced monocytic differentiation also manifested itself in the absolute number of cells. G-CSF reduced the number of $CD14^+$ cells by 38.4% from (137 ± 15.2) $\times 10^3$ to (84.5 ± 14.3) $\times 10^3$, and the number

of F4/80^{+} cells by 24.9%, from (166.4 ± 13.6) ×10^{3} to (125 ± 11.9) ×10^{3} (Fig. 3.39 E). When bone marrow cells were cultured with G-CSF the number of Gr-1^{+} cells was not different from cultures stimulated with G-CSF and CD137. This was unexpected as CD137 had reduced the percentage of Gr-1^{+} cells.




B
Gr-1
CD14
F4/80
CD137-Fc
CD137-Fc +G-CSF
G-CSF
Fc
Fc+G-CSF
12.4%
79.2%
82.1%
23.3%
54.1%
67.2%
38.6%
20.2%
37.2%
23.1%
37.0%
C
% of Gr-1+ cells
CD137-Fc
Fc
G-CSF
CD137-Fc + G-CSF
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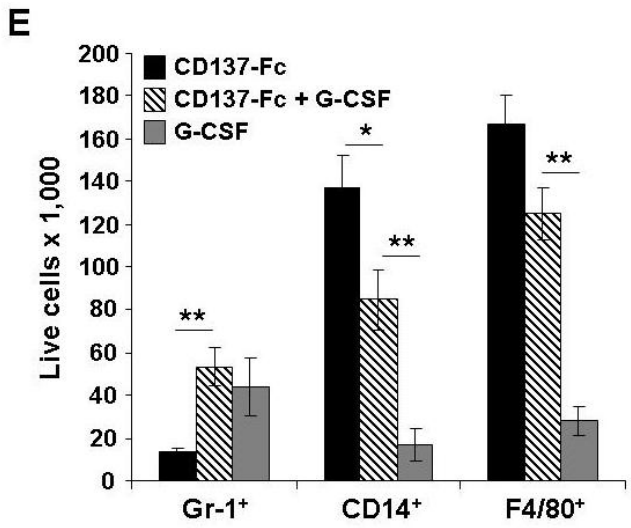


Figure 3.39. G-CSF and CD137 compete for inducing granulocytic and monocytic differentiation, respectively. 2×10^6 BALB/c bone marrow cells at a density of 10^6 cells/ml were cultured for 7 days on plates that had been coated with 10 μg/ml of Fc or CD137-Fc protein. Where indicated, G-CSF was added daily at 10 ng/ml or other stated concentrations. Differential cell counts **(A)** with a modified Wright stain were performed on a minimum of 500 cells. These data are representative of two experiments with similar results. The percentages of $Gr\text{-}1^+$ cells **(C)** and $CD14^+$, $F4/80^+$ cells **(D)**, as well as absolute cell numbers **(E)**, determined by flow cytometry on day 7, are depicted as means ± SE of 7 independent experiments, with **(B)** shown as a representative. *, $p<0.05$; **, $p<0.01$.

G-CSF induced granulocytic differentiation and CD137-induced monocytic differentiation are dose-dependent. Bone marrow cells were grown for 7 days on plates coated with a fixed concentration (10 μg/ml) of CD137-Fc or Fc protein, and G-CSF was added daily at 0, 1, 10 or 100 ng/ml. In the absence of G-CSF two thirds of the cells expressed CD14 and/or F4/80 and 10% expressed Gr-1. With increasing concentrations of G-CSF the percentage of granulocytes increased and the percentage macrophages decreased (Fig. 3.40 A). However, G-CSF at 100 ng/ml did not induce more granulocytic differentiation than at 10 ng/ml, implying a saturation point between these two concentrations. In a parallel experiment G-CSF was kept constant

at 10 ng/ml, and the coating concentrations of CD137-Fc and Fc were varied from 0, 5, 10 and 20 μg/ml. In the absence of CD137 58.2% expressed Gr-1 and 5.8% expressed CD14 (Fig. 3.40 B). Increasing concentrations of CD137 reduced granulocyte and increased correspondingly macrophage numbers. The pattern was similar for F4/80, though the difference between 0 and 20 μg/ml CD137 was less pronounced than for CD14 (Fig. 3.40 B).

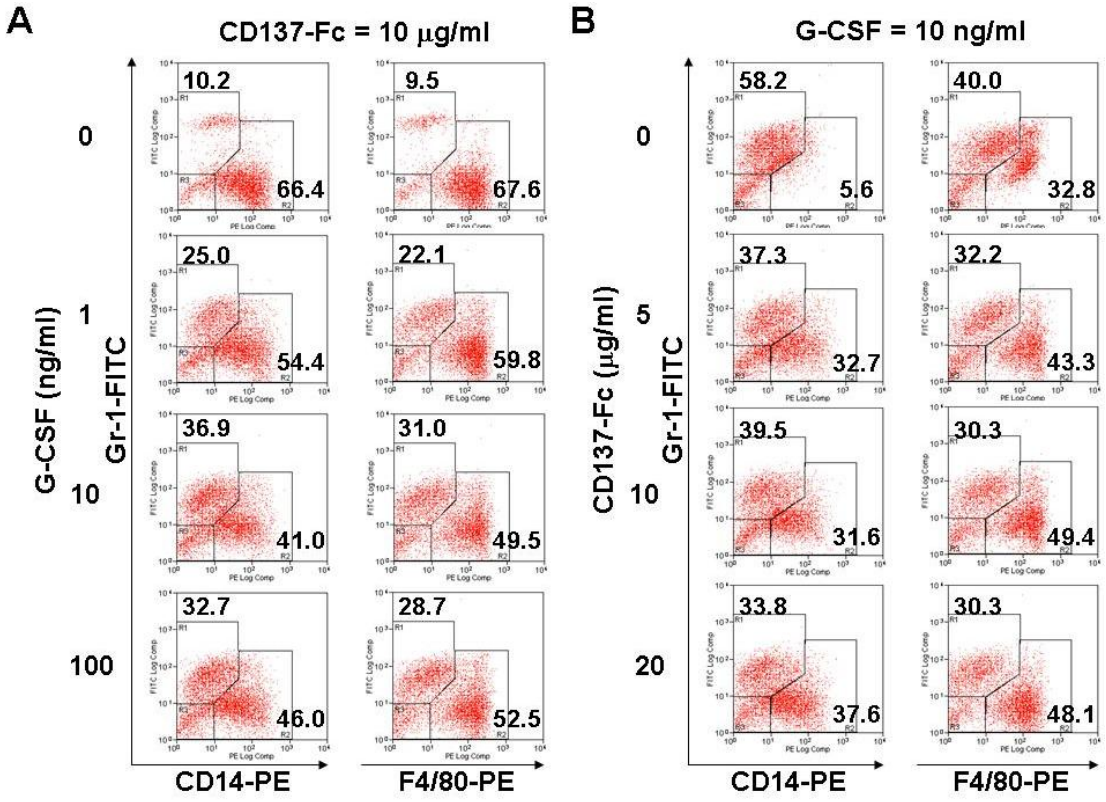


Figure 3.40. Dose response of the combination of G-CSF and CD137 protein. Cells were treated with a fixed concentration of CD137-Fc at 10 μg/ml and varying the concentration of G-CSF at 0, 1, 10, or 100 ng/ml **(A)**; or a fixed concentration of G-CSF at 10 ng/ml and varying the concentration of CD137-Fc at 0, 5, 10, or 20 μg/ml **(B)**, and flow cytometry was performed for Gr-1, CD14 and F4/80 on day 7. These data are representative of two experiments with similar results.

The CD137-induced switch towards monocytic cell differentiation at the expense of granulocytes could also be verified by esterase staining. Naphthol AS-D chloroacetate, a substrate for specific esterase, stains cells of the granulocytic lineage purple, and these cells were predominant in fresh bone marrow cells (Fig. 3.41). Also clearly visible in fresh bone marrow cells are the ring-shaped nuclei, typical for murine neutrophils. After a 7-day exposure to immobilized CD137-Fc, most cells stained black with α-naphthyl acetate, a substrate for non-specific esterase indicating differentiation towards the monocytic lineage. The concomitant increase in cell size and the kidney-shaped nuclei which are typical of monocytes further support CD137-mediated monocytic lineage differentiation. These results are in line with the data obtained by differential cell counting and flow cytometry (Fig. 3.39 A-D; Fig. 3.40 A, B).

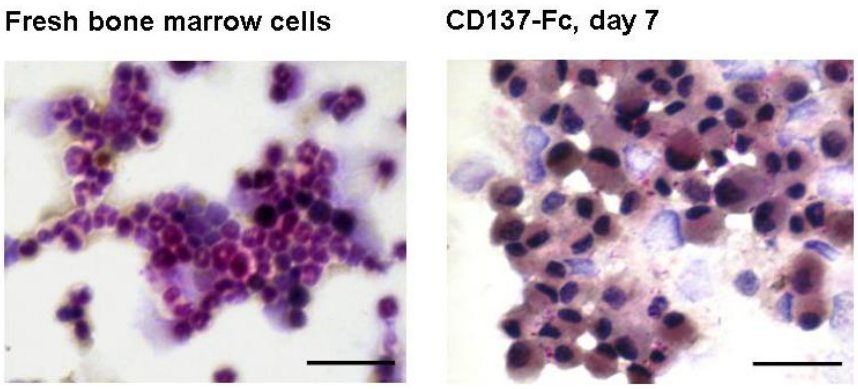


Figure 3.41. Esterase staining. Fresh murine bone marrow cells, or cells which were cultured on plates coated with 10 μg/ml CD137-Fc protein for 7 days, smeared on glass slides, fixed with citrate-acetone-formaldehyde solution, and stained for non-specific esterase (black, macrophages) and for specific esterase (purple, granulocytes). The slides were counterstained with hematoxylin and mounted with cover slips. Magnification: 630×. Scale bar: 25 μm. This figure is a representative of

three independent experiments.

Since bone marrow cells are a heterogeneous population of cells, we will investigate the function of CD137 and G-CSF in early myelopoiesis by using purified CMPs and GMPs in the future, and use Ly6G-specific Ab (clone 1A8) instead of Gr-1, which recognize both Ly6G and Ly6C.

3.4 CD137 supports Flt3L induced bone marrow derived monocytic DC differentiation

In previous sections, we have shown that, at least in murine system, CD137 alone could not induce the differentiation of bone marrow cell to DCs (Section 3.2.7); and CD137 could not induce the proliferation of immature or mature DCs (Section 3.1.8 and unpublished data from our lab). However, CD137 is reported to be able to mature and activate DCs (Wilcox *et al.*, 2002; Kim *et al.*, 2002; Laderach *et al.*, 2003; Lippert *et al.*, 2008). So we investigated whether CD137 had any effect on DC differentiation in the presence of Flt3L, which is a potent bone marrow derived DCs inducer (Naik *et al.*, 2005).

3.4.1 CD137 and Flt3L cooperatively induce morphological changes, survival and proliferation of murine bone marrow cells

Bone marrow cells from BALB/c mice were treated with immobilized CD137-Fc or single dose of 100 ng/ml Flt3L, or with both. Cells treated with Fc or Fc + Flt3L were used as controls. Morphologically, the combination of Flt3L + CD137-Fc resulted in an increase of cell numbers, of both the adherent and suspension cells compared to CD137 alone or Flt3L alone (Fig. 3.42).

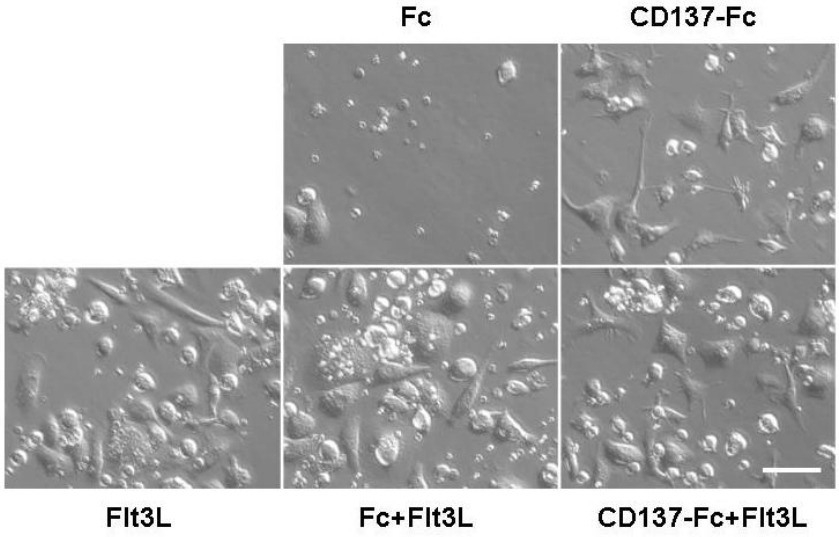


Figure 3.42. CD137 and Flt3L cooperatively induce morphological changes of bone marrow cells. 2×10^6 BALB/c bone marrow cells were cultured in wells that had been precoated with 10 μg/ml CD137-Fc or Fc, in the presence or absence of 100 ng/ml Flt3L. Photographs were taken on day 7 at magnification of 200×. Scale bar: 50 μm.

More importantly, the combination treatment of Flt3L and CD137 on bone marrow cells showed additive effects on cell survival and proliferation. Flt3L could induce bone marrow cell proliferation as reported before (Jacobsen *et al.*, 1995), and it showed a similar ability to maintain bone marrow cell survival and proliferation as CD137 (Fig. 3.43 A, B). When combined with immobilized CD137-Fc, the viable cell number on day 7 was slightly higher than the sum of that of Flt3L alone and CD137-Fc alone (3.5×10^5 vs. $(1.9+1.2) \times 10^5$) (Fig. 3.43 A). The same situation could be found regarding to the cell proliferation (CPM: 1683.2 vs. (824.6+769.0)) (Fig. 3.43 B). There was no difference in cell numbers and proliferation between Fc + Flt3L and Flt3L alone indicating that the Fc domain of the CD137-Fc recombinant protein had no influence (Fig. 3.43 A, B).

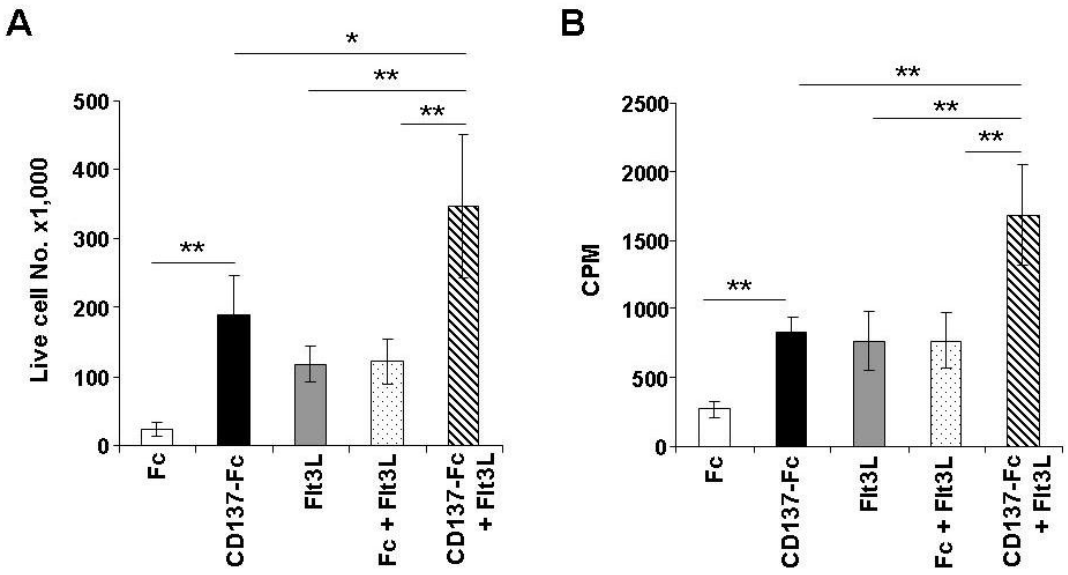


Fig. 3.43. Flt3L and CD137 additively induce survival and proliferation of bone marrow cells. (A) 2×10^6 BALB/c bone marrow cells at a density of 10^6 cells/ml were cultured in wells that had been precoated with 10 μg/ml Fc or CD137-Fc, in the presence or absence of 100 ng/ml Flt3L. Cells were harvested on day 7, and cell counts were determined by trypan blue staining. Depicted are means ± SD of 4 counts. **(B)** Bone marrow cells were cultured at 1×10^5 per well for 7 days in 96-well plate that had been precoated with 10 μg/ml of Fc or CD137-Fc in the presence or absence of 100 ng/ml Flt3L. Cells were labeled with 0.5 μCi 3H-thymidine for the last 24 h, and the rate of proliferation was determined with a scintillation counter. Depicted are means ± SD of triplicate measurements. *, $p<0.05$, **, $p<0.01$. This figure is a representative of four independent experiments with similar results.

3.4.2 CD137 supports Flt3L induced MoDC differentiation from bone marrow cells

The expression of the murine DC marker CD11c was used as a tool to assess the bone marrow derived DCs differentiation. Bone marrow cells treated with CD137-Fc or Flt3L alone resulted in 21.8% and 21.7% of $CD11c^+$ cells, respectively. Interestingly, the combination of CD137-Fc and Flt3L could additively increase the $CD11c^+$ population to 37.7% (Fig. 3.44 A). MHC-II^+ cells were also increased from 6.5% to 49.5% on day 7 by addition of Flt3L (Fig.3.44 B). Therefore, CD137 supports Flt3L

induced bone marrow derived monocytic DCs differentiation. On the other hand, addition of Flt3L to CD137-Fc-treated bone marrow cells decreased $CD14^+$ cells from 43.9% to 34.6% and $F4/80^+$ cells from 82.2% to 46.0% (Fig. 3.44 B). This indicated that Flt3L, suppressed CD137-induced macrophage differentiation from bone marrow cells. Flt3L may direct the monocytic cells to further differentiate to DCs rather than to macrophages. However, here we only examined the expression of murine DC surface marker CD11c together with MHC-II, which are not exclusively expressed on DCs. The functional analyses of CD137-Fc + Flt3L differentiated cells, such as allogeneic MLR, endocytosis assay and migration assay, need to be performed in the future.

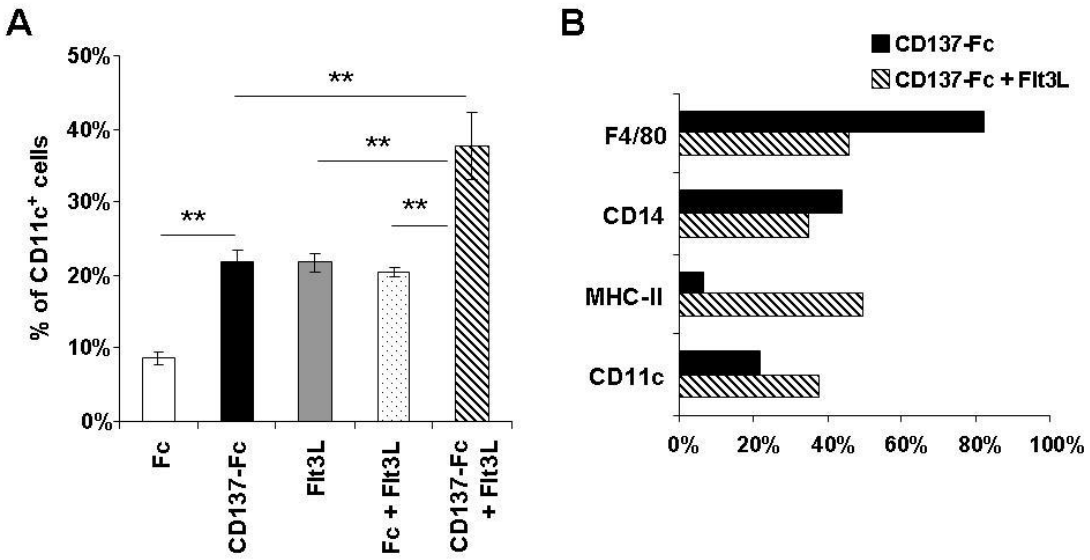


Fig. 3.44. CD137 supports Flt3L induced bone marrow derived DCs differentiation. 2×10^6 bone marrow cells at a density of 10^6 cells/ml were cultured in wells that had been precoated with 10 μg/ml Fc or CD137-Fc, in the presence or absence of 100 ng/ml Flt3L. Cells were harvested on day 7 and subjected to flow cytometric analysis of CD11c **(A)**, F4/80, CD14 and MHC class II expression **(B)**. Depicted are the percentages of positive cells, with means ± SD of triplicates in (A); **, $p<0.01$. This experiment was performed three times with comparable results.

In summary, CD137 can work additively with Flt3L to promote proliferation and differentiation of bone marrow cells to phenotypically DC-like cells.

3.4.3 CD137 does not further enhance Flt3L induced proliferation and differentiation of HSPCs

We also invested the effect of combination of CD137 and Flt3L on Lin^-, $CD117^+$ cells. However, the additive effects we observed on total bone marrow cells were not found on Lin^-, $CD117^+$ cells, in terms of cell proliferation (Fig. 3.45 A) and DC differentiation (Fig. 3.45 B). One possibility is that the effect of Flt3L on Lin^-, $CD117^+$ is dominant and masks the effect of CD137, since in the bone marrow Flt3, the receptor of Flt3L, is only expressed by the progenitor cells, and Flt3L is known as a very potent growth factor and DC differentiation factor of HSC (Jacobsen et al., 1995; Adolfsson et al., 2001).

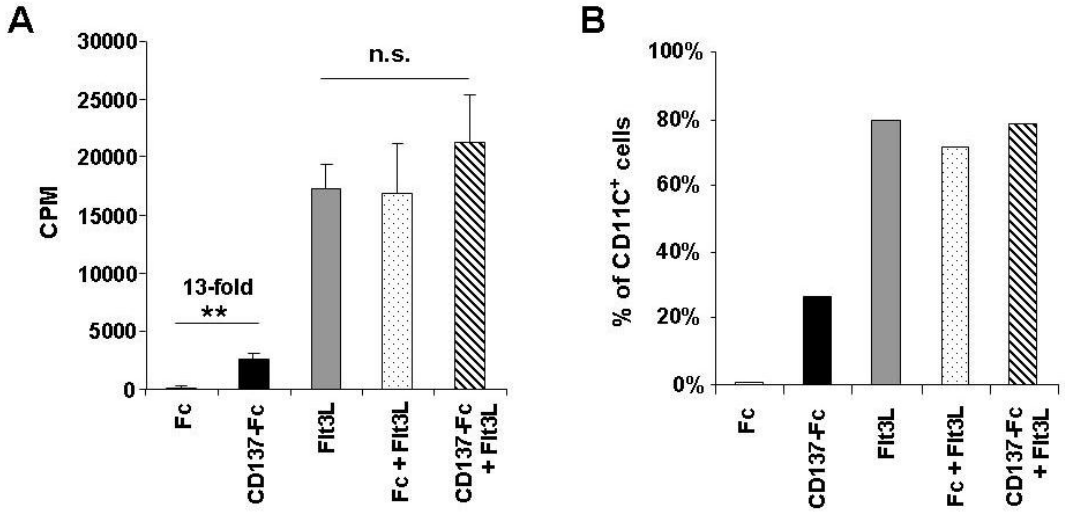


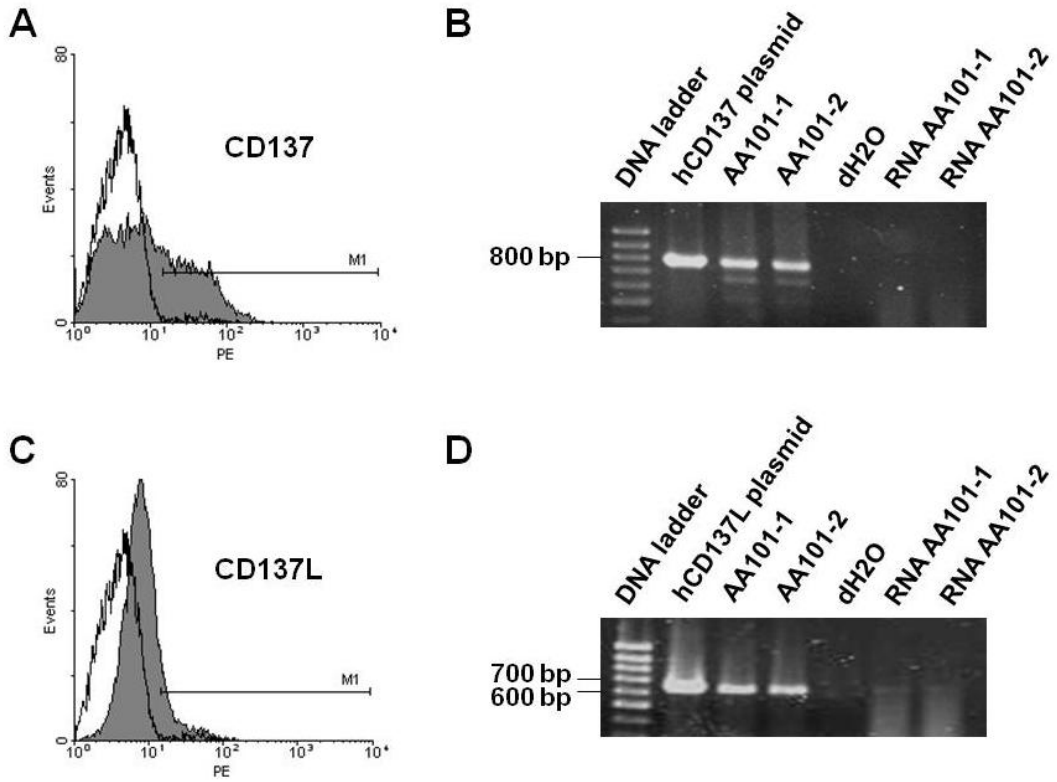
Figure. 3.45. CD137 shows no additive effect with Flt3L on Lin^-, $CD117^+$ cells.

Lin^-, $CD117^+$ cells were cultured for 7 days in wells that had been precoated with 10 μg/ml of Fc or CD137-Fc, in the presence or absence of 100 ng/ml Flt3L. **(A)** Cells were cultured in 96-well plate at 5×10^4 cells/well, and labeled with 0.5 μCi ^{3}H-thymidine for the last 24 h, and the rate of proliferation was determined with a scintillation counter. Depicted are means ± SD of triplicate measurements. **, $p<0.01$; n.s., not significant. **(B)** Cells were cultured in 12-well plates at 5×10^5 cells/well and harvested on day 7. The expression of CD11c was determined by flow cytometry.

3.5 CD137 expression is positively regulated during infection

3.5.1 Bone marrow stromal cells may not be the source of CD137 in the bone marrow

AA101 cells, a human bone marrow derived stromal cell line, were found to express CD137 (Fig. 3.46 A, B). The expression level was not homogenous among the cells, rather the expression correlated with the cell density (confluency) (Fig. 3.46 E; Table 4). Interestingly, AA101 cells also express CD137L (Fig. 3.46 C, D) and its expression level was relatively stable (Fig. 3.46 E; Table 4). We tested two murine bone marrow stromal cell lines, ST-2 and MS-5. However, both of them expressed neither murine CD137 nor murine CD137L (Fig. 3.47).



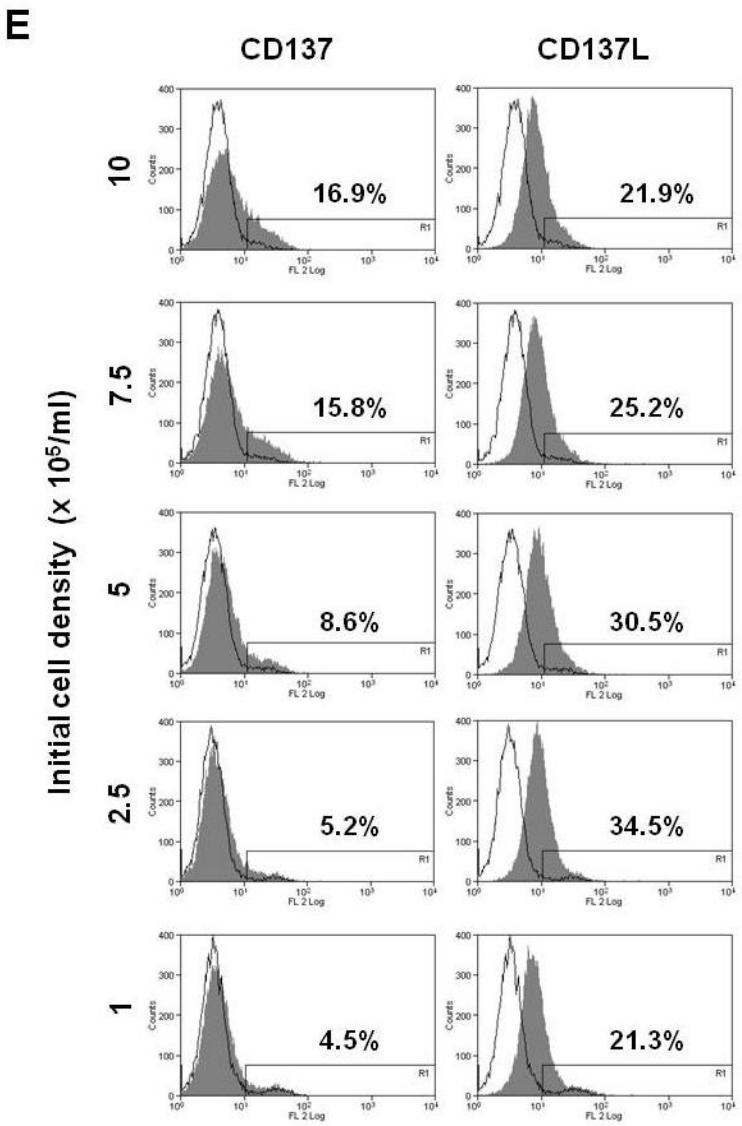


Figure 3.46. AA101 cells express CD137 and CD137L. AA101 cells, a human bone marrow derived stromal cell line, were stained with PE-conjugated anti-human CD137 mAb (Clone 4B4-1) **(A)** or PE conjugated anti-human CD137L mAb (Clone 5F4) **(C)** or their isotype control PE-conjugated anti-mouse IgG1 (Clone MOPC-21), and analyzed by flow cytometry. Expression of CD137 mRNA **(B)** or CD137L mRNA **(D)** was determined by RT-PCR using cDNAs from two batches of AA101 cells. Cloned hCD137 plasmid or hCD137L plasmid were used as positive controls, respectively. Reactions with no template (water) or RNA from AA101 cells that was not reverse-transcribed served as negative controls. GeneRuler DNA ladder (Fermentas) was used as a size standard. PCR products were analyzed on a 1% TAE agarose gel. **(E)** AA101 cells were seeded in 6-well plate with the initial density at 1, 2.5, 5, 7.5, or 10 $\times 10^5$ cells/ml. Cells were harvested after 2 days of culture and analyzed by flow cytometry with PE-conjugated anti-CD137 (Clone 4B4-1) and anti-CD137L (Clone 5F4). In (A), (C) and (E), Black line: Isotype, mouse IgG1 (clone MOPC-21); Gray, filled curve: anti-CD137 (Clone 4B4-1) or anti-CD137L (Clone 5F4). These experiments were performed three times with similar results.

Table 4. CD137 expression on AA101 cells is proportional to the cell density.

Initial cell density (10^5/ml)	$CD137^+$ %	$CD137L^+$ %
10	16.9	21.9
7.5	15.8	25.2
5	8.6	30.5
2.5	5.2	34.5
1	4.5	21.3

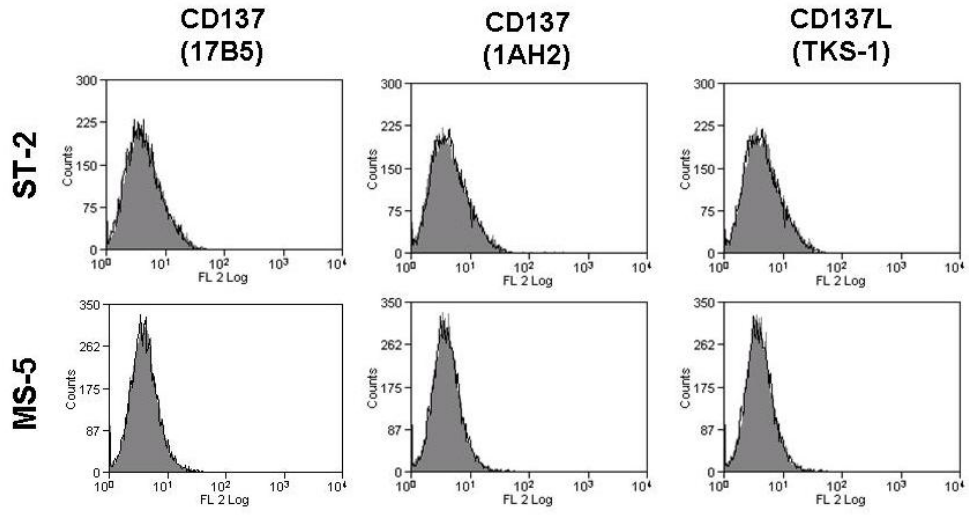
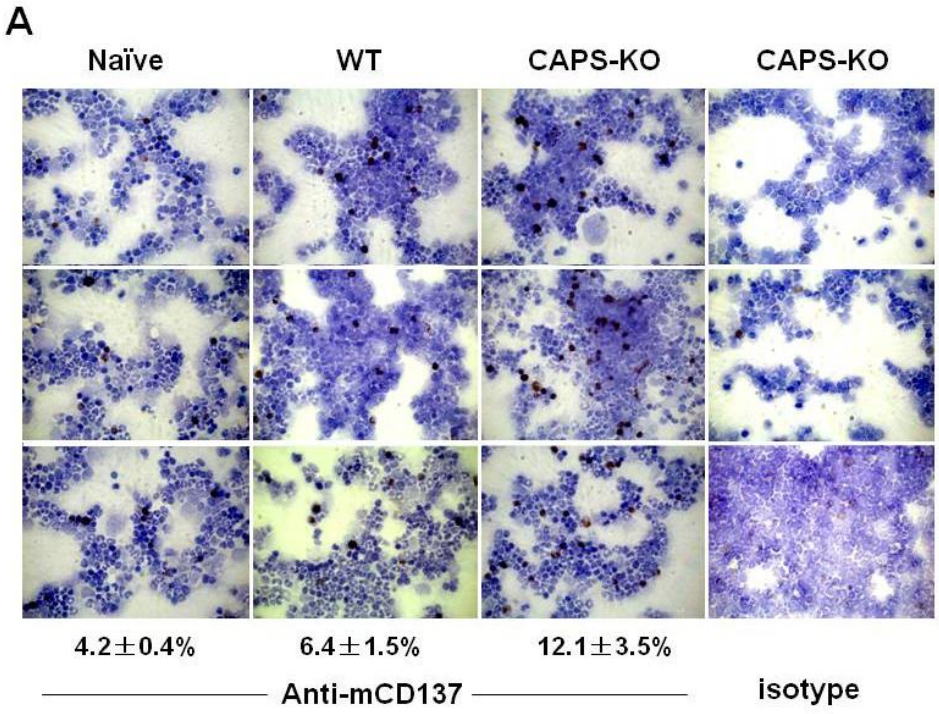


Figure 3.47. ST-2 and MS-5 cells express neither CD137 nor CD137L. ST-2 (upper panel) and MS-5 cells (lower panel), two murine bone marrow stromal cell lines, were stained with PE-conjugated anti-mouse CD137 mAbs (Clone 17B5 and 1AH2) or PE conjugated anti-mouse CD137L mAb (Clone TKS-1) or their respective isotype controls, and analyzed by flow cytometry. Black line: Isotype; Gray, filled curve: anti-CD137 (Clone 17B5) or anti-CD137L (Clone TKS-1). This experiment was performed three times with same results.

3.5.2 Bacterial Bordetella pertussis infection increases the number of $CD137^+$ cells in the bone marrow

Despite the fact that the type of cells that expresses CD137 in the bone marrow is unknown, interestingly, we found bacterial infection could induce CD137 expression in total bone marrow cells (Fig. 3.25 D).

In bones of healthy BALB/c mice we could detect CD137 expression by immunocytochemistry but the number of $CD137^+$ cells was increased more than 50% (from 4.2±0.4% to 6.4±1.5%) upon infection with wide type *B. pertussis* bacteria (Fig. 3.48 A). More interestingly, when mice were infected with capsule gene knockout *B. pertussis* (CAPS-KO) strain, which was much less virulent than WT, the percentage of $CD137^+$ cells were further increased to 12.1±3.5%. The regulation of CD137 expression was confirmed by RT-PCR at mRNA level (Fig. 3.48 B). The immunohistochemistry staining of EDTA-decalcified formalin fixed paraffin embedded bone slides, which had less system background, also confirmed that *B. pertussis* infection increased $CD137^+$ cells in the bone marrow (Fig. 3.48 C). This implied that the number of $CD137^+$ cells in the bone marrow could be regulated the infection, and type and virulence of the infection may play a role in triggering the regulation.



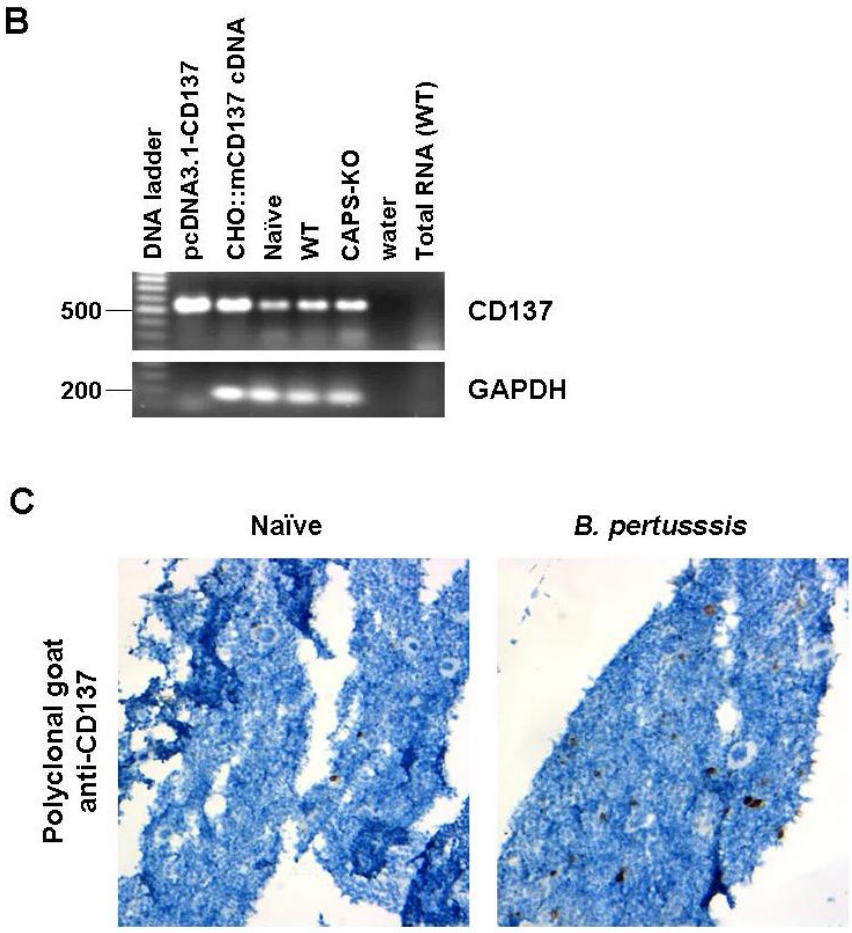
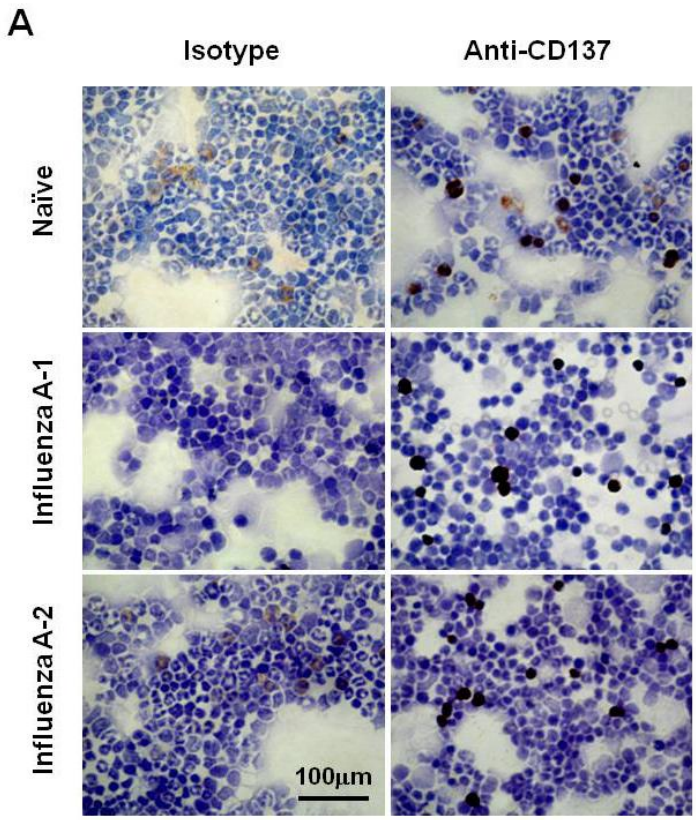


Figure 3.48. *B. pertussis* infection leads to an increase of CD137$^+$ cells in the bone marrow. BALB/c mice were infected with *B. pertussis* wild type or capsule gene knockout (CAPS-KO) strain. Naïve BALB/c mice from the same batch were used as control. Mice were euthanized at day 3 post infection, and femur bones were harvested. **(A)** Immunocytochemical detection of CD137 expression (brown stain). Bone marrow cells were smeared onto glass slides and fixed with absolute methanol. Cell smears were stained with anti-CD137 mAb (clone 17B5) or its isotype control Syrian hamster IgG (BioLegend). Magnification: 200×. The numbers indicated are the percentages of stained cells in the total population as means ± SD of triplicates of countings. **(B)** Expression of CD137 mRNA was determined by RT-PCR using cDNAs from bone marrow cells. Cloned murine CD137 plasmid (pcDNA3.1-CD137) and cDNA from murine CD137 transfected CHO cells (CHO::mCD137) were used as positive controls. Reactions with no template (water) or RNA from WT *B. pertussis* infected bone marrow cells that was not reverse-transcribed served as negative controls. Comparable amounts of cDNA were verified by amplification of GAPDH. GeneRuler DNA ladder (Fermentas) was used as a size standard. PCR products were analyzed on a 1% TAE agarose gel. **(C)** Immunohistochemical detection of CD137 expression (brown stain). Harvested femur bones were decalcified with EDTA, fixed with formalin, and embedded in paraffin. The sections were stained for CD137 using

the polyclonal goat anti-CD137 antibody (R&D Systems), and goat IgG was used as an isotype control. Magnification: 100×. These experiments were performed three times with comparable results.

3.5.3 Virus Influenza A (H1N1) infection increases number of CD137$^+$ cells in the bone marrow

Next we looked at viral infection, and influenza A (H1N1) infection was used as a model. Similar as *B. pertussis* infection, the number of CD137$^+$ cells in the bone marrow of C57/BL6 mice increased upon influenza A infection (Fig 3.49 A, B).



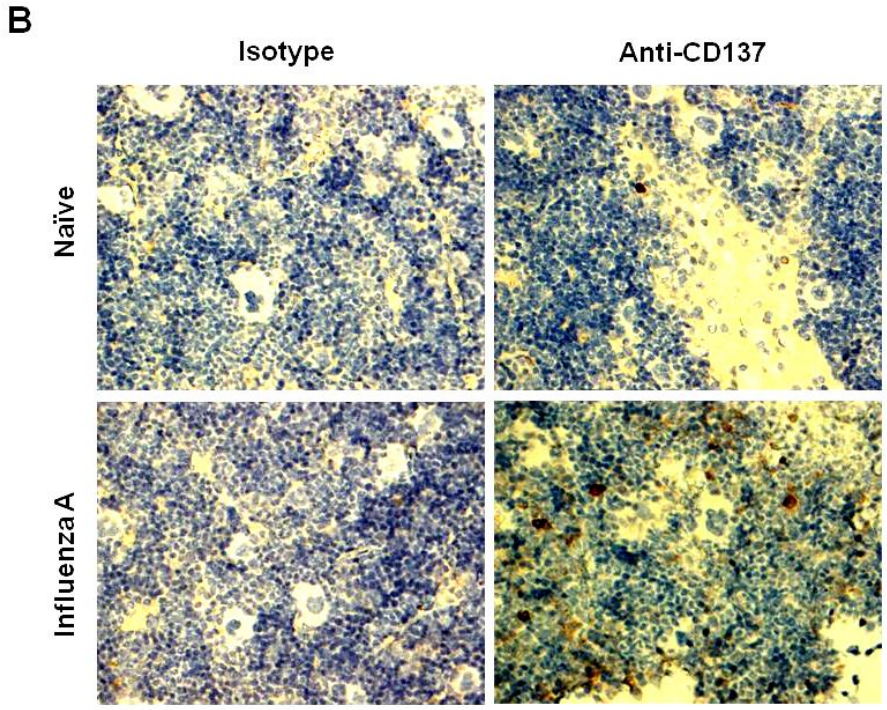


Figure 3.49. Influenza A infection leads to an increase of CD137$^+$ cells in the bone marrow. C57/BL6 mice were infected with Influenza A (H1N1). Naïve C57/BL6 mice from same batch were used as control. Mice were euthanized at day 4 post infection, and femur bones were harvested. **(A)** Immunocytochemical detection of CD137 expression (brown stain). Bone marrow cells were smeared onto glass slides and fixed with absolute methanol. Cell smears were stained with anti-CD137 mAb (clone 17B5) or its isotype control Syrian hamster IgG (BioLegend). Magnification: 200×. **(B)** Immunohistochemical detection of CD137 expression (brown stain). Femur bones were decalcified with EDTA, fixed with formalin, and embedded in paraffin. The sections were stained for CD137 using the polyclonal goat anti-CD137 antibody (R&D Systems), and goat IgG was used as an isotype control. Magnification: 100×. These experiments were performed two times with comparable results.

3.5.4 I.p. injected LPS increases the number of CD137$^+$ cells in the bone marrow

We also investigate the situation of systemic inflammation by i.p. injection of LPS (*E. coli* 0111:B4). C57/BL6 mice that had been injected with 1 mg/kg or 5 mg/kg LPS had increased CD137$^+$ cells in the bone marrow at 24 h post-injection compared to mice that had been injected with PBS (Fig. 3.50 A, B).

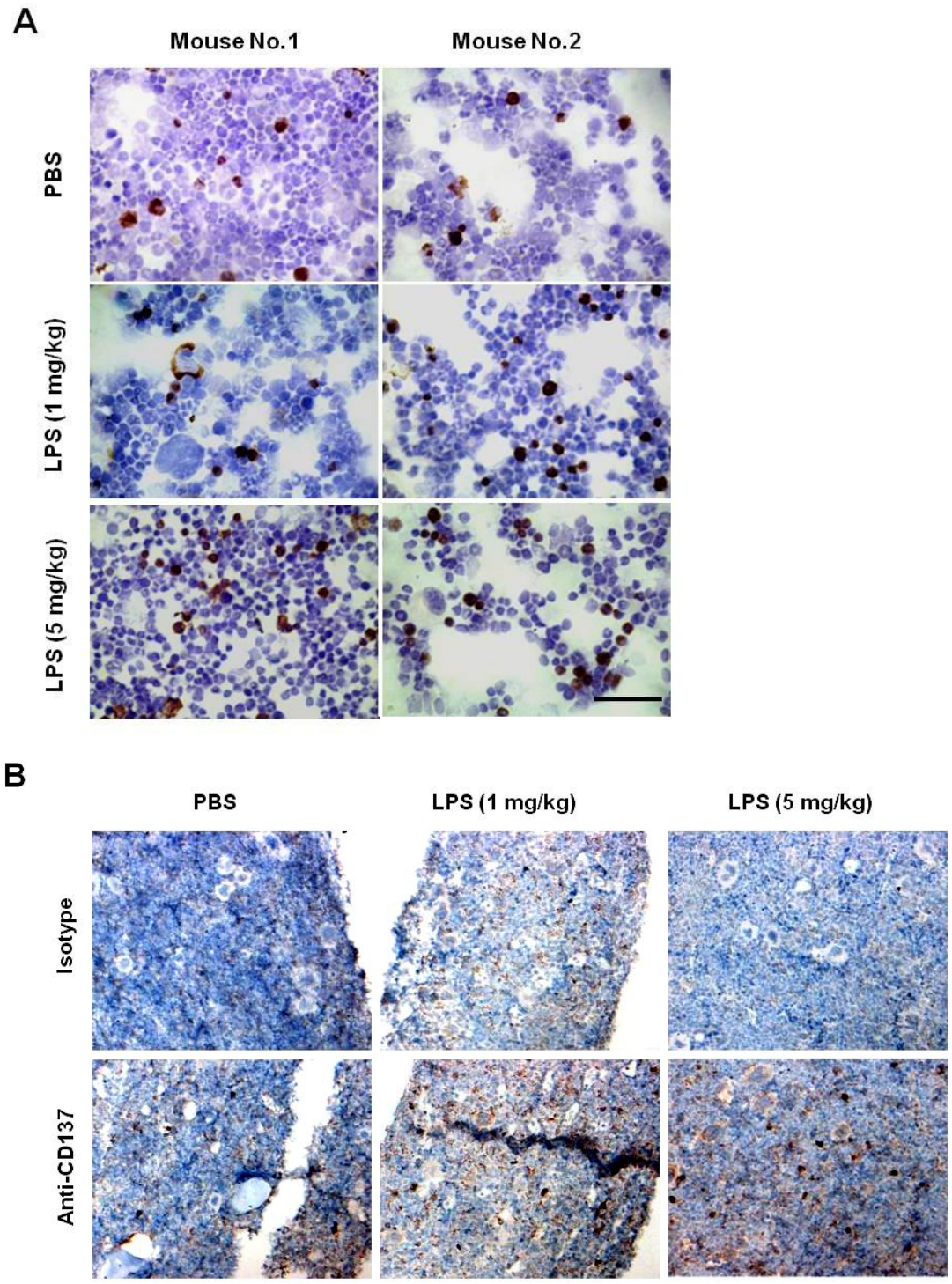


Figure. 3.50. I.p. injection of LPS leads to an increase of CD137^{+} cells in the bone marrow. C57/BL6 mice were i.p. injected with LPS from *E. coli* 0111:B4 (Sigma) at 1 mg/kg or 5 mg/kg in 100 µl PBS. Mice injected with 100 µl PBS were used as controls. Mice were euthanized after 24 h, and femur bones were harvested. **(A)** Immunocytochemical detection of CD137 expression (brown stain). Bone marrow cells were smeared onto glass slides and fixed with absolute methanol. Cell smears were stained with anti-CD137 mAb (clone 17B5) or its isotype control Syrian hamster

IgG (BioLegend). Magnification: 200×. Scale bar: 100 μm. **(B)** Immunohistochemical detection of CD137 expression (brown stain). Femur bones were decalcified with EDTA, fixed with formalin, and embedded in paraffin. The sections were stained for CD137 using the polyclonal goat anti-CD137 antibody (R&D Systems), and goat IgG was used as an isotype control. Magnification: 100×. These experiments were performed two times with comparable results.

3.5.5 CD137 expressed on activated T cells can induce proliferation of bone marrow cells

Since activated T cells are well known to express CD137, we would like to test whether there were T cells in the bone marrow, and whether they express CD137. Flow cytometric analysis of CD3 on total bone marrow cells in the healthy naïve BALB/c mice showed that, although the frequency was very low, about 1% nucleated bone marrow cells were T cells (Fig 3.27 B in Section 3.2.6).

Next we checked the ability of activated CD137 expressing T cell in inducing proliferation of bone marrow cells. When bone marrow cells from wild type (WT) C57/BL6 mice were cocultured for 5 days with activated T cells, but not resting T cells, from both WT and CD137-deficient C57/BL6 mice (Fig. 3.51 A), the proliferation of bone marrow cells was triggered. The activated T cells from WT mice had significantly higher potential to induce bone marrow cell proliferation than their counterparts from CD137-deficient mice (Fig. 3.51 B). This indicates that activated T cells have ability to induce proliferation of bone marrow cells, and that CD137 plays a role in this process.

Combined with the fact that bacterial and viral infections and systemic inflammation increased the number of $CD137^+$ cells in the bone marrow, we hypothesize that the CD137 expressing cells in the bone marrow are likely to be activated T cells that migrated there from inflammatory tissues or from draining lymph nodes, and that these activated T cells are able to induce hematopoiesis in the bone marrow through the CD137 receptor / ligand system.

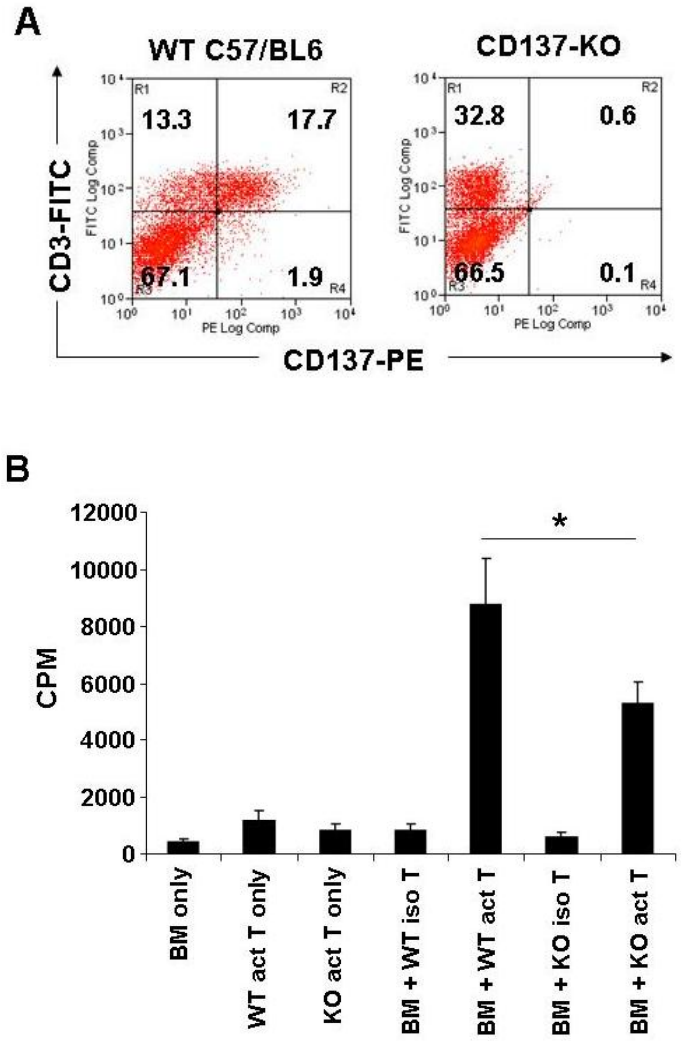


Figure 3.51. Activated T cells could induce bone marrow cell proliferation. (A) Splenocytes from C57/BL6 WT or CD137-deficient (KO) mice were cultured with 2.5 μg/ml Concavalin A for 48 h, and harvested and analyzed for CD137 expression by flow cytometry. **(B)** T cells were purified from splenocytes of WT or CD137-deficient

C57/BL6 mice by MACS using Pan T cell isolation kit (Miltenyi) and seeded into plate that had been precoated with 5 μg/ml agonistic rat anti-mouse CD3 mAb (clone 17A2) and 5 μg/ml of anti-mouse CD28 mAb (Clone 37.51) or their isotype controls rat IgG2b and Syrian hamster IgG, respectively, both at 5 μg/ml. After 48 h, the cells were harvested and cocultured with fresh bone marrow cells from wide type C57/BL6 mice at the ratio of T : BM = 1:1 in round-bottom 96-well plate for another 5 days. The wells containing only bone marrow cells or activated T cells from WT or CD137-deficient mice were used as single cell type controls. The cells were labeled for the last 24 h with 0.5 μCi ^{3}H-thymidine. The rate of proliferation was determined as ^{3}H-thymidine incorporation with a scintillation counter. Depicted are means ± SD of triplicate measurements. *, $p<0.05$; BM, bone marrow cells; act, anti-CD3 and anti-CD28 activated; iso, isotype control antibody treated; WT, wild type C57/BL6 mice; KO, CD137-dificient C57/BL6 mice. This experiment was performed three times with comparable results.

CHAPTER 4 DISCUSSION

The CD137 receptor / ligand system is involved in a number of different immune activities (Sica *et al.*, 2000; Croft, 2003; Watts, 2005), and reverse signaling by CD137L has been documented in monocytes, DCs, B cells and T cells where it has been shown to regulate activation, survival, proliferation and apoptosis (Schwarz, 2005). Our current study adds hematopoietic progenitor cells to this list, and demonstrates that CD137L signals also regulate hematopoiesis. CD137 and its ligand are found to be expressed by bone marrow cells and progenitor cells, respectively; and that they are involved in proliferation and differentiation of hematopoietic progenitor cells.

4.1 What have we learned about the role of CD137L signaling in hematopoiesis?

CD137 protein promoted survival (Fig. 3.3 A; 3.17), colony formation (Fig. 3.5; 3.22; 3.23) and proliferation (Fig. 3.3 B, C; 3.4; 3.19) of hematopoietic progenitor cells, in both man and mouse. In the murine system, CD137 was more potent than G-CSF (Fig. 3.17A). The activity of CD137 lasted for more than 3 weeks after a single dose, while the short half-life of G-CSF required it to be added daily. CD137-induced proliferation of bone marrow cells and Lin^-, $CD117^+$ cells resulted in an increase in cell numbers and in colony formation. The colonies formed in response to CD137L signaling were CFU-GM and CFU-M, i.e. myeloid precursor cells, indicating that

CD137-induced proliferation was accompanied by a differentiation of HSPCs towards the myelo-monocytic lineage. Myeloid differentiation was supported by the increase in CD11b expression.

Members of the TNF and TNF receptor superfamilies are primarily known for their functions in the regulation of immune responses rather than their roles in hematopoiesis. However, several members of these two superfamilies have been shown to be involved in the growth and differentiation of hematopoietic progenitor cells. For instance, CD95 and TNF related apoptosis inducing ligand (TRAIL) are of critical importance for lymphopoiesis and myelopoiesis (Greil *et al.,* 2003). TNF can induce or inhibit hematopoiesis, depending on its dose and interaction with other growth factors (Jacobsen *et al.*, 1994). CD27 inhibits myelopoiesis and may so counteract the CD137L activity (Nolte *et al.,* 2005). Our study adds CD137 and its ligand to this list. Support for this notion comes from the fact that mice injected with anti-CD137 antibodies have a 10-fold increased number of bone marrow cells with a hematopoietic stem cell phenotype (Niu *et al.*, 2007). Further, mice deficient in CD137 have enhanced numbers of multipotential, erythroid, granulocyte macrophage progenitor cells in bone marrow, spleen and blood (Kwon *et al.* 2002). If one accounts the increase in myeloid progenitor cells to a missing differentiation signal which normally would be delivered by CD137 then these observations fit nicely to our data showing that CD137 induces differentiation to mature myeloid cells, i.e. monocytes and macrophages. This interpretation could also explain why transgenic mice

overexpressing CD137L on APC have a 3-fold elevated number of macrophages in the spleen (Zhu *et al.* 2001). However, Lee and colleagues reported recently that at steady state CD137 and CD137L limit myelopoiesis and the development of DCs by *in vivo* study in CD137-deficient mice. It seems contradictory to our studies and previous reports, but it may just reveal the two sides of CD137 in regulating the hematopoiesis based on the different immune activation states. This will be discussed in more detail in section 4.5.

4.2 Into what lineage does CD137 drive differentiation of HSPCs?

Like most other growth factors that induce proliferation, CD137L agonists also influence differentiation of hematopoietic progenitor cells, specifically to the myeloid lineage, and within the myeloid lineage to monocytes, as evidenced by the increased CD11b and CD14 expression (Fig. 3.6), respectively. We could observe no effect of CD137-Fc on erythroid, megakaryocytic or lymphoid differentiation, not even when cytokines were added which induce erythroid or megakaryocytic differentiation (Jiang *et al.*, 2008b). Independent evidence against lymphoid differentiation comes from previous data where CD137 protein has been shown to induce cell death in T cells (Schwarz *et al.*, 1996; Michel *et al.*, 1999). Though CD137L signaling enhances proliferation of preactivated B cells, it has no effect on proliferation of resting B cells (Pauly *et al.*, 2002). These data imply that CD137 is not a general hematopoietic growth factor. Rather, it leads specifically to an amplification of monocytic cells.

Monocytes can give rise to DCs or macrophages. It has already been demonstrated that recombinant CD137 protein or anti-CD137L antibodies can induce activation, survival and proliferation in peripheral monocytes (Langstein *et al.*, 1998, 1999a, 1999b; Ju *et al.*, 2003). Our study extends these reports by showing that CD137 not only acts on mature monocytes but induces hematopoietic progenitor cells to grow and to differentiate to monocytes and macrophages. In man, macrophage differentiation is evidenced by increased phagocytosis, high IL-10 secretion, absence of IL-12 and dendritic cell markers (CD1a, CD83, CD209) (Smith *et al.*, 1998). In mouse, although in Lin^-, $CD117^+$ cells equal amounts of macrophages (CD14, F4/80) and DCs (CD11c) markers are induced by CD137 protein (Fig. 3.28), CD137L signals does not direct differentiation towards DCs, as shown by the inability of CD137-exposed cells to induce T cell proliferation in a MLR, and to secrete IL-12. Instead, high levels of IL-10 and increased phagocytic activity show that CD137L signaling induces macrophage differentiation in hematopoietic progenitor cells (Smith *et al.*, 1998). The spindle-like morphology of the cells is also more consistent with a macrophage than a DC differentiation. The differentiative influence of CD137L signaling towards macrophages was specific since it did not enhance numbers of lymphoid, megakaryocytic or erythroid cells.

CD137L crosslinking has been reported previously to support DC maturation. But in these studies DCs were derived from human umbilical cord blood $CD34^+$ cells or peripheral monocytes via culture with cytokine cocktails for 1 to 7 days before

exposure to CD137 protein (Kim *et al.* 2002; Laderach *et al.* 2003; Lippert *et al.* 2008). Our findings are not contradictory to these reports, rather they demonstrate wider biological activities of CD137 in myelopoiesis. While CD137 enhances maturation of immature DCs derived from hematopoietic progenitor cells, it will induce macrophage differentiation in hematopoietic progenitor cells when given as a first and sole signal. All these data suggest that CD137 may be a general growth and differentiation factor for monocytic cells, and are in line with the increased number of myeloid cells in a transgenic mouse overexpressing CD137 (Zhu *et al.*, 2001).

In addition, our data are partly in agreement with earlier studies which demonstrated that CD137 protein in combination with M-CSF increases proliferation of bone marrow-derived osteoclast progenitors but inhibits M-CSF + receptor activator of nuclear factor κ ligand (RANKL)-induced osteoclast differentiation (Saito *et al.*, 2004; Shin *et al.*, 2006a). Some of the osteoclast progenitor cells that proliferated in response to CD137 together with M-CSF in the study by Saito *et al.* may also have been contained within the Lin^-, $CD117^+$ cell population of our study (Saito *et al.*, 2004). What is surprising however is that CD137 protein inhibited osteoclast differentiation while it promoted macrophage differentiation, since both cell types are closely related if not identical. This difference could be due to the different starting cell populations or the presence of M-CSF.

4.3 Is CD137 a general growth factor and activator for monocytic cells?

By summarizing our results we found that CD137L agonist augment the proliferation of HSPCs and induce the differentiation to CFU-GM (GMP) in the first step (Fig. 3.5; 3.23); and then induce further differentiation to macrophages at the expense of granulocytes (Section 3.1.6; 3.2.7; 3.3.4). Combination of CD137 with G-CSF and Flt3L cooperatively or additively induces survival and proliferation of bone marrow cells (Fig. 3.37; 3.43). CD137 and G-CSF antagonize each other in inducing differentiation of macrophages and granulocytes (Section 3.3.4), while CD137 supports Flt3L induced DC differentiation (Fig. 3.44). Recent work from our lab also shows that CD137 alone can induce differentiation of human peripheral monocytes into DCs and can induce the maturation of the immature DCs (unpublished data from Shaqireen D/O K. M. M., and Schwarz H.).

It seems that CD137L signaling generally sustains the process of myelopoiesis starting from CFU-GM. It supports the differentiation of macrophages, monocytes and DCs depending on the environment and especially the presence of other hematopoietic growth factors. A proposed schematic summary of CD137-induced myelopoiesis is depicted in Fig. 4.1. In addition, previous studies have shown that CD137 is able to induce activation and maturation of monocytes and DCs. Therefore, CD137 can be recognized as a general growth factor and activator for myeloid cells, and in particular, of monocytic cells.

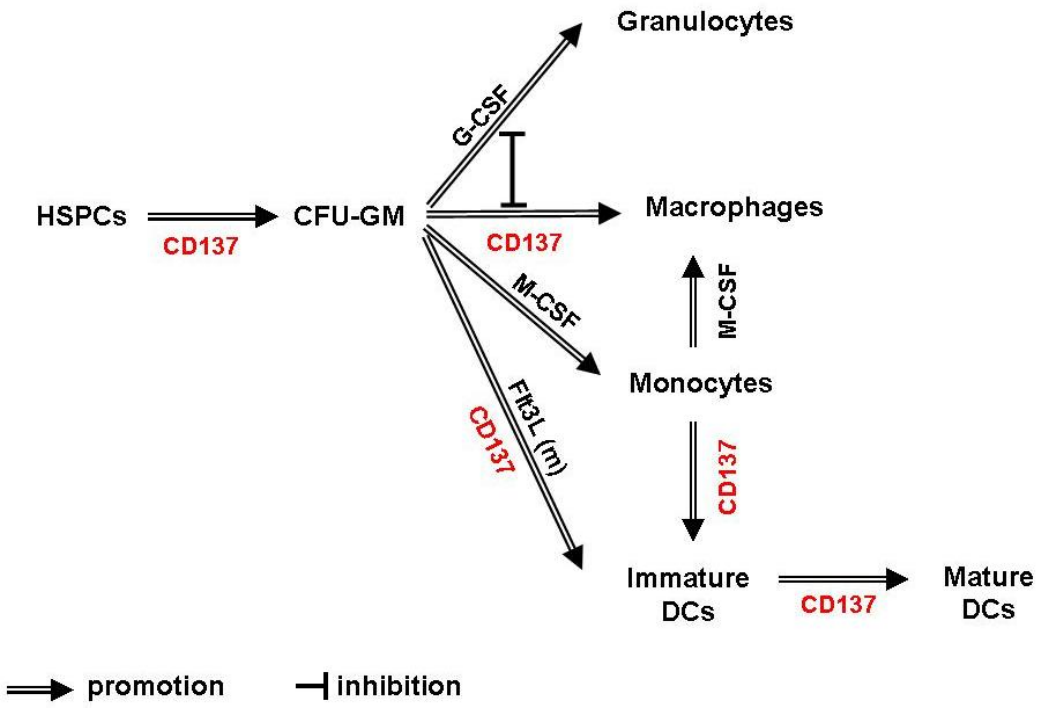


Figure 4.1. Proposed schematic summary of CD137-induced myelopoiesis. (m), specific to the murine system.

4.4 Are CD137^{+} bone marrow cells T cells?

CD137 is expressed on a small subset of total bone marrow cells (Fig. 3.1 A; Fig. 3.25 B, D), and the next important question we want to address was the nature of the CD137-expressing cells in the bone marrow.

Since we have shown that CD137 expression in the bone marrow can induce hematopoiesis, or more specifically monocytopoiesis, we first looked at the bone marrow stromal cells, which are well known to provide a unique niche to support the hematopoiesis in the bone marrow (Heissig *et al.*, 2005; Yin and Li, 2006). Although AA101 cells, a human bone marrow derived stromal cell line, expresses CD137 and

the expression seems to be under a certain regulation (Fig. 3.46), we could not detect CD137 expression on ST-2 and MS-5 cells, two murine bone marrow stromal cell lines (Fig. 3.47). This suggested that bone marrow stromal cells were unlikely the source of CD137 expression in the bone marrow. CD137 detected on AA101 cells may be an epigenetic effect of cellular transformation. CD137 may merely be a tumor antigen expressed by the AA101 cell line as its expression has been documented in several cancers, including osteosarcoma (Lisignoli *et al.*, 1998), lung cancer (Zhang *et al.*, 2007), non-Hodgkin's lymphoma (Furtner *et al.*, 2005), among others.

We then turned to T cells, since CD137 was originally found on activated T cells, and activated T cell can quickly upregulate CD137 expression (Kwon *et al.*, 1989; Schwarz *et al.*, 1993). It is well known that T cell progenitors leave the bone marrow and enter the thymus at a very early stage of T cell development (Fowlkes and Pardoll, 1989). CD137 expression on T cells is strictly activation dependent (Schwarz *et al.*, 1995), therefore, if the CD137-expressing cells in the bone marrow are T cells, they are likely to be circulating activated T cells that have been migrated back to the bone marrow.

CXCR4 is an important chemokine receptor of HSCs for receiving homing signal to the bone marrow based on a gradient of CXCL12 (SDF-1) which is released by bone marrow stromal cells (Aiuti *et al.*, 1997; Peled *et al.*, 1999; Kollet *et al.*, 2001). It has been shown that, during an infection, activated T cells also upregulate their CXCR4

expression to migrate back to the draining lymph nodes and thymus. And similar to HSCs and plasma cells, T cells can undergo transendothelial migration in response to CXCL12 *in vitro*, with memory T cells responding better than naïve T cells (Cinamon *et al.*, 2001). Therefore, it is tempting and reasonable to speculate that CXCR4-expressing activated T cells, that also express CD137, could migrate back to the bone marrow too, based on the CXCL12 gradient, similar as the HSCs.

In fact, some studies have already shown that activated $CD4^+$ cells, migratory memory T cells, and $FoxP3^+$ regulatory T cells could migrate back to the bone marrow compartment (Menteiro *et al.*, 2005; Rosa and Pabst, 2005; Kim, 2006). Rosa and Pabst (2005) have shown that under physiological conditions, mature CD4 and CD8 T cells undergo extensive migration from blood to the bone marrow and vice versa, and that bone marrow can function as a secondary lymphoid organ for both CD4 and CD8 cells, as well as a preferential homing site for memory T cells (Rosa and Pabst, 2005). This is further proven by Li and colleagues (2007), who have shown that bone marrow is a preferential homing site for autoreactive T cells in type 1 diabetes; and that bone marrow could serve as a reservoir for memory T cells (Li *et al.*, 2007). Monteiro and colleague (2005) have demonstrated that activated $CD4^+$ bone marrow T cells produce hematopoietic-related cytokines and essential for hematopoiesis stimulation during infection and hematologic recovery after bone marrow transplantation. The authors therefore conclude that the "normal hematopoiesis" does not reflect a basal level bone marrow activity, but it is

antigen-induced state maintained by constant activation of bone marrow $CD4^+$ T cells (Monteiro *et al.*, 2005). The bone marrow T cells only comprise ~ 3 – 8% of nucleated bone marrow cells (Zeng *et al.*, 2002; Westermann and Pabst, 1992), which correlates well with the low frequency of $CD137^+$ cells in the bone marrow (Fig. 3.1 A; Fig. 3.25 B).

In our *B. pertussis* and Influenza A H1N1 infection models a direct effect of the pathogens on bone marrow cells can be excluded since both pathogens infect the lung and not the bone marrow. Instead, we hypothesize that T cells in the lung or in lung-draining lymph nodes become activated in response to the infection and induce expression of CXCR4 and CD137, which plays an important role in anti-pathogen immune responses in the lung (Lin *et al.*, 2009; Harrison *et al.*, 2006). These CD137-expressing T cells then migrate back to the bone marrow and interact with HSPCs and induce proliferation and myelo-monocytic differentiation. The newly generated monocytic cells may then leave the bone marrow and migrate to the inflammation site and assist in the clearance of the pathogens under the help of Th1 cytokines.

However, we could not exclude the possibility that the CD137-expressing cells in the bone marrow are other immune cells, such as NK cells or macrophages, which have been reported to express CD137 at low level (Melero *et al.*, 1998; Lin *et al.*, 2008; Kienzle and von Kempis, 2000). The true identity of CD137-expressing bone marrow

cell requires further studies in the future. A multi-color flow cytometric analysis will be a good start.

4.5 Does CD137L signaling influence myelopoiesis differently during steady state conditions and during immune responses?

As stated at the end of section 4.1, the function of CD137L signaling in myelopoiesis seems to be opposite at activated immune states and steady state conditions.

4.5.1 CD137 induces myelopoiesis during infection

CD137 activation is found to be associated with various immune activation conditions.

Bacterial and mycobacterial infections are reported to induce CD137 expression. Upregulation of CD137 in osteoblastic cells was observed by infection with *Mycobacterium bovis bacillus Calmette-Guérin* (BCG), *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus*, and by treatment with lipopolysaccharides and heat-killed BCG *in vitro* (Saito et al., 2004). *In vivo* studies show that *E. coli* infection causes a 10-fold increase in the number of $lin^{-}c\text{-}kit^{+}sca\text{-}1^{+}$ cells in the bone marrow, and an increased number of CFU-GM (Zhang *et al.*, 2008), which fits nicely to CD137-induced colony formation from HSPCs, with mainly CFU-GM (Fig. 3.5; 3.22; 3.23). By using CD137-deficient mice, Lee and colleagues have shown that

CD137 is required for rapid clearance of *Listeria monocytogenes* infection by inducing rapid infiltration and activation of neutrophils and monocytes (Lee *et al.*, 2005, 2009). Curto and colleagues have showed that mycobacteria *M. avium* and *M. tuberculosis* could productively infect microglia, a macrophage-like cell in brain, and induce the expression of CD137. It is interesting that the expression of CD137 appears much earlier and stronger, and is maintained longer with the more virulent mycobacterium (Curto *et al.*, 2004). In line with these findings, our *B. pertussis* infection model also revealed that the expression of CD137 was upregulated in the bone marrow, and was correlated to the virulence of the bacterial strains (Fig. 3.48).

During influenza viral infection, CD137 is reported to be induced on lung T cells, transiently or sustained depending on the severity of the infection. Endogenous but not transgenic over-expressed CD137L plays a critical role in protecting mice from severe effects of influenza disease (Lin *et al.*, 2009). Mice infected with cytomegalovirus (CMV) showed defects in hematopoiesis in the bone marrow with increased numbers of progenitor cells (personal communication with Degli-Esposti M. A.). This observation may somehow relate to the CD137 expression, if we consider that influenza A infection causes an increased number of CD137-expressing cells in the bone marrow (Fig. 3.49), which could potentially induce the proliferation of HSPCs in the bone marrow.

Our results show that inflammation induced by systemic administration of high levels

of endotoxin such as LPS from *E. coli* can increase the number of CD137-expressing cells in the bone marrow (Fig. 3.50). It is also reported that LPS preferentially induces CD137L expression on human MoDCs, and that the sorted CD137L^{hi} cells could potently augment T cell expansion and survival (Lee *et al.*, 2003).

Furthermore, inflammation caused by thermal injuries and sepsis often lead to monocytosis accompanied with neutropenia (Santangelo *et al.*, 2001; Shoup *et al.*, 1998). Since CD137 is able to drive HSPCs away from granulocytic but towards monocytic differentiation (Fig. 3.39; 3.40; 3.41), perhaps CD137 plays an important role in burn induced inflammation. Interestingly, CD137 expression on human T cells is also upregulated by DNA damaging agents such as anti-cancer drugs, or γ-irradiation (Kim *et al.*, 2002).

The circumstances discussed above; including viral infection, bacterial infection, mycobacterial infection, high level of endotoxin, thermal injury, and DNA damage; can be recognized as danger signals or sensed as stress by the host. The immune system becomes activated in response to such danger signals. CD137 seems to play a significant role in initiating and sustaining the immune activation. Among all the events induced by CD137L signaling, the induction of myelopoiesis in the bone marrow by CD137^{+} cells seems to be an indispensable one.

4.5.2 CD137 limits myelopoiesis and the development of DCs at steady state

Recently, Lee and colleagues (2008) found CD137L expression on HSCs, differentiating CMPs and GMPs, and CD137 expression was inducible on activated myeloid progenitors. They claimed that CD137 and CD137L had a function to limit myelopoiesis and the development of DCs by showing that steady-state numbers of GMPs, myeloid lineage cells, and mature DCs were higher in CD137- and CD137L-deficient mice; and absence of interaction between CD137 and CD137L led to enhanced differentiation into DC lineages (Lee *et al.*, 2008).

The discrepancies between Lee *et al.*'s and our study and previous studies may reflect the different function of CD137 / CD137L in regulation of hematopoiesis, particularly myelopoiesis and DC development at different physiological conditions. In order to allow clearance of the pathogen, CD137L signaling tends to induce myelopoiesis in an inflammatory condition, while it tends to inhibit myelopoiesis at steady state to maintain the balance and homeostasis. This is partially confirmed by the follow-up study on the consequence of CD137 on DC survival and function during an activated immune state. Choi and colleagues, (2009) reported that, during LPS induced inflammation and *Propionibacterium acnes* infection, CD137 functioned as a survival factor for DCs by maintaining high level of Bcl-2 and Bcl-X_L (Choi *et al.*, 2009).

One very recent review has discussed this issue as well (Wang *et al.*, 2009). The author was concerned about the differences between Lee *et al.*'s and our studies. Lee *et al.* (2008) tested the effect of CD137-Fc on myeloid progenitors over 4 days of

culture, and showed no effect of CD137-Fc on WT cells (Lee *et al.*, 2008). However, we showed little effect of CD137-Fc on expansion of bone marrow cells in the first few days of culture, but CD137-Fc treatment resulted in a dramatically enhanced bone marrow cell expansion at 7-12 days of culture (Jiang *et al.*, 2008a, 2008b). Nevertheless, we believe that different timing (early vs. late) may not be the main reason for the discrepancy. The transient signaling may come closer to the truth; because expression of CD137 at high level occurs only transiently during acute inflammation, which is likely the situation that our experiments mimick. In contrast, the experiments of Lee and colleagues dealt with the steady state situation with low and constant CD137 expression.

4.6 Are HSPCs differentiated by CD137 regulatory macrophages or MDSCs?

Our studies show that, by *in vitro* culture with immobilized CD137 protein for 7 days or 10 days in murine and HSPCs differentiate to macrophages with a highly phagocytotic and IL-10^{high}IL-12^{low} profile (Fig. 3.10; 3.11; 3.29; 3.31). In an allogeneic MLR, these cells cannot induce T cells proliferation (Fig. 3.12; 3.30), and more importantly, they suppress the T cell proliferation induced by allogeneic mature DCs (Fig. 3.33). These data suggest that *in vitro* CD137 expanded cells are immunosuppressive. There are three major groups of myeloid cells that possess immunosuppressive functions – tolerogenic DCs, regulatory macrophages and MDSCs.

Our results showed that CD137-treated cells are not likely DCs, ruling out the possibility that the cells are tolerogenic DCs.

Macrophages are also important effector cells in tumor immunity besides CTL and NK cells. Depending on the phenotype, macrophages can either promote or prevent tumor progression. According to the traditional M1 / M2 classification, the so-called classically activated macrophages are activated by IFN-γ and LPS, and have an IL-12^{high}, IL-10^{low} phenotype, whereas alternatively activated macrophages are activated by IL-4 and IL-13 and have an IL-12^{low}, IL-10^{high} phenotype (Gordon, 2003). According to the new grouping system based on homeostatic functions, the so-called regulatory macrophages produce high levels of the immunosuppressive cytokine IL-10, and also downregulate IL-12 production (Gerber and Mosser, 2001), and the ratio of IL-10 to IL-12 could be used to define regulatory macrophages (Mosser and Edwards, 2008). The morphology and phenotypes of CD137-differentiated HSPCs fit the description of regulatory macrophages or alternatively activated macrophages quite well. Moreover, since the differentiated cells produce high level of MCP-1 and IL-8 in the murine and human system (Fig. 3.32 A; 3.13), respectively, the cells are believed to be activated. However, they do not produce M-CSF and IL-6 (unpublished data from Tang Q. and Schwarz H.), which is usually seen in classical activated monocytes/macrophages. This in turn indicates that the differentiation and activation of murine monocytic cells by CD137 is not through the classical pathways.

Because there were immature myeloid cells in the culture of CD137-treated HSPCs, these cells could also be MDSCs. Movahedi and colleagues described MDSC can be divided into two subpopulations including monocytic cells and polymorphonuclear (PMN) cells (Movahedi *et al.*, 2008). However, Gallina and colleagues showed that MDSCs are mainly inflammatory monocytes but not granulocytes (Gallina *et al.*, 2006). It is reported that MDSC expressed CD11c ($CD11b^+CD11c^+Gr\text{-}1^+IL\text{-}4R\alpha^+$), and the MDSC have mixed M1 and M2 type characteristics (Umemura *et al.*, 2007). These characteristics are quite similar to our CD137-treated cells. They are macrophage like cells, most of them are $CD11b^+$, and some of them co-express CD11c (Fig. 3.28), and they can effectively suppress T cell proliferation induced by mature DCs. A study from our lab also indicated that the CD137-treated human monocytes exhibit mixed characteristics of M1 and M2 macrophages in terms of the prolife of cytokine secretion, signature gene expression, and surface receptor expression (unpublished data from Toh K. L. and Schwarz H.).

The preliminary results from the follow-up study found that the supernatant from CD137-Fc treated Lin^-, $CD117^+$ cells could partially suppress T cell proliferation in an allogeneic MLR too, but with less extent (unpublished data). This indicated that both soluble mediators and cell-cell interactions were essential for the suppressive function. In preliminary studies the neutralizing anti-IL-10 antibody has not shown any effect on reversing the suppressive function (unpublished data), suggesting that the classical immunosuppressive cytokine IL-10 may not be one of the soluble factors mentioned

above. The detailed immune suppressive mechanisms of CD137 differentiated HSPCs require further investigation.

In shot, the cells augmented and differentiated *in vitro* from HSPCs by CD137L signaling could be regulatory macrophages or MDSCs or a mixture. However, we still don't know whether this is also the case *in vivo*. If the answer is yes, then this population of regulatory macrophages or MDSCs could be a potential target for cancer immunotherapy. The patients should have better immune response against tumors by preventing the generation or by a direct depletion of these regulatory cells.

4.7 Is CD137-induced myelopoiesis beneficial or deleterious for the host?

As discussed and speculated above, after the host senses danger signals, the expression of CD137, probably on T cells, is upregulated. The activated CD137-expressing T cells migrate back to bone marrow and induce myelopoiesis. The newly generated monocytes then leave the bone marrow and enter the circulation. Whether these cells are beneficial or deleterious depends on their immunological phenotypes: immunostimulatory or immunoregulatory.

As shown in Results under section 3.2.8, *in vitro* treatment of HSPCs with CD137 leads to suppressive cells; however, *in vivo* the situation is very likely more complex. The characteristics of the differentiated cells usually depend on the microenvironment.

In a long lasting immune activation, e.g. a sustained infection, high levels of inflammatory cytokines such as TNF (Mosser and Edwards, 2008) are maintained at the inflammation site. Under the influence of these inflammatory cytokines and the associated microbial pathogens or cellular stress, the CD137-induced monocytic cells would go through maturation and activation and elicit anti-microbial effector functions. Of course, the myelopoiesis induced by CD137 in this situation is beneficial for the host in fighting the infection. On the other hand, if this happens in the context of an autoimmune reaction, the disease is getting worse with the influx of new differentiated inflammatory macrophages.

However, in contrast to the situation of an infection and inflammation, in a tumor microenvironment, high levels of immunosuupressive cytokines such as IL-10 and TGF-β are maintained by the tumor cells, tumor stromal cells, and / or tumor infiltrating immune cells, while the production of Th1 cytokines such as TNF and IL-12 is inhibited (Dumont and Arteaga, 2002; Sierra, 2005). In the tumor microenvironment, the CD137-induced cells would differentiate into regulatory macrophages or tumor associated macrophages (TAM), which dampen the immune response and in turn are beneficial for tumorigenesis. This may be one of the mechanisms that some tumor cells express CD137 as neoantigen, in order to induce the local myelopoiesis and yield more TAM to gain advantage for growth. Interruption of this process could lead to the inhibition or eradication of tumors.

In summary, CD137-induced myelopoiesis is an outcome of stress conditions. It is a natural response that the host requires more leukocytes to deal with these conditions. Whether the outcome is beneficial or deleterious for the host depends on the microenvironment and cytokine milieu that the newly generated cells would encounter after they enter the circulation.

4.8 At what differentiation stage do HSPCs respond to CD137?

Both the $CD34^+$ cells in human system and the Lin^-, $CD117^+$ cells in murine system contain progenitor cells of different stages of development ranging from multipotent long-term hematopoietic stem cells (LT-HSCs) to already committed progenitor cells (Ogawa *et al.*, 1991; Adolfsson *et al.*, 2001; Christensen *et al.*, 2001). So the next question we asked was at what stage the progenitor cells start to respond to CD137.

The most primitive cells are embryonic stem (ES) cells, and CD137 showed no significant impact on murine and human ES cells (Fig. 3.34; personal communication by Kadereit S.).

Long-term HSCs (LT-HSCs) are defined by their ability to give rise to the lymphoid and myeloerythroid lineages for life after transplantation into lethally irradiated recipients while short-term HSCs (ST-HSCs) and committed CFUs have more limited self-renewal capacity and are capable to respond to stimuli within 6 – 14 days

(Christensen *et al.*, 2001; Capmany *et al.*, 1999). The fact that CD137 protein induced proliferation, colony formation and myeloid differentiation of $CD34^+$ cells within 14 days, and of Lin^-, $CD117^+$ cells within 7 days, suggests that the responding cells were ST-HSCs and myeloid progenitor cells, including CFU-GM and CFU-M. Whether more primitive progenitor cells such as LT-HSCs can also be activated by CD137 will need to be determined in future studies.

We found both G-CSF and CD137 are able to induce proliferation of hematopoietic progenitor cells and an increase in cell numbers with comparable potencies. The combination of G-CSF and CD137 generated significantly ($p<0.05$) more cells during a 7-day culture period than either factor alone but had neither a synergistic nor an additive effect on the number of live bone marrow cells (Fig. 3.37), which implies that either CD137 or G-CSF alone is sufficient for ensuring growth or survival of the responding progenitor cells, and the two factors are largely exchangeable for these effects. The nature of responding cell population is not known. The fact that CD137 protein and anti-CD137L antibodies can influence differentiation of granulocytes, monocytes and DCs indicates that the target cell for CD137 is a progenitor cells which can give rise to all three cell types, which would be CFU-GM or an even more primitive progenitor cell. The fact that CD137 and G-CSF compete in determining differentiation pathways also suggest that the responding cell population is CFU-GM or earlier.

However, CD137 and Flt3L showed much weaker cooperative effects on the proliferation and differentiation of Lin^-, $CD117^+$ cells than that of bone marrow cells (Fig. 3.44; 3.45). The effect of Flt3L on Lin^-, $CD117^+$ seems to be dominant and it masks the effect from CD137. Combined with the fact that Flt3, the receptor of Flt3L, is expressed mainly by the HSPCs in the bone marrow, the CD137 responding cells should be committed progenitor cells rather than the true stem cells.

Our preliminary data showed that as early as CMPs and one of their direct progeny GMPs (equivalent to CFU-GM) can respond to immobilized CD137-Fc protein effectively in terms of morphological changes. We will investigate the details in the future and further look into multi-potential progenitors (MPPs) and ST-HSCs.

4.9 How can a small population of CD137 and CD137L expressing cells in bone marrow cause such wide-spread functional effects?

Only few cells in the bone marrow stained positive for CD137, and only 1 – 3% of $CD34^+$ cells were found to express CD137L (Fig. 3.1). These low expression levels are contrasted by the comparatively large biological response of $CD34^+$ cells upon crosslinking of CD137L.

One possible explanation is that CD137L may be expressed on a small subpopulation of cells which can proliferate very potently upon CD137L crosslinking. Or

crosslinking of CD137L on the few cells which express it leads to secretion of soluble factors which then induce proliferation of a larger number of cells which do not need to express CD137L. IL-8 is likely to be one of the candidates among the various hematopoietic growth factors, since CD137L signaling leads to substantial IL-8 release (Fig. 3.13), and neutralizing IL-8 partially blocked CD137-induced proliferation of $CD34^+$ cells (Fig. 3.14). The partial dependency of CD137L signaling also indicates that besides IL-8 there are other signaling pathways involved, which require further investigation.

Alternatively, the detection methods used were not sensitive enough, thereby underestimating expression levels of CD137 and CD137L. Similar to us, Lee and colleagues (2008) also showed that the expression level and the frequency of CD137 on the hematopoietic progenitor cells were very low (Lee *et al.*, 2008). It is a fact accepted among our peers that CD137L expression is difficult to detect, as its expression is transient, and it can be released from cells by metalloproteinase cleavage (Salih *et al.*, 2001). Furthermore, Lin and colleagues (2009) have managed to show that the level of endogenous CD137L that is protective in severe influenza infection is very low, and below the limit of detection at the protein level by flow cytometry and immunofluorescent staining, although detectable at the mRNA level (Lin *et al.*, 2009).

Everything has its two sides and the immunity is not an exception. The balance of the immune system needs to be carefully considered when we apply immunotherapy. For

example, transfer of an optimal number of naïve T cells protects against an otherwise lethal dose of infection, but transfer of too many T cells results in disseminated inflammation and results in death likely due to CD8 T cell-mediated pathology. CD137 receptor / ligand system shows the same characteristic. Intranasal delivery of CD137L by recombinant adenovirus marginally improves survival of CD137L -deficient mice at low dose, but exacerbates disease at high dose (Lin *et al.*, 2009).

The expression of inducible costimulatory molecule pairs such as CD137 - CD137L is under tight regulation *in vivo*. Perhaps they have been acquired by the immune system to handle progressively more virulent infections, so as to maintain the effector CD8 T cell responses when required, but avoid excessive immunity when the danger signal is readily cleared. All these facts suggest that it is not the absence or presence of CD137 / CD137L causes all the effects, rather the magnitude of or the changes in CD137 / CD137L expression determines the outcomes. The subtle change in CD137 / CD137L expression level makes a huge impact on the balance of homeostasis and immune response. Thus, the positive regulation of CD137 expression in the bone marrow during infection and inflammation (Section 3.5.2 – 3.5.4) should be considered to be important for host defense.

4.10 Future studies

As we discovered that CD137L signaling plays a role in myelopoiesis more questions

emerged. Five main aspects are currently under investigation or going to be studied in future.

First, we would like to further explore the function and underlying mechanisms of the immunosuppressive cells differentiated from HSPCs by CD137. Several possible mechanisms of regulatory macrophages or MDSC suppressing T cell activation have been proposed. 1) They have direct suppressive functions on T cells: e.g. they produce reactive nitrogen compounds (peroxynitrites) that nitrosylate CD8 and the TCR on T cells to block T cell activation (Nagaraj *et al.*, 2007); 2) they express coinhibitory molecules such as PDL-1; 3) they release IL-10 to induce immature DCs becoming tolerogenic DC; 4) they suppress IL-12 release to abolish NK cell function; 5) they produce ROS or RNS to induce apoptosis in T cells.

Second, the identity of $CD137^+$ cells in the bone marrow need to be confirmed. *In vivo* transfer of activated T cells into WT or CD137-deficient mice will be a good attempt to start. The composition of the bone marrow, spleen and the peripheral blood of the hosts will be analyzed. At the same time, a methodology that can sensitively detect the low frequency of $CD137^+$ cells among the bone marrow cells in combination with other lineage markers needs to be established.

Third, We are going to look at the function of CD137 in granulopoiesis, the other arm of myelopoiesis besides monocytosis. The antagonism of CD137 and G-CSF on each

other in inducing differentiation of GMPs will be analyzed in details using immnuofluorescent tracking system at the single cell level together with a collaborator. Since I will sort out the subpopulations within the Lin^-, $CD117^+$ cells in this project, the identity of the earliest responder cells among the progenitor cells at different stages will be explored as well.

Fc protein of human IgG1 had no effect on the proliferation or cell survival of murine Lin^-, $CD117^+$ cells, same as PBS controls (Fig. 3.17, 3.18, 3.19). However, since monocytes, macrophages and DCs express Fc receptors, Fc protein may have some influence on monocytic cell differentiation, as we have seen in Fig 3.6A and 3.27A. Therefore, a recombinant CD137 protein without the Fc part needs to be developed. To remove the Fc portion on CD137-Fc simply by enzymatic cleavage of the linker between them may not be a good choice, because the proteases are not specific enough to give a clean cut. This may lead to the cleavage of extracellular domain of CD137 too. For purification purpose, CD137 could be fused with other affinity tags, such as His-, HA-, or GST-tag. Other Fc-free systems, such as expressing CD137 on cell surface (Fig. 3.26B) or immobilizing CD137-Fc on protein A-coupled beads (Fig 3.4B), could also be applied to figure out whether the differentiation of HSPCs is induced by CD137 alone or with the combinantion of Fc.

Last but most important, We have to find a recipe to delivery CD137 *in vivo*. Since crosslinking CD137L requires oligomerized CD137, CD137 protein has to be

immobilized onto a delivery vector, which is small enough and stable enough to deliver CD137 into the bone marrow compartment. Functional efficacy need to be monitored in the animal models to evaluate the functions of CD137-induced myelopoieisis *in vivo*.

CHAPTER 5 CONCLUSION

The detection of CD137 and CD137L expression in bone marrow, and the induction of proliferation, colony formation, increase in cell numbers and differentiation of HSPCs – $CD34^+$ cells in the human system, and Lin^-, $CD117^+$ cells in the murine system – identify novel biological activities for CD137 and its ligand, indicating a role for this receptor / ligand system in hematopoiesis. This is further confirmed by demonstrating that the number of $CD137^+$ cells in the bone marrow increases during viral and bacterial infection and inflammation. CD137 and G-CSF or Flt3L function cooperatively on promoting survival and proliferation of HSPCs, although they drive divergent differentiation pathways. This study extends previous knowledge that signals through CD137L induce activation, proliferation and survival in monocytes, and enhance maturation of DCs, and thereby shows that CD137 and CD137L regulate multiple aspects of hematopoiesis, in particular of myelopoiesis.

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APPENDICES

Appendix I. Buffers and Solutions

PBS (working concentration, 1 ×)

8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L H_2O, pH 7.4

FACS buffer

0.5% FBS, 0.02% NaN_3 in PBS, pH 7.4

MACS buffer

0.5% FBS, 2 mM EDTA in PBS, pH 7.4

RBC lysis buffer

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

ELISA wash buffer (PBST)

0.05% Tween-20 in PBS

Appendix II. List of antibodies used in the study

Antibody	Clone	Source
Rat anti-mouse CD137L (PE, biotin)	TKS-1	eBioscience
Hamster anti-mouse CD137 (PE)	17B5	eBioscience
Armenian hamster IgG (PE, FITC, biotin)	eBio299Arm	eBioscience
Hamster anti-mouse CD3e (PE, FITC)	145-2C11	eBioscience
Rat anti-mouse CD4 (FITC)	GK1.5	eBioscience
Rat anti-mouse CD11b (PE)	M1/70	eBioscience
Rat anti-mouse CD11c (PE, FITC)	N418	eBioscience
Rat anti-mouse CD14 (PE)	Sa2-8	eBioscience
Rat anti-mouse CD19 (PE)	6D5	eBioscience
Rat anti-mouse CD80 (PE)	16-10A1	eBioscience
Rat anti-mouse CD86 (PE)	PO3.1	eBioscience
Rat anti-mouse CD117 (c-kit) (FITC)	2B8	eBioscience
Rat anti-mouse CD117 (c-kit) (PE)	3C1	Miltenyi
Rat anti-mouse Gr-1 (Ly6C/G) (PE, FITC)	RB6-8C5	eBioscience
Rat anti-mouse Gr-1 (Ly6C/G) (biotin)	RB6-8C5	BioLegend
Rat anti-mouse Ly6G (FITC)	1A8	BD Pharmingen
Rat anti-mouse F4/80 (PE)	BM8	eBioscience
Rat anti-mouse MHC-II (I-A/I-E) (PE, FITC)	M5/114.15.2	eBioscience
Rat anti-mouse Sca-1 (Ly6A/E) (APC)	D7	eBioscience
Rat IgG2a (PE, FITC, biotin)	eBR2a	eBioscience
Rat IgG2b (PE, FITC, biotin)	eB149/10H5	eBioscience
Rat anti-mouse CD3 (LEAF)	17A2	BioLegend
Hamster anti-mouse CD28 (LEAF)	37.51	BioLegend
Rat anti-mouse GM-CSF (LEAF)	MP1-22E9	BioLegend
Rat IgG2b (LEAF)	RTK4530	BioLegend
Syrian hamster IgG (LEAF)	SHG-1	BioLegend
Mouse anti-human CD137L (PE)	5F4	Biolegend
Mouse anti-human CD137L (PE)	C65-485	BD Pharmingen
Mouse anti-human CD137 (PE)	4B4-1	BD Pharmingen
Mouse anti-human CD137 (biotin)	BBK-2	NeoMarkers
Mouse IgG1 (unconjugated, PE)	MOPC-21	Sigma
Mouse IgG1 (FITC, APC)	MOPC-21	eBioscience
Mouse anti-human CD3 (PE)	UCHT1	eBioscience
Mouse anti-human CD11b (PE)	ICRF44	eBioscience
Mouse anti-human CD11b (PE)	M1/70.15.11.5	Miltenyi
Mouse anti-human CD14 (PE)	61D3	eBioscience
Mouse anti-human CD19 (PE)	HD37	eBioscience
Mouse anti-human CD34 (PE, FITC)	AC136	Miltenyi
Mouse anti-human CD41 (PE)	HIP8	eBioscience

Mouse anti-human CD45 (PE)	5B1	Miltenyi
Mouse anti-human CD68 (PE)	Y1/82A	eBioscience
Mouse anti-human CD83 (PE)	HB15e	eBioscience
Mouse anti-human CD209 (DC-SIGN) (FITC)	eB-h209	eBioscience
Mouse anti-human CD235a (PE)	HIR2	eBioscience
Mouse anti-human HLA-DR (APC)	LN3	eBioscience
Mouse anti-human IL-8 (unconjugated)	6217.11	Sigma
Goat anti-human IgG (Fc specific) (FITC)	polyclonal	Sigma
Streptavidin (HRP)	-	Sigma
Extravidin (Cy3)	-	Sigma
Goat anti-rat IgG (Cy2)	polyclonal	Millipore

Appendix III. Primers and cycling conditions for RT-PCR

Target gene	Primers and sequences	T_A (ºC)	Cycle No.	Product length (bp)
m *CD137*	mCD137-F: 5'-ATG GGA AAC AAC TGT TAC AAC G-3' mCD137-R: 5'-ACA GAA ATG GTG GTA CTG GGA G-3'	57	40	518
m *CD137L*	MurCD137L-SEN.F: 5'-GCA CTG AAA GCT TGC CGC CAT GGA CCA GCA CAC ACT T-3' MurCD137L-SEN.R: 5'-GAA GGA GAA TTC TCA TTC TCA TTC CCA TGG GTT GTC GGG-3'	55	30	961
m *GAPDH*	GAPDH-F: 5'-TGG TAT CGT GGA AGG ACT CAT GAC-3' GAPDH-R: 5'-ATG CCA GTG AGC TTC CCG TTC AGC-3'	57	25	190
h *CD137*	CD137 SEN.F HindIII: 5'-TGT GCA AGC TTC CAC CAT GGG AAA CAG CTG TTA CAA C-3' CD137 SEN.R EcoRI: 5'-ACT GAA TTC TCA CAG TTC ACA TCC TCC TTC-3'	57	40	793
h *CD137L*	hCD137L-F: 5'-GGA ATA CGC CTC TGA CGC TT-3' hCD137L-R: 5'-TGT GAA GAT GGA CGC CCA G-3'	57	40	650
h *FcγR*	FCGR1A-F: 5'-ATG TGG TTC TTG ACA ACT CTG C-3' FCGR1A-R: 5'-GAC AGA TAT TCC TGC TGA TGT G-3'	56	40	550
h *TCF7L2*	TCF7L2-F: 5'- GAT GAC CTA GGC GCC AAC GA-3' TCF7L2-R: 5'- TTT GGG GTC TAC GTC GGC TG-3'	59.5	40	585
h *WNT5A*	WNT5A-F: 5'-AGA AGT CCA TTG GAA TAT TAA GCC C-3' WNT5A-R: 5'-CGT TGT TGT GCA GGT TCA TGA-3'	56	40	666
h *FcεR1A*	FCER1A-F: 5'-CTC CTG CCA TGG AAT CCC CT-3' FCER1A-R: 5'-AGC CAG TAC TTC TCA CGC GG-3'	59.5	40	607
h *Fibronectin*	FN1-F: 5'-ACG GGA GCC TCG AAG AGC AA-3' FN1-R: 5'-TTC CAC TCT CCT CGG CCG TT-3'	59.5	40	737
h *SOCS1*	SOCS1-F: 5'-GGT AGC ACA CAA CCA GGT GGC A-3' SOCS1-R: 5'-AAG GAG CTC AGG TAG TCG CGG A-3'	61.5	40	618
h *TGF-α*	TGFA-F: 5'-CCT GTT CGC TCT GGG TAT TGT G-3' TGFA-R: 5'-CGG TTC TTC CCT TCA GGA GG-3'	59.5	40	428
h *Cyclophilin*	cyclophilin-F: 5'-GTC CAG CAT TTG CCA TGG AC-3' cyclophilin-R: 5'-GAC AAG GTC CCA AAG ACA GC-3'	55	30	200

m = mouse, h = human, T_A = annealing temperature.

Appendix IV. The antibody list and the map of cytokine antibody array

	A	B	C	D	E
1	POS	POS	POS	POS	Blank
2	NEG	NEG	NEG	NEG	Blank
3	Eotaxin-2	FAS ligand	Fractalkine	G-CSF	GM-CSF
4	Eotaxin-2	FAS ligand	Fractalkine	G-CSF	GM-CSF
5	IL4	IL5	IL6	IL9	IL10
6	IL4	IL5	IL6	IL9	IL10
7	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG
8	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG
9	SDF-1-alpha	TARC	TCA-3	TECK	TIMP-1
10	SDF-1-alpha	TARC	TCA-3	TECK	TIMP-1
	F	**G**	**H**	**I**	**J**
1	axl	BLC	CD30L	CD30/TNFRSF8	CD40
2	axl	BLC	CD30L	CD30/TNFRSF8	CD40
3	IFN-gamma	IGF-BP-3	IGF-BP-5	IGF-BP-6	IL1-alpha
4	IFN-gamma	IGF-BP-3	IGF-BP-5	IGF-BP-6	IL1-alpha
5	IL12-p40/p70	IL12-p70	IL13	IL17	KC
6	IL12-p40/p70	IL12-p70	IL13	IL17	KC
7	MIP-1-alpha	MIP-1-gamma	MIP-2	MIP-3-beta	MIP-3-alpha
8	MIP-1-alpha	MIP-1-gamma	MIP-2	MIP-3-beta	MIP-3-alpha
9	TNF-alpha	sTNF RI	sTNF RII	TPO	VCAM-1
10	TNF-alpha	sTNF RI	sTNF RII	TPO	VCAM-1
	K	L	M	N	
1	CRG-2	CTACK	CXCL16	Eotaxin	
2	CRG-2	CTACK	CXCL16	Eotaxin	
3	IL1-beta	IL2	IL3	IL3 Rb	
4	IL1-beta	IL2	IL3	IL3 Rb	
5	Leptin R	Leptin	LIX	L-Selectin	
6	Leptin R	Leptin	LIX	L-Selectin	
7	PF4	P-Selectin	RANTES	SCF	
8	PF4	P-Selectin	RANTES	SCF	
9	VEGF	Blank	Blank	Blank	
10	VEGF	Blank	POS	POS	

Appendix V. Publications

International Journal Papers

1. 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* 26:2372-2381.

2. Jiang D, Chen Y, Schwarz H. 2008. CD137 induces proliferation of murine hematopoietic progenitor cells and differentiation to macrophages. *J. Immunol.* 181:3923-3932.

3. Jiang D, Schwarz H. 2009. G-CSF and CD137 cooperatively induce proliferation of bone marrow cells but antagonize each other in promoting granulocytic or monocytic differentiation. *Submitted*

Conference Posters

1. Jiang D., Chen Y., and Schwarz H. *CD137 induces proliferation of murine hematopoietic progenitor cells and differentiation to macrophages.* 1st Singapore Symposium of Immunology. Singapore. January 14-16, 2008.

2. Jiang D., Yue P. S., Drenkard D., and Schwarz H. *Induction of proliferation and monocytic differentiation of human CD34$^+$ cells by cd137 ligand signaling*. 1st Singapore Symposium of Immunology. Singapore. January 14-16, 2008.

3. Jiang D., Chen Y., and Schwarz H. *CD137 induces proliferation of murine hematopoietic progenitor cells and differentiation to macrophages.* 22nd Annual Meeting of European Macrophage & Dendritic Cell Society – Diversity and Plasticity of the Innate Immune Response. Brescia, Italy. September 18-20, 2008.

4. Jiang D., Yue P. S., Drenkard D., and Schwarz H. *Induction of proliferation and monocytic differentiation of human CD34$^+$ cells by cd137 ligand signaling*. 22nd Annual Meeting of European Macrophage & Dendritic Cell Society – Diversity and Plasticity of the Innate Immune Response. Brescia, Italy. September 18-20, 2008.

5. Jiang D., and Schwarz H. *G-CSF and CD137 cooperatively induce proliferation of bone marrow cells but antagonize each other in promoting granulocytic or monocytic differentiation.* 39th Annual Scientific Meeting of Australasian Society for Immunology. Gold Coast, Queensland, Australia. December 6-10, 2009.