Institute of Child Health

MUTZ-3: A potential in-vitro model for the generation of Dendritic-like cells

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ABSTRACT

Dendritic Cells (DCs) are key regulators in autoimmunity. DCs function as antigen presenting cells (APCs) and are present in trace amounts in virtually all organs of the human body. DCs activate and regulate naive T cell function by inspecting T cell function to identify specific T cells for the antigen presented on the DC surface as peptide-MHC molecules.

Currently DCs harvested from blood and bone marrow possess individual variability in function and phenotype expression. Thus the need for standardised DC model is essential to enable more efficient and controllable studies into DC function as key immunoregulatory cells.

Previous studies have shown myeloid DCs generated from MUTZ-3 myeloid leukaemic cell line precursors to be a potentially suitable model for DC function studies.

In this study we aimed to establish MUTZ-3 as a model for DCs by incubating with various cytokines (TPO, IL-3, FLT3L, MCSF and SCF). The phenotype of the MUTZ-3 was analysed by flow cytometry to ensure the monocyte surface marker, $CD14^+$, was optimally expressed. Further differentiation of these monocytes to immature DC was investigated by stimulating the MUTZ-3 with GM-CSF and IL-4.

The results obtained are preliminary and require repeating, however, most have been shown to be reproducible. It was observed that cytokines can differentiate MUTZ-3 to optimise $CD14^+$ monocytic expression. In addition, GM-CSF and IL-4 were shown to differentiate these monocytes to immature-DC. Finally, retinoic acid was used to differentiate the MUTZ-3. However, it is vital to continue investigating to establish the most efficient method for $CD14^+$ expression is optimised.

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ABBREVIATIONS

α-MEM	Alpha-modified Minimum Essential Medium Eagle
AML	Acute Myelogenous Leukaemia
APC	Allophycocyanin
ATRA	All-Trans Retinoic Acid
CD	Cluster of Differentiation
CSF	Colony Stimulating Factor
DC	Dendritic Cell
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FLT3L	FMS-like tyrosine kinase 3 ligand
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HBSS	Hanks' Buffered Solutions
IL-3	Interleukin-3
IL-4	Interleukin-4
LPS	Lipopolyssacharide
MAb	Monoclonal Antibody
MCSF	Macrophage Colony Stimulating Factor
MHC	Major Histocompatibility Complex
MSE	Monocyte-Specific Esterase
MPO	Myeloperoxidase
TNF-γ	Interferon-γ
TPA	Tetradecanoyl-phorbol-13-acetate
TPO	Thrombopoietin
TRAP	Tartrate Resistant Acid Phosphatase
PBMo	Peripheral Blood Monocytes
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
RA	Retinoic Acid
rpm	Revolutions per minute
v/v	Volume to volume
w/o	Without

CHAPTER 1

Introduction

1.1 The role of Dendritic Cells (DCs)

DCs function as antigen presenting cells (APCs) and are present in trace amounts in virtually all organs of the human body. DCs activate naive T cells *in vivo* and *in vitro* by inspecting T cell function to identify specific T cells for the antigen presented on the DC surface as peptide-MHC molecules (Howard et al., 2004). Some DCs are equipped with additional mechanisms to regulate the response of the T cells they activate, while others can interact with B cells and modify B cell responses (Dubois et al., 1999). DCs can also decommission T cells if they present a threat to the integrity of the host, thus if the tolerogenicity of DCs could be reliably exploited, they could be administered for autoimmunity, while enhancing their immunogencity could enable them to act against tumour-associated antigens (Liu et al., 2001; Morrison, III et al., 2003; Mahnke and Enk, 2005).

1.1.1 Major function of DCs

DCs have been identified from various populations and defined due to their origin and general properties. All types of DCs appear to pass through several levels of maturation during their life-span. Immature DCs express low levels of major histocompatibility complex (MHC) class II and co-stimulatory molecules; however, the surface expression of these glycoproteins is dramatically up-regulated during maturation in response to appropriate stimuli. Additionally, terminally differentiated DCs express specific maturation markers, such as MHC Class II and CD83 on human mature DCs (Frasca et al., 2003; Rescigno et al., 1998; Kawamura et al., 2005; Moghaddami et al., 2005).

Peripheral DCs are specialised to endocytose or macropinocytose antigens and pathogens, whereas maturating DCs lose this ability but significantly enhance their stimulatory properties for naive CD4 T cells. Research has shown that liver-derived DCs and lymphoid-derived DCs are also able to down-regulate immune responses (Takahara et al., 2002; Dieckmann et al., 2005; Li et al., 2005). However, these contrasting subtypes of DCs are represented almost exactly by their immature phenotype (i.e. $CD1a^{+}CD14^{-}$, $CD40^{-}$, $CD54^{-}$, $CD80^{-}$, $CD86^{-}$ and low Class II^{+}).

Immature DCs exist in peripheral tissues, where they monitor foreign antigens and microbial pathogens. Once activated, immature DCs undergo maturation and migrate to the lymph nodes. DC maturation involves the redistribution of MHC molecules from intracellular endocytic compartments to the cell surface, a selective reduction of antigen acquisition and a significant increase in the surface expression of clusters of differentiation (CD) molecules. During maturation DC morphology alters with the development and reorganisation of the cytoskeleton to form dendrites, as well as the surface expression of various chemokine and integrin receptors. These enable the mature DCs to prime naive T cells and initiate primary T cell-mediated immune responses (Ross et al., 2000; Al Alwan et al., 2001). Thus, DCs play a crucial role during the initiation and regulation of immune responses.

1.1.2 Sources of DCs

Dendritic cells are most commonly harvested from bone-marrow or peripheral blood, for experimental and clinical studies. (Ashton-Chess and Blancho, 2005; Sato et al., 1998). This method provides a reasonable number of monocyte-derived DCs to be obtained. Such DCs remain stable and can be matured, which is preferable for the

purpose of immunotherapy. Harvesting and deriving DCs by this method also reduces the possibility of infection or immunogenic reaction that might exist when proteins such as foetal calf serum (FCS) are added to maintain DCs in culture (Lehner et al., 2005). This is because any addition to cells in culture requires careful aseptic technique and also proteins such as FCS can clump in suspension and this can lead to increased debris. Despite this method being the common one, DCs have still not been standardised. Research shows that DCs generated by monocyte derivation are dependent on certain cytokines as well as the culture media, though the role of culture media in DC generation is unclear (Encabo et al., 2004; Masterson et al., 2002) .

There are at least two distinct sources of DC precursors present in human blood: the myeloid and lymphoid DC precursors (Sato et al., 1998). However, DCs have a great deal of plasticity in their expression and influence of T cell responses, thus the concept of different DC lineage and function has been increasingly studied.

1.1.3 DC phenotype expression

Research has shown that the DC precursor cells are non-adherent and negative for MHC class II whilst mature DCs expressed high levels of MHC class II, displayed dendritic morphology (e.g. dendrites) and were efficient stimulators of allogeneic T lymphocytes (Reid, 1997; Moghaddami et al., 2005; Kawamura et al., 2005; Hubert et al., 2004; Frasca et al., 2003). Mature DC phenotype has been characterised by surface expression of $CD14^{-}CD1a^{+}$ or $CD14^{+}CD11c^{-}$, high MHC class II, $CD80^{+}$, $CD83^{+}$ and $CD86^{+}$.

In DCs, unlike in other APCs, the MHC class II peptide complexes are transported to the cell surface during maturation, therefore immature DCs express lower MHC class II on their surface as most molecules remain in late-endosomal and lysosomal compartments whereas in mature DCs, almost all MHC class II molecules are expressed at the cell surface.

1.2 Cytokines

Cytokines include lymphokines produced by lymphocytes, monokines produced by monocytes and macrophages and interleukin produced when one cytokine from a leukocyte acts on other leukocytes. Cytokines are soluble extra-cellular hormone-like proteins that act as intracellular chemical mediators to regulate the intensity and duration of immunity, inflammation, and haematopoiesis by acting locally in a paracrine or autocrine rather than endocrine manner, over short distances and short time spans at very low concentration. They bind to specific membrane receptors, causing the cell to alter gene expression via second messengers such as tyrosine kinases. Cytokines can vary in activity, however many are redundant in their activity, meaning similar functions can be stimulated by different cytokines, whilst others are produced in cascade when one cytokine stimulates the production of another. Cytokines are also able to act synergistically when two or more cytokines are required for a specific effect, or in contrast, antagonistically when the cytokines oppose each others activities (Zou and Tam, 2002; Masterson et al., 2002).

1.2.1 Cytokine receptors

Growth factors and cytokines are often linked due to their similar receptors, for example, growth hormone is considered by many to be a cytokine because the growth

hormone receptor is similar to the cytokine receptors. The subfamilies of cytokines are formed either by their function or histology. For example interleukins (IL) IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF) are classed in a family due to their unique cytokine-specific receptor.

Haematopoietic cytokine receptors have been characterised as having two subunits, one cytokine-specific and one signal transducing. Thus, for example, these two subunits can dimerise when either GM-CSF or IL-3 bind with low affinity while another subunit signal transducer increases cytokine-binding affinity. This dimerised unit associates with tyrosine kinases in the cytoplasm which through phosphorylation activates transcription of effector proteins. GM-CSF and IL-3 act on haematopoietic stem and progenitor cells to induce monocyte activation and immature DC differentiation. GM-CSF has been shown to be the key cytokine for the final maturation process of DCs, while macrophage colony-stimulating factor (MCSF) has been shown to be ineffective (Zou and Tam, 2002).

MSCF, Stem cell factor (SCF) and FLT3-ligand (FLT3L) are early acting cytokines which share similar receptors that have tyrosine kinase activity and are expressed on primitive progenitor cells. Research using suspension cultures and assays of human $CD34^+$ haematopoietic cells suggests that addition of either SCF or FLT3L leads to higher DC production than with only GM-CSF and IL-4 (Encabo et al., 2004). However, it is still unclear whether FLT3L, MCSF and SCF are act as solely synergistic factors that enhance DC production by expanding already committed lineage-restricted precursors, or whether they act as permissive factors by inducing proliferation of very primitive haematopoietic cells that then become capable of

responding to GM-CSF and IL-4. GM-CSF and IL-4 function to differentiate mature and functional myeloid DC from early human progenitors (Kelly-Welch et al., 2005). This function is said to be diminished in the absence of early acting cytokines. Research on bone marrow cell lines has shown early acting cytokines such as MSCF, SCF and FLT3L cooperate with GM-CSF to induce and maintain long-term DC differentiation (Sumimoto et al., 2002; Hope et al., 2000). FLT3L has been shown to substantially increase the number of myeloid DCs in the blood of mice and that it also increases the numbers of myeloid DCs. FLT3L is known to act synergistically with thrombopoietin (TPO) to induce *in vitro* generation of large numbers of $CD14^+$ monocytes and $CD11c^+$ immature DCs (Hope et al., 2000).

1.2.2 The effect of cytokines on DC lineage

Immature DC are found in peripheral tissues and are characterised by high ability to acquire antigens, high intracellular MHC class II expression, and low expression of co-stimulatory molecules such as CD86. When DC maturation occurs, the acquisition of antigens is down-regulated, while the surface expression of co-stimulatory molecules is up-regulated and MHC class II molecules are translocated from intracellular compartments to the cell surface. As DCs are rare *in vivo*, most of the DC maturation studies have been conducted *in vitro* (Weigel et al., 2002; McRae et al., 2000). DCs can be harvested *in vivo* from blood or bone-marrow, however, the *in vitro* administration of the hemopoietic growth factors (such as FLT3L, IL-3 and GM-CSF) permits a greater number of DCs to be generated *in vitro*. Increasingly research on DCs is being conducted on larger populations of DCs from either $CD34^+$ proliferating progenitors or $CD14^+$ non-proliferating monocytic precursors (Zou and Tam, 2002; Ratta et al., 1998).

Human $CD34^+$ haematopoietic progenitor cells that have been purified from blood or bone-marrow have been shown to differentiate into DCs after stimulation with cytokines GM-CSF and IL-4. However, both SCF and FLT3L have been able to increase the DC yield as they probably expand the precursor cells, rather than inducing DC differentiation.

Studies on progenitor cells show that monocytes are able to develop into macrophages or develop directly into DCs. Blood monocytes can be induced to differentiate into a form of DCs. The treatment of these monocytes with GM-CSF and IL-4 produces monocyte-derived DC with an immature DC phenotype, and these immature DCs can undergo maturation when stimulated by other signalling molecules such as lipopolyssacharide (LPS) (Espuelas et al., 2005).

Figure 1. Myeloid-related DC development pathways. *This illustration, courtesy of Berlex, depicts the diverse pathways for DC formation. The two myeloid-derived pathways: one directly from blood monocytes without division and the other by division and differentiation of a common myeloid precursor cell, all under the influence of GM-CSF.*

As seen in figure 1, myeloid DCs of are close relatives of macrophages and may be generated by different developmental pathways. Much research is continuously being conducted to establish whether these differences in origin influence the biological function of DCs. The DCs generated by each lineage have been shown to display functional specialisations as well as presenting antigens to activate naive T cells. These differences make it harder to standardise DC functions.

1.2.4 Clinical importance of DCs

DCs are the only APC that can initiate immune response. Therefore, science aims to exploit this attribute to enable DCs to be used for antigen-specific immunotherapies for cancers and infectious diseases (Grunig et al., 2005; Hayashi et al., 2005). Thus monocyte-derived immature DCs isolated from a patient are loaded with antigen using synthetic peptides, dead tumour cells or infected cells after which they are matured and injected into patients during clinical trails (Figure 2).

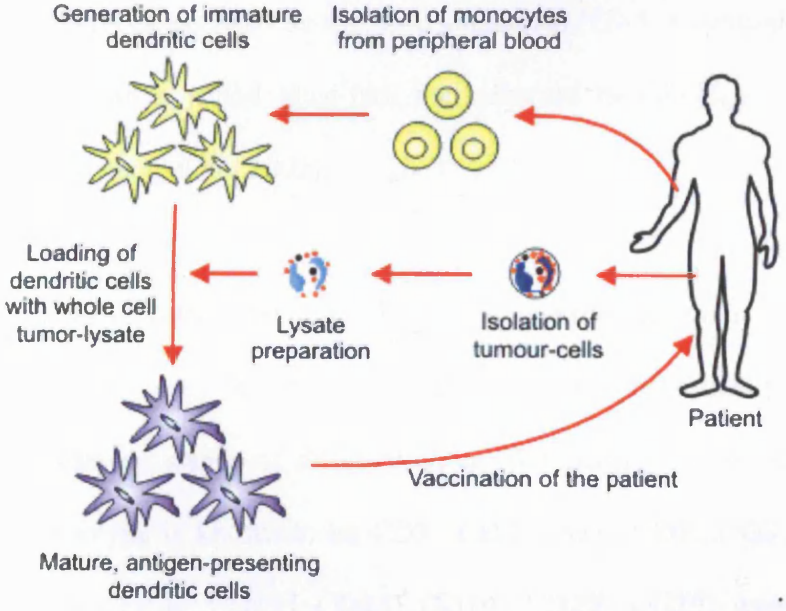

Figure 2. Shows the use of DCs in clinical applications. (H.Bohnenkamp, 2004) *Vaccination with DCs leads to protective immunity against infections and tumours.*

1.2.5 The importance of a dendritic cell model

A DC-like model cell line would provide a standard on which to base investigations. This is because DCs are of heterogeneous hemopoietic lineage, in that subsets from different tissues have been shown to possess considerable variability in morphology, phenotype and function. Therefore, recent studies are aimed at developing DC-like cell line models to establish reproducible and efficient methods to generate adequate numbers of fully functional DCs for clinical applications (Berges et al., 2005).

1.3 MUTZ-3 as a potential DC-like model

New *in vitro* research by Masterson *et al.* shows that myeloid DCs, generated from leukaemic cell line precursors expressing $CD34^+$, can develop into Langerhan or interstitial DCs. Masterson *et al.* have suggested that this may also share a potential for differentiation to DC-like APCs, thus providing a ready supply of DC precursors from which DCs can be efficiently generated. This cell line has been derived from tumours of myeloid lineage and named MUTZ-3: a continuous human leukaemia cell line with a round blast-like non-adherent morphology that grows as single cells (Masterson et al., 2002).

MUTZ-3 (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany) is a cell line containing mixed population of myeloid cells at varying stages of differentiation. The DSMZ cell catalogue states that MUTZ-3 phenotype is known to be $CD3^-$, $CD4^-$, $CD5^-$, $CD7^-$, $CD8^-$, $CD10^-$, $CD11b^-$, $CD13^+$, weak $CD14^+$, $CD15^+$, $CD16^-$, $CD19^-$, $CD20^-$, $CD25^-$, weak $CD30^+$, $CD33^-$, $CD34^+$, $CD38^+$, $CD41a^-$, $CD42b^-$, $CD56^-$, $CDw65^+$, $CD68^+$, $CD71^-$ and low MHC-Class II^+. MUTZ-3 also express monocyte-specific esterase (MSE), myeloperoxidase (MPO)

and tartrate resistant acid phosphatase (TRAP) enzymes, thus exhibiting monocytic behaviour (Hu et al., 1996). Hu *et al.* have also shown that MUTZ-3 can undergo sustained and continuous growth with the addition of cytokines to the culture medium and must be grown in conditioned medium for proliferation and survival. GM-CSF, M-CSF, SCF and IL-3 have been shown to be the most effective growth factors in inducing proliferation and differentiation of MUTZ-3. Also, Hu *et al.* exposed MUTZ-3 to phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and the physiological all-trans retinoic acid (ATRA), and both had growth-inhibitory and differentiation-inducing effects on MUTZ-3 (Hu et al., 1996).

As mentioned above, MUTZ-3 cell line can only undergo sustained and healthy growth when conditioned medium is present. This conditioned medium is sourced from a secondary cell line: The 5637 cell line is an epithelial-like adherent cell line which has been described as producing various growth factors (e.g. SCF, IL-1, IL-6, GM-CSF, etc) and though the exact cocktail of factors is unknown, the medium from 5637s is crucial for the development and growth of the MUTZ-3 cell line (Masterson et al., 2002).

MUTZ-3 has been reported to down-regulate CD14 expression when stimulated by IL-4 and GM-CSF and thus is valuable for investigating the complex cytokine and molecular signalling pathways of DCs (Hu et al., 1996; Quentmeier et al., 1996). This cell line has also been shown to acquire a phenotype consistent with either interstitial or Langerhan-like DCs ($CD83^+$) when stimulated by specific cytokine cocktails. Masterson *et al.* have suggested that MUTZ-3-derived DCs display the functional MHC complexes and antigen markers, therefore, MUTZ-3 cells could provide an

unlimited source of $CD34^+$ DC progenitors which could be used for research into cytokine-induced DC differentiation and DC functional studies (Hu et al., 1996; Steube et al., 2000; Quentmeier et al., 1996; Kharfan-Dabaja et al., 2005; Fazekas De St et al., 2002).

1.4 Aims

Most studies on DC function and molecular pathways have been conducted on DCs harvested from human or mouse blood and bone-marrow. These possess too much individual variability and the process to purify DCs from blood is inefficient with only a limited number of cells being available for research despite taking a relatively long time to process the blood or bone-marrow. Thus the need for a standardised DC model is essential to enable more efficient and controllable studies into DC function as key immunoregulatory cells.

Previous studies have suggested MUTZ-3 to be a potentially suitable model for DC function studies(Reid, 1997). Therefore it is important to independently establish a model to generate a supply of dendritic-like cells based on today's understanding of DCs and their progenitor lineages.

As DCs cannot proliferate or differentiate further, it is important to maintain a model cell line which could viably be grown in mass and induced to differentiate into DCs when required for DC studies. Therefore, we aim to bulk MUTZ-3 cell stocks and induce differentiation of MUTZ-3 precursors to monocytes. As CD14 is a key marker expressed on the surface of monocytes, we aim to optimise the yield of $CD14^+$ monocytes from MUTZ-3 cultures using various cytokine cocktails. Also, we aim to demonstrate that MUTZ-3 monocytes can be differentiated to immature DC using GM-CSF and IL-4.

This initial data will help us optimise a model for the study of dendritic cell signalling pathways & thus molecular events initiating adaptive immunity.

CHAPTER 2

Materials and Methods

2.1 Solutions and Materials

Complete αMEM$^+$	SIGMA® αMEM containing 20% (v/v) FBS, 10μg/ml penicillin/streptomycin and 10% (v/v) conditioned medium of cell line 5637[1]
Complete αMEM$^-$	SIGMA® αMEM containing 20% (v/v) FBS and 10μg/ml penicillin/streptomycin
Complete RPMI	GIBCO™ RPMI 1640 containing 10% (v/v) FBS and 10μg/ml penicillin/streptomycin + 2mM L-Glutamine
Formaldehyde	3.7% (v/v) formaldehyde in HBSS. (Sourced from Jane Rasaiyaah)
Complete HBSS	Containing 10% filtered human serum and 0.1% sodium azide
Retinoic Acid	1μM, 100nM, 10nM in complete αMEM +/- conditioned medium of cell line 5637. (Sourced from Jane Rasaiyaah)
Cytokines: TPO, IL-3, FLT3L, MCSF, SCF	Bought as 2μg each from www.immunotools.com Each diluted in αMEM to give 25ng/ml. 40μl stored in 1ml cryogenic vials at -80°C.

[1] **5637** is an epithelial-like adherent cell line from human urinary bladder carcinoma established from the primary bladder carcinoma of a 68-year-old man in 1974. Cells described to produce several growth factors (e.g. SCF, IL-1, IL-6, G-CSF, GM-CSF, etc.)

Conjugated antibodies and their specificity for flow cytometry

CD1a	PE-conjugated anti-human IgG
CD3	APC-conjugated anti-human IgG
CD13	PE-conjugated anti-human IgG
CD11c	APC-conjugated anti-human IgG
CD14	APC-conjugated anti-human IgG
	PE-conjugated anti-human IgG
CD19	PE-conjugated anti-human IgG
CD45	APC-conjugated anti-human IgG
Class II	PE-conjugated anti-human IgG
Secondary Antibody	PE-conjugated anti-human IgG

N.B:

All conjugated antibodies were purchased from BD Pharmigen™ in 2ml vial. These were diluted as required at the time of use – this was dependant on their effectiveness which was tested by a titration of antibody concentration (data not shown) - majority of the antibodies were diluted 10µl-15µl in 50µl of HBSS at the time of use.

The non-conjugated monoclonal antibodies (CD1a, CD3, CD11c, CD14, CD45, CD83, CD86, Class I and Class II) were sourced from Jane Rasaiyaah.

2.2 Preparation of 5637 conditioned media

5637 adherent cell line was maintained alongside the MUTZ-3 and its medium stored in 5ml aliquots at -80°C for use in complete αMEM$^+$.

The 5637s were cultured in complete RPMI for 7 days at 37°C till 95% confluency. The culture medium was aspirated from the 150mm dish into a 50ml Falcon tube. Then 4ml trypsin was added to the dish and incubated for 5-7 minutes till the cells where visibly in suspension. This trypsinised suspension was quenched in 3ml complete RPMI in a 15ml Falcon tube. The cells where harvested by centrifugation of the cell suspension for 10 minutes at 1200rpm to remove debris. The previously aspirated medium was also centrifuged and the supernatant transferred to a fresh 50ml Falcon tube and centrifuged again to remove all cell and debris content. The cell pellet from the quenched suspension in the 15ml Falcon tube was then resuspended to split the cells 1:2 dilution in complete RPMI and returned to the incubator (37°C, 5% CO_2) to culture for 7 days. Finally the conditioned medium from the 50ml falcon tube was transferred to 5ml Bjoux tubes and stored at -80°C till required.

2.3 MUTZ-3 culture

At 95% confluency, MUTZ-3 non-adherent cell culture was aspirated from T25 flask into a 15ml Falcon tube and centrifuged for 10 minutes at 1200rpm. The supernatant was discarded and the cell pellet resuspended for a 1:2 split by resuspending cells in 4ml complete αMEM$^+$. Finally 2ml/flask was transferred to 2 fresh T25 flasks containing 8ml media complete αMEM$^+$. These flasks where incubated horizontally for 4-5 days at 37°C and 5% CO_2 saturation with a density of $1.5x10^6$ cells/flask.

2.4 Cell Counting

Viable cells were counted using a Neubauer Improved Haemocytometer by trypan blue (0.4%, Sigma®) exclusion and phase contrast microscopy.

2.5 Staining MUTZ-3 for phenotypic expression

The MUTZ-3 cells were bulked up to 95% confluency in a T25 flask before being analysed by flow cytometry.

MUTZ-3 cells were transferred into a 15ml Falcon tube, counted and $6x10^5$ cells were centrifuged at 1200rpm for 10 minutes. The supernatant was disposed of and the cell pellet resuspended in 600µl of complete HBSS (100µl/stain). After incubating for 50-60 minutes on ice, the cells were transferred to 10 wells of a 96-well round-bottomed plate (100µl/well). Leaving one well unstained, the remaining 9 wells were individually incubated for 60 minutes with primary non-conjugated monoclonal antibodies. Then the cells were washed by pipetting 50µl complete HBSS into each of the 10 wells and the plate centrifuged at 1800rpm for 5 minutes before flicking the supernatant off carefully so as to not lose cell pellets - this was repeated 3 times. Then (PE labelled) secondary antibody was pipetted into each of the 5 wells and this was incubated on ice for 60 minutes (figure 3) before being fixed and analysed by flow cytometry (see section 2.9). This experiment was repeated three times to ensure the viability of data.

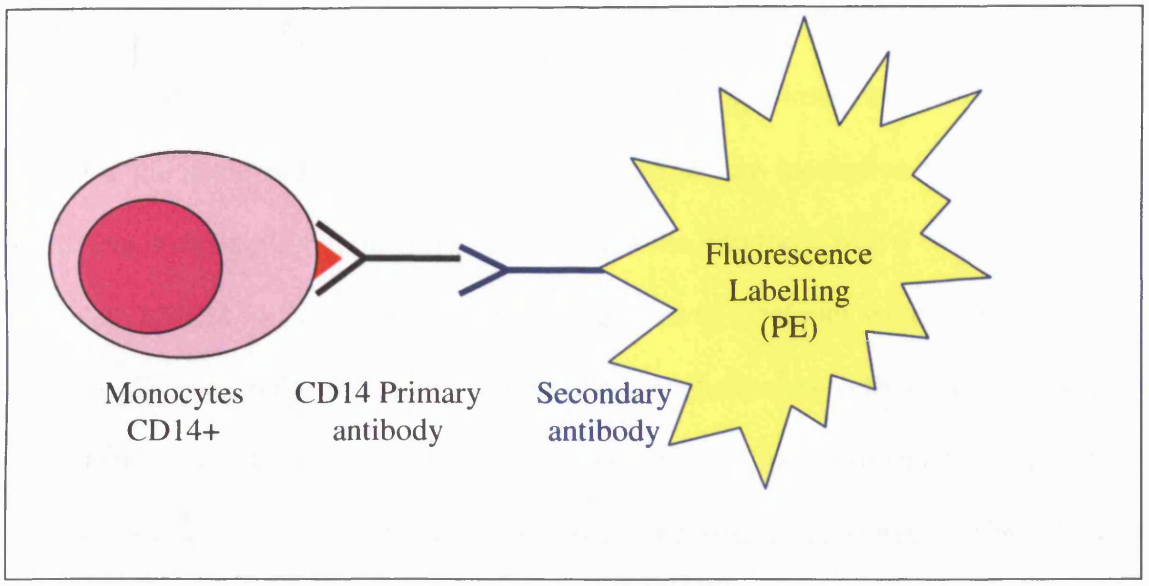

Figure 3. Flow cytometry using non-conjugated antibodies. *Monocytes expressing, for example CD14$^+$, are incubated with primary monoclonal antibodies for an hour followed by 1hr incubation with secondary antibodies that are conjugated to florescence markers.*

2.6 Preparation of MUTZ-3 for 10 day stimulation by cytokines with conditioned medium

MUTZ-3 cells were aspirated from the T25 flask into a 15ml Falcon tube and counted. Then $6x10^5$ cells were centrifuged for 10 minutes at 1200rpm and resuspended in 6ml complete αMEM$^+$. $1x10^5$ cells/well were seeded into 6 wells of a 12 well plate. Next 10µl (25ng/ml) of the relevant cytokines (see figure 4) were added to each well as follows:

- To the first well all 5 cytokines (TPO, IL-3, FLT-3L, MCSF and SCF) were added.
- To subsequent 5 wells, only 4 cytokines were added - leaving out 1 different cytokine in each well.

The 12 well plate was incubated for 2 days at 37°C with 5% CO_2 after which 1ml complete αMEM$^+$ was added to each well and 10µl of cytokines were added to each well (in the same order as before). After further 2 day incubation the cells were transferred respectively from the 12 well plate wells into those of a 6 well plate, 1ml complete αMEM$^+$ was added to each well and 10µl of cytokines were added to each well (in the same order as before). After 3 days incubation the cells were transferred from their wells into 6 separately labelled 50mm dishes. Each dish had 1ml complete αMEM$^+$ added and 10µl of cytokines added to each dish in the correct order. These plates were incubated for 2 days before being FACS stained (see figure 4).

2.6.1 Preparation of MUTZ-3 for 10 day stimulation by cytokines w/o conditioned medium

The MUTZ-3 cells were prepared as explained above, but complete αMEM$^+$ was replaced at every stage by complete αMEM$^-$.

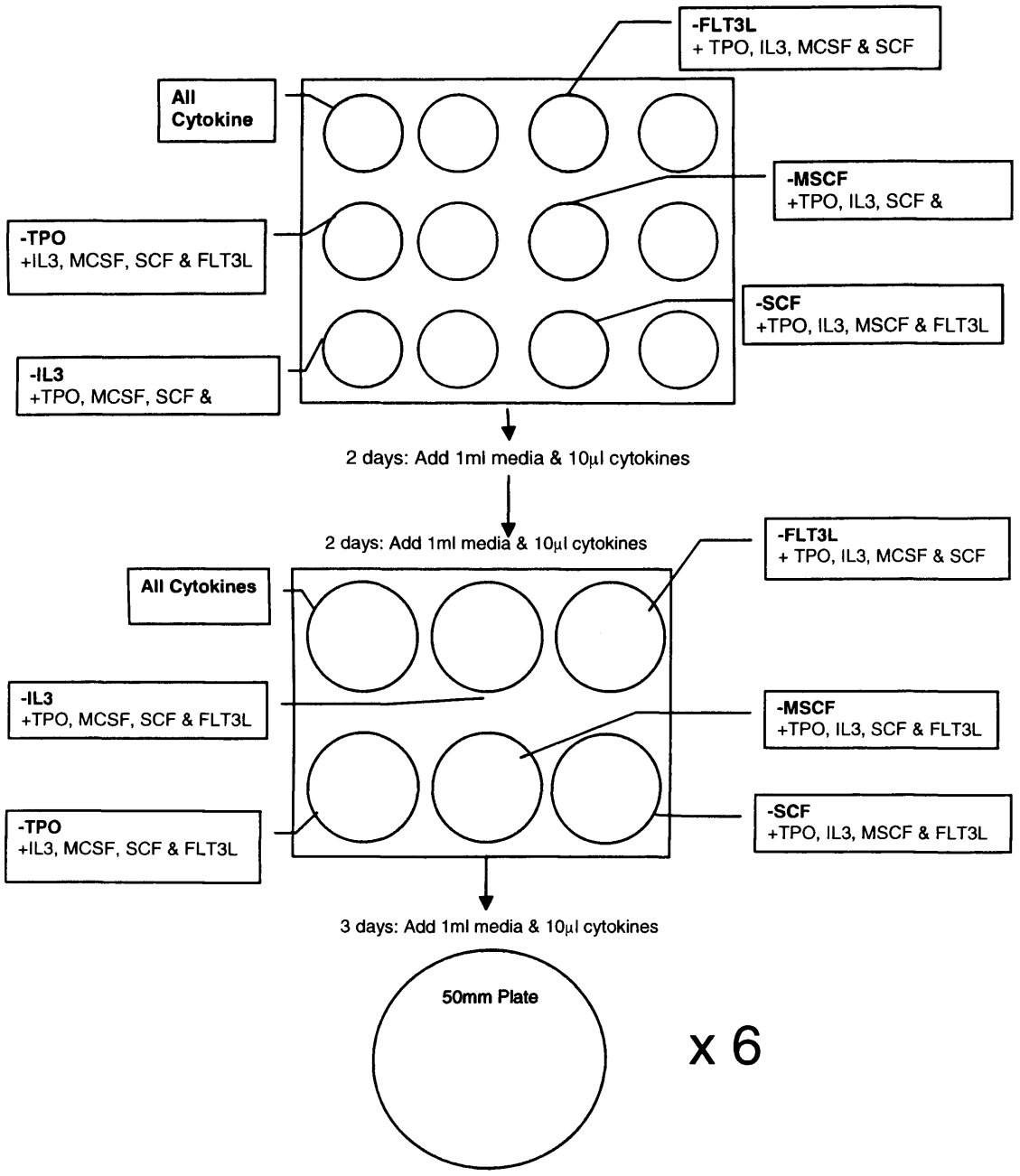

Figure 4. Diagram to show the various stages and setup of MUTZ-3 stimulated by cytokines for 10 days.

2.7 Inducing differentiation of MUTZ-3 monocytes to immature DC

MUTZ-3 cell line was stimulated with the addition of the 5 cytokine cocktail by pipetting 10µl of 25ng/ml concentration of each cytokine into a culture of $1x10^5$cells. This was repeated every 2 or 3 days for a total of 10 days, during which the cells were moved from a 12 well plate well to a 6 well plate to a 50mm dish (as explained previously). Then GM-CSF (100ng/ml) and IL-4 (10ng/ml) were pipetted and incubated for 7 days at 37°C and 5% CO_2. These plates were then counted and the cells analysed by non-conjugated antibody single staining and flow cytometry.

2.8 Preparation of MUTZ-3 for 12 hour and 24 hour stimulation by retinoic acid with conditioned medium

MUTZ-3 cells were aspirated from the T25 flask into a 15ml Falcon tube. The cells were counted, centrifuged for 10 minutes at 1200rpm and resuspended in complete αMEM^+ so as to have $4x10^5$ cells/ml to ensure sufficient cells for 3 stains (each stain requiring $1x10^5$ cells). Then 1ml was seeded into six 100mm plates. These plates were labelled as follows:

[Retinoic Acid]	**12 hr incubation**	**24 hr incubation**
1µM	$4x10^5$ cells total	$4x10^5$ cells total
100nM	$4x10^5$ cells total	$4x10^5$ cells total
10nM	$4x10^5$ cells total	$4x10^5$ cells total

2.8 1 Series dilution of RA

The plates then had 9ml of RA titre added to them as shown in the table above. The varying concentrations of RA were prepared by taking 100µl from the 100µM RA stock and adding to 9.9ml of complete αMEM^- to give a new concentration of 1µM.

Next a 1ml aliquot of 1μM RA was added to 9ml complete αMEM$^-$ giving a new concentration of 100nM. Finally, a 1ml aliquot of 100nM RA was added to 9ml of complete αMEM$^-$ giving a concentration of 10nM (figure 5).

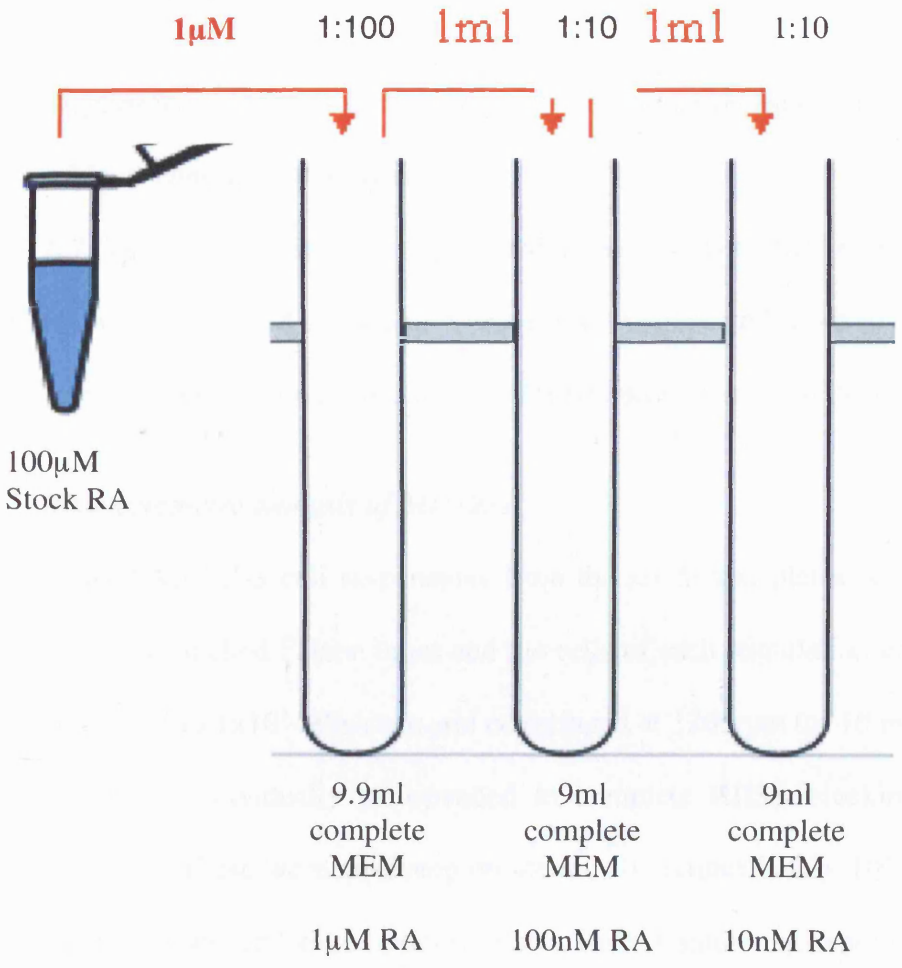

Figure 5. Dilution series of retinoic acid. *The stock RA was diluted and each tube pipetted into a 100mm culture dish into which 1ml of 4x10^5 cells was added.*

The dishes were incubated for 12hrs and 24hrs: at each time point the cell suspensions from the three RA concentrations were individually counted before being centrifuged at 1200rpm for 10 minutes.

Each cell pellet was then resuspended in 300µl complete HBSS blocking antibody for 1hr after which 100µl was aliquoted into a labelled 96-well plate to which conjugated antibodies were added and incubated for 1hr. Then the cells were fixed and analysed by flow cytometry (section 2.9).

2.8.2 Preparation of MUTZ-3 for 12 hour and 24 hour stimulation by retinoic acid w/o conditioned medium

The MUTZ-3 cells were prepared as explained above, but using the complete αMEM$^-$ instead of complete αMEM$^+$. The above procedure was repeated twice so a total of 12 dishes were incubated (3 for 12hrs and 3 for 24hrs) before being FACS stained.

2.9 Flow cytometry analysis of MUTZ-3

The stimulated MUTZ-3 cell suspensions from the six 50mm plates were aspirated into 6 separately labelled Falcon tubes and the cells of each stimulation counted. The cells were diluted to $1x10^5$ cells/stain and centrifuged at 1200rpm for 10 minutes. The cell pellets were individually resuspended in complete HBSS blocking antibody (100µl per stain). These were incubated on ice for 60 minutes before 100µl/well was transferred into a 96 well round-bottom plate. Then a saturating amount (50µl) of conjugated antibodies diluted in complete HBSS was pipetted individually to each well (see section 2.1). This was conducted with the lights off to ensure minimal degradation of the conjugated antibody fluorescence, and the plate was then wrapped in foil before being incubating on ice for 50-60 minutes after which 50µl complete HBSS was added to each well and the plate centrifuged at 1800rpm for 5 minutes. The supernatant was then flicked out of the plate, being careful not to flick out cell pellets and also avoid drops re-entering wells. A second wash by adding 50µl complete HBSS and centrifuging at 1800rpm for 5 minutes followed by flicking off

supernatant ensured any contamination through unbound or unspecific binding did not occur. Finally, to fix the cells, 50µl complete HBSS and 100µl of 3.7% (v/v) formaldehyde in complete HBSS was individually pipetted into each well and the cells transferred to a labelled flat-bottomed 96 well plate. The plate was then wrapped in foil, stored in the fridge and run on the FACS machine within 5 days. (N.B: the cells were carefully resuspended each time when adding solution to wash or fix).

The flow cytometry was performed for 10,000 events per sample on a BD Biosciences BD FACSArray™ bio-analyser using a green (532nm) and red (635nm) laser (PE 575nm, APC 600nm) and analysed using WinMDI software.

This bio-analyser is able to compensate for any cross-channel spill-over, and was setup so that the unstained and single colour positive controls for each channel (CD45APC and CD13PE) were adjacent to each other. These were followed by the negative isotype stains for each channel (CD3APC, CD19PE).

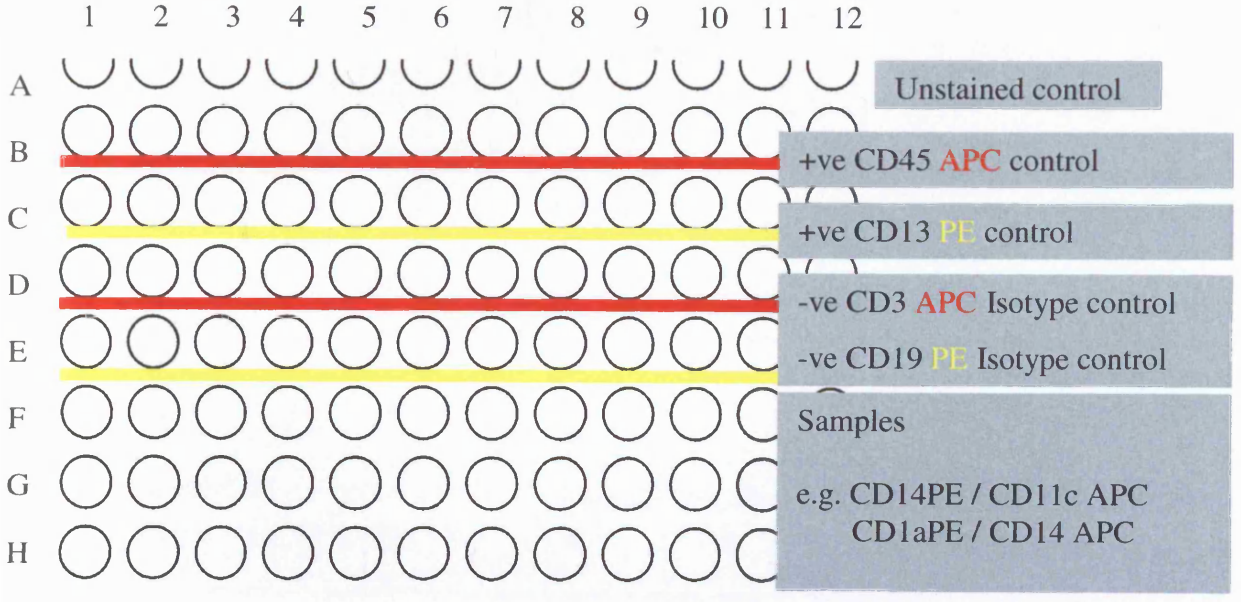

Figure 6. 96-well layout for FACS. *Diagram shows the contents and order of the wells to enable the FACS machine to compensate for spill-over and ensure correct flow cytometry analysis.*

CHAPTER 3

Preparation of MUTZ-3 for phenotype staining

3.1 Introduction

3.2 Results and Discussion

3.0 Preparation of MUTZ-3 for phenotype staining

3.1 Introduction

MUTZ-3 has been shown to exhibit dentritic-like properties when induced by growth factors. It is crucial however, to understand the phenotype of MUTZ-3 pre-stimulated by additional growth factors. It was crucial to understand the phenotype of the initial population of MUTZ-3 against which further stimulated MUTZ-3 phenotypes could be compared.

3.2 Results and Discussion

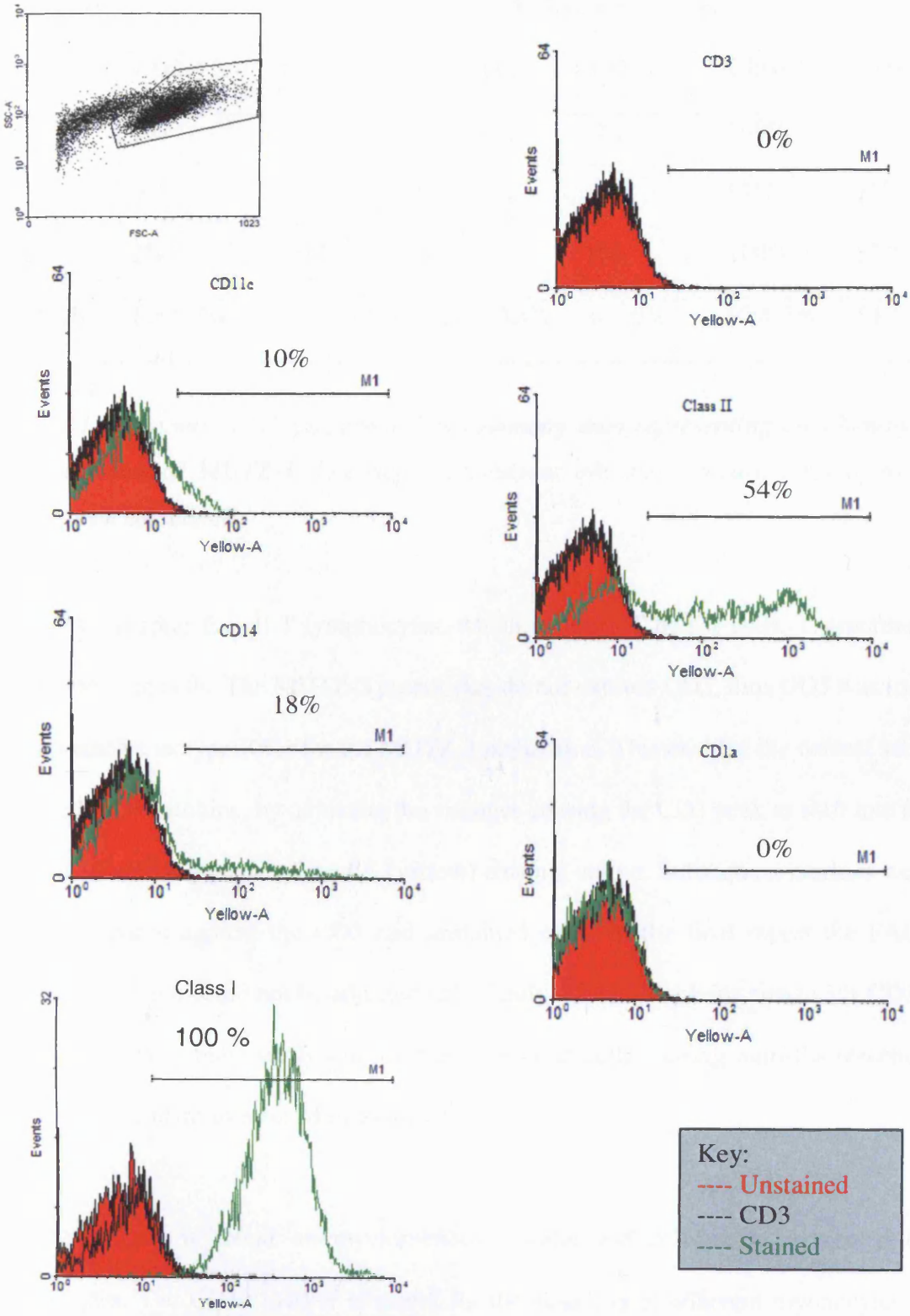

Figure 7. Phenotypic stain of MUTZ-3 cells. *Flow cytometry analysis of the initial population of MUTZ-3 grown to 95% confluency in a T25 flask. This was repeated three times.*

Data set	Surface Marker Expression					
	CD3	**CD14**	**CD11c**	**CD1a**	**Class I**	**Class II**
1	0%	18%	10%	0%	100%	54%
2	0%	23%	10%	0%	91%	55%
3	2%	19%	11%	0%	100%	52%
MEAN	1 ± 0.7%	20 ± 1.5%	10 ± 0.3%	0 ± 0%	97 ± 3%	54 ± 0.9%

Table 1. Summary of the repeated flow cytometry data representing the phenotype of unstimulated MUTZ-3. *The mean percentages with the standard error of mean have been calculated.*

CD3 is a marker for all T lymphocytes, which includes T helper cells, T suppressor and cytotoxic cells. The MUTZ-3 monocytes do not express CD3, thus CD3 was used as a negative isotype (0%) for the MUTZ-3 population. This enabled the correct setup of the FACS machine, by adjusting the voltages causing the CD3 peak to shift into the initial 10^1-10^2 log phase of the PE (yellow) channel output. Subsequent markers were then compared against the CD3 and unstained cells. In the final repeat the FACS machine voltage could not be adjusted sufficiently. Therefore giving rise to 2% $CD3^+$, however, this is most likely due to debris or dead cells causing auto-fluorescence, hence the standard of error of mean of ± 0.7%.

CD14 antigen is found on myelomonocytic cells and is strongly expressed on monocytes. The CD14 marker is useful for the detection of adherent myonocytes in normal peripheral blood and in disease states such as the identification of leukaemia and lymphoma cells of monocytic and myelomonocytic origin. It is present on blast

cells from patients with myelomonocytic leukaemia. CD14 is weakly expressed on B-lymphocytes and neutrophils, but not expressed on T-lymphocytes, red blood cells and platelets. Thus, we would expect to have expression of CD14 on the monocytes present in the MUTZ-3 population; hence $CD14^+$ is seen to be weakly expressed at only 20 ± 1.5%.

CD11c is a specific marker for monocytes and DCs, thus it is weakly expressed (10 ± 0.3%) in the mixed population of MUTZ-3. This suggests some cells in the MUTZ-3 population are exhibiting monocytic expression. Thus $CD14^+CD11c^+$ monocytes are present in the initial pre-stimulated MUTZ-3 culture.

CD1a is a human epidermal immature DC marker related to class I MHC molecules and responsible for presenting glycolipid and lipid antigens to T cells. However, the mixed population of MUTZ-3 are all $CD1a^-$ (0%), hence there are no immature DC present.

All haematopoietic cells express Class I surface antigen. All the initial pre-stimulated MUTZ-3 population expressed Class I (97 ± 3%) because MUTZ-3 is a haematopoietic cell line. MHC class II, however, is only expressed by a smaller subset of haematopoietic cells, hence 54 ± 0.9% Class II^+ cells. This is due to most the Class II remaining intracellular till the cells differentiate and mature further to mature DCs.

We can conclude that the phenotype of unstimulated MUTZ-3 precursors is low Class II^+, Class I^{++}, weak $CD14^+$ and even weaker $CD11c^+$.

CHAPTER 4

Inducing differentiation of MUTZ-3 by stimulating with cytokines

4.1 Introduction

4.2 Results and Discussion

4.0 Inducing differentiation of MUTZ-3 by stimulating with cytokines

4.1 Introduction

MUTZ-3 can be induced to behave DC-like when stimulated with growth factors such as cytokines. Indeed, the MUTZ-3 cell line must be cultured in conditioned media of cell line 5637 which are described as releasing various undefined cytokine growth factors such as SCF, IL-1, IL-6, GM-CSF, etc. It was important to establish the phenotype of MUTZ-3 after stimulation with specific cytokine cocktails. Therefore, the MUTZ-3 cell line was stimulated with the addition of 5 cytokines: TPO, IL-3, MSCF, SCF and FLT3L (see chapter 2 for further details).

4.2 Results and Discussion

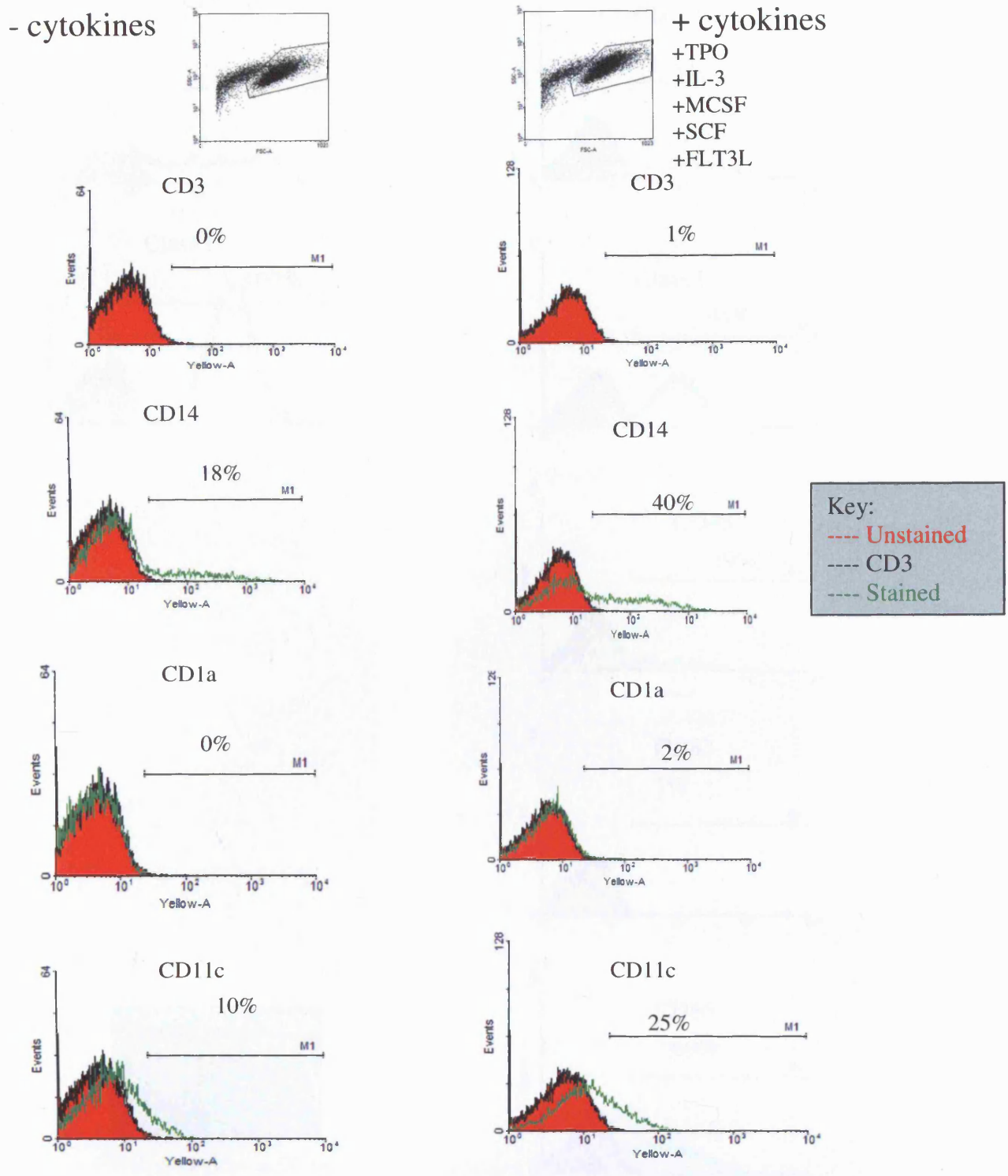

Figure 8. Phenotypic stain of MUTZ-3 cells before and after stimulation with cytokines. *Flow cytometry analysis of MUTZ-3 for the expression of CD3 (negative isotype control), CD1a, CD11c and CD14.*

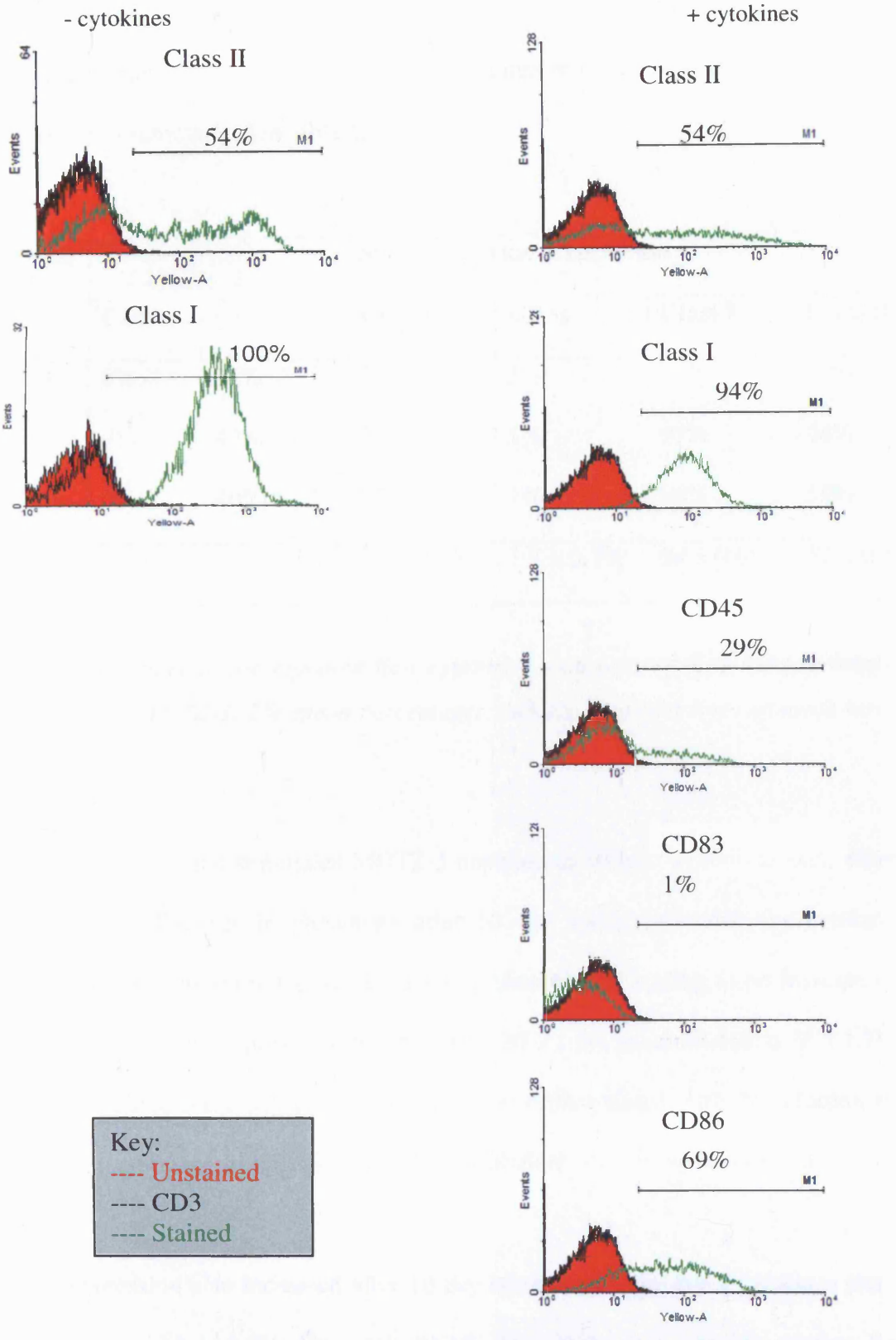

Figure 9. Phenotypic stain of MUTZ-3 cells before and after stimulation with cytokines. *Flow cytometry analysis of MUTZ-3 for the expression of Class I, Class II, CD45, CD83 and CD86.*

The 10 day stimulation of MUTZ-3 with cytokines was repeated three times. This data has been summarised in table 2.

Data set	Surface Marker Expression					
	CD3	**CD14**	**CD11c**	**CD1a**	**Class I**	**Class II**
1	0%	35%	28%	3%	95%	52%
2	0%	40%	25%	1%	93%	54%
3	0%	40%	27%	1%	94%	54%
MEAN	0 ± 0%	38 ± 1.7%	27 ± 0.9%	1.7 ± 0.7%	94 ± 0.6%	53 ± 0.7%

Table 2. Summary of the repeated flow cytometry data representing the phenotype of stimulated MUTZ-3. *The mean percentages with the standard error of mean have been calculated.*

Both unstimulated and stimulated MUTZ-3 populations were compared to each other to establish differences in phenotype after 10 day stimulation with the cytokine cocktail. The results show the MUTZ-3 had differentiated, leading to an increase of $CD14^+$ monocytes by approximately 20%, from 20 ± 1.5% unstimulated to 38 ± 1.7% stimulated. Thus, some MUTZ-3 precursors had differentiated with the addition of cytokines, thereby increasing the monocyte population.

CD11c expression also increased after 10 day stimulation with the 5 cytokines from 10 ± 0.3% to 27 ± 0.9% after stimulation. As CD11c is a specific marker for monocytes and DCs, this 17% increase can be attributed to increased differentiation of MUTZ-3 precursors to monocytes induced by addition of the cytokines.

CD1a is a marker for immature DC. CD1a expression did not increase significantly (<2%), suggesting no differentiation of monocytes to immature DC.

Class I expression was decreased by ~3% from 97 ± 3% to 94 ± 0.6% after stimulation., though Class II expression did not change significantly with a <1% decrease from 54 ± 0.9% pre-stimulation to 53 ± 0.7% post-stimulation, thus there were no mature DC present.

CD45 is a leukocyte common antigen which is expressed by the various subtypes of T cells and B cells as well as monocytes and macrophages. CD45 is present as two isoforms depending on exon banding, and these are denoted CD45RA and CD45RO. For this investigation only CD45RA was available to stain with, and after stimulation with cytokines $CD45RA^+$ is 29%, which can be attributed to the monocyte population. However, as only CD45RA was used, 29% only represents half the $CD45^+$ expression. Hence, it is important to repeat this stain with CD45RO and CD45RA or CD45, which contains both isoforms.

CD83 is a maturation marker for DC which is not expressed (~0%) in the mixed population of MUTZ-3 after stimulation with all 5 cytokines. Therefore, suggesting that the 10 day cytokine stimulation only induces the cells to differentiate to monocytes and not induce maturation to mature DC. CD86 is a monocyte and DC marker which is expressed (~70%) which can be attributed to the monocytes induced by the cytokine stimulation.

CHAPTER 5

Stimulation of MUTZ-3 differentiation by GM-CSF & IL-4

5.1 Introduction

5.2 Results and Discussion

5.0 Stimulation of MUTZ-3 differentiation by GM-CSF & IL-4

5.1 Introduction

MUTZ-3 is a myeloid cell line, therefore, to establish whether the mixed population of MUTZ-3 can be differentiated to the immature DC-like phenotypic expression, the MUTZ-3 were stimulated for 10 days by the 5 cytokines (TPO, IL-3, SCF, M-CSF, FLT3L) and then a further 7 day stimulation with GM-CSF (100ng/ml) and IL-4 (10ng/ml) which are known to induce differentiation to immature DC (figure 10).

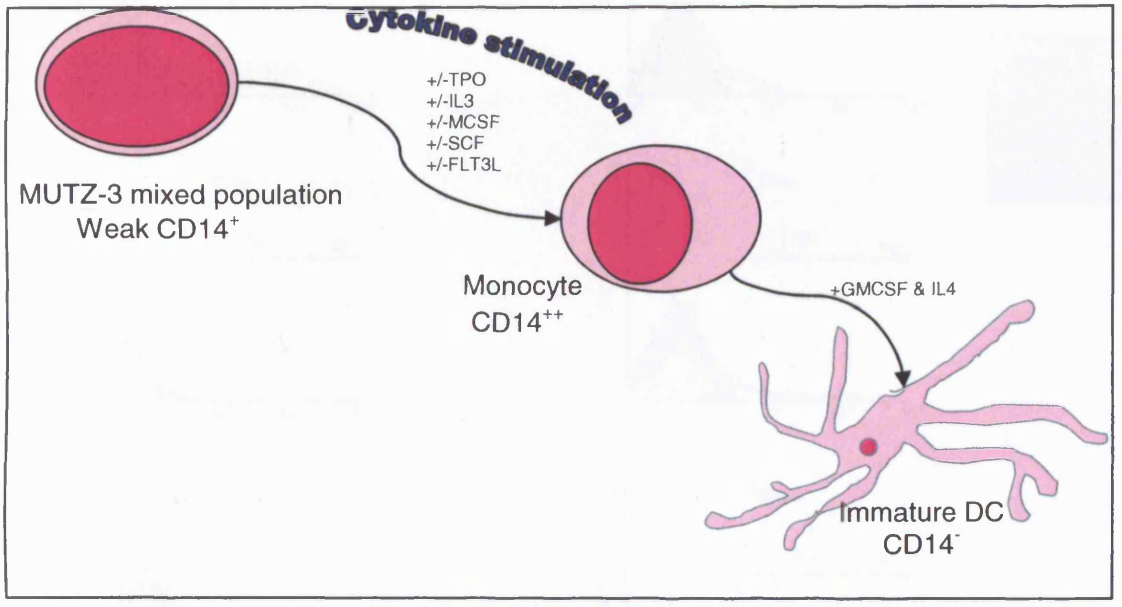

Figure 10. Differentiation of MUTZ-3*. Diagram showing differentiation of MUTZ-3 induced by GM-CSF and IL-4.*

5.2 *Results and Discussion*

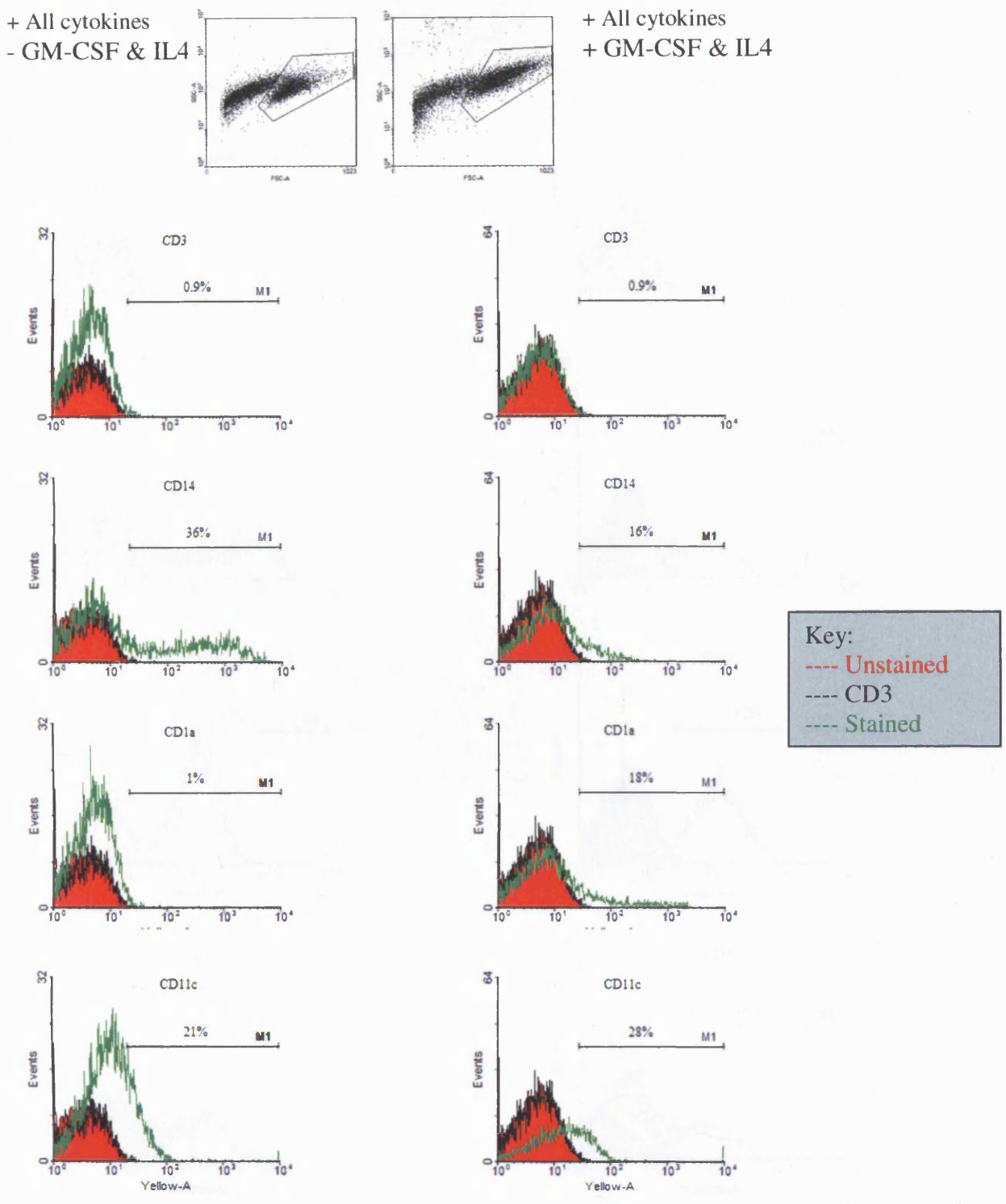

Figure 11. MUTZ-3 7 day stimulation of MUTZ-3 with GM-CSF and IL-4 after 10 day stimulation with TPO, IL-3, SCF, MCSF and FLT3L.

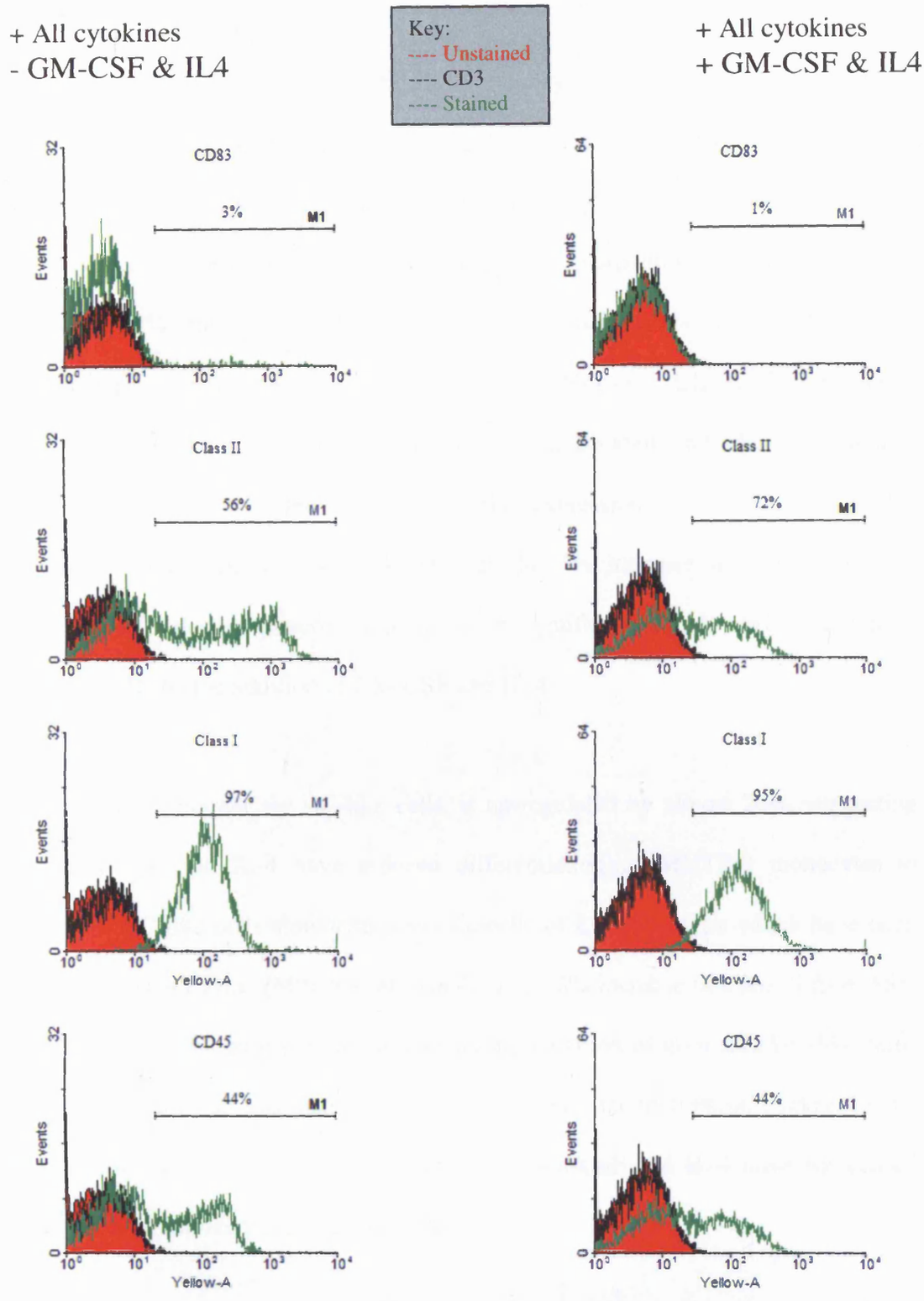

Figure 12. MUTZ-3 7 day stimulation of MUTZ-3 with GM-CSF and IL-4 after 10 day stimulation with TPO, IL-3, SCF, MCSF and FLT3L.

The GM-CSF and IL-4 induce the MUTZ-3 monocytes to differentiate into immature DC-like cells (Kelly-Welch et al., 2005; Reid, 1997). Immature DC are characterised by $CD14^{-}CD11c^{+}$, low Class II and $CD83^{-}$ phenotype. In our investigation, this phenotype is observed by the down-regulation in expression of monocyte marker CD14 by 20% from 36% to 16% after stimulation with GM-CSF and IL-4. Also, CD11c expression increases 7% from 21% to 28%. However, $CD11c^{+}$ 21% relates to MUTZ-3 after 10 day stimulation with the cytokine cocktail, and when compared to data from table 2, which shows the mean CD11c expression is 27 ± 0.9% after 10 day cytokine stimulation, it is unclear whether the 7% increase in $CD11c^{+}$ in this experiment is due to experimental errors or significant due to differentiation to immature DC by the addition of GM-CSF and IL-4.

Also $CD1a^{+}$, a marker for DC-like cells, is up-regulated by almost 20%, suggesting that GM-CSF and IL-4 have induced differentiation of MUTZ-3 monocytes to immature DC-like cells similar to dendritic cells of LC phenotype which have been shown as $CD14^{-}CD1a^{+}$ (Mitsui et al., 2004). The 16% increase in Class II from 56% to 72% can also be attributed to the increased population of immature DC-like cells. CD45 expression remains unchanged (44%), however, the maturation marker, CD83, is not expressed significantly (<3%), therefore GM-CSF and IL-4 have not caused maturation of immature DC to mature DC.

CHAPTER 6

Stimulating MUTZ-3 by varying cytokines

6.1 Introduction

6.2 Results and Discussion

6.2.1 Spill-over compensation

6.0 *Stimulating MUTZ-3 by varying cytokines*

6.1 Introduction

Up till now the MUTZ-3 were stimulated with all 5 cytokines, however, this is a very expensive procedure as cytokines cost a great deal for a very small amount – each vial of 2ml cytokines, once diluted to 25ng/ml, was sufficient for only two 10 day stimulations. It was important to establish which of the 5 cytokines was definitely required to in the cytokine cocktail to induce differentiation of MUTZ-3 to monocytes.

IL-3, MCSF & SCF are potent haematopoietic growth factors which have been suggested to activate monocytes in immunoregulatory roles. Also IL3 activity is known to require co-stimulation with other cytokines, while FLT3L cannot stimulate differentiation without synergising with other CSFs and interleukins to induce growth and differentiation. TPO is known to be a lineage specific growth factor which or may not induce monocyte formation in MUTZ-3. Thus, to reduce the amount of cytokines required for the differentiation of MUTZ-3 to monocytes, the cytokine content of each sample was varied by removing only one cytokine from the cocktail of five cytokines (see figure 4).

So far all the experiments were conducted with the cells and cytokines being in complete αMEM^{+}. However, to test the significance of the 5637 conditioned medium on the growth and differentiation of MUTZ-3, two experiments were conducted in parallel: one using complete αMEM^{-} and one using complete αMEM^{+}.

6.2 Results and Discussion

6.2.1 Spill-over compensation

To prevent spill-over contamination during the flow cytometry the wells were set up as described in chapter 1 with an unstained well followed by a CD45 APC positive stain and a CD13 PE stain for each of the two (red/yellow) channels. CD13 and CD45 are both antigens that are expressed on monocytes, granulocytes and their haematopoietic precursors, thus make ideal positive controls for these investigations (figure 13).

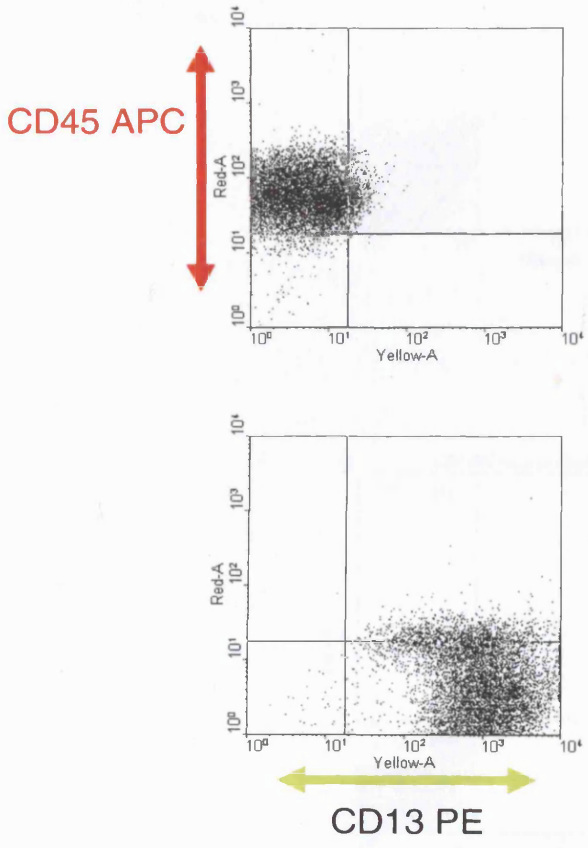

Figure 13. Positive controls. *The CD45APC and CD13PE conjugated antibodies were used as positive controls to allow for compensation during flow cytometry, as >97% of the MUTZ population is CD45$^+$ and CD13$^+$.*

Also, there were two single stains for the known negative isotypes: CD3APC and CD19PE. These were used along with an unstained to adjust the FACS machine channel voltages to shift the population into the lower left quadrant (i.e. negative expression).

Figure 14 shows that the FACS machine was set up to ensure ~100% of the negative isotype were in the lower left. The 1% in other quadrants represents auto-fluorescence.

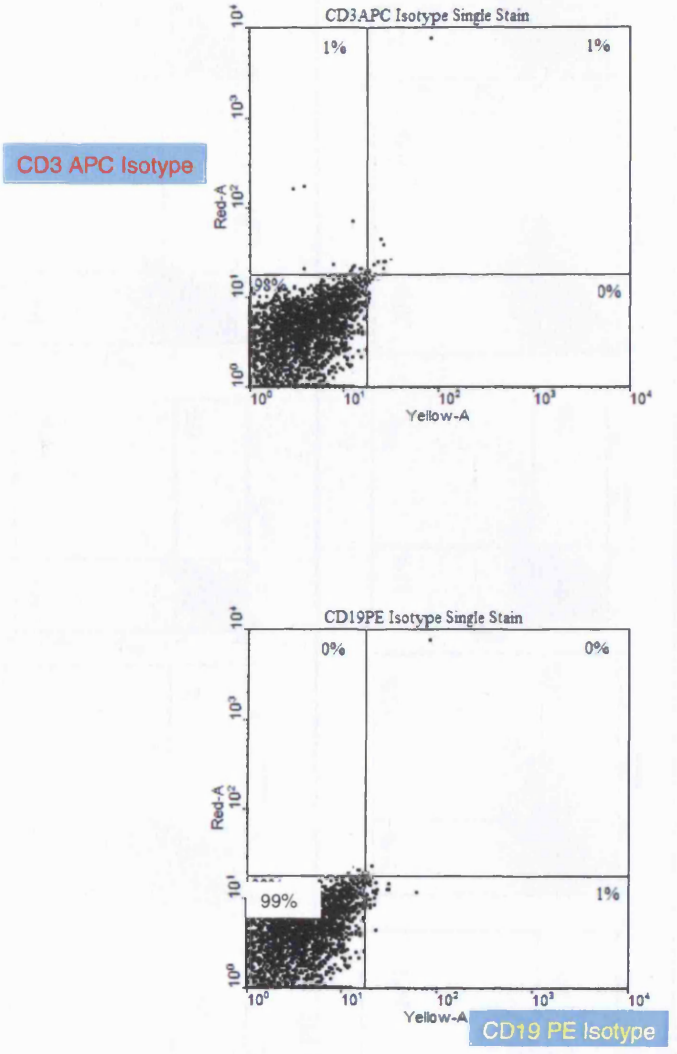

Figure 14. Negative Isotype controls. *The CD3APC and CD19PE conjugated antibodies were used as negative isotype controls.*

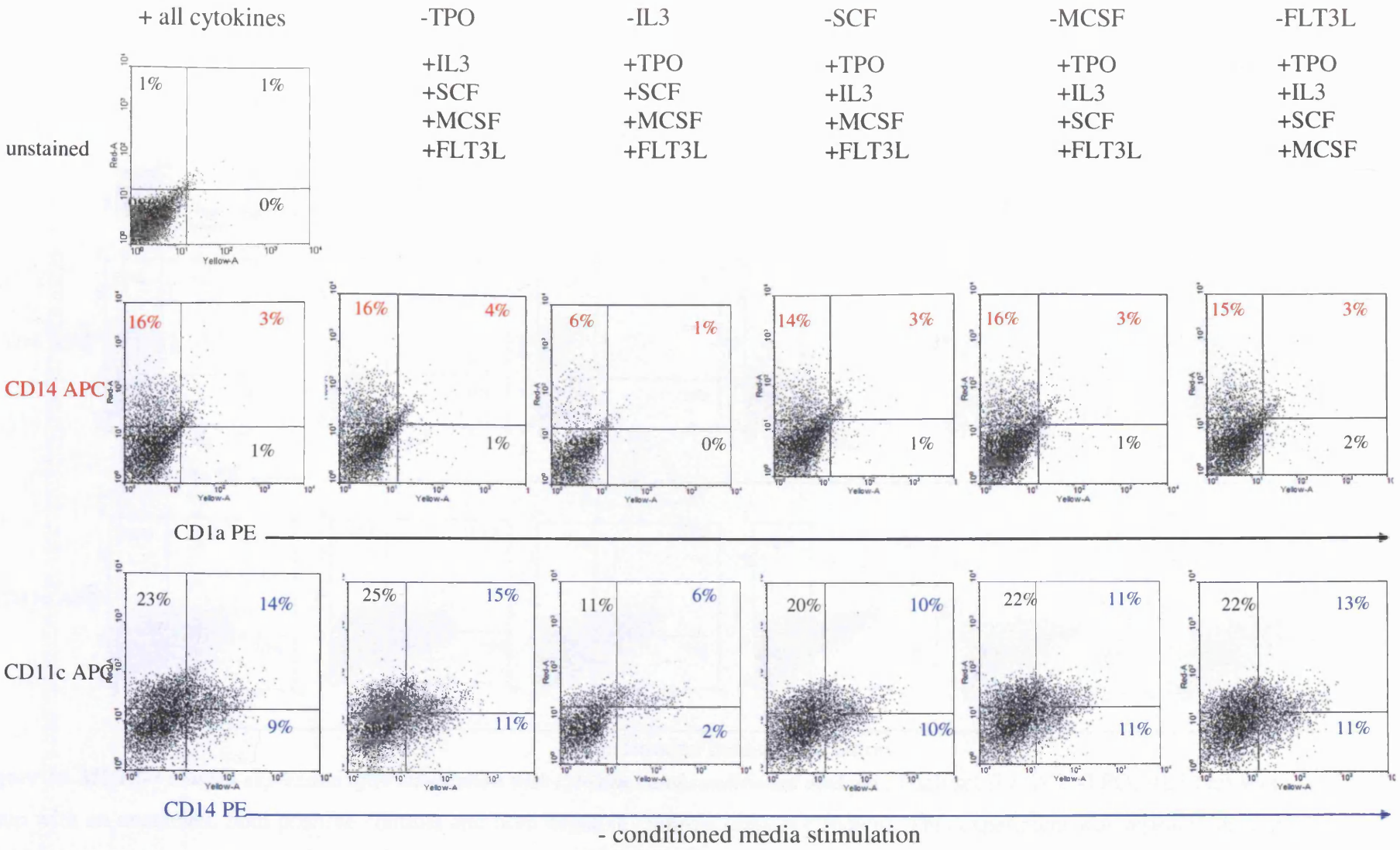

Figure 15. MUTZ-3 antigen expression after stimulation with cytokines and w/o conditioned medium. Each set (i.e. All, -TPO, -IL3 etc) was individually setup with an unstained, both positive controls and both negative controls (data not shown).

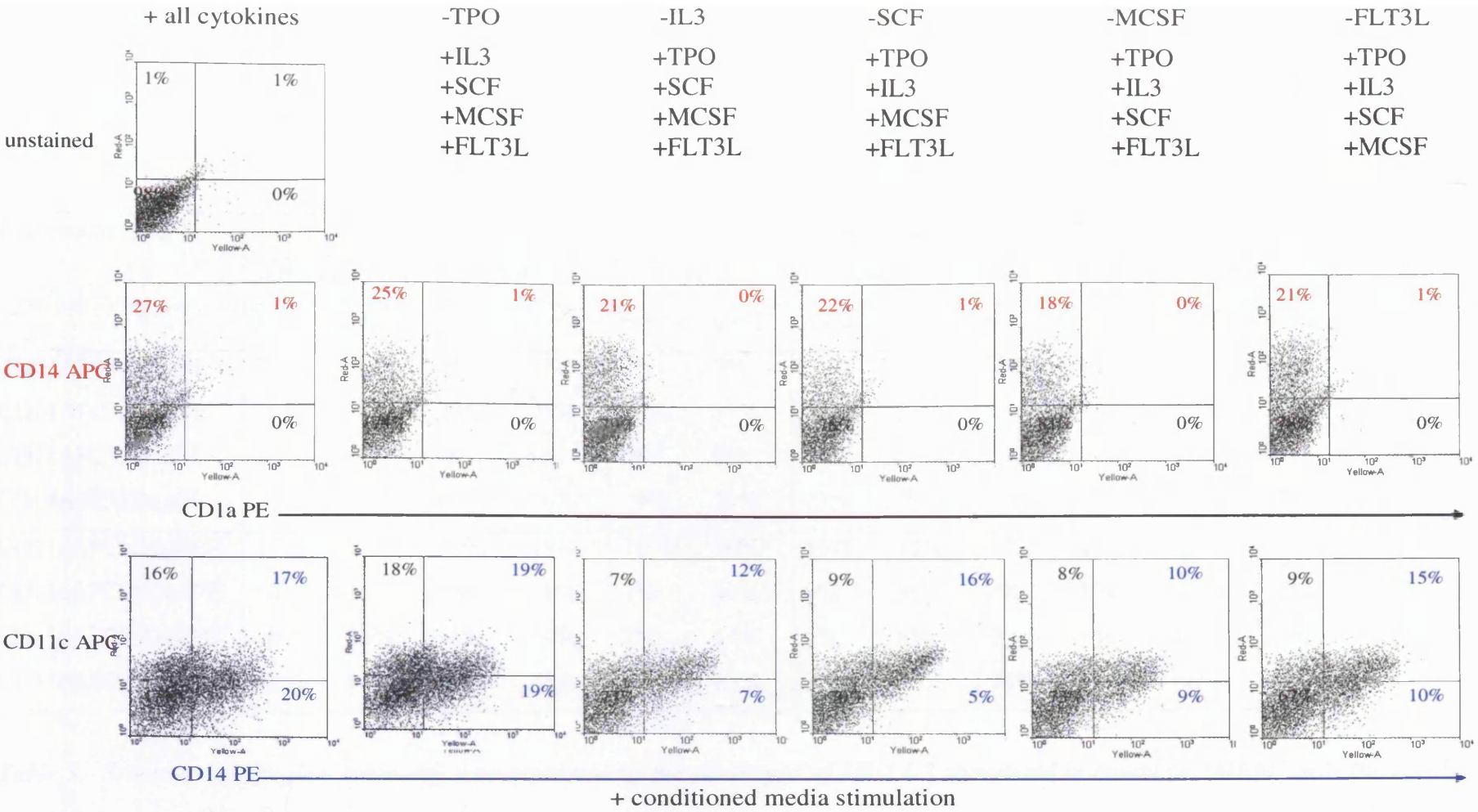

Figure 16. MUTZ-3 antigen expression after stimulation with cytokines and conditioned medium . Each set (i.e. All, -TPO, -IL3 etc) was individually setup with an unstained, both positive controls and both negative controls (data not shown). This experiment was repeated once more and the data is summarised in table 3.

Expression	*Data Set for +conditioned medium*											
	All Cytokines		**-TPO**		**-IL3**		**-SCF**		**-MCSF**		**-FLT3L**	
	n = 1	n = 2	n = 1	n = 2	n = 1	n = 2	n = 1	n = 2	n = 1	n = 2	n = 1	n = 2
$CD14APC^{+}CD1aPE^{+}$	2%	1%	4%	1%	0%	0%	3%	1%	3%	0%	3%	1%
$CD14APC^{+}CD1aPE^{-}$	28%	27%	28%	25%	20%	21%	25%	22%	25%	18%	26%	21%
$CD14APC^{-}CD1aPE^{+}$	0%	0%	1%	0%	0%	0%	1%	0%	1%	0%	1%	0%
$CD14APC^{-}CD1aPE^{-}$	70%	72%	67%	74%	79%	79%	73%	78%	71%	81%	69%	78%
$CD11cAPC^{+}CD14PE^{+}$	16%	17%	20%	19%	12%	10%	16%	16%	10%	12%	15%	15%
$CD11cAPC^{+}CD14PE^{-}$	16%	16%	18%	18%	7%	10%	9%	8%	8%	8%	9%	10%
$CD11cAPC^{-}CD14PE^{+}$	20%	20%	18%	19%	7%	14%	5%	6%	9%	9%	10%	10%
$CD11cAPC^{-}CD14PE^{-}$	48%	47%	45%	45%	73%	65%	70%	70%	73%	71%	66%	65%

Table 3. Summary of the flow cytometry data representing the phenotype of MUTZ-3 stimulated in complete αMEM^{+} (n is the number of repeats).

The experiments were only repeated once, and though the data is mostly consistent, this investigation must be repeated further to be conclusive. Therefore, the mean and errors cannot be calculated as that might not correctly represent the expression of the MUTZ-3. Unfortunately, due to technical errors, the repeat experiment using –conditioned medium failed (hence no data shown).

Firstly, the main difference in the two sets of data above is that the incubation was conducted with and without conditioned medium. This had a major effect on the growth and differentiation of the cells as can be seen by $CD14^+$ expression increasing 2-fold when conditioned medium was used.

	complete αMEM$^+$ (+ conditioned medium)	**complete αMEM$^-$ (- conditioned medium)**
Initial Cell count (seeding cells)	$1x10^5$ cells/ml	$1x10^5$ cells/ml
Cell count after 10 day incubation	$5.5x10^5$ cells/ml	$2.8x10^5$ cells/ml

Table 4. MUTZ-3 cell count. *Cells were counted before and after incubation with cytokines cocktails.*

Initially the same numbers of cells were seeded in all the wells, however, after 10 days incubation it can be seen that the doubling time with conditioned medium is ~5 days as opposed to ~7 days w/o conditioned medium (table 4). This suggests that conditioned media from the 5637 cell line contains various unspecified growth factors that are essential for the efficient growth and differentiation of MUTZ-3 cells.

Secondly, by comparing the two sets of data, it can be observed that absence of TPO has no significant influence on the monocyte population, as $CD14^+$ remains at ~25%. IL-3, however, appears to be essential to induce monocyte differentiation as there is a 5% drop from ~25% to ~20% $CD14^+$ expression when IL-3 is removed. SCF, MCSF and FLT3L appear to have a lesser though significant effect on inducing monocytic

$CD14^+$ expression. $CD11c^+$ expression is also greatly reduced by almost 15% in the absence of IL-3, SCF, MCSF and FLT3L, while $CD1a^+$ is very weak (1%) with or without the cytokines no matter which is removed.

The data also shows the majority of monocyte-like cells produced by the stimulation with these 5 cytokines are both $CD14^+$ and $CD11c^+$ simultaneously, hence the shift in the population to the top right quadrant. Also, without TPO the $CD14^+CD11c^+$ population appears to be 5% greater (20%) than if TPO is contained in the cytokine cocktail (15%). However, this data is inconclusive as ideally these experiments must be repeated a third time.

CHAPTER 7

Stimulating MUTZ-3 with retinoic acid

7.0 Stimulating MUTZ-3 with retinoic acid

7.1 Introduction

As cytokines are expensive and inducing DC-like or monocytic expression in MUTZ-3 using cytokines is also very time consuming (almost 4 weeks from stockpiling and stimulating cells till analysis by flow cytometry), it was crucial to test whether any other factors might be able to induce monocyte differentiation.

7.1.1 Cell differentiation by Retinoic Acid

Thus retinoic acid (RA) was tested. RA is most commonly used in experiments to study neural cell differentiation and gene activation. RA is a vitamin A derivative whose pleiotropic effects are suggested to be mediated through specific nuclear RA receptors (RARs). RA and RARs have been shown to play a key role in the haematopoietic system by inducing the proliferation and differentiation of haematopoietic progenitors. The exact mechanisms by which RA and RAR regulate haematopoietic differentiation are largely unknown; however, research suggests that RARs play a crucial role in the terminal differentiation of myeloid precursors as over-expression of RARs has been shown to suppress myeloid cell differentiation (Hu et al., 1996; Geissmann et al., 2003). As RA has been shown to induce terminal granulocytic differentiation of the HL-60 myeloid leukaemia cell line, it was assumed that it would stimulate the differentiation of other human acute myelogenous leukaemia (AML) cell lines. Therefore, as MUTZ-3 is a human leukemia acute promyelocytic cell line it might be possible to induce greater monocytic differentiation by incubating MUTZ-3 with RA than when MUTZ-3 are stimulated by cytokines. Thus, this investigation was conducted with a series of incubations at 12hrs and 24hrs with the RA titre being varied to establish the optimum time and concentration of RA required to induce monocytic expression.

7.2 Results and Discussion

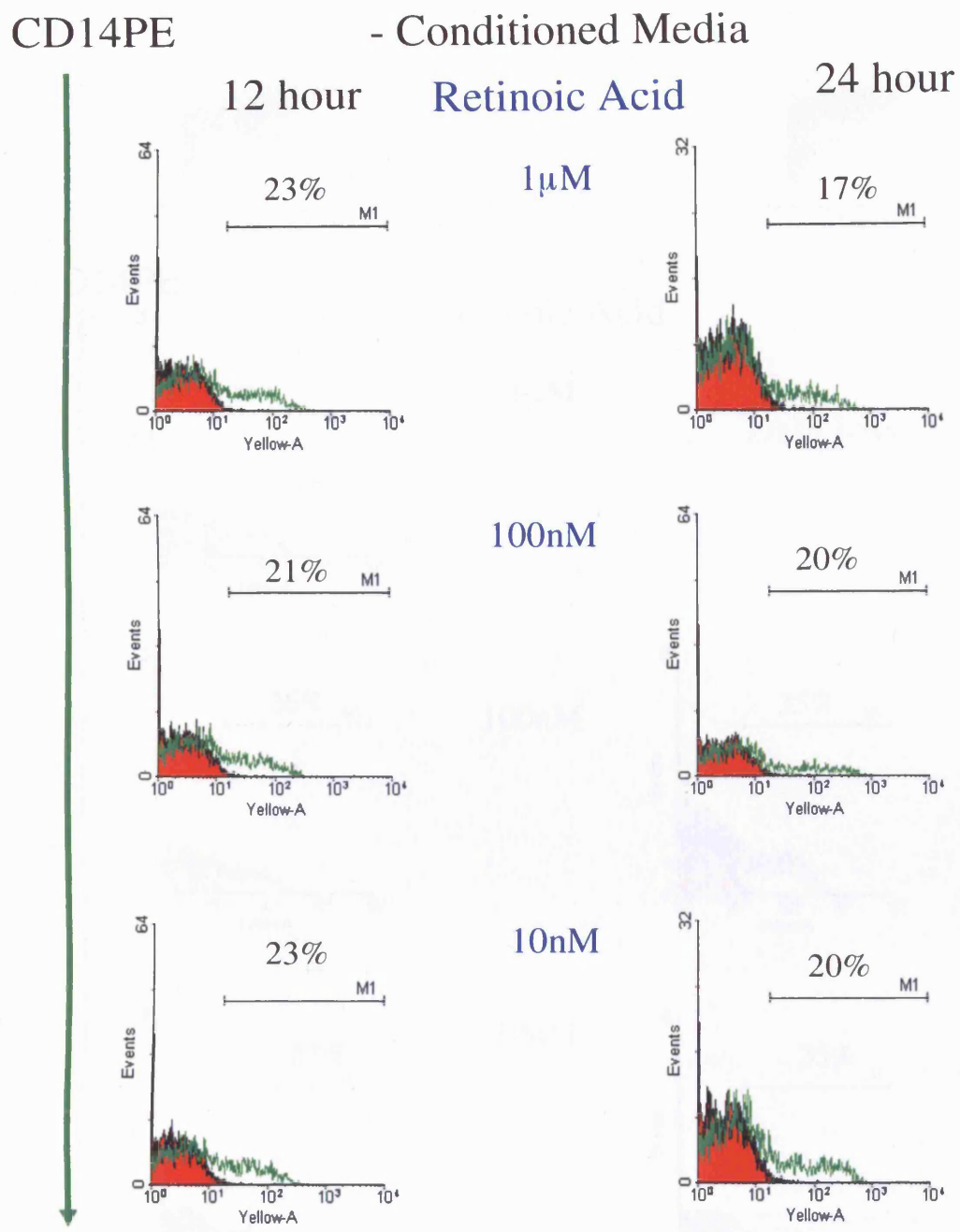

Figure 17. MUTZ-3 after incubation with retinoic acid and complete αMEM. *There was no control due to the limited number of cells available for this experiment. Ideally, the control should have been MUTZ-3 incubated without RA for 12hrs and 24hrs in complete αMEM.*

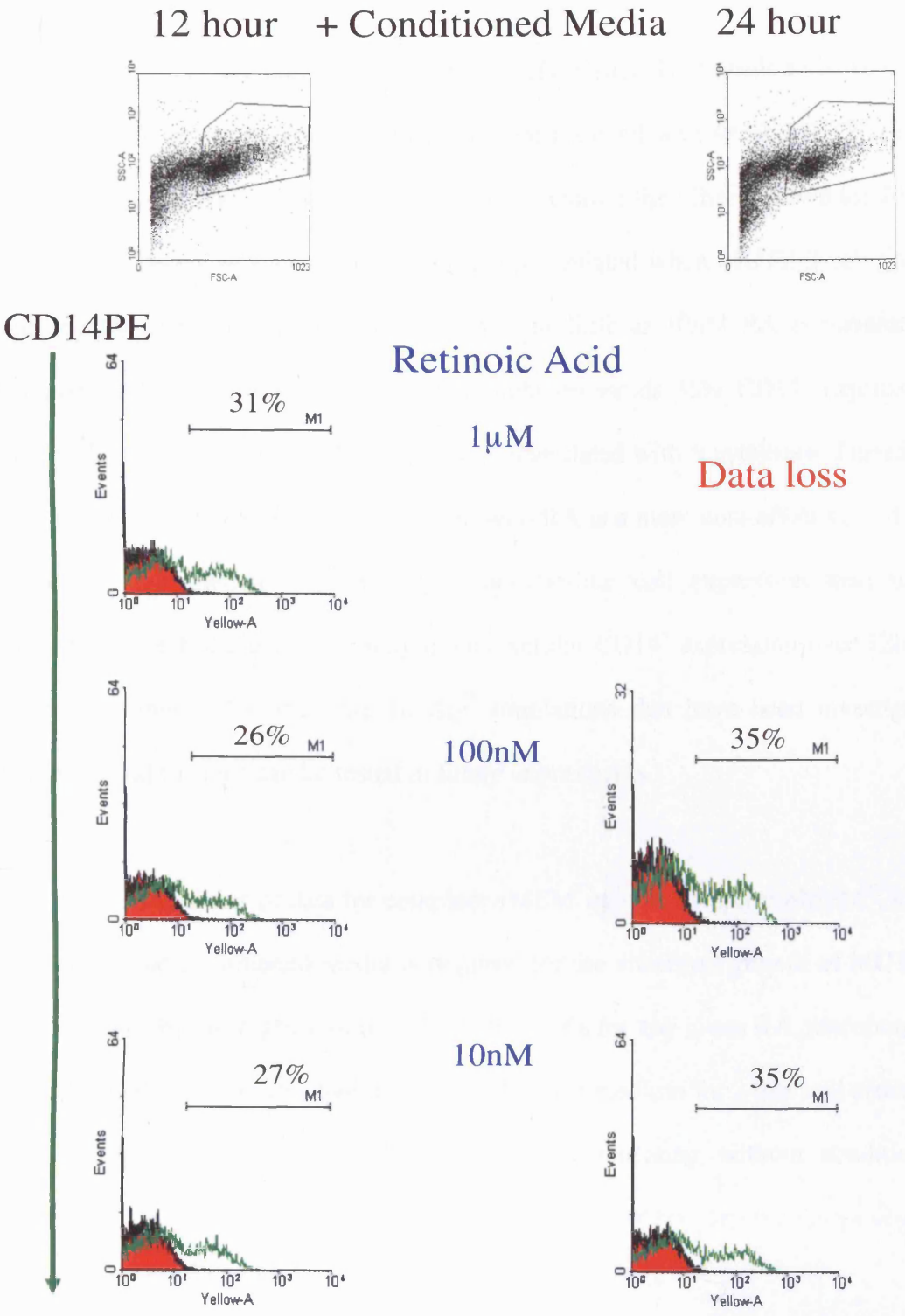

Figure 18. MUTZ-3 phenotype after incubation with retinoic acid and complete αMEM+. *There was no control due to the limited number of cells available for this experiment. Ideally, the control should have been MUTZ-3 incubated without RA for 12hrs and 24hrs in complete αMEM+. However, the initial phenotypic stain (CHAPTER 3) does represent the MUTZ-3 phenotypic expression without RA but in complete αMEM+.*

This data is preliminary and requires further study. Crucially it needs to be repeated with proper controls, i.e. four 100mm dishes, each seeded with $4x10^5$ MUTZ-3 cells and two incubated with and without conditioned medium for 12hrs and two for 24hrs. However, it does show $CD14^+$ expression is up-regulated when MUTZ-3 cell line is incubated for 24hrs in complete αMEM^+ with as little as 10nM RA concentration. This ~10% increase in $CD14^+$ after RA stimulation yields 35% $CD14^+$ expression, which is the same as when the MUTZ-3 were stimulated with 5 cytokines. Therefore, it is appears from this data, that stimulation with RA is a more cost-effective and less time consuming method of inducing monocytic-like cell expression than using cytokines. Though the cytokines may induce similar $CD14^+$ expression over 12hr or 24hr stimulations rather than the 10 day stimulations that have been investigated (chapters 4 and 6). This can be tested in future experiments.

Comparing the two sets of data for complete αMEM^- against complete αMEM^+, it can be observed that conditioned media is required for the enhanced growth of MUTZ-3 and this shown by an increase of the $CD14^+$ by ~10% for any given RA concentration at 12hr incubation. Also, incubating with conditioned medium for 24hrs still produces a 10-15% increase yield of $CD14^+$ than when incubating without conditioned medium.

The data for complete αMEM^- shows that when incubating MUTZ-3, with 1μM concentration of RA for 24hrs, the $CD14^+$ yield decreases by ~5% than if the incubation is for only 12hrs. Therefore suggesting that 1μM RA may potentially be inducing the differentiation of the $CD14^+$ monocytes to immature or mature DC-like.

Hence, future investigations should study whether Class II expression is increased or CD86 and other such maturation markers are expressed. Also RA might induce cells to differentiate to macrophages, however, when the plates where checked using the phase contrast microscope there were no significantly visible clumps of adherent cells and this is a classic attribute of macrophages.

CHAPTER 8

Discussion

8.1 Measuring MUTZ-3 differentiation

8.2 Effects of varying cytokine cocktail on MUTZ-3 monocytic expression

8.3 Differentiation of MUTZ-3 monocytes by GM-CSF and IL-4

8.4 Effect of LPS on MUTZ-3

8.5 MUTZ-3 differentiation by Retinoic Acid

8.6 Future investigations

8.7 The future of DC models

DCs play a vital role in immunoregulation, thus extensive research into DC gene expression and function is being conducted to establish methods for drug administration and cures to infections. As DCs play such an important role in disease control and autoimmunity, it is important to have a large on-demand supply of DCs in order to efficiently establish their functions. However, DCs are currently studied after being harvested from bone marrow or peripheral blood. Also DCs can vary in their expression and function depending on their origin, so it is crucial to establish a model for DC generation as this will enable greater control on the variability of DCs.

A recent study to establish a model for DCs, conducted by Masterson *et al.*, demonstrated that the human myeloid leukaemia cell line MUTZ-3 has the ability to acquire DC-like phenotype when stimulated by cytokines, despite cytokine responsiveness being low on most of the other cell lines they tested. MUTZ-3 is a $CD34^+$ cell line and responds to GM-CSF, IL4 which induce monocytic and DC-like expression both *in vivo* and *in vitro*. This research has shown MUTZ-3 can behave as $CD34^+$ DC precursors. Masterson *et al.* have shown that MUTZ-3 stimulated with a cocktail of cytokines acquired phenotypes consistent with that of Langerhan and intestinal DC (see chapter 1).

In our investigations, cultured MUTZ-3 cell line was stimulated over 10 days with a cocktail of cytokines: (25ng/ml of each) TPO, IL3, MSCF, SCF and FLT3L. This stimulation was repeated but removing individual cytokines to establish the significance of each of the 5 cytokines, and the MUTZ-3 were stimulated with GM-CSF (100ng/ml) and IL-4 (10ng/ml) for 7 days, which Masterson *et al.* have shown to induce immature DC-like phenotype.

8.1 *Measuring MUTZ-3 differentiation*

MUTZ-3 myeloid cell line expresses weak $CD14^+$ suggesting the cell line can be induced to increase $CD14^+$ monocyte population but stimulation with growth factors. MUTZ-3 stimulated by cytokines for 10 days were observed to have increased $CD14^+$ expression by 20% and the MUTZ-3 phenotype represented that of monocytes. However, this increase in CD14 may have been greater if a monocyte-specific progenitor population of MUTZ-3 was stimulated rather than the mixed MUTZ-3 population, which contains progenitors for granulocytes and other myeloid cells. Due to the cells being incubated for 10 days or more (as is the case when GM-CSF and IL-4) the presence of some dead cells and debris might have caused auto-fluorescence, hence why the negative controls (e.g. CD3APC and CD19PE) have shown a 1-3% expression. Also, there were some initial complications with the setup of the FACS machine, though these were later resolved with some careful tweaking of the voltage and gating settings, thereby allowing for correct spill-over compensations to be made to ensure the data was viable. This can be seen by the CD45APC and CD13PE quadrant plots in figure 1. Also, the two fluorescence markers APC and PE have do not overlap in emission wavelengths, thus there could have been no erroneous spill-over fluorescence between channels.

8.2 *Effects of varying cytokine cocktail on MUTZ-3 monocytic expression*

IL-3, MCSF & SCF are potent haematopoietic growth factors known to regulate immunological responses. The biological activities of IL-3 are reported to differ between species but IL-3 is known as a key molecule to activate monocytes, though this may depend upon co-stimulation with other cytokines. Our data shows that when IL-3 was removed from the cytokine cocktail stimulation, the yield of $CD14^+$

decreased by ~20% and $CD11c^+$ by ~15%. This suggests that IL-3 is a major factor for inducing $CD14^+CD11^+$ monocyte expression in MUTZ-3. IL-3 has been implicated in the differentiation of early non-lineage-committed haematopoietic progenitor cells into granulocytes, macrophages and megakaryocytes. IL-3 is a priming factor for haematopoietic cells that enhances their responsive to factors such GM-CSF. IL-3 can also increases expression of CSF receptors, thus potentially enhancing the effect of other cytokines or growth factors.

MCSF is produced by endothelial cells, monocytes and granulocytes but is a key factor in regulating the growth and differentiation of monocytes. MCSF synergises with IL-3 to stimulate the proliferation and differentiation of haematopoietic cells into macrophages, and GM-CSF to suppress the generation of macrophage populations (Sumimoto et al., 2002; Hope et al., 2000). This explains why the removal of MSCF from the cytokine cocktail also decreased $CD14^+$ yield by ~20% and CD11c by ~15%.

SCF stimulates the proliferation of myeloid, erythroid and lymphoid progenitors in bone marrow cultures and has been shown to act synergistically with other colony stimulating factors to stimulate the differentiation of myeloid lineage cells (Hope et al., 2000; Hagihara et al., 2001). The removal of SCF from the cytokine cocktail reduces the expression of $CD14^+$ by ~20% and $CD11c^+$ by ~10%.

FLT3L regulates proliferation of early haematopoietic cells and research by Hagihara *et al.* shows that FLT3L alone cannot stimulate proliferation or differentiation without synergises with other CSFs and interleukins. Therefore, removing FLT3L from the stimulation cocktail reduced $CD14^+$ by ~15% and CD11c by ~10%, suggesting that

FLT3L has an effect though this is probably enhanced further by the presence of the other cytokines(Hagihara et al., 2001).

TPO is a lineage specific growth factor which appears to have minimal effect on inducing monocytes. This is observed by only a 1-2% reduction in $CD14^+$ with the removal of TPO from the cytokine cocktail. This 1-2% could be attributed to auto-fluorescence and therefore is not significant.

Further experiments will be required to test the minimal amount of cytokines required to induce $CD14^+CD11c^+$ monocyte expression. Also, it is important to establish the phenotype of MUTZ-3 if only stimulated with each individual cytokine before testing with varying cytokine cocktails – adding an extra cytokine each time to the cocktail. This will clarify the synergistic or antagonistic response of the cytokines.

8.3 Differentiation of MUTZ-3 monocytes by GM-CSF and IL-4

GM-CSF regulates the majority of CSF activities and can be associated as a complex with proteoglycans to be stored in a biologically inactive form. Although its release mechanism is unknown, it might be linked to the presence of other cytokines or growth factors. As GM-CSF was initially isolated as a macrophage growth factor it is implicated as a major factor in the development of macrophage and immature DC progenitor cells. GM-CSF has been shown to trigger irreversible differentiation of monocytes (Hope et al., 2000). However, GM-CSF can act synergistically with MCSF to suppress the differentiation. Hope *et al.* also reported IL-4 synergistically acts with GM-CSF in controlling cell growth and differentiation. The exact mechanism by

which these factors regulate the differentiation of cells is still unknown, but they are implicated in maturation of monocyte-derived immature DCs.

Our MUTZ-3 cells showed $CD14^+$ and $CD11c^+$ down-regulation by ~20% and ~15% respectively, suggesting the monocytes have differentiated into immature DCs. However, the cells were first incubated with the cytokine cocktail containing MCSF, which may have partially suppressed the differentiation of monocytes to immature DC. Therefore, repeating this experiment with the removal of MSCF might enhance differentiation to immature DCs. Also, the maturation marker $CD83^+$ expression remains unchanged after GM-CSF and IL-4 stimulation, suggesting the cells have not matured after differentiating to immature DCs. However, Class II^+ expression increases 20%, which may be due to the down-regulation of the factors involved in antigen internalisation thus increasing expression of MHC Class II.

Cell morphology was also altered after the 7 day incubation and clear dendrite extensions were observed on many cells suggesting the formation of immature DC, though most cells remained in the veiled form.

8.4 Effect of LPS on MUTZ-3

LPS activates macrophages and innate immunity as well as inducing maturation of immature DC to mature DC. Therefore, to test whether MUTZ-3 would differentiate to mature-DC, a 24hr incubation of MUTZ-3 with LPS was conducted (data not shown). However, the cells number in the initial MUTZ-3 population was low, and this might account for the lack of expression of maturation markers $CD86^+$ and $CD83^+$. However, this experiment should be repeated using cells that have been stimulated by the cytokine cocktail and GM-CSF and IL-4 to optimise the immature

DC population. The addition of LPS to immature DC population should stimulate maturation into mature DC.

Recent studies have shown that the CD14 forms a receptor for LPS and LBP, however, as MUTZ-3 only expresses $CD14^+$ weakly there might not be sufficient LPS/LBP receptors present over the 24hrs incubation to induce maturation of MUTZ-3. Therefore, to enhance the CD14+ receptor expression the MUTZ-3 should be treated with the cytokine cocktail before incubating with LPS but not GM-CSF or IL-4 as these down-regulate CD14 expression. It would also be better to incubate MUTZ-3 for longer than 48hrs with LPS (100ng/ml) or for less than 48hrs with a lower concentration. LPS has been shown to strongly enhance $CD14^+$ in 45hr cultures of purified monocytes (Espuelas et al., 2005).

8.5 MUTZ-3 differentiation by Retinoic Acid

Retinoic acid (RA) is a derivative of vitamin A and has been established as a major proliferation and differentiation factor.

Recent studies using retinoic acid to differentiate cells of human leukaemic promyelocytes HL-60 showed that significant terminal cell differentiation occurred only after a minimum exposure to retinoic acid. Indeed research by Onodera shows that granulocytes will differentiate even with RA concentration as low as 1nM. Therefore, a similar effect might be observed when myelomonocytic progenitor cells are stimulated with RA (1nM), hence why I tested the effect of RA at varying concentrations at 12hrs and 24hrs.

Indeed, RA stimulated monocytic expression of $CD14^+$ over 24hrs which is a far shorter time and cheaper process than incubating MUTZ-3 with cytokine cocktails. However, this stimulation will need repeating to establish the optimum concentration and length of incubation required to induce monocytic expression without inducing further differentiation to macrophage or immature-DC.

I would recommend incubating MUTZ-3 for 24hrs in complete αMEM^+ with RA titres of concentrations 3nM, 5nM and 7nM to establish the minimum amount of RA required to induce the $CD14^+$ expression and to reduce any differentiation past this stage to immature DC. However, to test this, the MUTZ-3 would need to be cultured for longer to increase stocks and ensure a minimum of $1x10^6$ cells/ml (i.e. 10 stains). Then analyse double-stained cells by flow cytometry for the expression of the following antigens: CD3, CD14, CD11c, CD1a, CD83, CD86, Class I and Class II.

8.6 Future investigations

Crucially it is important to repeat all the experiments conducted throughout this project to confirm the findings and optimise the conditions to increase monocytic expression of MUTZ-3. It would be ideal to be able to reduce the incubation period for the MUTZ-3 and also reduce the amount of cytokines required so that only one or two of the cytokines are needed (e.g. IL3 and SCF). Also, it would be interesting to test the interactions between cytokines and RA to establish which combinations will provide the highest yield of MUTZ-3 monocytes.

It is also advisable to sort the initial mixed population of MUTZ-3 so as to harvest and grow the monocyte progenitors only, which could be experimented on to develop the

DC-like model. This may increase the yield of monocytes and DC-like populations after stimulation by cytokines and/or RA.

In the long term, further studies into the molecular signalling pathways that enable cytokines and RA to induce MUTZ-3 would allow us to better understand other molecules that might optimise the MUTZ-3 monocyte yield.

Additionally, MUTZ-3 could be incubated at varying lengths with cytokines and other growth factors to test their effect on differentiating this myeloid cell line. Research on other cell lines suggests the use of viral vectors to regulate translation or expression of various intracellular-produced receptors or signalling molecules. Regulation of these molecules might increase the harvest of monocytes which could be used in DC studies.

8.7 The future of DC-like models

Dendritic cells are rare leukocytes that are uniquely potent in their ability to present antigens and stimulate naive T cells as well as initiating primary immune responses. This property has prompted their recent application to therapeutic cancer vaccines as well as potential uses in HIV and TB cure development (Durrant and Ramage, 2005). DCs have been developed *ex vivo* to specifically express tumour antigen and then administered as cellular vaccines to induce protective and therapeutic anti-tumour immunity in animal studies and recent human trials.

Currently most DC cells are harvested from peripheral blood, Langerhan cells, thymic cells and bone-marrow, but DC from these sources exhibit individual variability

(Weigel et al., 2002; Ratta et al., 1998; Mitsui et al., 2004). These models are likely to apply with local variations to the DCs found in similar tissue; for example, the Langerhan model has been used to study DC function and life-cycle, however, the DC found in other stratified epithelia, such as in the lung can vary from those of the Langerhan, for example having varying rates of turnover in the lung as well as rapid response mechanisms to recruit DCs into the tissue on antigen entry. It is unclear though if any one model can apply to DCs found in other sites and whether blood DC behave in the same way as intestinal DC or dermal DC.

Human $CD34^+$ haematopoietic progenitor cells have been purified from bone-marrow and can be induced to proliferate and differentiate into DC when cultured with GM-CSF and interferon-γ (TNF-γ). These DC have been characterised by there dendritic morphology and DC-like surface phenotype, i.e. the expression of MHC class II^+, $CD1a^+$, $CD4^+$, $CD80^+$, $CD86^+$, $CD83^+$ and $CD14^-$ (Moghaddami et al., 2005).

However, new research conducted on cells such as $CD34^+$ MUTZ-3 by Masterson *et al.* is enabling the development of a cellular model for DC behaviour and biological function in the hope of being able to clearly understand and study the molecular pathways that lead to antigen presentation by DCs, and how this may lead to therapeutic effects in our fight against all diseases. We have shown MUTZ-3 precursors to be efficiently stimulated to provide a viable source of $CD14^+$ monocytes. Therefore, MUTZ-3 represents a model for study of DC differentiation and maturation. Indeed MUTZ-3 could be used as an unlimited supply of $CD34^+$ DC precursors to explain DC pathways and mechanisms.

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