Université de Montréal

The myeloid-specific transcription factor PU.1 as an effector of GM-CSF signaling in myeloid development

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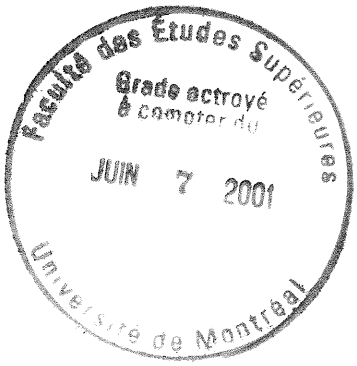

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Présenté par :

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SUMMARY

The hematopoietic system is a complex and dynamic system that leads to the establishment of eight known lineages each having a specific and fundamental role in our organism. The myeloid lineage leads to the development of granulocytes and macrophages. These cells act as our first line of defense against foreign particles as they participate in the innate immune response. In addition, macrophages also have a role in our adaptive immune response by acting as antigen presenting cells for certain T cells.

Hematopoietic growth factors have been shown to control the growth of hematopoietic cells by binding to their specific receptors expressed on the surface of specific cells. Some hematopoietic growth factors, also known as cytokines, are lineage-specific while others support the growth of multiple lineages. The granulocyte-macrophage colony-stimulating factor (GM-CSF), as long been established as a critical factor involved in the growth, survival and differentiation of hematopoietic precursor cells with important additional effects on myeloid development. For example, GM-CSF enhances neutrophilic response to antigens as well as increasing the phagocytic activity of macrophages. For these reasons, it is now regularly used in chemotherapy to alleviate the strong side-effects chemotherapeutic drugs have on our defensive system.

Hematopoietic transcription factors also play a pivotal role in the development of our hematopoietic system. As for hematopoietic growth factors, some hematopoietic transcription factors have a more restricted specificity while others have a more general contribution. For example, GATA-1 is known to play a crucial role in erythroid development while PU.1

is involved in myeloid development. These factors help in determining cell fate by controlling the expression of lineage-specific genes.

Through knockout studies, PU.1 has been shown to play an essential role in myeloid development due in part to its ability to activate critical myeloid-associated genes.

In this study, we demonstrate how the myeloid-specific transcription factor PU.1 acts as an effector of GM-CSF signaling in myeloid development. We show that PU.1 levels increase dramatically in response to GM-CSF as soon as six hours post-GM-CSF stimulation. This increase in PU.1 levels also precedes myeloid differentiation. In addition, ectopic expression of PU.1 is able to bypass GM-CSF signaling in the induction of myeloid differentiation.

Finally, we show that PU.1 is also required for survival of pluripotent cells, as well as primary bone marrow cells, in response to GM-CSF. For the first time, we show that an external signal implicates directly the activation of a lineage-specific transcription factor to favor lineage-specific development.

RÉSUMÉ

Notre système hématopoïétique est un système de développement dynamique dont le rôle est de maintenir un niveau suffisant de cellules sanguines fonctionnelles afin d'assurer la survie de notre organisme. C'est ainsi que le système hématopoïétique donne lieu aux huit différentes lignées cellulaires connues. Les cellules sanguines différenciées incluent les erythrocytes ou globules rouges, les mégakaryocytes qui donneront lieu aux plaquettes, les granulocytes et macrophages, les basophiles et eosinophiles, ainsi que les cellules lymphoïdes B et T. Tous trouvent leur origine à partir d'une population de cellules souches hématopoïétiques.

La différenciation de cellules hématopoïétiques primitives en granulocytes et monocytes matures constitue un processus de développement complex appelé la myélopoïèse. Le développement de ces cellules primitives en monocytes et granulocytes fonctionnels est en partie controlé par une myriade de facteurs de transcription dont le rôle est d'activer l'expression de gènes essentiels à une ou plusieurs lignées. Ces facteurs de transcription peuvent agir de façon positive ou négative à la régulation de gènes codant entre autres pour des facteurs de croissance, leurs récepteurs, pour des molécules d'adhésion, pour des enzymes ou bien pour d'autres facteurs de transcription.

Le développement de cellules myéloïdes primitives et de leur différenciation subséquente en cellules myéloïdes matures est également controlé par un vaste réseau de facteurs de croissance hématopoïétiques. Tout comme les facteurs de transcription, ces facteurs de croissance, ou cytokines, peuvent être spécifiques à une lignée particulière ou bien peuvent avoir une

contribution plus générale. Par exemple, le G-CSF (granulocyte-colony-stimulating factor) est un facteur de croissance essentiel au développement granulocytaire alors que l'IL-3 (interleukin-3) soutient la prolifération et la survie de plusieurs lignées.

Quelle est l'importance d'étudier un processus tel que la myélopoïèse? Environ 120 millions de nouveaux neutrophiles sont générés chaque jour chez l'adulte normal. Ces neutrophiles jouent un rôle fondamental dans notre système de défense. En effet, les neutrophiles agissent comme première ligne de défense face à l'invasion de particules étrangères. Ils participent à notre système immunitaire inné principalement par leur action phagocytaire.

Les macrophages quant à eux, jouent un rôle similaire c'est-à-dire qu'ils participent également à notre système immunitaire inné en éliminant toutes particules étrangères par phagocytose. Cependant, ils possèdent une caractéristique additionelle. Ils jouent également un rôle dans notre système immunitaire adaptif en agissant comme cellules présentatrices d'antigènes aux différentes cellules lymphoïdes T.

Chaque cellule est exposée à une multitude de signaux présents dans son environnement. La cellule se doit d'être en mesure de reconnaître et de répondre de façon spécifique à cette panoplie de signaux externes. Elle accompli ceci de plusieurs façons. Premièrement, afin de répondre à un signal particulier, la cellule se doit d'exprimer le récepteur spécifique au signal reçu. Ensuite, elle doit être en mesure d'intégrer et d'interpréter ce signal afin de le transformer en une réponse biologique concrète. Pour cela, la cellule doit posséder la machinerie interne nécessaire à la traduction et la transformation du signal.

Les facteurs de croissance sont un exemple de signal externe présent dans l'environnement d'une cellule hématopoïétique. Ces facteurs de croissance sont connus pour être impliqués dans la régulation de la prolifération et de la survie cellulaire. Ces facteurs se lient à leurs récepteurs appropriés exprimés à la surface de certaines cellules. Généralement, cette liaison induit l'activité catalytique du récepteur qui donne suite à l'activation de plusieurs voies de signalisation. Ces différentes voies de signalisation ciblent différents facteurs de transcription qui sont associés à différentes réponses biologiques correspondantes.

Ces facteurs de transcription, dont l'activité est induite par l'induction de voies de signalisation spécifique, vont être responsables du contrôle de l'expression de gènes essentiels à la détermination du destin cellulaire. Vu l'importance de réguler de façon étroite l'expression de tels gènes, l'activité des facteurs de transcription se doit d'être sous haute surveillance. Le temps d'expression ainsi que l'endroit d'activation des facteurs de transcription deviennent donc des variables importantes. Un facteur de transcription responsable de la destinée d'une cellule se doit d'être exprimé dans la bonne cellule et d'être actif au bon moment dans le processus de développement de cette cellule. Souvent, l'activité et l'expression de ces facteurs de transcription sont contrôlés par des facteurs externes tels que les facteurs de croissance. Un modèle qui se veut de plus en plus populaire, malgré qu'aucun exemple définitif n'ait été encore démontré, suggèrerait qu'un signal externe serait en mesure d'affecter directement l'activité d'un facteur de transcription qui lui se voudrait essentiel à la détermination de la destinée cellulaire.

Le facteur de transcription myélo-spécifique PU.1 est un membre de la famille Ets. Il fût découvert simultanément de deux façons différentes. D'un

côté, il fût identifié à partir d'une librairie de cDNA de macrophages comme étant un facteur pouvant lier avec haute affinité, le promoteur du gène MHC classe II 1-Ab. D'autre part, il fût identifié comme site d'intégration préféré du provirus SFFV (Spleen Focus Forming Virus), un provirus associé à une érythroleucémie induite par le virus Friend. Cependant, il est important de mentionner que PU.1 n'a pas été impliqué dans l'induction de leucémies humaines en partie dû au fait que son locus ne correspond pas à un site fréquent de translocation chromosomique.

La famille de facteurs de transcription Ets est caractérisée par son domaine de liaison à l'ADN (domaine Ets). Ce domaine basique situé en C-terminal lie des séquences riches en purines GGAA/T dénommées boîtes PU. Ets-1, Ets-2 et Spi-B sont d'autres exemples de facteurs appartenant à cette grande famille.

PU.1 est un facteur de transcription exclusif au système hématopoïétique. Son rôle dans le développement hématopoïétique semble assez bien décrit. La suppression complète du gène est associée à une absence complète de macrophages et de cellules B matures. Par contre, des monocytes et des précurseurs de cellules B sont présents mais en nombre réduit. Il y a également présence de granulocytes mais au développement et au fonctionnement anormal. Les cellules érythrocytaires ne sont, quant à elles, pas affectées. Ces résultats démontrent bien le rôle primordial de PU.1 dans le développement des cellules B ainsi que dans le développement myéloïde. Cela en fait un excellent candidat comme déterminant génétique dans l'engagement du destin cellulaire.

Le GM-CSF (granulocyte-macrophage colony-stimulating factor) est un facteur de croissance hématopoïétique impliqué dans la prolifération, la

survie et le développement de cellules primitives hématopoïétiques avec des effets additionels importants sur le développement myéloïde. C'est justement par son implication déterminante dans le développment des cellules granulocytaires et monocytaires que le GM-CSF est de plus en plus utilisé à des fins cliniques. Le GM-CSF fait partie de la grande famille des cytokines qui comprend entre autres l'Epo, Steel Factor, l'IL-3 et le G-CFS. Le GM-CSF lie son récepteur qui est exprimé spécifiquement à la surface de certaines cellules et cette liaison active différentes voies de signalisation qui se traduisent par différentes réponses biologiques.

Le récepteur du GM-CSF appartient à la super famille des récepteurs de cytokines. Il est composé d'une chaîne α et d'une chaîne β. La chaîne α est spécifique au récepteur et lui confère une basse affinité de liaison à son ligand. La chaîne β est commune à d'autres récepteurs tels les récepteurs de l'IL-3 et de l'IL-5. Elle ne possède aucune capacité de liaison. Par contre, en s'associant à la chaîne α, elle confère à celle-ci, une haute affinité de liaison au GM-CSF. De plus, c'est la chaîne β qui est principalement responsable d'activer les différentes voies de signalisation. En effet, son domaine cytoplasmique comporte de nombreux résidus de tyrosines responsables du recrutement de différents facteurs, ainsi que de deux boîtes situées à proximité du domaine membranaire qui sont responsables du recrutement de Jak2, le facteur actif dans le complexe. Les voies de signalisation qui découlent de l'activation du récepteur par son ligand sont bien décrites. Il est connu que certaines voies conservées, telles les voies MAPK, Jak-STAT et PI3K, sont activées suite à l'activation du récepteur et que ces voies sont impliquées dans des réponses de survie et de prolifération myéloïde. Par contre, rien n'est bien établi en ce qui concerne la différenciation myéloïde.

Aucun facteur de transcription impliqué directement dans la détermination du destin cellulaire n'a été identifié clairement. Bien que l'on connaisse des exemples de facteurs responsables d'activer des gènes qui eux, sont spécifiques à une lignée, très peu d'informations concernant les événements transcriptionels conduisant à la détermination du destin cellulaire sont connues. Le rôle de PU.1 dans le développement myéloïde est bien établi. Cependant, jusqu'à quel point PU.1 est-il déterminant pour qu'une cellule primitive prenne la décision de s'engager de façon irréversible vers une destiné myéloïde?

L'importance de PU.1 et du GM-CSF dans le développement myéloïde à été clairement, mais indépendemment démontrée. Cependant, il n'existe aucune documentation démontrant le lien possible entre ces deux facteurs dans le développement myéloïde. Dans cette étude, nous démontrons clairement que le facteur de transcription myélo-spécifique PU.1 agit comme effecteur du signal par le GM-CSF dans le développement myéloïde. En d'autres mots, l'activation de PU.1 et son effet déterminant dans le développement myéloïde sont directement liés à l'activation du récepteur du GM-CSF par son ligand.

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LISTE DES ABBRÉVIATIONS

aa	"Amino acid"
Ab	"Antibody"
APC	"Antigen presenting cell"
Asp	"Aspartic acid"
AS-PU.1	"antisense-PU.1"
BCl-2	"B-cell lymphoma-2"
BFU-E	"Blast-forming-unit-erythrocytes"
CBF	"CREB binding factor"
CBP	"CREB binding protein"
cDNA	"Complementary DNA"
C/EBP	"CAAT/enhancer binding protein"
CFU-E	"Colony-forming-unit-erythrocytes"
CSF	"Colony-stimulating factor"
Epo	"Erythropoietin"
ERK	"Extracellular regulated kinase"
ES cells	"Embryonic stem cells"
FOG	"Friend of GATA"
G-CSF	"Granulocyte Colony-Stimulating Factor"
GFP	"Green fluorescent protein"
Glu	"Glutamic acid"
GM-CSF	"Granulocyte/Macrophage Colony-Stimulating Factor"
GMR	"GM-CSF receptor"
GTP	"Guanine tri-phosphate"
HSC	"Hematopoietic stem cells"
IL-3	"Interleukin-3"
IL-5	"Interleukin-5"

LMO2	"Lim-only protein-2"
Jak	"Janus-kinase-3"
JNK	"Jun kinase"
mAb	"Monoclonal antibody"
M-CSF	"Macrophage-colony-stimulating factor"
MHC	"Major histocompatibility class"
mRNA	"Messenger RNA"
MCSV	"Murine-stem-cell-virus"
MSCV-neo	"Murine-stem-cell-virus-neomycin"
PU.1	"Purine-rich-1"
PEST	"Proline-glutamic-serine-threonine"
PI3K	"Phosphatidyl-inositol-3 kinase"
PKC	"Protein kinase C"
PTP1D	"Protein tyrosine phosphatase 1D"
Rb	"Retinoblastoma"
RTK	"Receptor tyrosine kinase"
RT-PCR	"Reverse transcriptase-polymerase chain reaction"
SCL	"Stem cell leukemia"
SF	"Steel factor"
SFFV	"Spleen Focus Forming Virus"
SH2	"Src homology domain 2"
Sp1	"Specificity protein-1"
STAT	"Signal transducer and activator of transcription"
Syp	see PTP1D
TBP	"TATA-box binding protein"
TFIID	"Transcription factor II D"
Tyr	"Tyrosine"
μg	"microgram"
μL	"microliter"

CHAPTER 1

INTRODUCTION

1 The hematopoietic system

Hematopoiesis is a dynamic developmental process that ensures a sufficient supply of terminally differentiated blood cells for the survival of the animal. The mature blood cells include erythrocytes or red blood cells, megakaryocytes which form the platelets, and monocytes and lymphocytes which have a major role in our defense mechanism. All originate from a population of self-renewing pluripotent stem cells (HSCs).

1.1 Primitive hematopoiesis

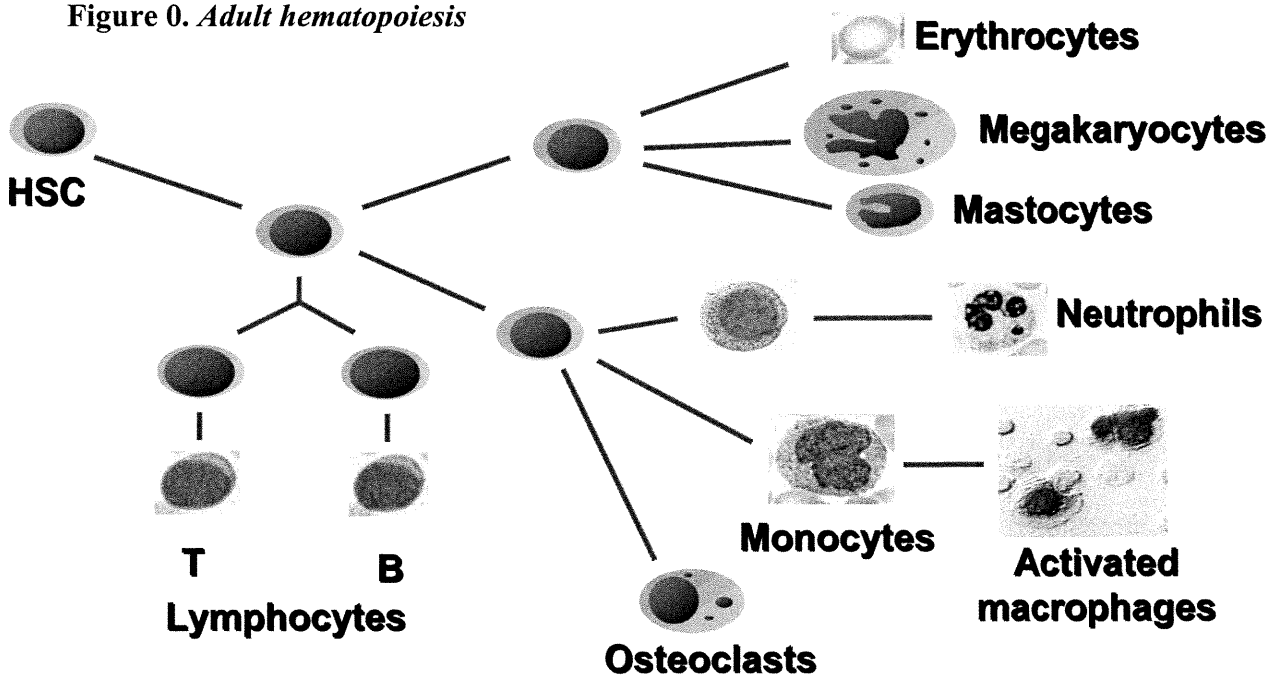
The hemangioblast is the proposed common precursor for the hematopoietic and endothelial cell lineages. The initial development of the hematopoietic lineage cells during mouse embryogenesis occurs in the yolk sac of the late primitive streak, day 7.5 embryo where blood islands develop in the mesenchyme of the visceral yolk sac (Cumano *et al.*, 1996; Medvinski *et al.*, 1996). These blood islands contain primitive, nucleated erythrocytes expressing embryonic globin, primitive macrophages, megakaryocytes, and multipotential progenitors. The network of blood islands increases with time and by embryonic day 9, when circulation begins in the embryo, hematopoietic stem cells that arose in the yolk sac migrate to the embryo and colonize the fetal liver where definitive hematopoiesis begins (Choi, 1998; Moore and Metcalf, 1970). Although the magnitude and complexity of liver hematopoiesis differs from yolk sac hematopoiesis, the latter consisting primarily of erythroid development, the stem cells of the yolk sac are fully functional to give rise to all hematopoietic lineages, both myeloid and

lymphoid. There is then a final shift to the bone marrow, which is the primary site of hematopoiesis in the adult.

1.2 Definitive hematopoiesis

Adult hematopoiesis, which primarily takes place in the bone marrow and the spleen, ensures that all blood cell lineages are produced sufficiently in the adult body. A small population of self-renewal pluripotent hematopoietic stem cells produces the eight different fully matured cell types : the lymphoid B and T cells, erythroid cells, megakaryocytes, neutrophils, monocytes, eosinophils, and basophils. As hematopoietic stem cells progress through the different differentiation steps, they become more and more restricted until they reach an irreversible committed faith and become specific, fully differentiated blood cell (Figure 0).

Figure 0. ***Adult hematopoiesis***



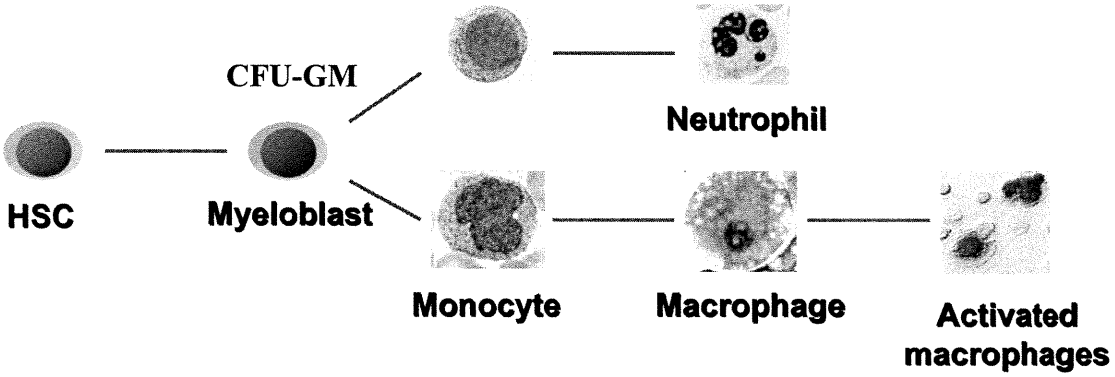
2 Myelopoiesis

Myelopoiesis is a complex process by which primitive blood precursors differentiate into fully matured, functionally active, granulocytes and monocytes (Figure 1). The development of these mature granulocytes and monocytes from hematopoietic precursor cells is controlled by a myriad of transcription factors which regulate the expression of essential genes, some more lineage specific, some more widely expressed (Ward *et al.*, 2000, Bello-Fernandez *et al.*, 1997). Such transcription factors can act both positively and negatively to regulate the expression of a wide range of important genes, including growth factors, their receptors, enzymes, adhesion molecules, and transcription factors themselves. Several genes expressed specifically by immature myeloid cells have been extensively studied (Ness *et al.*, 1993). From this work, the transcription factors C/EBPα, CBF, c-Myb and PU.1 have emerged as key regulators of gene expression during early myelopoiesis and myeloid lineage commitment.

The production of myeloid precursor cells, and subsequently fully matured granulocytes and monocytes, is also regulated by a network of hematopoietic growth factors and cytokines. One of these, the granulocyte colony-stimulating factor (G-CSF), is a major regulator of neutrophilic granulocyte production (Ward *et al.*, 2000). It increases the proliferation, survival, maturation and functional activation of cells of this lineage. On the other hand, the granulocyte-macrophage colony-stimulating factor, which plays an important role in granulocytic cells, also has important functions in macrophage proliferation, survival and differentiation through signaling by its receptor.

An important goal remains to understand in greater detail how these various transcription factors act in concert with signals emanating from cytokines and their receptors to influence these various steps of maturation. From the pluripotent hematopoietic stem cells, to a committed myeloid progenitor, to myeloid precursors, and ultimately to mature and fully active granulocytes and monocytes.

Figure 1. – *Myelopoiesis.*



2.1 Adaptive and innate immunity

Any immune response involves, firstly, recognition of the pathogen, and secondly, mounting a reaction against it for elimination. The different types of immune responses fall into two categories: innate (or non-adaptive) and adaptive responses. The important difference between these is that an adaptive immune response is highly specific for a particular pathogen. In effect, the

adaptive immune system "remembers" the infectious agent and can prevent it from causing any disease at later times. Lymphocytes are wholly responsible for the specific immune recognition of pathogens and initiate an adaptive immune response.

On the other hand, leukocytes, of which there are several types, act as a first line of defense against infection, thus mediating innate immune responses. Cells involved include monocytes, macrophages and granulocytes.

2.2 Biological functions of granulocytes

Neutrophils constitute over 90% of the circulating polymorphs and around 120 billion new neutrophils with a lifespan of only 48 hours are generated every day by a healthy adult. Neutrophils participate in innate immune responses mainly through phagocytosis of foreign particles. They posses lysosome granules which contain acid hydrolases, myeloperoxidase and muramidase (lysozyme). When ingested organisms contained in vacuoles fuse with the lysosomes, they are immediately degraded by the strong acids present (Alberts, 1996).

2.3 Biological functions of macrophages

Macrophages actively participate in the innate immune response mainly by phagocytosis of foreign particles or even tumor cells. These phagocytic cells contain specialized lysosomes that fuse with newly formed phagocytic

vesicles, exposing phagocytosed microorganisms to a barrage of enzymatically produced, highly reactive molecules of superoxide, hypochlorite, as well as a concentrated mixture of lysosomal hydrolases. Macrophages express a variety of receptors in part responsible for antigen recognition, triggering of extracellular killing and phagocytosis. Macrophages can also play a role in adaptive immune responses by acting as antigen–presenting cells (APC) and inducing the functional activity of certain T cells. Unlike neutrophils, which survive for only a few days, macrophages can persist for months outside the bloodstream where they can be activated by signals to resume proliferation (Steinman *et al.*, 1988).

3 Cell signaling and growth factor signaling

Any given cell in a multicellular organism is exposed to many different signals from its environment. The cell must respond to this variety of stimuli selectively, according to its own specific character acquired through progressive cell specialization in the course of development. Thus a cell may be programmed to respond to one set of signals by differentiating, to another set by proliferating, and to yet another by carrying out some specialized function. The specific way a cell reacts to its environment varies, first, according to the set of receptor proteins expressed that direct a particular subset of available signals and, second, according to the intracellular machinery by which the cell integrates and interprets the information that it receives.

3.1 Signaling pathways

Most extracellular signals are mediated by ligands binding to receptors on the surface of target cells. There are three known classes of cell-surface receptors: ion-channel-linked, G-protein-linked, and enzyme-linked. The last two types of receptors will be the focus of this section.

Signals received at the surface of cells by G-protein receptors or enzyme receptors are often relayed to the nucleus, where they alter the expression of specific genes thereby altering the behavior of cells. Elaborate sets of intracellular proteins form the relay systems. The majority of these proteins are one of two kinds: proteins that become phosphorylated by protein kinases, and proteins that are induced to bind GTP when the signal arrives. These proteins in turn generally cause the phosphorylation of downstream proteins as part of a *phosphorylation cascade* (Murdoch *et al.*, 2000). Many different but conserved signaling pathways have been identified. The Ras pathway leads to c-fos activation and induction of genes involved in proliferation. The PI3K pathway has several biological effects such as promoting survival through the Akt protein (Cantley *et al.*, 1999). There is also the Jak-STAT pathway involved in signaling from a number of tyrosine-kinase-associated-receptors, including those of growth factors and various cytokines that act on hematopoiesis (Ward *et al.*, 2000).

3.2 Growth factors signaling

Growth factors are proteins that regulate cell proliferation by stimulating resting cells to undergo cell division. Growth factors are specific for cell type, acting only on a cell that displays the appropriate cell-surface receptors. Growth factors that act only on a few cell types include the epidermal growth factor, platelet-derived growth factor, nerve growth factor, fibroblast growth factor and the interleukins. A common signaling pathway operates for many growth factors. The primary event, binding of the growth factor to its cell-surface receptor, is followed by activation of tyrosine-kinase catalytic activity of the intracellular domain of the receptor tyrosine kinase (RTK). For many growth factor receptors, activation of the catalytic activity of the kinase domain is thought to occur as a result of ligand-induced receptor dimerization. Following receptor activation, a wide variety of signaling pathways can be activated each generating a specific biological response.

3.3 Hematopoietic growth factors

Studies on hematopoietic growth factors have shown that these growth factors control the growth, survival and differentiation of hematopoietic progenitor cells and bind to specific receptors that are expressed on the surface of immature hematopoietic cells found in bone marrow (Metcalf, 1987). Some hematopoietic growth factors are lineage specific whereas others support multiple lineages. For example, erythropoietin (Epo) supports the growth of erythroid cells (Lin *et al.*, 1996) only whereas interlukin-3 (IL-3) supports the growth of multiple lineages (Sparrow *et al.*, 1987) . These growth factors are

also called colony-stimulating factors (CSFs). They are synthesized by various cell types (endothelial cells, fibroblasts, macrophages, lymphocytes) and, for example, their concentration in the blood typically increases rapidly in response to tissue infection thereby increasing the number of phagocytic cells released from the bone marrow into the blood stream. CSFs not only operate on the precursor cells to promote production of differentiated progeny, they also activate the specialized functions of the terminally differentiated cells. Recombinant hematopoietic growth factors are now being used in human patients to stimulate the regeneration of hematopoietic tissue and to boost resistance to infection.

Table I. – *Hematopoietic growth factors and their target cells.*

Growth factors	Responding hematopoietic cells
Epo	Erythroid progenitors
G-CSF	Granulocytes
IL-7	Lymphoid stem cells
IL-3	Pluripotent precursor cells, megakaryocytes
GM-CSF	Pluripotent precursor cells, myeloid cells
IL-5	B cells, eosinophils
M-CSF	Macrophages
IL-2	T cells

Receptors for hematopoietic growth factors belong mostly to a large receptor family called the cytokine receptor superfamily. This type of receptor will be discussed in greater detail in chapter 8.

4 Gene expression and transcription factors

The specific set of genes expressed in a cell will determine its identity (muscle cell, nerve cell, hematopoietic cell, etc.) as well as allowing the cell to undergo specialized function responsible for its identity (contraction, influx transmission, phagocytosis, etc.). It is therefore essential for the cell to keep a close watch on gene expression. For this reason, regulation of expressed genes becomes a critical task for each cell. In some cases, it is when genes are inappropriately expressed that undesired biological consequences are observed such as development of cancer.

4.1 Regulation of gene expression

Since gene expression is what determines, in part, cellular identity and appropriate cellular function, it needs to be tightly regulated. Regulation of gene expression exists at many levels: transcription, translation, and post-translation. Transcriptional regulation includes chromatin remodeling and DNA methylation, which interferes with DNA accessibility. There can also be competition between transcription factors for specific DNA sites. This brings up a very important aspect of transcriptional regulation: transcription factors. Transcription factors are key accessory proteins that are crucial for initiation of transcription and gene expression. Transcription factors need to be controlled by a tight regulation program for proper gene activation. They can either activate or repress directly or indirectly gene expression. To have an influence on a particular gene, a transcription factor first needs to be

expressed in the cell. It also needs to be in a functional conformation and in most cases, interacting partners must also be available.

4.2 Transcription factor functioning

The majority of transcription factors are able to bind DNA directly on specific consensus sequences. Some can bind by themselves while others need to bind as a complex. For example, PU.1 and GATA-1 can bind directly to DNA (Fisher *et al.*, 1991; Clore *et al.*, 1994) but c-jun, c-fos, C/EBPs, or SCL may need the presence of interacting partners. In some cases, transcription factors are unable to bind DNA directly and are recruited in proximity of the promoter by other factors. Examples are FOG, LMO2, or CBP/p300 (Tsang *et al.*., 1997; Merika *et al.*, 1998; Chrivia *et al.*, 1993). This necessity to regulate gene expression by forming specific complexes with various partners confers flexibility to the whole transcription mechanism as well as great control and tight regulation of the process.

4.3 Transcription factor modifications

Another way of controlling transcription and gene expression is by physically modifying transcription factors. Phosphorylation is to date, the most frequent protein modification. By adding phosphate residues to a transcription factor, it is possible to interfere with its ability to bind DNA (Hardy *et al.*, 1993) or to interact with partners and activate transcription. In some cases, phosphorylation accomplishes the opposite by enhancing transactivation

properties (Hagmeyer *et al.*, 1993) and promoting protein interaction (Eisenbeis *et al.*, 1995). Protein phosphorylation results from extracellular signals that activate signaling pathways where protein kinases play a determining role.

4.4 Specific expression of transcription factors

Another important aspect of transcription factor regulation is the fact that some factors need to be present at a particular time in a particular place. Their expression also needs to be time restricted. Often, the expression of these types of transcription factors is influenced by environmental signals. There exist many examples of these in the course of hematopoiesis. The hematopoietic system is indeed highly dependent to extracellular stimuli such as cytokines and growth factors to dictate cell fate and cell development. Linking these environmental signals with tissue-specific transcription factor activation and specific gene expression is becoming an attractive model and a challenging one (Shivdasani and Orkin, 1996).

4.5 Hematopoietic transcription factors

Development of the different hematopoietic cell lineages from a small population of hematopoietic stem cells is highly dependent on extracellular signals, as well as the intrinsic properties of the cell that confers its ability to recognize and respond to these signals. It is why tissue-specific transcription factors may have a crucial role in "transforming" these environmental signals

into biological responses such as proliferation, survival, or differentiation. These tissue-specific transcription factors were primarily discovered from chromosomal translocations seen in some types of leukemia or by gene targeting and promoter studies. These transcription factors play a determining role in the development of the different hematopoietic lineages, some having a more restricted specificity, while others have a more general contribution.

4.6 Cell-lineage specific transcriptional regulation

There are several examples of lineage-specific transcription factors. For example, GATA-1, a zinc-finger protein, is essential for erythropoiesis and expression of most erythroid-specific genes such as the Epo receptor, SCL, β-globin, and itself (Shivdasasani *et al.*, 1996). Lymphoid development is also highly dependent on one "master" transcription factor, Ikaros. It is another zinc-finger protein specifically expressed in lymphoid cells and mice knock-outs for the Ikaros gene are completely depleted of mature B and T cells as well as their progenitors while other lineages are not affected (Georgopoulos *et al.*, 1994). Finally, myeloid cell development is also highly dependent on an essential transcription factor, PU.1 (Anderson *et al.*, 1998, DeKoter *et al.*, 1998). The transcription factor PU.1 is specific to the myeloid and lymphoid lineages and is an important regulator of many B cell and myeloid cell genes (Hromas *et al.*, 1993). PU.1 is further discussed in the following chapter.

5 The transcription factor PU.1

5.1 Discovery

Experimental retrovirally-induced leukemia in mammals or birds have been fruitful systems for the identification of oncogenes and proto-oncogenes directly involved in neoplastic processes and consequently in the control of normal cellular growth and differentiation. The transcription factor PU.1/Spi-1/Sfpi-1 has been characterized by virtue of its activation in murine erythroleukemic processes (Moreau-Gachelin *et al.*, 1998). Studies revealed that the provirus SFFV (Spleen Focus Forming Virus) integrates in a preferential genomic locus in 95% of the Friend tumors induced by SFFV. This locus corresponds to the region that encodes the normal Spi-1 protein. In addition, the integration of SFFV at the Spi-1 site is concomitant with the massive expression of a unique 1.4-kilobase messenger RNA corresponding to the Spi-1 protein. This proviral integration is associated with the massive proliferation of proerythroblastic cells in the spleen and liver which are blocked in their differentiation process at the late BFU-E (blast-forming unit-erythrocytes) or CFU-E (colony-forming unit-erythrocytes) stage (Shibuya *et al.*, 1983).

At about the same time, another group elaborated a strategy to identify from a macrophage cDNA library, a protein susceptible of interacting with the promoter of the murine MHC class II 1-Ab gene (Klemsz *et al.*, 1990; Goebl *et al.*, 1990). This led to the cloning and the functional characterization of a transcription factor which appeared identical to, and later confirmed to be, Spi-1 (Moreau –Gachelin *et al.*, 1990; Goebl*et al.*, 1990).

Although PU.1 was first isolated as a gene activated in murine erythroleukemia, so far it has not been implicated in the pathogenesis of human leukemia. The PU.1 gene has been mapped to 11p11.00, not a frequent site of chromosomal translocations found in human leukemia.

5.2 A member of the Ets family

PU.1 belong to a family of transcription factor known as the Ets family (Seth *et al.*, 1992). The Ets domain is a highly conserved region that specifies its ability to bind to DNA. It is a basic region at the COOH terminal part made up of 85 amino acid. This basic amino acid-rich region mediates DNA binding by a winged α helix-turn-helix configuration (Karim *et al.*, 1990) which recognizes a consensus sequence 5'-GGAA/T-3' known as PU boxes which are purine rich, hence its name (Pio *et al.*, 1995; Kodandapani *et al.*, 1996). We also find ets-1, ets-2, erg, elk-1, fli-1 and Spi-B as part of the same family of transcription factors. Members of the family identified to date share between 36 and 97% sequence identity with the Ets-1 (Watson *et al.*, 1988) DNA-binding domain and their specificity in DNA binding is dependent on sequences outside the GGAA core. Members of the Ets family of transcription factors have been found in species ranging from lower invertebrates to humans (Ray *et al.*, 1990).

5.3 Structure

5.3.1 The transactivation domain

There are no conserved structural motifs which characterize protein domains involved in the regulation of transcription, but only some conserved biochemical features such as a high content in negatively charged amino acid residues, hydrophobic regions, high glutamine residue content, or proline-rich domains.

The amino-terminal part of PU.1 (aa 1 to 102) is acidic due to the preponderance of acidic (16% asp and glu) over basic amino acids. It constitutes a functional transactivator domain when fused to a heterologous DNA binding domain. It contains a glutamine-rich sequence (31%) between amino acids 70 and 100 which is involved in the transcriptional activity of PU.1 since its deletion suppresses the PU.1-induced transactivation in reporter assays (Shin *et al.*, 1993).

5.3.2 The PEST domain

The central part of the protein (aa 102 to 158) is characterized by its high content in proline (P), glutamic acid (E), serine (S), and threonine (T). It is believed that this PEST region could be involved in the rapid turnover of the protein as well as a protein-protein interaction region. The PEST domain also contains a phosphorylation site at serine 148 (Ghoda *et al.*, 1992; Salama *et al.*, 1994).

6 Role of PU.1 in hematopoiesis

6.1 The lymphoid/myeloid-specific transcription factor

PU.1 is a transcription factor found exclusively in cells of the hematopoietic system. Erythroid cells express PU.1 at a very early stage of their development, but PU.1 protein disappears before maturation (Cheng *et al.*, 1996). PU.1-null mice are born with apparently normal erythrocytes, suggesting that PU.1 does not play a dominant role in development of this lineage. The peripheral blood reticulocyte count of PU.1-null pups is considerably elevated compared to normals, and their extruded nuclei and other debris clutter the blood. Most of these phenomena suggest secondary effects on the erythroid lineage in PU.1-null mice, although direct effects due to lack of the gene at early stages of development have not been ruled out (Schuetze *et al.*, 1993).

Northern analysis indicated that PU.1 is highly expressed in cells of myeloid and B- lymphoid lineages. It functions exclusively in a cell-intrinsic manner to control the development of granulocytes, macrophages, and B and T lymphocytes. It was demonstrated that mutation of the PU.1 gene causes a severe reduction in myeloid progenitors and that knockout mice die shortly after birth, often of infectious diseases due to severe neutropenia (Scott *et al.*, 1994; McKercher *et al*, 1996). They show a complete absence of monocytes/macrophages and mature B cells while neutrophils are present but reduced in numbers and showed significant abnormalities such as being Mac-1 negative, rendering them nonfunctional (Oikawa *et al.*, 1999).

These results demonstrate that PU.1 is an important regulator in the development of cells in the hematopoietic system and that its expression pattern makes PU.1 a candidate for a genetic determinant of lineage commitment and stage progression in blood cell development.

6.2 Transcriptional regulation in myeloid development

Consistent with the finding that PU.1 has a major role in myeloid development, multiple myeloid genes have been found to be regulated by PU.1. Many studies show PU.1 acting as a transcriptional regulator through binding as a monomer to the minimal tetranucleotide GGAA identified in regulatory elements of viral gene, as well as in promoters or enhancers of genes specific of myeloid and lymphoid cells.

6.3 PU.1 and myeloid promoters

PU.1 is implicated in the control of expression of an increasing number of myeloid specific genes. Myeloid promoters in general appear to differ from what has been described previously for tissue specific promoters (Goodbourn *et al.*, 1988). In general, a relatively small upstream region, usually a few hundred base pairs, is capable of directing cell type specific expression in tissue culture studies (Pahl *et al.*, 1992). In general, they lack a TATA box or a defined initiator sequence. Consistent with this lack of TATA box, these promoters often are dependent on a functional Sp1 site, which not only

mediates activity, but also specificity and inducibility with differentiation. Of the myeloid genes that have been shown to function in transgenic studies to date, several of them including CD11b (Pahl *et al.*, 1993), the M-CSF receptor α chain (Zhang *et al.*, 1994), the GM-CSF receptor α chain (Hohaus *et al.*, 1995), the lysozyme enhancer (Ahne *et al.*, 1994), the scavenger receptor (Moulton *et al.*, 1994), c-fes (Heydemann *et al.*, 1996), and PU.1 itself (Chen *et al.*, 1995), have important functional PU.1 sites. Mutations of these critical PU boxes which abrogate PU.1 binding, decrease promoter activity significantly in PU.1 expressing cells. These studies strongly suggest that PU.1 is a critical factor in directing myeloid expression.

Table II – ***Myeloid gene targets regulated by PU.1***

Gene targets
CD11b (Mac-1 alpha)
M-CSF receptor
Fc-gamma-R1
lysozyme
CD18 (Mac-1 beta)
macrophage scavenger receptor
neutrophil elastase
G-CSF receptor
GM-CSF receptor PU.1
c-fes
myeloperoxidase
proteinase-3
IL-1 beta

It is important to note that PU.1 does not act alone in regulating myeloid gene expression. Other major regulators have now been identified. Of them is the family of C/EBP factors. Studies have suggested that in the hematopoietic system, C/EBP factors are specifically expressed in myeloid cells (Natsuka *et al.*, 1992), and may hence be important in myeloid development. It was shown that important C/EBP binding sites are located in the proximal promoter regions of the M-CSF, G-CSF, and GM-CSF receptors. Interestingly, the C/EBP and PU.1 sites are separated by as little as 15 to 80 bases and C/EBPα and PU.1 have been shown to physically interact with each other (Hsu *et al.*, 1994).

6.4 Protein interactions

As mentioned above, most myeloid gene promoters lack a TATA box. Interestingly, PU.1 has been found to physically interact with TBP, a component of the TFIID, *in vitro* (Hagemeier *et al.*, 1993), via its activation domain. An attractive hypothesis is that PU.1 may recruit the basal transcription machinery to myeloid promoters which in general lack TATA boxes, but the functional significance of this interaction remains to be determined. In this same study, it was shown that PU.1 can also interact physically with Rb. It has been shown that hypophosphorylated Rb can negatively regulate the activity of another Ets factor, Elf-1, in the course of T-cell activation. Because Rb becomes hypophosphorylated during myeloid differentiation, it could possibly regulate PU.1 function in these cells. PU.1 has also been shown to interact with the basic leucine zipper (bZIP) transcription factor c-jun (Behre *et al.*, 1999). In fact, c-jun promotes macrophage differentiation by inducing the M-CSF promoter, in collaboration

with PU.1 (Behre *et al.*, 1999). Furthermore, it was recently demonstrated that PU.1 interacts directly with GATA-1, a zinc finger transcription factor required for erythroid differentiation. This interaction interferes with normal GATA-1 function and normal erythroid gene expression thereby blocking terminal erythroid differentiation (Rekhtman *et al.*, 1999).

6.5 Regulation

The regulated development of the different hematopoietic cell lineages from a common precursor involves fundamental changes in gene expression, resulting in each cell type expressing a characteristic complement of genes necessary for its function. This is achieved through the action of transcription regulators specifically expressed in each cell type. However, it is becoming clear that another level of regulation is superimposed on this pattern of combinatorial activation, which involves repression of genes specific for one lineage by transcription factors promoting other lineages. This is illustrated by the incompatibility of the myeloid phenotype with GATA-1 expression. Ectopic GATA-1 expression represses myeloid gene expression through its ability to directly interact with the PU.1 Ets domain and thereby interfering with PU.1 function (Nerlov *et al.*, 2000*)*. Another striking example is the possible role of the Rb protein in regulating PU.1 activity again by directly interacting with it although experiments to date have failed to show an effect of Rb on PU.1 function on myeloid promoters (Hagemeir *et al.*, 1993). An emerging concept is suggesting that the stoichiometry of directly interacting but opposing transcription factors may be a crucial determinant governing processes of normal differentiation and malignant transformation. In another study, the CREB binding protein (CBP) was found, through the yeast two-

hybrid system, to physically interact with PU.1. In addition, CBP enhanced PU.1-mediated transcription of multimerized PU-box luc reporter constructs, suggesting that CBP acts as a coactivator for the transcription factor PU.1. Since the amount of CBP is limited in cells (Tanaka *et al.*, 1997), CBP may mediate positive and negative cross talk between PU.1 and other transcription factors in the process of hematopoietic cell differentiation. It was also found that Goosecoid is specifically able to bind PU.1, but not other members of the Ets family, and in that way suppressing blood formation in primitive hematopoiesis (Konishi *et al.*, 1999).

Recently, one group showed for the first time, possible regulation of Ets family members function through a well-conserved mechanism, phosphorylation. Phosphorylation of transcription factors is a key link between cell signaling and the control of gene expression. It was reported that phosphorylation regulated DNA binding of the Ets-1 transcription factor by reinforcing an autoinhibitory mechanism (Cowley *et al.*, 2000).

6.6. PU.1 in myeloid development

Little is known about the transcription factors that mediate lineage commitment of multipotent hematopoietic precursors. Although a number of transcription factors that regulate the expression of cell type-specific genes are well characterized, little is known about the transcriptional events that lead to lineage commitment of multipotent progenitors. PU.1 involvement in monocytes/macrophages and granulocytes differentiation has been well established. The question is to what extent is it important for myeloid development. Gene inactivation studies in mice demonstrated the ability of

PU.1-/- progenitors to commit to the monocytic-granulocytic lineage . Along with *in vivo* flow cytometry and cytochemical analysis of primary neonate liver cells, it showed that commitment to the monocytic and granulocytic lineages occurs in the absence of the transcription factor PU.1 (McKercher *et al.*, 1999; Henkel *et al.*, 1999). But gene inactivation studies in mice are of somewhat limited value in addressing this question because of commonly encountered gene redundancies and difficulties in defining the earliest committed progenitors. Another group has shown that commitment of multipotent progenitors to the myeloid lineage is indeed mediated by PU.1. Using an alternative *in vitro* system, based on the generation of primary transformed hematopoietic cells, they have shown that PU.1 instructs these transformed cells to exit the multipotent state and to differentiate into myeoblasts. This process becomes irreversible within a couple of days of forced expression of an active PU.1 form and is preceded by the down-regulation of GATA-1, a factor incompatible with myeloid gene expression (Nerlov *et al.*, 1998). Although the question addressing the extent of the role of PU.1 in myeloid commitment is still under investigation, one fact remains clear: terminal myeloid differentiation requires the transcription factor PU.1.

It has been previously demonstrated using gene targeting that there is a multilineage defect in the generation of monocytic and granulocytic cells in PU.1-/- embryos (Anderson *et al.*, 1998). PU.1 knock-outs are viable at birth and could be kept alive for days by housing them in a sterile environment with the administration of antibiotics. These animals completely lacked monocytes and mature B cells. Although neutrophilic cells were observed, they were Mac-1-negative, reduced in number and functionally incompetent. RT-PCR analysis of embryonic stem (ES) cells indicates that some early myeloid genes can be expressed in the absence of PU.1, but expression of late myeloid genes

is blocked (Olson *et al.*, 1995). There is also a complete absence of fully matured F4/80 positive monocytic cells.

Studies, again using PU.1 gene targeting, demonstrated that PU.1 mutation severely reduces but does not eliminate myeloid progenitors (Henkel *et al.*, 1999). Rescue by ectopic PU.1 expression leads to an increase in progenitor cell as well as the appearance of normal, fully differentiated myeloid cells (DeKoter *et al.*, 1998). Therefore, PU.1 controls both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors.

7 Granulocyte-macrophage colony-stimulating factor

7.1 The cytokine superfamily

Granulocyte-macrophage colony-stimulating factor is a hematopoietic growth factor involved in the regulation of proliferation, survival, and differentiation of hematopoietic progenitor cells with some additional effects on the monocyte/macrophage system. It belongs to the cytokine superfamily that includes colony-stimulating factors such as the interleukins (IL-3, IL-1, IL-6, etc.), G-CSF, Steel factor (SF) and Epo, to name only a few. The cytokine has four α-helical regions. The region nearest to the N-terminal end interacts with the beta common chain (β_c) of its receptor while the α-helical domain nearest to the C-terminal end confers receptor specificity by binding only to the α sub-unit, specific for its own receptor .

7.2 Clinical applications

Until recently, GM-CSF has been regarded exclusively as a hematopoietic growth factor simply involved in proliferation and differentiation of different hematopoietic progenitors and of certain, more committed, hematopoietic cells. Based on recent studies, GM-CSF is no longer being investigated as a simple hematopoietic growth factor only. It has become apparent that GM-CSF also has multiple activities for clinical applications (Armitage, 1998). It plays an important role in inflammatory processes. It contributes to the regulation of the immune response through effector cells such as granulocytes, macrophages or lymphocytes (Wing *et al.*, 1989) and by enhancing dendritic cell growth and function (Gosselin *et al.*, 1993). GM-CSF has also been shown to enhance antibody-dependent cell cytotoxicity (ADCC) of granulocytes and monocytes (Wing *et al.*, 1989). Because monoclonal antibodies represent a potentially extremely effective form of cancer treatment by acting on tumor-associated surface structure, GM-CSF could further enhance the efficiency of therapeutic mAb.

Another approach for using the immunostimulating activities of GM-CSF is the combination with anticancer vaccines (Young *et al.*, 1995). This strategy is based on recent development in cancer immunology which demonstrated the critical role of T cells in anticancer responses and the antitumor activity of cancer vaccines in animal models and patients.

Antigen recognized by T cells are peptide fragments of intracellular proteins that are bound to the MHC molecules and then expressed on the cell surface. When administered with antigenic peptides, GM-CSF is able to elicit both a

specific antibody and cellular immune response, thus representing a potent adjuvant for the generation of immune responses to foreign proteins as well as peptides derived from a self-tumor antigen. The importance of using GM-CSF in this setting is further emphasized by the fact that peptides alone may not be immunogenic whereas in combination with GM-CSF, a strong delayed-type hypersensitivity reaction (DTH) response can be detected (Caux *et al.*, 1992).

GM-CSF, in combination with IL-4, is the most important cytokine for the development of dendritic cells. Dendritic cells are highly specialized to initiate specific immune responses by presenting antigens to lymphocytes. Consequently, the dendritic cell network plays a central role in the induction of T cell- as well as B cell-mediated immunity and therefore may serve as a natural adjuvant in future strategies for specific immunotherapy with tumor antigens (Sallusto *et al.*, 1994).

It can be concluded that the therapeutic potential of GM-CSF by far exceeds its known role as a hematopoietic growth factor because of its essential effects within the immune system.

These days, GM-CSF is being tested and used in the prevention and treatment of fungal infections (Jones *et al.*, 1999), in hematopoietic stem cell transplantation (Croockewit *et al.*, 1999), severe mucositis (Hejna *et al.*, 1999), and antitumor vaccine strategies (Gaudernack *et al.*, 1999) to name a few applications.

8 The Granulocyte-macrophage colony-stimulating factor receptor

8.1 Superfamily

The superfamily of cytokine receptors is now very large. These cell-surface glycoprotein receptors are characterized by a common structural feature: the presence of at least two fibronectin-like folds in the extracellular domain formed by four conserved cysteine residues, which was first identified in the growth hormone receptor, and a typical WSXWS motif in the juxtamembrane region (Basan *et al.*, 1990). These receptors enable cells to communicate with the extracellular environment by responding to signal generated in the vicinity or in other parts of the organism.

8.2 Structure

Cloning of the receptor components and the reconstitution of functional receptors for IL-3, IL-5, and GM-CSF has shown that the receptors are heterodimers composed of a cytokine-specific α chain (IL-3Rα, IL-5Rα, GMRα) and a common β chain (β_c) (Kitamura *et al.*, 1991). The human α subunit is 378 amino acids in length (Gearing *et al.*, 1989), most of which constitutes the extracellular domain, whereas the cytoplasmic tail has only 54 amino acids. The β_c subunit comprises 881 amino acids with a 432 amino acid cytoplasmic tail (Hayashida *et al.*, 1990).

Although the human β_c subunit has no binding capacity by itself, it forms a high-affinity receptor complex by association with the low-affinity α chains. Besides its role in high-affinity binding, the β_c chain plays a major role in IL-3-, IL-5-, and GM-CSF-mediated signal transduction (Sakamaki *et al.*, 1992). Therefore, the common use of the β_c chain subunit by all three cytokines explains the observed partial functional redundancy of these cytokines (Miyajima *et al.*, 1992). The cytoplasmic domain of the common β chain includes a membrane-proximal region containing a proline-rich sequence termed box1 that is highly conserved among several cytokine receptors and serves as a binding site for the cytoplasmic tyrosine kinase Jak2. A second conserved cytokine receptor element, box2, occurs at approximately 60 amino acids C-terminal to the transmembrane domain (Smith *et al.*, 1997). The membrane-distal region of the β_c contains several tyrosine residues involved in the induction of several different signaling pathways. It is important to note that the receptor for GM-CSF does not posses intrinsic catalytic activity. Indeed, Jak2 binding is critical for all the biological functions expressed by GM-CSF. It is Jak2 that is responsible for tyrosine phosphorylation of the cytoplasmic tail of the β chain (Doyle and Gasson 1998).

Another interesting point is that when the entire cytoplasmic domain of the α subunit is deleted, intracellular signaling is blocked, indicating that the α chain is also involved in signaling (Takaki *et al.*, 1994, Lia *et al.*, 1996).

8.3 Expression

Cell surface expression of the GM-CSF receptor (GMR) alpha chain was examined using anti-GMR alpha and flow cytometry. GMR alpha was readily

detectable on both blood monocytes and neutrophils. In adherence- depleted normal bone marrow, two separate populations expressed GMR alpha. The most positive cells were predominantly macrophages, whereas the cells that expressed less GMR alpha were largely myelocytes and metamyelocytes. A small population of lin-CD34+ or CD34+CD38- cells also expressed GMR alpha, but they were not capable of significant growth in colony-forming assays. In contrast, the majority of lin-CD34+ and CD34+CD38- cells were GMR alpha-, yet they produced large numbers of myeloid and erythroid colonies in the same assay. These studies indicate that GMR alpha is expressed on certain lineages throughout hematopoietic development; however, progenitors that express the receptor may have a reduced capacity to proliferate in response to hematopoietic growth factors (Jubinsky *et al.*, 1994; van Dijk *et al.*, 1998).

8.4 Signaling pathways

Cytokines have specific biological functions, including proliferation, differentiation, and functional modulation, in target cells expressing their cognate receptors. Thus, most cytokine receptors are coupled to multiple signaling pathways, which act in concert to govern the functional specificity of a particular cytokine. Members of the class I subgroup of the cytokine receptor superfamily do not posses intrinsic tyrosine kinase activity. They do interact with one or more nonreceptor tyrosine kinases (Watanabe *et al.*, 1993). Stimulation by ligand binding results in rapid and reversible tyrosine phosphorylation of multiple proteins including the receptor itself. The common β chain of the granulocyte-macrophage colony-stimulating factor, IL-3 and IL-5 receptors is the major signaling subunit of these receptors,

coupling ligand binding to multiple biological activities. Various cytoplasmic regions are associated with distinct signaling pathways which in turn illicit different biological responses. However, the α subunit has been shown to stimulate a mitogenic response to GM-CSF (Weiss *et al.*, 1993).

8.4.1 The Jak-STAT pathway

As mentioned above, the GM-CSF receptor possesses conserved domains known as box1 and box2. These domains have been shown to be crucial for the ability of the receptor to generate a proliferative response, and box1 has been shown to be the site of Jak2 interaction (Quelle *et al.*, 1994). Phosphorylation of tyrosine residues of the cytoplasmic tail and other signaling molecules in response to GM-CSF is dependent on Jak2 (Watanabe *et al., 1996).* Deletion of box1 or use of a dominant-negative Jak2 leads to an inability to stimulate transcription of c-fos, and inhibits proliferation upon GM-CSF stimulation (Watanabe *et al.*, 1996). Members of the Jak family of kinases are known to phosphorylate and thereby activate latent transcription factors, termed signal transducers and activators of transcription (STATs). These proteins are normally located in the cytosol, but upon receptor activation, they bind to the receptor, where they can then be phosphorylated by receptor-associated Jak kinases. Upon phosphorylation, STAT dissociates from the receptors and forms homodimers and heterodimers with other activated STATs. The dimer then translocates to the nucleus, where it is able to bind its cognate DNA binding site and activate transcription (Ihle *et al.*, 1996). Dominant-negative forms of STAT5 reduced expression levels of c-fos and inhibited the proliferative response of BaF3 cells to GM-CSF stimulation, indicating the significant role for STAT5 in GM-CSF (and IL-3) signal

transduction. Interestingly, one group has shown the importance of the 29 amino acid intracellular domain of the α subunit for activation of Jak2 and determination of which homologue of STAT5 is activated (Doyle *et al.*, 1998).

8.4.2 The MAPK pathway

Other studies have implicated the distal region of the cytoplasmic domain of the β_c in the activation of cascade events involving Shc (Kavanaugh *et al.*, 1994). , Ras (Watanabe *et al.*, 1996) , Raf, mitogen-activated protein kinase, and for induction of the c-fos and c-jun mRNAs. Shc is an adaptor protein that contains the SH2 (Src homology 2) domain and the phosphotyrosine interaction domain (Pellici *et al.*, 1992). It is associated with phosphotyrosine residues of several growth factor receptors being phosphorylated, and binding to Grb2, which in turn activates Ras by recruiting the Ras-guanine nucleotide exchange factor Sos (Lowenstein *et al.*, 1992). Tyrosine phosphorylation of PTP1D (also known as Syp or SH-PTP2), a cellular protein tyrosine phosphatase which contains two SH2 domains, also results in its association with Grb2 and is therefore thought to induce the activation of Ras. It has been reported that GM-CSF induces tyrosine phosphorylation of PTP1D and its association and its association with Grb2 and the p85 subunit of phosphatidyl-inositol 3'-kinase (PI3K) (Welham *et al.*, 1994). Ras activation ultimately results in activation of the MAPK pathways (Erk, JNK) that ultimately induces c-fos and c-jun expression resulting in proliferative and anti-apoptotic responses (Kinoshita *et al.*, 1995).

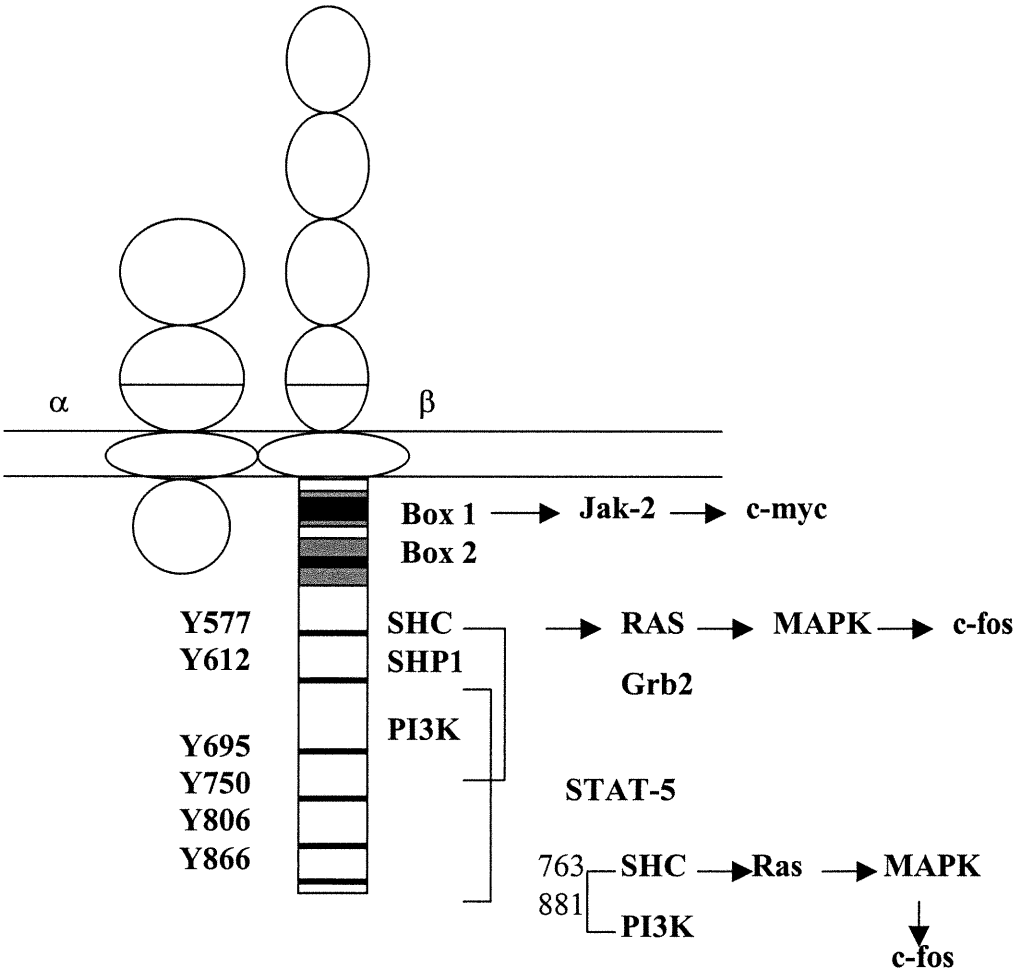
Despite extensive studies on the mapping of particular biochemical signaling pathways to specific regions of the cytoplasmic domain of β_c, the correlation of these pathways with different types of biological responses is less understood. But a recent study investigating the role of cytoplasmic tyrosine residues of the common β chain using a series of β_c mutants helped in shedding light on the matter.

8.5 Definition on the role of tyrosine residues

Substituting all eight cytoplasmic tyrosines together, but leaving box1 and box2 intact, severely impaired the potential of the β_c to transduce various signals, thereby indicating the critical requirement of β_c tyrosines for signaling. Because this mutant was still able to activate Jak2, loss of this function likely did not result from unfavorable changes in structural integrity (Itoh *et al.*, 1997). Different mutants lacking only one tyrosine did not show any significant defect in activating the c-fos promoter or in growth promotion indicating some redundancy among the different tyrosine residues. Therefore, a series of mutants was constructed by adding back each and every tyrosine. Among the eight tyrosine of the β_c, three tyrosine, Tyr577, Tyr612, and Tyr695, are involved in and can individually induce PTP1D (Syp) phosphorylation, its association with Grb2, and activation of the putative downstream Raf and Erk pathway, resulting in transactivation of the c-fos promoter. Tyr577 is also necessary for Shc phosphorylation and ultimately Ras activation. The activation of the Jun kinase (JNK) was also mediated by the same set of tyrosines as that needed for phosphorylation of PTP1D and the induction of c-fos (Itoh *et al.*, 1997).

The activation of STAT5 by GM-CSF was also seen to depend on β_c tyrosines, but the requirement differed. Tyr450 and Tyr452 played a substantial role in phosphorylating STAT5. It should be noted that Tyr612, Tyr750, Tyr806, and Tyr866 were also involved in STAT5 activation again suggesting some redundancy between tyrosine residues. Although no precise work on linking specific tyrosines residues to a differentiation response has been presented, it was however shown that the membrane-distal region of the β_c (starting at Tyr626) was involved in generating such a biological response, inducing progenitor cells to differentiate into granulocytes and monocytes (Smith *et al.*, 1997).

Figure 2. Signal transduction by the GM-CSF receptor.



8.6. GM-CSF in myeloid development

It is well understood that one of the major functions of the GM-CSF cytokine is the inhibition of apoptosis in its target cells, both in mature blood cells and in early progenitors (Brandt *et al.*, 1994). However, the signaling pathways utilized by GM-CSF to overcome death signals have only recently started to become evident. The targets important for cellular survival induced by GM-CSF seem to be proteins of the anti-apoptotic bcl-2 gene family. It was found that GM-CSF induces the expression of A1, a novel hematopoietic-specific homologue of Bcl-2 (Lin *et al.*, 1993). This survival response seems to be mediated by the Ras/Erk pathway (Itoh *et al.*, 1996), the Protein kinase C (PKC) (Rajotte *et al.*, 1992), and the PI3K/Akt pathway (Martens *et al.*, 1998).

As mentioned before, GM-CSF favors differentiation of multipotent progenitors into myeloid cells by, in part, activating myeloid-associated genes. GM-CSF also favors myeloid proliferation. It is because of these properties that GM-CSF is now being used and tested for many different diseases that are associated with a down-production of myeloid cells such as granulocytes and monocytes. Surprisingly though, GM-CSF receptor knockout (GMR-/-) mice develop to adult state with no apparent disadvantage. This implies a possible redundancy of the signaling pathways such that macrophages can develop in mice lacking GM-CSF signaling. These macrophages, however, exhibit decreased adhesion and decreased uptake of colloidal carbon (Scott *et al.*, 1998).

It is widely acknowledged that the GM-CSF receptor also modifies various effector functions in myeloid cells, such as cellular migration and respiratory burst in granulocytes (Coffer *et al.*, 1997). In addition, GM-CSF directly

enhances the function of antigen presenting cells and therefore elicits potent tumoricidal activity in macrophages both *in vitro* and *in vivo* (Pardoll *et al.*, 1995, Grabbe *et al.*, 1992, Witmer-Pack *et al.*, 1987)

Finally, exposure of purified bone marrow cells or of the multipotent FDCP-Mix cell line to GM-CSF irreversibly favors granulocyte and macrophage development. These studies, however, failed to document whether the effect of GM-CSF is permissive or instructive for macrophage formation.

9 Myeloid development

As shown in the previous sections, the differentiation of hematopoietic stem cells into specialized blood cells involved global changes in gene expression, resulting in expression of a characteristic set of genes in each mature cell type. These global changes have their influence at every level. Changes in hematopoietic growth factors and growth factor receptor expression, in receptor activation leading to a specific signal being transduced along a specific signaling cascade, in transcription factor regulation and expression, and finally, in the activation of specific sets of genes which eventually characterize the particular cell type. As we can see, the long road from one hematopoietic stem cell to a fully differentiated, functional specialized cell involves many different players at many different levels. Most of the work on GM-CSF signaling has focussed on the regulation of proliferation and cellular survival. As of yet, no study has addressed GM-CSF-dependent pathways that lead to macrophage differentiation.

10 Conclusion

The predominant roles of PU.1 and the GM-CSF receptor in myeloid development have been independently demonstrated: PU.1 favors myeloid development by inducing myeloid-associated genes and by interacting with negative regulators of myeloid differentiation and GM-CSF, through activation of its receptor and subsequent induction of different signaling pathways, plays an essential role in triggering myeloid-associated biological responses.

Recently, one group showed that PU.1 and the GM-CSF receptor play distinct roles in late-stage myeloid cell differentiation (Anderson *et al.*, 1999). They restored the expression of G-, GM-, and M-CSF receptors in PU.1-deficient cells using retroviral vectors. The expression of these receptors merely allowed a PU.1-deficient cell line to survive and grow in the relevant growth factor. When PU.1 expression is restored, F4/80+, and Mac-1+ macrophage development is also restored reinforcing the idea that availability of PU.1 is crucial for normal myeloid development. One hypothesis can be made from this study which the authors fail to foresee. Differentiation of myeloid progenitors is indeed highly dependent on PU.1 availability, but it could also mean that PU.1 is the effector of GM-CSF signaling in myeloid differentiation since PU.1 knock-out cells that ectopically express the GM-CSF receptor fail to differentiate into mature neutrophils and macrophages. By reintroducing PU.1 in this system, we are also reintroducing the missing link for myeloid development by GM-CSF.

11 Objective of this work

The objective of this work is to ask whether the myeloid-specific transcription factor PU.1 acts as an effector of GM-CSF signaling in myeloid development. An important question in hematopoiesis is how hematopoietic cells are able to translate environmental signals into specific biological responses such as cell fate. This is what this work tries to demonstrate. This study is based on the hypothesis that GM-CSF signaling leads to an upregulation of PU.1, therefore favoring myeloid development. PU.1 levels are assessed by Western blotting and PU.1 function through immunodetection of its target gene product CD11b. The role of PU.1 downstream of the GM-CSF receptor is determined by ectopic expression of PU.1 in the sense and anti-sense orientation.

CHAPTER 2

MATERIALS AND METHODS

Retrovirus production and infection

The murine anti-apoptotic Bcl-2 factor, PU.1 and anti-sense PU.1 were all introduced in the murine stem cell virus expression vector. 293 VSV G packaging cells were transiently transfected with the respective cDNAs through the calcium phosphate DNA transfer method. One day before transfection, the packaging cells were split 1:3 in 15mm culture dishes. On the day of transfection, 400μL of DNA-$Ca_3(PO_4)_2$ precipitate containing 25μg of DNA was added to the cultures and incubated overnight. The cells were then washed with PBS and resuspended in Dulbecco's Modified medium plus 10% FCS. The culture medium was collected after 48 hours and centrifuged 90 minutes at 24 000 rpm to obtain, in the supernatent, high titer (10^9 PFU/ml) amphotropic viruses.

For retroviral infection, 10^6 exponentially growing cells (FDCP-Mix) were pre-sensitized with 2μg/ml of polybrene for 24 hours and co-cultured with virus-producing clones for another 24-48 hours. Nonadherent FDCP-Mix cells were separated from the infected 293 fibroblast cells. A polyclonal population was analyzed 7 days after selection with neomycin (G418) at 1mg/ml. BOSC 23 cells used to infect bone marrow cells were also transfected by the calcium-phosphate precipitate method.

Cell culture and treatments

The murine multipotent hemopoietic FDCP-Mix A4 cell line was grown and maintained in Iscove Modified Dulbecco's Medium (IMDM) with 20% (vol/vol) horse serum (HS; GIBCO, Grand Island, NY) and 150ng/ml IL-3 and passed at every second day at $1.0x10^5$/ml. Differentiation induction of

FDCP-Mix cells along the granulocyte/macrophage pathway was performed as followed: cells from cultures maintained in 150ng/ml IL-3, which were in logarithmic growth phase were washed three times in IMDM plus 20% (vol/vol) horse serum to remove the cytokine. Cells were then resuspended to a concentration of 1.0-2.0x10^5 cells/ml in IMDM plus 20% (vol/vol) fetal bovine serum (FBS; Biomedia), low IL-3 concentrations (1.5ng/ml) and mGM-CSF (50U/ml). Cells were then incubated at 37°C in a gassed incubator containing 5%CO_2 in air. Short-term (5-7days) differentiation experiments using the stable FDCP-Bcl-2 cell line did not require the presence of IL-3 in the culture medium. Where indicated, FDCP-Bcl-2 cells were treated with 10μM of MG132 proteasome inhibitor (CalBiochem, La Jolla, CA). Stable FDCP-Mix transfectants, unless under constant selective pressure, should be kept a maximum of two weeks in culture in order to retain the gene of interest they are harboring.

Stable mouse fibroblast NIH 3T3 harboring the hGM-CSF receptor (GMR) were maintained in IMDM supplemented with 10% (vol/vol) fetal calf serum and passed every second day at 3.0-6.0x10^4/ml in presence of the selectable marker G418 (200μg/ml). Where indicated, cells were maintained in serum-free medium supplemented with 1% bovine serum albumin (BSA; Sigma, St-Louis, MO) and 180μg/ml iron saturated transferrin (Tf; CalbioChem, La Jolla, CA), as described previously (Rajotte *et al.*, 1990). Experiments on the GM-CSF receptor using this cell line were performed using 200pmol/L of human recombinant GM-CSF.

The packaging cell line 293 vesicular stomatitis virus G protein (VSV G) was a gift from Dr Guy Sauvageau (IRCM, Montréal, Canada). This cell line is maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) with 10% (vol/vol) inactivated fetal calf serum (FCS), 1μg/ml of tetracycline

(Sigma; Oakville, Ontario, Canada), 2μg/ml of puromycin (Aldrich-Sigma), and 0.3mg/ml of neomycin. Cells are passed at $1x10^6/80cm^2$ culture dish when 90% confluent. After transfection, all antibiotics are removed from the medium.

Murine BOSC 23 cells used to produce ecotropic viruses and infect bone marrow cells, were maintained in genetecin/puromycin/tetracycline (gpt) selective media [83% DMEM, 10% dialyzed FCS, 10mg/ml of Xanthine, 10mg/ml of Hypoxanthine, 10mg/ml of Mycophenolic acid. 5mg/ml of Thymidine, 0.5mg/ml of Aminopterin, and 1M HCl] and passed at 1:3-1:4 every 3 to 4 days to prevent cell clumping which occurs when the cells are passed at low density or when allowed to become overconfluent.

Plasmids

The human GMRα cDNA was cloned in the expression vector pME18S. The cDNA for human β_c (KH97) was cloned in the same vector, but without the neomycin-selectable gene. Both were gifts from Dr Toshio Kitamura (DNAX, Palo Alto, CA).

The murine anti-apoptotic factor Bcl-2, the different deletants of the intracellular domain of the human GM-CSF receptor and the anti-sense PU.1 cDNAs were transferred in the murine stem cell virus vector (MSCV) with the selectable neomycin marker. The different deletants were inserted BamH1/Bgl II while Bcl-2 and AS-PU.1 were inserted at the EcoR1 site. The PU.1 gene was also cloned in the expression vector MSCV-neo in this same manner. In addition, it was cloned in the murine stem cell virus along with the gene

coding for the green fluorescent protein (MSCV-GFP). The human α chain of the GM-CSF receptor was also cloned (BamH1/Bgl II) in this same expression vector. HA-tagged ubiquitin, a 700 base pair insert, was cloned in the pBlueScriptSK- expression vector at the SacI site and was a gift from Dr Sylvain Meloche (Institut de recherches cliniques de Montréal, Montréal, Canada).

Transfections

The human GMRα cDNA and β_c (KH97) were cotransfected by the calcium phosphate method in NIH 3T3 cells at a ratio of 1:10, respectively. One day before transfection, 200 000 cells were seeded in a 60mm tissue culture dish. On the day of transfection, 400μL of DNA-$Ca_3(PO_4)_2$ precipitate containing 12μg of DNA was added to the culture and incubated for 6 hours. Cells were then washed and fed with culture medium. Two days after transfection, the cultures were expanded 1:10 and Genetecin (G418) was added at a concentration of 500μg/ml as described previously (Rajotte blood 88 p.2906).

Stable NIH-GMR transfectants were transiently co-transfected with PU.1-GFP and HA-ubiquitin in the same manner but in a 150mm tissue culture dish and using 75μg of total cDNA in the calcium phosphate precipitate. Approximately 50-60% of NIH cells showed green fluorescence under the fluorescent microscope 24 hours after transfection.

BOSC 23 cells were also transfected by the calcium-phosphate method. Thirty-six hours before transfection, BOSC cells are washed with PBS and resuspended in DMEM plus 10%FCS. The day following transfection, cells

are washed and resuspended in new medium consisting of IMDM, 15%FBS, 100ng/ml of SF, 5ng/ml of IL-3, and 10ng/ml of IL-6. This medium is the same as for bone marrow pre-stimulation medium. Forty-eight hours after transfection, BOSC cells are cocultivated with bone marrow cells.

Cytospin

$1.0x10^5$ cells of each specific condition under study are collected and resuspended in 100μL of IMDM plus 20% horse serum. Cells are fixed on microscopic plaques by spinning for 5 minutes at 400rpm using the Cytospin3® (Fisher; Nepean, Canada). Fixed cells are then treated with 700μL of Wright stain and 700μL of Wright stain buffer and let to sit at room temperature for 5 minutes. After washing, fixed cells are examined under conventional microscope for qualitative assay.

Western blotting analysis and immunoprecipitation

For immunoblot analysis, cells were lysed in lysis buffer [10mM Tris at pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 10% NP40] plus protease inhibitors [phenylmethyl sulfonyl fluoride (PMSF) (100mM), pepstatine (10μg/μL), DTT (1M), leupeptin (1μg/μL), aprotinin (10μg/μL), chymotrypsin (1μg/μL), antiparin (1μg/μL)]. Protein concentrations were determined using the dying (Dc) protein assay kit (Bio-Rad). Samples were solubilized and boiled for 5 minutes in SDS sample buffer plus 10% β-mercaptoethanol for a total volume of 36μL containing 30μg of proteins. After sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE) for 90 minutes at 120V using 10% polyacrylamide resolving gels, proteins were electrophoretically transferred onto Immobilon polyvinyldiene difluoride (PVDF) membrane (Millipore, Bedford, MA), using 300mA for 2 hours at 4°C in 25mM Tris, 192mM glycine, 0.05% SDS, and 20% methanol. Membranes were blocked in 5% non-fat dry milk plus 0.05% Tween-20 in Tris-buffered saline (TBST) for 1 hour before antibody incubation. Blots were then incubated with the primary antibody at the appropriate dilution [PU.1: 1/300, c-jun: 1/10 000, Sp1: 1/200, PTP1D: 1/2500, HA: 1/2000] for 1 hour at room temperature or overnight at 4°C in TBST-5% milk. After washing twice for 10 minutes in TBST, blots were incubated 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody before adding ECL substrate solution and exposing on Kodak-Omat film (Eastman Kodak, Rochester, NY). In some experiments, blots were stripped with 62.5mM Tris-HCl, pH 6.8, 2% SDS, 100mM β-mercaptoethanol at 60°C for 1 hour, reblocked, washed, and reprobed.

For immunoprecipitation, cells were lysed with lysis buffer as above plus 5μM N-ethylmaleimide plus the protease inhibitors. 200μg of protein extracts was used for each condition. To the extracts, 20μl of Protein G/Plus Protein A Agarose beads (CalBiochem) and 3μg of primary antibody against PU.1 were added. The volume was completed to 1mL of NP40 buffer [20mM Tris-HCl at pH 8.0, 1% NP40, 137mM NaCl, 10% Glycerol, 1mM EDTA, 1mM PMSF]. After a 4 hour incubation at 4°C on a Varimix apparatus, the samples were washed three times with NP40 buffer, solubilized in SDS sample buffer, and boiled for 5 minutes. The eluted proteins were resolved on SDS-PAGE and immunoblot analysis performed as described above

Cell surface antigen analysis and cell sorting

Monoclonal antibodies against murine cell surface antigen were the following: rat anti-Gr-1, rat anti-F4/80, and rat anti-Cd11b (American Type Culture Collection; Rockville, MD). The secondary antibody, a goat anti-rat phycoerythrin (GAR-PE) (CEDARLANE; Hornby, Canada), was used at the recommended concentrations.

For surface marker staining, $1x10^5$ cells were washed once with PBS supplemented with 2%FCS. The cells are then labeled with the appropriate dilution of the primary antibody ($10\mu g/10^6$cells) for 30 minutes on ice in a total volume of 100μL of PBS(2%FCS) plus 10% normal goat serum (Sigma-Aldrich, Oakville, Ontario, Canada) and 1% human IgG (Sigma). The cells were then washed twice with PBS(2%FCS) and then labeled with a secondary antibody for 30 minutes on ice. Negative controls were labeled with the second antibody alone. After a final wash, cells are analyzed on the FACS-Scan® flow cytometer (Becton Dickinson, San Jose, CA).

Bone marrow culture and infection

After three days of treatment with 5-Flurouracil (5-FU), mice were sacrificed and their bone marrow was collected. Bone marrow cells were negatively selected against Cd11b and Gr-1 and cultured in IMDM plus 10%FCS, 2%BSA, 200μg/ml of Transferrin prepared for culture, 1U/ml of Epo, 5ng/ml of IL-3, $5x10^{-5}$M of monothioglycerol, 50% methyl cellulose (1% final), 50ng/ml of SF, and 5ng/ml of IL-6. Before infection, bone marrow cells are cultured at 1x106 cells/ml in a pre-stimulation media containing IMDM plus

15%FBS, 100ng/ml of SF, 5ng/ml of IL-3, and 10ng/ml of IL-6. Bone marrow cell cultures were then infected with MSCV-neo, MSCV-PU.1 and MSCV-ASPU.1 viral particles from producing BOSC cells and left in normal medium for a period of two days. Polybrene (8μg/ml) was also included in the media. The selection drug G418 (100mg/ml) was then added overnight. After 48 hours of infection time, a 24-hour recovery period in pre-stimulation media was required. Cells are then plated back on semi-solid methylcellulose. Sixty-percent (vol/vol) of mGM-CSF or 40% of SF (vol/vol) was then added for up to 3 days. Cells were then analyzed by flow cytometry for survival response.

Apoptosis assays

Apoptosis was assessed by flow cytometry analysis of cells labeled with Annexin V-FITC (1μg/ml) (CalbioChem), shown to be an early marker of apoptosis. Immediately before acquisition, cells were incubated 10 minutes on ice following the addition of 2μg/μL of 7-actinomycin D (CalbioChem).

CHAPTER 3

RESULTS

1 GM-CSF induces myeloid differentiation of FDCP-Mix cells.

FDCP-Mix cells were chosen as our experimental system. It is the only known pluripotent cell line that behaves like CD34+ primitive cells by differentiating in the presence of GM-CSF. One inconvenient was the difficulty to use FDCP-Mix cells in gene transfer experiments. When cultured in the presence of 100U/ml of IL-3, FDCP-Mix cells rapidly proliferate and undergo a 100-fold increase in cell number within 7 days. Throughout this time, about 95% of the cells have a primitive blast morphology and there is very little spontaneous differentiation. At low IL-3 concentration (1U/ml), only limited proliferation is seen, but a greater proportion of mature cells is present, including a few mature neutrophils. In the presence of low IL-3 concentration and 50U/ml of GM-CSF, there is a slight increase in cell number, two to three days following stimulation. Eventually, little or no increase is seen and the population of primitive cells progressively declines. Neutrophils typically have multilobed nuclei and light blue to colorless cytoplasm containing variable sizes and number of azurophilic granules while macrophages are much larger in size, contain many vacuoles, and have a small nucleus lining the edge of the cell membrane (Heyworth *et al.*, 1990). Morphological analysis of these cultures shows that cells develop into mature neutrophils and macrophages only, in response to GM-CSF. In the presence of GM-CSF, maturing neutrophils can be seen as early as day 1, while macrophages appear much later and reach significant numbers by day 7 only (Figure 3). This can also be shown quantitatively by flow cytometry analysis. Cells maintained in 20%FBS and stimulated with 50U/ml of GM-CSF were analyzed every second day for the presence of the cell-surface markers F4/80 (mature macrophages) and Gr-1 (granulocytes). As can be seen from Figure 4A, and in

agreement with morphological data, F4/80 positive cells increased on day 5 of GM-CSF stimulation and were as high as 40% by day 7 of stimulation, while Gr-1 positive cells can be detected as early as day 1 (Figure 4B).

Thus, FDCP-Mix cells differentiate into mature granulocytes and macrophages when exposed to high concentrations of GM-CSF and low levels of IL-3. Upon complete IL-3 withdrawal, the cells start to die by apoptosis despite the presence of GM-CSF (data not shown). Furthermore, the cells differentiate completely into mature granulocytes and macrophages. To avoid this problem, and because of the desire to exclude completely IL-3 and its biological effects from future differentiation experiments, we infected the FDCP-Mix parental cell line with the anti-apoptotic factor Bcl-2 (MSCV-Bcl2). These cells can survive in the presence of GM-CSF and in the absence of IL-3 for many days. After 5 days in culture containing 50U/ml of GM-CSF, 20%FBS, and IMDM, and no IL-3, 85% of the total cell population was still viable (data not shown).

2 GM-CSF induces an early increase in PU.1 levels that precedes macrophage differentiation.

Because of the importance of the transcription factor PU.1 in myeloid differentiation, we addressed the question whether PU.1 is induced in FDCP-Mix cells undergoing granulocyte and macrophage differentiation in response to GM-CSF. Nuclear extracted proteins (30μg), from FDCP-Bcl2 cells induced to differentiate with 50U/ml of GM-CSF in IMDM plus 20%FBS, were subjected to Western blotting with a PU.1 antiserum. Sample at day 0 corresponds to nuclear extracts from cells simply maintained in IL-3 (100U/ml) with no GM-CSF addition. PU.1 protein levels show a significant

increase as early as day 1 (Figure 5A). This sudden rise is then followed by a steady, progressive increase in PU.1 protein levels.

We then tested for other possible transcription factors that could be influenced the same way as PU.1 by GM-CSF signaling. The transcription factor c-jun is known to interact with PU.1 to activate the expression of some myeloid-specific genes including the M-CSF receptor and the IL-1β gene in macrophages (our group, unpublished data). Western blot analysis of nuclear extracts (30μg) from FDCP-Bcl2 cells exposed to the same conditions as above, shows that c-jun protein levels dramatically increase on day 5 (Figure 5B). This late increase corresponds to a stage when myeloid differentiation is well underway and differs from the pattern seen with PU.1 protein levels. Similarly, the tyrosine phosphatase Syp and the Sp1 transcription factor show late induction by GM-CSF (Figure 6). In contrast, the transcription factor C/EBPβ is not affected by GM-CSF stimulation and serves as a control for protein loading (Figure 5C).

Since only PU.1 exhibits an increase on day 1, we then narrowed down our kinetic study by assaying PU.1 protein levels within hours of GM-CSF induction.

3 PU.1 levels increase rapidly in response to GM-CSF

Using the same experimental approach as for figure 5A, we stimulated FDCP-Mix cells with 50U/ml of GM-CSF at time points ranging between 0 and 6 hours. As can be seen, PU.1 is induced at 1 hour post-stimulation and shows a dramatic increase at 6 hours (Figure 7A). This rapid increase could suggest a post-transcriptional mechanism. Recent evidence suggests that many transcription factors are regulated through degradation via the proteasome

machinery (Nawaz *et al.*, 1999). Thus a possible mechanism in the regulation of PU.1 could involve protein stabilization by GM-CSF signaling. To investigate this possibility, we added to the media 10μM of the proteasome inhibitor MG132. Thirty minutes later, we stimulated the cultures with GM-CSF for the indicated times (Figure 7B). Interestingly, Western blot analysis of the different extracts shows that PU.1 protein levels are initially very high, even in the absence of GM-CSF. As GM-CSF is added for longer periods, PU.1 levels show a gradual increase.

Proteins destined to be degraded by the proteasome machinery are heavily tagged by the ubiquitin molecule (Chau *et al.*, 1989; Goldberg and Rock, 1992; Goldberg, 1995). The more the protein is tagged, the faster its degradation. For this reason, we tried to identify whether PU.1 is normally associated with ubiquitin. For this, we co-transfected NIH3T3 cells stably expressing the human GM-CSF receptor with PU.1 linked to the green fluorescent protein (GFP) and the ubiquitin molecule linked to HA. Forty-eight hours after transfection, around 60% of NIH3T3 cells were fluorescent under the microscope. NIH3T3 cells were then stimulated with human GM-CSF for 24 hours. Controls correspond to cells maintained in BSA/transferrin media with no GM-CSF addition. By co-immunoprecipitation (see materials and methods) we attempted to evaluate the extent of ubiquitination of PU.1 with or without human GM-CSF. Preliminary results were obtained but the experiment needs to be optimized before presenting any conclusive results.

It is important to note that this regulation of PU.1 by the proteasome machinery represents an initial hypothesis that is still under investigation. Whether or not conclusions can be drawn from figure 7B will be discussed later.

4 The increase in PU.1 protein levels can be the cause or the consequence of macrophage differentiation.

How does the kinetics of PU.1 induction relate to the differentiation model previously shown? The kinetics of PU.1 induction observed by Western blot analysis precedes significant macrophage differentiation, which only appears at around day 5 of GM-CSF stimulation as shown by morphological and cytometry analysis. In contrast, the rise in PU.1 protein levels is rapid, occurring within one day of stimulation. This shows that the increase observed is not a consequence of macrophage differentiation. Rather, it represents an initial event that could be the cause of macrophage differentiation or alternatively, that it represents a parallel phenomenon.

5 Ectopic PU.1 expression is sufficient to drive macrophage differentiation

In order to address directly the question whether PU.1 acts downstream of the GM-CSF receptor, we proceeded to gain-of-function and anti-sense experiments. We reasoned that ectopic expression of PU.1 could bypass GM-CSF signaling in myeloid differentiation. Western blot analysis shows that FDCP-Mix cells, infected with a recombinant retrovirus expressing PU.1, greatly overexpress the protein (Figure 8A). In parallel, PU.1 overexpressing cells show an increased staining for CD11b, a target gene of PU.1 (Figure 8B).

FDCP cells overexpressing PU.1 were induced to differentiate with GM-CSF and cell differentiation was analyzed by flow cytometry. F4/80 positive cells (Figure 9A) were quantified at the indicated times ranging from 0 to 7 days. Interestingly, at time 0, where cells are maintained in IL-3, almost half of the

total cell population is positive for the macrophage-specific cell-surface marker F4/80. Furthermore, GM-CSF did not induce an increase in F4/80 staining as observed in parental cells. This spontaneous differentiation clearly shows that overexpressing PU.1 in FDCP-Mix cells bypasses the required differential signaling by GM-CSF. It also suggests that PU.1 may act downstream of GM-CSF signaling in macrophage differentiation. Following this, we then examined if we could get similar results for granulocytic differentiation.

6 Ectopic PU.1 expression enhances and accelerates granulocytic differentiation in response to GM-CSF.

Again, FDCP-BCl2 cells overexpressing PU.1 were induced to differentiate with 50U/ml of GM-CSF for different time periods. Cell populations were analyzed by flow cytometry. As shown in Figure 9B, cells expressing the granulocyte-specific cell-surface marker Gr-1 are of greater proportion in populations overexpressing PU.1 as compared to populations overexpressing the empty MSCV-neo vector. The difference further increases at later time points. This clearly shows that ectopic PU.1 expression enhances and accelerates granulocyte differentiation in response to GM-CSF although it does not seem to be sufficient as in the case of macrophage differentiation.

Thus, gain-of-function experiments confirm that elevating PU.1 levels in multipotent cells is sufficient to bypass differentiation signals delivered by the GM-CSF receptor. These results suggest one of two possibilities: either that PU.1 acts downstream of the GM-CSF receptor, or alternatively, that PU.1 activates pathways that are parallel to those triggered by the GM-CSF receptor.

In order to directly address these possibilities, we generated FDCP-Mix transfectants with a targeted down-regulation of PU.1 protein levels through the use of an anti-sense construct cloned in the MSCV retroviral vector.

7 Decreased PU.1 levels prevent the survival of FDCP cells in response to GM-CSF.

As shown by western blot analysis (Figure 8A), FDCP-Mix cells overexpressing the anti-sense PU.1 have very reduced PU.1 protein levels. These cells also show reduced CD11b staining, a target gene of PU.1 (Figure 8B). FDCP-Mix cells ectopically expressing the anti-sense PU.1 were induced to differentiate in the presence of 50U/ml of GM-CSF. At different time periods, viable cells in FDCP-ASPU.1 populations were counted by trypan blue staining and compared with FDCP cell populations expressing the empty vector (Figure 10A). By day 4, a significant difference in the survival response can be observed that becomes more significant at later times. This suggests that PU.1 is important in directing FDCP-Mix cell survival in response to GM-CSF. FACS analysis of annexin V positive cells on day 3 of GM-CSF stimulation indicated that FDCP cells overexpressing PU.1 survive well while most anti-sense PU.1 transfectants fail to do so (Figure 10B). In contrast, these cells survive in IL-3 indicating a specific requirement in PU.1 function, downstream of the GM-CSF receptor.

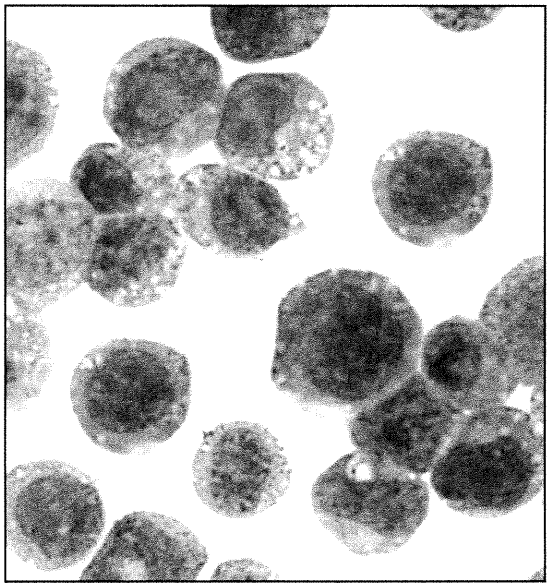
Finally, RT-PCR was performed using ASPU.1 cells in order to address the question whether failure to survive in response to GM-CSF was due to lack of expression of GMR α and β chains. As shown in Figure 10C, both α and β

chains were present in these cells at levels that were not significantly different from those of control cells.

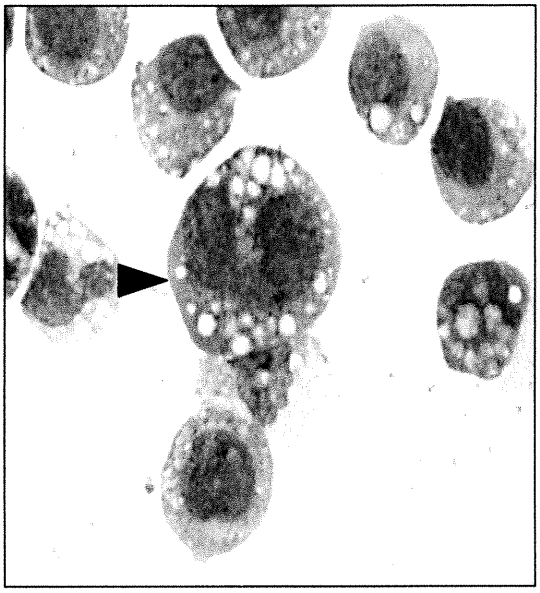
These results corroborate previous data obtained on bone marrow studies performed in our laboratory, showing that PU.1 is required for the survival of primary bone marrow cells in response to GM-CSF (Figure A1). Primary bone marrow cells infected with the anti-sense PU.1 (Figure A2) show a higher proportion of annexin V positive cells compared to primary bone marrow cells infected with the empty MSCV vector. Results shown represent bone marrow cells cultured in GM-CSF for a period of 2 days.

In summary, our results indicate that PU.1 function is required downstream of the GM-CSF receptor to sustain cell survival and to drive macrophage and granulocyte differentiation.

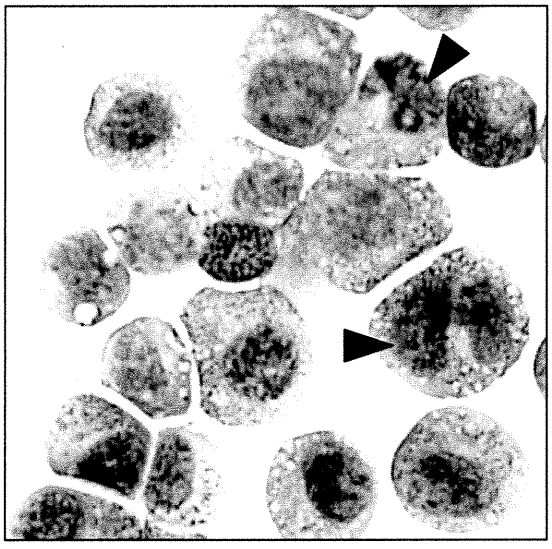
Day 0 in IL-3



Day 1 in GM-CSF



Day 3 in GM-CSF



Day 7 in GM-CSF

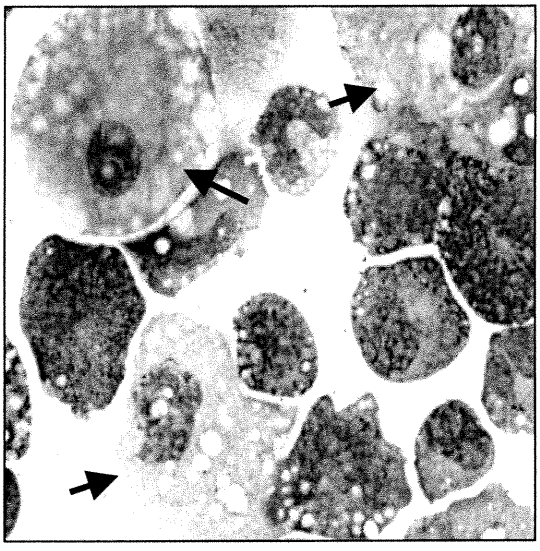
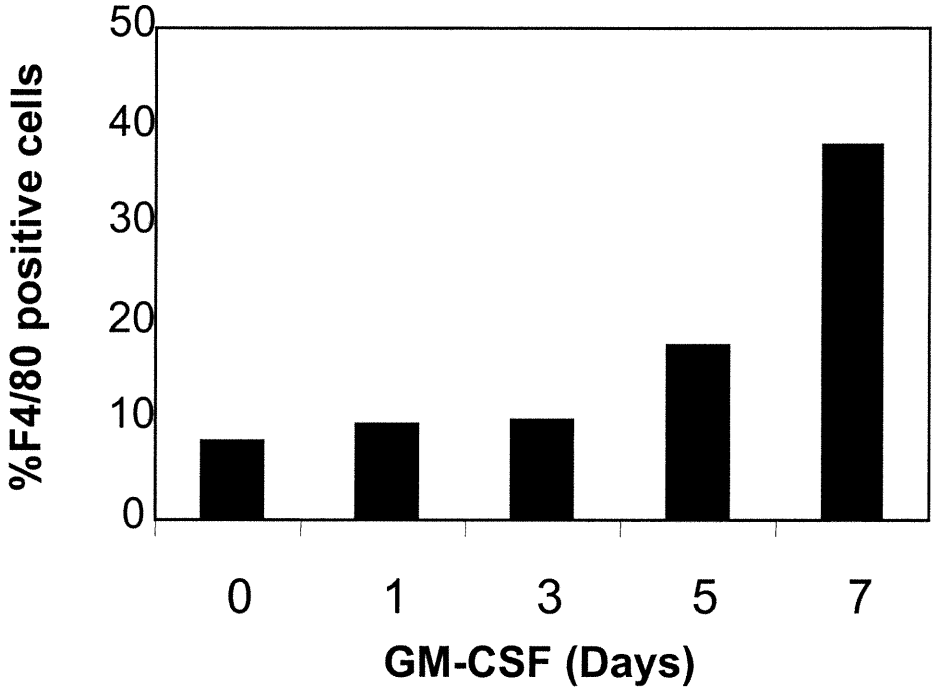


FIGURE 3

A


%F4/80 positive cells
50
40
30
20
10
0
0
1
3
5
7
GM-CSF (Days)

B

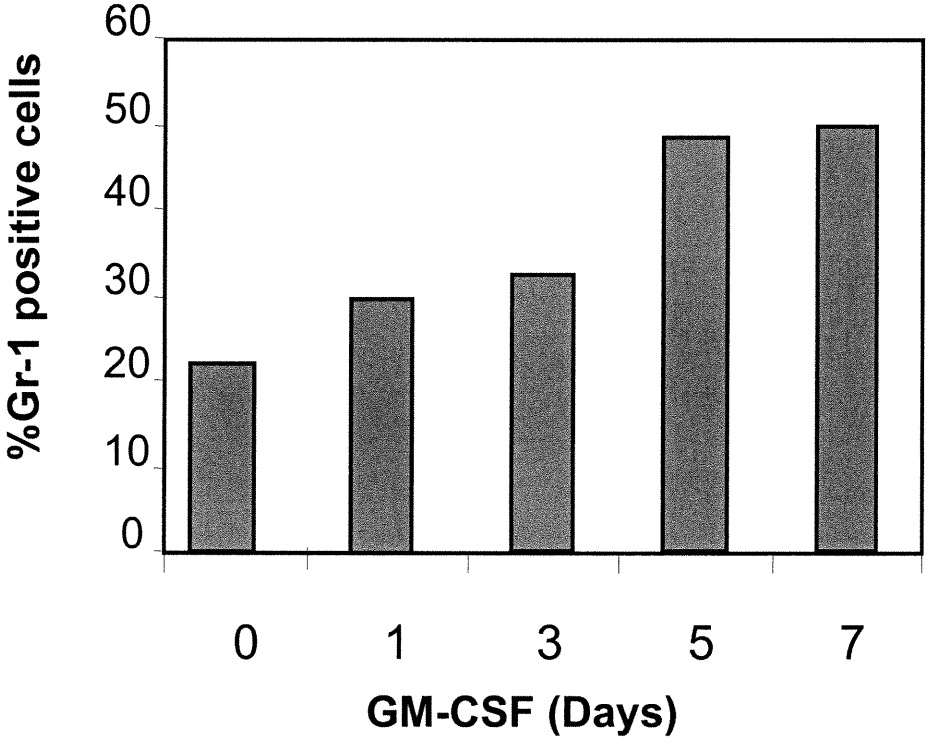

%Gr-1 positive cells
60
50
40
30
20
10
0
0
1
3
5
7
GM-CSF (Days)

FIGURE 4

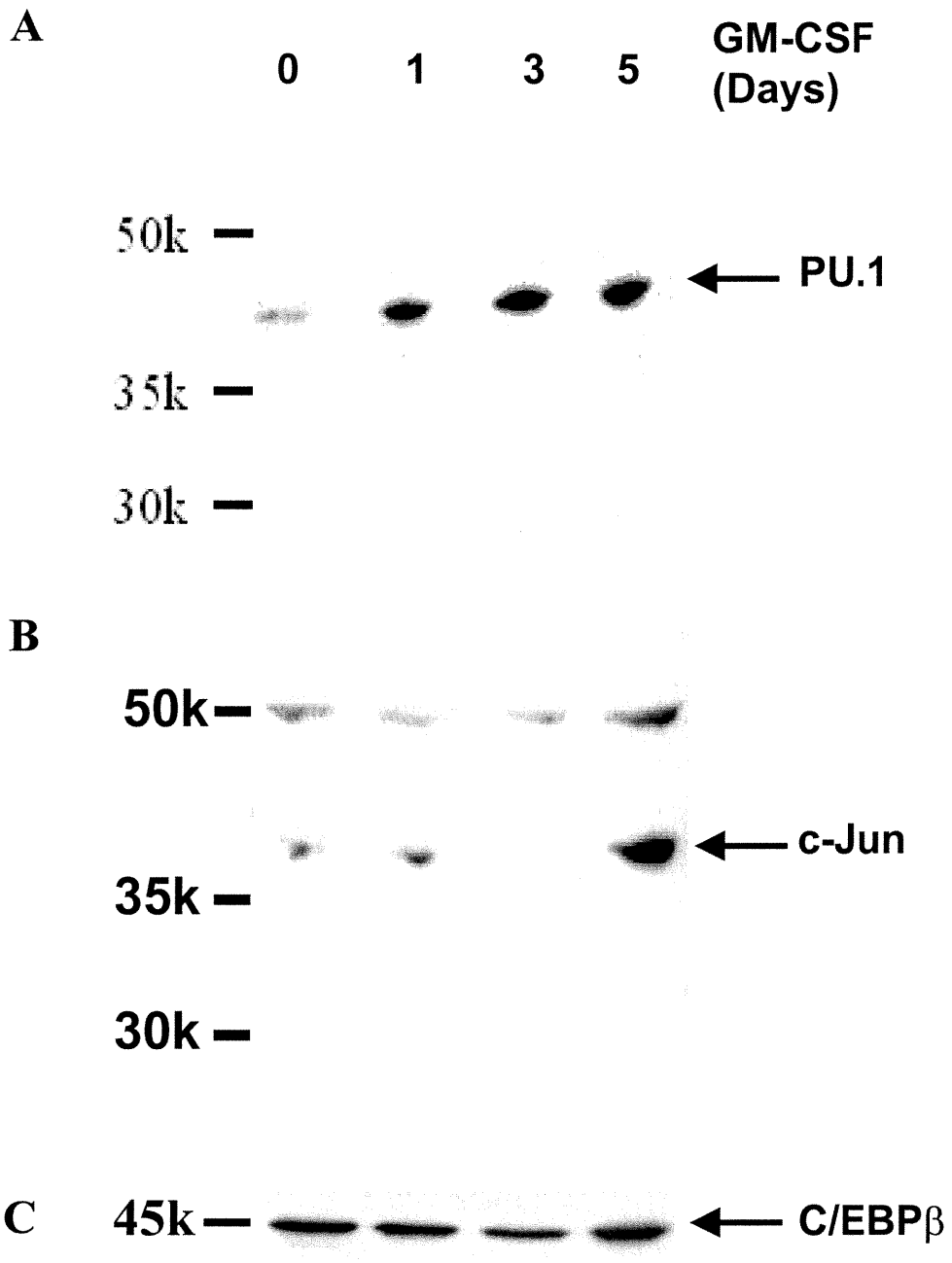


FIGURE 5

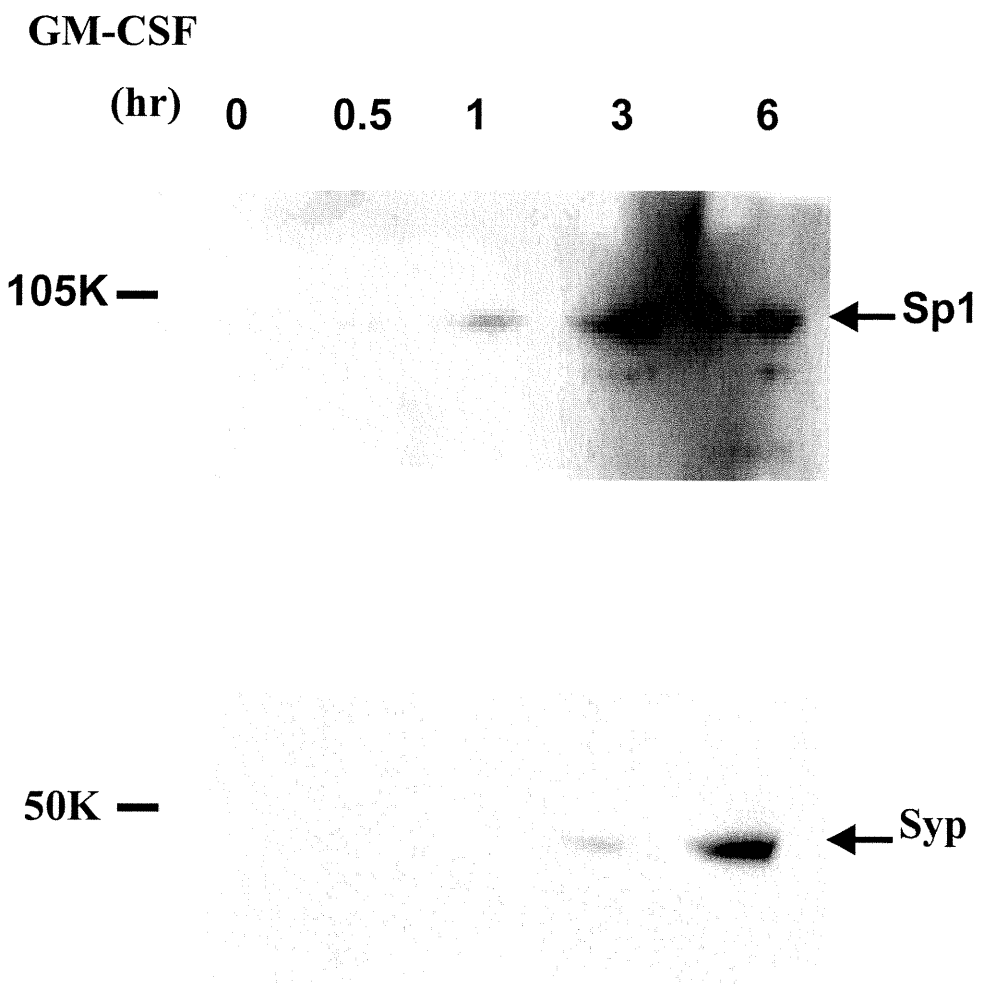


FIGURE 6

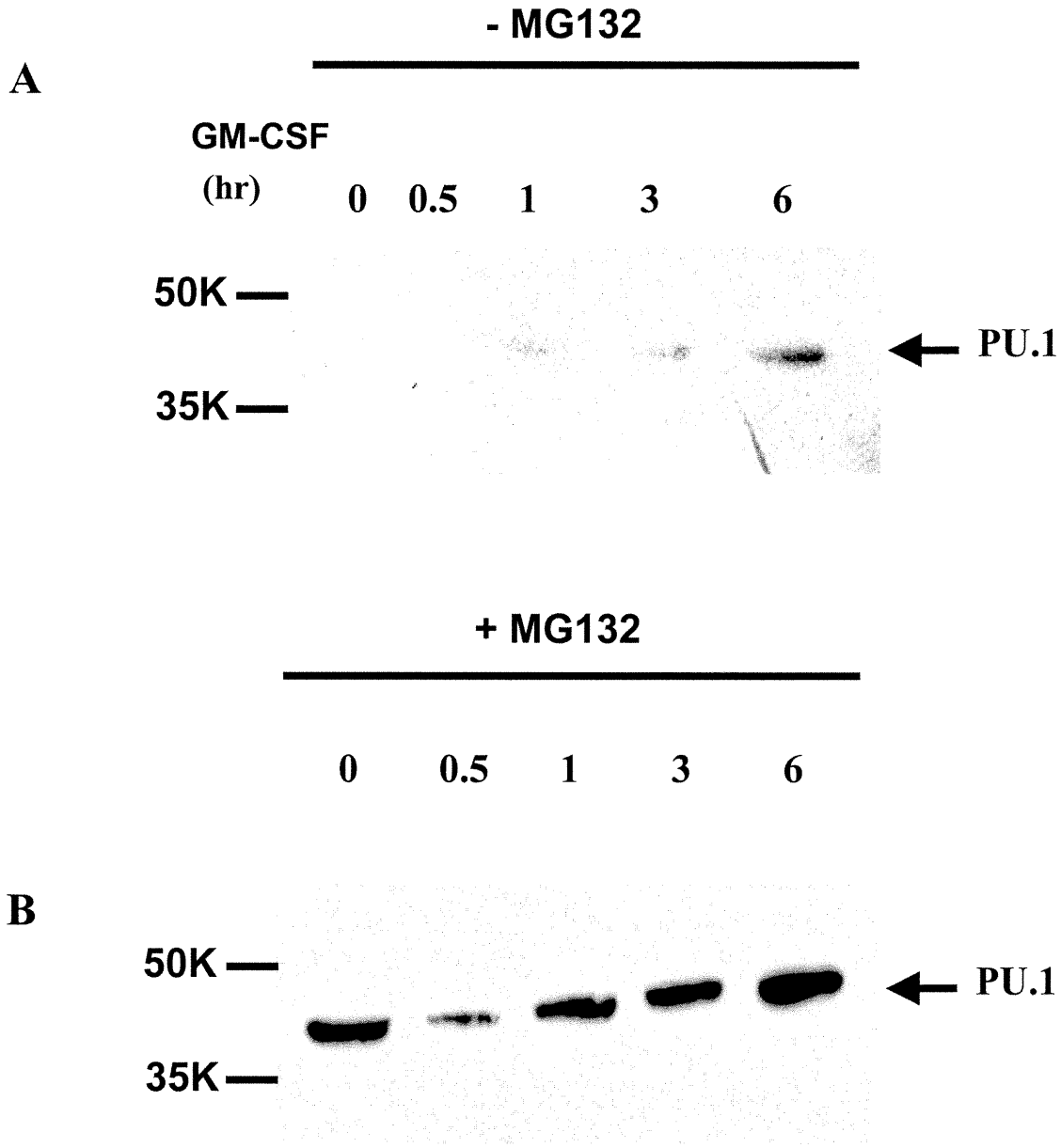
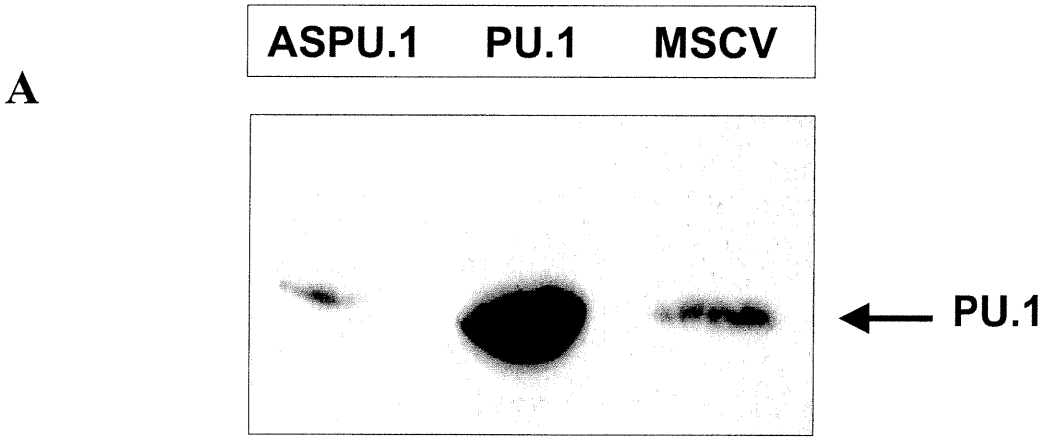


FIGURE 7


A
ASPU.1
PU.1
MSCV
PU.1

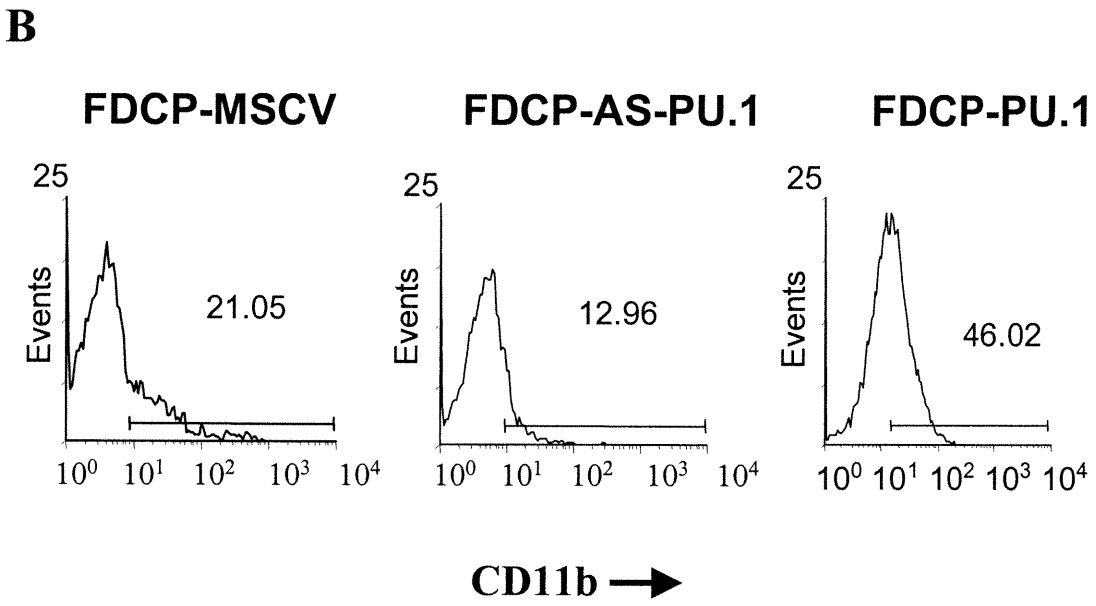

B
FDCP-MSCV
FDCP-AS-PU.1
FDCP-PU.1
25
Events
21.05
12.96
46.02
10^0 10^1 10^2 10^3 10^4
CD11b

FIGURE 8

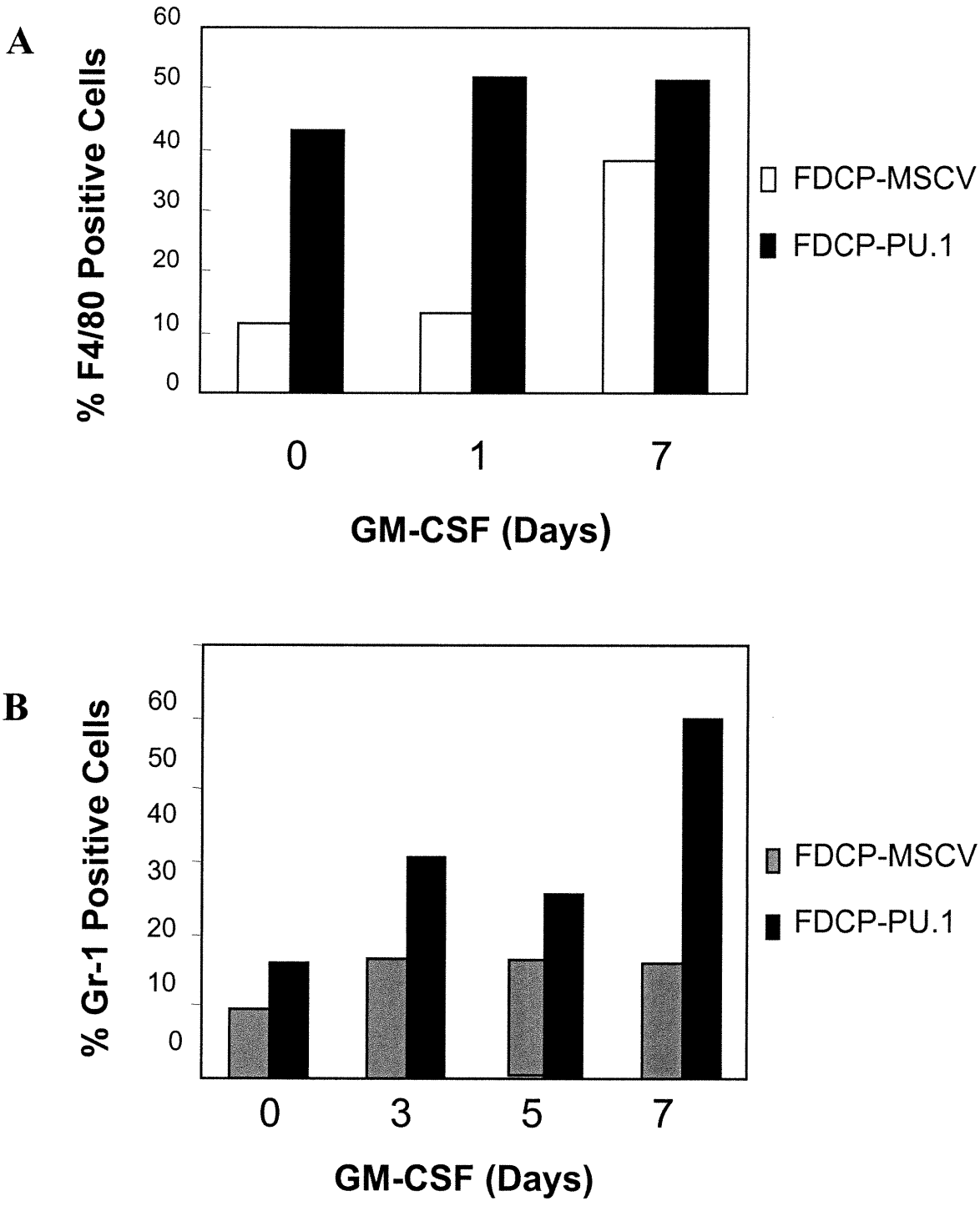
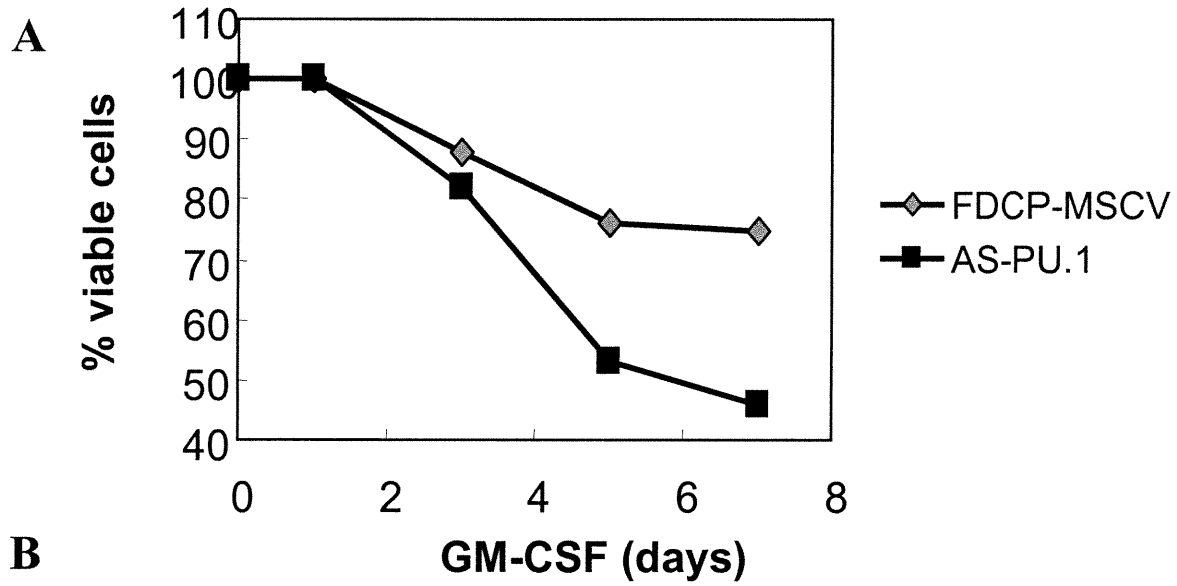
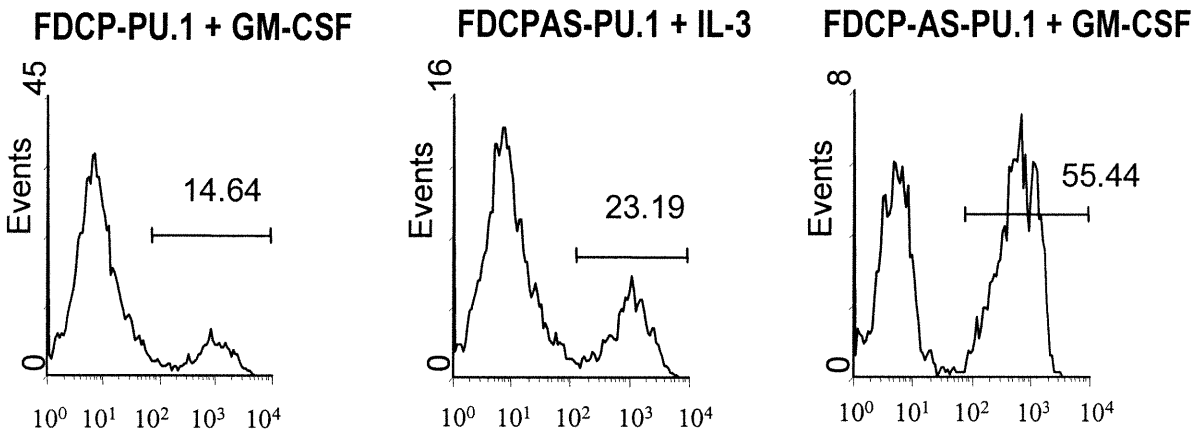

A
% F4/80 Positive Cells
60
50
40
30
20
10
0
0
1
7
GM-CSF (Days)
FDCP-MSCV
FDCP-PU.1
B
% Gr-1 Positive Cells
60
50
40
30
20
10
0
0
3
5
7
GM-CSF (Days)
FDCP-MSCV
FDCP-PU.1

FIGURE 9


A
% viable cells
110
100
90
80
70
60
50
40
0
2
4
6
8
GM-CSF (days)
FDCP-MSCV
AS-PU.1


B
FDCP-PU.1 + GM-CSF
FDCPAS-PU.1 + IL-3
FDCP-AS-PU.1 + GM-CSF
Events
45
16
8
0
14.64
23.19
55.44
10⁰ 10¹ 10² 10³ 10⁴

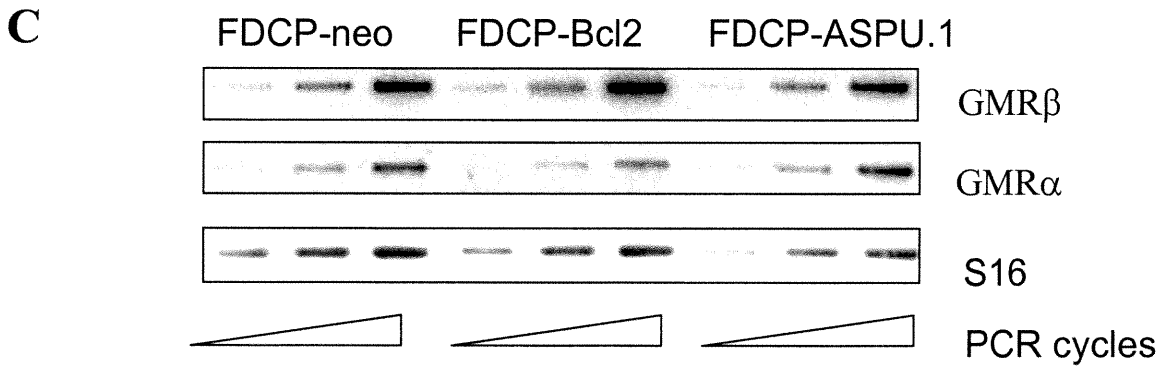
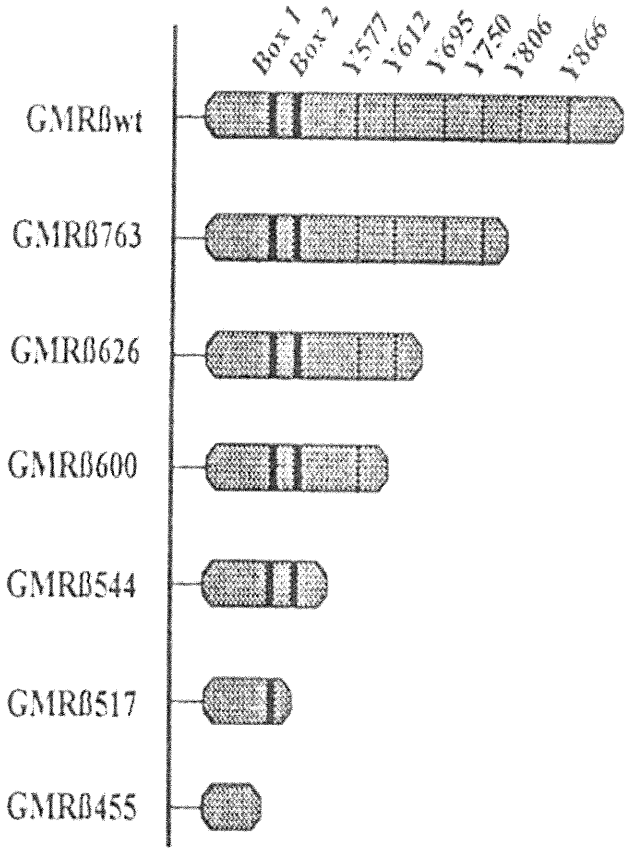

C
FDCP-neo
FDCP-Bcl2
FDCP-ASPU.1
GMRβ
GMRα
S16
PCR cycles

FIGURE 10



Domaines intracellulaires des délétants de la chaîne GMRß

FIGURE 11

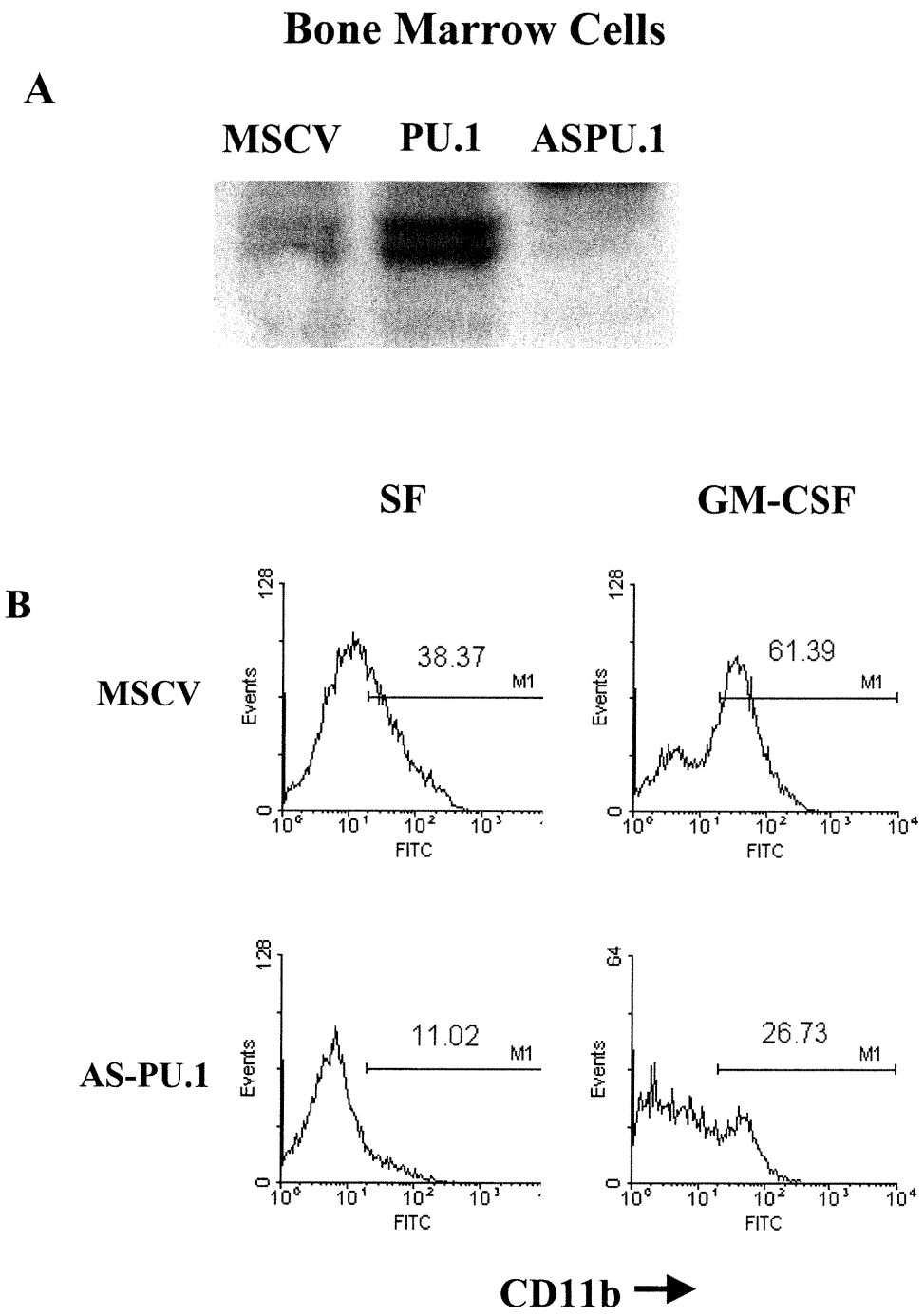

Bone Marrow Cells
A
MSCV
PU.1
ASPU.1
SF
GM-CSF
B
MSCV
AS-PU.1
38.37
61.39
11.02
26.73
M1
Events
FITC
CD11b

FIGURE A1

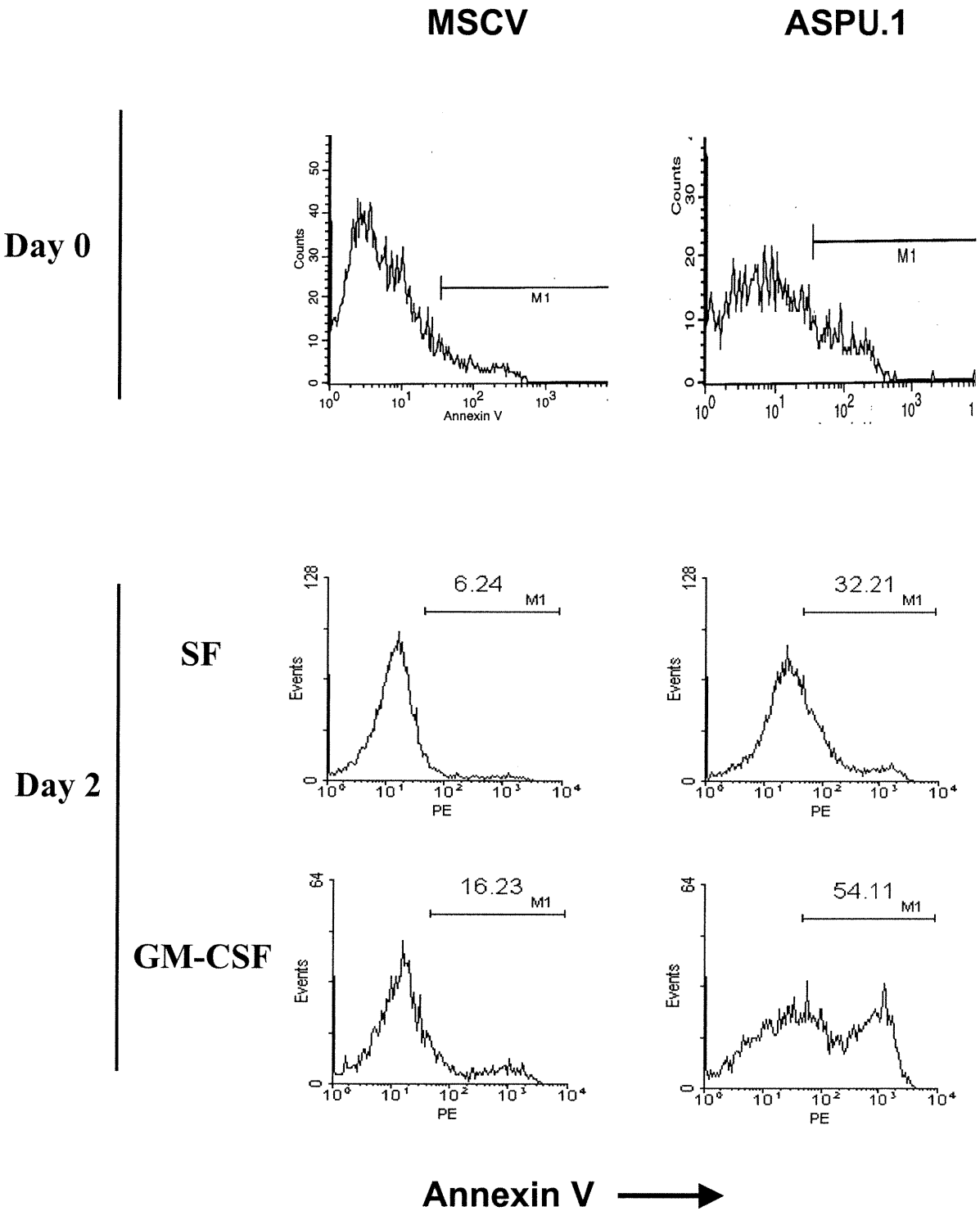

MSCV
ASPU.1
Day 0
Counts
M1
Annexin V
Day 2
SF
GM-CSF
6.24
32.21
16.23
54.11
Events
PE
Annexin V

FIGURE A2

FIGURE LEGENDS

Figure legends

Figure 3. Morphological analysis of FDCP-BCl-2 cells.

FDCP-BCL-2 cells were cultured in differentiation media containing IMDM plus 50U/ml of GM-CSF and 20% (vol/vol) FBS at different time points. At day 0, cells are maintained in IMDM plus 150ng/ml of IL-3 and 20% (vol/vol) horse serum. Granulocytes appearing as early as day 1 are indicated by arrowheads while macrophages appearing only by day 7 are indicated by arrows.

Figure 4. Granulocyte and macrophage differentiation pattern in response to GM-CSF.

FDCP-BCl-2 cells were cultured as in figure 3. **A** Cells harvested at the indicated time periods and stained with the macrophage-specific cell surface marker F4/80 were analyzed by flow cytometry. **B** Cells were stained with the granulocyte-specific cell surface marker Gr-1. As controls, cells were stained with the secondary antibody alone and gating was set on 1% or less of control cells.

Figure 5. Detection of PU.1 and c-jun protein levels in FDCP-BCl-2 cells.

Western blotting of nuclear extracts (30μg) from FDCP-BCl-2 cell cultures stimulated with 50U/ml of GM-CSF. Aliquots of purified nuclear extracts were fractionated on a 10% polyacrylamide gel and transferred to a PVDF membrane. The membrane was probed with a monoclonal antibody of appropriate dilution as described in "Materials and Methods". Antibody reactions were detected using horseradish peroxidase-conjugated with the appropriate antibody. **A** Detection of PU.1 protein levels with a PU.1 anti-serum, **B** Detection of c-jun and **C** Detection of C/EBPβ which is unaffected

by GM-CSF stimulation and serves as a control for protein loading. Shown on the left are molecular weight markers expressed in kilodaltons.

Figure 6. Detection of Sp1 and Syp in FDCp-BCl-2 cells.

Western blot analysis of nuclear extracts (30μg), form FDCP-BCl-2 cell cultures exposed to 50U/ml of GM-CSF. As in figure 5, aliquots of purified extracts were fractionated on a 10% polyacrylamide gel and transferred on PVDF membrane. Shown here are the detection of Sp1 and Syp protein levels at early time points of GM-CSF stimulation by probing the membrane with appropriate primary and secondary antibody dilutions as described in "Materials and methods".

Figure 7. Early induction of PU.1 protein levels by GM-CSF in FDCP-BCl-2 cells.

Western blot analysis of nuclear extracts (30g) from FDCP-BCl-2 cells cultured in differentiation media containing 50U/ml of GM-CSF. **A** Detection of PU.1 protein levels at very early time points using the appropriate antibody concentrations. **B** Cultures were pre-incubated with the proteasome inhibitor MG132 (10μM final) for 30 minutes before resuspension in the differentiation media containing 50U/ml of GM-CSF and MG132 (10μM final). Shown here is the detection of PU.1 protein levels at early time points from these cell cultures exposed to GM-CSF and the proteasome inhibitor MG132. The arrows on the right show the 40-kd PU.1 protein.

Figure 8. Retroviral-mediated transfer of PU.1 in the sense and antisense orientation in FDCP-Mix cells.

A Western blot analysis showing the detection of PU.1 protein levels in retrovirally- infected FDCP-Mix cells. Packaging 293 VSVG cells were

transfected with the anti-sense PU.1, sense PU.1, or the empty MSCV vector alone as described in "Materials and methods". After 72 hours, viral particles were collected and concentrated by ultracentrifugation. FDCP-Mix cells were infected accordingly. **B** Immunodetection analysis showing the functionality of the anti-sense PU.1 on endogenous PU.1. Cells were stained with the myeloid-specific cell surface marker CD11b, a target gene of PU.1, and analyzed by flow cytometry..

Figure 9. Ectopic PU.1 expression increases granulocyte and macrophage differentiation in response to GM-CSF.

FDCP-Mix cell populations ectopically expressing the PU.1 transcription factor or the empty MSCV-neo vectors were cultured in differentiation media as above. **A** Cells were stained with the macrophage-specific cell surface marker F4/80 and analyzed by flow cytometry. **B** Cells were stained with the granulocyte-specific cell surface marker Gr-1 and analyzed by FACS.

Figure 10. Survival of FDCP-Mix cells in response to GM-CSF.

A FDCP-Mix cells expressing either the empty MSCV vector or the AS-PU.1 were stimulated with GM-CSF for different time periods. Cell survival was assayed by trypan blue counts. **B** In parallel, FACS analysis for annexin V staining for detection of apoptotic cells was performed on cell cultures ectopically expressing either the empty vector, PU.1 , or AS-PU.1, and exposed to GM-CSF (50U/ml) or IL-3 (150ng/ml) for a period of 3 days. **C** RT-PCR detecting expression of GMR α and β chains in ASPU.1 and control cells.

Figure 11. Intracellular cytoplasmic domains of the different deletants of the β chain of the GM-CSF receptor.

Figure A1. Retrovirally-infected primary bone marrow cells.

A Western blot analysis of retrovirally-infected primary bone marrow cells ectopically expressing PU.1, AS-PU.1, or the MSCV vector, showing PU.1 levels in the different transfectants. **B** Flow cytometry analysis for CD11b positive cells in two different primary bone marrow cell populations, one expressing the empty MSCV vector and the other expressing AS-PU.1. Staining with CD11b, which is a gene target of PU.1, allows to test the efficiency of the AS-PU.1 on endogenous PU.1 function.

Figure A2. Increased apoptosis in primary bone marrow cells expressing a PU.1 antisense in response to GM-CSF.

Primary bone marrow cell populations were exposed for 2 days to GM-CSF or SF. Cells were stained with annexin V to detect apoptotic cells and analyzed by flow cytometry.

CHAPTER 4

DISCUSSION AND CONCLUSION

Understanding how pluripotent stem cells undergo progressive restriction of lineage potential and acquire the characteristics of mature, terminally differentiated cells is central to developmental biology. Hematopoietic stem cells and differentiated progenitors are among the best studied and have contributed an important model system for cell differentiation. In this context, hematopoiesis is the dynamic process by which blood cells acquire defining phenotypes as a result of coordinated, cell-specific gene expression.

The development of mature granulocytes and monocytes from hematopoietic precursor cells is controlled by a myriad of transcription factors that regulate the expression of essential genes that make up the specific identity of the newly formed blood cell. Work on myeloid differentiation has revealed the intricate and often essential roles played by various transcription factors – both those specific to the myeloid lineage, as well as those more widely expressed – in the control of differentiation (Tenen *et al.*, 1997).

The production of granulocytes and monocytes from myeloid precursor cells is also regulated by a network of hematopoietic growth factors and cytokines. One of these, GM-CSF, is a major regulator of granulocyte and mostly monocytes production, increasing proliferation, survival, maturation, and functional activation of cells of this lineage.

It is presumed that the bulk of signals emanating from cytokine receptors ultimately converge in the nucleus to affect myeloid gene transcription, largely via stimulation, suppression, and collaboration with a range of transcription factors. Examples of cytokine-receptor-activated transcription factors include c-jun (de Groot *et al.*, 1997)), c-fos and components of the MAPK pathways (Itho *et al.*, 1996), and STATs (Larner *et al.*, 1993). Interestingly, all these transcription factors regulate the expression of genes

involved in cell proliferation and cell survival. The mechanism responsible for commitment of multipotent hematopoietic progenitors to cells of each blood lineage is still unclear. Evidence indicates that growth factors are essential for hematopoietic development (Whetton and Dexter, 1993). However, the precise role played by these proteins, whether to direct multipotent cells down a particular cellular pathway or to merely support the survival of cells that have intrinsically selected a specific hematopoietic lineage, remains controversial.

In embryonic development, it is firmly established that commitment is extrinsically regulated by position effects or inductive gradients and does not occur by random chance. There is evidence that commitment events in the initiation and continued production of hematopoietic populations depends on, and is presumably initiated by, the activation of a succession of nuclear transcription factors, beginning with SCL and LMO2 (Porcher *et al.*, 1996) and then involving more lineage-restricted transcription factors such as GATA-1 (Linette *et al.*, 1994) and PU.1 (Zon, 1995; Tenen *et al.*, 1997). The extrinsic agents responsible for activation of these nuclear transcription factors are at present unknown.

1 The myeloid-specific transcription factor PU.1 acts as an effector of GM-CSF signaling in myeloid development.

The pluripotent FDCP-Mix cell line (Heyworth *et al.*, 1990) represented an appropriate model for our study and allowed us to show that PU.1 acts as a downstream effector of GM-CSF signaling in myeloid differentiation. In response to IL-3, FDCP-Mix cells undergo rapid proliferation with little or no

spontaneous differentiation. At low IL-3 concentration and in the presence of GM-CSF, FDCP-Mix cells start to differentiate, first in granulocytes, and then in monocytes and macrophages. This biological response represents well the differentiation response of primitive CD34+. Eventually, cell cultures contain few remaining myeloblast precursor cells while differentiated myeloid cells predominate.

The rationale for stably introducing the anti-apoptotic BCl-2 factor in our FDCP-Mix cell line for the differentiation experiments was of two reasons. First, cell cultures could be kept alive long enough in GM-CSF only, without the requirement of low IL-3 concentrations. Because the IL-3 and the GM-SCF receptor share a common cytoplasmic β sub-unit, this allowed us to exclude completely any overlapping biological effect that could be influenced by the presence of IL-3. Second and most importantly, the presence of the anti-apoptotic BCl-2 factor allowed us to bypass the survival function required for completion of the differentiation program, thus permitting the use of the AS-PU.1 in our differentiation assays. It is important to note that Bcl-2 infection does not affect the differentiation pattern observed in the parental FDCP-Mix cell line as determined by morphological and FACS analysis (data not shown). In addition, it is clear why FDCP-Mix cells, and not Bcl-2 infected cells, were used in survival experiments.

In this study, we show that PU.1 protein level dramatically increase in response to GM-CSF and that this increase which appears as early as day one of stimulation, precedes any significant macrophage differentiation. Gain and loss of function experiments allowed us to determine that PU.1 acts downstream of GM-CSF signaling in differentiation assays and is indeed a downstream effector of GM-CSF signaling in myeloid differentiation.

We also show similar results for myeloid cell survival. Indeed. FDCP-Mix cells cultured in IL-3-deficient media, not expressing the BCl-2 factor and with decreased PU.1 levels, fail to respond to GM-CSF and die by apoptosis while surviving well in IL-3. In contrast, cells overexpressing PU.1 survive well in response to GM-CSF. This clearly shows that PU.1 is important in directing short-term FDCP-Mix cell survival in response to GM-CSF and its function is a specific requirement downstream of the GM-CSF receptor.

Although no previous studies have addressed this question it is interesting to speculate on which mechanisms would PU.1 protect cells from apoptosis. One mechanism could be that PU.1 affects in some way, Bcl-xl, a member of the anti-apoptotic Bcl-2 family. Interestingly, in Bcl-xl knockout mice, hematopoietic cells seem to be predominantly affected by the mutation. Could it be that survival or apoptotic signals converge to Bcl-xl in hematopoietic cells? Another interesting possibility could involve another member of the Bcl-2 family, MCL1. It was previously shown that GM-CSF induces MCL1 in hematopoietic cells. Could PU.1 be an intermediate in the signaling pathway leading to MCL1 induction?

Finally, it is interesting to compare our survival results with the ones obtained from mice ablated for PU.1. As oppose to mice knockouts, FDCP-Mix cells are well characterised. ASPU.1 infection did not completely eliminate PU.1. For this reason, cells still expressed the GM-CSF receptor (a known target of PU.1) but at levels low enough to observe a difference in the survival response. This is in contrast with mice knockouts for PU.1 where the GM-CSF receptor is undetectable making cells non-responsive to GM-CSF and therefore non-responsive to its survival signaling. This indicates a direct implication of PU.1 in myeloid cell survival that is, in part, independent of GM-CSF receptor gene targeting.

2 Mechanism of upregulation of PU.1 levels by GM-CSF.

The fact that PU.1 protein levels increase very rapidly brought us to hypothesize that it might be regulated via degradation. This post-transcriptional mechanism of regulation occurs within minutes of a positive signal (Nawaz *et al.*, 1999). Proteins destined to be degraded by the proteasome machinery become heavily ubiquitinated. This tagging signals the cell that the protein is unwanted and needs to be rapidly disposed of. Using the proteasome inhibitor MG132, we showed that PU.1 protein levels normally at low concentrations are highly increased by MG132. These results suggest that PU.1 protein levels may be normally regulated by degradation via the proteasome in steady state conditions. To assess whether PU.1 undergoes covalent ubiquitination, co-immunoprecipitation experiments were performed. Future experiments will address the question whether PU.1 is covalently linked to ubiquitin, and whether GM-CSF modifies this process. When optimized, these experiments will allow us to confirm whether or not protein degradation is the mode of regulation of PU.1 by GM-CSF signaling in myeloid development.

In addition, the induction of PU.1 could also be attributed to a transcriptional mechanism. Indeed, PU.1 has been shown to upregulate the transcription of its own gene (Chen *et al.*, 1995). Northern blotting, in preliminary experiments, shows that PU.1 mRNA increases as early as 6 hours after GM-CSF stimulation (data not shown). Finally, it is also possible that PU.1 function is modulated by phosphorylation in response to GM-CSF.

Transcription factors that play a critical role in development processes often have multiple mechanism of regulation. Because these factors are often determinant, it is essential that the cell keep a close watch on their activity

hence the necessity and convenience of having different ways of regulation. For example, the SCL/Tal-1 transcription factor has a deterministic role in erythroid differentiation (Tsai *et al.*, 1989). Because of its critical importance in erythroid development, SCL is regulated by both transcriptional and post-transcriptional mechanism. Indeed, it was found that Epo elicited a rapid, dose-related increase in TAL1 mRNA by increasing transcription of the gene and stabilizing one of its mRNAs. In addition, Epo induced phosphorylation of nuclear TAL1 protein (Brandt *et al.*, 1995). As for SCL, PU.1 has a similar deterministic role in the development of the myeloid lineage. Due to its critical importance, it is therefore possible that PU.1 is also regulated through similar mechanisms.

In parallel, we sought to determine which signaling pathway is involved in the observed induction of PU.1 following GMR activation by its ligand. Knowing that murine GM-CSF does not activate the human receptor, we generated a series of intracellular deletants of the human β chain of the GM-CSF receptor (Figure 11). The human α chain as well as the different human β deletants were introduced in the murine FDCP-Mix cell line by retroviral-mediated gene transfer. Using the same differentiation assays, this time by activating the ectopic receptor with the human GM-CSF ligand, we hope to pinpoint the region of the β chain, and hence the signaling pathway associated with this region, that would be responsible for PU.1 induction and myeloid differentiation. It is also important to see if the α chain could also play a role in PU.1 induction and myeloid development using the same approach.

3 Regulation of myeloid cell development: intrinsic versus extrinsic regulation.

The debate surrounding the issue of whether or not hematopoietic stem cell commitment and differentiation are orchestrated in a cell-intrinsic or cell-extrinsic fashion is in many ways akin to the nature versus nurture debate that typifies discussions of human personal potentials. Simply put, the question is as follow: is unilineage commitment the result of a cell-autonomous or internally driven program, or rather, is it the consequence of a cell responding to an external, environmentally imposed agenda? This question is the essence of our study.

Much attention has revolved around the role played by hematopoietic growth factors in the developmental process: do they play an instructive and a deterministic role, or are they simply permissive or selective, meaning that they only allow for the survival and proliferation of independently committed cells? The use of cell lines and the study of their behavior in response to cytokines to explain this dilemma may be misleading. For example, the elicitation of granulocytes from a pool of multipotent cells by the addition of G-CSF would seem to argue for an instructive role for G-CSF (Stoffel *et al.*, 1997). However, the result could equally well be explained by suggesting that G-CSF is selecting for the survival and proliferation of a subpopulation of cells that have been already programmed for neutrophil development (by cell-intrinsic/stochastic means), with the other cells, with different lineage potential, either remaining undeveloped, unexpanded, or simply dying.

Another significant component is likely provided by the transcription factor profiles of individual multipotent cells. If growth factors play an instructive role in lineage commitment they are likely to modulate lineage-specific transcription factors such as GATA-1 and PU.1. However, most investigations to date have focussed on either proliferation functions of growth factors (MAPK, Ras, AP-1,c-myc) (Smith *et al.*, 1997) or their survival functions (PI3K, Akt, Bax/Bcl-2) (Martens *et al.*, 1998). But there is little evidence of their impact on tissue-specific transcription factors.

This growing debate in hematology of whether or not lineage commitment is a cell-intrinsic or cell-extrinsic mechanism (Orkin, 1996) reveals the importance of my study. For the first time, I show that external signaling by GM-CSF results in the direct activation of the tissue-specific transcription factor PU.1 to trigger a cell-intrinsic differentiation program mediated by PU.1. This differentiation response results exclusively in the differentiation of granulocytes and macrophages. The main argument in this study is the fact that PU.1 is directly affected by GM-CSF signaling and not by some parallel, independent pathway and that the differentiation response is mediated by PU.1 activation.

This work provides direct evidence that hematopoietic growth factors can influence cell fate through their effect on tissue-specific transcription factors. However, one cannot help feeling that the all or none instructive versus selective debate is no longer the question. One possible question is whether or not more primitive cells follow the same deterministic mechanism as more lineage restricted cells. It is now important to piece together the different molecular components, cell-intrinsic and cell-extrinsic, and understand the profile of their interactions.

Conclusion

We have shown that the myeloid-specific transcription factor PU.1 acts as a downstream effector of GM-CSF signaling in myeloid development. We clearly demonstrated that PU.1 protein levels rapidly increase in response to GM-CSF and that this increase precedes significant macrophage differentiation. Using gain and loss of function experiments, we showed that PU.1 acts downstream of the GM-CSF receptor in triggering a differentiation response. Furthermore, we showed similar results for cell survival response, that is, PU.1 function is a requirement downstream of GM-CSF signaling in directing cell survival.

This study illustrates for the first time how external signaling might have a deterministic effect on lineage commitment by directly influencing the cell-intrinsic differentiation machinery mediated by lineage-specific transcription factors. As of now, the extrinsic agents responsible for the activation of these nuclear transcription factors were unknown. The GM-CSF cytokine represents the first case reported whereby a hematopoietic growth factor directly induces a lineage-specific transcription factor, PU.1, involved in the cell-intrinsic differentiation program.

However, the ongoing debate featuring permissive versus instructive roles for environmental signals is far from over. What is true for more developmentally restricted progenitor cells may not hold for multipotent, more primitive cells. And following physiological insults, when cell proliferation needs to respond rapidly to bleeding or infection, and where certain cell types are more needed over others, what is then the mechanism of differentiation of progenitor cells? Hematopoietic commitment is likely to be extrinsically regulated. But there is only limited evidence and probably only limited opportunities for

hematopoietic regulators to be involved in these events. In other words, once the differentiation window is reinforced, these cytokines are likely to resume a more common role in cell survival and proliferation. This present study is a clear example of how an external signal is received, interpreted, and translated into a specific differentiation response. It is likely that the determination of the other hematopoietic cell lineages will follow a similar mechanism of action: GATA-1 and eryrythrocyte differentiation or Ikaros and lymphoid differentiation. The race is on.

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