ACTIVITIES OF THE CYTOKINE RECEPTOR CD137 IN MULTIPLE MYELOMA

KOH LIANG KAI

(B.Sc (Hons), NUS)

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ABSTRACT

Multiple myeloma is an incurable hematological malignancy derived from B cells, and characterized by bone destruction and multiple organ dysfunctions. CD137 and its ligand are members of the Tumor Necrosis Factor (TNF) Receptor and TNF superfamilies, respectively. CD137 enhances proliferation and survival in healthy B cells. Since CD137 can be expressed as a neoantigen by certain B cell lymphomas we hypothesized that CD137 may act as a growth factor for B cell lymphomas. Surprisingly, we found that CD137 has the opposite effects in multiple myeloma (MM) cells, where it inhibits proliferation and induces cell death by apoptosis. In contrast, CD137 does not significantly affect or enhance proliferation or survival in non-MM B cell lymphoma lines. Further, secretion of IL-6 and IL-8 is also enhanced in MM but not in non-MM cell lines in response to CD137. A selective elimination of malignant B cells in MM patients by CD137 could help to slow down disease progression and reduce the doses (and hence side effects) in conjunction with conventional treatment regimes.

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LIST OF ABBREVIATIONS

aa	Amino acid
AAD	Amino-actinomycin D
AICD	Activation induced cell death
AO	Acridine orange
APC	Antigen presenting cell
BCR	B cell receptor
BMSC	Bone marrow stromal cell
BSA	Bovine serum albumin
CD137-Fc	Recombinant human CD137 protein
CD137L	CD137 ligand
CHO	Chinese hamster ovary
CLL	Chronic lymphocytic leukemia
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DR	Death receptors
EB	Ethidium bromide
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ESR	Erthrocyte sedimentation rate
FACS	Fluorescence activated cell sorter
FasL	Fas ligand

FBS	Fetal bovine serum
Fc	Fc portion of an antibody
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocynate
FISH	Fluorescence in situ hybridization
Fv	Variable domains of the Fab portion of an antibody
GC	Germinal centre
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HAT	Hypoxanthine, aminopterin, thymidine
H-CAM	Homing-associated cell adhesion molecule
ICAM	Intracellular adhesion molecule
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor - 1
IL	Interleukin
IP_3	Inositol 1, 4, 5-triphosphate
ISS	International staging system
JNK	jun-N-terminal kinase
LDH	Lactate dehydrogenase
LFA	Lymphocyte function-associated molecule
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorter
MAPK	Mitogen activated protein kinases
MEK	MAPK/Erk kinase

MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MM	Multiple myeloma
MRI	Magnetic resonance imaging
mRNA	Messenger ribosomal nucleic acid
N-CAM	Neural cell adhesion molecule
NF-κB	Nuclear factor-κB
NIK	NF-κB inducing kinase
NK	Natural killer
PBS	Phosphate buffered saline
PBST	PBS with 0.05% Tween-20
PCL	Plasma cell leukemia
PE	Phycoerythrin
PIP3	Phosphotidylinositol 3, 4, 5-triphosphate
PI3K	Phosphotidylinositol-3 kinase
PLCγ	Phospholipase Cγ
RNA	Ribosomal nucleic acid
SAPK	Stress-activated protein kinase
sCD137	Soluble CD137
SCID	Severe combined immuodeficiency
SHP-1	Src-homology 2 domain phosphatase-1
TAA	Tumor associated antigen
TCR	T cell receptor
TGF-β	Transforming growth factor - beta

TMB	3, 3', 5, 5' - tetramethylbenzidine
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	Tumor necrosis factor receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
VLA	Very late antigen

1. INTRODUCTION

1.1 MULTIPLE MYELOMA

Multiple myeloma (MM) is a malignancy of terminally differentiated plasma cells that primarily reside at multiple sites in the bone marrow, as well as, at the extramedullary sites in the later stages of the disease. It is characterized by the excessive secretion of monoclonal immunoglobulins (IgG, IgA, IgD, or IgE) into the serum and/or urine by monotypic plasma cells (Kuehl and Bergsagel, 2002). Bone destruction is frequently caused by the intricate interactions that occur between the myeloma cells and bone-marrow microenvironment (Kyle and Rajikumar, 2004), leading to the activation of signaling pathways stimulating tumor growth, and ultimately resulting in a multitude of symptoms and organ dysfunction, including bone pain, fractures, hypercalcemia, renal failure, anaemia, and an increased susceptibility to infections (Bataille *et al.*, 1995; Bommert *et al.*, 2006).

MM accounts for 20% of all new hematological malignancies, making it the second most prevalent blood cancer (Selina, 2003). Epidemiology indicates both an increasing incidence and earlier age of onset for the disease (Chen-Kiang, 2005), with an average prognosis of approximately 33 months (Piazza *et al.*, 2007). Treatments currently available, including the administration of drugs like

thalidomide, bortezomib, lenalidomide, have only resulted in an improvement in the overall survival of patients (Trudel *et al*., 2007, Palumbo *et al*., 2009), with no cure currently in sight. Even stem cell transplantation, which has been shown to provide long-term remission, suffers from both an increased treatment-related mortality, and a high rate of relapse (Bensinger, 2004).

Despite advances in MM therapy, it remains an incurable hematological malignancy characterized by frequent early responses inevitably followed by treatment relapse. Relapses tend to result in progressively shorter response durations, with higher proliferative fractions and lower apoptotic rates, underscoring the emergence of drug resistance, hence contributing to the majority of death of MM patients, with median survival time ranges from six to nine months (Richardson *et al*., 2007). While unprecedented response rates have been achieved via combination therapy with the immunomodulatory drug thalidomide, proteasome inhibitor bortezomib, with traditional chemotherapeutic drugs like dexamethasone (Trudel *et al*., 2007, Palumbo *et al*., 2009), relapse rates remain universal and are the reason why alternative therapeutic strategies must be developed. Therefore, an approach that allows targeting and selective killing of cancerous MM cells remains highly desirable.

1.3 GENETICS OF MULTIPLE MYELOMA

MM presents complex heterogenous cytogenetic abnormalities, with the majority of patients exhibiting hyperdiploid karyotypes (Smadja *et al*., 1998). The usage of interphase fluorescence in situ hybridization (FISH) has also led to the observance of other forms of aneuploidy, such as patients with hypoploid, near-diploid, pseudodiploid or near-tetraploid chromosome numbers, with the rearrangement of the *IgH* gene, trisomy 1q, and 13q deletion, being the most frequent chromosomal changes (Hee *et al.*, 2006).

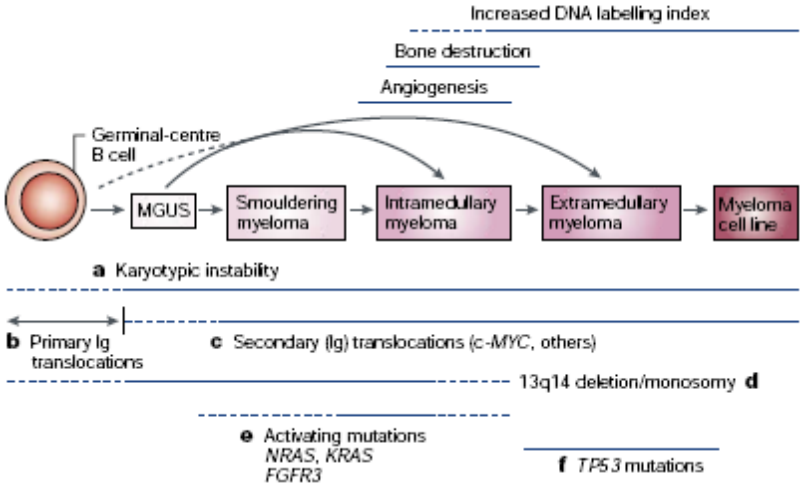

Figure 1. Molecular pathogenesis of myeloma: multiple oncogenic events. Diagram adapted from Kuehl and Bergsagel (2002).

While one of the strongest predictors of MM disease outcome is the t(4;14)(p16;q32) genetic marker (Keats *et al*., 2006), myeloma pathogenesis itself relies upon multiple oncogenic events which are detailed in Figure 1. As B cells are inherently genetically unstable, due to the many DNA breaks necessary for maturation, genetic alterations at 14q32 in the Ig heavy-chain site frequently occur. These lead to errors in switch recombination or somatic hypermutation, resulting in secondary Ig translocations which aid in MM progression (Kuehl and Bergsagel, 2002).

Another late stage progression event that occurs is the translocation of the prominent oncogene c-MYC, resulting in an enhanced proliferation of the tumor. Aberrant methylation of tumor suppressor genes like p16, SHP1, and E-cadherin might also be involved in the progression of monoclonal gammopathy of undetermined significance (MGUS), the pre-malignant lesion of MM, to full-fledged multiple myeloma (Chim *et al*., 2007). Although MGUS is now easily diagnosed by a simple blood test, the prevention of progression to malignancy, or even prediction of when the tumor turns malignant, is still not possible with current medical technology.

1.3 BIOLOGY OF MULTIPLE MYELOMA

The bone marrow microenvironment, consisting of extracellular matrix (ECM) proteins, bone marrow stromal cells (BMSC), vascular endothelial cells, osteoclasts, and lymphocytes, is believed to play an important role in the homing, proliferation and terminal differentiation of myeloma cells (Kibler *et al.*, 1998). It is this direct physical interaction of the MM cells with the BMSCs, within the bone marrow microenvironment, depicted in Figure 2, that leads to the activation of various signaling pathways, and the secretion of numerous cytokines and growth factors.

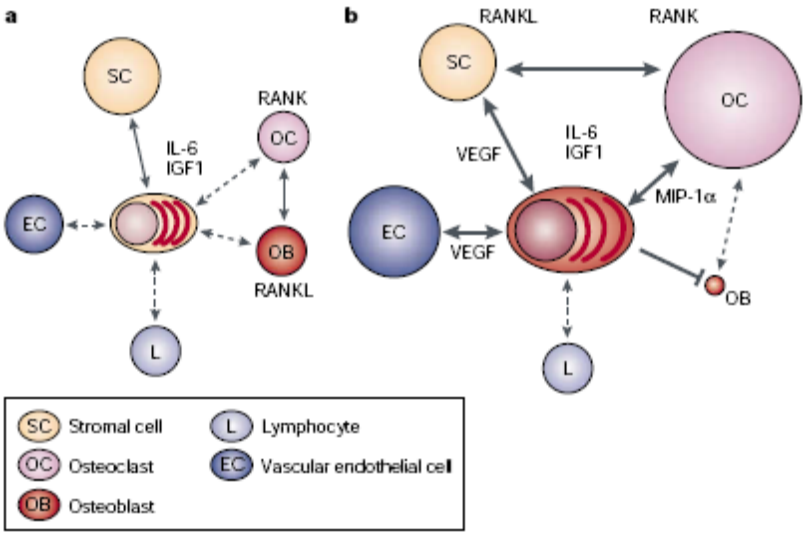

Figure 2. The bone marrow microenvironment in multiple myeloma.
Solid arrows reflect well-defined interactions while dashed lines reflect poorly defined interactions. **a)** A normal plasma cell. **b)** A multiple myeloma tumor cell and its interactions with five types of BMSCs. The sizes of the circles reflect apparent relative changes in the number and/or activity of the BMSCs. Diagram adapted from Kuehl and Bergsagel (2002).

Transforming growth factor-beta (TGF-β) is one of these cytokines, and is observed in high levels in multiple myeloma patients. This in turn induces interleukin-6 (IL-6) secretion, a pivotal MM growth factor (Krytsonis *et al.*, 1998; Cook *et al.*, 1999; Hayashi *et al.*, 2004). Various studies have consistently demonstrated that stimulation of IL-6 dependent signaling pathways, by oncogenic mutations and the bone marrow microenvironment, not only protect MM cells from apoptosis induced by different stimuli (Bommert *et al.*, 2006; Barille *et al.*, 2000), but also markedly increased the spontaneous proliferation in some MM lines by as much as 151% (Kovacs, 2006).

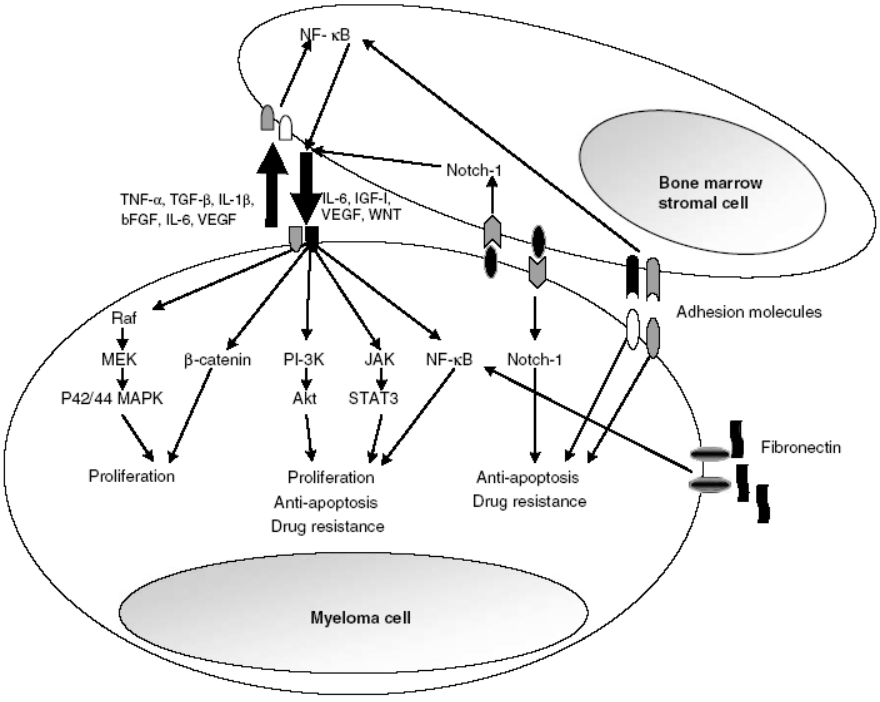

Figure 3. Essential cytokines in the proliferation and survival of MM cells. Diagram adapted from Van De Donk *et al.* (2005).

Other cytokines that are upregulated upon the binding of the myeloma cells to BMSCs include IL-8, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-1), tumor necrosis factor α, and stroma-derived factor-1. These cytokines are thought to aid in proliferation, angiogenesis, drug resistances, upregulation of adhesion molecules, and the induction of an immunocompromised status (Shapiro *et al.*, 2001; Sirohi and Powles, 2004; Pellegrino *et al.*, 2005), but most importantly, they also induce IL-6 production from BMSCs, establishing a potent autocrine feedback loop that promotes tumor progression in the bone marrow (Van De Donk *et al*., 2005), as depicted in Figure 3.

While interaction with the ECM may be the reason why plasma cells are specifically retained in the bone marrow, it is the actions of adhesion molecules, like H-CAM, VLA-4, ICAM-1, N-CAM, and LFA-3, which mediate the homing of myeloma cells as well as adhesion to bone marrow stromal cells (Teoh and Anderson, 1997; Cook *et al*, 1997). These adhesion molecules also play an important role in the regulation of MM cell growth and survival within the bone marrow microenvironment, tumor cell egress form the bone marrow with the development of plasma cell leukemia (PCL), and lastly, metastatic seeding at extramedullary sites (Urashima *et al*, 1997).

1.4 DIAGNOSIS AND STAGING OF MULTIPLE MYELOMA

Patients who present with unexplained anemia, kidney dysfunction, high erythrocyte sedimentation rate (ESR) and serum protein, are usually asked to undergo blood and urine protein electrophoresis, so as to allow detection of the presence of Bence Jones protein, a urinary paraportein composed of free light chains. As this paraprotein is an abnormal immunoglobulin produced by the tumor, quantitative measurements are required to establish a diagnosis and in disease monitoring, although in very rare cases, the myeloma may be of a non-secretory nature (Kyle and Rajkumar, 2009).

Once a preliminary diagnosis of multiple myeloma has been arrived at, additional workup tests usually follow, such as, radiological skeletal bone surveys whereby a series of X-rays of the skull, axial skeleton, and proximal long bones are taken. Myeloma activity may manifest as lytic lesions, where resorption of local bone mass occurs, or punched-out lesions on the skull. A more sensitive alternative to the simple X-ray, is magnetic resonance imaging (MRI), which may supersede the skeletal survey. A bone marrow biopsy is also usually performed in order to estimate the percentage of bone marrow occupied by plasma cells (Kyle and Rajkumar, 2009).

Lastly, immunohistochemistry and cytogenetic analysis may also be undertaken, to detect myeloma cells which are typically $CD19^-$, $CD38^+$, $CD45^-$, $CD56^+$, $CD138^+$, as well as, to provide prognostic information. A standardized diagnostic criteria, as detailed in Table 1, was created in 2003 by the International Myeloma Working Group, for symptomatic myeloma, asymptomatic myeloma and MGUS, as well as other related conditions (Kyle and Rajkumar, 2009).

Table 1. Diagnostic criteria for multiple myeloma

	Symptomatic myeloma	Asymptomatic myeloma	MGUS
Serum paraprotein	Present	> 30 g/L	< 30 g/L
Clonal plasma cells (on bone marrow biopsy)	> 10%	> 10%	< 10%
Evidence of end-organ damage	1. Hypercalcemia (corrected calcium >2.75 mmol/L) 2. Renal insufficiency (attributable to myeloma) 3. Anemia (haemoglobin <10 g/dL) 4. Bone lesions (lytic lesions, or osteoporosis) 5. Frequent severe infections (>2 a year) 6. Amyloidosis 7. Hyperviscosity syndrome	No myeloma-related organ or tissue impairment	No myeloma-related organ or tissue impairment

Traditionally, MM patients have been staged according to the Durie-Salmon system, and although, some doctors still use this system, newer diagnostic methods are rendering it obsolete. Published by the International Myeloma Working Group in 2005, the International Staging System (ISS) for multiple myeloma relies mainly on the detection of levels of albumin and beta-2-microglobulin in the blood (Greipp *et al*., 2005). As detailed in Table 2, this system divides cases of myeloma based only on these levels.

Table 2. International Staging System for multiple myeloma

	Stage I	**Stage II***	**Stage II***	**Stage III**
Serum beta-2 microglobulin	< 3.5 mg/L	< 3.5 mg/L	3.5 -5.5 mg/L	> 5.5 mg/L
Albumin	> 3.5 g/L	< 3.5 g/L	-	-
Median survival	62 months	44 months	44 months	29 months

*Not stage I or II

1.5 STRUCTURE AND EXPRESSION OF HUMAN CD137

CD137 (also known as 4-1BB, *induced by lymphocyte activation*) is a cytokine receptor, that belongs to the tumor necrosis factor receptor (TNFR) superfamily. It was first identified in the murine system, in 1989, by screening of concanavalin

A-activated T cells (Kwon & Weissman, 1989). Isolation of its human homologue, from activated human T lymphocytes, was subsequently accomplished in 1993 (Schwarz *et al*., 1993).

The gene encoding human CD137 is located on chromosome band 1p36, alongside four other members of the TNFR superfamily; CD30, OX40, TNFR-2 and TRAMP/Apo3. The CD137 protein has a calculated molecular mass of 27 kDa, and consists of 255 amino acids (aa). The first 17 aa form a signal peptide, while the subsequent 169 aa comprise the extracellular domain. The transmembrane and cytoplasmic domains, which is essential for cellular signal transduction, are composed of the next 27 aa and last 42 aa, respectively (Schwarz *et al.*, 1997). CD137 exists either as a type I transmembrane glycoprotein with three cysteine-rich motifs in the extracellular domain (Mallett and Barclay, 1991; Schwarz *et al.*, 1993), or as a soluble protein, that is produced from a mRNA splice variant (Setareh *et al.*, 1995; Michel *et al.*, 1998).

CD137 is present on the surface of primary T lymphocytes, where its expression is strictly activation dependent (Schwarz *et al*., 1995). Dendritic cells (DC), natural killer (NK) cells, monocytes, and follicular dendritic cells (FDC) in germinal centres, are other examples of immune cells that also express CD137 (DeBenedette *et al*., 1995; Melero *et al*., 1998; Schwarz *et al*., 1995; Wilcox *et al*., 2002; Heinisch *et al*, 2000; Heinisch *et al*., 2001; Pauly *et al*., 2002). CD137

may also be expressed at sites of inflammation, by vascular endothelial cells lining the walls of blood vessels (Drenkard *et al.,* 2007), as well as, by some malignant tumors, in particular, Reed-Sternburg cells in Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), osteosarcoma, rhabdomyosarcoma, and pancreatic cancer (Broll *et al*., 2001; Lisignoli *et al*., 1998; Ringel *et al*., 2001; personal communication, Schwarz H).

1.6 CO-STIMULATORY SIGNALLING EFFECTS OF CD137

CD137 delivers potent co-stimulatory signals to activated T lymphocytes, acting as an important survival factor, by maintaining cellular division and facilitating differentiation into effector and memory cells (Kim *et al*., 1998; Takahashi *et al*., 1999; Hurtado *et al*., 1997). Once the initial activating signals are received through the T cell receptor (TCR) and CD28, it is believed that CD137 is upregulated, and subsequently interacts with APC-expressed CD137L. This in turn causes a signal to be transduced into the APC to enhance pro-inflammatory cytokine secretion, thereby providing additional co-stimulatory signals to the T cells; enhancing both clonal proliferation and survival in $CD8^+$ T cells, and the latter in $CD4^+$ T cells (Langstein *et al*., 1998; Lane *et al*., 1999; Langstein *et al*., 1999; Cheuk *et al*., 2004).

Tumor necrosis factor receptor-associated factor (TRAF) 2 is an essential component and link to the various downstream signaling pathways of CD137, with the modulation of TRAF 1 (Jang *et al*., 1998). Activation of TRAF 2 by CD137 leads to its trimerization, which in turn activates the mitogen activated protein kinases (MAPKs). As detailed in Figure 4, the initial CD137 signal eventually results in the activation of jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and the p38 MAPK pathways (Dempsey *et al*., 2003). Although the exact pathway linking the initial CD137 signal and nuclear factor-κB (NF-κB) is unclear, NF-κB inducing kinase (NIK) has been proposed to play a pivotal role in the activation of NF-κB. As a result, the expression of the anti-apoptotic proteins Bcl-XL and Bfl-1 are upregulated, and together with a signal from the TCR, CD137 is thus able to aid in the prevention of activation induced cell death (AICD), and costimulate interleukin (IL)-2 production respectively (Lee *et al*., 2002).

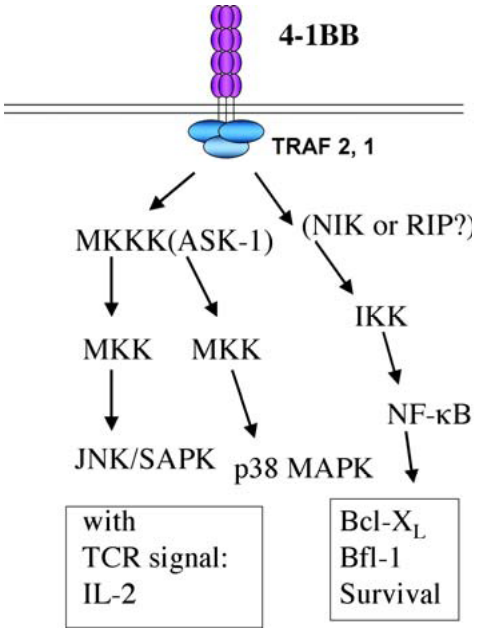

Figure 4. CD137 (4-1BB) signaling pathways. Diagram adapted from Watts *et al* (2005).

1.7 STRUCTURE AND EXPRESSION OF HUMAN CD137 LIGAND

CD137 ligand (CD137L, or 4-1BBL) is a type II transmembrane glycoprotein that belongs to the TNF superfamily, and is presumed to exist as a homotrimer (Smith and Baglioni, 1987; Rabu *et al*., 2006). The gene encoding human CD137L has been mapped to chromosome 19p13.3, with the protein itself comprising 254 aa (Alderson *et al*., 1994).

CD137L is constitutively expressed by antigen presenting cells (APC), including

mature dendritic cells, monocytes and macrophages (Salih *et al*., 2000; Jung *et al*., 2004; Palma *et al*., 2004; Laderach *et al*., 2003; Lee *et al*., 2003). While some B cell lines also express CD137L constitutively, primary B cells only do so upon activation (Zhou *et al*., 1995; Palma *et al*., 2004). CD137L expression has also been observed in activated T cells, and some human carcinoma cell lines, notably, colonic, lung, breast, ovarian, and prostate (Laderach *et al*., 2003; Lee *et al*., 2003; Schwarz, 2005).

1.8 BIDIRECTIONAL SIGNALLING OF THE CD137:CD137L SYSTEM

Like many other members of the TNFR superfamily, the CD137:CD137L system is capable of bidirectional signal transduction; the ability to transduce signals through both the receptor and its corresponding ligand (Schwarz, 2005). Reverse signaling, which specifically refers to signal transduction through the ligand, is possible as most TNF family members are expressed as membrane proteins with cytoplasmic domains (Lotz *et al.,* 1996; Eissner *et al.,* 2004). It is this bidirectional signaling capability that allows for extensive cross-talk to be mediated by the CD137:CD137L system, not only between many leukocyte subpopulations, but also between immune, and non-immune cells. Thus, in functional terms, these molecules would be described more aptly as co-receptors as opposed to their historical designation as ligands (Schwarz, 2005).

CD137L signal transduction into DCs is purported to enhance antigen presentation, by upregulating CD11c, CD80, CD86, and major histocompatibility complex (MHC) class II, as well as by increasing the production of IL-6 and IL-12. (Kim *et al.*, 2002; Futagawa *et al.*, 2002; Laderach *et al.*, 2003; Lippert *et al*., 2008). As a result, the immune response mounted by DCs after CD137L crosslinking is enhanced. In the case of monocytes, CD137L signaling enhances proliferation and endomitosis, due to increased monocyte colony stimulating factor (M-CSF) secretion. Reverse signaling into monocytes also promotes adherence and secretion of proinflammatory cytokines such as TNF, IL-6, IL-8, and IL-12 (Langstein *et al*., 1998; Langstein *et al*., 1999; Langstein and Schwarz, 1999).

While the effects of CD137L signaling on APCs are costimulatory in nature, in T lymphocytes, the same reverse signaling is inhibitory; proliferation is reduced and apoptosis is increased (Schwarz *et al*., 1996). As CD137L expression on T cells is strictly activation-dependent, it has been postulated that the physiological function of this protein may be to down-regulate T cells when they are no longer required (Goodwin *et al*., 1993). Detailed in Figure 5 are some of the diverse effects of the CD137:CD137L system.

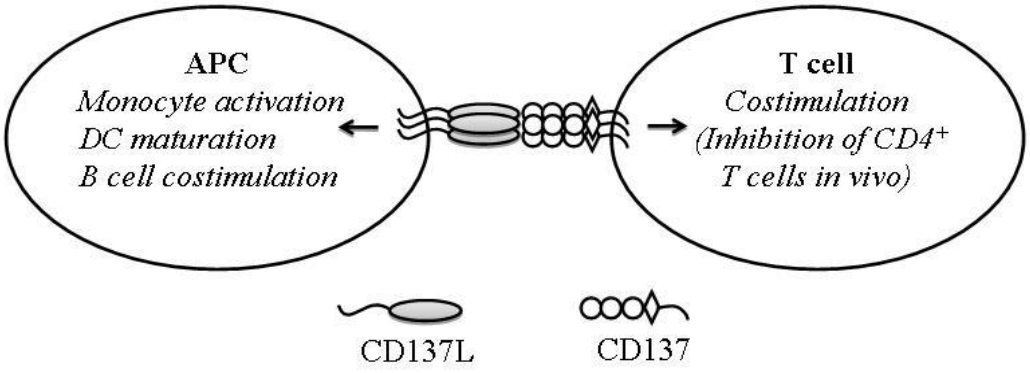

Figure 5. Bidirectional and reverse signaling of the CD137:CD137L system. Reverse signaling into APCs is activating in nature, while CD137L signal transduction into T cells induces apoptosis. Conversely, signaling via CD137 into T cells results in costimulation. Diagram adapted from Thum *et al.* (2008).

Upon the cross-linking of the constitutively expressed CD137L on B lymphocytes by FDC-expressed CD137, B cell proliferation as well as Ig synthesis are enhanced (Pauly *et al.*, 2002; Lindstedt *et al.*, 2003). Apart from delivering growth and survival signals to the B cells via ICAM-1 mediated cell contact, FDCs also present antigens in the form of iccosomes to the B cells and play an essential role in the clonal selection of B cells with high-affinity B cell receptors (Pauly *et al.*, 2002). Due to similarities to the CD40:CD40L system, which mediates T cell help to B cells after the first antigen encounter, the CD137:CD137L system may mediate co-stimulation of B lymphocytes during affinity maturation (Schwarz, 2005).

1.9 CD137/CD137L IN TUMOR IMMUNOTHERAPY

As cell-mediated responses are essential for the elimination of cancer cells by the immune system, the immune evasion exhibited by tumors is often times frustrating. This immune evasion is generally accomplished by one or more of the following means: down-regulation of MHC I molecules, suppression of immune inhibitory molecule expression, and the total absence of recognizable tumor antigens (Cheuk *et al.,* 2004). In order to circumvent these obstacles, numerous alternative strategies involving the activation of T cells, using co-stimulatory molecules of the B7:CD28 signaling pathway, have been developed. Since the effects of CD137:CD137L signal transduction are assumed to co-stimulate $CD8^+$ T lymphocytes, thereby up-regulating the targeting of tumor cells, an increasing number of studies are starting to focus on the CD137:CD137L system as a viable candidate for anti-cancer immunotherapy.

In the earliest murine models used, the eradication of established sarcoma and mastocytoma tumors following the direct injection of anti-CD137 monoclonal antibodies (mAbs) was observed (Melero *et al*., 1997). Equally encouraging are the many successes observed in numerous other murine models, and one of the latest phase I clinical trial of a humanized anti-CD137 mAb, even if most *in vivo* triumphs have not translated well into human clinical trials (McNamara *et al*., 2008; Son *et al*., 2008). Anti-CD137 mAbs have also seen usage in combination

with mAbs against CD40 and TNF-related apoptosis inducing ligand (TRAIL), as well as, with engineered drug-resistant haematopoietic cells (Uno *et al.,* 2006; McMillin *et al.,* 2006).

Apart from the direct injection of anti-CD137 mAbs, another approach that has been investigated is the adoptive transfer of T cells. In this technique, T lymphocytes were first costimulated *ex vivo* through the CD137 signaling pathway in conjunction with various other costimulatory molecules, and then adoptively transferred into mice. In the melanoma model used, a 60% cure rate was achieved, while in the fibrosarcoma model, survival was significantly prolonged (Strome *et al*., 2000).

Various groups have also worked on developing whole cell vaccines against many different murine cancer models. Specifically, three main methods have been adapted for use. Firstly, cell lines were transduced with the CD137L gene and then injected into mice, resulting in the development of long term immunity against the wild-type tumor (Guinn *et al*., 1999; Guinn *et al.,* 2001). Another approach involved the co-transfection of primary DCs with human CD137L and the tumor associated antigen (TAA) HER-2/neu, and using them as APCs in order to generate HER-2/neu-specific cytotoxic T lymphocytes (CTLs) (Grunebach *et al.,* 2005). Lastly, single chains of Fv fragments of an anti-CD137 mAb gene

were transduced into the tumor cells, resulting in the vaccinated mice rejecting the established wild-type tumor (Ye *et al*., 2002; Yang *et al*., 2007).

Stragery	Cancer type	Comments	Outcome
Adoptive transfer of *ex vivo* costimulated T cell	Melanoma A9P	T cells from tumor bearing mice were stimulated *in vitro* through CD28 and 4-1BB pathway and adaptively transferred into mice bearing A9P tumor	60% cure rate was achieved
	Fibrosarcoma MCA 205	T cells from tumur-draining lymph nodes were costimulated *ex vivo* using α-CD3, α-CD28 and α-4-1LL Ab, and transferred into syngeneic mice bearing MCA205 tumor	Pulmonary metastases significantly reduced, survival was prolonged
Therapy using α-4-1BB Ab	Sarcoma Ag104A	α-4-1BB Ab was injected i.p.	Large established tumor was eradicated
	Mastocytoma P815	α-4-1BB Ab was injected i.p.	Large established tumor was eradicated
	Fibrosarcoma MCA 205	α-4-1BB Ab was injected i.p.	Four and two of groups of five mice were cured
	Glioma GL261	α-4-1BB Ab was injected i.p.	Prolongation of survival and cure of disease in two of five mice
	Metastatic colon carcinoma	IL-12 plus α-4-1BBL Ab were used	Animal bearing simultaneous hepatic and multiple pulmonary meastases were quantitatively cured
	Breast cancer JC	Local 4-1BBL gene delievery in combination with IL-12	It was able to eradicate established tumour with a survival rate 78%
Whole cell vaccine	Mastocytoma P815	P815 cells were transduced with 4-1BBL gene and injected into mice	Developed long-term immunity against wild-type tumor
	Sarcoma Ag104A	Ag104A cells were transduced with 4-1BBL gene and then injected into mouse	Developed long-term immunity against wild-type tumor
	B-cell lymphoma A20	Mice injected with 4-1BBL expressing A20	No tumors were formed for the 150 days follow-up period
	Squamous cell carcinoma NRS1	4-1BBL cDNA was first induced into NRS1 cells	Syngeneic mice acquired specific immunity against wild-type tumor
	T-cell lymphoma EL4	4-1BBL was transfected into EL4 cells, cells were then injected into mice	It was able to induce tumor regression
	Colon cancer MCA26	MCA26 cells was first injected into the liver. Adenoviral vector was used to transduce IL-12 and 4-1BBL DNA into the established liver tumor	The survival were improved than the control group
	Liver metastases of breast cancer	Intratumor adenoviral mediated gene transfer of the 4-1BBL	Survival rate was 72%
	Melanoma K1735	Single chain of Fv fragments of a α-4-BB mAB gene was transduced into the tumor cells	Vaccinated mice rejected established wild-type tumor

Figure 6. Summary of CD137/CD137L in murine models of tumor immunotherapy. Diagram adapted from Cheuk *et al*. (2004).

1.10 MULTIPLE MYELOMA AND THE CD137:CD137L SYSTEM

Thus far, no death domains, such as in CD95, death receptor (DR)4, and DR5, have been observed in the cytoplasmic regions of CD137 or CD137L, or even in the TNF/TNFR superfamily groups that CD137:CD137L system is classified together with (Croft, 2003). Hence, the use of CD137 or CD137L has traditionally been limited to the augmentation of the immune system, and not in the direct eradication of tumors. That is slowly changing, with reports emerging that present findings of apoptosis in resting primary T and B cells, and in anti-CD3 stimulated lymphocytes, by the direct action of the CD137:CD137L signal transduction pathway (Michel *et al.,* 1998: Schwarz *et al*., 1996).

In B cells however, current findings paint a vastly different picture; cross-linking of B cell-expressed CD137L results in activation and an enhanced survivability, while CD137 on FDCs has been observed in contributing to an increased rate of survival of some B cell lymphomas (Park *et al*., 2004). Hence, it was assumed that the CD137L signal would also contribute to the enhanced and uncontrolled growth of MM cells, a B cell malignancy. Unexpectedly, preliminary results actually showed that the CD137L signal transduction resulted in an inhibition of proliferation, and even induced cell death, in the MM cell lines tested. If true, this data suggests that the cross-linking of CD137L on MM cells might represent a novel method to

specifically target MM cells for destruction.

1.11 MULTIPLE MYELOMA AND FOLLICULAR DENDRITIC CELLS

The majority of B cell lymphomas originate from the germinal centre (Kuppers *et al*., 1999), which is also where follicular dendritic cells (FDCs) are located. These germinal centre stromal cells are able to contribute to lymphoma generation by preventing apoptosis as well as by promoting the proliferation of transformed B cells. Tumorigenesis of these B cells can occur via selection for additional genetic changes or through adaptation to the protumorigenic environment provided by the FDCs; it is also probable that the FDCs provide the growth factors required for the metastasis of these cancer cells (Park and Choi, 2005).

It has also been shown conclusively, via immunohistochemistry, that FDCs in the germinal center express CD137 strongly (Pauly *et al*., 2002). As previously mentioned, CD137:CD137L reverse signaling promotes proliferation in B lymphocytes and B cell lymphomas. Thus, the presence of CD137 on the FDCs might also indicate a similar proliferation inducing function for B lymphocytes. On this basis, it would appear that any interaction of B cell lymphoma cells, including MM, with CD137 expressing FDCs

should logically induce growth and survival. However, in direct contradiction to these facts, are the preliminary results showing that the effects of CD137:CD137L interaction in MM cells are in fact inhibitory in nature.

In light of the above, it would be interesting to investigate the mechanisms behind this unforeseen occurrence. By isolating and immortalizing FDCs, if the same apoptotic effect is seen in the MM cell lines *ex vivo*, it might point to the presence of other factors, or perhaps even a synergistic effect only present in the germinal center microenvironment.

1.12 OBJECTIVES OF STUDY

The specific objectives of this study are:

a. to investigate the expression of CD137/CD137L on multiple myeloma cells
b. to characterize the inhibitory effects of CD137 on multiple myeloma cells in detail
c. to investigate the different effects of CD137 on multiple myeloma cells compared with other non-MM B cell lymphomas
d. to develop an effective method of delivery for *in vivo* CD137 anti-MM therapy
e. to generate a stable follicular dendritic cell line, followed by characterization of the CD137:CD137L signaling effects between FDCs and MM cells

2. MATERIALS AND METHODS

All materials were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated.

2.1 CELL LINES

The RPMI-8226 MM cell line, Raji, a Burkitt's lymphoma cell line, and Sp2/mIL-6, a transfected murine B cell line secreting murine IL-6, were obtained from ATCC (Manassas, VA, USA). The diffuse large B cell lymphoma lines, DOHH-2 and SUDHL-4, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). These five cell lines were routinely cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS), henceforth referred to as RPMI-10 (refer to Appendix I).

The newly established EBV-negative SGH-MM5 and SGH-MM6 human MM cell lines (CD10+, CD19-, CD20-, CD38+, CD40+, CD45+, CD56+, CD138+) were a generous gift from Dr Charles Gullo (Department of Clinical Research, Singapore General Hospital). The patient-derived MM cells were maintained in IMDM with 10% heat-inactivated FBS, henceforth referred to as IMDM-10 (refer to Appendix I).

MM5-HS, the original SGH-MM5 cell line rendered HAT-sensitive by chronic administration of 8-azaguanine, was a gift from Dr Paul MacAry (Department of Microbiology, National University of Singapore). Similar to the original SGH cell lines, this HAT-sensitive cell line was also maintained in IMDM-10, with the addition of 5 μM 8-azaguanine.

All of the above mentioned cell lines were passaged every 2-3 days and kept in a humidified incubator at 37^{o}C with 5% CO_2.

2.2 RECOMBINANT PROTEINS AND ANTIBODIES

The recombinant human CD137-Fc protein was purified from supernatants of stably transfected CHO cells by protein G sepharose, as described previously (Schwarz *et al*, 1996). Human IgG1 Fc fragment was purchased from Chemicon (Temecula, CA, USA).

Table 3. List of antibodies used

Antibody	Clone	Source
Mouse IgG1 κ isotype (PE-conjugated)	MOPC-21	Sigma-Aldrich (St Louis, MO, USA)
Mouse anti-human CD137 (PE-conjugated)	4B4-1	BD Pharmingen (Franklin Lakes, NJ, USA)
Mouse anti-human 4-1BB ligand (unlabelled)	5F4	Biolegend (San Diego, CA, USA)
Mouse anti-human 4-1BB ligand (unlabelled)	41B436	Alexis Biochemicals (Switzerland)
Mouse IgG1 κ isotype (unlabelled)	MOPC-21	Sigma-Aldrich (St Louis, MO, USA)
Goat anti-mouse IgG (PE-conjugated)	-	Sigma-Aldrich (St Louis, MO, USA)
Donkey anti-mouse IgG (PE-conjugated)	-	eBioscience (San Diego, CA, USA)
Mouse anti-human IgG (Fc-specific)	GG-7	Sigma-Aldrich (St Louis, MO, USA)
Mouse anti-human CD3 (FITC-conjugated)	UCHT1	Biolegend (San Diego, CA, USA)
Mouse anti-human CD14 (PE-conjugated)	61D3	eBioscience (San Diego, CA, USA)
Mouse anti-human CD31 (FITC-conjugated)	WM59	eBioscience (San Diego, CA, USA)
Mouse anti-human CD303 (PE-conjugated)	AC144	Miltenyi Biotec (Germany)
Mouse anti-human FDC (unlabelled)	Ki-M4	Abcam (Cambridge, MA, USA)

2.3 FLOW CYTOMETRIC ANALYSIS

Aliquots of cultured cells (2-3 x 10^5) were stained with respective fluorochrome conjugated antibodies in phosphate buffered saline (PBS) containing 0.5% BSA and 0.1% sodium azide, hence forth referred to as FACS buffer (refer to Appendix II), for 1 h at 4^{o}C in the dark. Cells were then washed twice with FACS buffer and resuspended in 400 µl FACS buffer. Flow cytometry was performed on a FACSort (Becton Dickinson, San Jose, CA) with CellQuest (Becton Disckinson) data acquisition and analysis software. Non-specific staining was controlled by isotype-matched antibodies.

2.4 COATING OF CD137-Fc AND Fc PROTEIN

Recombinant human CD137 protein was prepared as a fusion protein tagged with the Fc portion of human IgG1 molecule. Human IgG Fc protein and PBS were used as controls in all experiments.

2.4.1 Coating on Plates

96-well and 24-well plates (Nunc, Rosklide, Denmark) were coated with 50 µl and 500 µl, respectively, of CD137-Fc or Fc protein diluted in PBS to a final concentration of 10 µg/ml per well. The plates were then incubated at 4^{o}C overnight, and the wells washed with PBS before addition of cells.

2.4.2 Coating on Beads

SPHERE^{TM} Protein G-coated polystyrene particles (Spherotech, Lake Forest, IL) were incubated at 4^{o}C overnight with 1.5 μg of CD137-Fc or Fc protein per 10 μl of beads. The beads were then washed twice with PBS before being plated into round bottom 96-well plates together with the cells. Plate-coated protein was used concurrently, and PBS-treated beads as the background control.

2.4.3 Multimerization via Anti-Human Fc Antibody

96-well plates were first coated with 50 μl of 10 μg/ml of anti-human Fc antibody, clone GG-7, at 4^{o}C overnight, before washing with PBS, and blocking with 100 μl FBS at 37^{o}C for 1 h. 50 μl of 10 μg/ml of CD137-Fc or Fc protein were then added to the wells and incubated for a further 1 h at 37^{o}C, before the addition of the cells.

2.5 DEATH AND APOPTOSIS ASSAYS

Live and dead cell counts were performed with a haemocytometer after staining with trypan blue. Apoptotic and necrotic cells were stained by 10 μg/ml ethidium bromide (EB) and 3 μg/ml acridine orange (AO) at a ratio of 1 μl AO/EB solution per 10^6 cells, and viewed under the IX81 microscope (Olympus, USA).

Annexin-V externalization was detected using the Annexin-V Apoptosis Detection Kit (BD Pharmingen, USA) according to the manufacturer's protocols. In brief, cells were stained with Annexin-V and 7-aminoactinomycin D (7-AAD) and left to incubate in the dark. Apoptotic analysis was then accomplished via flow cytometry and Summit 4.2 software (CyAN^{TM}, DakoCytomation, Denmark).

2.6 PROLIFERATION ASSAYS

Cells were pulsed with 0.5 μCi of [^{3}H]-thymidine (Perkin Elmer, Waltham, MA, USA) for the last 16 h of the culture period. The cells were then harvested into a Packard Unifilter Plate using a MicroMate 196 Cell Harvester (Packard Instruments, Meridien, CT, USA) and dried at 56^{o}C for 1-2 h, after which 20 μl of MicroScint^{TM} solution (Perkin Elmer, Waltham, MA, USA), Radioactivity was measured using the TopCount liquid scintillation analyzer (Packard Instruments, Meridien, CT, USA).

2.7 CELL CYCLE ANALYSIS

Cells were first pelleted, followed by fixation and permeabilization by adding 1.8 ml of cold 70% ethanol drop-wise, with continuous vortexing. Nucleic acid content in the cells were stained with 0.25 μg 7-AAD (BD Pharmingen,

USA) per 10^6 cells, for 10 min before flow analysis. Cell cycle analysis was performed on via flow cytometry (Cyan™, DakoCytomation, Denmark) and analyzed with ModFit LT software (Verity Software House, Topsham, USA).

2.8 SANDWICH ELISA

Levels of IL-6, IL-8, VEGF and TGF-β present in the cell culture supernatants were determined by human DuoSet ELISA Development Kits (R & D Systems, Minneapolis, MN), according to the manufacturer's protocol. All measurements were performed in triplicate.

2.9 ISOLATION OF MM CELLS FROM PATIENT BONE MARROW ASPIRATES

Primary patient cells, which were a generous gift from Dr Chng Wee Joo (Department of Haematology-Oncology, National University Hospital), were derived from bone marrow aspirates taken from patients suffering frommultiple myeloma. CD138+ cells were isolated by using the MACS CD138+ Plasma Cell Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturer's protocols. In brief, CD138-expressing cells were magnetically labelled with a monoclonal anti-human CD138 antibody, and then passed through a magnetic column. The CD138 enriched fraction was then eluted from the column.

2.10 GENERATION OF A STABLE, CD137-EXPRESSING FOLLICULAR DENDRITIC CELL (FDC) LINE

Tonsils were obtained from patients undergoing tonsillectomies courtesy of Dr Lynne Lim (Head Neck Surgery, National University Hospital). Only enlarged tonsils from otherwise healthy patients were utilized; inflamed tonsils were summarily rejected due to the elevated levels of activated T cells, which would also have expressed high levels of CD137.

Two different methods were utilized in order to achieve immortalization of the cell line; transfection with telomerase-expressing vectors, and hybridization with already immortalized cell lines.

2.10.1 Plasmids

The pBABE-neo and pBABE-hTERT retroviral preparations were gifts from Dr Prakash Hande (Department of Physiology, National University of Singapore). Both plasmids contain the ampicillin resistance gene, and are selectable by neomycin. Transfection with pBABE-hTERT enables the expression of catalytic subunit of human telomerase, while pBABE-neo is its empty vector counterpart. Both vectors were amplified in *Escherichia coli* DH5α competent cells (Invitrogen, Carlsbad, CA, USA) before use.

2.10.2 Preparation of Single-Cell Suspension from Whole Tonsil

The whole tonsil was placed in a gentleMACS C Tube (Miltenyi Biotec, Germany) together with 3 ml of PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% BSA, henceforth referred to as MACS Buffer (refer to Appendix I). Additionally, 300 units/ml of Collagenase type IV (Worthington Biochemical, Lakewood, NJ), and 0.004% DNAse I (United States Biological, Swampscott, MA, USA), were included in the mixture. Tissue dissociation was accomplished with the aid of the gentleMACS Dissociator (Miltenyi Biotec, Germany), by running the preset spleen tissue dissociation protocol twice. The resulting cell suspension was filtered through a 30 μm cell strainer (BD Biosciences, San Jose, CA, USA), washed with PBS, and the pelleted cells resuspended in Hepes-free RPMI 1640 media.

2.10.3 Selection of CD137-Expressing Cells

Density gradient centrifugation with Histopaque®-1077, and CD3+ depletion via MACS CD3 MicroBeads, was first performed before selecting CD137+ cells utilizing the MACS CD137 MicroBead Kit (Miltenyi Biotec, Germany) according to the manufacturer's protocols.

In brief, the cell suspension was overlaid on Histopaque®-1077 and centrifuged at 400g for 30 min. The interphase fraction was collected and used for CD3 depletion. CD3+ cells were magnetically labelled with a monoclonal anti-human CD3 antibody and passed through a magnetic column. The flow through was subsequently magnetically labelled with a monoclonal anti-human CD137 antibody and again passed through a magnetic column. The bound cells were eluted, centrifuged at 300g for 10 min, and resuspended in Hepes-free RPMI-10 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen, Carlsbad, CA).

2.10.4 Transfection of CD137-Expressing Cells

Transfection was performed using Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. A DNA: Lipofectamine^{TM} ratio of 1 μg : 1 μl was used for 10^7 cells per ml per 24-well

plate. Cells were transfected either with pBABE-hTERT, or with the empty vector pBABE-neo as control. 48 h post-transfection, selection antibiotics, in the form of 1 mg/ml Geneticin® (Gibco-Invitrogen, Carlsbard, CA, USA), was added and maintained for approximately 14 days, with medium changes every 3-4 days.

2.10.5 Formation of FDC Hybridomas

Two different HAT-sensitive cell lines were chosen for fusion with the CD3-depleted, CD137-enriched tonsil cells: Sp2/mIL-6, as described previously (Harris *et al*, 1992), and MM5-HS.

The CD137-enriched cells were mixed with the respective HAT-sensitive cell lines in a 3:1 ratio, and centrifuged to give a cell pellet. Cells were washed with Hepes-free RPMI 1640 media and centrifuged again. Subsequent steps were maintained at 37^{o}C. The pellet was loosened by flicking the base of the tube. 1 ml of polyethylene glycol 1500 (Roche, Nutley, NJ, USA) was added per 10^8 cells and left to stand for 1 min. A total of 20 ml Hepes-free RPMI 1640 media was then added very slowly, drop-wise and with constant agitation, in the following format: 1 ml in the 1st min, 4 ml in the 2nd min, and 15 ml in the 3rd min. The fused cells are then centrifuged and gently resuspended in Hepes-free RPMI-10 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and HAT Media Supplement.

2.10.6 Selection of CD137-Expressing FDCs

Both transfected cells and fused cells were left to grow at 37^{o}C at 5% atmospheric CO_2 and were selected over two weeks. In both cases, growth and confluency of the cultures indicated either a successful transfection or creation of a hybridoma. In order to sieve out any immortalized non-FDC cells, flow cytometry analysis was performed.

Prospective immortalized FDCs were stained to check for the presence of CD14 (monocyte marker), and CD303 (DC marker), and the absence of CD3 (T cell marker), and CD31 (endothelial cell marker). Additionally, the cells

were sorted by fluorescence-activated cell sorting (FACS) after staining with an anti-human FDC monoclonal antibody, clone Ki-M4, and a PE-conjugated anti-mouse antibody.

2.11 STATISTICAL ANALYSIS

Statistical significance was determined by a two-tailed Student's t-test for both pair-wise comparisons and comparisons across more than two groups.

3. RESULTS

After initial results showed an unexpected inhibitory effect on MM cell growth and survival, preliminary experiments revolving around the characterization of the effects of CD137 on the proliferation and viability of the MM cell lines were planned. To establish a molecular basis for this premise, Section 3.1 details the expression profiles of both CD137 and CD137L on all six of the cell lines used. The following two sections go on to describe the inhibition of MM cell proliferation after treatment with CD137-Fc, as well as CD137-induced cell death. These results suggest an induction of apoptosis in the CD137-treated MM cells. As the data presented in Section 3.4 shows an up-regulation of pro-survival cytokines, experiments in the following section were subsequently planned to substantiate the hypothesis that these survival signals do not prevent CD137-induced apoptosis. Possible alternatives to enable immobilization of the recombinant CD137-Fc protein, so as to allow successful crosslinking of the CD13L, were also looked into, in the eventuality of moving into an *in vivo* test setting.

As FDCs contribute to lymphoma generation by preventing apoptosis, and promoting the proliferation of transformed B cells, an immortalized FDC line would be an invaluable tool in the study of MM cell interactions. Thus, the last section details the generation of a FDC line from primary human tonsillar tissue.

3.1 B CELL LYMPHOMA CELL LINES EXPRESS CD137L BUT NOT CD137

The molecular basis for B cells to receive costimulatory signals from CD137 is the constitutive expression of CD137L by primary B cells (Jung *et al*., 2004; Zhou *et al*., 1995). Therefore, in any examination regarding the effects of CD137 on B cell lymphoma cell lines, the first step would be to determine CD137L expression. For this study, the Burkitt's lymphoma Raji, the non-Hodgkin's lymphoma SUDHL-4, the B cell lymphoma DOHH-2, and the three multiple myeloma cell lines SGH-MM5, SGH-MM6, and RPMI-8226, were selected. All six cell lines express CD137L constitutively, but none of them expresses CD137 at any significant level (Figure 7), a situation identical to that of primary B cells (Jung *et al*., 1995; Zhou *et al*., 2004).

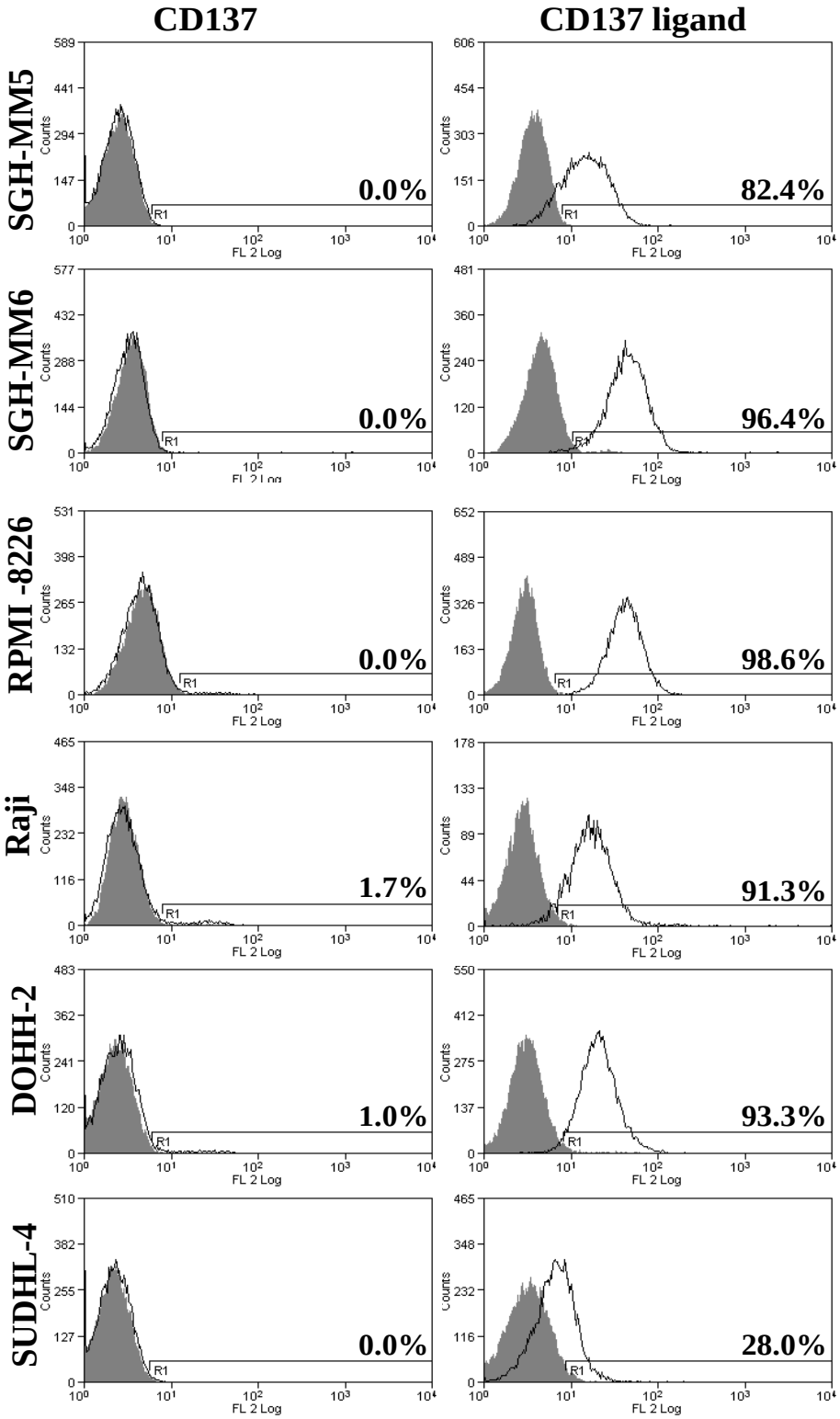

Figure 7. CD137L is expressed by B cell lymphoma cell lines. Cells were stained with PE-conjugated monoclonal antibodies against CD137 (clone 4B4-1), or anti-CD137L (clone 41B436), displayed as open histograms, or their isotype control (clone MOPC-21), displayed as filled histograms.

3.2 CD137 INHIBITS PROLIFERATION OF MM CELLS

The reverse signal transduction activity of CD137 was tested in the B cell lines, as CD137L crosslinking has been shown to enhance the proliferation of preactivated B cells (Pauly *et al*., 2002; Pollock *et al*., 1994). Either monoclonal antibodies specific for CD137L, or a recombinant fusion protein consisting of the extracellular domain of CD137 and the constant domain (Fc) of IgG1 (CD137-Fc), were selected for use as tools to crosslink the CD137L on the cells. As negative controls, isotype-matched unspecific antibodies or a recombinant Fc protein were chosen, respectively. These proteins were immobilized onto tissue culture plates by coating the plates at 4^{o}C overnight, to enable crosslinking of the CD137L on the lymphoma cells.

No significant effect of CD137L stimulation on the proliferation of the Raji, DOHH-2, and SUDHL-4 cells over four days was observed, as assessed by ^{3}H-thymidine incorporation (Figure 8). In contrast, the proliferation of the three MM cell lines SGH-MM5, SGH-MM6, and RPMI-8226, was profoundly decreased by CD137, by as much as three-fold. This inhibitory effect was most visible at the final timepoint of 96 h (Figure 8). The lower count per min (CPM) figures exhibited by the MM cell line RPMI-8226 could be attributed to its lower proliferative index in general, as compared to the other, much faster growing B cell lines.

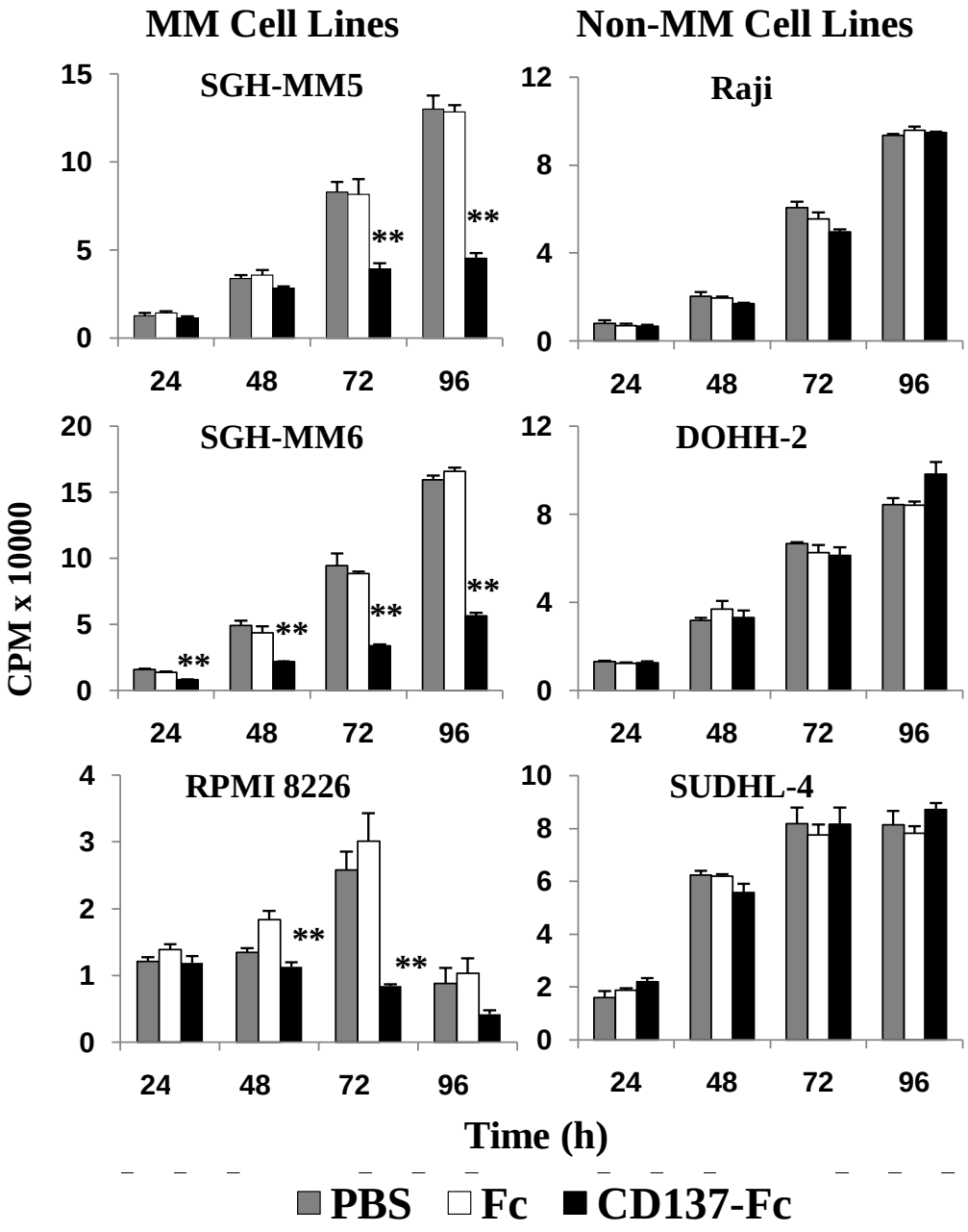

Figure 8. CD137 inhibits proliferation in MM, but not in non-MM cells. Cells were cultured on plates coated with 10 µg/ml of Fc or CD137-Fc protein or on uncoated plates (PBS). After the indicated times, proliferation was determined via ^{3}H-thymidine incorporation. Depicted are means ± standard deviations of triplicate measurements. * $p < 0.05$, ** $p < 0.01$. This experiment is representative of three independent experiments with similar results.

3.3 CD137 INDUCES CELL DEATH IN THE MM CELL LINES BY APOPTOSIS

The next question to be answered, in order to investigate the mechanism behind the inhibition of proliferation, was whether CD137L ligation of MM cells induced cell death. As exhibited clearly in Figure 9, the percentage of dead cells was increased up to two- to three-fold in MM cells after culturing for 72 h on CD137-Fc when compared to both Fc or PBS (Figure 9). Viability of the non-MM B cell lymphoma cell lines was not affected by CD137L signalling.

In order to determine if the reduction in cell viability was due to CD137-Fc induced MM cell apoptosis, Annexin-V and 7-AAD staining was performed. This staining revealed significant increases in the percentages of both early apoptosis (Annexin-V^+, 7-AAD^-), and late apoptosis or necrosis (Annexin-V^+, 7-AAD^+) after 24 h treatment with CD137-Fc (Figure 10). Apoptotic rates of the non-MM cell lines remained unchanged, consistent with the results from the proliferation and viability assays (Figures 8, 9).

Induction of apoptosis was visually correlated by staining the cells with ethidium bromide and acridine orange. Extensive chromatin condensation and membrane blebbing, classical hallmarks of cells undergoing apoptosis, were

exhibited by the MM cells after treatment with CD137-Fc (Figure 11A, B). Cell cycle analysis further confirmed CD137-induced apoptosis by the appearance of a sub-G1 peak. In addition, the cell cycle analysis also revealed some evidence of cell cycle arrest in the S phase (Figure 12).

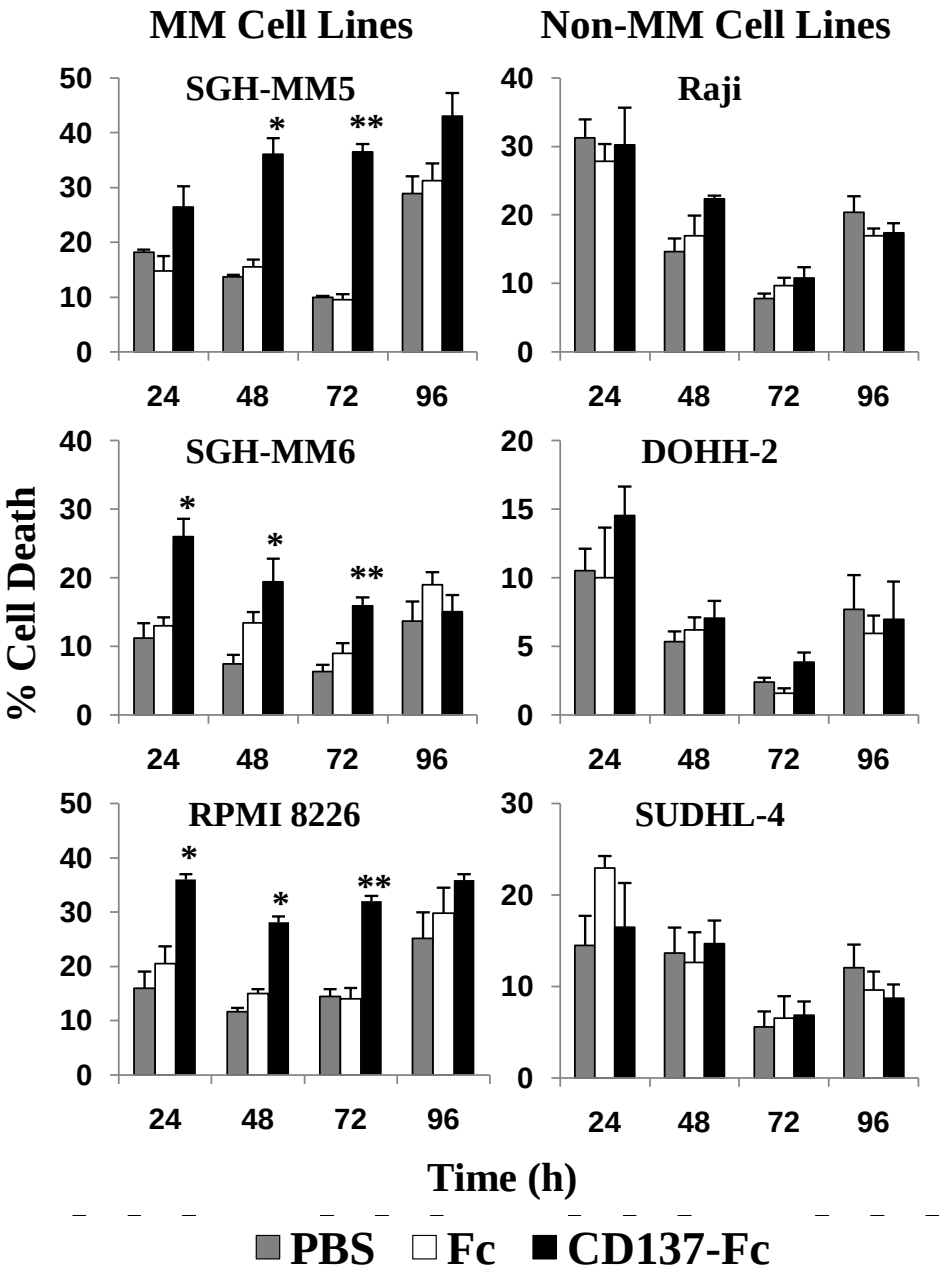

Figure 9. CD137 induces cell death of MM, but not of non-MM cells. Cells were cultured on plates coated with 10 µg/ml of Fc or CD137-Fc protein or on uncoated plates (PBS). Cell viability was determined after 24, 48, 72, and 96 h via trypan blue staining. Depicted are means ± standard deviations of triplicate measurements. * $p < 0.05$, ** $p < 0.01$. This experiment is representative of three independent experiments with similar results.

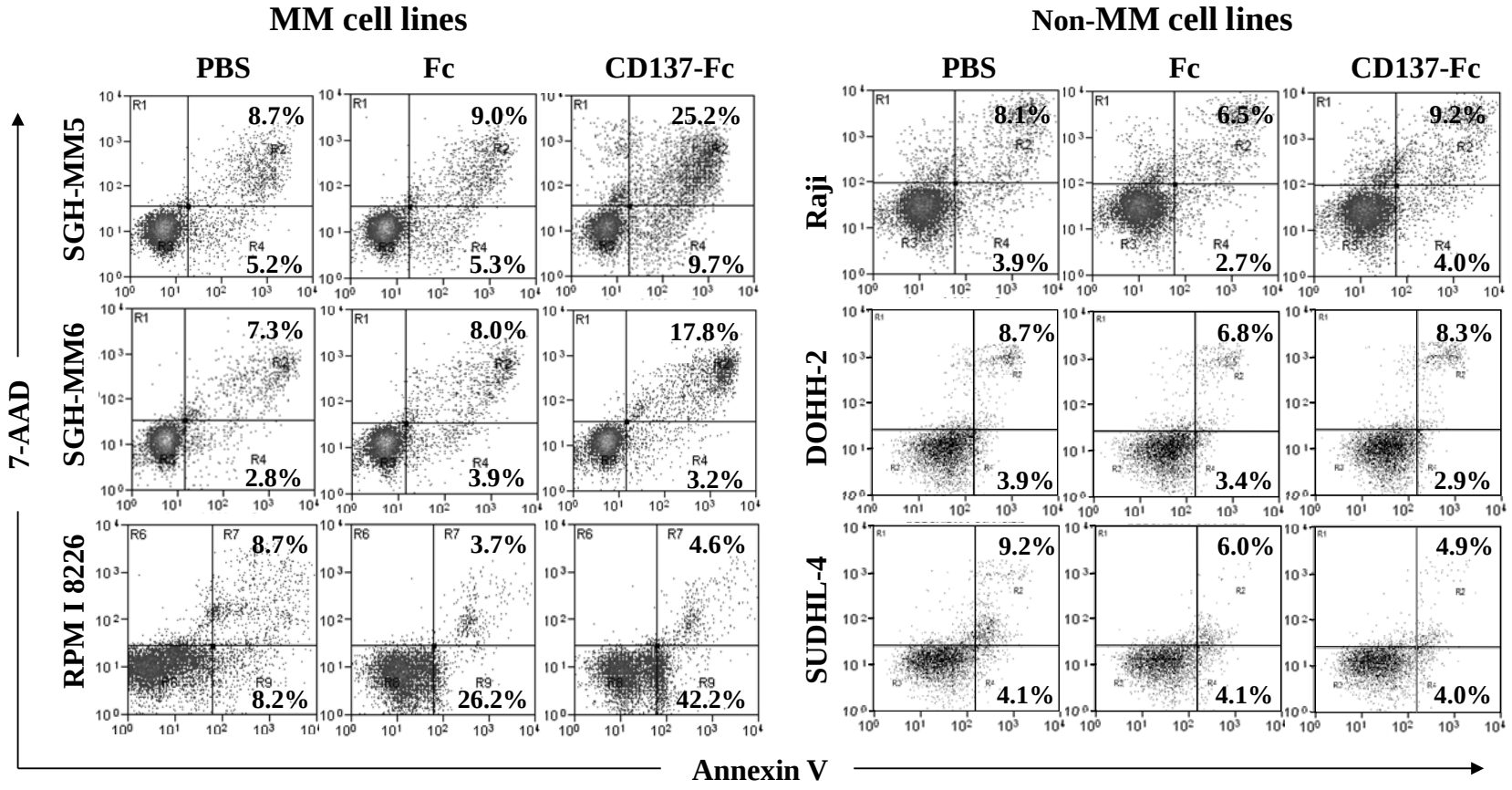

Figure 10. CD137 induces apoptosis in the MM cell lines. SGH-MM5 cells at a density of 10^6 cells/ml were cultured on plates coated with 10 μg/ml of Fc or CD137-Fc protein or on uncoated plates (PBS). After 24 h, the cells were stained with Annexin V and 7-AAD. Similar results were obtained for the other MM cell lines.

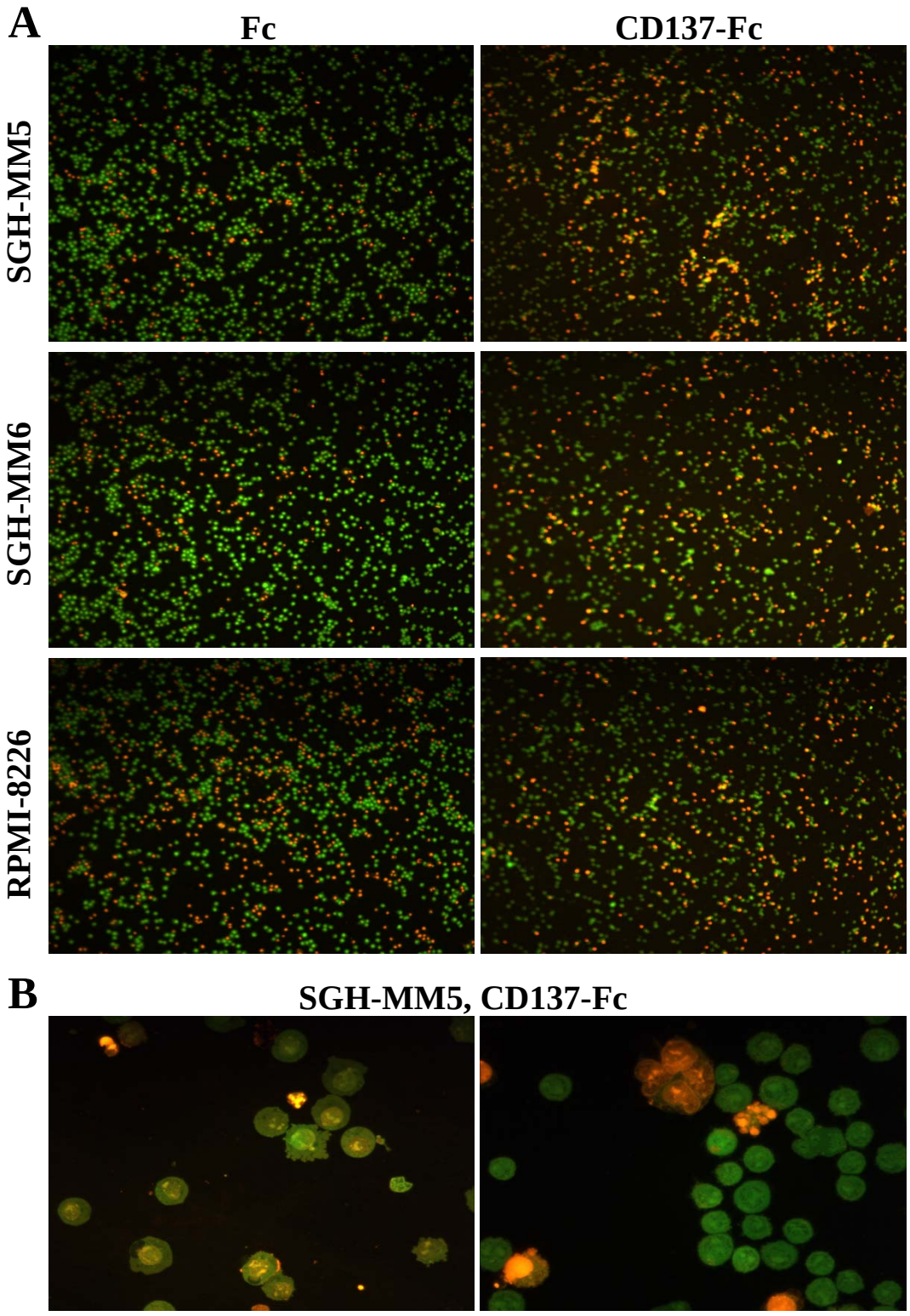

Figure 11. CD137 induces chromatin condensation and membrane blebbing in MM cells. SGH-MM5 cells at a density of 10^6 cells/ml were cultured on plates coated with 10 μg/ml of Fc or CD137-Fc protein or on uncoated plates (PBS). (A) Cells were stained with Acridine Orange (green) and Ethidium Bromide (red). Photographs were taken at a magnification of 40x. (B) CD137-Fc treated SGH-MM5 cells of B at a magnification of 200x. This experiment was performed three times with similar results.

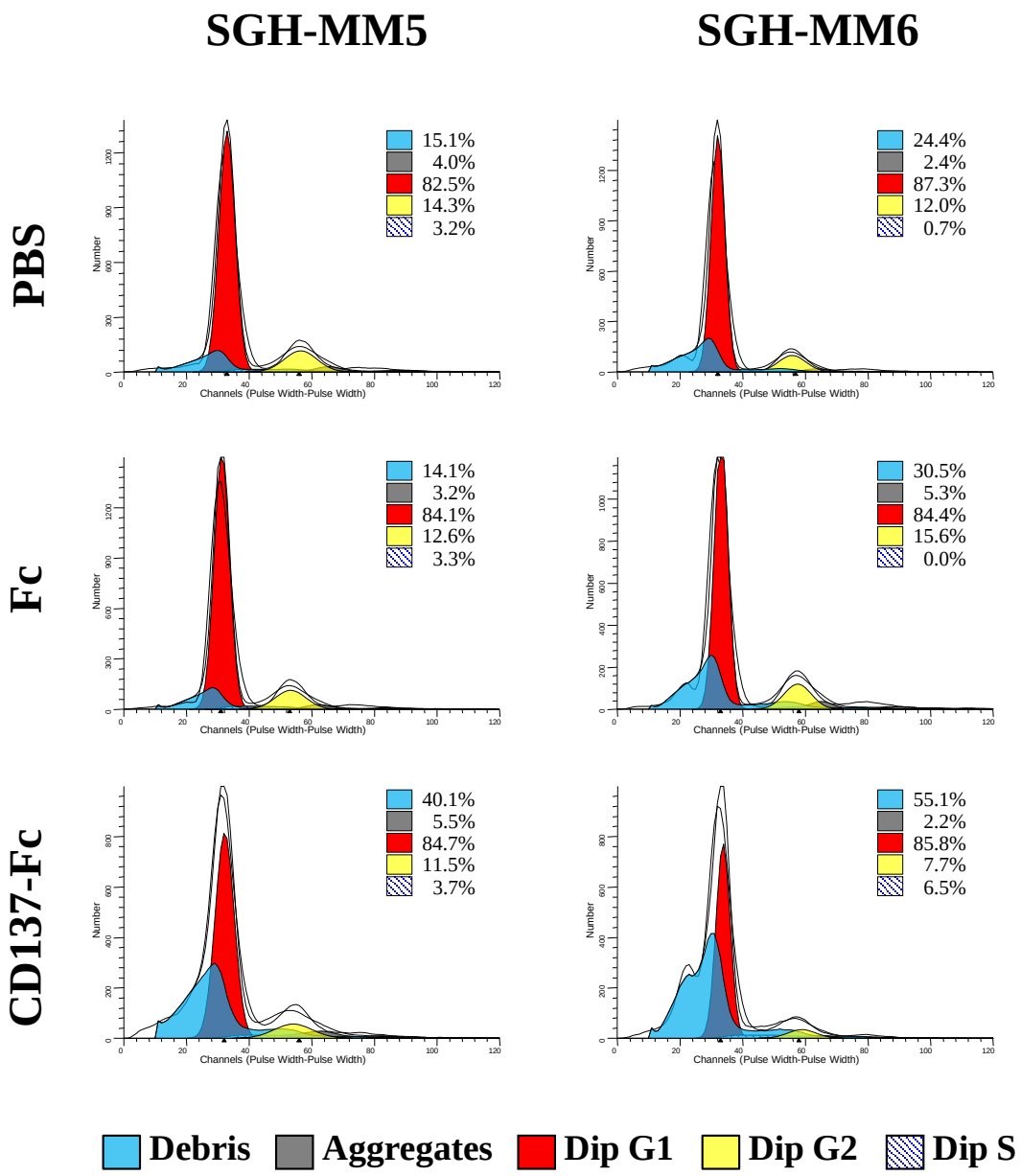

Figure 12. CD137 induces apoptosis and cell cycle arrest in the S phase. Cells at a density of 10^6 cells/ml were cultured on plates coated with 10 μg/ml of Fc or CD137-Fc protein. Nucleic acid content in the cells were stained with 7-AAD, and cell cycle analysis was subsequently performed. Dip G1, G2 and S refer to the diploid G1, G2 and S phases of the cell cycle. This experiment was performed three times with similar results.

3.4 ENGAGEMENT OF MM CELLS VIA CD137 RESULTS IN THE EXPRESSION OF PRO-SURVIVAL CYTOKINES

The processes of proliferation, survival and death of healthy and malignant B cell lymphoma cells are crucially influenced by cytokines. MM cells are no exception to this rule, in particular, IL-6 and IL-8 are important growth and survival factors, and their production is enhanced upon interaction of MM cells with BMSCs (Kline *et al*., 2007; Kawano *et al*., 1988; Kawano *et al*., 1995). VEGF is crucial in regulating angiogenesis, while TGF-β is often secreted by tumor cells to blunt an anti-tumor immune response, or to increase the cells' threshold for the induction of apoptosis (Lauta, 2003; Chen *et al*., 2001).

Treatment with CD137L agonists promoted a strong up-regulation of both IL-6 and IL-8 after 24, 48, or 96 h, that was not observed in the non-MM cell lines (Figures 13, 14). With the exception of IL-8 in RPMI-8226 cells, levels of these two cytokines were either below the detection limit or produced in negligible amounts in the control conditions. VEGF secretion was enhanced moderately in both the MM and non-MM cell lines by CD137-Fc (Figure 15), whereas levels of TGF-β were not affected significantly or uniformly (Figure 16). The 10-fold higher increase of IL-6 secreted by RPMI-8226 cells, as compared to the other MM cell lines, could be attributed to the higher CD137L presence on the cellular membrane (Figure 7). Similarly, the 5-fold higher increase of TGF-β secreted by RPMI-8226 cells in comparison with its MM counterparts, could be due to the higher unstimulated basal level present.

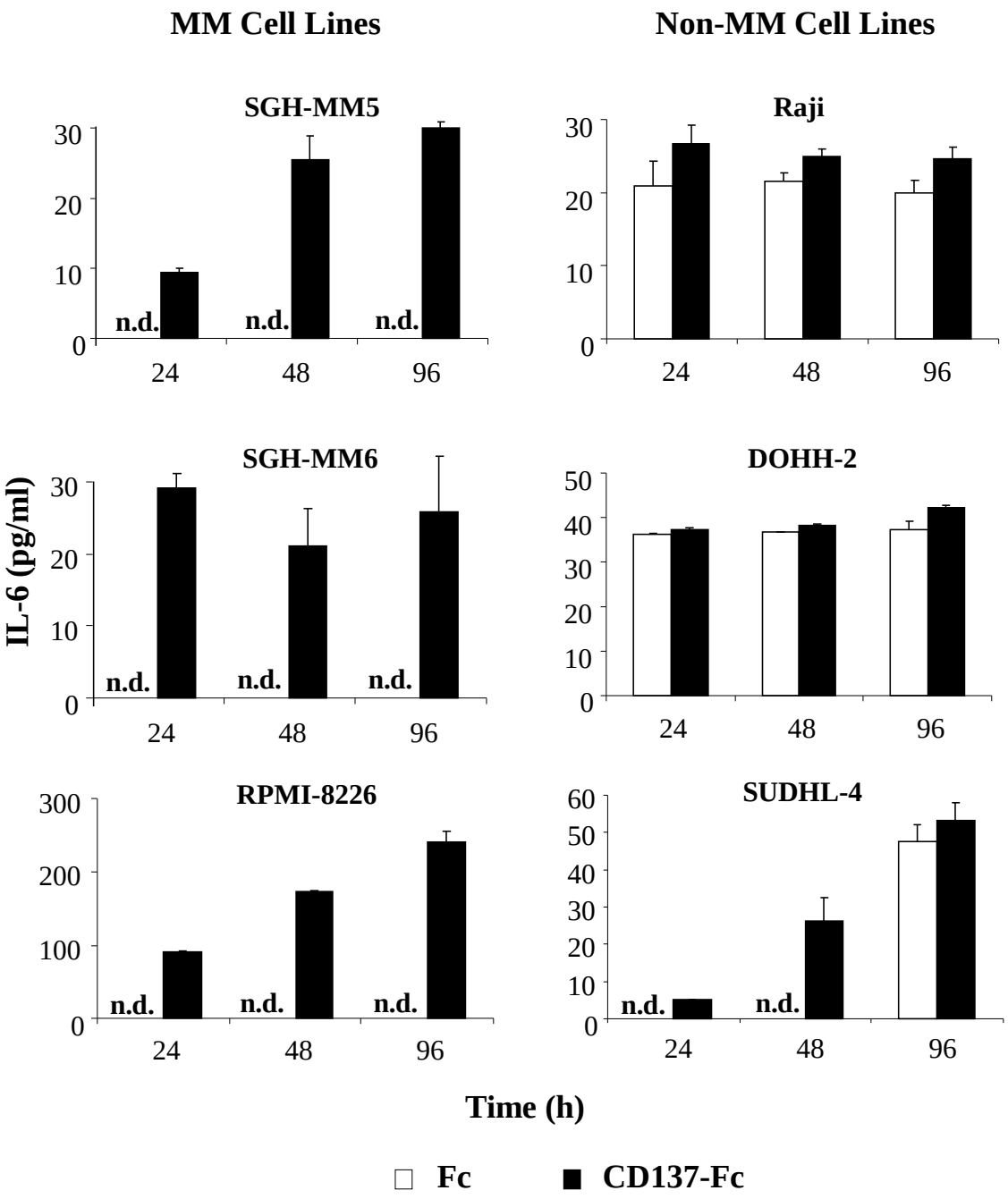

Figure 13. CD137 upregulates IL-6 in MM, but not in non-MM cell lines. Cells at a density of 10^6 cells/ml were cultured on plates coated with 10 µg/ml of Fc or CD137-Fc protein. IL-6 concentrations in 24, 48, and 96 h cell supernatants were determined by ELISA. Depicted are means ± standard deviations from triplicate measurements. n.d. represents IL-6 levels below detection limits. This experiment was performed three times with similar results.

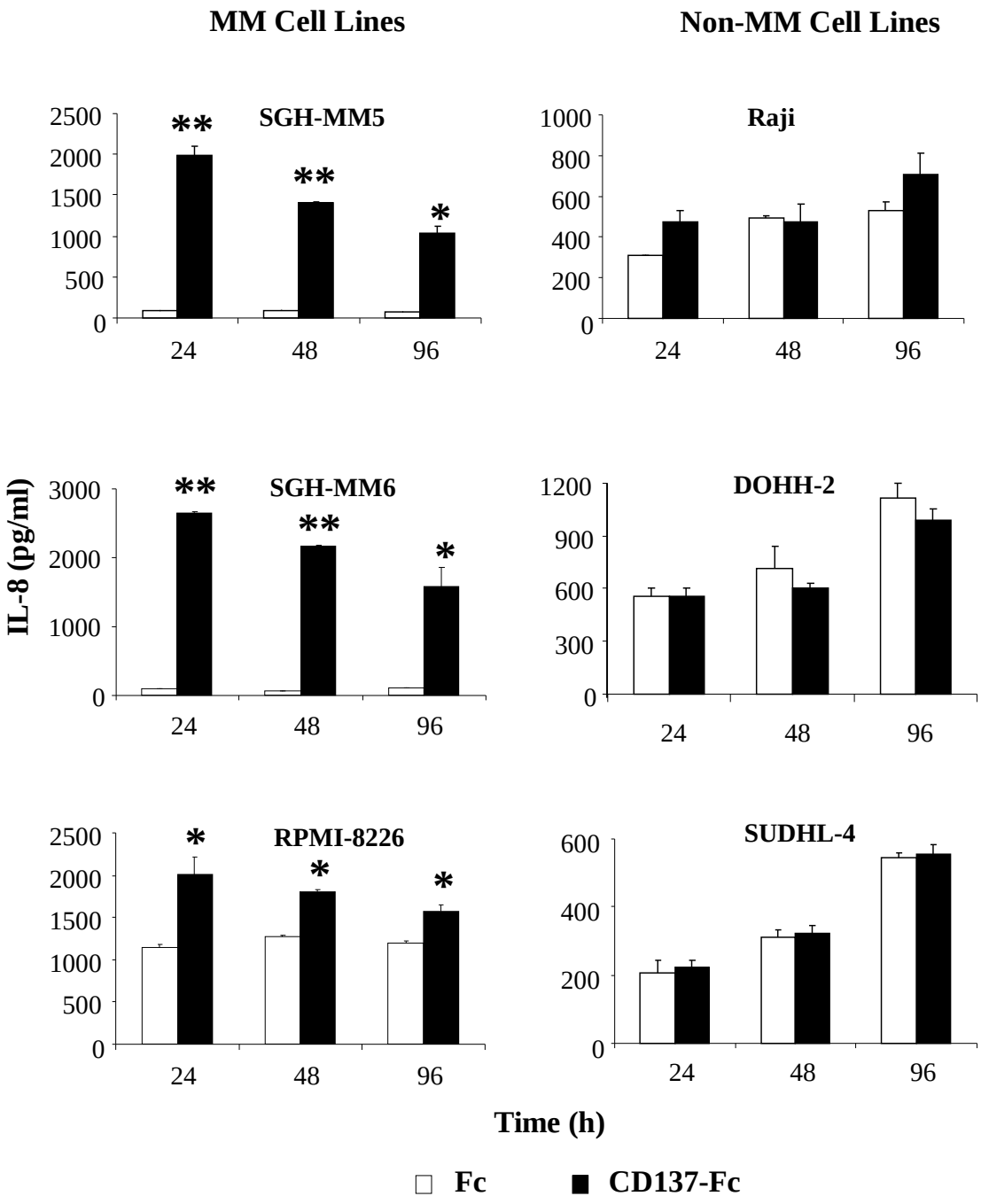

Figure 14. CD137 upregulates IL-8 in MM, but not in non-MM cell lines. Cells at a density of 10^6 cells/ml were cultured on plates coated with 10 µg/ml of Fc or CD137-Fc protein. IL-8 concentrations in 24, 48, and 96 h cell supernatants were determined by ELISA. Depicted are means ± standard deviations from triplicate measurements. * $p<0.05$, ** $p<0.01$. This experiment was performed three times with similar results.

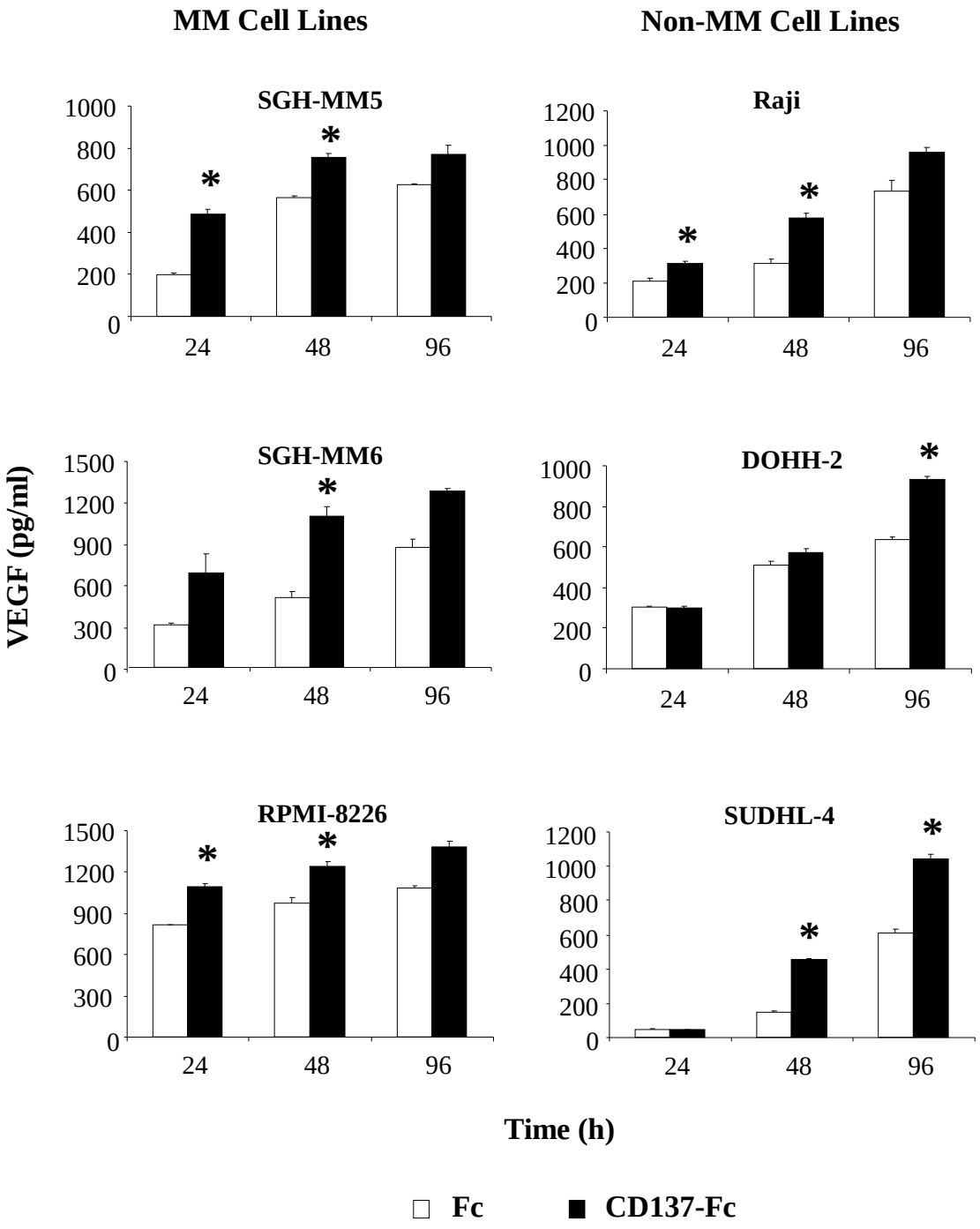

Figure 15. CD137 upregulates VEGF in both MM and non-MM cell lines. Cells at a density of 10^6 cells/ml were cultured on plates coated with 10 μg/ml of Fc or CD137-Fc protein. VEGF concentrations in 24, 48, and 96 h cell supernatants were determined by ELISA. Depicted are means ± standard deviations from triplicate measurements. * $p<0.05$. This experiment was performed three times with similar results.

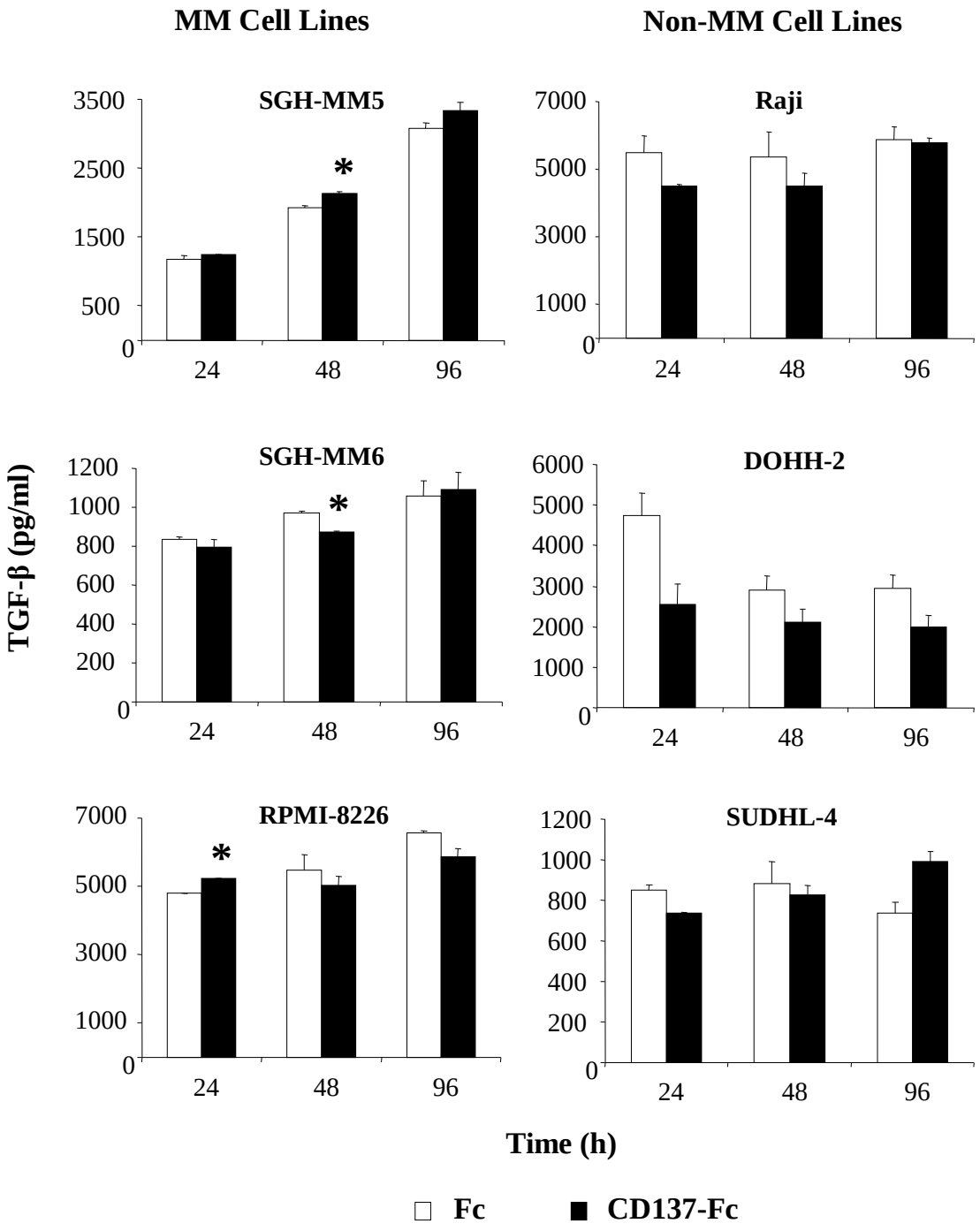

Figure 16. CD137 has no effect on TGF-β production in both MM and non-MM cell lines. Cells at a density of 10^6 cells/ml were cultured on plates coated with 10 μg/ml of Fc or CD137-Fc protein. TGF-β concentrations in 24, 48, and 96 h cell supernatants were determined by ELISA. Depicted are means ± standard deviations from triplicate measurements. * $p<0.05$. This experiment was performed three times with similar results.

3.5 SURVIVAL SIGNALS DO NOT PREVENT CD137-INDUCED APOPTOSIS OF MM CELLS

The observation that CD137 treatment strongly induced up-regulation of IL-6, a potent MM survival factor, came as a great surprise, especially given its simultaneous induction of apoptosis in the MM cell lines. Therefore, an important next step was to determine if IL-6 interferes with the CD137L-induced cell death. IL-2, the classical lymphocyte growth and survival factor was also included.

Even in the presence of IL-6 or IL-2, treatment with immobilized CD137-Fc protein still triggered the induction of apoptosis in the MM cells. Neither cytokine was able to rescue the MM cells from CD137-induced apoptosis (Figure 17). In addition, blocking the IL-6 receptor by neutralizing antibodies had no effect on CD137-induced apoptosis in MM cells (personal communication, Schwarz H).

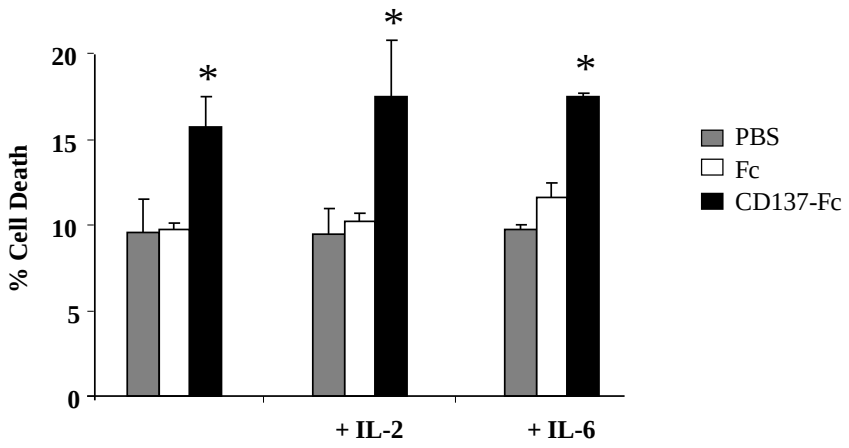

Figure 17. CD137-induced MM cell death is not inhibited by IL-6 or IL-2. SGH-MM6 cells at a density of $1.2x10^6$ cells/ml were cultured on plates coated with 10 μg/ml of Fc or CD137-Fc protein or on uncoated plates (PBS). Cell viability was determined after 24 h via trypan blue staining. Depicted are means ± standard deviations from triplicate measurements. * $p<0.05$. This experiment was performed three times with similar results.

3.6 REQUIREMENT OF IMMOBILIZATION OF CD137L AGONISTS

As the addition of recombinant CD137-Fc protein in a soluble form showed no discernable effects on both the induction of cell death and the reduction of live cell numbers (Figure 18A), it was concluded that cross-linking of the CD137L was essential. There was also no observable difference in the numbers of live and apoptotic cells between uncoated wells (PBS) and Fc protein-coated wells, thereby demonstrating the lack of influence of the Fc control protein (Figure 18B).

In order to overcome the potential problem whereby CD137 crosslinking is rendered ineffective due to the protein not being immobilized and thereby undergo multimerization successfully, in both an *in vivo* and a clinical setting, the decision to attempt the immobilization of the CD137-Fc protein on microbeads was made. This is an especially vital component of the study, since without a means of immobilizing the protein, CD137-induced apoptosis would not occur. An alternative method, via the use of an anti-human Fc antibody to capture and thus to enable the multimerization of the recombinant CD137-Fc protein was also adopted. In both techniques, no significant induction of either MM cell death (Figures 19A, B), or IL-8 (Figures 20A, B) was observed.

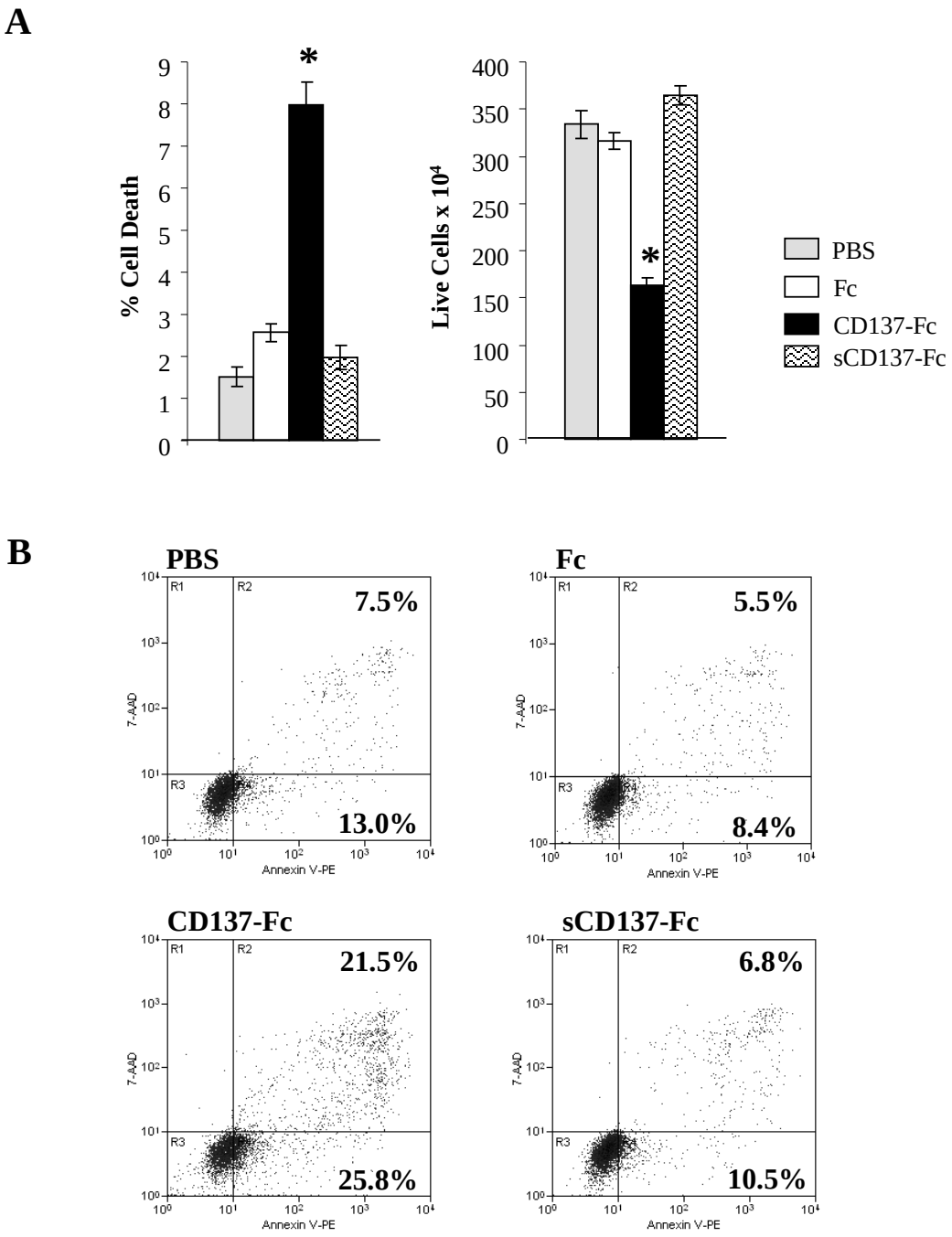

Figure 18. Requirement of immobilization of CD137. SGH-MM5 (A and B) cells at a density of 10^6 cells/ml were cultured on uncoated plates (PBS), or on plates coated with 10 µg/ml of Fc, CD137-Fc, or to which Fc or CD137-Fc proteins were added soluble at 10 µg/ml. (A) Percentage of dead cells (left panel) and number of total live cells (right panel) were determined after 24 h via trypan blue staining. (B) Extent of apoptosis of cells in (A) was determined by Annexin V and 7-AAD staining. Depicted are means ± standard deviations of triplicate measurements. * $p<0.05$. These experiments are representatives of three independent experiments with similar results.

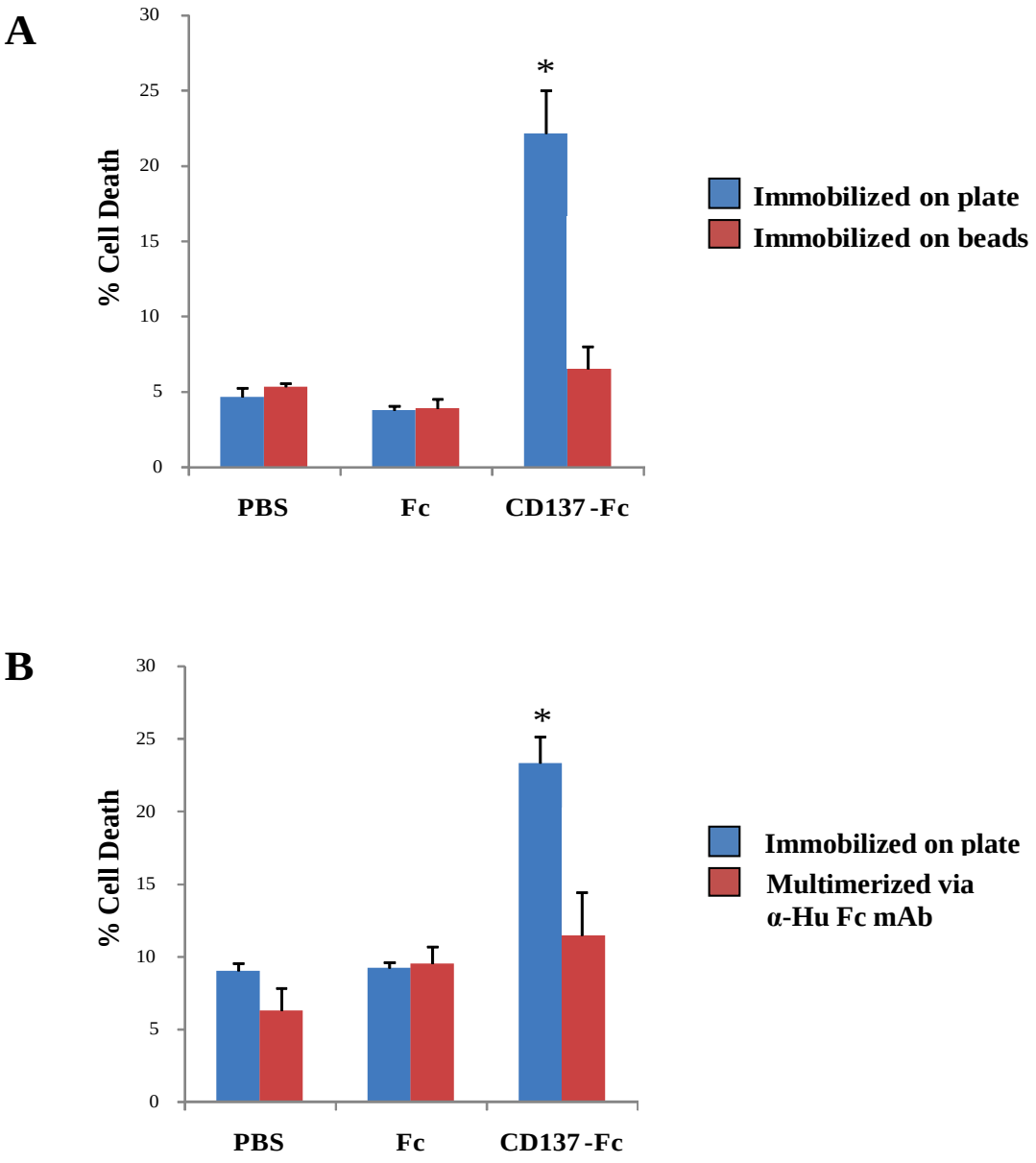

Figure 19. CD137 immobilized on beads or multimerized via α-Hu Fc mAb does not induce cell death in SGH-MM5 cells. SGH-MM5 cells at a density of 10^6 cells/ml were cultured on uncoated plates (PBS), or on plates coated with 10 μg/ml of Fc, CD137-Fc, for 24 h. Fc and CD137-Fc protein were also immobilized via (A) protein G-coated beads (B) α-Hu Fc mAb (clone: GG-7). Depicted are means ± standard deviations of triplicate measurements. * $p<0.05$. These experiments are representatives of three independent experiments with similar results.

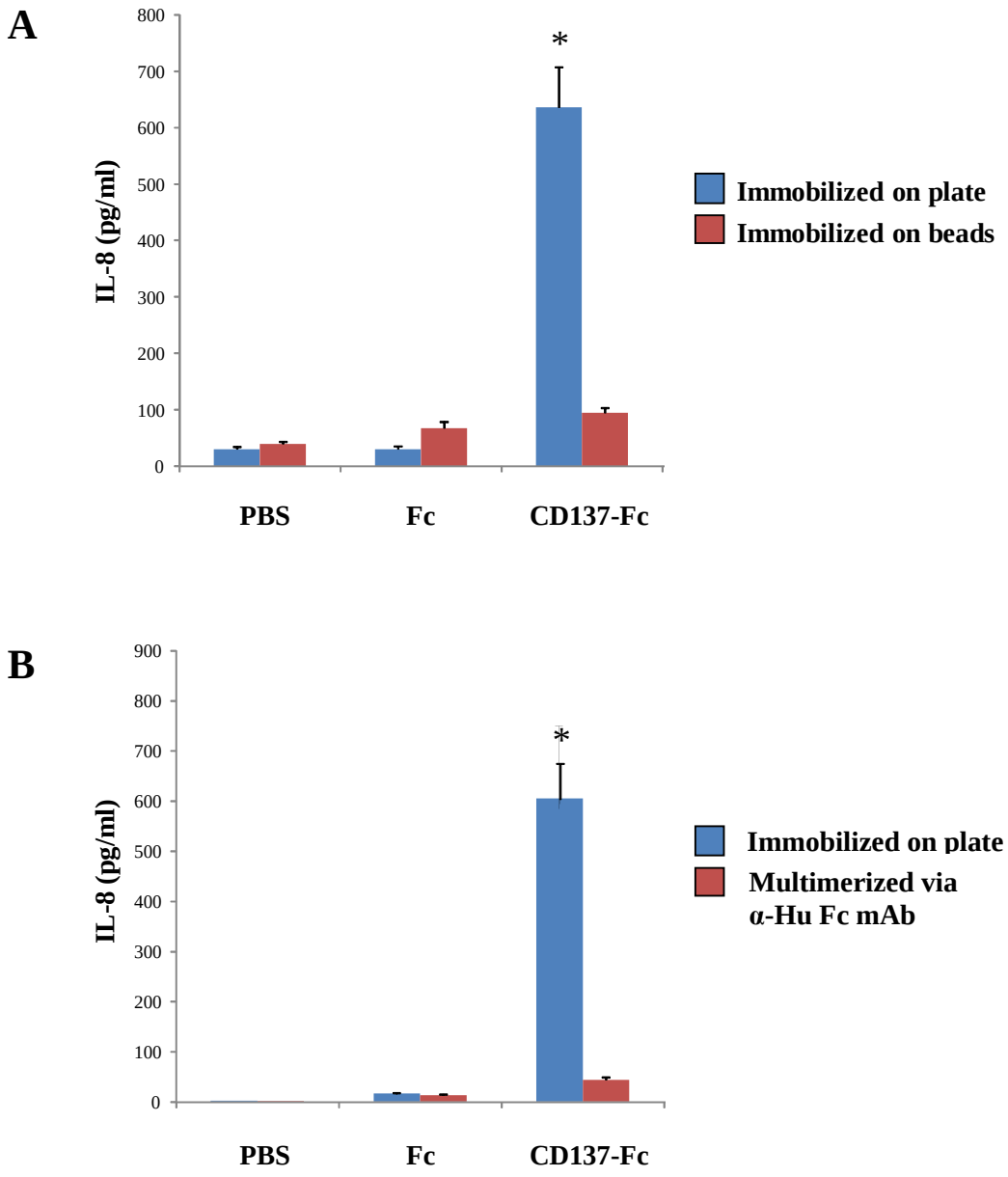

Figure 20. CD137 immobilized on beads or multimerized via α-Hu Fc mAb does not induce IL-8 production in SGH-MM5 cells. SGH-MM5 cells at a density of 10^6 cells/ml were cultured on uncoated plates (PBS), or on plates coated with 10 μg/ml of Fc, CD137-Fc, for 24 h. Fc and CD137-Fc protein were also immobilized via (A) protein G-coated beads (B) α-Hu Fc mAb (clone: GG-7). Depicted are means ± standard deviations of triplicate measurements. * $p<0.05$. These experiments are representatives of three independent experiments with similar results.

3.7 GENERATION OF A STABLE, CD137-EXPRESSING FDC LINE

To investigate and characterize the effects of CD137:CD137L signaling between FDCs and MM cells, CD137-expressing FDCs were first isolated from primary human tonsillar tissue, and then immortalized via two different methods; transfection and cell fusion.

The selection of CD137-expressing FDCs was divided into three main phases; tissue dissociation, density gradient centrifugation, and magnetic cell sorting. After each phase, a sampling was taken and stained for a variety of cell markers. These markers included CD14, a monocyte/FDC marker, CD3, a T cell marker, CD31, an endothelial cell marker, KiM4, a FDC marker, as well as CD137. The samples were stained with both CD14 and KiM4 so as to help identify which fraction in each of the phases the FDCs were primarily isolated in. CD31 was chosen, as a literature review of previous FDC isolation protocols (Muñoz-Fernández *et al*., 2006; Kim *et al*., 1994) had revealed that endothelial cells co-isolated should also be in the same density gradient fraction as the FDCs. Lastly, staining was performed for CD137 and CD3 so as to ascertain the extent of activated T cell contamination.

As the cell isolation progressed, there was an enrichment of both CD14 and CD31-positive cells, indicating that the fraction chosen for sampling, and further isolation, very likely held the FDCs, as evidenced by the correlating presence of endothelial cells. While the increased percentage of $CD137^+$ cells

was encouraging, the increased $CD3^+$ population pointed towards a larger extent of activated T cell population, which as previously mentioned, would also express CD137 (Figure 21). KiM4 was not stained for to verify FDC enrichment, post CD137 magnetic selection, as it is a murine-produced antibody, similar to the PE-conjugated anti-CD137 mAb used to pull out the $CD137^+$ cells. The anti-mouse PE-conjugated antibody would have labelled the anti-CD137 mAb as well, leading to a false positive result.

Post-isolation, two techniques were employed in the immortalization of the FDCs. The first method involved the manipulation of cellular DNA via transfection, so as to allow the expression of the catalytic subunit of human telomerase. Cell fusion was the second technique performed. Fusion with two different HAT-sensitive cell lines to form hybridomas was carried out. Both transfected and fused cells were left to grow and were selected over two weeks. Unfortunately, in both cases, sorting and staining for the presence of KiM4 showed that none of the surviving cells post-selection were immortalized FDCs (data not shown).

The isolation was performed thrice more. However, in order to reduce the amount of activated T cells present, only non-inflamed tonsils were used. An additional step whereby $CD3^+$ cells were depleted by positive magnetic selection was also included in the protocol, prior to $CD137^+$ magnetic selection. While this improved protocol resulted in cells that survived the

antibiotic selection process after transfection or fusion, the resulting cells were still not hardy enough to survive the sorting process.

In summary, the objective of generating a stable, CD137-expressing FDC line was not accomplished. Although a streamlined and effective protocol for the isolation and enrichment of FDCs was formulated and proven, cell viability was still an issue post-immortalization.

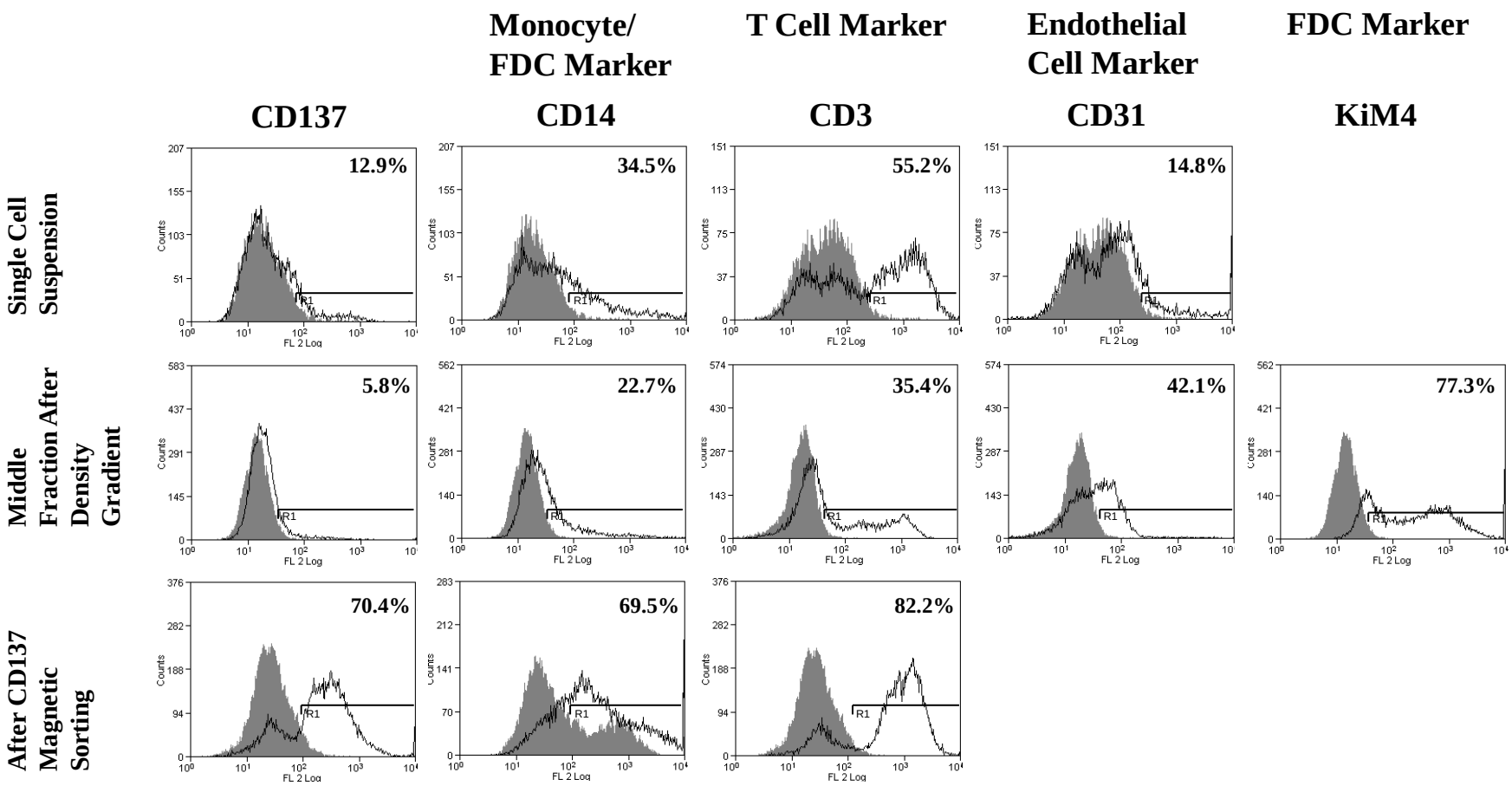

Figure 21. Expression levels of CD137, CD14, CD3, CD31, and KiM4 in the samples after each phase of FDC isolation. Cells were stained with monoclonal antibodies against CD137 (clone 4B4-1), CD14 (clone 61D3), CD3 (UCHT1), CD31 (clone WM59), or KiM4 (clone KiM4), displayed as open histograms, or their isotype control (clone MOPC-21), displayed as filled histogram.

4. DISCUSSION

In this study, some unexpected activities of CD137L crosslinking on MM cell lines were identified. Some of these activities include the observations that CD137L reverse signalling inhibited proliferation, induced apoptosis and secretion of IL-6 and IL-8 selectively in the MM cell lines, but not in the non-MM B cell lymphoma cell lines.

These data comes as a surprise, especially since CD137 has been shown to enhance the activation and proliferation of primary B cells (Pauly *et al*., 2002; Pollok *et al*., 1994). Thus, it was a logical step in formulating the hypothesis that CD137 would also enhance the proliferation of B cell lines, even more so as CD137 can be expressed as a neoantigen by certain B cell lymphomas (personal communication, Thum E; unpublished data). In theory, the ectopic expression of CD137 could enable malignant B cells to send and receive growth and survival signals in an autocrine or paracrine manner, which under normal physiological conditions are delivered by CD137-expressing T helper cells, or follicular dendritic cells (Pauly *et al*., 2002; Lindstedt *et al*., 2003).

In the following sections, a hitherto unrecognized role of CD137 and CD137L in MM cell biology will be elucidated and discussed, in particular, the

induction of apoptosis and the up-regulation of pro-survival cytokines. A potential underlying mechanism how CD137 induces cell death in MM cells will also be proposed. Alternate methods to enable successful CD137:CD137L crosslinking in an *in vivo* or clinical setting will be discussed as well. This will be followed by a dissection of the FDC isolation and immortalization process, as well as, an analysis of the advantages and implications derived in studying the interactions of MM cells and FDCs. Lastly, this chapter will conclude with suggestions for possible directions for future works.

4.1 ACTIVATION INDUCED CELL DEATH AS A POSSIBLE MECHANISM OF CD137-INDUCED CELL DEATH

CD137L signalling induced the secretion of IL-6 and IL-8 specifically in the MM cell lines but not in the non-MM B cell lymphoma lines. VEGF is also induced by CD137L signals, albeit to a lower extent, and in both the MM and non-MM cell lines. IL-6 has been shown to be essential for MM growth and for protection from apoptosis (Kawano *et al*., 1988; Kawano *et al*., 1995), and increased IL-6 levels in MM patient sera correlate with disease progression (Lauta, 2003; Kabelitz *et al*., 1993). Similarly, IL-8 supports MM growth, and IL-8 secretion by bone marrow stromal cells also correlates with MM progression (Kline *et al*., 2007). VEGF is a potent growth factor for MM cells,

and supports MM growth by inducing angiogenesis (Dankbar *et al*., 2000; Podar *et al.,* 2004). As all three cytokines listed above have been shown to be pro-survival cytokines in MM, these results are at first sight surprising, especially in the face of increased apoptosis rates in the MM cell lines tested.

Secretion of pro-survival cytokines and NF-κB activation (personal communication, Schwarz H) clearly indicates cellular activation by CD137L signalling. A signal that is able to induce both cell death and activation at the same time seems to be a contradiction. A possible mechanism that might explain these conflicting effects could be due to activation-induced cell death (AICD), whereby the frequency of AICD is positively correlated to the degree of cell activation (Kabelitz *et al*., 1993). AICD is a well known phenomenon that has been described frequently in activated T cells, and to a lesser extent in B cells, at various stages of development after activation (Daniel *et al*., 1997; Berad *et al*., 1999; Dubravka and Scott, 2000).

In B lymphocytes, AICD is a highly regulated event initiated by crosslinking of the B cell receptor (BCR), where the complex interplay between survival and death signals determines the B cell fate and, consequently, the immune response (Dubravka and Scott, 2000). In normal B cells, BCR crosslinking results in an initial activation of Src family members, followed subsequently

by a divergence of the BCR signaling pathway into several different signaling cascades, namely, phospholipase Cγ (PLCγ), Ras, and phosphotidylinositol-3 kinase (PI3K) (Berridge, 1997). PLCγ activation by phosphorylation results in the release of the second messengers, inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerl, that mediate a small and sustained increase of cytosolic free calcium; essential for DNA synethesis, and subsequently, B cell proliferation to proceed (Yamada *et al.*, 1993). A rapid elevation of cytosolic free calcium on the other hand, seems sufficient to induce growth inhibition and apoptosis in a variety of B cell lines (Muthukkumar *et al*., 1993; Genestier *et al*., 1994; Bonnefoy-Berad *et al*., 1994; Graves *et al*., 1996). The second signal transduction cascade initiated by BCR crosslinking is mediated by Ras activation, whereby a sequential phosphorylation cascade, involving Raf-1, MAPK/Erk kinase (MEK), and mitogen-activated protein kinase (MAPK), is activated. Subsequent translocation of MAPK into the nucleus then induces transcription, or in some cases, might be required for the induction of apoptosis (Graves *et al*., 1996). Finally, the activation of the Src family kinases leads to PI3K activation, which in turn phosphorylates phosphotidylinositol 4, 5-bisphosphate (PIP_2), leading to the phosphotidylinositol 3, 4, 5-triphosphate (PIP3) second messenger. The downstream target of PI3K, PKB/Akt then acts upon $p70^{S6K}$, an enzyme implicated in mRNA translation (Fruman *et al*., 1998; Campbell, 1999). Inhibition of the PI3K pathway on the other hand, has been shown to induce

G1 arrest and apoptosis, alluding to a correlation between BCR-mediated inactivation of PI3K/p70^{S6K} pathway and BCR-mediated apoptosis (Carey and Scott, 2001). For the sustained proliferation upon the engagement of the BCR, a second co-stimulatory signal, that is provided by T helper cells, is needed. Without this second signal, BCR engagement results in abortive activation, anergy, or deletion (Parry *et al*., 1994). In conclusion, extensive BCR stimulation can lead to either B cell proliferation, in the presence of a co-stimulatory signal, or growth arrest and apoptosis/AICD in the absence of T cell help.

The fact that MM cells are not rescued from CD137L agonist-induced cell death, by the addition of IL-2 or IL-6, also supports AICD as a viable hypothesis. Another factor in favour of the AICD hypothesis, is that this same mechanism has been suggested as the most plausible explanation for anti-CD40 induced B cell death (Funakoshi *et al*., 1994; Szocinski *et al*., 2002). CD40 shares many similarities with CD137, and indeed, anti-CD40 antibodies have been shown to have a direct cytotoxic effect in various B cell malignancies, including in MM (Tai *et al*., 2004; Law *et al*., 2005). Finally, CD40 stimulation, similar to CD137 ligand, has been shown to induce apoptosis or proliferation in different cell types despite the activation of the NF-κB pathway in both instances (Vonderheide *et al.*, 2007). Phase I clinical trials with CD40 agonists are currently being conducted on MM patients with

encouraging results (Vonderheide *et al*., 2007), and while the exact mechanism of anti-CD40 induced B cell death is not know, AICD has been one of the mechanisms postulated to be at the root of it.

Both the MM and non-MM cell lines expressed similar levels of CD137L. The observation of an enhanced VEGF secretion in both cell line types after CD137L crosslinking demonstrates that the CD137 reverse signalling pathway is also functional in the non-MM cell lines. Inhibition of proliferation, induction of apoptosis, and IL-6 and IL-8 secretion however, were specific to the MM cell lines. Although the molecular basis of this difference in biological responses between the MM and non-MM cell lines is still unknown, a possible explanation could be provided by the recent finding that many MM tumors have NF-κB constitutively activated (Keats *at al*., 2007; Annunziata *et al*., 2007). Thus, any additional stimulation, for example by CD137 or by anti-CD40 antibodies, may therefore induce AICD in MM cells, especially when

both the CD137 ligand and CD40 signalling systems are known to be part of a group of co-stimulatory molecules known to activate B cells (Pauly *et al*., 2002).

As AICD may take place when the surrounding environment is favourable for cell survival and proliferation (Szocinski *et al*., 2002), this would explain why the upregulation of pro-survival cytokines like IL-6 and IL-8 after CD137-Fc treatment is not able to prevent cell death. The effects, shown previously, of CD137-Fc treatment on MM cells could suggest that the CD137:CD137L system may help to regulate the activity and survival of normal, terminally differentiated plasma cells in the bone marrow. Excessive signalling through CD137L might result in plasma cells undergoing apoptosis in the form of AICD, as plasma cells by nature usually have a short lifespan of two to three days. Although this might point to why MM cells die when treated with CD137-Fc, there is no data currently available showing the effects of CD137 reverse signalling on primary plasma cells. Neither has the expression of CD137 in the bone marrow microenvironment been shown previously. Therefore, more work has to be done in these areas to prove the theory of CD137-induced MM cell death as AICD and that this phenomenon occurs in normal plasma cells as well.

4.2 CD137L AGONISTS NEED TO BE IMMOBILIZED IN ORDER FOR THE INDUCTION OF CELL DEATH

All of the experiments depicted in the previous section, required the immobilization of the CD137-Fc protein onto tissue culture plates for successful crosslinking of the CD137L on MM cells, and thus enabling the induction of cell death. Initially, it was thought that immobilization of the protein merely served as a way to concentrate the CD137, allowing a lower concentration to be used than otherwise needed. In this way, all one would need to deliver the CD137-Fc, would be a simple injection at the site of interest. However, attempts to replicate CD137-induced apoptosis were unsuccessful when the CD137-Fc protein was added into the culture medium soluble. This poses a pretty grave problem when the experiments are moved into an *in vivo* setting.

Thus, two alternatives were tested out in an attempt to circumvent this problem. Namely, immobilization on microbeads, and multimerization of the CD137 protein via an α-Hu Fc mAb. However, as shown in the section above, both methods did not yield any significant induction of cell death, or secretion of IL-8. Observation of the MM cells under magnification, via the use of a light microscope, likewise did not exhibit any increased signs of early apoptosis.

The failure of the CD137-coated microbeads to increase the apoptotic rates in the MM cells could be postulated to be due to a low efficiency of protein adsorption onto the beads. This was unlikely, as there have been many reports in the past few decades demonstrating the use of microbeads to bind various proteins by simple adsorption (Wide and Porath, 1966; Weliky and Weetall, 1965; Monthony *et al*., 1977; Jolley *at al*., 1984). What might have happened, which is also outlined as a point of caution in the manufacturer's technical notes, was that leaching of the coated CD137-Fc protein might have occurred. And as shown earlier, this would have resulted in a much higher proportion of soluble CD137 than assumed, resulting in a loss of successful CD137:CD137L signalling and thus a decrease in MM cell death. Thus, in future experiments, it would be wise to bind CD137-Fc covalently, to ensure a more permanent and stable immobilization. From this reasoning, one might therefore infer that a certain signalling threshold would have to be exceeded, before the inhibitory effects of CD137 can be observed.

Although efforts were made to ensure a good mix of CD137-coated beads and MM cells by pipetting the mixture thoroughly, the establishment of a consistent contact geometry between microbeads and cells could not be confirmed. Based on previous reports (Wei *et al*., 1999; Puig-de-Morales *et al*., 2004), this lack of a precise contact geometry might have resulted in a spatial orientation mismatch of the CD137 and CD137L. Thus, the spherical

architecture of the beads, as compared to the relatively flat surface of the bottom of a cell culture plate well, itself might have limited the actual density of CD137-Fc available to crosslink the CD137L expressed on the MM cells. This might have been compounded by the fact that the size of the microbeads, at a diameter of 4 μm, would have provided a much smaller contact surface area for CD137:CD137L crosslinking. The actual contact area between the MM cells and the microbeads would most likely also be smaller than the apparent contact area, considering the local roughness of the cell surface. Therefore, some portion of the CD137 protein coated on estimated contact area might not have been able to reach the CD137L molecules, despite favourable diffusion.

Previously, it had been shown that 3 μm and 1 μm bead diameter thresholds were essential for effective antigen-dependent degranulation and integrin-mediated adhesion of cytotoxic T lymphocytes, respectively (Mescher, 1992; Ganpule *et al*., 1997). Thus based on the available literature, it was assumed that a 4 μm diameter microbead would have provided sufficient contact area for successful CD137:CD137L crosslinking. The minimal contact area required might correspond to a spatial limit for recruiting CD137L and/or other signal transduction molecules (Wei *et al*., 1999). In order to ensure sufficient contact, one might have to increase the size of the beads used. But this would bring another problem into the foreground; any bead size larger

than 4 μm would cause problems with regards to delivery into the site of interest in an *in vivo* setting, as it could clog capillaries. Therefore, it appears that another alternative needs to be found for a successful *in vivo* CD137:CD137L crosslinking.

Next, an attempt was made to multimerize the CD137-Fc protein via the use of an α-Hu Fc mAb. This experimental step was supported by previous literature showing that two trimeric Fas ligands or CD40 ligands were sufficient to trigger apoptosis or B cell proliferation, respectively, and that a higher degree of multimerization further enhanced CD40 ligand stimulation (Holler *et al*., 2003). This was also supported by data showing that two cross-linked CD137L trimers were sufficient to trigger co-stimulatory signals, with a higher degree of oligomerization leading to enhanced co-stimulation (Rabu *et al*., 2005). Hence, the higher-order multimerization of the ligand, previously achieved by the direct immobilization of the CD137 protein to the tissue culture plates, was replicated by using an α-Hu Fc mAb to capture the CD137-Fc.

Since an induction of MM cell death or IL-8 was not obtained, a logical assumption would be that the α-Hu Fc mAb had failed to multimerize the CD137-Fc protein. However, other recombinant proteins, containing the Fc

portion of human IgG, had also been previously successfully immobilized onto cell culture plates for use in cell adhesion assays, via an anti-human IgG antibody, demonstrating that this technique does in fact work (Mizuno *et al*., 1997). Additionally, an ELISA testing for the presence of sCD137 in the coating supernatant, after incubation with the α-Hu Fc mAb, also revealed an expected and corresponding drop in CD137 levels (data not shown), thereby demonstrating the successful capture of the CD137-Fc by the α-Hu Fc mAb onto the cell culture plate.

Due to the specificity of the antibody to the Fc region of the recombinant protein, it is unlikely that the CD137-Fc is being multimerized in such a way that it would block the CD137:CD137L interaction. But the mAb might multimerize the CD137-Fc in such a way that the density of the recombinant protein might not be sufficient to trigger an apoptotic signal. Theoretically, each of the binding domains on the α-Hu Fc mAb should be able to bind one molecule of CD137-Fc. However, not all of the Fc domains in CD137-Fc may be simultaneously available for binding. What could be happening is that when a CD137-Fc molecule is captured by one of the anti-Hu Fc antibodies, it might be sterically blocking the access of another CD137 molecule to an adjacent Fc-binding domain. Another possibility is that the CD137 molecules that are bound by one anti-Hu Fc mAb molecule might be hindering other CD137-Fc molecules from being captured by an adjacent α-Hu Fc mAb on the cell culture plate (Ghose *et al*., 2006).

4.3 TROUBLESHOOTING IMPROVEMENTS MADE AND RECOMMENDED IN THE ISOLATION AND IMMORTALIZATION OF FDCs

To investigate the effects of FDC on CD137:CD137L reverse signalling in MM cells, an enriched FDC fraction was prepared from human tonsillar tissue. The protocol adopted for use in the FDC isolation, consisted of commonly used methods, including the enzymatic digestion of the tonsillar tissue followed by centrifugal separation through Percoll or BSA (diluted in PBS to form specific density layers) density gradients. With the use of a specific α-Hu FDC mAb, it is possible to obtain a 95% homogeneous FDC fraction via cell sorting (Kim *et al*., 1994). However, the number of FDCs purified in this way, would have been insufficient to perform any experiments extensively. Furthermore, it would have been extremely difficult to dissociate *in vivo* bound B cells from the freshly isolated FDCs. Therefore, in order to surmount these obstacles, it was decided to establish a FDC cell line to facilitate this process.

Digestion was performed with type IV collagenase instead of other recommended enzymes so as to preserve the structural integrity of the CD137 expressed on the FDC cell surface. DNAse I was added in subsequent isolations to decrease the viscosity of the enriched fractions. Both Percoll and

BSA density gradient centrifugations were carried out, but cell separation via Histopaque was eventually chosen, both for fractions that were visibly more separated, as well as for convenience of preparation.

For the first two isolations, tonsillar tissues were used without consideration of their inflammation status. Upon discovering the extent of activated T cell contamination within the enriched FDC population, it was decided that only non-inflamed tonsils would be used. In addition, an extra step was taken to limit the levels of CD137-expressing, activated T cells by performing a CD3 magnetic assisted depletion prior to the CD137 positive magnetic selection. Although recommended, cell sorting was not immediately carried out before immortalization of the FDCs, as a visual inspection under microscopic magnification had revealed that the FDCs were not as numerous or as healthy as hoped for. Hence, immortalization of the FDCs was performed, and the cultures left to expand, in the hope that cell sorting would then become a viable option.

Although one of the aims of this project was to generate a stable FDC cell line, followed by characterization of the CD137:CD137L signalling effects between FDCs and MM cells, the isolation and immortalization protocols were not as successful as hoped for. In addition, the use of the HAT-sensitive multiple

myeloma cell line, MM5-HS, would seem contradictory to this aim. Since CD137 has already been shown to induce apoptosis in MM cells, it would seem counter-intuitive to attempt a cell fusion of CD137-expressing FDCs together with a CD137L-expressing MM cell line. The implications of doing so did not go unnoticed. The selection of this MM cell line was in part due to its ready availability, as well as a stop-gap measure in lieu of an appropriate cell line. Eventually, the Sp2/mIL-6 cell line was obtained and used in place accordingly, as previously described (Harris *et al*., 1992).

A major reason for the failure to successfully generate a stable CD137-expressing FDC cell line could be attributed to the low numbers of isolated FDCs. In each of the tonsillectomies, only one tonsil sample was accessible for use. Based on the fact that the percentage of FDCs in human tonsillar tissue only ranges from 5 to 8% (Sprenger *et al*., 1995), and the small mass of each tonsil, it would be highly difficult to extricate sufficient quantities of viable FDCs for use, even assuming a 100% rate of isolation. The use of both tonsils would logically double the number of FDCs obtained, and greatly increase the chances of success.

4.4 ADVANTAGES AND IMPLICATIONS IN STUDYING THE INTERACTIONS BETWEEN B CELLS, MM CELLS AND FDCs

FDCs trap immune complexes with their abundant complement and Ig Fc receptors within the B-cell follicles, a critical process in the development of high-affinity, isotype-switched Ab responses (Tew *et al*., 1997). FDCs play important roles in germinal centre (GC) B cell proliferation, survival, and differentiation in both antigen-dependent and independent manners (Burton *et al*., 1993, Tew *et al*., 1997). FDCs also seem to supply numerous non-specific stimuli during GC responses for the generation of an optimal B cell response (Kosco-Vilbois, 2003), as well as, various potent accessory signals to prevent apoptosis and aid in B cell selection during receptor affinity maturation processes (Imal and Yamakawa, 1996; Szabo *et al*., 1997; Liu *et al*., 1989; Koopman *et al*., 1994; Kosco *et al*., 1992). The finding that FDCs express high levels of CD137 on the cell surface, is not only in agreement with recent data exhibiting the effects of CD137 on the activation of GC B cells (Pauly *et* al., 2002), it also represents an additional way for the FDCs to interact with both the CD137L-expressing GC B cells (Lindstedt *et al*., 2003) and MM cells.

Interestingly, both T cells and FDCs, the two main regulators of the humoral response, have been shown to express CD137. T cells regulate the B cell

response by activating the proliferation and differentiation of B cells, as well as, eliminating autoreactive B cell clones, while FDCs present antigens and allow for the selection of high-affinity clones (Hollmann and Gerdes, 1999). In addition to providing survival signals, some FDCs have also been shown to express FasL, and may also participate in the apoptosis of GC B cells (Hur *et al*., 2000). In the light of these findings, and of the results presented above showing CD137-induced apoptosis in MM cells, a malignancy of B cell origin, it would therefore be interesting to determine if FDC-expressed CD137 is able to either compound, or rescue MM cells from CD137-induced cell death.

Notch signalling regulates cell fate during development and differentiation in many cell lineages, including lymphocytes (Artavanis-Tsakonas *et al*., 1999). It has been reported that Notch signalling plays critical roles in B cell development (He and Pear, 2003). In addition, recent data has also provided the observation that Notch signalling is able to affect the activation and differntiation of mature B cells to antibody-secreting cells (Thomas *et al*., 2007; Santos *et al*., 2007), revealing the importance of Notch signalling to the generation of plasma cells, and possibly MM cells. FDCs have been shown to not only express Notch ligands, but recent data has also pointed to the protection of GC B cells from apoptosis by Notch signalling (Yoon *et al*., 2009). Since the data presented above have already shown the apoptosis-

inducing effects of CD137 on MM cells, Notch signalling might provide a molecular basis to understanding FDC-MM cell interaction, in the event that a CD137-expressing FDC cell line is unable to induce MM cell death.

These observations on Notch signalling may have significant implications in treating B cell lymphomas, including multiple myeloma. The majority of B cell lymphomas originates from the GC (Stevenson *et al*., 1998), in particular, follicular lymphoma, nodular lymphocyte predominant Hodgkin's lymphoma, and classic Hodgkin's lymphoma (Carbone *et al*., 2009), and remains dependent on the GC microenvironment (Petrasch *et al*., 1992). It has been speculated that stromal microenvironments may contribute to the development of drug resistance in B cell lymphomas (Bohen *et al*., 2003). On the other hand, although no studies have been conducted to date with regards to the effect of Notch signalling on MM cells, it might yet turn out that targeting both the CD137 and Notch signalling pathways deserves consideration as candidates for the treatment of multiple myeloma, in combination with current therapies.

Cross-linking of CD137L by CD137 has been shown to induce apoptosis in lymphocytes (Schwarz et al., 2006; Michel *et al*., 1999). In a transgenic murine model, where CD137L was exclusively produced by APCs, T cell

numbers remained unchanged, whereas B cells were depleted. No B cell follicles could be detected either. The increased interaction between CD137-expressing FDCs and CD137L-expressing B cells may have resulted in an enhanced depletion of B cells, and thus lack of formation of follicles (Lindstedt *et al*., 2003). These results are in direct contradiction to previous data where CD137:CD137L interaction results in B cell proliferation. Therefore, functional studies concerning the CD137:CD137L signalling are important for better understanding of the interaction between FDCs, B cells, and MM cells. Hence, the generation of a CD137-expressing FDC cell line is an important first step in working towards this aim.

4.5 FUTURE WORKS

4.5.1 Synergistic Effects of CD137 and Chemotherapeutic Drugs on MM Cell Death

In addition to treating the MM cell lines with CD137-Fc, the culture media will also include various concentrations of chemotherapeutic drugs such as thalidomide, dexamethasone, and doxorubicin. Lactate dehydrogenase (LDH) assays will then be conducted as a readout of cell death.

4.5.2 Verification of Key Results with Patient MM Cells and Healthy B Cells

For validation of the observations made with the MM cell lines, MM patient plasma cells will be extracted from bone marrow aspirates, while B cells from healthy volunteers will be isolated from their buffy coat.

4.5.3 Identifying Mechanisms and Signalling Cascades Involved in MM Cell Migration

A major problem in multiple myeloma is the migration of malignant cells from the blood to the bone marrow, the accumulation of MM cells in the bone, and

their destruction of healthy bone tissue. Although very little is known about the signals that lead to this aberrant migration, Rho family GTPases have been shown to play a pivotal role in the migration and attachment of leukocytes. To this end, the function of Rho GTPases will be characterized, as well as, determining if CD137 interferes with MM cell migration and signalling.

4.5.4 Development of a Formulation of CD137 for *in vivo* Experiments

Soluble CD137-Fc shows little to no effect in the inhibition of MM cell lines. The above data has also shown that both the immobilization of CD137 on microbeads or its capturing via α-Hu Fc mAbs are not viable options. An alternative would be to employ a "Cell-Ghost", where a CD137-expressing cell line is incubated in a hypotonic solution. Upon bursting of the cell, its intracellular contents are released. Passage through a magnetic column would then ensure that only the membrane, with all of its associated proteins, including CD137, is selected, forming the "Cell-Ghost".

4.5.5 Murine MM Models

Two different models will be considered for the *in vivo* phase of the experiments; a xenograft and human bone chip SCID mouse MM model.

These models will hopefully aid in the determination of the inhibitory effects of CD137 on MM cell growth *in vivo*.

SGH-MM5 or SGH-MM6 cells will be injected subcutaneously into nude mice (Epstein and Yaccoby, 2005). Upon tumor development, one group of mice will be treated with the *in vivo* formulation of immobilized CD137-Fc. Two other groups will be treated with the control Fc protein and normally immobilized CD137-Fc respectively. Tumor growth will be checked and measured, while the effect of CD137 or anti-CD137L mAb treatment will be evaluated in a Kaplan-Meyer plot.

Although technically more demanding, the human bone chip SCID mouse MM model is physiologically more accurate, as it allows the MM cells to interact with the bone marrow microenvironment that is essential for human MM growth (Tassone *et al*., 2005; Harousseau *et al*., 2004). Femurs and tibia from week 19 to 23 gestational foetuses will be implanted subcutaneously into both hind flanks of 6 to 8 week old SCID mice (McCune *et al*., 1988). Four weeks later, MM cells will be labeled by transfection with green fluorescent protein (GFP), and injected directly into the right flank human bone chip implant, while PBS is injected into the left implant. At two week intervals after treatment, the mice will be evaluated for MM proliferation and establishment of disease in the human bone chip.

5. CONCLUSION

CD137 treatment of MM cells inhibits proliferation and induces cell death via apoptosis. This happens despite the induction of the pro-survival cytokines IL-6 and IL-8. Since CD137 functions as a co-stimulatory molecule on B cells, the results presented above suggest AICD as a possible mechanism of cell death. In addition, the significant inhibitory effects exerted by CD137 are exclusively seen with MM cell lines, and not with non-MM B cell lymphoma lines. If a similar selective targeting and elimination of MM cells in patients could be achieved, that could help to slow down the progression of the disease. It could also be used in conjunction with conventional treatment regimes such as thalidomide and dexamethasone, and thereby allowing a reduction in the dose of chemotherapy and hence reduced side effects. A reduction in the dosage needed to achieve efficacy would allow patients to lengthen their use of therapeutic agents while reducing the frequency and/or severity of side effects.

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APPENDIX I - MATERIALS FOR CELL CULTURE

1. Iscove's Modified Dulbeccos Medium (IMDM)

To prepare 1L of media:

Item	Quantity	Source
IMDM powder	17.7 g	Sigma-Aldrich (St Louis, MO)
L-glutamine (100x)	10 ml	Gibco, Invitrogen (Carlsbad, CA)
Sodium bicarbonate	3.02 g	US Biological (Swampscott, MA)
MiliQ water	1 L	-

The medium was then sterile-filtered through a 0.22 μm filter membrane.

2. IMDM-10

To prepare 500 ml of media:

Item	Quantity	Source
IMDM media	450 ml	-
Foetal Bovine Serum	50 ml	Biowest (Nuaville, France)

3. RPMI-1640

To prepare 1L of media:

Item	Quantity	Source
RPMI powder	16.35 g	Sigma-Aldrich (St Louis, MO)
L-glutamine (100x)	10 ml	Gibco, Invitrogen (Carlsbad, CA)
Sodium bicarbonate	2.0 g	US Biological (Swampscott, MA)
MiliQ water	1 L	-

The medium was then sterile-filtered through a 0.22 μm filter membrane.

4. RPMI-10

To prepare 500 ml of media:

Item	Quantity	Source
RPMI-1640 media	450 ml	-
Foetal Bovine Serum	50 ml	Biowest (Nuaville, France)

5. Phosphate Buffered Saline (PBS)

To prepare 1 L of solution:

Item	Quantity	Source
NaCl	8 g	Sigma-Aldrich (St Louis, MO)
KCl	0.2 g	Sigma-Aldrich (St Louis, MO)
Na_2HPO_4	1.44 g	Sigma-Aldrich (St Louis, MO)
KH_2PO_4	0.24 g	Sigma-Aldrich (St Louis, MO)
MiliQ Water	1 L	-

The solution was sterilized by autoclaving after its pH was adjusted to 7.4.

6. MACS buffer

To prepare 1 L of buffer:

Item	Quantity	Source
PBS	1 L	-
Bovine Serum Albumin	5 ml	Biowest (Nuaville, France)
EDTA	4 ml	1st Base (Singapore)

The medium was then sterile-filtered through a 0.22 μm filter membrane.

APPENDIX II - MATERIALS FOR FLOW CYTOMETRY AND ELISA

1. FACS buffer

To prepare 500 ml of buffer:

Item	Quantity	Source
FBS	2.5 ml	Biowest (Nuaville, France)
NaN_3	0.1 g	Sigma-Aldrich (St Louis, MO)
PBS	500 ml	-

2. 0.05 M Phosphate Citrate buffer (for TMB substrate solution)

To prepare 100 ml of buffer:

Item	Quantity	Source
0.2 M Na_2HPO_4 solution	25.7 ml	Sigma-Aldrich (St Louis, MO)
0.1 M citric acid	24.3 ml	Sigma-Aldrich (St Louis, MO)

The pH of the solution was adjusted to 5.0, and the volume was topped up to 100 ml with MiliQ water.

3. TMB substrate solution

To prepare 10 ml of solution:

Item	Quantity	Source
TMB tablet	1 tablet	Sigma-Aldrich (St Louis, MO)
0.05 M phosphate citrate buffer	10 ml	-
30% H_2O_2	2 μl	Kanto Chemicals (Japan)

4. PBST

To prepare 1 L of solution:

Item	Quantity	Source
Tween-20	500 μl	Bio-Rad (Hercules, CA)
PBS	1 L	-