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Cell-to-cell interactions in EML cell differentiation: The role of diversity and heterogeneity in cell populations

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Cell-to-cell interactions in EML cell differentiation: The role of diversity and heterogeneity in cell populations

By

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ABSTRACT

The differentiation of hematopoietic stem cells to mature cell types is a multistep, branching process that generates the various cell types of the entire blood system. Our laboratory and others recently found that the heterogeneity of cell phenotypes that is present even in a clonal population of nominally identical cells plays a central role in cell fate choice and commitment of multi-potent progenitor cells. In this project I analyzed the precise dynamics of such cell population heterogeneity during differentiation and asked whether there is a specific heterotypic communication between the cells given that individual cells exhibit distinct behaviors. For this purpose I examined cell fate commitment of the hematopoietic progenitor cell line EML into the myeloid lineage at single-cell resolution using flow cytometry to monitor the induction of the differentiation marker, CD11. First I found that individual EML cells of a clonal cell population begin the differentiation process at disparate time points despite the simultaneous stimulation of differentiation and the uniformity of the culture environment. This asynchrony suggests an intrinsic heterogeneity also with respect to the response kinetics which further increases the diversity of the cells. Specifically, dissection of this differentiation process along the CD11b axis revealed that the differentiating signal led to a splitting of the uniform population into three discrete subpopulations at an intermediate stage. Second, I found that all three subpopulations were necessary to sustain a maximal differentiation rate of the entire population. Thus these subpopulations do not act independently but instead, communicate with each other. I determined that this communication is mediated via soluble signals and that it significantly affected the global differentiation kinetics of population. Finally, I have also begun to identify the nature of the soluble factor that mediates this interaction and discovered that production of IFN-γ during myeloid differentiation accelerates the process. These observations indicate that differentiation of a uniform cell population of cells from a precursor state to a mature state involves a non-cell-autonomous behavior that is mediated by secreted factors.

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Symbol	Definition
HSC	Hematopoietic stem cells
MNC	Mononuclear cells
CD34	Glycoprotein marker for stem cells
SLAM	Signaling lymphocyte activation
SCA 1	Stem cell antigen 1
C KIT	Tyrosine kinase receptor which ligand is SCF
SCF	Stem cell factor
CFC	Colony forming cell
TPO	A membrane-bound glycoprotein
CMP	Common myeloid progenitors
EPO	Erythropoietin
IL-3	Interleukin 3
IL-7	Interleukin 7
LNK	B-cell Linker-protein
IL-6	Interleukin 6
IL-11	Interleukin 11
Flt-3	Kinase receptor regulating hematopoiesis
TGF-β	Transforming growth factor beta
ST-HSC	Short-term hematopoietic stem cells
LT-HSC	Long term hematopoietic stem cells
CLP	Common lymphoid progenitor

MEP	Megakaryocite/erythrocyte progenitors.
EML	Hematopoietic stem cell line
atRA	All-trans retinoic acid
RAR	Retinoic acid receptor
GM-CSF	Granulocyte macrophage colony stimulating factor
GATA1	Transcription factor involved in erythroid development
PU.1	Transcription factor involved in myeloid development
TF	Transcription factor
GRN	Gene regulatory network
FACS	Fluorescence-activated cell sorting
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet-derived growth factor
EGF	Epidermal growth factor
CCL3	Macrophage inflammatory protein-1α
CCl4	Chemokine ligand 4
CXCL10	C-X-C motif chemokine 10
TNF α	Tumor necrosis factor alpha
BHK/MKL	SCF producing cell line
IFN γ	Interferon gamma

CHAPTER ONE: INTRODUCTION

1.1 Stem cells are important for tissue homeostasis in multicellular organisms.

Stem cells exist in virtually every adult tissue and by differentiating into the appropriate mature cell type play a key role in tissue homeostasis. Accordingly, they are capable of self-renewal (cell division without loss of proliferative potential) and differentiation into a variety of mature cell types (multipotency) [1, 2]. In mammals, stem cells are particularly important to maintain tissues with lineages that have a short lifespan (e.g., blood, intestine, skin) by continuously supplying newly differentiated cells. The importance of stem cells, however, is not restricted only to maintaining such kinds of "dynamic" tissues. For example, especially in primitive multicellular organisms, such as hydra and planarian [3, 4] , the regulated proliferation and differentiation of stem cells serve to replace differentiated cells that die through the normal course of aging and allows for the regeneration of entire damaged tissues through injury. Thus, stem cells are at the heart of both creating and maintaining cellular diversity. They play a tightly regulated role both in development and in regeneration in the adult.

Most of the research in the field has been dedicated to embryonic stem cells (considered totipotent cells) due to their capacity for the production of all tissue layers and, consequently, all tissue types. One of the best models for the study of adult stem cells is perhaps the hematopoietic stem cells (HSCs). A single HSC can give rise to all lymphoid, myeloid and erythroid cell lineages, and repopulate an ablated hematopoietic system [5–7]. Besides the blood system, similar paradigms appear to apply to non-hematopoietic tissues as well, including the liver, intestinal epithelium, endothelium, skeletal muscle and brain [1, 8]. Surprisingly, cancer biologists have described cells within a variety of different tumors with stem cell properties, so

called cancer stem cells: rare cells that can give rise to an entire tumor, sustain tumor growth and accounts for malignancy. The cancer stem cells share many features of tissue-specific stem cells [9, 10]. Stem cell therapy may be one day used to promote healing and regeneration in the emerging field of regenerative medicine. This potential of clinical impact creates the need to better understand the biology of stem cell population dynamics since obviously, only as population can stem cells exert their function in the tissue of multicellular organisms.

One of the most widely model for stem cell research has been the hematopoietic system. It has attracted much interest not only as a research model for multipotency and differentiation but also for therapeutic strategies because of the success in the transplantation of hematopoietic stem cells (HSC) and reconstitution of the blood system[5].

1.2 Hematopoietic stem cell characterization reveals inherent heterogeneity and multiple multiple subpopulations.

HSCs are frequently isolated from umbilical cord blood cultures with a frequency of just under one in 1 million mononuclear cells (MNCs), and are less frequent in the adult bone marrow (one in 3 million MNCs) or mobilized peripheral blood (one in 6 million MNCs) [11]. Most effort has been focused on characterizing cell surface markers that can identify those cells with true functional stem cell properties. Perhaps the most familiar marker is CD34, a glycoprotein present on the cell surface of stem and progenitor cells which is used to enrich stem cells in the context of stem cell mobilization and collection for HSC [6, 12]. In mice transplantation studies, use of combinations of markers highly enrich for HSCs, yielding a cell population where 20% have the ability to repopulate the blood of a recipient mouse – the functional definition of stem cell [13–15]. Usually, selection of these cells starts with the depletion of bone marrow cells that express any lineage-specific markers, followed by the positive selection of Sca-1+c-kit+ cells (Lin−Sca-

1+c-kit+ cells or LSK cells)[11]. Recently, the family of signaling lymphocyte activation molecule (SLAM) were found as important discriminators between true HSCs and primitive progenitor cells, holding promise for a simpler combination of three markers that can effectively enrich for HSCs as current, more complex combinations [16].

Despite the efforts to quantitatively identify and isolate HSCs there is no phenotypic assay that directly identifies and measures HSCs on a cell per cell basis. Instead, the best way to measure such complex cell populations rely on functional assays in animals [11, 17]. The most popular assay for the identification of *progenitors* (as opposed to stem cells) of the blood has been colony-forming cell (CFC) assays which measure cells that are partially committed, that is "positioned" in the developmental hierarchy somewhere between HSCs and the mature blood cells [18–20] and still can give rise to defined sets of blood lineages – but not all (only HSC are capable of generating all cells). These progenitor cells readily produce detectable blood cell colonies in a semisolid medium, like methylcellulose, after incubation for several weeks. Cell fractionation studies have shown that such cells of intermediate maturity detected in these assays are not true HSCs, contributing only very slightly and temporarily to hematopoietic recovery after transplantation [21, 22].

1.3 Different molecular cues govern Hematopoietic Stem Cells self-renewal or differentiation.

Although a large variety of soluble factors and their receptors have been studied for HSC differentiation and self-renewal, few receptors/ligands have been shown to play well-defined, essential roles in their function. To date we know that SCF/c-Kit and TPO/c-Mpl are two of the receptor/ligand pairs that are important for HSC. Studies using isolated mouse HSCs have shown that both c-Kit and c-Mpl are expressed on highly purified HSC [23–25] and genetic deletion of

either TPO or c-Mpl leads to a reduction of HSC numbers [26, 27]. TPO signaling in HSC occurs via an association of ligand-bound c-Mpl with the adaptor protein Lnk, an intracellular protein that inhibits the action of many cytokine mediated signaling pathways, including SCF, EPO, IL-3, IL-7, as well as TPO [28, 29]. Detailed studies of Lnk-/- mice have revealed not only the increased absolute numbers of HSC but also the increased repopulation potential of individual HSC in competitive repopulation assays, suggesting that Lnk is a negative regulator of HSC self-renewal and that TPO signaling is a positive regulator of HSC self-renewal [28].

Functional effects of many cytokines, including IL-3, IL-6, IL-11, Flt-3 ligand, in combinations with either SCF and/or TPO have been reported [30, 31]. For almost all the cytokines, culturing HSCs with different combinations of them results in increased survival and proliferation of cells. Besides the common hematopoietic cytokines, developmental signaling pathways have also been shown to be relevant for adult HSC. Purified Wnt3a treatment *in vitro* increases self-renewal of mouse adult HSCs [32]. Overexpression of constitutively active *β*-catenin, an intracellular transducer of Wnt signaling, in mouse HSCs followed by long-term culture results in dramatic expansion of functional HSC as measured by both cell surface markers and transplantation assays.

In contrast to the high turnover of lineage restricted progenitors, most of HSCs reside in the 'quiescent' G0 phase of the cell cycle [33]. TGF-*β*/Smad signaling is one of the pathways that is responsible for maintaining quiescence of HSCs. *In vitro* studies have shown that TGF-*β* inhibits HSC proliferation without inducing apoptosis and neutralization of TGF-*β* seems to release early hematopoietic progenitor cells from quiescence [34].

Another receptor involved in the maintenance of HSC quiescence is Ang-1/Tie2. Tie2 is a tyrosine kinase receptor highly expressed on endothelial cells and HSCs [35] and evidence shows that Tie2 is indispensable to maintain the HSC pool in adult BM [36].

The above is a summary of a few salient findings to recall the general concept that maintenance and differentiation of hematopoietic stem and progenitor cells is strongly modulated by secreted cytokines. This will become relevant in the later part of this thesis. One point worth mentioning here is that in the above studies, there is little awareness of heterogeneity of cell populations, that is, the cell population being examined as target of a given cytokine is tacitly considered to consist of identical replicates of individual cells. Biochemical studies on the signaling pathways accordingly typically regard entire dishes of cultured cells as a homogenous mass and lysates of populations are analyzed for abundance of proteins, thus (more often than not) abnegating the explicit notion of cell population heterogeneity.

1.4 The differentiation of hematopoietic stem cells consists of a successive chain of multiple of multiple binary decisions and can be studied in cell culture systems.

Hematopoietic differentiation is a complex process in which multiple functionally and morphologically distinct cell types arise from a population of multipotent hematopoietic stem cells [37][Fig. 1]. In the canonical model, hematopoiesis begins with self-renewing long-term hematopoietic stem cells (LT-HSCs) which later develop into short-term hematopoietic stem cells (ST-HSCs)[5]. These ST-HSCs have the ability to differentiate into multipotent progenitors (MPPs) which further differentiate into either Common Lymphoid Progenitors (CLP) or the Common Myeloid Progenitors (CMPs). These CMPs are the stage that is of relevance for my studies. These multipotent progenitors in turn face a binary choice, differentiating into the granulocyte/macrophage progenitors (GMPs) or megakaryocite/erythrocyte progenitors (MEPs) [38].

Thus, the entire differentiation program is a hierarchical, multi-step process consisting of subsequent binary developmental branch points. At such points, a multipotent cell generally has

to decide between two cell fate (lineage) choices such that it ultimately produces a defined set of phenotypes. Between a CMP and a final state phenotype (Myeloid or Erythroid), we find intermediate stage progenitors. Each of the stages in the hematopoietic differentiation cascade (stem cells, progenitors and mature cells) has a distinct gene expression profile that is established and maintained by specific combinations of transcription factors and chromatin remodeling components [39]. This process has been intensely studied *in vivo* and *in vitro* using different model organisms, and many putative molecular pathways involved have been identified[7]. However, most studies, as is common place in biology, analyze "snapshots" at some time intervals of whole cell populations. The integrated dynamics underlying this entire process of how a heterogeneous cell population with cells in phenotype *A* becomes a population of cells with phenotype *B* is not explicitly explored in such approaches and remains unclear.

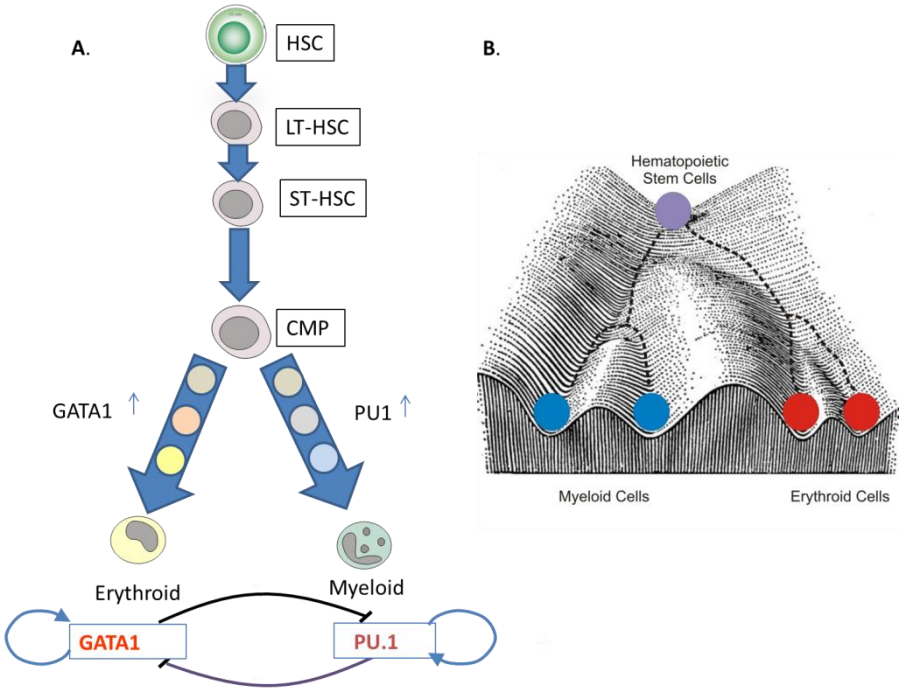

Fig. 1. HSC differentiation is governed by the GATA1-PU.1 gene circuit. A. HSC differentiation into myeloid cells is a complex process composed by different states. **B.** The Waddingtonian epigenetic landscape shows the existence of different theoretical stable states.

The EML murine cell line has proven to be a well suited in vitro model system for studying many aspects of cell fate decisions in the hematopoietic system. This multipotent hematopoietic cell line has multi-lineage potential and corresponds roughly to the MPP or CMP stage (depending on culture conditions). It was generated from murine bone marrow cells by the ectopic expression of a dominant negative retinoic acid receptor (RAR) molecule [40]. In these cells, myeloid differentiation is specifically blocked by the dominant negative RAR protein; nevertheless, this block can be overcome by culture in the presence of IL-3 and all trans-retinoic acid (atRA). Under these conditions, EML cells differentiate predominantly into promyelocytes and granulocyte maturation can be stimulated by granulocyte-macrophage colony-stimulating factor (GM-CSF). They can also be differentiated into the erythroid lineage with the appropriate signals, including erythropoietin. Thus, EML is an in vitro model for a binary branch point. In this work I will focus on myeloid commitment to the myeloid (macrophage/neutrophil) lineage.

For the CMP stage, the molecular circuit that drives the binary decision is composed of the two transcription factors (hereafter, TF) GATA1 and PU.1. They form the core of a circuitry that controls the myeloid/erythroid fate decision. Induced overexpression of PU.1 or knockout of GATA1 on CMP cells promotes myeloid lineage differentiation, leading to a constellation that is characterized by the gene expression pattern ($GATA1^{low}$, $PU.1^{high}$); on the other hand, over expression of GATA1 or suppression of PU.1 induces erythroid differentiation, which is associated with the inverse pattern ($GATA1^{high}$, $PU.1^{low}$) [41, 42]. These results are explained by the fact that both transcription factors form a mutual inhibition circuit, suppressing each other's activity through direct protein-protein interactions [43]. At the CMP state, they hold an unstable balance ($GATA^{mid}$=$PU.1^{mid}$) that is prone to be tipped to either side, namely, to the ($GATA1^{low}$, $PU.1^{high}$) or the ($GATA1^{high}$, $PU.1^{low}$) state. In dynamical systems theory such dynamics – two stable states separated by an unstable (balanced) state - is called bistability. The undecided state

exhibits low expression of both opposing TFs, GATA-1 and PU.1[38]. Such a balanced state has now been found to be a general feature of the multipotent state facing a binary fate decision in many other progenitor cells of other tissue [44]. External signaling events together with intrinsic fluctuations are key factors that influence this delicate balance between both transcription factors, allowing the progenitors to be tipped into either direction, and thereafter robustly differentiate down one or the another developmental path [38, 45, 46].

1.5 Hematopoietic stem cell differentiation is governed by the non-linear dynamics of a dynamics of a gene regulatory network.

Cell types in general (as well as in the hematopoietic system) are discrete, mutually exclusive and stable phenotypic states. These distinct cellular states include immature stem and progenitor cells as well as the fully differentiated mature cell. Each of them is characterized by a specific gene expression program which we can now approximately measure in the form of transcriptomes [47]. Beyond this, the problem of how stem cells determine their own fate and how they then establish the gene expression program, defined by the gene activity status across the ten thousands of loci in the genome, is still unsolved[44]. Regarding the fate choice of a multipotent cell, two major hypotheses have been proposed[48]. One hypothesis is that fate decision is “cell-intrinsic”, according to which stem cell commitment is triggered by a preexisting intra-cellular program. Which program is chosen depends on signals that tip the cell to either one or on pure chance (stochastic event). The other hypothesis assumes an ”instructive” process in which differentiation is caused by instructive signals received from outside the cell, which precisely tells the progenitor cell which gene to turn on and off in order to commit to a particular lineage.

A unification of these two phenomenological hypotheses is possible when we look at stem cell commitment through the lenses of non-linear dynamics of a molecular interaction network, or more precisely, a Gene Regulatory Network (GRN) [Fig.2]. This GRN determines the expression levels of the *N-genes* that it encompasses at a given time point (for a better explanation [47]). It is in this genome-wide network that the two aforementioned circuit of the two opposing fate decision controlling TFs (such as GATA1 and PU.1) must be envisioned to be integrated. Thus, we can extend the two-gene circuit that give rise to bistability to a broader picture that encompasses all the genes in the genome – for ultimately, during differentiation the transcriptome shifts from one state *A* to another state *B* in which thousands of genes will change their expression levels. The basic ideas are summarized in Figure 2.

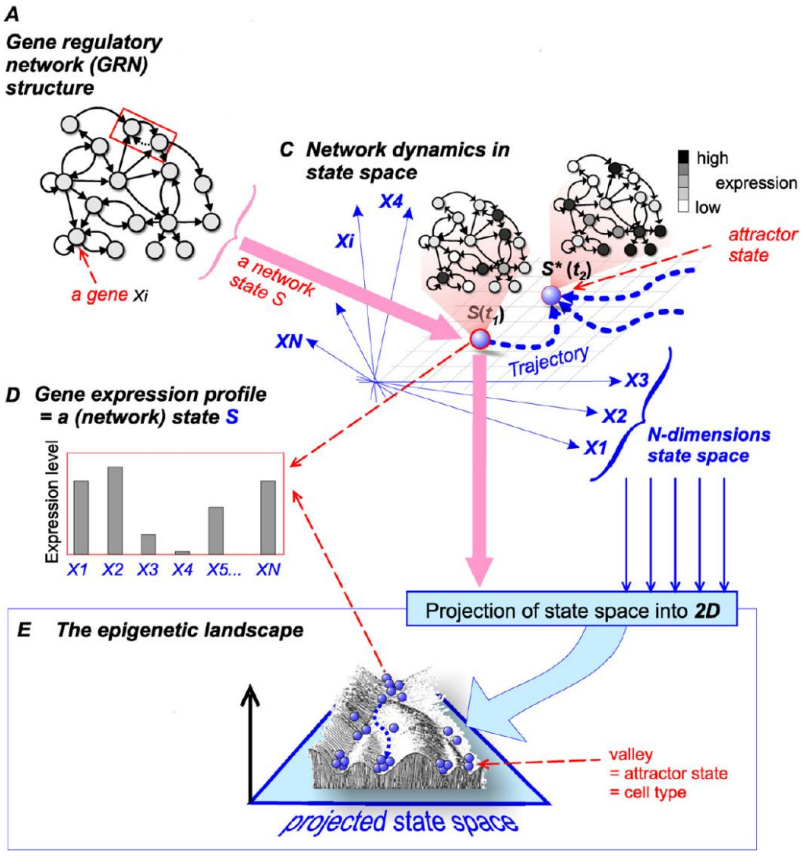

Fig 2. Schematic representation of a GRN. (A) GRN that via network dynamics represents the N-dimensional state space (C) in which each point is a state defined by its gene expression profile (D), into a quasi-potential landscape (E).

In this network, the connection and interdependence between N regulatory molecular elements establish an *N*-dimensional system whose dynamics control the trajectory of the cell differentiation process [FIG 2.C]. In a mathematical sense, the cell moves in an N-dimensional "state space" in which each point represents a particular gene expression profile. The regulatory interactions now impose heavy constraints on how the expression levels of individual genes can change, which maps mathematically in the highly constrained way in which the cell, or the point in the state space, can move: It moves only along predestined trajectories. This is the integrated dynamics of the network[49].

A trajectory is not only determined by the nature of the interactions but is highly influenced by gene expression noise, which confers certain stochasticity to the course of the trajectory. However**,** the system dynamics are not totally erratic due to the presence of well-established interactions that keep the trajectories towards stable points. In our two-gene circuit of mutually repressing genes (where here the repression is a constraining interaction [50]) implies a well-ordered and predictable dynamics, resulting in particular trajectories where several stable steady states are observed to which the system (point in state space representing the cell) is literarily "attracted". This sense of directionality in our system typically starts at a binary decision of the undecided state and ends in the state of the committed lineage that is robust, that is, unlike the aforementioned balanced state, resistant to perturbations because it cannot be tipped into another state anymore. These destination states, as well as any observable, discernible (meta)stable cell types thus has been referred to as attractor states [50].

For this work it suffices to know that the attractor model affords an explanation for the directionality of the differentiation trajectory in gene expression state space and also offers a conceptual framework to unite the cell-intrinsic model (where attractor states represent the preexisting, genomically hard-wired gene expression programs) with the instructive model

(where gene regulatory interactions allows regulatory signals to propagate information to the specific set of genes that must be altered to facilitate an attractor transition).

In summary, we can view HSC differentiation as the hierarchical change in the gene expression profile driven by the dynamics of the GRN in which developmental trajectories undergo binary branching and drive cells from less stable attractors to increasingly stable ones to ultimately give rise to the various stable (terminally differentiated) phenotypes.

Thus, in the more general, larger genome-wide picture, we have not just two stable states as explained for the bistable circuit but thousands. Conceptually, such *multistability* and the associated switch of phenotype (attractor transitions) observed during the differentiation process can be mapped into Waddington's epigenetic landscape metaphor that he proposed around 1950, to explain the successive cell fate branching from a pluripotent cell to the discrete phenotypes of the differentiated cells [50, 51][FIG 2 E]. Waddington's epigenetic landscape can be viewed as the projection of an *N*-dimensional state space (for a GRN of *N* genes) into two dimensions (the XY plane). This then allows the third dimension (vertical z-axis) to serve as the "elevation" to assign to each XY position (a state of the GRN, or equivalently, the associated gene expression pattern) a value that reflects the "stability" (roughly speaking) of a given GRN state that is represented by that respective position in the XY plane. This creates a landscape in which the more stable GRN states, hence, gene expression patterns, and consequently, cell phenotypes, are in the valleys; whereas the less stable or unstable states are on the hills. The valleys are the attractors of the GRN, and thus, the stable observable cell types. Conversely, mountains and peaks are unstable states, separating the stable ones. The cell now can be represented by a rolling ball, as Waddington already suggested which seeks stable steady states as it rolls down the valleys to the lowest points.

The epigenetic landscape as a whole thus depicts the entire set of possible stable states (valleys) that exist in the GRN and that the cell can in principle achieve during development; however, it also shows the ease or difficulty with which these potential attractors can be reached. At each point, the cell faces constraints that predefine a set of adjacent ("reachable") phenotypes. This is captured in Waddington's landscape metaphor where the "watersheds" represent the binary decision points at which the cell has only two possibilities (binary decision) – which of course is due to the constraints of the dynamics in the GRN.

1.6 The importance of Waddington's epigenetic landscape: more than just a metaphor. metaphor.

The term "*epigenetic*" has been coined recently to group non-genetic events (most of them covalent chromatin and DNA modifications) affecting cell development, activity and evolution [52]. Waddington's "epigenetic landscape" is similar to what physicists call an "epigenetic state" [50], a system-level stable state that arises from genetic interactions, which in turn directly reflects Waddington's original meaning.

1.6.1Properties of cell fate regulation

Assuming that cell phenotypes are attractors of complex GRNs explains different cell behaviors that are hardly explained by linear molecular pathways. It explains why cell-type specific genome-wide expression profiles, defined by the values of N-genes, are so reliably established during differentiation: The self-organizing and self-stabilizing property of GRN is a natural feature conferred by attractors. Hence, cell-type specific gene expression patterns are robust to noise, re-establishing themselves after small perturbations (imposed changes of expression levels of individual genes) and can be reached, in principle, via an almost infinite number of paths [49,

53]. At the same time, they are capable of undergoing discontinuous transitions to other specific stable expression profiles via genome-wide changes of gene expression. These kinds of transitions occur when cells encounter the proper cell fate regulatory signals that, via branching signal transduction pathways change the expression of a specific set of genes of the network, or due to sufficiently high random fluctuations of gene expression levels. As shown before, attractor transitions underlie the cell phenotype switching during development [49, 54, 55].

1.6.2. The "descend" from pluripotent embryonic cell to the mature cell type

The epigenetic landscape offers an integrative view of development. Pluripotent embryonic stem cells are in a metastable state at the highest point in a landscape. They roll down the landscape as cells multiply and swarm out to fill the state space of the genomic network, driven by gene expression noise and regulatory interactions, and come to rest as they occupy the various valleys and subvalleys in the "lower regions" of the landscape, representing terminal differentiation [50]. The associated inevitable loss of "potential energy" is consistent with the progressive "lineage-restriction" in the course of development. The discernible intermediate states of maturation that correspond to metastable multipotent progenitor cells (like HSC) are represented by small groves located at watersheds, where a cell has to make a binary fate decision to roll further down into either one of the two adjacent valleys. Experimental and theoretical analysis of bipotent progenitor or stem cells are consistent with this picture [44]. In other words, development is the distribution of cells into a set of ("low energy") attractors and their balanced occupation by cell populations.

1.6.3 To determine the relative occupation of the valleys of the epigenetic landscape cells must talk to each other

But another layer of regulatory network that will be treated later is important for this project, namely, that of *cell-to-cell communication* which is implemented by paracrine, iuxtacrine and humoral interaction. In terms of the formal model of the epigenetic landscape, cell-cell interaction can be absorbed in that model as a means by which the relative occupancy of neighboring attractors by cells is regulated. This information is not contained in the regulatory network that establishes the landscape topography. With this notion of relative occupancy of attractors we move into the domain of single-cell behavior (state dynamics) to that of entire cell populations. The relative occupation of distinct attractors by a given number of cells is of course important for tissue homeostasis because it maps into the cell-type composition of a given tissue (Mathematically, such cell-to-cell regulation can be formalized as a modification of the landscape topography that deepens an attractor.) Taken together, the epigenetic landscape is the stage on which the play of cell fate decisions and cell type maturation is choreographed. Its detailed topography must be such as to guide the coordinated production of the appropriate proportions of distinct cell types at the right place and time. Driving this graphical representation further, each of the expanding number of cells, rolling down the landscape during development, has to be channeled to the appropriate valleys and subvalleys without jumping into unphysiological side valleys – which may happen due to gene expression noise (see below).

It is important to keep in mind that the epigenetic landscape is more than a metaphor—it is mathematically derived from the dynamics of the gene regulatory network; The GRN of a particular genome maps into one landscape and each geographic position in it represents a unique gene expression profile, i.e., a cell state, in the high-dimensional state space of the genome wide network. Then, one may speculate, evolution of development fine-tunes the wiring

diagram of the genomic network to shape the topography of the epigenetic landscape such as to guarantee a smooth flow of the multiplying cells down into the destined valleys of mature cell types without leaving them behind at inappropriate positions.

1.7 Clonal cell populations are heterogeneous.

The term heterogeneity of a cell population here refers to the cell-to-cell variability observed in populations of cells of the nominally identical types. In our framework of the epigenetic landscape, these cells, since representing the same cell type, sit in the same attractor state. But due to gene expression noise (random fluctuations of gene activities cause by the stochastic nature of chemical reactions) each cell is slightly different from each other at a given time point since they differ from each other (slightly) with respect to the expression level of every gene. Thus a cell population is then not a sharp point at the bottom of a valley (attractor state) as with Waddington's ball, but rather a cloud of point around the attractor state

This of course is consistent with our everyday experience: As all experimentalists know, it is inevitable that every cell population, even if clonal (consisting of genetically identical cells), is phenotypically heterogeneous. Thus, we speak of "non-genetic heterogeneity" to distinguish this phenomenon from phenotypic differences due to somatic mutations in individual cells [56] Heterogeneity of traits is due to heterogeneity in the expression level of genes. The realization that such cell-to-cell variability is independent of genetic differences but is also a valid source of heterogeneity challenges the classic default assumption of cell homogeneity and key concepts in modern molecular biology and evolution [49].

Non-genetic heterogeneity can be readily shown by observing the statistical distribution of cell populations with respect to a given marker. Our group has previously shown that a multipotent blood progenitor cells, including the EML cell that I will use, shows a bell-shape like

distribution when SCA1 expression is measured by flow cytometry [57]. While this is, as said above, ubiquitous and nothing particular, our group found that the observed cell population heterogeneity in the abundance of specific proteins per cell is not just noise in the sense of "gene expression noise" as traditionally assumed but reflects functional differences between the individual cells. Moreover, this variability is not only in the expression of a given marker, but also with respect to the genome-wide gene expression profile. This could mean that the heterogeneity observed with respect to SCA1 in EML cells reflects fluctuations in the entire transcriptome, which gives reason to believe that the outlier cells in the broad distribution do not reflect short lived deviations from the average behavior of the population (which would be the case if the heterogeneity is simply due to gene expression noise); instead, those cells reflect a particular phenomenon involving slow, correlated (transcriptome-wide) fluctuations that could be important for the development of a given phenotype [48].

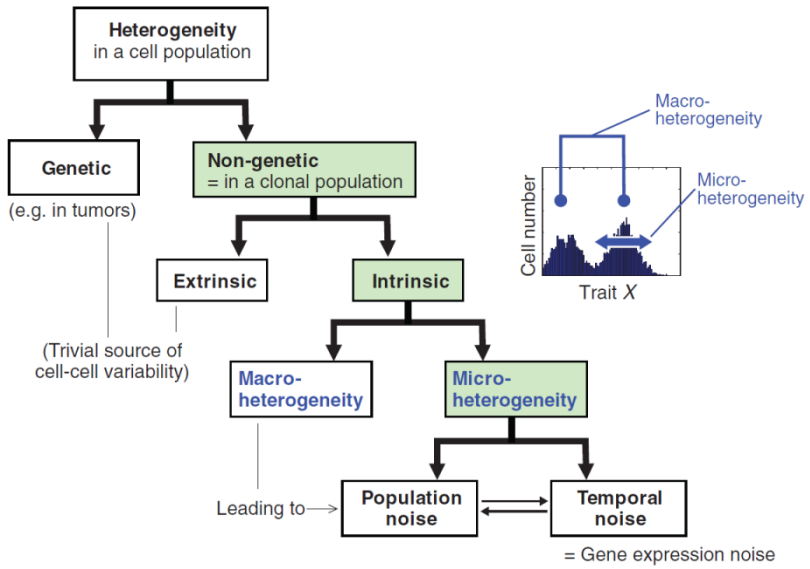

Fig 3. Schematic representation of population heterogeneity organized into a hierarchy of dichotomies: genetic versus non-genetic heterogeneity; extrinsic versus intrinsic non-genetic heterogeneity; macro- versus micro-heterogeneity within intrinsic non-genetic heterogeneity; and population versus temporal noise within micro-

heterogeneity. The inset represents a flow cytometry histogram that reveals a bimodal distribution, which reflects two distinct subpopulations.

This was evident when the progenitor cells were FACS sorted for the outliers with extremely high or low SCA1 expression and then induced to differentiate; outliers with high SCA1 expression committed preferentially to the myeloid lineage, whereas cells with low SCA1 expression developed into proerythrocytes [57]. From this experiment we learn two relevant lessons that will play a role in this thesis: first, SCA1 is a marker on EML that allows for the observation of cell population heterogeneity and second, this heterogeneity shows that the outlier cells with respect to SCA1 expression are "primed" to one phenotype or another, pointing to a non-trivial role for clonal cell population heterogeneity. Thus, not all cells in an apparently homogenous population are equally ready to respond and differentiate when presented with the differentiation signal.

Nonetheless, this striking finding does not yet give full account of the behavior of the entire population as a whole. A particular point that has not been addressed and may affect the differentiation of the population as a whole by cell-to-cell communication: Do the functionally distinct cells, such as the outliers, "communicate" with each other?

1.8 Cell-to-cell interactions lead to non-cell-autonomous behavior

The relevance of cell-to-cell interactions during cell development is of course not a novel idea. In his pioneering theoretical work on pattern formation Turing proposed a mechanism whereby a spatially uniform state is destabilized by the cell-to-cell interactions, leading to the formation of a spatially periodic pattern [58, 59]. However, such interactions has not yet been

much considered in the domain of work of Kauffman's overarching idea of the coexistence of multiple stable attractors in genetic networks that correspond to different cell types [60]. Only recently has Kaneko proposed a theoretical model for multicellular development in which cells are attractors but which includes as a crucial element, cell-to-cell communication by soluble factors [61]. In the hematopoietic system, it is well known that phenomena like T-cell maturation in the thymus or B-cell maturation in the spleen (including negative and positive selection) depend on physical cell-to-cell interactions in order to eliminate auto-reacting lymphocytes [62]. However, such interactions typically occur between distinct cell types (e.g. T cells and dendritic cells) and very few examples are known of cell-to-cell interactions within the same cell lineage but between phenotypically distinct cells states produced by non-genetic heterogeneity. Kirouac et al. have shown the relevance of inter-cellular interactions in umbilical cord blood progenitors which involves the production of stimulatory growth factors, such as VEGF, PDGF, EGF and serotonin and monocyte-derived inhibitory factors CCL3, CCL4, CXCL10, TGFB2, and TNFSF9 [63]. These soluble factors form an intercellular feedback loop involved in the maintenance, self-renewal and differentiation of hematopoietic progenitors, underscoring the importance of the interdependence of cells in a population and supporting the idea for the cells' non-autonomous behavior. Thus, molecular interactions at the population level are important for shaping cell population structures during development. However, cell-cell communication is normally studied for signaling either between nominally distinct cell types (heterotypic interaction) or between cells of the same type (autocrine signaling; or homotypic interaction). With the notion of population heterogeneity introduced here we can now have heterotypic interactions within apparently uniform cell populations. Such interaction would make an apparently cell-autonomous process, such as differentiation, non-cell autonomous and has not been explicitly studied.

1.9. Overall philosophy and Specific Aims of this project

The above formal conceptual framework whose signature element is – in one sentence - that discrete cell states, including intermediate stages as well as differentiated cell types, are attractor states of the network (valleys in the epigenetic landscape). This framework offers a novel motivation for addressing a set of questions. These questions would not have been posed without this formal framework but simply based on ad hoc reasoning (as is common place in cell biology). Moreover, while the theoretical framework may appear unnecessary to some, it actually adds value and those readers who appreciate "systems thinking" will see the connection between the dynamical systems framework and the specific experiments that I will present. These experiments would have been cumbersome and non-intuitive to motivate using traditional "linear pathway-based" thinking of cell biology. However, it would go beyond the scope of this thesis to explicitly establish this connection between my experimental approach and the dynamical systems concepts using mathematical modeling. Thus, at the moment I am limited to test the qualitative consistency of experimental outcomes with the conceptual framework.

The specific experimental goals of this thesis can be framed as addressing the following questions:

QUESTION (1): Can single-cell resolution monitoring of myeloid differentiation in clonal EML cell populations using flow cytometry detect heterogenization in the response to the differentiation signal, or even, reveal the existence of distinct intermediate states?

RATIONALE: The model of a rugged epigenetic landscape predicts a hidden heterogeneity with respect to internal priming to differentiation that may be exposed when cells are stimulate to exit the current attractor states and move to another attractor state.

QUESTION (2): If we discover intermediate subpopulations (which increase the population heterogeneity) do cells of these subpopulations engage in (heterotypic) communication that affects the population–level differentiation kinetics, and in what sense (acceleration or slowing down)?

RATIONALE: Preliminary work (published in the Supplementary Information of [51] suggest a sigmoidal state transition kinetic that is indicative of cell-cell dependency. We will focus on detecting communication by soluble signals because this can be done readily in simple experiments. As explained in the section 1.3., given the abundance of cytokines that can influence stem cell fate determination and differentiation, a focus on soluble factors is warranted (although direct physical cell-cell contact is likely to also be involved).

QUESTION (3): If there is cell-cell communication, what cytokine may mediate such interactions? While this is clearly a "fishing expedition" using new methods for rapid screening of potential candidates may point us to the right direction.

RATIONALE: With a vast literature on soluble factors that have been demonstrated to influence myeloid differentiation and efficient screening methods it should be possible to narrow down the list of candidates for future detailed studies.

CHAPTER 2: MATERIAL AND METHODS

2.1 Cell line and cell culture.

2.1.1 EML hematopoietic multipotent progenitor cell line as system.

The EML multipotent hematopoietic cell line was generated from murine bone marrow by ectopic expression of a dominant negative retinoic acid receptor (RARα) molecule [40]. EML cells proliferate in the presence of stem cell factor (SCF, the ligand for the c-kit receptor) and generates lymphoid, myeloid, and erythroid progenitors in the presence of different cytokines [40]. It also expresses multiple surface markers characteristic of primitive hematopoietic progenitors[22]. Myeloid differentiation is specifically blocked in EML cells by the dominant negative RAR α protein, but this block can be overcome by culturing in the presence of IL-3 and all trans-retinoic acid (atRA). Under these conditions, EML cells differentiate predominantly into promyelocytes, and terminal neutrophil maturation can be stimulated by substituting granulocyte-macrophage colony-stimulating factor (GM-CSF) for interleukin- 3 (IL-3) and SCF after three days. Erythroid differentiation from EML can be occurred by adding erythropoietin (EPO) to the media. By the addition of erythropoietin (EPO) and the reduction of stem cell factor (SCF) erythroid progenitors are able to proliferate and differentiate into red blood cells [40]. A small proportion (10–15%) of macrophages can be detected in cultures grown under these conditions. Because of these characteristics, EML-C1 cell line appears to be the closest in vitro approximation of hematopoietic stem cells to date, and it provides a unique model system for studying the molecular control of the survival, proliferation and differentiation of hematopoietic stem cells [37].

2.1.2 EML cell culture.

EML-C1 cells were maintained in Iscove's modified Dulbecco's medium (IMDM), 20% (vol/vol) horse serum plus 12% (vol/vol) BHK/MKL conditioned medium. BHK/MKL cell line was established by transfecting a Baby Hamster Kidney cell line with an expression vector containing a cDNA encoding the secretory form of mouse SCF. Conditioned medium from BHK/MKL cell line was used for maintaining EML-C1 cells as the source of mouse SCF [40].

2.2 Differentiation of multipotent EML cells into myeloid lineage.

To differentiate EML cells into myeloid cells, EML cells were suspended in growth medium containing 10 ng/ml IL-3 (Sigma) and 10 μM ATRA (Sigma) at an initial cell density of 2.0 x 10^5 cells/ml in day 0. After incubation for 3 days at 37°C in 5 % CO2/95 % air conditions, cells were resuspended in IMDM + 20 % Donor Horse Serum + 2 %(v/v) BHK/MKL conditioned medium + 1 % glutamine + 10 ng/ml IL-3 (Sigma) + 10 μM ATRA (Sigma) + 10 ng/ml mGM-CSF (Peprotech). Cells were further incubated at 37°C for two days after which additional 10ng/ml mGM-CSF (Peprotech) was added. After 6 or 7 days of incubation myeloid cells were observed qualitatively using Wright-Giemsa stain.

2.3 Biological assessment of differentiation

2.3.1 Wright-Giemsa staining for myeloid cell identification.

Wright-Giemsa staining was used to identify multiple cell types present in EML culture cells containing cells differentiated toward the myeloid lineage. Wright-Giemsa staining uses a thiazine-eosin formula to differentially stain cells in which the nucleus will appear dark purple while the cytoplasm will appear light purple to pink.

In a sectioned area of a 96 well plate, wells were coated with 0.1 % (w/v) poly-L-lysine and allowed to sit for 1 hour. After rinsing with PBS three times, 100 µl of cell culture suspension at 1-5 x 10^5 /ml cell density were added and centrifuged at 200 g (1000 rpm) for 3 minutes at a low braking deceleration. The cell suspension was then aspirated from wells and allowed to dry. Once dried, 50 µl of 0.4 % (w/v) Wright-Giemsa staining solution (Sigma) was added for 1 minute. Without removing the Wright-Giemsa staining solution, 50 µl of 1x phosphate buffer, wells were observed under the microscope for cell type identification.

2.3.2 Cell surface marker (antigen) distribution using flow cytometry

Using flow cytometry, we can monitor the distribution of cell surface markers on the undifferentiated EML cells and also the erythroid and myeloid differentiated cells. Sca-1 and c-kit are surface protein markers found on the undifferentiated EML cells. As the progenitor cells differentiate down on myeloid lineage the expression of these markers decreases gradually until it disappears. On the other hand, there are some lineage markers on the surface of particular developmental lineages that can be used to identify specific lineages. For instance, CD11b (Mac-1) is expressed mainly on myeloid cells and used as a marker for myeloid lineage [57]. During differentiation, expression of this antigens increase relative to undifferentiated EML progenitor cells. Cell surface protein immunostaining and flow cytometry measurements were performed using standard methods as described in [57]. Briefly, the antibodies Sca-1–PE (BD Pharmingen) and c-kit–FITC (BD Pharmingen) were used at 1:1,000 dilutions in ice-cold PBS plus 1% fetal calf serum with 0.01% NaN3. Appropriate isotype control antibodies (BD Pharmingen) were used to establish background signal caused by non-specific antibody binding. Propidium iodide staining was used to identify dead cell population so that could be removed from analysis. We can use antibodies CD11b-FITC (BD Pharmingen) in similar procedure.

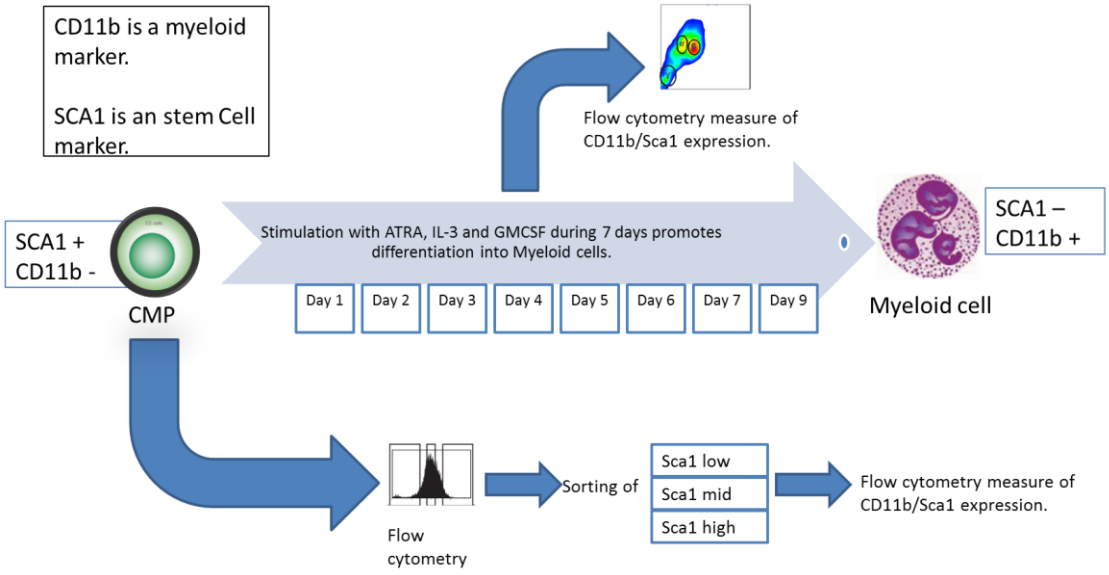

Fig 4. Graphical representation of the experimental set up. Myeloid differentiation was observed after 7 days of differentiation. We measured by flow cytometry the expression of CD11b and Sca1 during the entire process of differentiation. Also, CMP where sorted in three different fractions (Sca1 low, mid and high) to observe if the original cell heterogeneity was implicated in myeloid differentiation.

2.4 Luminex array for the detection of soluble factors

The xMAP technology is a flow cytometric method in which different antibodies can be coupled to fluorescent microspheres that have a unique spectral identity depending on the ratio of different fluorochromes, allowing the simultaneous quantification of different analytes. The used sample was conditioned medium from cells that after 4 days of myeloid differentiation where sorted for α, β and γ. 500 uL of conditioned medium was collected from each subpopulation culture and send to the Luminex facility at Eve Technologies, Calgary, AB.

To obtain a first hint of what mediator may account for the observed modulation of the rate we will measure the presence and concentration of 32 different mouse cytokines using Luminex. In brief, Luminex is essentially an immunoassay, adapted for multiplexing by using microspheres for the simultaneous detection of several analytes at the same time. This assay is commercially available and comprises the following cytokines: Eotaxin, G-CSF, GM-CSF,

IFNγ, IL-1α, IL-1β, IL-2, IL4, IL-3, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, VEGF.

CHAPTER 3: RESULTS

3.1. EML cell differentiation into myeloid lineage is a multistep process that exhibits exhibits distinct intermediate meta-stable states.

Clonal cell populations are heterogeneous, meaning that the individual cells are, despite being genetically identical (isogenic) phenotypically not identical. The cells exhibit "cell-to-cell" variability with respect to phenotypic traits such as the expression level of a given gene. Such heterogeneity is not merely "gene expression noise" but influences the behavior of cell populations as an entity and the response to a given environmental condition. Cell population heterogeneity with respect to gene expression can be quantified by analyzing the dispersion in the expression levels of a cell surface expressed marker within a cell population using flow cytometry. This instrument displays the expression level of a protein in each individual cell of a population and computes a histogram for the measured population. The population heterogeneity can be observed as the broad distribution in a bell shape histogram (micro-heterogeneity) or as a multimodal distribution (macro-heterogeneity), the latter indicating presence of multiple subpopulations due to multistability [56]. Here we monitored how the heterogeneity per se, that is, the structure of the population as an entity changes during the myeloid differentiation process.

Flow cytometry analyses of EML cells were used to monitor the progress of myeloid cell differentiation, which was induced using standard protocols involving IL-3 and ATRA (see section 2.2), by measuring the two markers, Sca-1 and CD11b. Sca-1 is a marker of non-differentiated (stem or progenitor) cells, and is slowly down-regulated during differentiation, whereas CD11b is a marker of myeloid differentiation. However, the single-cell level resolution of these two markers in this differentiation has not been analyzed in detail, we found consistently

that at day 3-4 the population, originally broad but unimodal, split into a tri-modal population in the Sca-1/CD11 state space (phase plane) [Fig 4 A and B].

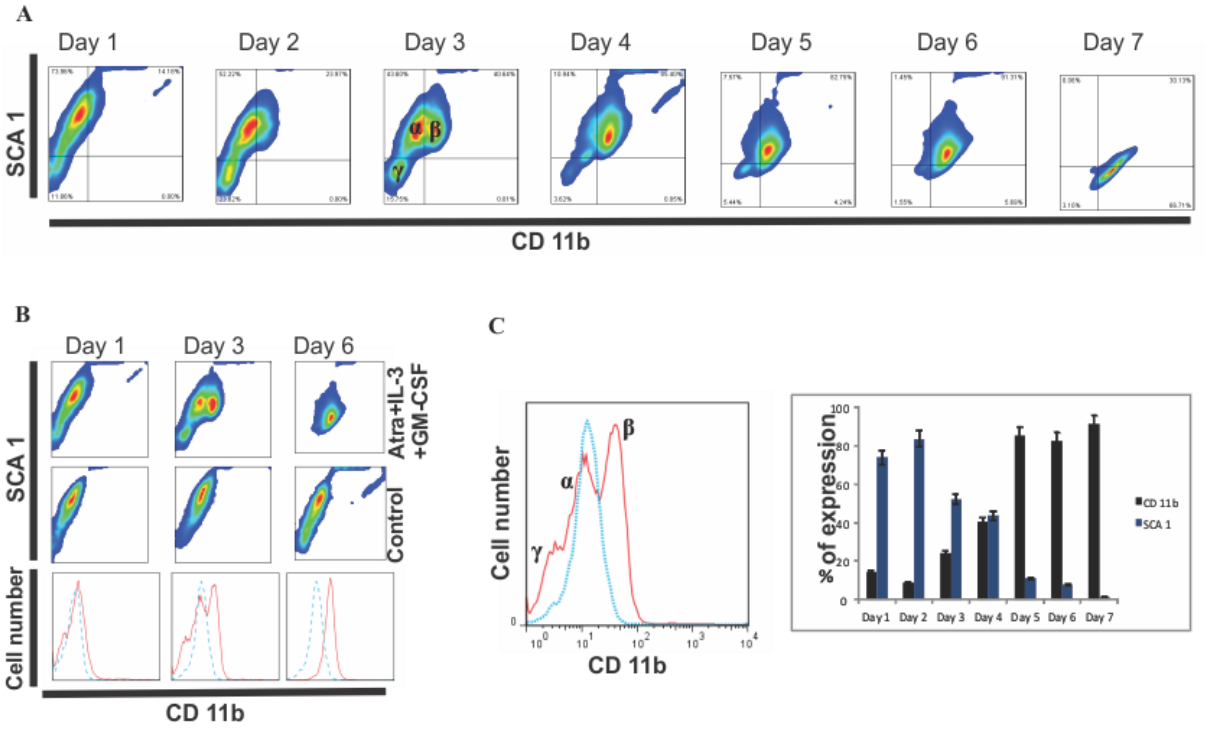

Fig 5. CD11b and SCA-1 expression on EML cells cultured with ATRA, IL-3 and GM-CSF to differentiate into myeloid cells. **A.** The differentiation process takes almost 8 days to complete and at day 3 the coexistence of three different subpopulations with respect to the expression of CD11b and SCA-1 is observed. **B.** These three subpopulations are also observed in the histogram as multimodality (cells differentiating to myeloid in red and non-differentiating control cells are in blue). Hereafter we will denote the three subpopulations as α, β and γ (**C**). The bar graph shows averages of triplicates of one representative experiment for the changes in the expression (as % of the cells in the respective subpopulations) of SCA1 and CD11b.

Thus, the single cell distribution splits into three subpopulations ("substates") with different expression profiles after 3 days of differentiation which we define as follows: **α** ($CD11b^-$,$SCA\text{-}1^+$), **β** ($CD11b^+$,$SCA\text{-}1^+$) and **γ** ($CD11b^-$,$SCA\text{-}1^-$) [Fig 4 B and C]. (Herein, the label + or – indicate the relative, HIGH or LOW state of expression in this discrete behavior). Later on, the broadening of the population disappears and the population consolidates in a single population of committed $CD11b^+$, $SCA\text{-}1^-$ cells. Thus the tri-state configuration at day 3 is metastable. The coexistence of these three distinct metastable intermediate states was corroborated by differences

between cell granularity (SSC in FACS) and size (FSC), showing that the three different subpopulations were also different for size and cell content [Fig 5 C].

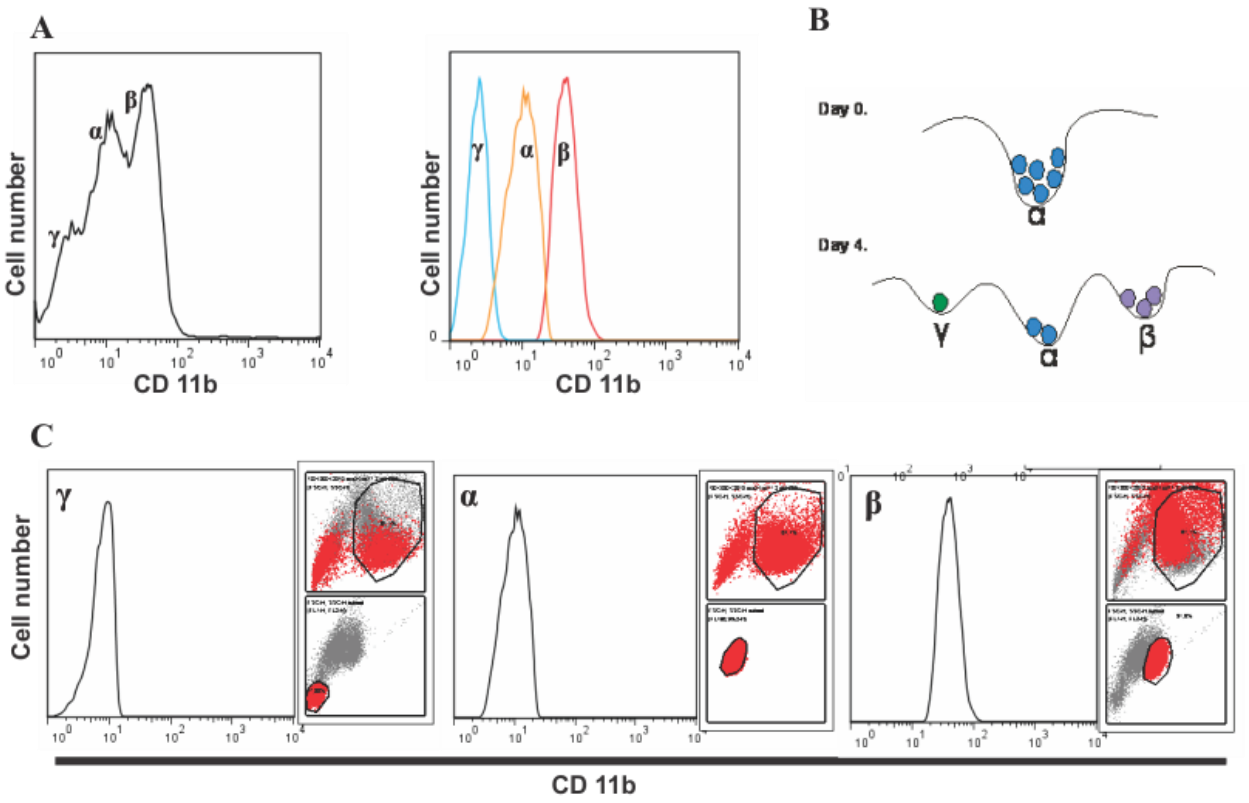

Fig 6. Myeloid differentiation exhibits multistablity during the differentiation process despite starting from a clonal (homogenous) population. **A.** The observed multistability reflects the switch between metastable states before the achievement of the myeloid phenotype, which can be decomposed in the three different states α, β and γ. **B**. This is schematically illustrated on the right as valleys in a "quasi-potential landscape". **C**. Besides CD11b expression multistability is also observed at the morphology level based on granularity and size (small insets, horizontal axis=cell size (FSC); vertical axis = granulatrity (SSC).

3.2 The observed multistability is related to the inherent heterogeneity of progenitor populations and affects differentiation kinetics

The observed multistability at day 3-4 could be related to the underlying diversification dynamics - the very mechanism that generates the clonal cell heterogeneity in the first place that is seen in non-induced populations (but is now accentuated during differentiation) - as our group

has previously published [57]. To establish the relationship between this newly exposed functional heterogeneity which may represent distinct intermediate stages of differentiation, and the underlying diversification tendency of static (uninduced populations) we used the FACS (fluorescence-activated cell sorter) to sort the 3 segments in the original SCA-1 smooth bell-shape distribution as previously reported [57]: SCA1-low, SCA-1-mid and SCA1-high. We asked whether these three fractions would be distinct in generating the three intermediate subpopulations that we see at day 3-4 of myeloid differentiation. We then induced myeloid differentiation as above in each of these three fractions [Fig 6].

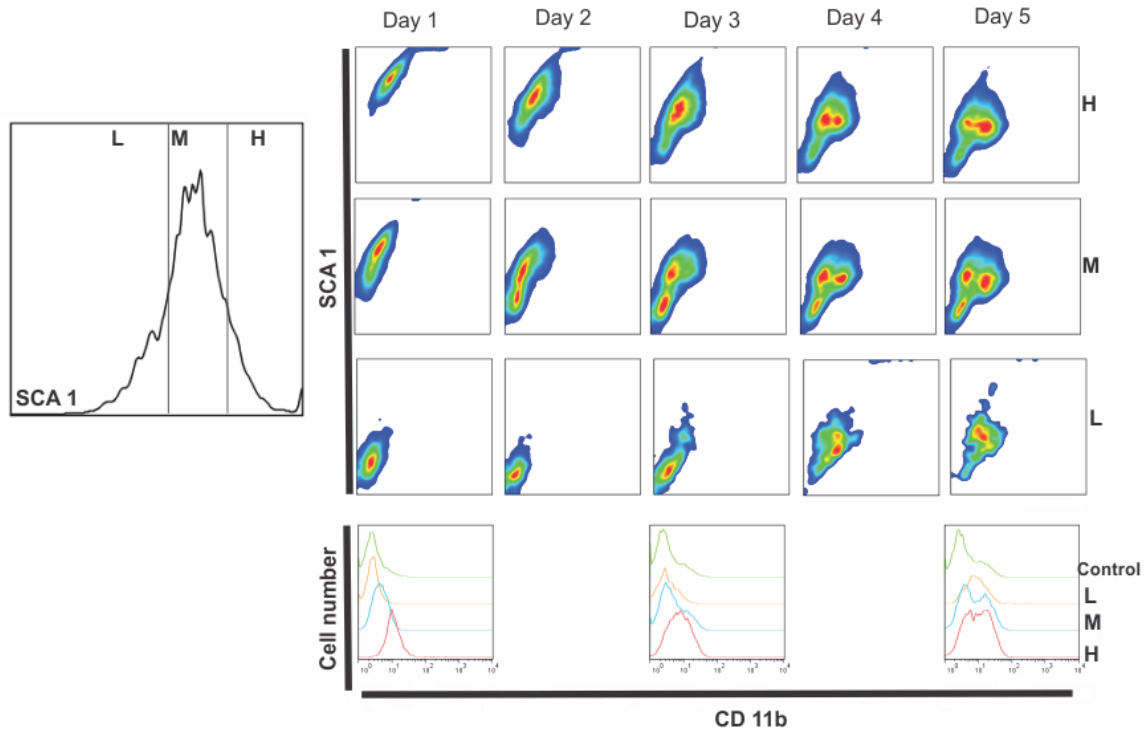

Fig 7. Myeloid development in the three FACS-sorted SCA-1 fractions. All three fractions (SCA-1 High, Low and Medium) were able to develop the three observed sub populations after additional three days of differentiation(at day 7). But they did so to distinct degrees: The SCA-1 High had a pronounced β subpopulation whereas the SCA- low fraction mostly occupied the γ subpopulation.

All three fractions were able to populate the observed α, β and γ subpopulations during the myeloid developmental process. However, the rates and proportions were different for the different segments. We observed a tendency of SCA1-high cells (known to be prone to

differentiate into myeloids, see [57]) to generate more efficiently the β subpopulation, whereas SCA-1 low cells (which are prone to become erythroid) tended to generate γ cells. The SCA-1 mid populated α, β and γ sub-populations equally.

3.3 EML cell differentiation into myeloid cells is influenced by communication between the between the substates.

The evident multistability (the presence of discretely distinct subpopulations) at day four of myeloid differentiation of EML progenitor cells led to our hypothesis that the diversity of cell states influences development because of the ability of the different subpopulations to communicate with each other (heterotypic interaction). Such heterotypic interaction would be novel and unexpected, since clonal populations were not expected to exhibit such clear-cut discrete diversification during cell phenotype change. This would also be different from what has been known as "autocrine" signaling which actually may be homotypic (symmetric) interaction between different individuals of the *same* phenoytpe. The possibility of heterotypic interaction due to the presence of transient subtypes creates the conditions for a more complex dynamics. Our hypothesis is grounded in the notion that cell state transitions often follow a non-linear kinetics, apparent as sigmoidal (rather than inverted-exponential) kinetics of state transition, for which cell-to-cell interactions (non cell-autonomous phenomena) offer the most straightforward explanation. To evaluate the influence of individual subpopulations, α, β, and γ on the kinetics of differentiation of the entire population we first sorted the three subpopulations α, β and γ and let them continue myeloid differentiation separately [Fig 7A].

We found that cell differentiation in the α subpopulation, when isolated, was significantly delayed in comparison with the original (parental) culture, suggesting that presence of all subpopulations is critically important for a normal rate of differentiation [Fig 7A]. This, in turn,

confirms that there should be some type of interaction between the three sub-populations modulating the differentiation process.

Thus, to study the influence of the other subpopulations in differentiation we evaluated the influence of humoral cell-to-cell interactions on the CD11b expression marker for differentiation progression by culturing the subpopulations pairwise in the Transwell Filters as described in methods section. With this set-up physical interactions between cells of the respective subpopulations are prevented, allowing the cells to interact only via secreted soluble factors. Such humoral interaction between pairs of subpopulations, α:β and α:γ revealed substantial differences in the differentiation rates of EML cells, showing that depending on which subpopulations are interacting, the rate by which the differentiation marker CD11b is expressed is altered. Specifically, presence of the β subpopulation alone was able to significantly delay differentiation of cells in α subpopulation compared to the constellation with α alone and α, β and γ all together. Paring α with γ also delayed the differentiation. These results suggest that in EML progenitor cells normal differentiation is a process that requires cell-to-cell communication between different metastable states that are generated along the path of the very differentiation process itself and that modulate the rate of CD11b expression.

It is interesting that the α subpopuation when held alone was faster than when receiving signal from the β and γ subpopulations but that when both (β and γ) are present (the entire population) these two subpopulations cancelled their inhibitory function. This regulatory constellation, where either β or γ but not both together, have an inhibitory output, i.e. where presence or absence of both (equality of both input values) lead to inhibition, constitutes an "exclusive-nor" logical function (XNOR) – a rare logical function in biological regulation (which typically uses AND, OR, or NOT-IF). The study of the meaning of such regulatory modality is beyond the scope of this thesis.

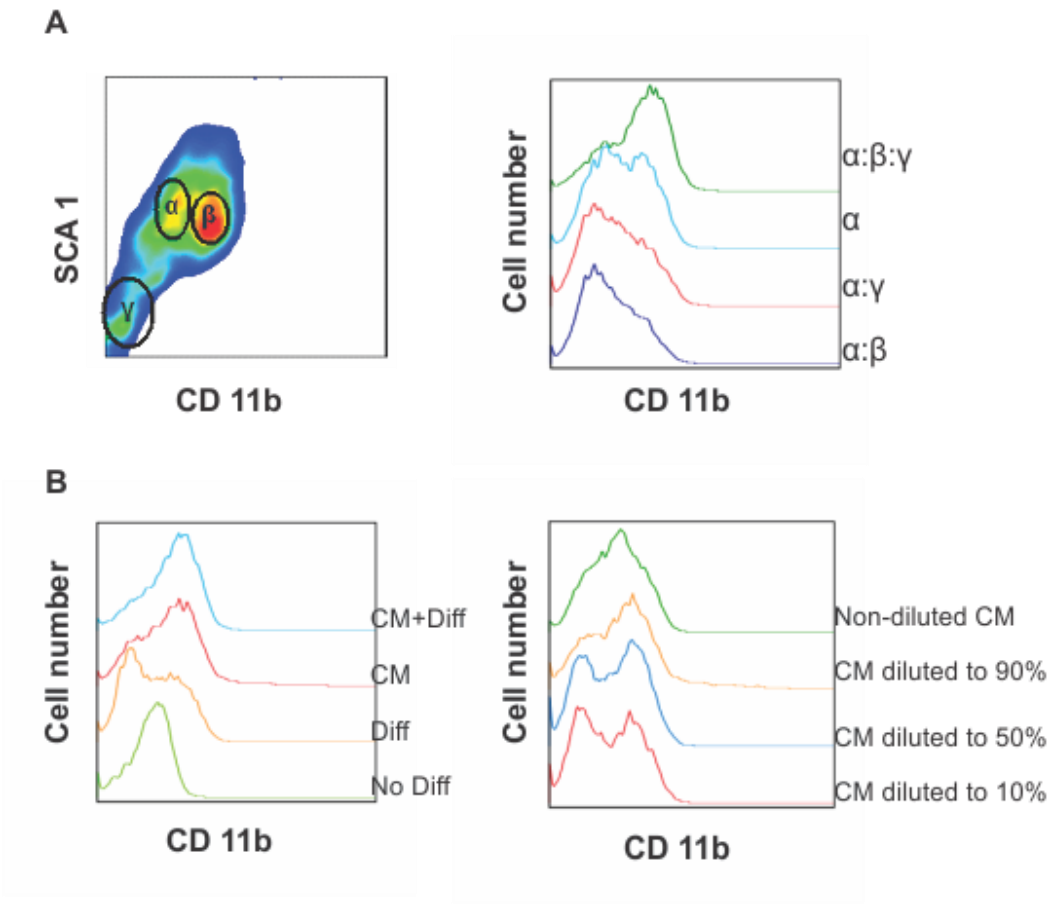

Fig 7. C**o-culture between the three subpopulations in Transwell reveals a role for cell-to-cell interactions on myeloid differentiation via soluble factors**. **A**. after 24 hour post-sorting and culturing CD11b expression for α subpopulation was modulated depending on which "partner" subpopulation is in the other chamber. **B.** EML cells where stimulated with conditioned medium collected from myeloid differentiated cells and CD11b expression was monitored after 48 hours of culture (left panel). Note that supernatant treated cells increased the rate of CD11b expression compared to the cells treated with the standard differentiation medium (ATRA, IL-3 and GM-CSF). This effect was dose-dependent (B, right panel).

To see if these distinct modulating activities of the three subpopulations was present in the initial smooth bell-shape distribution (micro-heterogeneity), cells from the population fractions with the highest, mid and lowest 15% SCA1 expression level (denoted henceforth as Sca-1low, Sca-1mid and Sca-1high fractions) were isolated from a clonal population by FACS and co-cultured in Transwell cultures in the pairwise combinations shown in Fig 7A. The SCA1-mid fraction was able to repopulate all subpopulations. However, when these SCA1-mid fraction

cells were co-cultured with cells from the SCA1-high fraction, differentiation was significantly delayed compared to the control and co-culture with the other fractions [Fig 8]. This may suggest that, during myeloid differentiation, the SCA1high fraction in basal (unstimulated) populations behaves similarly to the β subpopulation during myeloid differentiation. In fact as this lab has previously shown, SCA1-high cells in un-stimulated cultures are most primed to commit to the myeloid lineage corresponding to the fact that in the case of induced myeloid differentiation, the β subpopulation is most advanced in terms of differentiation along the myeloid (CD11b) axis.

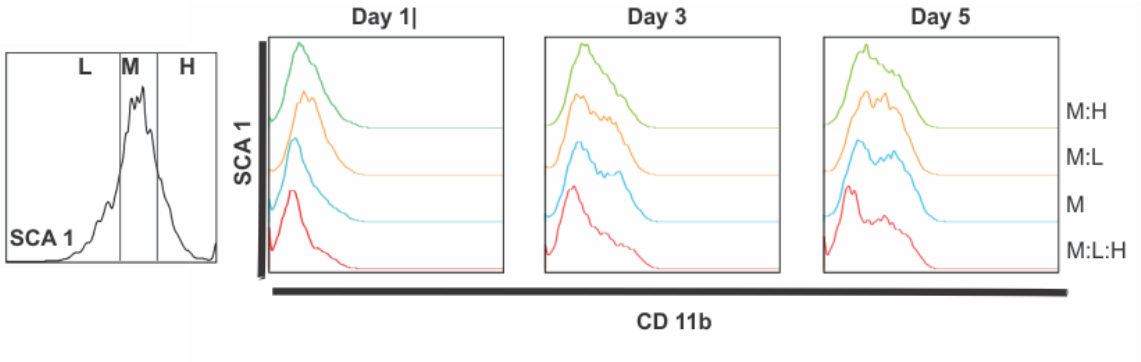

Fig 9. CD11b expression is delayed when subfractions of the initial mono-modal SCA-1 bell-shape distribution are sorted in the three fractions (high, mid, low Sca1 – see Fig 3) and allowed to interact. Shown is the CD11b expression on cells of the SCA-1 Mid (M) fraction when they are co-cultured with the other two fractions (SCA-1 Low (L) and High (H)). A significant reduction in the expression of CD11b was observed when cells of the SCA-1 Mid fraction was cultured with cells from the SCA-1 High fraction.

To confirm that the observed interactions affecting the differentiation kinetics is mediated by soluble factors, we next, instead of using the Transwell culture system, simply examined conditioned media: we took conditioned medium of unsorted ("bulk") differentiated myeloid cells (day 7 after differentiation induction with standard protocol) and added this medium, produced by maximally myeloid differentiated cells, to EML cells. 48 hours after treatment of the EML cells with the conditioned medium from myeloid differentiated cells we observed an increase in the expression of CD11b [FIg.7]. This differentiation stimulation effect was

significantly greater than that conveyed by the regular differentiation medium alone which has been optimized for inducing myeloid differentiation (Fig 7B, left panel). The latter makes it unlikely that the additional boost was due to residual differentiation inducers in the conditioned medium. Also, the effect in differentiation was dose-dependent (Fig 7B, right panel), suggesting that soluble factors are playing a possible role on myeloid cells differentiation and maturation.

3.4 Myeloid differentiation is regulated by IFN gamma expression

Now that we have a robust system that exhibits a phenotype change (modulation of the rate of differentiation) due to transferable (soluble) factors, we set out to determine if there are differences in the production of soluble factors between the three different subpopulations α, β, γ [Fig. 3A]. We used the Luminex system (core facility service) to evaluate the presence of 32 different soluble factors known to be involved in differentiation and inflammation in the medium conditioned by the three sorted subpopulations (α, β and γ) 4 days after myeloid differentiation was induced on EML cells. (Luminex uses antibody coated barcoded beads to multiplex the immunodetection of multiple cytokines in solution)

We screened for 32 soluble factors, and found that 12 were differentially expressed by one of the three subpopulations with respect to the controls (cells without any differentiation treatment and "bulk" cells during 7 days of myeloid differentiation).

Most of the highly expressed soluble factors are involved in acute stages of the inflammatory process, involving activation of lymphocytes and early stages of infection [Table 1]. Of particular interest was the differential expression of IFN γ in the α subpopulation. After reviewing literature and experimental evidence we choose to focus on this soluble factor as possible candidates that regulate myeloid differentiation and maturation. Therefore, EML cells were stimulated with IFN γ for 7 days and again SCA 1 and CD11b surface expression was

measured to see if these factors are able to promote myeloid differentiation in presence of atRA (as explained in chapter 1, atRA is used to reverse the "biological lock" imposed to EML cells due the overexpression of the retinoid receptor). IFN γ increased SCA1 expression, as measured by flow cytometry but no basal induction of myeloid differentiation was observed by the expression of CD11b, indicating that it may prime the cells (given that intermediate high SCA1 expression correlates with myeloid priming in these EML cells, see section 3.2.) and that continued myeloid differentiation requires the presence of additional factors. But surprisingly, when myeloid differentiation of EML cells was induced using the standard protocol (IL-3, GM-CSF and atRA, see Chapter 2) but in the presence of IFN γ, cells were developed in myeloid cells at faster rates than EML cells stimulated only with the standard protocol [Fig 9].

Intriguingly, the γ subpopulation which seems to be a state that manifests some temporary "detour" (in the CD11b/SCA1 plane) during differentiation was significantly reduced to almost no appearance [Fig 9 B]. This is consistent with the differentiation-promoting effect of IFNγ: by preventing cells to visit a temporary (aberrant) state along the trajectory, that is manifest as the g subpopulation, IFNγ may accelerate differentiation but it can by itself not sustain the entire fate commitment as does the standard protocol. To ensure that the observed activity is IFNγ specific we also inhibited this activity by adding a neutralizing anti-IFNγ mAb, and found that IFNγ was also able to increase SCA1 expression which, as we have learned in the lab, marks the priming towards the myeloid fate as opposed to the erythroid fate although it is ultimately down-regulated in the terminally differentiated myeloid cells. TNF α was also evaluated but no difference was observed respecting to controls.

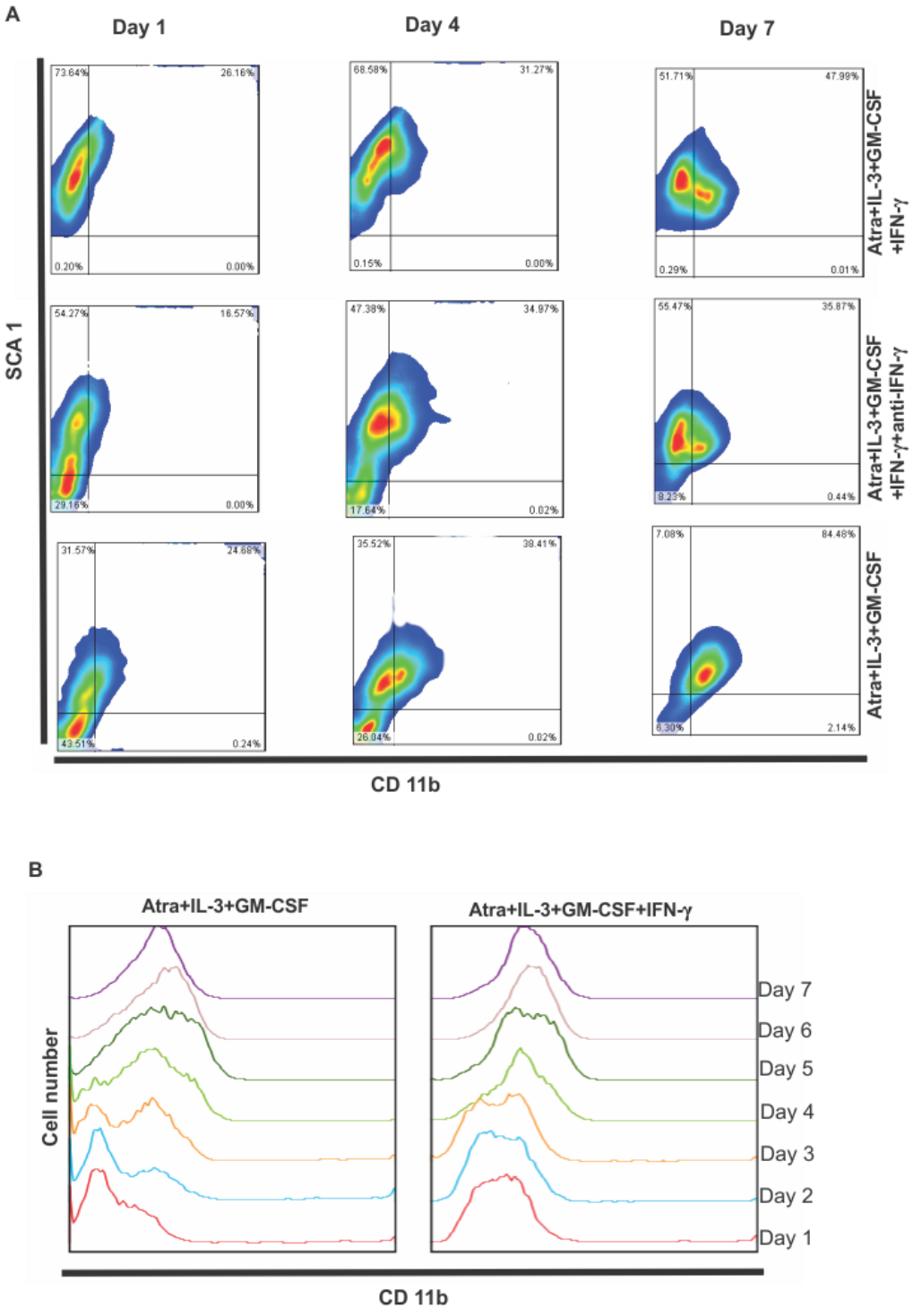

Fig 10. **IFN γ promotes SCA1 and CD 11b expression during myeloid differentiation**. **A.** IFN γ was added after myeloid differentiation was induced and after 2 days was able to induce higher expression of CD 11b+ subpopulation (**B**). IFN γ visibly limited the appearance of the γ subpopulation when compared to the standard differentiation protocol. Both of these phenomena were neutralized by an anti-IFNγ mAb.

Soluble factor	Biological role in hematopoietic cells differentiation	A	β	γ	Reference
IFN-gamma	*Modulates self-renewal of repopulating human hemopoietic stem cells*	•			[64]
IL-5	*Granulocites differentiation and activation during infection and inflammation.*	•			[65]
IL-9	*Lymphocyte differentiation, maturation and proliferation.*	•			[66, 67]
IL-12	*Lymphocyte differentiation, maturation and proliferation.*	•	•		[68]
IL-13	*Allergic inflammation mediated by Th2 immune response.*	•			[69]
IL-15	*NK cells differentiation.*	•			[70]
IL-17	*Increases granulopoiesis by stimulating SCF binding.*	•	•		[71]
KC	*Neutrophil stimulation during inflammation.*	•	•		[72]
MCP-1	*Monocites chemo attractant*	•			[73]
MIP-1alpha	*Granulocytes pro-inflammatory inductor.*	•			[74]
MIP-1beta	*Granulocytes pro-inflammatory inductor.*	•			[74]
TNF-alpha	*NK cells differentiation and maturation*	•			[12]

Table 1. Luminex array of cytokines and growth factors for conditioned medium. Over 12 different cytokines where differentially expressed by the three different subpopulations respecting to the differentiation medium and regular medium. Many of them have been involved in bone marrow hamotopoiesis and myeloid. The dot indicates detection of the respective cytokine in the indcated subpopulation above background level.

CHAPTER 4: DISCUSSION AND CONCLUDING REMARKS

4.1 Cell population heterogeneity within one lineage is dynamic and affects differentiation differentiation rate.

HSC differentiation has been extensively studied previously [38] and the influence of soluble factors in cell fate decisions in general is well recognized. Nonetheless, the role of cell population heterogeneity during cell differentiation has not been explored with respect to the secreted factors. That differentiating cells secrete factors that influence fate decision is well known, for instance the case of Th1/Th2 differentiation is a well-known paradigm. These two sister lineages secrete distinct sets of cytokines that suppress the other lineage, thus strengthening the bifurcation into two discrete lineages. But here obviously distinct cell types are the source of the distinct cell-fate controlling factors. By contrast, in this project we show that in an apparently uniform cell population of only one type when stimulated to differentiate first distinct states within the same lineage arise, and then they engage in cell-cell interaction that modulates the rate (rather than destination) of differentiation.

Specifically, we show that in EML cells myeloid differentiation *rate* is highly influenced by cell population heterogeneity, revealing the existence of meta-stable states during the differentiation process and the importance of those for the normal developmental process [Fig 4]. Four days after myeloid differentiation was induced, three different subpopulations (respecting to their CD11b and SCA1 expression) appeared and developed from a single ("smooth") Gaussian distribution SCA1. After seven days, most of the cells were CD11b positive and revealed signs of myeloid differentiation (by this time SCA1 expression decreased and Wright-Giemsa showed cytoplasm granules and polymorphonuclear cells). The existence of multiple subpopulations

during intermediate stages of differentiation is most likely a joint manifestation of multi-stability of the underlying GRN and of stochastic fluctuations of gene expression that produces the micro-heterogeneity (broadening of the peaks in the histogram) interaction between cells.

4.2. Cell population heterogeneity allows "priming" of a subset of cells

Microheterogeneity in clonal cell populations is due to cell-to-cell differences with respect to all the genes across the genome, i.e. to transcriptome fluctuations which, nonetheless, are constrained by the attractor state, which in turn is due to the specific GRN architecture and will guarantee stable phenotypic identity. Thus, a clonal cell population represents more or less a "cloud" of points in state space – a swarm of slightly different cells that on average preserve the same cellular identity. But, at the same time, this (micro)heterogeneity, that is, the cell-cell variations within one cell population of one nominal type, permits the generation of (temporarily) "primed cells" – cells that are near the border of an attractor and hence, most ready to exit the attractor. These are the cells that respond first when the population is exposed to differentiation stimuli. We think that this initial heterogeneity with respect to responsiveness to differentiation cues accounts for the augmented heterogeneity, up to the point that the population splits into the discretely distinct three subpopulations (α, β and γ).

4.3. Identity of the subpopulations generated during the differentiation process

In collaboration with Mitra Mohtahedi in the lab, we sought to determine the identity of each sub-population (α, β and γ). We expected that they are not only distinct from each other with respect to surface expression of CD11b and SCA1 (the defining markers) but also to the expression of other genes. In fact, qPCR measurements showed that mRNA expression of the

transcription factor PU.1 (which promotes myeloid differentiation – see Chapter 1) was higher for the β subpopulation (CD11b+/SCA1-) and that the TF, GATA1 (which promote the opposite lineage, the erythroids) was significantly higher expressed in γ (CD11b-/SCA1-) compared to α. Both subpopulations are cells on the way towards differentiation: the (β) nearing the myeloid commitment (as intended by the stimulation), whereas the (γ) subpopulation appears to represent cells that temporarily move towards the erythroid fate [75].

The GATA1-PU.1 gene circuit has been shown to play a central role in the regulation of HSC fate decisions, including in the commitment and differentiation to either the myeloid (GATA1-/PU,1+) of the erythroid (GATA1+/PU.1-) lineages. Thus, we can conclude that, even if myeloid differentiation was the desired fate to be induced by IL3/GM-CSF, one encounters, at least temporarily, erythroid-like cells in the γ subpopulation (as suggested by their high GATA1 expression). This could be due to the existence of cells primed in the opposite direction of this binary decision, namely, cells with a higher probability to develop into the fate opposite from the intended one. To test for the existence of primed cells we FACS sorted the untreated cell population with respect to the basal SCA1 distribution (into SCA1-low, SCA1-mid and SCA1-high as explained in Fig 7) and induced myeloid differentiation. After 7 days of differentiation (which is as complete as one can do in this in vitro system) we observed that each fraction was primed to reproduce some of the three subpopulations described at day 4 of differentiation (α, β and γ); SCA1 low populated most of the γ subpopulation, SCA1 high did the same but for β and SCA1 mid was able to populate all of the three sub-populations respectively [Fig 6]. Thus, these intermediate subpopulations are latently preexisting states that are transiently occupied – and thus, is consistent with the formal framework of the epigenetic landscape: A differentiation cue may perturb the progenitor cell population into neighboring attractors many of which however

are unstable states – such that ultimately, the cloud of cells on the epigenetic landscape 'flows" down the predestined trajectories ("valleys") towards stable attractor states representing mature state.

4.4. Identity of the soluble mediator of the cell-cell interactions

Cell-to-cell variability within clonal population of nominally identical cells (which also appear to be phenotypically uniform) has been a subject of research for more than half of the century [44, 61, 62]. More recently, such heterogeneity has been interpreted as gene expression noise and understood as an essential factor for embryonic development [47] notably in immune cells maturation and vascular development [reviewed in 47, 49]. However, most of the previous work in cell-cell communication focuses on heterotypic interactions between nominally and phenotypically distinct cell lines communicating with each other by physical interactions or through soluble factors. However, due to the assumption of homogeneous clonal cell populations, few studies have been conducted to examine the putatively interactions which would represent homotypic interactions between clonally identical cells. The intriguing feature in our case is that the heterogeneity of cell types required for heterotypic interaction arises dynamically from within and as transient stages during the differentiation process.

To evaluate the role of the transient multistability during cell development we sorted α, β and γ four days after myeloid differentiation was induced, and using transwell cultures we limited their interactions to that which can be mediated by soluble factors. 48 hours after FACS sorting and transwell culture we found that CD11b expression for the α subpopulation (~ close to the starting population) was being modulated by the influence of the other three subpopulations [Fig 7] as follows: if α was co-cultured with either β or γ, then the expression of the differentiation marker CD11b 48 hours after decreased compared to the regular differentiation

program (when all the three subpopulations are present and interacting). This result indicates that population heterogeneity is an important factor for proper rate of differentiation and highlights the role of cell-to-cell interactions between transiently created subpopulations within one originally more homogeneous population.

To determine the identity of the factors that mediates the interaction we performed a Luminex screen in the conditioned medium collected from culture of the three subpopulations separately. Among the 32 soluble factors tested, cytokines ranging from inflammatory to differentiation factors were detected. We found that among them 12 cytokines were differentially expressed in one of the subpopulations. Most of them were highly expressed in α subpopulation (Table 1). As explained in Table 1, the majority of the produced factors are involved in granulocyte maturation and activation but, IFNγ, which has been related to type 2 inflammatory process and lymphocyte acute response, has not been reported as an agent that modulates myeloid differentiation. We observed that IFNγ accelerated the rate of appearance of CD11b+ cells and increased SCA1 expression in the early stage, while at the same time limited the transient appearance of the γ subpopulation.

4.5. A new role of soluble mediator: canalization of the differentiation process

Thus, while IFNγ alone is not an instructive cytokine that can induce the progenitor cell's fate decision and begin of the differentiation, it streamlines the trajectory through the rugged epigenetic landscape by smoothening the path (=Waddington's chreods [51]) towards the destination attractor while preventing the veering off into other fates that are part of the fate-repertoire of a multipotent progenitor cell. In yet other words borrowing from Waddington's

imagery, we can say that IFNγ production by the α subpopulation (once differentiation is induced by the "standard cytokines") helps to *canalize* myeloid cell development, thus ultimately improves efficiency and accuracy of lineage commitment and differentiation. In a broader perspective, the ruggedness of the epigenetic landscape that Waddington proposed as a metaphoric conceptual aid [51], and that we now understand as a mathematical construction of the quasi-potential landscape of the gene regulatory network [50] poses a challenge for development in that cells can easily get lost in side valleys as they descend. Such cells get trapped in abnormal attractors and it has been proposed that this is the fundamental origin of cancer: This model would nicely explain why cancer start from "cancer stem cells", that is, cells that appear to be "blocked" in their differentiation [76]. Perhaps it is no coincidence that interferons were among the first therapies of cancer that achieved cure of cancer. They act not by killing cancer cells but by inducing differentiation [77]. Unfortunately the whole picture is more complex since interferons are effective only in a small subset of tumors.

4.2 Concluding remarks

Differentiation has been thought of as a linear process where a stem cell population differentiates to a given phenotype going through different metastable states of maturity. Our results gives further credence to the existing idea that myeloid differentiation is a "non-linear" process where multistability arises due to the non-linear interactions of the underlying gene regulatory network that governs this process. The multistability emerges only after the induction of differentiation and accounts for the additional "heterogenization", namely, in our case the splitting of the progenitor cell population in to the three subpopulations. This leads to a multi-step nature of differentiation whose rate of progress is strongly influenced by differential production of specific soluble factors. This means that cell-to-cell communication during differentiation plays a major

role in heterogeneous clonal cell populations, where the interplay of differences between the cells creates complex microenvironments.

Clonal cell population heterogeneity has been shown previously to be a general phenomenon that is pronounced in stem cell populations, but its implications are not clear. Here we show one biological function of heterogeneity and that a particular form, manifesting the multi stability during differentiation is not only an epiphenomenon but also is a key element with regulatory function in cell development of myeloid cells. This implies that robustness during development is not only a property of cell intrinsic phenotype change but also extrinsic, namely contributed by the heterogeneity of cell populations.

It's reasonable to say that cell development doesn't simply represents a gene directed program in which genes switch on or off in a linear cascade the genes. Instead we think that while the cell-intrinsic genetic makeup influences differentiation, cell-to-cell communication is also an important machinery that keeps a robust developmental process going. This implies that the non-genetic cell heterogeneity that underlies such (heterotypic) cell-cell communication is a critical phenomenon for cell differentiation.

More specifically, our results offer additional evidence supporting the idea that clonal cell heterogeneity generates diversity of phenotypes in a heterotypical manner despite starting form one cell type, hence increases the combinatorial complexity which represents a considerable source of biological creativity.

This thesis shows a novel principle of regulation of cell development based on a well-characterized system, EML cell differentiation into the myeloid lineage. Although only

marginally discussed, the overall framework benefitted enormously from the theories of complex dynamical systems.

Coming back to the questions that motivated the project (Chapter 1), I can summarize the answers that this series of experiments provides: (1) I confirmed the existence of intermediate states during differentiation that increases population heterogeneity compared to that of the starting cell population, (2) I established the role of cell-cell interactions and (3) have begun to characterize the specific mediators.

However, this work also opens many new questions which I hope to address in future work: For instance, one will immediately ask: how generalizable are our findings? I hypothesize that similar principles apply to other differentiation processes since our theoretical framework contains only elementary "first principles" of dynamical systems theory and nothing that is specific to EML. The gene regulatory network imposes so many dynamical constraints such that it produces very rugged epigenetic landscape with multiple local attractors (multistability) and thus differentiation which entails the change of expression of thousands of genes across the genome would be an inherently inefficient process. Cell-cell interactions may have evolved to help canalize cells through this rugged landscape to efficiently guide cells to the attractors of the mature cell types.

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