

The monocyte system in haematological malignancies

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Scientific environment

This thesis was initiated in January 2016. The work was conducted at the Department of Safety, Chemistry, and Biomedical laboratory sciences, Faculty of Engineering and Science, Western Norway University of Applied Sciences, and at the Leukemia Research Group at the Department of Clinical Science, Faculty of Medicine, University of Bergen, Bergen, Norway.

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Abstract

Monocytes consist of classical, intermediate, and non-classical monocytes. In haematological malignancies, such as multiple myeloma and acute myeloid leukaemia, monocytes are affected by both the disease itself and the treatment patients receive. The overall aim of this thesis was to explore monocyte subpopulations in clinical settings of haematological malignancies by (i) elucidating the important pre-analytical factors to properly identify the subsets, (ii) monitoring monocyte regeneration after stem cell transplantation in patients with haematological malignancies, and (iii) investigating the effects of immunomodulatory drugs on metabolism and cytokine secretion.

We demonstrated that K₂EDTA, ACD-A, and Li-Heparin blood sampling tubes perform similarly regarding the distribution of monocyte subpopulations. In contrast, both the monocyte concentration and relative values could be significantly affected by choice of blood sampling tubes and decreased sample volume. Our results demonstrate that monocytes regenerate very early after stem cell transplantation, before the normalisation of other cell populations. This is true for multiple myeloma patients, as well for patients with other haematological malignancies, mainly acute myeloid leukaemia. The immunomodulatory drugs thalidomide, lenalidomide, and pomalidomide, which are therapeutically important in haematological malignancies, altered monocyte metabolism, especially when cells were cultured with LPS. Lenalidomide had a stronger effect on monocyte metabolism than the other two drugs. Furthermore, all three drugs decreased TLR4-induced mediator release, with the strongest effect for pomalidomide, whereas lenalidomide, and especially, thalidomide, had weaker effects.

In conclusion, carefully standardizing blood sampling procedures is important to further develop monocyte analysis as a clinical tool, and the associations between monocyte subset variations and various diseases suggest additional studies should be conducted to clarify whether the analysis of monocyte subsets should be incorporated into routine clinical handling. IMiDs can alter monocyte metabolism and communication, but the strength of these effects differs between the IMiDs.

List of publications

Article I: Standardisation of sampling and sample preparation for the analysis of human monocyte subsets in peripheral blood

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Article II: Circulating monocyte subsets in multiple myeloma patients receiving autologous stem cell transplantation – A study of the preconditioning status and the course until posttransplant reconstitution for a consecutive group of patients

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Article III: A pilot study of circulating monocyte subsets in patients treated with stem cell transplantation for high-risk haematological malignancies

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Article IV: Effects of immunomodulatory IMiDs drugs on normal monocytes: a comparison of thalidomide's, lenalidomide's, and pomalidomide's effects on monocyte metabolism and cytokine response to toll-like receptor 4 ligation

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Abbreviations

ADP	Adenosine diphosphate
AML	Acute myeloid leukemia
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
APC	Antigen presenting cells
ATP	Adenosine triphosphate
CD	Cluster of differentiation (e.g., CD14)
CCR	C-C chemokine receptor (e.g., CCR1)
CCL	C-C motif chemokine ligand (e.g., CCL2)
CLL	Chronic lymphocytic leukemia
CLR	C-type lectin receptors
CRAB	Calcium renal failure anemia bone lesions
CSR	Class switch recombination
CXCL	C-X-C motif chemokine ligand (e.g., CXCL 10)
CXCR	C-X-C chemokine receptor (e.g., CXCR 3)
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DNA	Deoxyribonucleic acid
HSP	Heat shock protein
Ig	Immunoglobulin
IL	Interleukin (e.g., IL-6)
ILC	Innate lymphoid cells
IMiD	Immunomodulatory drugs
iNKT	Invariant natural killer T cells
LPS	Lipopolysaccharide
MAIT	Mucosal associated T cell
MDSC	Myeloid derived suppressive cell
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MDS	Myeloid dysplastic syndrome
miR	MicroRNA
M-SCF	Macrophage-Stem cell factor
NETS	Neutrophil extracellular traps
NK	Natural killer cell

NLR	Nucleotide-binding oligomerisation domain leucine-rich-repeat containing receptors
PAMP	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptor
ROS	Reactive oxygen species
STAT 3	Signal transducer and activator of transcription 3
TCR	T-cell receptor
Tfh Cell	T follicular helper (Tfh) cell
TNF	Tumor necrosis factor
TLR	Toll-like receptor

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1. INTRODUCTION

The theoretical background of this thesis is focused on cell-mediated immunity, especially on monocytes, and two haematological malignancies, acute myeloid leukaemia (AML) and multiple myeloma (MM).

1.1. THE IMMUNE SYSTEM - A HOST DEFENSE SYSTEM

The immune system is a complex and interactive network of surface barriers/organs, immunoregulatory soluble mediators (e.g., enzymes, histamine, antibodies, cytokines, and soluble adhesion molecules) and immune cells [1]. Additionally, the immune system is a part of fundamental physiological processes, including development, reproduction, and wound healing, and it is important in regulating metabolism and functions of the central nervous system and cardiovascular system [1-6].

The immune system can be divided into innate immunity and adaptive immunity [1, 6], but there are multiple interactions between these two systems. The innate immune system is crucial for the early detection of foreign invaders and is the first line of defence; it also alerts and shapes the adaptive immune system, which involves the antigen-specific reactivity of B and T lymphocytes [6].

1.1.1. The innate immune system

The innate immune system can be divided into surface barriers, immunological soluble mediators, and cellular components. The plasma components include the complement system [7], acute phase proteins (e.g., C-reactive protein, CRP) [8, 9], and cytokines [10], including chemokines [11, 12]. The cellular innate immune system [13-15] consists of leukocytes, such as polymorph-nucleated granulocytes (e.g., neutrophils, basophils, and eosinophils), mast cells, monocytes, macrophages, dendritic cells, natural killer (NK) cells, and NK T-cells (NKT). The cells of the innate immune system express germ-line-encoded receptors called pattern recognition receptors (PRRs) that bind specifically to highly conserved pathogen-associated molecular patterns (PAMPs) only present on microorganisms. PAMPs are typical constituents of microbial cell-wall components, nucleic acids or metabolic products. PRR-PAMP ligations activate the

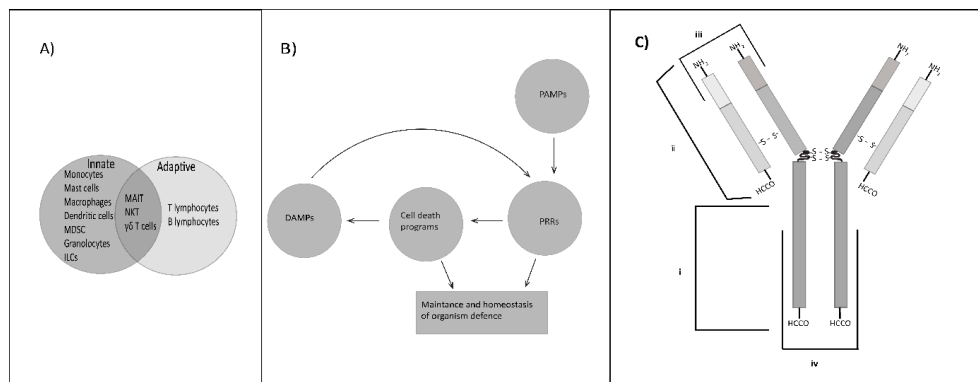


Figure 1. Illustration of the immune system's essential components. **A)** Immunocompetent cells (adapted from [16]). **B)** Pattern recognition receptors (PRR), which are key players in maintaining homeostasis and organism defence (adapted from [17]). **C)** The general immunoglobulin structure. Each immunoglobulin molecule consists of two heavy (i) and two light (ii) chains. Both types of chains contribute to the hyper-variable antigen-binding region (iii), and the fragment antigen-binding (Fab) component includes both these hypervariable regions. The two heavy chains also form a constant part, referred to as the constant or complement-binding Fc region (iv) (adapted from [18]). Abbreviations: **DAMPs**, damage-associated molecular patterns; **PAMPs**, pathogen-associated molecular patterns; **PRRs**, pattern recognition receptor; **ILCs**, innate lymphoid cells; **MAIT**, mucosal-associated T-cell; **MDSC**, myeloid-derived suppressor cells; **NKT**, natural-killer T-cells.

innate cell. Endogenous danger molecules, called damage-associated molecular patterns (DAMPs), are released or expressed by stressed, damaged, or dying cells, such as uric acid, mitochondrial DNA, extracellular ATP, heat-shock proteins (HSPs), amyloid β , and S100 molecules, for example, S100A8, also known as myeloid-related protein-8 or MRP-8, and S100A9, also known as MRP14, as well as serum amyloid A (SAA), high-mobility group box 1 protein (HMGB1), and extracellular matrix proteins [19, 20]. DAMPs can activate the innate immune system by interacting with PRRs (see Figure 1b). An overview of the four PRR classes' important characteristics and the corresponding PAMPs and DAMPs are presented in Table 1. The PRRs-PAMPs/DAMPs cause innate cell activation by initiating signalling through various downstream pathways [17, 21, 22]. For example, membrane-bound TLR4 is one out of the ten TLRs (TLR1–TLR10) identified in humans and can bind to, and thereby become activated by, for example, lipopolysaccharide (LPS), a PAMP expressed by gram-negative bacteria [21]. Endogenous DAMPs, such as high-mobility group box-1 protein (HMGB1), heat-shock proteins (HSPs), histones, and extracellular matrix components (e.g., hyaluronic acid and biglycan), can act as TLR4 agonists [21].

Table 1. The four classes of pattern-recognition receptors (PRRs) and their corresponding PAMPs and DAMPs. Only some examples within each class are shown (for a comprehensive overview, see reference [17]).

PRR class	Example PRRs	Corresponding PAMPs
Toll-like receptors (TLRs)	TLR2-2	Peptidoglycan (bacteria)
	TLR4	Lipoarabinomannan (mycobacteria) Lipopolysaccharide (LPS) (gram-negative bacteria) Fungal mannans (Candida species)
	TLR5	Envelope proteins (respiratory virus)
	TLR8	Flagellin (flagellated bacteria) ssRNA viruses (herpes simplex virus)
C-type lectin receptors CLRs	Mannose receptor	Fungal mannans (e.g., Candida species)
	Mannose-binding lectin	Repetitive oligosaccharides (bacteria and fungi)
NLRs	NOD1	Muramyl tripeptide peptidoglycans (gram-negative bacteria)
	NOD2	Muramyl dipeptide peptidoglycans (gram-positive bacteria)
RIG-I helicase receptors	RIG-I	Short double-stranded dsRNA (e.g., paramyxoviruses)
	MDA5	Long double-stranded dsRNA (e.g., flaviviruses)

Abbreviations; **NLRs**, nucleotide-binding oligomerisation domain (NOD) leucine-rich repeat-containing receptors; **RIG-I helicase receptors**, retinoic acid-inducible gene I protein helicase receptors; **MAD5**, melanoma differentiation-associated protein 5.

TLR4 initiates downstream signalling through the adaptor proteins myeloid differentiation primary-response 88 (MyD88) and TIR-domain-containing adaptor-inducing IFN β (TRIF) [21]. Both MyD88 and TRIF recruit and activate mitogen-activated protein kinases (MAPKs) and I κ B kinase (IKK), leading to the activation of the transcription factor activator protein-1 (AP-1) and nuclear factor κ B (NF κ B), respectively, with the subsequent expression of proinflammatory cytokines [21]. TRIF also recruits and activates another cellular kinase, TNF receptor-associated factor (TRAF) family member-associated (TANK)-binding kinase 1 (TBK1), leading to the synthesis of type I interferon (IFN-I) by activating transcription factor IFN regulatory factor 3 (IRF3) [21]. The co-receptor CD14 increases LPS responsiveness by binding LPS and promoting LPS transfer to TLR4 [12]. CD14 is either membrane-bound or soluble (sCD14) [23]; it can bind several PAMPs and DAMPs (e.g., LPS, peptidoglycan, polyinosinic-polycytidylic acid, and DNA) and transfer them to the correct TLR or non-TLR (e.g., the purinergic P2X7 receptor for ATP) [23].

Inflammasomes are multimolecular complexes and, when activated, trigger the caspase-1-dependent proteolytic activation of the inflammatory cytokines IL-1 β and IL-18. The formation of inflammasomes is initiated a plethora of signals associated with pathogens,

sterile inflammation, and metabolic dysfunction and result in the activation of caspase-1. This leads to the proteolytic activation of IL-1 β and IL-18. IL-1 β signals through the receptor IL-1R. Examples of inflammasomes are NLR3 inflammasome [24, 25]. In summary, PAMPs/DAMPs' ligation to PRRs causes the activation of innate immune cells, illustrated by the TLR4 example. A brief overview of the innate cells and some of their important effector functions are presented in Table 2.

Table 2. A brief overview of the effector mechanisms for a selected subset of innate immunocompetent cells.

Cell Family Subsets/types	Effector functions of innate cells	Ref.
Neutrophils N1 N2 NBH	<ul style="list-style-type: none"> • Phagocytosis • Degranulation, cytokine production/release • Neutrophil extracellular traps (NETs/NETosis) • 'Amateur' antigen-presenting Cells? B-cell-helper properties? 	[26-29]
Eosinophils	<ul style="list-style-type: none"> • Degranulation, cytokine production/release • 'Amateur' antigen-presenting cells 	[29, 30]
Basophils	<ul style="list-style-type: none"> • Degranulation (i.e., IgE) • 'Amateur' Antigen-Presenting Cells 	[29, 31]
Mast cells	<ul style="list-style-type: none"> • Release of cytokines and mediators that contribute to increased vascular permeability and chemoattraction of innate immune cells • Migration and cytotoxic responses by T lymphocytes • Mast cell extracellular traps (MCETs) • APC, support an adequate ratio of activated-to-regulatory T-cells 	[32]
Monocytes Classical Intermediate Non-classical	<ul style="list-style-type: none"> • Phagocytosis • Antigen-presenting cells • Cytokine production/release • Tissue repair • Secretion of NETs 	[33-36]
MDSC PMN-MDSC M-MDSC E-MDSC	<ul style="list-style-type: none"> • Production of immunosuppressive mediators such as indoleamine 2,3-dioxygenase (IDO), IL-10, IL-4receptor, Arg-1 and prostaglandin E2 	[37, 38]
Macrophages M1 M2	<ul style="list-style-type: none"> • Phagocytosis • Professional antigen-presenting cells (APCs) • Cytokine production/release • ETosis 	[35, 39]
Dendritic cells pDCs cDCs mDCs	<ul style="list-style-type: none"> • Endocytosis • Production of cytokines and inflammatory mediators • Professional antigen-presenting cells (APCs) 	[40, 41]
ILCs family NK cells LTi ILCs1-3	<ul style="list-style-type: none"> • Secretory lysosome exocytosis (e.g., perforin) • Cytotoxic activity • Antibody-dependent cellular cytotoxicity (ADCC) • Cytokines 	[29, 42, 43]

Abbreviations: MDSC, myeloid-derived suppressor cells; PMN-MDSC: polymorphonuclear MDSC; M-MDSC: monocytic MDSC; E-MDSC: early-stage MDSC. pDCs, plasmacytoid DCs; cDCs, conventional DCs, mDCs: DCs derived from monocytes. ILCs, innate lymphoid cells; LTi, lymphoid tissue-inducer cells; NBH, B-cell-helper neutrophils.

Notably, PRR expression (e.g., TLRs) is not limited to innate immune cells; they also seem to be expressed by cells of the adaptive branch of the immune system [44, 45] and

can be activated by endogenous or natural ligands, as will be discussed later in this thesis.

The effector functions of innate cells include (i) receptor-mediated endocytosis, phagocytosis, macro-pinocytosis for the clearance of microbes, molecular degradation, and the presentation of antigenic peptides in a complex with MHC (the latter are described in more detail in Section 1.1.2); (ii) degranulation and the extracellular release of cellular contents to eliminate the pathogen (e.g., anti-microbial mediators); (iii) the secretion of ‘nets’ interlaced with anti-microbial mediators to capture and prevent microbial spread (NETosis, ETosis); (iv) the lysosomal exocytosis of, for example, perforin; and finally, (v) cytokine release (e.g., IL-6) to induce acute-phase proteins, such as C-reactive protein, the stimulation of antibody production, as well as effector T-cell development [1, 6, 28, 31, 32, 46].

1.1.2. The adaptive immune system: T-cells and B-cells

The inflammatory environment induced by innate immune responses will stimulate cells of the adaptive branch of the immune response (B- and T-cells) to proliferate and differentiate into cells with a range of functions appropriate for the existing challenge. B- and T-cells are derived from multipotent hematopoietic stem cells in bone marrow. B-cells differentiate and mature within the bone marrow, while T-cell precursors localise to the thymus for the final stages of their maturation. Both these adaptive lymphocytes display antigen receptors that result from the recombination, random insertion, deletion, and substitution of germline-encoded gene segments, thereby securing the diversity of antigen receptors [47]. The repertoire of expressed antigen receptors allows B- and T-cells to recognise foreign antigens. This self–nonself discrimination is a key feature of adaptive immunity; both B- and T-cells undergo tightly controlled and regulated stages during their maturation to secure central tolerance, and these steps include clonal deletion/selection, receptor editing, and genetic recombination (for a more detailed description, see [48-50]).

A typical feature of adaptive immune responses is that the effector response occurs later than the innate response due to the need for antigen-specific activation, leading to cell proliferation and differentiation before the effector response can occur [6]. Furthermore,

adaptive immunity involves the ability to remember previous infections, as both T- and B-cells produce long-term memory cells capable of responding faster if re-infection with the same agent occurs [51, 52]. However, the dogma that immunological memory is exclusive to the adaptive immune system has been challenged by evidence that innate immunity may also involve retaining memories of previous events (i.e., trained immunity) [53].

Products of the major histocompatibility complex (MHC) gene bridge the innate and acquired immune response: There are two primary classes of major histocompatibility complex (MHC) molecules: MHC classes I and II. The main task of professional antigen-presenting cells (i.e., dendritic cells [DCs], macrophages, B-cells and thymic epithelial cells) is detecting and presenting peptides (i.e., short protein fragments resulting from their intracellular degradation). Molecules encoded by the MHC gene binds and present self and foreign peptides at the cell surface for recognition by specific antigen receptors on T lymphocytes [54].

When receptors recognise pathogens by, for example, TLRs, APCs undergo maturation, in which global rearrangements of the endolysosomal system take place [55]. Following internalisation by the endocytic pathway, microbial proteins/constituents are transported to late endosomal compartments for processing into short peptides, loaded onto major histocompatibility complex class II (MHC-II) molecules, and subsequently exported to the plasma membrane. Cell surface peptide-MHC-II complexes (p-MHC-II) can activate CD4 T-cells and stimulate their differentiation into T-cell subsets (e.g., Th1 and Th2), which are essential for the proper activation of cytotoxic T-cells and the differentiation of B-cells [55].

Following molecular internalisation, DCs may present exogenous peptides on MHC class I molecules (MHC-I) and thereby activate naïve CD8 T-cells. This process is called cross-presentation. Internalisation, cross-presentation, and subsequent cell surface peptide-MHC-I complexes (p-MHC-I) seem important for both the activation of antigen-specific cytotoxic T-lymphocytes and the induction of self-tolerance [56]. All nucleated human cells, as well as platelets (but not erythrocytes), express MHC class I molecules [57]. MHC-I can then present pathogen-derived peptides from intracellular

pathogens, thereby enabling cells to alert the immune system to the presence of an invader. Pathogen-specific cytotoxic T lymphocytes monitor cell surface MHC class I molecules for pathogen-derived peptides and the elimination of infected cells [57].

T lymphocytes express the clonal-specific T-cell antigen receptors (TCRs) that recognise antigens only on the surface of other cells in the context of MHC class I and class II molecules (see above). Circulating immunocompetent T-cells scan MHC molecules in search of peptides derived from altered self (e.g., carcinogenic) or foreign (e.g., bacterial) proteins. TCRs survey both the intra- and extracellular environment, and their recognition of and binding to the peptide-MHC-complex activates T-cells [58]. However, bringing together TCRs and the cognate peptide-MHC, often referred to as signal 1, is only the start of a successful T-cell activation and response. Additional, second costimulatory signals, such as CD28's binding of CD80/CD86, often referred to as signal 2, are also necessary to establish cell-cell contact (i.e., the immunological synapse), and another required signal is APC-derived cytokines, often referred to as signal 3 (e.g., IL12) [59].

The T-cell immune system includes several inter-related and interacting T-cell subsets [60]. T-cells can be divided into four main classes based on when they left the thymus as immunocompetent cells: (i) naïve cytotoxic T-cells (TCR- $\alpha\beta$, CD8+), (ii) naïve helper T-cells (TCR- $\alpha\beta$, CD4+), (iii) thymus-derived regulatory T-cells (TCR- $\alpha\beta$, CD4+, FoxP3+) [61, 62] and (iv) $\gamma\delta$ T-cells (TCR- $\gamma\delta$) [63, 64]. Naïve T-cells can differentiate after antigen recognition into various subsets [6, 59, 65] with a variety of effector functions. Table 3 gives a brief overview of suggested T-cell subsets, their proposed effector functions, and their proposed cytokine secretion profile. The cytokines produced by each lineage of mature effector T-cells reinforce their developmental program through positive and negative feedback, acting on both naïve T-cells and the innate immune system [65, 66].

As described above, during acute infection, naïve T-cells give rise to effector T-cells that subsequently clear the pathogens. With antigen clearance, many antigen-specific T-cells die, and only a pool of the remaining highly diverse memory T-cells retain the capacity to respond to another challenge [58, 67]. Substantial differences between

various infections that may depend on the duration of antigen exposure, tissue localisation, or distribution of infection have been described [58, 67]. Suggested memory cell subsets include effector memory T-cells (T_{EM}), central memory T-cells (T_{CM}), and resident memory T-cells (TRM), which differ primarily in their capacity for self-renewal, circulation through secondary lymphoid organs, or presence in tissue [58, 67]

When activated, T-cells upregulate several chemokine receptors and ligands that facilitate their own migration to tissues, as well as the migration of other cells to the same compartment, such as dendritic cells [68]. Interferons also induce the production of CXCL9 and CXCL10 by DCs and stromal cells, resulting in inducing T-cells to upregulate CXCR3s, which are highly expressed in many memory T-cells. This allows rapid migration, independent of antigens, toward infected cells in outer T-cell areas and into lymph nodes' subcapsular sinus in response to infection. Hence, memory cells are recruited more quickly to APC compartments than naïve T-cells after infection [68].

Our overview of T-cell functions is far from complete, but taken together, these observations illustrate that the T-cell population includes a large number of interacting T-cell subsets with many different effector functions and different roles in the regulation of inflammation and immunity. The various subsets mediate their regulatory functions through cytotoxicity, the release of pro- or anti-inflammatory soluble mediators with direct effects on the function/proliferation/differentiation/maturation of other immunocompetent cells, and through the modulation of T-cell (subset) trafficking.

Table 3. Overview of some important T-cell subsets and some of their important functions.

T-cell subset	MHC restriction	Third signal*	T-cell subsets	Important functional characteristics (effector function/cytokine release/immunoregulation)	Ref.
CD8 T-cells	p-MHC-I	IL-12	Tc1	<ul style="list-style-type: none"> Releases perforin and granzyme, inducing apoptosis of infected cells. Destroys virally-infected or malignant cells. Produces TNF-α, INF-γ, (TC1). Produces IL-4, IL-5, IL13 (TC2). 	[69, 70]
		IL4	Tc2		
CD4 T-cells	p-MHC-II	IFN γ IL12	Th1	<ul style="list-style-type: none"> Helps B- and Tc-cells. Activates macrophages. Produces IFN-γ, lymphotoxins, IL-2, and TNF-α. 	[65, 71]
		IL4	Th2	<ul style="list-style-type: none"> Stimulates eosinophils, basophils, and mast cells. Stimulates B-cells to produce IgE and IgA. Releases IL-4, IL-5 and IL-13, TNF-α, IL-9, and IL-2. 	[65, 72]
		IL1 IL6 TGF- β IL-4 TGF- β	Th17	<ul style="list-style-type: none"> A proinflammatory T-cell subset. Produces IL-17A, IL-17F, IL-21, and IL-22. 	[73]
			Th9	<ul style="list-style-type: none"> Supports CD4+ T-cell expansion and survival; recruits mast cells. 	[74]
			Tfh	<ul style="list-style-type: none"> Produce IL-9 In follicles of the spleen and tonsils. Major B-cell helper cell. 	[75]
			Th3	<ul style="list-style-type: none"> Produce IL-4. Seems to have a role in maintaining thymic Tregs in the peripheral immune compartment by the secretion of TGF-beta 	[76]
		IL10	iTreg/TR1	<ul style="list-style-type: none"> Anti-inflammatory and immunosuppressive cells; cytotoxic effects. Produce IL-10. 	[65]
tTreg	p-MHC-II			The atrophied thymus attempts to balance the defective negative selection by relatively enhancing tTreg cell generation to maintain central T-cell tolerance in the elderly.	[63, 77]
$\gamma\delta$ T-cells	Annexin CD1d ULBPs		V γ 9V δ 2 V δ 3 V δ 1	<ul style="list-style-type: none"> Perforin and granzyme-dependent mechanisms Cytokine profile IFN-γ, TNF-α, IL-17, RANTES, and CXCL10 	[63, 77]

Abbreviations: tTreg, thymus-derived regulatory T-cells; p-MHC-I, peptide in complex with MHC class I; p-MHC-II, peptide in complex with MHC class II; ULBPs, UL16 binding proteins. *The presence of APC-mediated cytokines is often referred to as signal 3.

B (bursal or bone marrow-derived) lymphocytes express clonally diverse cell surface immunoglobulin (Ig) receptors. B-cells develop in the bone marrow from hematopoietic precursor cells and their development involves a continuum of stages [78, 79]. Early B-cell development in the bone marrow includes the rearrangement of immunoglobulin

gene segments to secure clonogenic diversity; V_H , D_H , and J_H rearrangements of the heavy chain (H-chain), along with V_L - J_L rearrangements of the light chain (L-chain) gene segments [78]. The final stage of early B-cell development in bone marrow is the formation of IgM molecules that are expressed on the cell surface. The developmentally intermediate and heterogeneous population of B-cells are termed transitional [80] or immature B-cells [78], and they leave the bone marrow and migrate to the spleen, where they finalise their development by differentiating into naive, follicular, or marginal zone B-cells [78]. Naïve cells circulate through peripheral blood and lymphoid tissues and die within days if they do not encounter a cognate antigen [81, 82].

Mature splenic marginal-zone B-cells can respond rapidly to T-cell-independent antigens and develop into short-lived plasma cells [80], a process thought to be supported by innate cells, such as B-cell helper neutrophils (N_{BH} cells) [27, 78, 83, 84]. After being presented with T-cell-independent antigens, marginal-zone B-cells migrate from the marginal zone to bridging channels and undergo a proliferation burst and differentiation to make loci of plasma blasts. This proliferative burst may be a requirement for plasma cell differentiation [84]. The plasma cells from this response do not always undergo a class switch (i.e., they remain IgM-expressing) and may be short-lived [80]. Additionally, the antibodies produced seem to be directed toward less complex antigens (e.g., polysaccharides, lipids), have lower affinity and have undergone less somatic hypermutation [80].

The majority of mature B-cells are follicular B-cells [80], located in the lymphoid follicles of the spleen and lymph nodes, where they participate in T-cell dependent antibody responses [80]. Follicular B-cells encountering antigen and T-cell help can become (i) short-lived plasma cells, or they can enter into a germinal centre response in which (ii) long-lived antibody-secreting plasma cells and (iii) memory B-cells are generated. Both of these last two subsets provide protection against reinfection. Germinal centres are formed around follicular DCs [85]. The regulation of germinal centre responses is complex and involves assistance from T follicular helper (T_{fh}) cells and regulation by Foxp3⁺ T follicular regulatory (T_{fr}) cells [85]. The T-cell-dependent activation of B-cells induces the somatic hypermutation/class-switch machinery, thus

changing the affinity of the B-cell receptor and isotype (e.g., IgM to IgG, IgA, or IgE) [80, 86].

The first line of defence against re-infection is pre-existing protective antibodies secreted by long-lived plasma cells (i.e., constitutive humoral memory). If constitutive memory is insufficient, pathogen-experienced memory B-cells are quickly reactivated to produce antibodies (i.e., reactive humoral memory), which is the second line of defence. The reactive humoral memory response is faster, of greater magnitude, and consists of antibodies of switched isotypes and higher affinity compared to the primary antibody response [52]. Several pathways to B-cell memory exist, and heterogeneity among memory B-cells is based on their produced Ig isotype [52]. Human B-cell functions are regulated by IL-21R/STAT3 signalling, and the threshold of STAT3 activation required for differentiation seems to be lower in memory compared to naïve B-cells. This last observation suggests that STAT3 is an intrinsic difference in the underlying mechanism of differentiation between naive and memory B-cells [87]. Stemness, longevity, and robust responsiveness are among the key characteristics of memory B-cells, and these cells make effector cells upon re-encountering pathogens while maintaining the memory state. IgG⁺ memory B-cells seem to have a greater predisposition to differentiate towards plasma cells than IgM⁺ memory B-cells, possibly reflecting that the IgM⁺ memory B-cell compartment comprises more stem cell-like cells and class-switched memory B-cells (e.g., IgG⁺ memory cells), corresponding to a more committed progenitor cell. This may be similar to memory CD8⁺ T-cells [52, 88].

Complement receptor 2 (CR2) on follicular DCs seems to be essential for the survival of IgG⁺ memory B-cells, while B-cell receptor signalling seems to be essential for IgG1⁺ memory, and T-bet or ROR α (transcription factors) are essential for the survival of IgG2a⁺ or IgA⁺ memory B-cells, respectively. Vaccinations and infections are known to produce stable IgG⁺ memory B-cell populations in humans; for example, one study detected IgG1⁺ memory B-cells specific for the influenza strain causing the 1918 pandemic in circulation 90 years after primary exposure [52, 89]. Furthermore, memory CD8⁺ T-cells maintain mitochondrial mass, providing these cells with a bioenergetic advantage during reactivation [52].

Antibodies were discovered more than 100 years ago, but the question of how they obtain their diversity was not answered until the 1970s with the detection of somatic hypermutation. The most ancient class of antibodies are IgM; the transmembrane form defines B-cells, and after B-cell stimulation, IgM is released into plasma as pentameres [90]. Other human Igs are IgA (dimer), IgG, IgD, and IgE [90, 91]. The T-cell-dependent activation of B-cells induces the somatic hypermutation/class-switch machinery, thus changing the affinity of the B-cell receptor and isotype (e.g., IgM to IgG, IgA, or IgE) [80, 86]. The general structure of Igs is two light chains and two heavy chains with an antigen-binding variable region and a constant region, as illustrated in Figure 1C; characteristics of the various immunoglobulins are summarised in Table 4.

Table 4. Secreted Igs families and general characteristics (adapted from [18]).

Family	FcR	Subclasses	Characteristics	Molecular form	Released	Complement activation
IgM			Primary response	Pentamer	Serum	Yes
IgD	Fc δ		Homeostasis	Monomeric	Serum, low	No
IgG	Fc γ R II, III	I, IgG1, IgG2, IgG3, IgG4	Secondary response, neutralise toxins and viruses	Monomeric	Serum, predominant isotype	Yes
IgE	Fc ϵ R		Allergic response	Monomeric	Serum, low	No
IgA	Fc α R	IgA1, IgA2	Mucosal response	Monomeric	Serum, saliva, breast milk	No

1.2. THE MONOCYTE SYSTEM AND ITS HETEROGENEITY

Monocytes are large mononuclear cells of the innate immune system and often described as highly plastic cells with the ability to function as both precursor and effector cells [92]. Monocytes constitute approximately 5–10% of peripheral blood leukocytes in healthy humans [93]. Monocytes are separated from granulocytes and lymphocytes in automated cell analysers based on their larger size, as well as their non-lobular nucleus and smaller/different granules when compared to granulocytes. Monocyte parameters used in routine clinical practice are their concentration in peripheral blood (absolute numbers), the percentage of monocytes among circulating leukocytes (relative values), and the monocytes-to-platelets ratio (MPR) [94], but the latter is not in clinical use in Norway.

Monocytes and monocyte subsets can also be identified based on CD14 expression (discussed in Section 1.1.1), a cell surface co-receptor for LPS [23, 95], and whether they express Fc-gamma receptor IIIa (CD16a); see Section 1.2.3 for further subset definitions. CD16a (Fc γ RIIIa) consists of (i) two extracellular Ig-like domains that bind to IgG, (ii) a transmembrane domain that associates with adaptor proteins containing an immunoreceptor tyrosine-based activating motif (ITAM) and induces downstream intracellular signalling, and finally, (iii) a short intracellular domain [96]. CD16a associated γ -chains are essential for signalling and stable expression, as well as targeting the receptor on the monocyte cell membrane [96]. Humans seem to express two Fc γ RIIIa (CD16a) allotypes that differ in a single amino acid residue at position 158; the isoform with valine at this position has high affinity for the IgG1 Fc domain, while phenylalanine, at the same position, results in low affinity [97]. CD16a seems to be important for antibody-dependent cellular phagocytosis and antibody-dependent cellular cytotoxicity by human monocytes [98].

Immunophenotyping circulating monocytes is now used in routine clinical practice for the detailed characterisation of circulating monocytes, especially for the diagnosis of monocytic leukaemias (i.e., chronic myelomonocytic leukaemia [CMML] and monocytic variants of acute myeloid leukaemia) [99].

1.2.1. Monocyte development from hematopoietic stem cells in bone marrow

The development and differentiation of monocytes are called monopoiesis [100-105]. One model of monopoiesis indicates that monocytes develop from a lineage-committed bone marrow progenitor referred to as the common monocyte progenitor [94, 102, 104, 105]. Another model based on murine studies suggests that distinct monocyte subsets arise from two independent pathways [100, 105]. This development uncertainty is probably also reflected by [106] uncertainty regarding the expression of various differentiation markers in the different steps of monopoiesis [94, 102, 107]. Common monocyte progenitors seem to express receptors for the cytokine macrophage-stem cell factor (M-SCF-R), and their development and survival seem to depend on M-SCF [108]. Other early monocyte markers seem to include CD64, CD11c, cytoplasmatic cyLysozome, cyCD68, CD36, and CD35 [109-111]. Mature monocytes typically

express CD300e, CD312, CD45, CD11b, HLA-DR, and CD14 [110, 111]. Both immature (e.g., CD64^{high} CD14^{negative}) and mature (e.g., CD300e^{high}) monocytes express CD62L, and CD62L^{positive} monocytes are mainly detected in blood and bone marrow, whereas CD62L⁻ monocytes are most abundant in lymph nodes and the spleen [112].

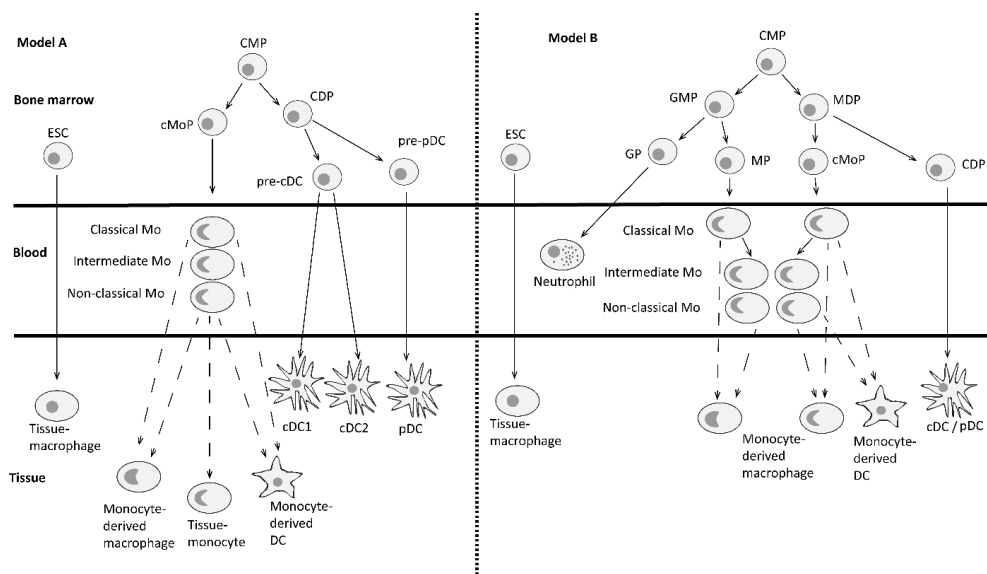


Figure 2. Models of monopoiesis. Model A: Monocytes differentiate from a common monocyte progenitor (cMoP), which arises from a common myeloid progenitor (CMP). Classical monocytes differentiate into either monocyte-derived macrophages, tissue-monocytes, non-classical monocytes via intermediate monocytes, or monocyte-derived dendritic cells, and non-classical monocytes are suggested to differentiate into macrophages (illustration adapted from [107]). **Model B:** Monocytes arise from both monocyte-DC progenitors (MDPs) and granulocyte-monocyte progenitors (GMPs) and provide heterogeneous populations of non-classical monocytes and monocyte-derived macrophages. Only classical monocytes differentiated from MDP via cMoP may differentiate into monocyte-derived DC (illustration adapted from [100]). Classical and non-classical monocytes are both suggested to contribute to monocyte-derived macrophages and monocyte-derived dendritic cells. The progenitor monocyte subset may contribute to specific subsets of either macrophages or dendritic cells [36]. **Abbreviations:** ESC, embryonic stem cell; MP, Monocyte-committed progenitors; GP, Granulocyte progenitor; cDC, conventional dendritic cells; pDC, plasmacytoid dendritic cells.

Murine studies suggest that differentiation into common monocyte progenitors is followed by differentiation in bone marrow into classical monocytes with subsequent conversion into intermediate monocytes (i.e., an intermediate state), followed by conversion into non-classical monocytes [113]. This is also supported by *in vivo* studies in humans [114, 115]. Computational models suggest that 1% of classical monocytes are converted into non-classical monocytes during homeostasis, which is consistent with a model where classical monocytes are either converted into non-classical monocytes via the intermediate stage and enter tissues or the lymphatic system or undergo apoptosis

[114, 115]. Non-classical monocytes may be close to senescence, and this may contribute to their pro-inflammatory phenotype. They also have other signs of senescence, such as altered miR-146 (a microRNA precursor) expression [116-120]. However, current data cannot exclude the existence of a lineage-restricted progenitor that differentiates directly into non-classical monocytes.

Monocytes as precursor cells: Monocytes, dendritic cells, and macrophages are all mononuclear phagocytotic cells, and monocytes were previously regarded as precursors that give rise to tissue macrophages and dendritic cells [121, 122]. Recent evidence indicates that there exist distinct monocyte subsets with different genetic, epigenetic, transcriptional, and metabolic arrangements committed to becoming macrophages and DCs [94]; this process is different from the previous theory that monocytes differentiated into macrophages or dendritic cells in response to an inflammatory environment [94]. However, monocytes are recruited to sites of inflammation and gain phagocytic functions, as well as phenotypic characteristics similar to dendritic cells and macrophages, leading to difficulty separating monocyte-derived cell subsets from other phagocytes [123]. Monocytes are also shown to preserve their monocyte-like phenotype within tissues [94, 123].

Animal studies suggest that during a steady-state, classical monocytes replenish resident peripheral monocyte-derived cells [124, 125]; these monocytes also show the highest migration in response to CCL2, CCL8, and CCL7 [126] and have a half-life in the circulation of less than one day in humans [115]. Monocytes are recruited to inflamed tissues at high rates, where they attract other immune cells by secreting cytokines and antimicrobial factors [127]. Monocyte migration studies have not detected any significant heterogeneity among intermediate monocytes or differences between intermediate and non-classical monocytes [128]. In contrast, intermediate and non-classical monocytes seem to have a distinct (i.e., slower) migration pattern when compared to classical monocytes.

Monocyte-derived dendritic cells: Conventional DCs seem to be distinct cellular lineages from monocytes and originate from a separate precursor cell (Figure 2) [107, 129]; however, monocyte-derived DCs may be generated by culturing monocytes in the

presence of granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4. This generates immature DCs that differentiate further into mature DCs by TNF- α stimulation [36, 130]. Transcriptomic analyses suggest that dermal CD14⁺ DCs, intestinal CD103-CD172a⁺ DCs, and 'inflammatory DC-expressing CD1c, CD1a, and CD14 are monocyte-derived cells [121, 131-134].

Monocyte-derived DCs may also invade tissue not normally maintained by monocyte-derived cells, and not only the skin and intestine [36], where they contribute to the control of immune response [135], are able to enhance Th2 cell response, and express a high variation cytokines (e.g., CCL-1, CCL-10) [136].

The exact contribution of monocyte subsets to monocyte-derived DCs during inflammation is unclear [36]. However, examples of increased numbers of CD16⁺ monocytes during inflammation have been associated with increased numbers of monocyte-derived DCs [137], and functional differences among monocyte-derived DCs generated from different subsets have been reported (e.g., more potent immune responses from DCs derived from classical monocytes and better immune tolerance from DCs generated from non-classical monocytes) [138-140]. It is suggested that CD16⁺ monocytes differentiate into alternative dendritic cells with poorer antigen-presenting function [140] and are more likely to induce T regulatory cells [141].

Monocyte-derived macrophages: Tissue-resident macrophages seem to originate from either embryonic precursors or differentiate from extravasated monocytes, which are the origin of several macrophage populations, such as Kupffer cells and splenic macrophages (Figure 2, panel A) [36, 94, 102, 107]. Macrophages are well adapted to their environment, obtain organ-specific functionalities, and contribute to the maintenance of tissue homeostasis, and the differentiation, proliferation, and function of macrophages are controlled by growth factor colony-stimulating factor CSF-1 and IL-34 [36].

Monocytes are capable of renewing macrophages and are associated with the slow replacement of embryonic macrophages in tissues, and monocyte-derived macrophages then obtain transcriptional similarities to embryonic macrophages, although some of the epigenetic, transcriptional, and functional difference are maintained [94]. Classical

monocytes are suggested to be continuously recruited to maintain local macrophage populations in homeostasis in some tissues, such as the intestine [142].

As with monocyte-derived DCs, there are reports of functional differences among monocyte-derived macrophages. Murine studies suggest that monocyte heterogeneity may underlie macrophage heterogeneity. The two main monocyte subsets in mice are suggested to give rise to either proinflammatory (M1) or anti-inflammatory macrophages (M2), each associated with a specific monocyte subset [100], suggesting monocytes may differentiate into distinct macrophages with different capacities to drive inflammatory responses or promote tissue repair, pre-determined by the subset of the progenitor monocyte [36]. Non-classical monocytes are suggested to be the source of wound healing macrophages [143], although the contribution of CD16⁺ monocytes in a steady state to the macrophage population is unclear [144]. Furthermore, these two macrophage phenotypes (M1 and M2) are regarded as being transient and occurring along a spectrum [145, 146], and several other factors seem to influence or drive monocyte-derived macrophage polarisation, including platelets [147], IL-4 [148], and microRNA [146].

The monocyte subsets also appear to function as macrophage precursors in different pathological conditions; for example, during infection, classical monocytes will rapidly invade tissue and generate macrophages with little tissue repair capacity [149, 150]. However, without infection, except, for example, during cardiac pressure overload, the preferential recruitment of non-classical monocytes/macrophages in cardiac tissue is observed [151]. The selective recruitment of specific monocyte subsets seems to be context-dependent and based on the nature of the challenge [152].

Monocyte-derived endothelial cells: Monocytes may also differentiate into endothelial cell-like cells [153]. CD16⁺ monocytes exposed to tissue factors showed increased expression of VE-cadherin, von Willebrand factor (VWF), and eNOS, while blocking β 1-integration inhibited this effect [153].

Monocyte-derived multinucleated giant cells: Monocyte-derived macrophages are also able to fuse and form multinucleated giant cells, such as osteoclasts and

Langerhans' cells [36, 154]. These macrophages arise from monocytes differentiated in the presence of M-CSF and the receptor activator of the NF- κ B ligand (RANKL) [36].

Langerhans cells are proinflammatory and formed in response to infection, and osteoclasts reside in the bone marrow and are important in bone homeostasis [154]. Although long believed a source of osteoclasts, it was confirmed when fluorescently labelled monocytes were recruited from circulation to the bone surface and differentiated locally into osteoclasts [155]. There are also reports of osteoclasts contributing to inflammation and immune responses [156].

Monocytes are also able to fuse into multinucleated giant cells in response to foreign material, such as medical implants [154]. The molecular mechanisms responsible for forming multinucleated giant cells are unknown, but the development of foreign-body giant cells and Langerhans' cells seem to be initiated by different cytokines [154]. Multinucleated cells can reabsorb mineralised tissue, such as bone; they bind and then degrade the various matrix components by secreting acid, followed by a cocktail of different proteolytic enzymes [36].

Classical monocytes have the highest propensity to differentiate into osteoclasts [157], although they may shift to intermediate and non-classical monocytes, as reported in patients with psoriatic arthritis, a chronic type of inflammatory arthritis characterised by severe bone erosion [157]. This is an indication that the intrinsic properties of isolated monocytes generate distinct osteoclasts rather than altered cytokine levels in the inflammatory environment [36]; for example, IL-17A decreased the osteoclasts formation of intermediate monocytes, however the bone resorption was not affected by IL-17A treatment of intermediate monocytes [158]. Intermediate monocytes are suggested to be more likely to fuse into multinucleated giant cells and be precursors of osteoclasts [159, 160]. The elevated number of CD16⁺ monocytes, especially intermediate monocytes, seem to play a critical role in osteoclastogenesis during inflammation, which may explain the observed osteoclast-associated bone loss in inflammatory disorders [36].

1.3. THE HETEROGENEITY OF MONOCYTE EFFECTOR CELLS

1.3.1. Monocyte subpopulations and heterogeneity

Several studies have shown that monocytes are heterogeneous; for example, they show differences in tetraspanin (membrane protein family) expression, the capacity to form multinucleated giant cells, phagocytic responses, cytokine secretion, and cellular migration, as well as cell surface receptor expression [94, 128, 160-164]. Monocytes are typically classified into the three subsets: classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺), and non-classical (CD14⁺CD16⁺) [34, 115, 164-172]. However, the detailed definition of each cell subset of each monocyte subset remains a matter of debate (see Table 5 for more detailed descriptions). The following sections will summarise some of the heterogeneity among monocytes reported in the literature.

Classical monocytes constitute the majority of circulating monocytes, usually 80–90% of peripheral venous blood monocytes [164]. Previously, data have suggested heterogeneity among intermediate monocytes [126, 173]. However, very recently, high-dimensional mass cytometry data identified one intermediate subset [174], but this finding suggests there is some heterogeneity among classical monocytes [174].

Single-cell RNA sequencing studies have shown that classical and non-classical monocytes form two separate clusters. On the other side, intermediate monocytes formed two clusters, in addition, some intermediate monocytes were included into the classical or non-classical monocyte clusters; this study strongly suggests that intermediate monocytes are a very heterogeneous group [173]. The first intermediate monocyte cluster was characterised by the expression of genes important to the regulation of proliferation, differentiation, and trafficking, such as MAX dimerisation protein 1 (MXD1), CXCR1, CXCR2, and vascular non-inflammatory molecule 2 (VNN2). The second intermediate monocyte cluster expresses genes important to cytotoxicity (e.g., PRF1, GNLY, and CTSW), in addition to the classical monocyte gene [173].

Recently, based on high-dimensional mass cytometry, the existence of eight human monocyte subsets were reported. Three subsets were found to fall within the non-classical monocyte population, while four subsets belong to the classical monocytes,

and interestingly, intermediate monocytes clustered together into one subpopulation [174].

Table 5. A selection of recently published papers on the topic of monocyte subsets and markers.

Monocyte subsets	Markers of identification	Ref.
Inflammatory monocytes (iMo) Patrolling monocytes (pMo) Immunosuppressive monocytes (M-MDSC) Trained monocytes SatM-expressing monocytes Neutrophil-like monocytes	CCR2 ⁺ CD14 ^{high} CD16 ^{low/neg} CX3CR1 ⁺ CD16 ^{high} CD14 ^{low} CD11b ⁺ CD14 ⁺ CD124 ⁺ PD-L1 ⁺ CCR2 ⁺ HLA-DR ⁻ CD14 ⁺ Dectin1 ⁺ CD36 ⁺ TLR4 ⁺ GM-CSF-R ⁺ NOD ⁺ Undefined in humans. Defined in mice. Undefined in humans. Defined in mice.	[94]
Classical monocytes Intermediate monocytes Non-classical	CD33 ^{high} , CD86 ^{+/high} , CD64 ⁺ , HLA-DR ⁺ , CCR2 ^{high} CD33 ^{high} CD86 ^{+/high} CD64 ⁺ HLA-DR ^{high} CCR2 ^{low} CD33 ^{low} CD86 ^{high}	[175]
Classical monocytes Intermediate monocytes Non-classical	CD300e ^{high} CD14 ^{high} CD16 ^{negative} CD300e ^{hi} CD14 ^{hi} CD16 ⁺ CD300e ^{hi} CD14 ^{low/-} CD16 ⁺	[111]
Classical monocytes Intermediate monocytes Non-classical	CD14 ⁺⁺ CD16 ⁻ CD14 ⁺⁺ CD16 ⁺ CD14 ⁺ CD16 ⁺⁺	[34, 165, 168-172, 176]*

*There is a great variation in the overall antibody panel and gating strategy applied, but all the studies identified monocyte subsets by CD14 and CD16 expression.

1.3.2. Monocytes as effector cells

Monocytes are key innate cells involved in eliminating invading bacteria, virus, fungi and, protozoa by, for example, phagocytosis and antibody-dependent cellular cytotoxicity (ADCC), and they perform important immunoregulatory functions through their cytokine/mediator release and antigen presentation [94, 103, 161, 162, 177]. As with other innate cells, monocytes detect PAMPs and DAMPs through PRRs like, for example, TLRs. These receptors dimerise after ligation and thereby activate the TLR-MyD88-NF-κB signalling pathway and induce pro-inflammatory or anti-inflammatory responses, which depend on the monocyte subset being involved [22]. Table 6 summarises important features of monocyte subsets as effector cells.

Phagocytosis is a process involved in the (i) ingestion and elimination of pathogens and (ii) the elimination of apoptotic cells, and it is important in (iii) tissue homeostasis [178]. The phagocytic response can be categorised into two main categories: (i) pathogen killing and digestion and (ii) antigen processing and presentation [178].

Table 6. Monocyte subsets as effector cells: A brief summary of important findings. The comparisons are to other monocyte subsets.

CD16⁻ versus CD16⁺ Monocytes		References
CD16 ⁻ monocytes are suggested to be migratory, while CD14 ^{low} CD16 ⁺ are patrolling monocytes.		[103, 127, 179]
CD16 ⁺ monocyte numbers are increased in the blood of patients with systemic infections, implying they play an important role in the immune system's rapid defence against pathogens.		[180, 181]
Antibody-mediated cellular cytotoxicity (ADCC) seems CD16-dependent.		[98]
CD16 ⁺ seems more efficient in activating T-cells than CD16 ⁻ monocytes.		[103]
CD16 ⁺ expresses higher levels of CX3CR1, supporting the idea that they migrate and adhere more than CD16 ⁻ monocytes to fractalkine/CX3CL1-secreting endothelium.		[182, 183]
Classical monocytes		
Migration	Migrate to CCL2 and CCL3 gradients.	[183, 184]
Chemokine receptor	Express higher levels of CCR1, CCR2, CCR5, CXCR1, and CXCR2 and lower levels of CX3CR1.	[161, 184, 185]
Phagocytosis	Differences in the phagosomal environment of the three monocytes subsets; classical monocytes exhibit alkaline phagosomes.	[186, 187]
APCs	Entry into tissue and lymph nodes causes the upregulation of MHC class II expression. Show the largest increases in MHC class II expression in response to IFN γ , GM-CSF, and IL-4. Probably able to cross-present cell-associated antigen to CD8 ⁺ T-cells. Suggested to induce Th1-cell-mediated immunity.	[103, 187-190]
Mediator Secretion	Secrete pro-inflammatory molecules (e.g., IL-6, IL-8, CCL2, CCL3, and CCL5).	[161, 177]
Efficient in producing ROS		[177]
Intermediate monocytes		
Chemokine receptor	Chemokine receptors, they express more CCR5 than classical monocytes and this likely accounts for their high susceptibility to HIV-1 infection	[182, 184, 191]
Phagocytosis	The intermediate monocytes seem to be the most efficient phagocytic subset with regard to CD16- and the complement-dependent phagocytosis of antibody-opsonised pathogens.	[162]
Differences in the phagosomal environment; intermediate monocytes have a phenotype between alkaline/acidic phagosomes.		[186]
APCs	Express the highest levels of antigen presentation-related molecules in steady-state	[161, 188]
Mediator Secretion	Secrete TNF- α , IL-1 β , IL-6, and CCL3 upon TLR stimulation.	[161, 177, 192]
Suggested as the main producers of IL-10 upon TLR stimulation.		[193]
Non-classical monocytes		
Chemokine receptor	Express high levels of CX3CR but low CCR2 levels.	[184, 185, 194]
Phagocytosis	Differences in the phagosomal environment: non-classical monocytes had more acidic phagosomes	[186]
A Slan ⁺ CXCR6 ⁺ intermediate subset showed to migrate towards CXCL16 as well as had increased efferocytosis capacity		[174]
APCs	Similar HLA-DR, DQ, and DP expression to classical monocytes but no observed upregulation when stimulated (e.g., IL-4).	[188]
Mediator Secretion	Antagonize classical monocytes and promote neutrophil adhesion at the endothelial interface via the secretion of TNF- α .	[128]
Do not reach the classical monocyte production levels of pro-inflammatory cytokines.		[163]
Are activated by viruses and immune complexes through the TLR7-TLR8-MyD88-MEK pathway to release TNF- α , IL-1 β , CCL-3, and CCL-4.		[116, 177, 195]
Involved in wound healing processes.		[196]

Phagocytic responses can be initiated by PRRs or through complement activation and antibody-mediated Fc γ R receptors [162, 197]. Classical and intermediate monocytes are suggested in the literature to be the most effective phagocytic cells, whereas non-

classical monocytes seem less effective [161, 162, 198]. Intermediate monocytes also seem to be the most efficient phagocytic subset with regard to CD16 (FcγRIIIa) and the complement-dependent phagocytosis of antibody-opsonised pathogens [162]. Further heterogeneity within the phagocytosis process for monocytes was supported by recent observations indicating differences in the phagosomal environment between the three monocytes subsets [186]. Classical monocytes and neutrophils exhibit alkaline phagosomes, while non-classical monocytes have more acidic phagosomes, and intermediate monocytes have a phenotype between the two [186].

Antibody-dependent cellular cytotoxicity (ADCC) is the mechanism by which Fc receptor-expressing effector cells recognise and kill antibody-coated target cells. Monocytes are suggested to be involved in antibody-mediated cellular cytotoxicity, which is CD16 (FcγRIII) dependent; this process seems to require cell-cell contact through β2 integrins and is mediated through the release of TNF-α [98]. Additionally, a recent study found that slan⁺ (6-Sulfo LacNAc, the carbohydrate modification of P selectin glycoprotein ligand 1) monocytes, but not CD14⁺ monocytes, increased in number and displayed highly efficient rituximab-mediated antibody-dependent cellular cytotoxicity almost equivalent to that exerted by NK cells [199].

Monocytes secrete a wide range of cytokines, including several chemokines, such as IL-6, IL-8, IL-1β, IL-1ra, and TNF-α [116, 163, 200-203]. TNF-α is released by all three monocyte subsets [163, 193, 203], whereas classical monocytes secrete higher levels of IL-6 and IL-1β than the two other subsets [163]. The intermediate monocyte subset releases both anti-inflammatory (e.g., IL-10) and pro-inflammatory cytokines, such as TNF-α and IL-1β. Both CD16⁺ monocyte subsets release high levels of TNF, CCL-3, CCL-4, CCL-5, IL-6, IL-8, and IL-1 [116]. See table 7 for an overview of some of the cytokines secreted by monocytes.

Table 7. An overview of important cytokines released by normal monocytes and their effects on other cells.

Cytokine*	Some important features	Ref.
Chemokines		
CCL1	Chemoattractant. Associated with a distinct form of M2 monocyte activation, which participates in the macrophage-dependent regulatory circuits of innate and adaptive immunity. Binds with CCR8.	[144, 204, 205]
CCL2/ MCP-1	Chemoattractant and the key monocyte-attracting chemokine; regulates monocyte migration and infiltration through CCR2; macrophage and T-cell activation; stimulates histamine release.	[144, 206, 207]
CCL3/ MIP-1 α	Chemoattractant. Proinflammatory activities. Negatively regulate the proliferation of hematopoietic stem/progenitor cells. Induce the differentiation of monocytes to osteoclasts. Possible role in leukemogenesis. Enhance leukocyte migration to tumour-draining lymph nodes and suppress tumour growth.	[144, 208-211]
CCL4/ MIP-1 β	Chemoattractant for monocytes/macrophages, T-cells. CCL4 induces the synthesis and release of other pro-inflammatory cytokines.	[144, 210, 212]
CCL7/ MCP-3	Chemoattractant, especially granulocytes and monocytes. Pleotropic effects: both pro- and anti-inflammatory responses.	[144, 213, 214]
CXCL1	Chemoattractant. Recruit and activate neutrophils. Role in inflammation, angiogenesis, tumorigenesis, and wound healing.	[144, 215, 216]
CXCL1 0/IP-10	Chemoattractant. Th1 response; Th1, CD8, and NK trafficking.	[144]
Interleukins		
IL1 β	Induces hematopoiesis and pro-inflammatory cytokines. When released from bone marrow plasma cells, induces IL-6 production from marrow stromal cells, activates B- and T-cell differentiation into Th17. Induces Breg cells in mouse spleen and mesenteric lymph nodes.	[217, 218]
IL1RA	Anti-inflammatory, IL-1 antagonist.	[217, 218]
IL6	Inducer of antibody production, CD8 ⁺ cytotoxic T-cell differentiation; CD4 ⁺ T-cell differentiation toward Th17 and myeloid cell development. Regulation and induction of acute-phase proteins in hepatocytes. Activates B-cells and the production of IgG, IgM and IgA. Hematopoiesis. Involved in osteoclastogenesis and bone reabsorption. <i>In vivo</i> ; synovial fibroblast proliferation and cartilage degradation. Cholinergic neuron survival and the induction of adrenocorticotrophic hormone synthesis.	[46, 218]
IL8/ CXCL8	Enhance angiogenesis and attract neutrophils, NK, T-cells, and MDSC. Hematopoietic stem cell mobilisation.	[218, 219]
IL10	Anti-inflammatory master regulator. Regulates and suppresses the expression of proinflammatory cytokines during the recovery phase of infection, which consequently reduces the damage caused by inflammatory cytokines. Has an immunosuppressive effect through APC or directly on T-cells. Suppression of IgE production and induce IgG.	[218, 220]
Other mediators		
MMP9	Controls the access of monocytes and T-cells to the vascular wall. Participates in pathological remodelling processes that involve inflammation and fibrosis. Enhances elastin degradation and induces plaque disruption. Decreases collagen synthesis and is involved in cardiac fibroblast migration.	[221-223]
TNF α	Important for host defence, initiates the pro-inflammatory response, and limits the duration of the response. Modulates multiple signalling pathways with wide-ranging downstream effects, including immunometabolism in monocytes. Promotes nitric oxide, necrosis, cytotoxicity, and expression of matrix metalloproteinases (MMPs). Increases MHC expression, activates macrophages, enhances tumour cell killing. Important in inhibiting autoimmune disease and tumorigenesis.	[218, 222, 224, 225]

*Released cytokine levels in supernatant for the listed cytokines was evaluated in Article IV

Monocytes as antigen-presenting cells: Experiments in mice indicate that monocytes seem important to the induction and regulation of CD4⁺ Th1 [190], Th2 [136], and Th17

[136, 226], as well as CD8⁺ T-cell [227] responses (reviewed in [127]). CD16⁺ monocytes are suggested to be more efficient T-cell-activating cells than classical CD16⁻ monocytes [103]. MHCs are important molecules, presenting antigens to T-cells. When comparing monocyte subsets, intermediate monocytes show the highest expression of MHC class II molecules, (HLA-DR, -DQ and -DP, and HLA-DM), and the lowest ratio of class II-associated invariant chain peptide (CLIP) and MHCII (the CLIP:MHC class II ratio) [188]. High cell surface expression of CLIP can be viewed as an indicator of less effective antigen presentation [188, 228], and these results imply this subset presents other peptide/MHCII complexes efficiently [188].

Additionally, the intermediate monocyte subset seems to be heterogeneous regarding the expression of HLA-DR (HLA-DR^{high} and HLA-DR^{mid} populations) [126], supporting the hypothesis that the intermediate monocyte subset is a heterogeneous group. In contrast, classical monocytes show the largest increase in MHC class II expression in response to IFN γ , GM-CSF, and IL-4 [188], whereas all subsets show decreased HLA-DR levels when exposed to IL-10 [188]. Taken together, these observations suggest intermediate monocytes are the most efficient constitutive antigen-presenting monocyte subset, whereas the classical subset becomes more important in response to inflammation.

Monocytes contribute to immunosurveillance and tissue repair: Immunosurveillance is the process whereby abnormal (neoplastic) cells are detected and destroyed. CD16⁺ monocytes are suggested to be especially important for immunosurveillance of the central nervous system [229] and within cancer immunosurveillance [230, 231]. In an ‘injury niche’, the tissue microenvironment emits signals to recruit and instruct cells of the inflammatory system to program repair and inflammation resolution. Although heterogeneous populations of monocytes (CD16⁻ and CD16⁺) are recruited from circulation in (i) vascular remodelling, (ii) muscle repair, and (iii) bone repair [232], the non-classical monocyte subset (CD16⁺) seems to be especially important for tissue repair [143, 195, 233, 234].

Trained innate immunity and monocytes: A body of evidence indicates that innate immune cells can mount (i) acute response to eliminate invading microbes or

transformed self-cells, (ii) innate adaptive memory responses (i.e., long-term responses), or (iii) immune tolerance [53, 235-238]. Currently, the concept of innate memory is a matter of debate and was recently suggested to be better described as adaptive but transient phenotypes not within the concepts of ‘memory’ as we know it from antigen-specific adaptive cells (T- and B-cells) [239]. This is somewhat in contrast to the view that trained innate immunity involves the full haematopoietic system (myelopoiesis) [240, 241]. However, innate memory (trained immunity) is suggested to exist in monocytes, macrophages, NK cells, and neutrophils [242, 243], and recently, in dendritic cells [244]. Additionally, a possible role for non-immune cells in harbouring long-lasting immunological memory has been suggested [242].

Enhanced monocyte reactivity upon a second encounter has been suggested for microbial particles (e.g., *Candida albicans*, a fungal pathogen, β -glucan, a component of the fungal cell wall, Bacille Calmette-Guérin, the BCG vaccine, and peptidoglycan, a component of the bacterial cell envelope [53, 245, 246], as well as for DAMPs, such as oxLDS, oxidised lipid droplets, HSP90, and S100A4) [247-250].

Trained monocytes are suggested to originate from inflammatory monocytes [94, 240, 241, 247]. Enhanced reactivity upon a second encounter in monocytes is associated with specific cell signalling pathways and metabolic and epigenetic rewiring [53, 94, 236, 237, 251, 252]. **The signalling pathways** identified to be involved in the induction of trained-monocytes include [241] (i) the dectin-1/AKT/mTOR/HIF-1 α signalling pathway, with the subsequent secretion of IL-6, IL-1 β , and TNF α [94, 245], and (ii) the NOD2/NF-kB pathway, with the subsequent secretion of IL-6 and TNF α [246]. **Epigenetic reprogramming** associated with trained immunity in monocytes includes monomethylation at histone H3 lysine 4 (H3K4me1), trimethylation (H3K4me3), and acetylation at H3 lysine 27 (H3K27ac) [252]. **Metabolic reprogramming** associated with innate memory will be discussed in Section 1.4.

1.4. IMMUNOMETABOLISM, MITOCHONDRIA AND MONOCYTES

Immunometabolism: Cellular metabolism serves three major functions: (i) generating energy (ATP) by catabolism, (ii) producing building blocks (termed biosynthesis or anabolism), and (iii) modulating cellular signalling (e.g., via mitochondrial activity) [253]. Activated immune cells have an increased demand for nutrients, but the type of nutrients needed is dependent on the functional requirements and the type of immunocompetent cell [253], as well as the pathogen and the tissue microenvironment [253]. The antimicrobial activities of immunocompetent cells seem to require specific metabolic programs, termed immunometabolism [240, 253-258].

Immune cells require energy (i.e., ATP) to survive [253]. ATP is preferably generated using glucose; however, it can also be generated via the use of lipids or amino acids [253, 259]. In glycolysis, glucose is turned into pyruvate, or lactate, within the cytoplasm. Pyruvate is transported to the mitochondria and subsequently converted into acetyl coenzyme A. Within the mitochondria, acetyl coenzyme A enters the tricarboxylic acid cycle, which produces electron carriers (e.g., NADH). Finally, NADH donates electrons to an electron transport chain that leads to the production ATP [253, 260]. Decreased ATP production increases AMP levels and thereby causes a shift from an anabolic to a catabolic state to sustain a high ATP/ADP ratio, while an increased AMP/ATP ratio activates AMPK (AMP-activated protein kinase), which decreases mTOR (rapamycin) activity and favours autophagy and the supply of nutrients to mitochondria [261]. Thus, the modulation of cellular metabolism will alter intracellular signalling and thereby influence the functional characteristics of cells. Additionally, extracellular ATP is part of the immune system; it is released during hypoxic conditions by necrotic cells, activated immune cells, and endothelial cells, acting as a pro-inflammatory 'danger' signal for innate cells, as a costimulatory signal to T-cells. At low concentrations, ATP has anti-inflammatory properties [262, 263]. For instance, in monocytes, ATP is released after stimulation with PAMPs, which induces IL-1 β and IL-18 secretion in an autocrine manner [264].

Mitochondria have been described as an especially central component of the immune system [265, 266]; mitochondrial DNA acts as a DAMP, and the mitochondrial outer

membrane is a platform for the NLRP3 inflammasome [266]. There exist several known signal transduction pathways between the cell and its mitochondria [267]: (i) calcium influx to the mitochondrial matrix and bursts of Ca^{2+} into the cytosol, (ii) mitochondrial production of reactive oxygen species (ROS), and (iii) alteration of the availability of Krebs cycle intermediates, such as acetyl-Coenzyme A, succinate, fumarate, and α -ketoglutarate [261].

Monocyte activation is associated with metabolic modulation. TLR4/LPS stimulation is often used as a model of acute monocyte activation and results in unique metabolic reprogramming [253, 268-271], with the upregulation of glycolysis and fatty acid synthesis and suppression of oxidative phosphorylation [271]. However, metabolic LPS effects seem to be dose-dependent. The suppression of oxidative phosphorylation seems to be linked to the induction of tolerance by higher LPS concentrations, whereas lower LPS doses associated with monocyte activation may increase both glycolysis and oxidative phosphorylation [272-275].

The glycolytic pathway is vital for monocyte function and monocyte-derived cells' differentiation and functions (see Table 8): (i) the glycolytic pathway influences monocyte differentiation into macrophages and dendritic cells [261, 276-278]; (ii) glycolysis is upregulated in monocytes in response to infection and is important for effector functions [279-281], and the Akt-mTOR-HIF-1- α pathway seems especially important [268, 276-278]; (iii) trained monocytes are mainly glycolytic [94, 245, 282].

Fatty acid oxidation plays a role in regulating the inflammatory properties of monocytes [94]. In glucose-deprived monocytes, oxidative metabolism has been found to be fuelled mainly by fatty acids, and they show comparable levels of cytokine production, migration rates, and phagocytosis during LPS activation as when glucose is present. Glucose-deprived monocytes also increase oxidative phosphorylation and decrease ROS levels [283].

Table 8. Overview of findings reported in some published articles.

Description of findings	Ref.
The glycolytic pathway influences monocyte differentiation into macrophages. Glycolysis inhibitors affect the expression of essential genes for macrophage differentiation and retained plasticity of macrophages.	[276]
Monocyte-derived CD137L-dendritic cells show higher glycolysis rates and Akt-mTOR1 activity than immature DCs. Glycolysis is vital for the expression of most co-stimulatory molecules and the secretion of inflammatory cytokines. Inhibition of mTORC1 after the induction of immature moDCs or CD137L-DCs differentiation always blocks the differentiation of DCs	[277]
The TLR4 activation of human monocyte-derived DCs seems to stimulate glycolysis and increased glucose consumption and lactate production	[278]
Monocytes upregulate genes involved in glycolysis in response to <i>C. Albicans</i> infection. C-type lectin signalling seems to trigger glycolysis when challenged by pathogens, and both glycolysis and the pentose phosphate pathway are then required for ROS production/	[279]
Monocytes in Chagas patients are mainly glycolytic, and monocyte glycolysis determines CD8+ T-cell functionality .	[280]
CD14 ⁺ monocytes from rheumatoid arthritis patients express IL-1 β , TNF α , IL-6, IL-27, CXCL10, and CXCL11, and are highly glycolytic, with increased expression of HIF1 α , HK, and PFKFB3, which is mediated by STAT3.	[281]
Amino acid transporter SLC7A5 and mediated amino acid uptake in monocytes influence cytokine production, and inhibition of SLC7A5 is associated with the downregulation of glycolysis during LPS activation.	[284]
In B-Glucan-trained monocytes, the induction of Dectin-1-Akt-mTOR-HIF-1- α leads to a shift toward glycolysis, as well as increased lactate production and TNF- α production.	[245]

There are scarce data available regarding the **metabolic profile of monocyte subsets**, namely classical, intermediate, and non-classical monocytes. Pathway analysis suggests differences in metabolic gene signatures, whereas classical monocytes express higher levels of genes involved in carbohydrate metabolism, priming them for anaerobic energy production. Non-classical monocytes express higher levels of oxidative pathway components and show higher mitochondrial routine activity [196].

Reactive oxygen species (ROS) are molecules containing oxygen (e.g., peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen), produced in all aerobic cells generated during, for example, mitochondrial oxidative metabolism. Intermediate monocytes show higher mitochondrial ROS production than the other monocyte subsets in malaria patients, although similar ROS levels have been reported for intermediate and non-classical monocytes [116, 285]. Classical monocytes show lower levels of ROS production [116].

The induction of trained immunity requires specific, complex metabolic modulations [240, 253, 286], possibly also at the hematopoietic progenitor level [287, 288]. Metabolites are suggested to play a part in the epigenetic programming of trained

immunity by acting as cofactors for enzymes, such as methylases, methyltransferases, and histone deacetylases [94].

Trained monocytes seem to exhibit impaired oxidative phosphorylation and lactate production, as well as disrupted tricarboxylic acid cycles at the citrate and succinate levels [94]. Citrate is withdrawn for fatty acid synthesis, while succinate activates HIF-1 α and consequently upregulates the expression of several pro-inflammatory cytokines, mainly IL-1 β and TNF- α [94, 245].

Immunometabolism is a complex field, and this overview regarding monocyte immunometabolism is far from complete. For some recent reviews, see [94, 106].

1.5. MULTIPLE MYELOMA

Multiple myeloma is a malignancy characterised by uncontrolled plasma cell proliferation, driven by intrinsic genetic abnormalities and extrinsic stromal cell support and usually detectable by monoclonal protein in blood or urine [289].

1.5.1. Epidemiology and classification of multiple myeloma

Multiple myeloma is the second most common hematologic malignancy and accounts for 1% of all cancers [289, 290]. The median age at the time of initial diagnosis is 70 years; only 35-40% of patients newly diagnosed with the disease are below 65 years of age, while approximately 25% are 65–74 years, and 35–40% are above 75 years of age [291]. Monoclonal gammopathy of undetermined significance (MGUS) refers to the detection of monoclonal immunoglobulin without any symptoms or signs of disease; it seems to be present in 2–3% of the population above 50 years of age and is associated with an annual risk of 1% for transformation into multiple myeloma.

Soldering multiple myeloma is characterised by the presence of monoclonal immunoglobulin in blood samples, increased (i.e., > 10%) plasma cells in bone marrow, and the absence of myeloma-related organ impairment. The overall risk of progression to multiple myeloma is approximately 10% per year for the first 5 years after diagnosis, 3% for the next five years, and thereafter, 1% per year [292, 293]. Globally, multiple myeloma was responsible for approximately 100,000 deaths with an age-standardised death rate of 1.5 per 100,000 in 2016, and between 1990 and 2016, myeloma incidence

rates increased by 126% [294]. MGUS, smouldering myeloma, and multiple myeloma has a slight male predominance [295-297]. African Americans have a higher prevalence than European Americans, but the two groups have similar transformation rates [298]. These observations suggest that genetic factors contribute to the development of multiple myeloma.

Multiple myeloma is usually characterised by bone marrow infiltration by abnormal plasma cells and is, thus, a bone marrow malignancy for most patients. The detection of circulating plasma cells in myeloma patients is usually associated with aggressive disease and reduced survival [299]. Studies of Ig gene sequences have shown extensive somatic hypermutation without interclonal variation, suggesting that multiple myeloma arises from postgerminal B-cells [300] and resembles memory B-cells [301]. However, malignant plasma cells seem to differ between patients; the analysis of CD19 and CD81 expression in newly diagnosed myeloma patients reveals that a majority have malignant plasma cells with an immunophenotype similar to fully differentiated plasma cells, whereas, for a minority of patients, plasma cells have a phenotype similar to less differentiated stages [302, 303].

1.5.2. The plasma cell population in multiple myeloma

A recent study of disease heterogeneity used single-cell RNA sequencing and observed extensive subclonal structures for 10 out of 29 patients. They also used this technology to identify rare malignant plasma cells in asymptomatic patients with early disease and patients with minimal residual disease after previous antimyeloma therapy [303]. Thus, clonal heterogeneity seems to be relatively common, and this heterogeneity (including genetic heterogeneity) seems to be reflected in the gene expression profiles.

A more detailed study of the plasma cell phenotype was performed by Robillard *et al.* [304]. They report that the predominant myeloma plasma cell immunophenotype lacks CD19 expression but shows increased expression of either CD56 or CD28. This is different from normal bone marrow plasma cells, which have high levels of CD19 alone or CD19 together with CD28/CD56. However, normal bone marrow also includes plasma cells that express one or both of CD28 and CD56 without CD19, or are completely devoid of all three surface markers. These observations clearly illustrate the

polyclonal heterogeneity of the normal bone marrow plasma cell population [304], whereas myeloma patients have a clonal bone marrow plasma cell population. The differential stage of this clonal plasma cell population may even have a prognostic impact regarding progression-free and overall survival rates. The presence of less mature plasma cells seems to be associated with an adverse prognosis, and later chemotherapy may represent a selection pressure with the preferential proliferation of a myeloma clone representing a distinct differentiation stage that is different from the initial majority of myeloma plasma cells [302]. Such myeloma plasma cell dynamics will usually favour the dominance of a less mature plasma cell phenotype compared to the dominant phenotype in the initial cell population

Development and progression of multiple myeloma: Multiple myeloma is characterised by the proliferation of abnormal plasma cells secreting a monoclonal immunoglobulin, also referred to as monoclonal protein or M protein [289, 305, 306]. The most important complications, also referred to as end-organ damage, are renal failure, bone marrow failure, especially with anaemia and, possibly, other cytopenias, hypercalcemia, and bone disease leading to skeletal pain and, possibly, pathological fractures. The therapeutic intention for most myeloma patients is disease stabilisation rather than a cure; as will be described below, this is also true for younger patients below 65–70 years of age who receive autologous stem cell transplantation [307-311]. The indications for treatment will usually be the development of end-organ complications.

Multiple myeloma can be preceded by MGUS [297] or solitary plasmacytoma [289]. MGUS can be a non-IgM, IgM, or light chain type [289]. The M-component should generally be below 30 g/L; for the light chain MGUS, the free light chain ratio is abnormal with increased levels of the involved light chain [289]. The number of clonal plasma cells in the patient's bone marrow should be <10%, and there should be no end-organ damage, soft tissue plasmacytomas, or evidence of lymphoproliferative disease or circulating plasma cells. [295]. The overall annual progression rate of non-IgM MGUS is 1% [289]. Finally, patients with MGUS have an increased risk of fractures, and there is an increased prevalence of MGUS among osteoporosis patients [312].

Solitary plasmacytomas are localised to bone or soft tissue, and they are defined as biopsy-proven monoclonal plasma cell lesions without evidence for end-organ damage, clonal plasma cells, or less than 10% clonal cells in the bone marrow [289]. The 3-year progression rate to multiple myeloma is 10% for plasmacytomas without clonal plasma cells in the bone marrow, 60% for bone plasmacytomas, and 20% for soft tissue plasmacytomas [289].

MGUS has progressed to either smouldering multiple myeloma or multiple myeloma when bone marrow plasma cells exceed 10% [289, 313]. Smouldering myeloma is defined by the following two criteria [289]. First, serum IgG or IgA exceeds 30 g/L, urinary monoclonal protein exceeds 0.5 g/24 hours, or clonal bone marrow plasma cells are 10–60%. Second, the absence of myeloma-defining events or amyloidosis is required [313]. Smouldering multiple myeloma has a 10% overall annual risk of progression to multiple myeloma, but this risk varies considerably between patients. The following factors are associated with an increased risk of progression [314-318]:

- A high percentage of bone marrow plasma cells.
- A highly abnormal serum free light chain ratio is associated with a high risk of progression in some studies.
- Increasing paraprotein levels or the presence of Bence-Jones proteinuria at the time of diagnosis.
- Circulating plasma cells.
- Immunoparesis with hypogammaglobulinemia.
- The genetic abnormalities t(4;14), del17p, +1q24, and hyperploidy.
- Increasing skeletal lesions.

More detailed criteria for progression have been defined for several of these risk factors, and prognostic models based on two or three of these factors have also been developed [289, 317].

The definition of multiple myeloma is generally based on (i) bone marrow plasma cells exceeding 10–60%, (ii) biopsy-proven bone or extramedullary plasmacytoma and either one end-organ damage (i.e., at least one of the four; hypercalcemia, renal insufficiency, anaemia or bone lesion) or at least one biomarker of malignancy (i.e., a bone marrow

plasma cell percentage of at least 60%, a highly abnormal free light chain ratio, or more than one skeletal lesion). More detailed diagnostic criteria for each of these events/biomarkers have been defined [289].

Equilibrium between osteoblastic and osteoclastic activity is skewed from normal bone maintenance toward bone loss during myeloma progression, and myeloma-induced osteolysis is caused by a disease-driven increase in osteoclastic activity [319, 320]. The disease will often start as a single or a few plasma cell tumours and later progress with the development of new osteolytic lesions [321, 322]. For some patients, the development of aggressive plasma cell leukaemia will be the final event [321, 323]; malignant plasma cells then migrate into circulation and cause a $\geq 20\%$ increase in circulating plasma cells with a risk of generating plasmacytomas at new locations outside the bone marrow [324].

A recent study described a possible role of the NLRP3 inflammasome and its downstream cytokines in the development of myeloma [325]; such a role in myeloma pathogenesis for the cytokine network and immunocompetent cells has also been suggested by immunogenetic studies [326]. These observations further suggest that immunocompetent cells, immunogenetic factors, or both are involved in the pathogenesis of multiple myeloma.

As described, above $\geq 10\%$ clonal bone marrow plasma cells, along with one or more events attributed to the underlying plasma cell proliferative disorder, are diagnostic criteria for multiple myeloma. The other events are end-organ damage, an involved:uninvolved serum free light chain ratio of ≥ 100 , more than one focal (e.g., skeletal) lesion on MRI examination, or clonal bone marrow plasma cells higher than 60% [289].

1.5.3. End organ damage in multiple myeloma: diagnostic criteria and indications for disease-stabilising treatment

End organ damage events in multiple myeloma include hypercalcemia, renal insufficiency, anaemia, and bone lesions [289]. First, hypercalcemia is defined as serum calcium values at 0.25 mM/L above the upper-normal limit or above 2.75 mM/L. Second, renal insufficiency is defined as creatinine clearance less than 40 mL/minute or

serum creatinine values higher than 177 mM/L. Third, anaemia is defined as haemoglobin values at least 20 g/L below the lower normal limit or below 100 g/L. Finally, bone lesions are defined as one or more osteolytic lesions detected on skeletal radiography, computed tomography (CT), or positron emission tomography (PET)-CT [289].

The standard recommendation is that patients with $\geq 60\%$ plasma cell involvement on marrow examination should be diagnosed and treated as having multiple myeloma irrespective of whether the CRAB criteria are fulfilled [289]. The reason is that 90% of these patients will progress to symptomatic disease within two years, and the median time to progression is only 7 months [289]. The percentage of plasma cells can then be based on bone marrow aspirate or biopsy examination, and if there is a discrepancy, the higher of the two values should be used [289]. The prognostic importance of the high bone marrow plasma cell percentage was first demonstrated in a small study [327] but was confirmed [327, 328].

Genetic abnormalities in multiple myeloma: Many myeloma patients show clonal heterogeneity with multiple subclones early in the disease, and this heterogeneity may reflect genetic heterogeneity [329, 330]. Certain abnormalities are regarded as primary or disease-initiating (e.g., translocations involving the IgH locus), whereas other abnormalities are regarded as secondary events associated with disease progression [331]. A wide range of genetic abnormalities has been detected in myeloma patients. First, translocations involving the immunoglobulin heavy chain gene (IgH) on chromosome 14q32 have been detected in up to 65% of patients [318, 330]. The two most common translocations are t(11:14) and t(4:14), while t(14:16) is less frequent [332]. Second, several chromosomal deletions have been detected in myeloma cells; the single most important abnormality is del17p13, which is associated with shorter survival and an adverse prognosis [333]. Finally, hyperdiploidy is detected in up to 60% of patients. The most important clinical characteristics of common genetic abnormalities are summarised in Tables 9 and 10 [314, 315, 329, 334].

An important question is whether the prognostic impact of a certain abnormality is influenced by the biological or genetic context in the myeloma cells or whether a factor

has a prognostic impact only if the patient receives a certain type of treatment. This question has been addressed for t(11;14), which has been regarded as a standard risk prognostic marker based on studies before the introduction of current therapies [335]. This study included 365 patients with t(11;14). These patients were compared with 730 matched control patients; 132 of the controls had non-(11;14) translocations, and 598 had no translocation. The median overall survival for these three patient subsets was 74, 50, and 104 months; these differences were statistically significant when excluding patients with the adverse 17p abnormality, for patients younger than 65 years of age, and patients receiving the present novel agent-based induction. However, the overall survival for patients with t(11;17) was reduced by advanced age, the presence of the 17p abnormality, and an advanced disease stage. This example illustrates that the prognostic impact of defined genetic abnormalities may be modified by the clinical or genetic context, as well as the overall situation of the patient, reflected in the International Scoring System (often referred to as ISS, based on serum β_2 -microglobulin and albumin serum levels), but in this case, the type of treatment did not seem to have a major impact. A recently published Revised-ISS system incorporates genetic abnormalities into the prognostic evaluation of newly diagnosed myeloma patients and is based on the original parameters: β_2 -microglobulin and albumin, the karyotype (i.e., the presence of genetic abnormalities), and serum lactate dehydrogenase levels [336]. This should be regarded as a simple and updated prognostic classification system suitable for routine clinical practice.

Another important question is whether the efficiency of certain antimyeloma drugs depends on the general clinical and biological context (i.e., the overall evaluation, as reflected in the Revised-ISS) or is mainly dependent on certain clinical factors and, especially, certain genetic abnormalities. This question has been addressed for the amplification of chromosome band 1q21. One study found that treatment including thalidomide improves the survival of patients lacking 1q21 amplification but not patients with the 1q21 amplification [337]. Another study reported that proteasome inhibitors, as well as immune-modulatory drugs (IMiDs), did not improve survival for patients with amplification 1q21, but these drugs were able to improve outcomes for myeloma patients with other abnormalities [338].

Table 9. The prognostic impact of selected genetic abnormalities in multiple myeloma [317, 318].

Abnormality	Frequency in newly diagnosed myeloma	Prognostic impact
del17p13	5-15%	<ul style="list-style-type: none"> • The most important single marker. • Negative prognostic factor or treatment based on proteasome inhibitors and IMiDs, but pomalidomide may be more effective. • Adverse prognosis, both with regard to progression-free and overall survival. Proteasome inhibitors can improve survival. Adverse prognosis for all IMiDs. • Favourable prognosis. • Conflicting results, but a negative impact has been described in certain studies. • Gain: Adverse prognosis both with regard to progression-free and overall survival both for proteasome inhibitor and ImiD-based therapy. May be directly implicated in resistance to bortezomib. • del1p32: Adverse prognosis. • Hyperdiploidy usually consists of numerical gains but with a few structural changes; usually relatively good survival, but the impact differs between abnormalities. The prognostic impact differs even between trisomies; trisomy 21 impairs but trisomy 3 and 5 seem to improve overall survival. • Standard prognosis unless associated with a documented negative prognostic markers. • May neutralise the adverse impact of del17 or t(4;14). • Shorter survival of patients with combined del(13q14) or del(1p32) abnormalities, decreased survival when two adverse abnormalities are present.
t(4;14)	15%	
t(11;14)	20%	
t(14;16)	2-3%	
Gain 1q21	30-40%	
Del(1p32)		
Hyperdiploidy or odd chromosomes	60%	
Number of genetic abnormalities		

To conclude, karyotyping should be regarded as an important tool for the prognostic evaluation of multiple myeloma patients, but it should only be a part of this evaluation, combined with other prognostic parameters. Genetic analyses may also become increasingly important to guide antimyeloma therapy in future clinical practice.

Diagnostic tools in multiple myeloma: Detailed recommendations for tools used in the diagnosis and monitoring of multiple myeloma were recently published [318]. These recommendations are based on the classification systems and biological characteristics described above. Among the most important recommendations are (i) the use of both bone marrow smears and biopsies to estimate the percentage of plasma cells in bone marrow, (ii) the use of an extended flow cytometric panel to distinguish between normal/reactive and monoclonal plasma cells, (iii) the use of karyotyping for extended prognostic evaluation, and (iv) the use of the Revised-ISS for prognostic evaluation. Guidelines for skeletal evaluation by imaging are also given [339, 340]. Although several studies suggest the analysis of gene expression profiles in malignant plasma cells

can be used for prognostic subclassification possibly in combination with the Revised-ISS [341-343], the guidelines do not recommend the use of this parameter for the diagnosis or monitoring of myeloma patients in routine clinical practice.

Table 10. Associations between genetic abnormalities and survival in a study [314] of 351 patients with smouldering myeloma.

Patient characteristics	Median progression-free survival	Overall survival
Genetic abnormalities		
t(4;14)	28 months*	105 months*
t(11;14)	55 months	147 months
Trisomies alone	34 months	
del17p	24 months	
Other abnormalities	55 months	
No detectable abnormality	Not reached	
All patients		135 months
Defined risk groups		
1. High risk: del17p, t(4;14)	28 months	51 months
2. Intermediate: trisomies	34 months	77 months
3. Standard-risk: t(11;14), t(14;16), t(14;20), trisomy+IgH translocations.	55 months	86 months
4. Low-risk: No cytogenetic abnormalities detected	Not reached	112 months

* Significantly different from t(11;14). The study included 154 patients (43.9%) with trisomies, 127 (36.2%) with IgH translocations, 14 (4%) with both trisomies and IgH translocations, 53 (15.1%) with no detected abnormalities, and 3 (0.9%) with monosomy13/del(13q) alone. The patients with IgH translocations included 57 patients with t(11;14), 36 with t(4;14), 11 with musculoaponeurotic fibrosarcoma (MAF) translocations and 23 with other or unknown translocation partners. The patients were included in the period of January 1991 to June 2010. A total of 219 patients showed progression during the observation period. The median period of follow-up was 82 months, and the patients' median age was 63 years at the time smouldering myeloma was diagnosed. The risk groups were defined based on the observations in the present study.

Treatment of multiple myeloma patients: Several drugs are now available or are in late clinical trials for the treatment of multiple myeloma, including second-generation proteasome inhibitors and IMiDs, monoclonal antibodies, histone deacetylase inhibitors, checkpoint inhibitors, and small molecules, have been included in a large number of drug combinations [344]. The treatment landscape and patient survival rates have changed over the past two decades, and patients now have improved median survival of 8–10 years [344]. A complete review of this complex scientific field is outside the scope of this thesis, as the focus is on the use of IMiDs in the treatment of multiple myeloma

Autologous stem cell transplantation in the treatment of multiple myeloma:

Autologous stem cell transplantation is currently considered the standard of care for fit,

newly diagnosed myeloma patients [344]. It was previously considered for patients below 65 years of age but is now also considered for fit elderly patients [344]. In elderly patients, one should consider reducing melphalan conditioning therapy and performance status, comorbidity should be carefully considered before the final decision is made. Pretransplant induction therapy usually consists of 3–6 cycles, the goal of which is to achieve disease control and decrease symptom severity, allowing successful stem cell collection [344]. The European guidelines state that the current standard should be a three-drug bortezomib-based combination, and most of these regimens include a steroid as the second drug [344]. The third drug was previously a conventional cytotoxic drug, such as doxorubicin or cyclophosphamide. However, the third drug can also be an IMiD; thalidomide was initially used, but lenalidomide seems to be more effective at achieving complete remission. However, a major concern when combining bortezomib with thalidomide or doxorubicin is the risk of thromboembolism [345]. The importance of a deep response provides the rationale for choosing a regimen with a strong antimyeloma effect and acceptable toxicity. The second-generation proteasome inhibitor carfilzomib has also been tried in combination with a steroid and either thalidomide or lenalidomide. This combination seems to achieve a complete remission rate of 18–24%. Despite these more recent results, the European guidelines conclude that standard treatment is a combination of bortezomib, a steroid, and IMiD or chemotherapy.

The recommended conditioning therapy is melphalan 200 mg/m²; this dose is reduced to 100–140 mg/m² for patients with renal failure, and a reduction should also be considered for patients above 65 years of age [344]. Conditioning regimens that include novel agents have only been investigated in single-arm studies [344]. Double transplantation should be considered for patients with high-risk disease [344]. The European guidelines recommend single transplantation to be considered for standard-risk patients with a response to the induction treatment better than a very good partial response [344].

Posttransplant consolidation therapy can be considered to increase the quality of the response [344]. Similar to the induction phase, the combination of a second-generation proteasome inhibitor and IMiD can be considered for maintenance therapy. Another

alternative is to use the original induction treatment. Consolidation can be administered in 2–3 cycles of a three-drug regimen [344].

Maintenance therapy has also been investigated. The optimal maintenance therapy should prolong remission without reducing the patient's quality of life and should not increase the development of multi-resistance or reduce overall survival [344]. The European guidelines recommend lenalidomide or thalidomide for two years or until progression; bortezomib is an alternative for high-risk patients [344]. Multiple retrospective studies have shown that continued chemosensitivity and remission duration after the first autotransplantation are the two most important prognostic factors for long-term disease stabilisation after salvage autotransplantation [344]. Several studies have also demonstrated that salvage transplantation is safe and has acceptable toxicity, and even a third transplantation may be possible for some patients [344]. The European guidelines recommend considering salvage transplantation after the first relapse in patients who have a minimum duration of remission of at least 18 months after the first autotransplantation; this cutoff can be extended to 24 months for patients receiving consolidation or maintenance therapy [344]. The patients should then receive pretransplant induction therapy with a triple combination, possibly including IMiD.

Allogeneic stem cell transplantation in multiple myeloma: The role of allogeneic stem cell transplantation is controversial in multiple myeloma, but the available studies suggest it could be considered for young patients with high-risk disease, who usually experience poor outcomes and easily develop chemoresistance [344].

First-line treatment of patients who cannot receive stem cell transplantation: Several therapeutic alternatives are possible for patients who are elderly or otherwise unsuitable for autologous stem cell transplantation [346]. One alternative is a thalidomide-based regimen (i.e., melphalan combined with prednisolone and thalidomide) or thalidomide in combination with cyclophosphamide and dexamethasone. However, major problems with both regimen are thromboembolic events and infections [345]. Lenalidomide- and carfilzomib-based regimens have also been suggested. Thus, IMiDs can also be considered part of the first-line treatment for these patients.

Treatment of relapsed multiple myeloma—alternatives to salvage autotransplantation: These patients should generally receive treatment with triple-drug combinations [347]. A wide range of combinations has been investigated in clinical studies, including the following combinations:

- Patients experiencing their first relapse after discontinuing treatment may receive treatment with triple combinations, including carfilzomib or daratumumab [348].
- Frail patients experiencing their first relapse after discontinuing treatment may be treated with ixazomib, lenalidomide, and dexamethasone or, alternatively, elotuzumab, lenalidomide, and dexamethasone [348].
- Patients experiencing their first relapse on maintenance therapy may also be treated with triple-drug combinations, similar to patients with relapse after discontinuation [347, 348].
- Patients experiencing a lenalidomide-resistant relapse may be treated with triple combination therapy: daratumumab, bortezomib, and dexamethasone [348]. Such patients may also respond to pomalidomide-based regimens [349, 350].
- An intensive regimen based on a proteasome inhibitor, IMiD, dexamethasone, and combinations of conventional cytotoxic drugs followed by autologous stem cell transplantation has been suggested [348]. Daratumumab or anthracycline-based regimen may be a less toxic alternative for patients with advanced or aggressive disease [348].
- Other combinations with less toxicity suggested for elderly (>75 years of age) and frail patients unfit for transplantation are karfilzomib and dexamethasone or daratumumab, bortezomib, and dexamethasone [348]. The choice of regimen should be based on the treatment received before the development of resistance.
- Lenalidomide combined with carfilzomib or ixazomib has been tried [348].
- It has been suggested that exceptional frail patients may benefit from steroids in combination with daratumumab, elotuzumab, or ixazomib, but one should remember that high-quality data collected after third-line therapy in elderly patients are unavailable [348].

- Even low-dose melphalan or cyclophosphamide combined with dexamethasone may be an alternative for elderly or unfit patients with advanced disease [348].

To conclude, the treatment of patients with advanced disease needs to be individualised; many different combinations have been suggested, and IMiDs are included in many of these alternatives. Documented resistance, as well as performance status and comorbidity, should be carefully considered [351]

1.6. ACUTE MYELOID LEUKAEMIA

Acute myeloid leukaemia (AML) is a heterogeneous bone marrow malignancy. Acute promyelocytic leukaemia (APL) is an AML variant with specific genetic abnormalities and requires specific treatment. In this thesis, we refer to the non-APL variants of the disease when we use the term AML.

1.6.1. The diagnosis of AML

The diagnosis of AML is usually made by the morphological detection of at least 20% immature myeloblasts in the bone marrow, except for AML accompanied by the chromosomal abnormalities t(15;17), t(8;21), inv(16), or t(16;16) and certain subsets of erythroleukemia [352, 353]. Flow cytometric immunophenotyping to characterise the expression of surface and cytoplasmic molecules is used to verify and characterise the blasts' myeloid phenotype in greater detail. Detailed flow cytometric characterisation may be used later to evaluate the treatment response by detecting minimal residual disease [352, 353]. Flow cytometric analysis is also essential for the diagnosis of AML with minimal differentiation, acute undifferentiated leukaemia [353], and acute megakaryoblastic leukaemia [352, 353].

The prognostic evaluation of AML patients: AML patients are usually classified as having a favourable, intermediate, or adverse prognosis based on their genetic abnormalities [352]. A summary of this genomic classification, according to European Leukemia Net (ELN), is presented in Table 11. Favourable prognosis means long-term AML-free survival of 60–70%, while intermediate is 40–50%, and adverse is approximately 20% long-term survival. Thus, there is a need to identify other pretreatment prognostic factors to improve this classification [352]. The genetic

classification reflects the risk of chemoresistant leukaemia relapse for patients receiving intensive and potentially curative treatment.

Table 11. The ELN prognostic classification of AML based on karyotype and molecular genetic analysis (APL is omitted) [352].

Prognostic subset	Abnormality
Favourable	<ul style="list-style-type: none"> t(8;21)(q22;q22.1); RUNX1-RUNX1T1 inv(16)(p13.1;q22) or t(16;16)(p13.1;q22); CBFβ-MYH11 Mutated NPM1 and no FLT3-ITD or Flt3-ITD^{low allelic ratio} (defined as ratio <0,5*) Biallelic mutated CEBPA
Intermediate	<ul style="list-style-type: none"> Mutated NPM1 and mutated FLT3-ITD^{high allelic ratio} (ratio ≥0,5*) Not mutated NPM1 and no FLT3-ITD or FLT3-ITD^{low allelic ratio}* (no adverse cytogenetic abnormalities) t(9;11)(p21.3; q23.3); MLLT3-KMT2A (MLL); detection of t(9;11) is decisive if rare, high-risk mutations are also detected. Cytogenetic abnormalities not classified elsewhere.
Adverse	<ul style="list-style-type: none"> t(6;9)(p23;q34.1); DEK-NUP214 t(v; 11q23.3); KMT2A (MLL) rearranged t(9;22)(q34.1;q11.2); BCR-ABL Inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EV11) -5 or del(5q); -7 -17/abnl(17p). Complex karyotype; defined as ≥3 chromosomal abnormalities but absence of t(8;21), inv(16), t(16;16) and t(9;11). Monosomal karyotype, defined as single monosomy (not the loss of X or Y), along with at least one additional monosomy or an additional chromosomal abnormality other than core-binding factor AML. Non-mutated NPM1 with FLT3-ITD^{high allelic ratio}. Mutated RUNX1 or ASXL with no concomitant favourable classification. TP53 mutation (often, concomitant complex cytogenetic abnormalities or monosomal karyotype).

* FLT3-ITD allelic ratio defined by ELN as FLT3-ITD/ FLT3-WT.

Age is also associated with decreased survival, which may, at least partly, be caused by the effect of age on the patient's ability to tolerate chemotherapy, a low frequency of favourable cytogenetic abnormalities, and a higher frequency of secondary AML [352]. Finally, response to the first induction cycle is also an important prognostic parameter; the need for more than one cycle to induce complete haematological remission is associated with an adverse prognosis [354].

Approximately 60% of AML patients have cytogenetic abnormalities, as certain abnormalities show a strong association with myelodysplastic syndrome (MDS) and are regarded as a marker for previous MDS [352]. Certain genetic abnormalities detected in

MDS are also associated with progression to secondary AML (e.g., del 17p, TP53 gene mutation, and mutations in RUNX1, ETV6, EZH2, and ASXL1). Mutated SRSF2, EZH2, and ASXL1 can also identify patients with primary myelofibrosis and at high risk of developing secondary AML [352, 353]. AML patients with such genetic abnormalities indicating a transformed haematological malignancy have an adverse prognosis [352]. In contrast, *NPM1* mutations, along with CBF and *KMT2A* rearrangements, are highly specific to *de novo* AML [352].

Complex karyotypes occur in 10–12% of patients, are associated with poor outcomes, and are defined as the presence of 3 or more chromosomal abnormalities in the absence of the favourable abnormalities t(8;21), inv(16) or t(16;16), and t(15;17) [353]. Cytogenetic evidence for clonal heterogeneity is associated with a complex karyotype and will further aggravate the prognosis [355]. A monosomal karyotype is also associated with very low AML-free survival [352]. As stated above, the high frequency of unfavourable genetic abnormalities in elderly patients contributes to their decreased survival [353]. Patients with genetic abnormalities not classified elsewhere are classified as intermediate; this group includes patients for whom an intermediate prognosis has been documented and uncommon abnormalities where the prognostic impact is unknown [353].

A large number of mutations has been detected in AML [356]. The prognostic impact of NPM1, CEBPA, and FLT3 is generally accepted, whereas the possible impact of FLT3 tyrosine kinase domain (TKD) mutations at codons D835 and I836 remains controversial [352, 353]. However, other mutations may also have a prognostic impact. The ELN classification includes molecular genetic abnormalities that should be part of the routine clinical evaluation of AML patients [352, 353], but it is still regarded as controversial whether, for example, the detection of a KIT mutation will worsen the prognosis of patients with t(8;21) or inv(16)/t(16;16) [352, 353].

1.6.2. Treatment of acute myeloid leukaemia

AML can only be cured by intensive chemotherapy, possibly combined with autologous or allogeneic stem cell transplantation [352]. This therapy has a relatively high

treatment-related mortality but can be used in patients up to 70–75 years of age if they do not have severe comorbidity [352].

Induction therapy: The aim of the initial induction treatment is to achieve disease control. The most common induction therapy is an anthracycline (e.g., daunorubicin 90 mg/m² daily for 1–3 days) combined with cytarabine infusion for 1–7 days; complete haematological remission (i.e., no morphological signs of AML in the bone marrow and normal cell counts in peripheral blood) is achieved in 60–80% of younger adults and in 40–60% of patients older than age 60 [352].

Consolidation therapy: Additional postremission consolidation is needed to eradicate residual disease, and several alternatives exist for this treatment. First, consolidation based on repeated cycles with high-dose cytarabine (3000 mg/m² twice daily on days 1, 3, and 5) is commonly used for patients younger than age 60 who have a favourable prognosis. Second, intensive treatment based on the combination of two or three antileukemic agents, usually including cytarabine, is another possibility. The consolidation will usually include two or three separate cycles. Third, autologous stem cell transplantation can be used for consolidation, especially in patients with a favourable prognosis. Finally, allogeneic stem cell transplantation can also be used as consolidation. This is the most effective but also the most toxic antileukemic treatment, and it is used especially for patients younger than age 70–75 without severe comorbidity and with an available suitable stem cell donor (i.e., an HLA-matched related or unrelated donor or, if possible, an HLA-identical sibling donor). Allogeneic stem cell transplantation is associated with early treatment-related mortality of 10–20%, and there is also a risk of later severe morbidity. This therapeutic strategy is used both for AML and for other haematological malignancies with a high risk of early chemotherapy-resistant relapse (e.g., high-risk MDS and high-risk lymphoproliferative diseases).

New strategies for the treatment of AML: Several new strategies are now available for the treatment of AML. First, midostaurin has been added to intensive chemotherapy and seems to cause a limited improvement in long-term AML-free survival in patients not receiving allogeneic stem cell transplantation [357, 358] Second, gemtuzumab ozogamicin (GO) is an antibody-toxin (calicheamicin) conjugate that targets

CD33⁺ AML, and recent studies suggest that adding GO is associated with improved relapse-free survival, especially in patients with a favourable prognosis and, possibly, intermediate-risk patients [359]. This drug may also be used in elderly patients up to age 70 [360, 361]. Third, CPX-351 is encapsulated in nanoscale liposomes of cytarabine and daunorubicin at a synergistic 5:1 molar ratio; this agent may become useful even for high-risk patients and may also be used in elderly patients [362, 363]. Finally, inhibitors of mutated IDH are also used in the treatment of this patient subset [364].

The possible use of IMiDs in AML: Lenalidomide has been considered for use in AML, but it is not a part of the generally accepted treatment protocol [365]. It has been tried in combination with conventional intensive chemotherapy and as monotherapy after allogeneic stem cell transplantation. As expected, posttransplant treatment was associated with increased graft versus host activity [366].

1.7. IMMUNEMODULATORY DRUGS

Thalidomide has beneficial effects against leprosy [367], as well as anti-angiogenic effects [368]. The beneficial effect against myeloma was detected two decades ago, and both thalidomide and its analogues, lenalidomide and pomalidomide, are currently used in myeloma treatment [369-372]. These drugs have antiangiogenic, immunomodulatory, anti-inflammatory, and antiproliferative effects [371, 372], and they are typically used in combination with steroids or proteasome inhibitors in myeloma treatment [373]. IMiDs have contributed to the improved overall survival of myeloma patients over the past two decades [374]

Cereblon is a direct target of IMiDs and has important intracellular effects:

Cereblon is a molecular target of IMiDs, and the therapeutic effects of IMiDs mediated by cereblon were reviewed recently [375]. Lenalidomide and pomalidomide bind more strongly to cereblon than thalidomide [376]. The following effects seem to contribute to the anticancer effects of IMiDs [375]:

- Altered expression by c-myc and p21.
- Lenalidomide binding induces ubiquitination and degradation of the transcription factors IKZF1 and IKZF3; both these factors are involved in B-cell maturation.

- Degradation of casein kinase 1 α ; this effect may lead to p53 activation, which seems to be a lenalidomide-specific.
- Altered epigenetic regulation partly mediated by altered JAK-STAT signalling.
- Altered cell cycle regulation.

Thus, IMiDs have a common molecular target, but they differ in their binding to this target and in the intracellular consequences of cereblon binding. As stated above, they probably also have cereblon-independent effects. These differences may explain why myeloma patients developing resistance to one IMiD may not be resistant to the antimyeloma effects of other IMiDs [349, 350], and it may also explain the differences in toxicity profiles between different IMiDs [313].

Functional effects of IMiDs mediated via cereblon: Cereblon binding is important to several effects of IMiDs, and these effects may contribute to these drugs' anticancer effects [375, 377]:

- There seems to be dysregulation of various immunocompetent cells in many cancer patients (e.g., B-cells, NK cells, T-cells, and dendritic cells), along with an increase in the number of immunosuppressive cells (e.g., regulatory T- and B-cells and MDSCs; see [369]). Quantitative and qualitative monocyte defects have been reported, including the reduced release of proinflammatory cytokines [378-386]. IMiDs may improve the balance between effector functions and immunosuppressive activity.
- IMiDs also seem to modulate cell adhesion mechanisms.
- IMiDs' antiangiogenic effects are linked to their cytokine modulation, which probably plays an important role in their anticancer effects.
- IMiDs alters epigenetic regulation of gene expression, and such mechanisms seem to be important to its cytokine-modulating effects.
- IMiDs alters oxidative stress with the generation of reactive oxygen species.
- IMiDs seem to form a complex with damage-specific DNA binding protein 1 (DDB1) and cereblon [375]; IKZF1 and CK-1 α degradation, as well as IL-2 up-regulation, are cereblon-dependent. The costimulation of proinflammatory cytokine release by activated T-cells thus seems to be a cereblon-mediated effect.

- Animal models suggest that IMiDs' therapeutic effect may also be induced through a cereblon independent pathway [387], but cereblon seems to be critical to the effects of IMiDs on ubiquitination and the stability of cereblon-interacting proteins [388]. The cereblon-independent effect seems to contribute to IMiDs' effects on inflammation/immunity.
- IMiDs can activate caspase-8 and, hence, have pro-apoptotic activity [389].

Thus, IMiDs have complex intracellular effects, as well as complex effects on interactions and communication between cells.

1.7.1. Anticancer effect of IMiDs – the chronic lymphocytic leukaemia experience

As described in detail in recent reviews, lenalidomide is regarded as a possible therapeutic target not only in multiple myeloma but also in other haematological malignancies, including AML and chronic lymphocytic leukaemia (CLL) [390-394]. The anticancer effects of lenalidomide have been studied in greater detail, especially in chronic lymphocytic leukaemia (CLL) [395], and several of the antileukemic mechanisms described in these studies may also be relevant to other haematological malignancies (Table 12) [152, 360, 376, 396-408]. The most important conclusion from CLL research is that IMiDs' anticancer effects are very complex, which is unsurprising considering the complex effects of these drugs on intracellular regulation and extracellular crosstalk between a wide range of cells. First, lenalidomide seems to have non-cytotoxic, antiproliferative, and direct effects on CLL cells, whereas the observation of complete AML remission after lenalidomide monotherapy suggests that the drug can also have direct cytotoxic effects on malignant cells. A direct antileukemic effect favours the use of lenalidomide to treat various malignancies.

Second, lenalidomide seems to increase the reactivity of several T-cell subsets, B-cells and NK cells. These cells may also be defective in malignancies other than CLL, and

Table 12. The use of lenalidomide in chronic lymphocytic leukaemia (CLL)— the effect of immunocompetent cells and their interactions with leukemic cells.

Effects on CLL cells
<ul style="list-style-type: none"> • Lenalidomide has no direct cytotoxic effect on leukemic cells [396], but it has an antiproliferative effect by binding to cereblon and, thereby, the induction of p21WAF1/Cip1. This effect is also seen <i>in vivo</i> during treatment with lenalidomide 5 mg daily [397]. Cereblon mediates antiproliferative effects for both lenalidomide and pomalidomide in malignant cells [376].
T-cell effects
<ul style="list-style-type: none"> • Decreased SOCS1 expression in CD4 and CD8 T-cells [398]. • Increased T-cell production of IL2, TNFα, and IFNγ, and this increased T-cell cytokine release is induced via cereblon by lenalidomide and pomalidomide [376]. • Increased CD8⁺ T-cell cytotoxicity. • Pomalidomide activates RhoA and enhances F-actin formation, and GTPase activation increases IL2 release [399]. • Lenalidomide and pomalidomide inhibits FOXP3 positive, CTLA4 positive, and CD25^{high} CD4⁺ T regulatory cell induction and proliferation, as well as inhibit their suppressive effect [400]. • CLL cells cause the global inhibition of CD11a/CD18 (αLβ2)-mediated T-cell migration through altered Rho GTPase activation signalling, adhesion, and motility, which is reversed by lenalidomide [402].
B-cells
<ul style="list-style-type: none"> • Lenalidomide seems to reverse the humoral immune defect in CLL [403].
NK cells
<ul style="list-style-type: none"> • Increased NK cell cytotoxicity through the enhancement of NK cell immune synapse formation [400, 408]. • Decreased SOCS1 expression [398].
Monocytes
<ul style="list-style-type: none"> • Lenalidomide inhibits the differentiation of nursing-like cells into an M1 phenotype with the high release of CCL2, IGF1, CXCL12, and HGF1 and has the ability to support T-cell proliferation [152, 404]. • Pomalidomide activates RhoA, enhances F-actin formation, and increases cell migration [360]. • Inhibit TLR4-induced cytokine release [400, 404].
Effects on the leukemic cell microenvironment
<ul style="list-style-type: none"> • Decreased VEGF and FGFb release and increased angiopoietin release [405] • Inhibited angiogenesis [405].
CLL-immunocompetent cell interactions
<ul style="list-style-type: none"> • Downregulated PD-1/PDL1 interactions between T-cells and leukemic cells. • Reduced nursing-like cell support to leukemic cells by downregulating CCL2, IGF1, CXCL12, and HGF1 [152]. • Impaired immunological synapse formation is reversed by lenalidomide[401]. • Lenalidomide after immunochemotherapy induces the long-term repair of T-cell immune synapses and improves the quality of T-cell responses [402, 406]. • Lenalidomide interferes with the expression of the costimulatory molecules CD40, CD80, CD83, CD86, and CD40L in CLL cells after <i>in vivo</i> treatment [396]. • Improved response to pneumococcal vaccines [407].

improved functioning through the use of lenalidomide may then enhance autologous immune reactivity against malignant cells. Third, lenalidomide alters the bone marrow microenvironment, and both AML and multiple myeloma should be regarded as bone marrow malignancies supported by nonleukemic stromal cells through local angioregulation, as well as the functions of local nursing cells (e.g., monocytes and endothelial cells) of bone marrow stem cell niches [409].

Finally, lenalidomide seems to favour antileukemic contact between immunocompetent and leukemic cells; it increases the expression of checkpoint molecules by leukemic cells and restores immune synapses. Defects in the formation of immune synapses are also observed in other haematological malignancies, such as human AML [409]. However, to the best of our knowledge, it is unknown whether lenalidomide or other IMiDs alter the AML cell expression of checkpoint molecules. Thus, although intensive conventional chemotherapy, as well as autologous and allogeneic stem cell transplantation, can alter the immune system and cause posttreatment CD4⁺ T-cell defects, patients receiving intensive chemotherapy have an operative immune system, and all immunomodulatory effects of lenalidomide described for CLL patients should be further investigated regarding their possible relevance in other (haematological) malignancies.

2. AIM OF THESIS

Monocytes are a heterogeneous cell population that can be classified into three main subsets. Monocytes are important for immunoregulation and have an important role in regulating inflammation, but they are also important for the support of normal cells and, possibly, leukemic haematopoiesis.

The main aim of this thesis was to explore the potential use of monocyte subset analyses in clinical settings, with a focus on haematological malignancies. This was done through three objectives: (i) to establish a standardised flow-cytometric method for analysing monocyte subset levels in venous peripheral blood, (ii) use this method to characterise monocyte reconstitution in patients with haematological malignancies receiving stem cell transplantation, and (iii) characterise the effects of IMiDs (i.e., anticancer agents used in the treatment of haematological malignancies) on human monocytes.

3. STUDY DESIGN, ETHICAL AND METHODOLOGICAL CONSIDERATIONS

3.1. STUDY DESIGN OF PAPERS I-IV

The study designs were selected to answer the research questions/aims in our respective studies [410]. The study design of papers I and IV are categorised within the concepts of basic studies and *in vitro* experimental studies. Paper I is a method development study investigating the improvement of flow cytometry and immunophenotyping methodology for monocyte subset characterisation. In paper IV, we investigate the cause-outcome relationships between dependent (i.e., cytokines and ECAR) and independent (i.e., IMIDs) variables. In contrast, papers II and III are prospective, observational longitudinal studies conducted on a cohort of consecutively recruited MM and AML patients, with strictly defined criteria for inclusion, and the control cohort were individuals recruited from healthy blood donors, matched for age and gender.

3.2. ETHICAL CONSIDERATIONS

All studies were conducted according to the Declaration of Helsinki and were submitted for review by the Regional Ethics Committee (REK Vest, Bergen, Norway: Paper I: 2015/1410, 2017/487; paper II: 2017/305, 2013/102; Paper III: 2015/1759, 2017/305, 2013/102; Paper IV: 2017/305, 2013/635). All samples were collected after written, informed consent. None of our research interventions involved a major risk or burden to the research subjects. Conventional venous sampling is not associated with a major risk or burden, and the sampling of the patients (i.e., AML and MM-patients) and healthy blood donors was often done at the same time as the clinical samples. Hence, there was no need for an additional needle insertion, only the collection of additional blood. Blood collection from subjects with pancytopenia should, however, be carefully monitored and minimised.

3.3. METHODOLOGICAL ASPECTS: THE PRE-ANALYTICAL PHASE

In laboratory medicine, pre-analytical concerns include sample collection, blood sampling tube additives, and the storage of blood samples [411]. Our findings in paper I regarding common pre-analytical [412-415] variables related to the (i) collection of insufficient sample volume, (ii) inappropriate sample tube types, and (iii) improperly stored samples, were important to the following papers, II–IV, as they helped establish

high quality standards and enhance data reproducibility. In our studies, phlebotomists collected blood samples in accordance with approved clinical protocols. All the patient material and the age- and gender-matched healthy control samples were collected in ACD-A anticoagulated blood sampling tubes and processed further within a specified time-period (papers II–III). Otherwise, for healthy donors, blood samples were collected in Li-Heparin, K₂-EDTA or ACD-A (paper I). Blood samples collected at the blood bank (papers I–IV) were first drawn into the diversion pouch, also called a sampling pouch, for blood collection bags before being transferred into blood sampling tubes (papers I–III) and submitted to the standardised flow cytometry protocol, or the blood bag was processed into buffy coats (paper IV) before being submitted to monocyte enrichment. Using a diversion pouch means that the initial flow of blood was directed into a pouch. In addition to serving as a temporary reservoir for whole blood for sampling, the redirection was also intended to prevent bacterial contamination from entering the primary blood collection bag [416, 417]. To avoid possible clot formation, one should collect blood samples from the sample diversion pouch within approximately four minutes per the vendor’s recommendations. The standard procedure used by the Haukeland University Hospital’s blood bank facility is to collect blood from the pouch within one minute. One could argue that there are differences in human blood components (e.g., monocyte numbers) when comparing standard blood collection procedures to redirection through a diversion pouch. However, to our knowledge, as well as in the literature, there are no such differences; the only exception is, perhaps, the risk of bacterial contamination.

3.4. METHODOLOGICAL CONSIDERATIONS: FLOW CYTOMETRY

Multicolour flow cytometry [418, 419] provides the possibility to inspect populations of cells at a single-cell level, allowing cell populations to be separated by morphology (scatter properties) and fluorochrome-conjugated antigen-specific antibodies (e.g., immunophenotyping) or specific fluorescent dyes (e.g., SYTOX Red Dead Cell Stain for cell viability assessment). **BD FACS Verse**, the applied flow cytometer in this thesis, is equipped with three lasers: violet (405 nm with 40 mW optical power), blue (488 nm with 20 mW optical power) and red laser (640 nm with 40 mW optical power) and eight

fluorescent detection channels, along with forward scatter (FSC) and side scatter (SSC). For papers I–IV, the configuration was set to use all the three lasers; violet, blue, and red laser-detected in 2, 4, and 2 channels, respectively (see Table 13).

Table 13. Overview of fluorochrome-conjugated antibodies used in papers I–IV related to FACSVerse configuration and a simplified overview of respective CD expression by human cells.

Antibody specificity	Fluorochrome	Clones	Laser	PMT Filters	CD marker expressed by
Anti-CD11b	V450	ICRF44	405 nm	nn	Monocytes, neutrophils, natural killer cells, granulocytes
Anti-CD45	V500	HI30	405 nm	nn	Monocytes, granulocytes, lymphocytes
Anti-CD14	Alexa 488	M5E2	488 nm	nn	Monocytes, neutrophils ^{low}
Anti-HLA-DR	PE	G46-6	488 nm	nn	Monocytes, DC, B-cells,
Anti-CD16	PerCPCy5-5	3G8	488 nm	nn	Monocytes, NK cells, Neutrophils
Anti-CD56	Alexa 647 / 700*	B159	640 nM	nn	NK-cells

*In paper I, a change in fluorochrome was necessary due to the viability assessment in panel C and the use of SYTOX Red Dead Cell Stain. Abbreviations: **PMT**, photomultiplier tubes

A standardised daily and monthly clean program was performed on the BD FACS Verse. Quality control routines and performance tracking (BD FACSuite™ CS&T research beads) indicated the flow cytometer performed well throughout our studies. In addition, our samples were run at a medium-to-low flow rate, which prevented a wider core stream, minimised data spreading (decreased CV for the population of interest), and hence, improved the quality of the data obtained. By applying appropriate compensation controls, spillover coefficients were determined and corrected for each experiment, taking into the account day-to-day variations. All compensations were performed by utilising BD™ CompBead Anti-Mouse Ig and a κ /Negative Control Compensation Particles Set. When compensation methods were compared using single-staining and beads, the generated compensation matrices were not significantly different from each other [420, 421] and had good accuracy in a 12-colour panel [422].

The selection of fluorochrome and CD-marker panels involves interlocked issues and is crucial for good quality data in flow cytometry. For instance, steric hindrance or fluorescence resonance excitation transfer between neighbouring fluorochromes (e.g., PE and PE-CY7, resulting in false PE-CY7 signals) could be problematic. In our panel (see Table 11), we minimised the use of tandem dye-conjugated antibodies. We could not, however, avoid the possibility of steric hindrance, as CD45, CD11b, HLA-DR, and

CD14 are all markers expressed in abundance and were used for the selection of monocytes in our gating strategy. However, in support of our targets and selected gating strategy, one recent publication presents optimised flow cytometry panels in which the monocyte panel does, indeed, contain anti - CD45, anti - HLA-DR, and anti - CD11 as a prerequisite for further monocyte gating (CD14⁺ and CD16^{-/+}) [423]. However, a more simple variant with HLA-DR⁺ as the only prerequisite was published recently [172]. We used a dump channel to avoid contamination by unwanted cell populations. Autessier *et al.* recommend using only one marker per channel to exclude populations when possible [422]. Our panel included CD56 for the exclusion of NK cells, which also express CD16 [165]. Moreover, in our studies, monocytes were sub-divided into three subsets based on CD16 and CD14 expression. This classification system has limitations, as CD14 seems to be expressed in a continuity, leading to subjective gating of the non-classical and intermediate subsets. CD16 expression is also changeable, making the identification of subsets difficult and impossible after, for example, cryopreservation (paper I). Several alternative markers for use in monocyte subset detection have been suggested (see Table 5).

The selection of specific antibody clones for the flow cytometry panel was based upon conducting an in-depth literature search and selecting the antibody clones most often used for the specific antigen of interest. One could argue that using this approach is a drawback in our studies. For example, newer and, perhaps, improved antibody clones may have been missed, and we cannot be sure that factors (e.g., temperature, ionic strength, pH levels, and incubation duration) affecting the antigen-antibody reaction are the same in our experiments or that such a selection can be made [424]. However, all our selected clones of antibodies are (i) known antibody clones used and published by other researchers in the field and (ii) properly titrated by us to find the optimal staining index for our experiments and the applied flow cytometer.

In conclusion, currently, within the flow cytometry methodology for monocyte characterisation, there seems to be (i) no agreement in the literature regarding which selection/exclusion markers should be used for proper monocyte subset identification and, hence, (ii) no agreement on standardising multicolour flow cytometry panels,

including, for instance, clones of antibodies and fluorochrome combinations. We have, however, taken measures in our studies to avoid several potential pitfalls of flow cytometry methodology.

3.5. METHODOLOGICAL CONSIDERATIONS: EXTRACELLULAR FLUX ASSAY

High throughput metabolic profiling by Seahorse extracellular flux assays made it possible to observe differences between quiescent, activated, and memory immune cells [425-427], evaluate the effects of changes in the cellular microenvironment [428-430], and assess the capability of drugs to alter metabolic phenotypes [431, 432]. As previously outlined, monocytes undergo concentration-dependent metabolic changes when activated. For instance, the LPS/TLR4-induced activation of monocytes may increase both glycolysis and oxidative phosphorylation [272-275]. Mitochondrial oxidative phosphorylation, measured as the oxygen consumption rate (OCR) [433, 434], is most likely a contributor to cellular ATP demand during monocyte activation. In paper IV, we assess whether immunomodulatory drugs (i.e., IMIDs such as thalidomide and analogues) display mitochondrial susceptibility; if they do, oxygen consumption is, thus, affected, and extracellular acidification increases accordingly as cells attempt to avoid mitochondrial insult through increased glycolytic flux [435].

XFe96 analysers measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells simultaneously in a 96-well plate format. The oxygen consumption rate is an established measure of mitochondrial function, while the extracellular acidification rate is an estimate of glycolytic activity under certain conditions.

In the following section, the principle of Seahorse XFe96 is briefly outlined. Pre-hydrated probes on the sensor cartridge are lowered into the wells of the 96-well plate, creating a microchamber in close proximity to the cellular monolayer, allowing differences in O₂ concentration (OCR: measured as picomol oxygen per minutes, pMol/min) and protons (ECAR: milli-pH units per minutes, mpH/min, are measured). The sensor cartridge also contains four injection ports that allow up to four stimulants or drugs to be injected into the wells of the cell plate in sequential order and the effect on OCR and ECAR to be measured in real-time.

In paper IV, Seahorse XFe Cell Mito Stress Test Kit as applied to provide information on mitochondrial function. Mitochondria are the power plants of cells, producing the majority of ATP through oxidative phosphorylation (OXPHOS), which refers to redox reactions (substrate oxidation) involving the electron transport chain and ATP synthase complexes. The kit contains the following compounds added sequentially in the following order through the injection ports: (i) oligomycin, (ii) carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP), and (iii) rotenon and antimycin A (see Figure 3 and its description for details of the reactions). Due to the added compounds, OCR was measured through the key parameters of mitochondrial function: (i) basal respiration, (ii) ATP production, (iii) proton leak respiration, (iv) maximal respiration, and (v) spare respiratory capacity. Simultaneously, ECAR values were measured consistently, allowing the calculation of OCR/ECAR ratios.

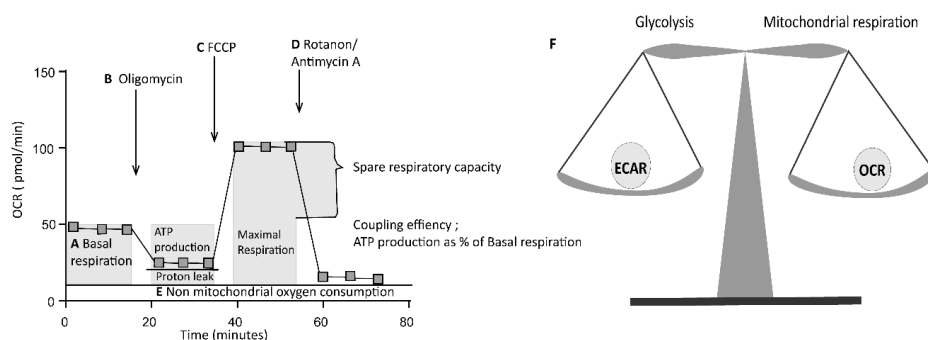


Figure 3. Overview of the Seahorse XF Cell Mito Stress Test. (A) Basal respiration is derived by measurements of baseline cellular oxygen consumption, from which non-mitochondrial oxygen consumption is subtracted (see E). (B) Oligomycin, an inhibitor of complex V (inhibition of final complex of OXPHOS), is added. The resulting decline in OCR is used to derive two parameters: (i) ATP-linked oxygen consumption (respiration), by subtracting the oligomycin rate from the baseline cellular OCR, and (ii) proton leak respiration, by subtracting non-mitochondrial oxygen consumption from the oligomycin rate. (C) Protonophore carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP), a mobile ion carrier, is added, leading to the collapse of the inner membrane gradient, driving the electron transport chain to function at its maximal rate. The maximal respiratory capacity is derived by subtracting non-mitochondrial oxygen consumption from the FCCP OCR. (D) Adding two inhibitors of the electron transport chain (ECT), antimycin A (a complex III inhibitor) and rotenone (a complex I inhibitor), shuts down the electron transport chain function and reveals non-mitochondrial respiration, allowing oxygen from the soundings to be accounted for, as it contributes to the OCR. (E) The mitochondrial reserve capacity is calculated by subtracting basal respiration from the maximal respiratory capacity. (F) The extracellular acidification rate (ECAR) is primarily a measure of lactate production and can be equated to the glycolytic rate (i.e., glycolysis), and ECAR is measured simultaneously with OCR in the Seahorse assay. Basal ECAR refers to the ECAR measured before the injection of oligomycin. The glycolytic reserve capacity is calculated by subtracting the basal ECAR from the oligomycin-induced ECAR.

Unlike OCR, ECAR is not unambiguously linked to extracellular acid production. Changes in extracellular pH are due to both lactate from anaerobic glycolysis and CO₂ from the Krebs cycle. From glycolysis, glucose is converted into lactate⁻ and H⁺, and from respiration, CO₂ export and hydration to H₂CO₃, leading to dissociation into HCO₃⁻ and H⁺. The pentose phosphate pathway also contributes to acidification, and experimental conditions allow determining the proportion of acidification between glycolysis and respiration. This includes the cell type and substrates provided, and acidification may be derived completely from either glycolysis or respiration. ECAR only determines anaerobic glycolysis, whereas glycolysis (glucose metabolised to pyruvate) includes pyruvate metabolism to acetyl Coenzyme A [436-438].

Seahorse data normalisation is vulnerable due to, for instance, changes in cell viability, mitochondrial density, or protein levels. However, normalisation is commonly achieved using either protein assay or cell counting [427, 430, 439, 440]. In paper IV, the values were normalised to protein content via BCA assay. One could argue that additional measurements of, for example, cell viability or the adjustment of data to cell numbers in paper IV would have strengthened data quality. However, to obtain reproducible data within the linear detection range of the XFe96 analyser, the following optimisation of the assay was performed: (i) titration of monocyte numbers, (ii) titration of oligomycin and FCCP concentrations, and (iii) titration of the LPS concentration.

We analysed a mixed subset of monocytes, and one could, therefore, claim that this introduced great heterogeneity to the examined monocytes. However, one could argue that isolating monocyte subpopulations based on CD14 and CD16 does not necessarily ensure homogeneity [174].

In conclusion, the Seahorse XFe cell analyser provides OCR and ECAR values for the same sample, while other ways to obtain these parameters may require performing two separate assays [441, 442]. The ability to show changes in both OCR and ECAR allow investigating the relationship between glycolysis and mitochondrial respiration in live cells and is a major advantage of this analysis. In contrast, the ability to identify site-specific changes (e.g., damage to components of the respiratory chain or anaplerotic enzymes) are reduced in this assay [443]. Furthermore, the advantage of XFe96 analysis

is that single wells are seeded with relatively few cells, can give information about mitochondrial functions, including basal respiration, ATP production, proton leak respiration, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration, with relatively high throughput.

Table 14. Overview of the literature: monocytes and the use of Seahorse methodology.

Cells	Kit / Focus	Monocyte source and isolation	Cell number	LPS	Incubation time (hours)	Ref.
Classical monocytes CD14+CD16- Monocytes	ECAR/OCR	<ul style="list-style-type: none"> • Buffy coats • Immunophenotyping • FACS 	100 000 cells/well	100 ng/mL	1 h	[444]
	Acute response OCR and ECAR	<ul style="list-style-type: none"> • PBONE MARRO WC • Ficoll-Paque • Immunomagnetic negative selection 	400 000 cells/100 µl	100 ng/mL	NT*	[283]
Classical monocytes CD14+CD16-	Cell mito stress test	<ul style="list-style-type: none"> • K₂EDTA-blood • Immunomagnetic negative selection of whole blood 	150 000 cells/well	NT*	Immediately on freshly-isolated monocytes	[430]
Classical monocytes CD14+CD16-	Acute response OCR and ECAR	<ul style="list-style-type: none"> • K₂EDTA-blood • Immunomagnetic negative selection of whole blood 	150 000 cells/well	10 ng/mL	30 min after monocytes isolated	
Classical monocytes CD14+CD16-	Glycolysis stress test	<ul style="list-style-type: none"> • K₂EDTA-blood • Immunomagnetic negative selection of whole blood 	150 000 cells/well (triplicate)	10 ng/mL 1 ng/mL	2 h 24h	[445]
Monocyte CD14+	ECAR, OCR and SRC	<ul style="list-style-type: none"> • Buffy coats • PBONE MARRO WC • Ficoll-Paque • Immunomagnetic positive selection 	200 000 Cells/well	NT* <i>C. Albicans</i>	2h 24h	[279]
Monocyte CD14+	Mitochondrial stress test	<ul style="list-style-type: none"> • ACD-blood • PBONE MARRO WC 	150 000 Cells/well	NT*	NT*	[427]
	Bioenergetic Health Index	<ul style="list-style-type: none"> • histopaque density gradient • Antibody bead selection 				

*NT, not tested

Finally, currently, within the Seahorse XFe cell analyser methodology for monocyte characterisation of energy metabolism, there seems to be no standardised methodology for monocyte subset characterisation (see Table 14 for an overview of the literature). The variety of experimental Seahorse XFe conditions used by investigators to measure mitochondrial function in monocytes makes comparison amongst studies challenging [279, 283, 427, 430, 444-446]

4.1 SUMMARY OF RESULTS

3.6. ARTICLE I

Standardisation of sampling and sample preparation for the analysis of human monocyte subsets in peripheral blood

Authors: Ida Marie Rundgren, Øystein Bruserud, Anita Rynningen, and Elisabeth Ersvær.

Summary of background

As important immune cells, monocytes are interesting in many clinical settings. However, to further develop monocyte analysis as a clinical tool, it is vital to improve and standardise sample collection and handling procedures. We aimed to investigate the effect of different blood sampling tube anticoagulants, insufficient sample volume, and cryopreservation on monocyte subset analysis.

Summary of study design and methodology

We analysed the distribution of monocyte subsets in blood sampling tubes with different anticoagulants, in insufficient and correctly filled blood sampling tubes, and in fresh and cryopreserved PBMC by flow cytometry.

Summary of results

We detected no significant differences in the various monocyte subpopulations between the different anticoagulated sampling tubes. However, there was a significant difference in the percentage of total monocytes between different anticoagulants, also observed in the absolute cell count.

For the K₂EDTA samples, no significant differences were detected between the differently-filled K₂EDTA tubes. For the differently-filled ACD-A samples, we detected a significant difference between the two samples of reduced sample volume regarding the percentage of total monocytes. For the monocyte subpopulations, a significant difference was detected between fresh and cryopreserved PBMCs with respect to the CD16⁺ monocyte subsets, which decreased.

3.7. ARTICLE II

Circulating monocyte subsets in multiple myeloma patients receiving autologous stem cell transplantation – A study of the preconditioning status and course until posttransplant reconstitution for a consecutive group of patients

Authors: Ida Marie Rundgren, Elisabeth Ersvær, Aymen Bushra Ahmed, Anita Rynningen, and Øystein Bruserud.

Summary of background

Autologous stem cell transplantation (ASCT) is the recommended strategy for managing multiple myeloma in patients younger than age 65–70. Monocytes are suggested to be involved in the pathogenesis of multiple myeloma, as contributors to multiple myeloma bone disease. We monitored the regeneration of monocyte subsets after autologous stem cell transplantation.

Summary of study design and methodology

Blood samples from multiple myeloma patients who received autologous stem cell transplantation were collected at different stages of treatment. All blood samples were directly subjected to flow cytometry analysis using a standardised protocol for monocyte subset detection.

Summary of results

MM patients also showed a wider range of monocyte subsets, both in absolute and relative values, compared to healthy controls. We continued by comparing patient samples at each collection time point by paired sample analysis. We observed a significant reduction in monocytes by conditioning therapy. Unsurprisingly, this was also detected in the monocyte subsets, and a further reduction during neutropenia was observed. Monocytes also show early regeneration, detected in absolute values, and in classical and non-classical monocytes. Monocytes reached normalisation levels before neutrophils and platelets.

3.8. ARTICLE III

A pilot study of circulating monocyte subsets in patients treated with stem cell transplantation for high-risk haematological malignancies

Authors: Ida Marie Rundgren, Elisabeth Ersvær, Aymen Bushra Ahmed, Anita Ryningen, and Øystein Bruserud.

Summary of background

Autologous and allogeneic stem cell transplantation is used in the treatment of high-risk haematological malignancies. Monocytes are probably involved in haematological reconstitution and posttransplant immunoregulation because they are a part of the bone marrow stem cell niche and, therefore, may influence outcomes after transplantation.

Summary of study design and methodology

We used a highly standardised flow cytometric method to characterise peripheral blood monocyte subset levels at different time points during the treatment of patients who received autologous stem cell transplantation.

Summary of results

During remission, patients showed a majority of classical monocytes and severe, early posttransplant monocytopenia. During the first few days after transplantation (i.e., during cytopenia), most of the circulating monocytes showed a non-classical phenotype, but later, most showed a classical phenotype. The patients showed a greater variation range of classical monocytes during remission and regeneration than healthy controls. The total peripheral blood monocyte levels normalised very early, before neutrophil reconstitution, and dominance of classical monocytes was reached within 2–4 weeks posttransplantation.

3.9. ARTICLE IV

Immunomodulatory drugs alter the metabolism and the extracellular release of soluble mediators by normal monocytes.

Authors: Ida Marie Rundgren, Anita Ryningen, Tor Henrik Anderson Tvedt, Øystein Bruserud, and Elisabeth Ersvær.

Summary of background

Immunomodulatory drugs (IMiD) are used in the treatment of haematological malignancies and have direct anticancer effects, but they also have indirect effects via cancer-supporting stromal cells. Monocytes are a stromal cell subset whose metabolism is modulated by the microenvironment, and they communicate with neighbouring cells through the extracellular release of soluble mediators. Toll-like receptor 4 (TLR4) is a common regulator of monocyte metabolism and mediator release. Our aim was to investigate IMiD's effects on these two monocyte functions.

Summary of study design and methodology

We compared the effects of thalidomide, lenalidomide, and pomalidomide on *in vitro*-cultured normal monocytes activated by lipopolysaccharide (LPS, a TLR4 agonist) or cultured in media alone. Metabolism was analysed by the Seahorse XFe 96 cell analyser. Mediator release was measured as culture supernatant levels by Luminex.

Summary of results

TLR4 was shown to regulate both monocyte metabolism and mediator release. Monocyte metabolism was altered by all three IMiDs, especially when cells were cultured with LPS, with the strongest effect for lenalidomide. IMiDs also decreased TLR4-induced mediator release, with the strongest effect for pomalidomide, whereas lenalidomide and, especially, thalidomide had weaker effects. IMiDs alter monocyte metabolism and communication, but the strength of these effects differs between the IMiDs.

4. GENERAL DISCUSSION

There has been growing evidence of and interest in the possible clinical uses of monocyte subsets analyses by flow cytometry for inflammation [447, 448], autoimmune diseases [449], infection [100, 450-457], and cancer [458-460]. Monocyte subpopulation analyses are also used in the diagnosis of chronic myelomonocytic leukaemia [458-460] and may contribute to the prognostic evaluation of patients' MDS [459, 460].

Monocytes seem to be involved in the development of multiple myeloma bone disease, which is characterised by osteoclast activation and the absence of functioning osteoblasts, with the final effect being bone resorption [159, 383, 461, 462]. CD16⁺ bone marrow monocytes in myeloma patients have distinct features compared with smouldering myeloma and MGUS patients; they overexpress IL21R, which may induce osteoclastic differentiation [159, 383, 385]. Activin A is a TGF- β cytokine that stimulates osteoclastogenesis; it is derived from bone marrow monocytes and mediates the osteoclastogenic effect of IL-3 in myeloma. Non-classical monocytes are a potential cytological marker for osteoclast precursors [159, 462]. Lenalidomide enhanced differentiation toward dendritic cells of both bone marrow and the peripheral blood monocytes of MM patients and increased the chemokine and cytokine production, degrading Ikaros, a transcription regulator of hematopoietic cell differentiation, and Aiolos, a transcription factor of lymphocyte differentiation [463].

Monocytes seem to be important in stem cell mobilisation and the regulation of hematopoiesis [409, 464], and they are a part of the bone marrow stem cell niche [465, 466]. A subset of monocytes characterised by the expression of the Tek tyrosine kinase receptor TIE-2 also seems important for the paracrine induction of angiogenesis in malignant diseases [467]. Such bone marrow cells have also been detected in various haematological malignancies [467]; in these cases, this monocyte subset seems to support the growth of malignant cells [467]. Bone marrow monocytes may, thereby, support the survival and growth of myeloma stem cells [468].

4.1. MONOCYTE REGENERATION AFTER STEM CELL TRANSPLANTATION

Patients included in the transplantation studies: In the second study, we investigated patients with multiple myeloma receiving autologous stem cell transplantation. The European guidelines recommend that cytogenetic analyses and bone marrow biopsies should be a part of the routine handling of patients with multiple myeloma [344], but unfortunately, these data were unavailable for our patients. However, we emphasise that the patients included in paper 2 represent all but one patient admitted for autologous stem cell transplantation from a defined geographical area during a defined time period. Our study should, therefore, be regarded as population-based and including unselected patients; for this reason, it is likely that our patients constitute a representative cohort.

Our patients were treated largely in accordance with the European guidelines published in 2018 after the end of our study; this statement is based on the following data (see Section 1.5):

- Our patients have an expected median age for an autotransplanted patient cohort. However, we did not perform transplants in patients older than age 65 at that time, whereas the present guidelines state that autotransplantation can be considered for older patients.
- In accordance with the guidelines, our patients receiving a second transplant had not received maintenance or consolidation therapy, had a previous posttransplant stabilisation lasting for at least 18 months, and received a second transplant in their first relapse.
- The patients were heterogeneous regarding their induction treatment, but we regard all regimens to be in accordance with the guidelines, both the drug combinations and the number of cycles.
- The European guidelines state that myeloma patients should achieve better than very good partial remission before transplantation; this was not the case for our patients, as they reached very good partial remission (17 patients) or partial remission (7 patients) according to the generally accepted criteria for response evaluation in

multiple myeloma [469, 470]. However, we emphasise that all our patients had an objective response to the induction treatment.

- We used standard melphalan conditioning therapy, as recommended.
- The time until haematological reconstitution was as expected.

Thus, even though our patient cohort was relatively small and genetic analyses were unavailable, we would regard it as representative because our study is population-based, and patients were treated in accordance with generally accepted European guidelines.

We would also regard the allotransplant recipients included in our third study/article to be representative even though the cohort was small [352]:

- We included consecutive and unselected patients planned for allotransplantation at the time of initial diagnosis.
- All allotransplanted patients reached pretransplant remission.
- All AML and MDS patients received standardised anthracycline/cytarabine induction treatment and cytarabine-based consolidation therapy.
- Although the conditioning therapy differed, we emphasise that we used well-known regimens for all patients.
- The time until haematological reconstitution was as expected.

However, we only investigated patients transplanted with matched sibling donors for high-risk bone marrow diseases, and our observations may, therefore, be representative only for this subset of allotransplant recipients.

Expected immunological reconstitution after autologous stem cell transplantation:

Immunological reconstitution after autologous stem cell transplantation has been reviewed previously, and similar observations have been made in various types of patients receiving this treatment [471]. First, the level of circulating B-cells is low for 3–18 months, and serum immunoglobulin levels (IgM up to 6 months, IgG 12–18 months, and IgA up to 36 months) are decreased for several months. *In vivo* antibody responses are also reduced for months to years. Secondly, the levels of circulating CD4⁺ T-cells can be low for months to years, whereas CD8⁺ T-cell levels usually normalise

earlier, after 3–18 months. T-cells also show functional abnormalities for 6 months to several years. Finally, NK cell levels and functions normalise within a few weeks.

Patients with early lymphoid reconstitution seem to have a more favourable prognosis than patients with late reconstitution (generally evaluated on day +15 or +30 posttransplant) [472-475]. This prognostic impact of early lymphoid reconstitution suggests that early posttransplant events are important for outcomes after autologous stem cell transplantation. Future studies should, therefore, clarify whether early monocyte reconstitution is important for the early lymphoid reconstitution or whether lymphocyte-monocyte crosstalk is important for the prognostic impact of lymphocyte levels.

Expected immune reconstitution after allogeneic stem cell transplantation:

Although allotransplanted patients have a long-lasting quantitative and qualitative T-cell effect [476-478], early lymphoid reconstitution reaching a level of at least $0.3 \times 10^9/L$ on day +15 and day 30 posttransplant is associated with positive outcomes after transplantation and improved survival [479]. Total monocyte counts also showed an association with prognosis in this study. In total, these observations suggest that immunological or inflammatory events early after posttransplantation are important for later outcomes. This hypothesis is supported by several other observations. First, pretransplant recipient characteristics are associated with outcomes after allotransplantation [480, 481]. Second, donor and graft characteristics are also associated with prognosis [482, 483]. Finally, the use of G-CSF therapy to support posttransplant engraftment has an adverse prognostic impact, especially for patients receiving conditioning therapy, including total body irradiation [484, 485]. Experimental studies suggest that irradiation increases the G-CSF responsiveness of immunocompetent cells [486]. As discussed in article 3, monocyte levels after allotransplantation are associated with prognosis. Based on our present study describing the early reconstitution of various monocyte subsets, researchers should investigate monocyte subset reconstitution in larger studies to clarify whether differences in early monocyte reconstitution are associated with prognosis and whether early monocyte targeting should be tried in allotransplant recipients.

4.2. THE POSSIBLE USE OF MONOCYTE SUBSET ANALYSES IN FUTURE CLINICAL MEDICINE

Several previous studies have described altered levels of circulating monocyte subsets in patients with a wide range of diseases. Some examples of observations in common human diseases are given below:

- *Alzheimer's disease.* Animal models suggest that monocyte subsets are altered in this disease, with a decrease in the peripheral blood levels of CCR2⁺ monocytes, which seems to have a neuroprotective effect [450, 451].
- *Cardiovascular events.* The absolute count of intermediate monocytes seems to predict cardiovascular events [452]. Statin-treated coronary artery disease is associated with increased non-classical monocytes, whereas acute stroke is associated with increased levels of circulating intermediate monocytes [453].
- *Cardiovascular disease and chronic renal failure.* Cardiovascular calcification is regarded as a predictor of cardiovascular events/disease in patients with chronic renal failure; this process of calcification is complex and involves several cells, including endothelium, vascular smooth muscle cells, fibroblasts, and monocytes/macrophages [454]. Furthermore, the proportion of circulating CD14⁺CD16⁺ monocytes is abnormally high in both dialysed and nondialysed patients with chronic kidney disease [455]. Thus, the intermediate monocyte subset, regarded as the most proinflammatory, is also regarded as a cellular marker of the chronic inflammation associated with cardiovascular disease and chronic renal failure [454].
- *Microbiota.* Circulating components derived from the microbiome and metabolites modified by bacteria can influence hematopoiesis [100], but to the best of our knowledge, it is unknown whether this mechanism can alter the levels of various monocyte subsets.
- *Effects of various infections.* The associations between infections and altered levels of monocyte subsets have been reviewed recently, and for more detailed information and additional references, we refer the reader to reference [453]. First, sepsis is associated with increased levels of circulating intermediate and, possibly,

nonclassical monocytes; this seems to be true both for adults and pediatric patients. The same is true for tuberculosis. Second, several viral infections are also associated with increased levels of intermediate or nonclassical monocytes, including dengue fever and hepatitis B and C.

- *Autoimmune and inflammatory diseases.* Increased levels of the intermediate monocyte subset have been described in active Chron's disease, rheumatoid arthritis, and asthma [453]
- *Drug effects.* Steroids decrease nonclassical monocyte levels but increase classical monocytes levels [456]. Whether statins contribute to the increased levels of nonclassical monocytes in patients receiving them for coronary artery disease needs further studies for clarification [453].
- *Pregnancy.* Variations in monocyte levels observed in pregnancy were reviewed recently [457]. Studies of monocyte subset levels in pregnancy have produced conflicting results, but patients with preeclampsia have increased peripheral blood monocyte counts, increased monocyte-lymphocyte ratios, and at the same time, increased levels of the CD14⁺⁺CD16⁺⁺ subset but decreased levels of the CD14⁺⁺CD16⁻ monocyte subset.

Taken together, these examples clearly show that levels of circulating monocyte subsets are altered in many human diseases. However, for most of these examples, it is unknown whether the altered subset levels have any predictive or prognostic impact, as well as whether the therapeutic targeting of abnormal monocyte subset levels is an effective strategy in any of these diseases.

5. CONCLUSION AND FUTURE PERSPECTIVES

There are several clear examples that altered levels of circulating monocyte subsets are altered in human diseases, but little is known whether any of these alterations have a predictive or prognostic impact. More studies are, therefore, needed to further elucidate whether targeting abnormal distributions of monocyte subsets is of potential therapeutic value in any of these diseases. Thus, additional studies should be performed to clarify whether analysing monocyte subsets should be incorporated into routine clinical protocols, especially in patients with infectious, autoimmune, or inflammatory diseases. The second important question is whether additional monocyte subsets exist; as discussed previously, heterogeneity has been reported for all three monocyte subpopulations, and a more thorough biological characterisation of circulation monocyte populations in various diseases is needed.

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Research paper

Standardization of sampling and sample preparation for analysis of human monocyte subsets in peripheral blood

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ABSTRACT

Introduction: Monocytes are important for innate immunity and include the classical (CD14^{bright}CD16^{negative}), intermediate (CD14^{bright}CD16^{dim}) and non-classical (CD14^{dim}CD16^{bright}) monocyte subsets. The quantification of these functionally different subsets in peripheral blood may become useful for diagnosis and follow-up in human diseases. The aim of the present study was to investigate how different pre-analytical parameters influence analysis of monocyte subsets in peripheral blood samples.

Methods: We determined relative levels of monocytes and monocyte subsets by flow cytometry of peripheral blood samples derived from healthy individuals. A gating strategy exclusively extracting viable CD14⁺ monocytes and focusing on the three monocyte subsets was applied. We investigated the effects of (i) various anticoagulants (i.e. Li-Heparin, ACD-A, K₂EDTA), (ii) insufficient filling of blood sampling tubes, (iii) cryopreservation. In addition, we analysed expression of the CCR2 chemokine receptor.

Results: The relative numbers of CD14⁺ monocytes depended on the anticoagulant used, whereas the fraction of the three monocyte subsets did not. Insufficient filling of blood sampling tubes altered the relative levels of monocytes out of leukocytes, but not the relative levels of the monocyte subsets. Finally, the fraction of CD14⁺ monocytes out of isolated peripheral blood mononuclear cells was not significantly altered by cryopreservation, but the relative percentages of monocyte subsets was altered (similar effects for ACD-A and K₂EDTA samples) and this was observed in correlation to a decreased CD16 expression.

Concluding remarks: Analysis of the monocyte subsets (i.e. classical, intermediate, non-classical) in peripheral blood samples requires a careful standardization of peripheral blood sampling and pre-analytic handling of the samples with respect to the anticoagulant used, filling of sample tubes, and cryopreservation of cells prior to analysis.

1. Introduction

Circulating monocytes (Jakubzick et al., 2017) represent around 10% of peripheral blood leukocytes (Swirski et al., 2009), and following extravasation they differentiate into macrophages or dendritic cells (Jakubzick et al., 2017) but they may also differentiate in endothelial direction (Arderiu et al., 2017). Based on their expression of CD14 (a cell surface co-receptor for lipopolysaccharide) and CD16 (the low affinity IgG receptor) the circulating monocytes can be divided into the three main subsets, termed classical (CD14^{bright}CD16^{negative}), intermediate (CD14^{bright}CD16^{dim}) and non-classical (CD14^{dim}CD16^{bright})

monocytes (Passlick et al., 1989; Franca et al., 2017; Ziegler-Heitbrock et al., 2010; Patel et al., 2017). Classical monocytes normally comprise > 90% of the circulating monocytes, release proinflammatory cytokines like IL-6, are phagocytic and mediate a wide range of responses to pathogens (Franca et al., 2017; Patel et al., 2017; Mukherjee et al., 2015; Skrzeczynska-Moncznik et al., 2008; Cros et al., 2010; Wong et al., 2011; Boyette et al., 2017). In contrast, the intermediate monocytes seem to release anti-inflammatory cytokines (e.g. interleukin (IL)-10) + and proinflammatory cytokines, e.g. tumour necrosis factor- α (TNF- α) and IL-1 β (Franca et al., 2017; Patel et al., 2017; Cros et al., 2010; Wong et al., 2011; Boyette et al., 2017). Finally, the non-

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classical subset seems to be involved in tissue repair; their capacity of cytokine release is less well characterized and they bind viruses as well as nucleic acids through their expression of Toll-like receptor (TLR) 7 and 8 (Patel et al., 2017; Skrzeczynska-Moncznik et al., 2008; Cros et al., 2010; Boyette et al., 2017). Monocytes are thus important members of the innate immune system, they function as immunoregulatory cells and animal studies suggest that functional reprogramming through epigenetic mechanisms will induce innate memory that is important for the defence against infections (Bekkering et al., 2015; Hamon and Quintin, 2016; Quintin et al., 2012). Taken together these observations suggest that the possible clinical use of monocyte subset analysis for the diagnosis and/or follow-up during treatment of human diseases should be further explored, e.g. for patients with inflammatory disorders (Rogacev et al., 2012; Williams et al., 2017), autoimmune diseases (Chara et al., 2015), severe infections and cancer (Greenberg, 2017; Selimoglu-Buet et al., 2017; Talati et al., 2017).

Pre-analytical factors may have a major impact on later analyses of peripheral blood samples (Lippi et al., 2015). Such factors can be collection of insufficient sample volumes, use of suboptimal anticoagulants or storage of samples (Kalra, 2004; Wallin et al., 2008; Plebani and Carraro, 1997; Carraro and Plebani, 2007; West et al., 2017). Patient-associated variables (e.g. fasting, physical exercise, genetic factors and previous infections) may also influence the results (Appleby et al., 2013; Da Silva Neves et al., 2015; Crosslin et al., 2013; Kahn et al., 2016). Such pre-analytical factors are important for quantification of total monocyte blood counts (Buttarelli, 2004), but to the best of our knowledge their impact on monocyte subset identification and quantification has only been addressed in two previous studies (Appleby et al., 2013; Ji et al., 2017), investigating differences between the two anticoagulants K₂EDTA and sodium citrate (Ji et al., 2017) and effects of delayed analysis (Ji et al., 2017) and cryopreservation (Appleby et al., 2013). Further development of monocyte subset analysis as a clinical tool will probably require a careful standardization of sampling and sample handling. In this context, we have investigated in more detail the effects of pre-analytical factors on the flow cytometric detection (i.e. relative levels of various monocyte subsets) of circulating monocyte subsets.

2. Material and methods

2.1. Ethical statement and study population

The study was conducted according to the Declaration of Helsinki, and was submitted for review by the local Ethics Committee (Regional Committees for Medical and Health Research Ethics, REC West, Bergen, Norway) (REK 2015/1410, 2017/487). The study was characterized as a laboratory quality improvement project, and the donor identity was not registered. Our data contains no information that can identify the individual sample donors. All donors were healthy adults volunteering after informed consent (see Supplementary Table 1).

2.2. Blood sampling

Well trained biomedical laboratory scientists performed the venipuncture of the antecubital vein for all experiments. For experiment A (see Supplementary Fig. 1) we standardized the procedure by performing all phlebotomies in the morning and instructed the donors to eat only a light meal before sampling, and everything was done to ensure a relaxed and comfortable atmosphere. Blood was collected by a closed-loop system directly onto K₂EDTA (4 mL, #367862, BD vacutainer, San Jose, CA, USA, spray dried), Li-Heparin (4 mL, #454029 Greiner Bio-one Vacuette®, Kremsmünster, Austria, spray dried) and ACD-A (9 mL, #248,368, BD Vacutainer, San Jose, CA, USA) blood sampling vacuum tubes from 22 donors. For all subsequent studies (see Supplementary Fig. 1, experiment B and C) blood sampling was

undertaken at the Blood Bank Services at Haukeland University Hospital (HUH, Bergen, Norway). We then used a pre-donation sampling bag for blood bags (TERUFLEX blood bag systems, TERUMO BCT, Lakewood, CO, USA), and blood was thereafter transferred to K₂EDTA (spray dried; 6 mL #456023, Greiner Bio-one Vacuette®, Kremsmünster, Austria,) or ACD-A (9 mL #248368, BD Vacutainer, San Jose, CA, USA) vacuum collection tubes. Blood sampling tubes were immediately turned over 6–10 times, incubated at room temperature for at least 15 min but not more than two hours after blood collection before further processing.

We followed the same highly standardized procedures in all these experimental studies of freshly isolated cells; for three donors we included analysis of cell viability (SYTOX™ Red Dead Cell Stain, Thermo Fischer Scientific, Invitrogen™, #S34859) and in all these studies the viability was very high and the fraction of dead cells considered to be negligible (data not shown).

2.3. Insufficient blood sample volume

K₂EDTA (6 mL) or ACD-A (9 mL) vacuum collection tubes were filled with insufficient sample volume (Supplementary Fig. 1, experiment B). Blood was collected in the pre-donation sampling bag for blood bags as described in Section 2.2; 1.5 mL (¼ of correct volume), 3 mL (½ of correct volume) or 6 mL (correct volume, 1/1) of whole blood from 9 donors were drawn into separate K₂EDTA vacuum tubes containing 1.8 mg/mL of dipotassium EDTA when filled with correct volume. Alternatively, 2, 4 or 9 mL were drawn into separate tubes containing ACD-A (8 donors). The resulting ratios of whole blood to ACD-A were 2:1 (¼ filled), 4:1 (½ filled) and 9:1 (correctly filled, 1/1), respectively.

2.4. Isolation of peripheral blood mononuclear cells (PBMC), cryopreservation and thawing

2.4.1. Isolation of PBMC

ACD-A (9 mL) or K₂EDTA (6 mL) blood sampling tubes were collected. Blood samples were diluted 1:2 in 0.9% sodium chloride (9 g/L # 884239, Fresenius Kabi, Oslo, Norway) and peripheral blood mononuclear cells (PBMC) collected from the interphase after density gradient separation (Lymphoprep™ #07801, Stemcell Technologies, Oslo, Norway, specific density 1.077 g/mL) with centrifugation at 800 × g for 30 min. Cells were washed twice at room temperature with 0.9% sodium chloride (Fresenius Kabi, Oslo, Norway) by centrifugation at 400 × g for 8 min. Cells were resuspended in 10 mL of phosphate-buffered saline (PBS, Department of microbiology, Haukeland University Hospital, Bergen, Norway). Approximately 1 × 10⁶ cells were processed for subsequent flow cytometry analysis, while the remaining cells were cryopreserved (see Supplementary Fig. 1, experiment C, and immunofluorescence staining protocol in Section 2.5.2).

2.4.2. Cryopreservation and thawing

The cells (1–2.5 × 10⁷ cells) were suspended in 1 to 2 mL RPMI 1640 (#R5886-500ML, SIGMA/Merck KGaA, Darmstadt, Germany) supplemented with 40% inactivated fetal bovine serum (FBS; #DE14-801F, LONZA, Basel, Switzerland) and an equal volume of RPMI 1640 plus 20% dimethyl sulfoxide (DMSO, #D2650-100ML, SIGMA/Merck KGaA, Darmstadt, Germany), for a final DMSO concentration of 10%. The cells were aliquoted immediately after (1 mL per freezing vial) and frozen by a controlled rate freezer before vials were transferred to liquid nitrogen. Each vial contained 0.5–1.25 × 10⁷ PBMC. All samples were handled by sterile techniques and frozen within 2 h.

After at least 3 weeks of storage, samples were analysed. The samples were then thawed by hand before RPMI 1640 was immediately added, samples were thereafter centrifuged and washed once in PBS (HUH, Bergen, Norway) before being stained for flow cytometry. We conducted a small-scale study to evaluate the effect of a short recovery

time post-cryopreservation on CD16 and CCR2 (CD192) expression. For each of the three healthy donors we collected ACD-A and K₂EDTA blood samples that were prepared for direct flow cytometry and for cryopreservation. After thawing the cryopreserved PBMC were both directly immunostained and seeded at 1×10^6 cells per well in a 24 well plate and incubated for 90 min at 37 °C in a humidified atmosphere of 5% CO₂. The plate was thereafter left on ice for 5 min to release adherent monocytes. The cells were collected by tilting the plate and rinsing each well by pipetting the medium from the bottom to the top of the well several times. Subsequently, the plate was washed once more with cold RPMI 1640 medium before collection and immunofluorescence staining of the cell; the collection of cells was controlled by light microscopy of the wells and detachment of the cells could then be confirmed.

2.5. Flow cytometric analysis of membrane molecule expression

2.5.1. Flow cytometry

All analyses were performed by an 8-parameter BD FACS Verse equipped with 404 nm, 488 nm and 640 nm lasers. Instrument quality control was done on a daily basis using BD FACSuite™ CS&T Research Beads (#650621, BD Biosciences, San Jose, CA, USA). For each run single stained compensation beads (#552843, BD Biosciences) were prepared and applied for compensation. Unstained samples served as gating controls. The concentrations used of each antibody were determined by titration, and for each sample at least 5000 monocytes based on SSC/FSC properties were counted and analysed by FlowJo software (Tree Star, Inc., OR, USA). The gating strategy is shown in Supplementary Fig. 2.

2.5.2. Immunostaining of whole blood samples and PBMC

Whole blood (100 µL) was added to flow cytometry tubes containing the appropriate antibody mixture and volume; cells were thereafter incubated for 30 min at room temperature in the dark and subsequently haemolysed according to the manufacturer's instructions (# 55589, BD Biosciences). Samples were thereafter washed once in 1% BSA (#10735086001, Roche, Basel, Switzerland) in PBS, centrifuged (200 × g, 5 min) before analysis. Alternatively, the PBMC concentration was adjusted to $0.5\text{--}0.75 \times 10^6$ cells per 100 µL staining buffer (PBS, HUH, Bergen, Norway) containing 1% BSA (#10735086001, Roche, Basel, Switzerland) and 10% Octagam (100 mg/mL, Octapharma, Lachen, Switzerland). The cells were thereafter added to tubes containing anti-human antibodies (see Section 2.5.3) and finally incubated in the dark on ice for 20 min before being washed once in PBS with 1% BSA and analysed by flow cytometry.

2.5.3. Antibody panels

As can be seen from Supplementary Fig. 1 mouse anti-human CD14 (clone M5E2), CD56 (clone B156), and CD16 (clone 3G8, all from BD Biosciences, San Jose, CA, USA) served as a basis for the monocyte subset identification in all our experiments. In experiment B and C the antibody panel included mouse anti-human CD45 (clone HI30), CD11b (clone ICRF 44) and HLA-DR (clone G46-6, all from BD Biosciences). K₂EDTA blood samples in experiment C included in addition a dead-viable marker (SYTOX™ Red Dead Cell Stain). The small-scale part of the cryopreservation study (N = 3; evaluation of a recovery time after cryopreservation and thawing) included staining with and analysis of mouse anti-human antibodies (all from BD Biosciences) specific for the following molecular markers; CD192 (CCR2, 48,607, BV421), CD45 (HI30, PerCpCy™5.5), CD16 (3G8, PE-Cy™7) CD11b (D12, BV510), CD56 (B156, Alexa 700) CD14 (M5E2, Alexa 488) and HLA-DR (G46-6, PE); but for these studies we also included SYTOX Red Dead Cell Stain and CountBright™ Absolute Counting Beads (Thermo Fischer Scientific, # C36950).

2.6. Differential blood count

Differential blood counts were estimated for the K₂EDTA and Li-Heparin blood samples in experiment A (see Supplementary Fig. 1). Blood samples were then analysed 15–45 min after collection by the ABX Pentra XL 80 hemocytometer (Horiba Medical, Kjeller, Norway). Quality control sample (ABX Minotrol CRP2, Horiba, Albany, NY, USA) was applied prior to analysis and at two hours intervals.

2.7. Statistical analyses

All statistical analyses were conducted using IBM SSP statistics 23. One-way analysis of variance (one way ANOVA) was used with monocyte subsets as grouping variables and post hoc tests to determine significance between groups. Paired sample t-test was used instead of one way ANOVA when appropriate. *P*-values below 0.05 were regarded as statistically significant. We did not compare data across the various experiments; hence, only within-subjects comparisons were performed.

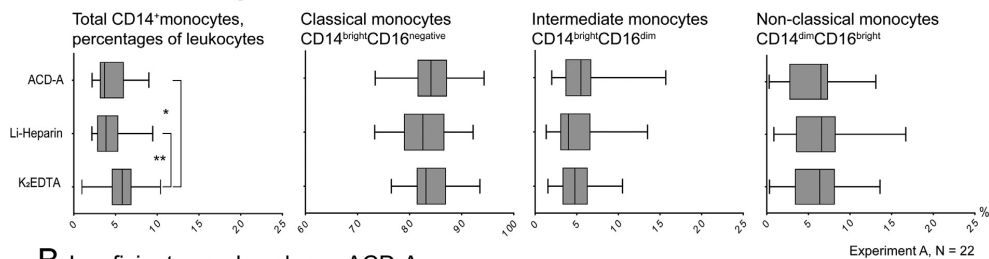
3. Results

3.1. Only the relative levels of total circulating monocytes but not the relative distribution of the three monocyte subsets differed between ACD-A, Li-Heparin and K₂EDTA samples

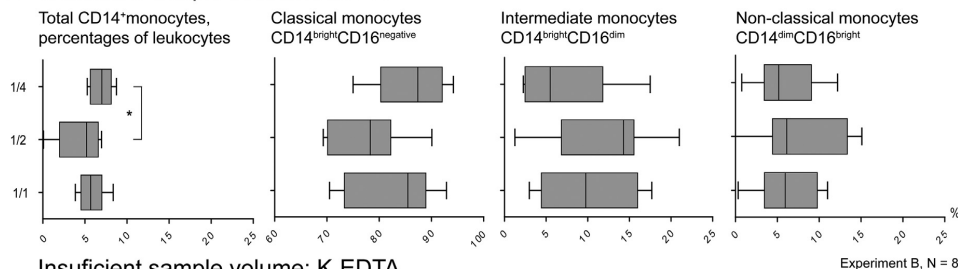
We investigated the relative representation of monocytes out of leukocytes as well as the monocyte subsets for 22 healthy donors when using three different anticoagulants; K₂EDTA, Li-Heparin and ACD-A, following protocol as described for experiment A (see Supplementary Fig. 1). The median percentages of CD14⁺ monocytes of leukocytes differed significantly between ACD-A (median 4.4%), Li-Heparin (median 3.9%) and K₂EDTA (median 5.8%; *p* = 0.006). In contrast, the median percentages of the classical monocyte subset (median relative levels 84.1, 82.6 and 83.1%, ACD-A, Li-Heparin, and K₂EDTA, respectively), the intermediate subset (median relative levels 5.5, 4.0 and 4.8%, ACD-A, Li-Heparin, and K₂EDTA, respectively) and the non-classical subset (median 6.5, 6.6 and 6.4%, ACD-A, Li-Heparin, and K₂EDTA, respectively) among total monocytes did not differ when comparing the three anticoagulants. Results are presented in Fig. 1A.

We also determined the absolute numbers of monocytes as well as the other leukocyte subsets and platelets when using differential blood cell counting by an automated hemocytometer (see Fig. 2), and this analysis showed higher absolute levels of monocytes for the anticoagulant K₂EDTA (median $0.57 \times 10^9/L$) than for Li-Heparinized (median $0.43 \times 10^9/L$, *p* < 0.0001). The absolute numbers of monocyte subsets, calculated from the differential blood cell count and the median relative percentages of monocyte subsets from the flow cytometry analysis, indicate higher levels of classical monocytes for the anticoagulant K₂EDTA (median $0.48 \times 10^9/L$) than for Li-Heparinized (median $0.39 \times 10^9/L$). This is in contrast to intermediate and non-classical monocytes, where results indicate equal relative levels irrespective of the anticoagulant used (Supplementary Table 2). The absolute numbers for lymphocytes and granulocytes were both within the laboratory reference ranges for healthy adults (Helse Bergen, 2018), however, both cell types was statistical significant altered by the use of Li-Heparin (Fig. 2). By calculating the neutrophil to lymphocyte ratio (NLR) a declined relationship from mean 1.3 for K₂EDTA to 0.99 for Li-Heparin was observed (*p* < 0.0001 by paired sample t-test; Fig. 2B). For the differential blood cell count, the largest difference was observed for platelets with median level of $254 \times 10^9/L$ in K₂EDTA samples versus $143 \times 10^9/L$ in Li-Heparin anticoagulated blood samples (*p* < 0.0001).

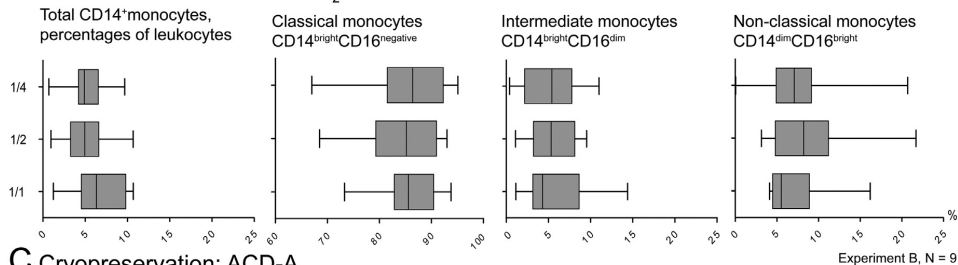
A Effect of anticoagulants



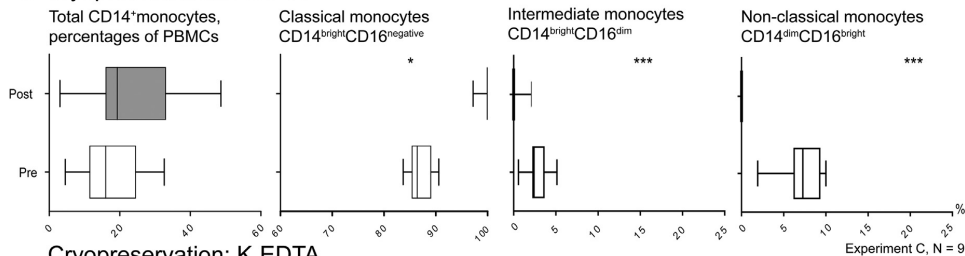
B Insufficient sample volume; ACD-A



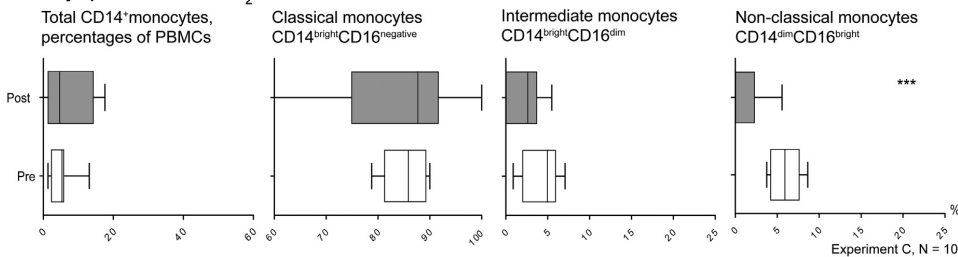
Insufficient sample volume; K₂EDTA



C Cryopreservation; ACD-A



Cryopreservation; K₂EDTA



(caption on next page)

Fig. 1. Effect of pre-analytical variables on the detection of monocytes and various monocyte subsets. Circulating leukocytes in peripheral blood samples from healthy individuals were studied. The figure presents the results for all three experiments (A, B and C) that are described in Supplementary Fig. 1; the experimental variables are given to the left in the figure. All the results are presented as box and whisker plots where the vertical line presents the median level, the boxes represent the upper and lower quartiles (the interquartile range); the two lines outside the box (the whiskers) represent the variation range. In all parts of the figure we present (from left to right) the results for analysis of CD14⁺ monocytes among total leukocytes and the percentages of classical CD14^{bright}CD16^{negative}, intermediate CD14^{bright}CD16^{dim} and non-classical CD14^{dim}CD16^{bright}. The number of individuals tested is indicated to the lower right in each part of the figure. (Panel A) Anticoagulants. We compared the effects of the three anticoagulants ACD-A, Li-Heparin, and K₂EDTA on the analysis of monocytes and monocyte subsets. (Panel B) Insufficient sample volume in ACD-A tubes (upper, N = 8) and K₂EDTA tubes (lower, N = 9). The tubes were either correctly filled (1/1) or filled with a reduced volume (½ and ¼ filled). (Panel C) Cryopreservation; monocyte distribution in ACD-A PBMC (upper, N = 9) and K₂EDTA PBMC (lower, N = 10). Post- and pre-cryopreservation relative levels are indicated. Statistically significant as *P* < 0.05 by One Way-ANOVA (Panel A and B) or paired sample *t*-test (Panel C); *p* < 0.01:**; *p* < 0.001:***.

3.2. The relative distribution of the monocyte subsets was not altered by a reduced blood sample volume

We collected blood from healthy adults onto ACD-A (N = 8) and K₂EDTA (N = 9) anticoagulation tubes; the tubes were filled with either correct volume (1/1) or reduced volumes, i.e. ½ and ¼ of the correct sample volume. Samples were further handled as described for experiment B (Supplementary Fig. 1). We compared the percentage of total CD14⁺ monocytes among total peripheral blood leukocytes for tubes filled with either the correct (1/1) volume or ½ or ¼ of the correct sample volume. For the ACD-A samples, the median percentages of CD14⁺ monocytes was 5.7%, 5.2% and 7.2% respectively; these differences were statistically significant (*p* = 0.044 by One Way-ANOVA). The same trend was seen for K₂EDTA anticoagulated blood with reduced sample volume (median 4.9% and 5.0% for ½ and ¼ respectively) compared with the correct sample volume (median 6.3%),

but was not statistical significant (Fig. 1B).

We also compared the percentage of classical, intermediate and non-classical monocyte subsets in ACD-A and K₂EDTA samples when the tubes were filled 1/1 or with reduced volumes corresponding to ½ and ¼ of the correct blood sample volume. The percentage of the various monocyte subsets did not differ between the various sample volumes, and this was true both for the ACD-A and K₂EDTA samples (Fig. 1B).

3.3. Cryopreservation of PBMC alters the relative distribution of monocyte subsets

Cryopreserved PBMC were stored for at least 3 weeks before thawing and subsequent flow cytometric analysis; ACD-A samples derived from 9 donors and K₂EDTA samples derived from 10 donors were analysed according to Protocol C and a viable/dead marker was

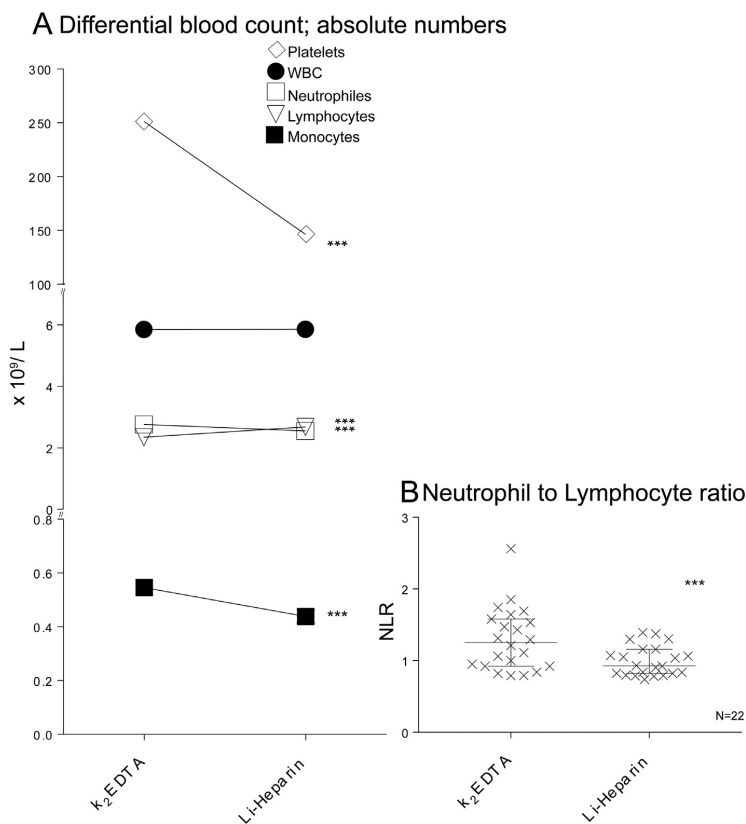


Fig. 2. Differential blood cell counts from K₂EDTA and Li-Heparin anticoagulated blood. Peripheral blood samples derived from 22 healthy individuals were investigated. Panel A indicate the median absolute numbers of platelets, total white blood cells (WBC), neutrophils, lymphocytes and monocytes (x 10⁹ cells/L). The differential blood cell counts were estimated by Pentra XL80. All counts except for the WBC showed a significant difference between K₂EDTA and Li-Heparin blood samples (***) (*p* = 0.0001, paired sample *t*-test). The data in panel B are presented as the NLR (neutrophil to lymphocyte ratio) for 22 healthy donors (X) of K₂EDTA and Li-Heparin blood samples. Horizontal line indicate median and vertical line indicate the 95% confidence interval. The difference between K₂EDTA and Li-Heparin samples was significant by paired sample *t*-test (***) (*p* = 0.0001).

included for the K₂EDTA blood samples (see Supplementary Fig. 1). The percentage of CD14⁺ monocytes among PBMC was not significantly altered by the cryopreservation; this was true both for ACD-A and K₂EDTA samples (Fig. 1C). In contrast, the percentage of the three main monocyte subsets among total monocytes was significantly altered after cryopreservation. For the ACD-A samples the classical monocyte subset increased from a median relative level of 86.4% before cryopreservation to 99.9% after preservation ($p = 0.013$, Fig. 1C), whereas the intermediate (median 2.4% before preservation) and non-classical (median 7.7% before preservation) monocyte subsets could not be detected after preservation ($p = 0.001$, Fig. 1C). For the K₂EDTA samples the statistically significant effect of cryopreservation was a decrease of the non-classical monocyte subset from 5.9% to 1.1% ($p = 0.001$, Fig. 1C). Finally, remaining adherent cells could not be detected by light microscopy of the wells after the recovery procedure; this observation makes selective loss of monocyte subsets due to plastic adhesion to be less likely (data not shown).

Our present study cannot explain our observations when analysing cryopreserved cells. However, based on the overall results from our present methodological studies we can conclude that; (i) flow-cytometric studies of monocyte subsets should preferably be done in fresh samples, (ii) analyses of fresh and cryopreserved cells cannot be directly compared, and (iii) if cryopreserved cells are analysed this require careful standardization of the methodology and a very careful interpretation of the observations.

3.4. Only cryopreservation but not different anticoagulants and insufficient sample volume alters CD16 expression by circulating monocytes

CD16 expression is an important marker for subclassification of monocytes (Passlick et al., 1989; Ziegler-Heitbrock et al., 2010), and for this reason the effects of pre-analytical factors on relative CD16 levels was investigated in more detail. Firstly, we investigated the effects of the three anticoagulants ACD-A, Li-Heparin and K₂EDTA on CD16 expression for monocytes derived from 22 healthy adults (see Section 3.1). There was no difference between these three anticoagulants with regard to CD16 (MFI, mean fluorescence intensity) expression (Fig. 3A). Secondly, differences in sample volumes (see Section 3.2) did not alter CD16 expression and this was true both when using ACD-A ($N = 8$) and K₂EDTA ($N = 9$) as the anticoagulant (Fig. 3B). Finally, in contrast to the above described results, cryopreservation and thawing altered CD16 expression (MFI) significantly, and this was seen both when using ACD-A and K₂EDTA as the anticoagulant. For ACD-A a significant decrease correspond to 40 times whereas for K₂EDTA the decrease was only 2 times (Fig. 3C). The additional experiments demonstrated that a short recovery period after thawing did not have any statistical significant effect on CD16 expression compared to cryopreserved and direct immunostained PBMC (Fig. 3D). However, the absolute numbers indicate loss of cells after the short recovery period of 90 min (see Supplementary Table 3). This small-scale study also suggests that the MFI of CD14 was doubled in PBMC compared to whole blood for both K₂EDTA and ACD-A samples (see Supplementary Fig. 3).

3.5. Monocyte CCR2 levels decrease due to both PBMC isolation and cryopreservation

The CCR2 chemokine receptor (CD192) is important for regulation of monocyte trafficking and has been used to aid classification of the three monocyte subsets (França et al., 2017). Previous studies have also shown that cryopreservation of cells seems to reduce the level of various cell surface molecules (Sasnoor et al., 2003). We therefore investigated the CCR2 levels in a small-scale experiments ($N = 3$) for whole blood samples and for PBMC before, immediately after cryopreservation and after a post-thawing recovery period. The CCR2 levels decreased after gradient separation of PBMC and this decrease was seen for all three monocyte subsets. However, no further decrease was seen

after cryopreservation and CCR2 could be detected mainly for the classical monocyte subset when investigating cryopreserved PBMC. This is illustrated in Fig. 4.

4. Discussion

The various monocyte subsets differ in their functions, membrane molecule expression and disease associations (Wong et al., 2012). It may therefore be useful to determine the absolute or relative levels of circulating monocyte subsets as a part of the diagnostic work, evaluation of disease activity or early detection of hematological and/or immunological toxicity during treatment. In the present study, we have therefore analysed how various pre-analytical parameters will influence the flow cytometric identification of monocyte subsets.

Our analyses were based on gating and thereby separation of viable blood cells from debris and dead cells using the forward- and side-scatter area (Supplementary Fig. 2). For PBMC and cryopreserved PBMC this viable cell gating was combined with a live/dead marker to identify viable cells only. Selection of monocytes was based on negative selection using CD56 (excluding natural killer cells, NK-cells) and positive selection using CD45 (only nucleus-containing hematopoietic cells), CD11b and HLA-DR (the last two being extensively expressed by monocytes). The classic, intermediate and non-classic monocyte subsets were then identified by their expression of CD14 and CD16.

Our blood sampling was carefully standardized. Phlebotomy was performed using a closed-loop system by routine laboratory personnel. The blood was harvested onto pre-sampling bags (experiment B and C; see Supplementary Fig. 1) and the blood from the pre-sampling bags was added to sampling tubes within 2 min and immediately after the glasses for routine analyses had been filled. Alternatively, for experiment A, we drew the blood directly into the blood sampling vacuum tubes similar to what was described in a previous study (Ji et al., 2017). These authors also investigated differences between the two anticoagulants K₂EDTA and sodium citrate upon monocyte subset identification (Ji et al., 2017), but they collected the whole blood first into a syringe before it was distributed equally to the EDTA and sodium citrate tubes; a methodology that may cause erroneous results due to a possible contamination of sample tubes with additives from previous tubes (Cornes et al., 2017).

K₂EDTA is today regarded as the anticoagulant of choice for routine analyses of peripheral blood cells (Ji et al., 2017; Zini and H. International Council for Standardization, 2014; Bruegel et al., 2015). It allows the best preservation of blood cell morphology and causes an irreversibly prevention of clotting (Banfi et al., 2007). Other anticoagulants are commonly used in research laboratories including ACD-A (Melve et al., 2016), CPDA (Citrate Phosphate Dextrose Adenine solution) (Ersvaer et al., 2015; Bruslerud and Lundin, 1987), heparin (Appleby et al., 2013), and sodium citrate (Ji et al., 2017), but for several studies detailed information is not given (Boyette et al., 2017; Chara et al., 2015; Selimoglu-Buet et al., 2017; Talati et al., 2017). We wanted to characterize and compare the effects of anticoagulants used in routine clinical practice, i.e. K₂EDTA, ACD-A and Li-Heparin.

Our results indicate that the choice of anticoagulant did not have any major effect on the relative distribution of monocyte subsets. However, the percentage of total monocytes relative to total leukocyte counts, as well as the platelet counts decreased significantly, especially in Li-Heparin when compared to K₂EDTA. Previous studies have demonstrated that heparin has an effect on platelets consistent with platelet activation (Gao et al., 2011), and the most likely explanation for the decrease in platelet and monocyte numbers when using Li-Heparin activation and aggregation of platelets leading to platelet-monocyte adhesion (Basavaraj et al., 2012). Li-Heparin should therefore be used with caution in experimental studies of monocyte function. ACD-A is the preferred anticoagulant used in transfusion medicine services, and it is reliable with regard to preservation of resting platelets for several hours without inhibition of their responsiveness (Pignatelli et al., 1995;

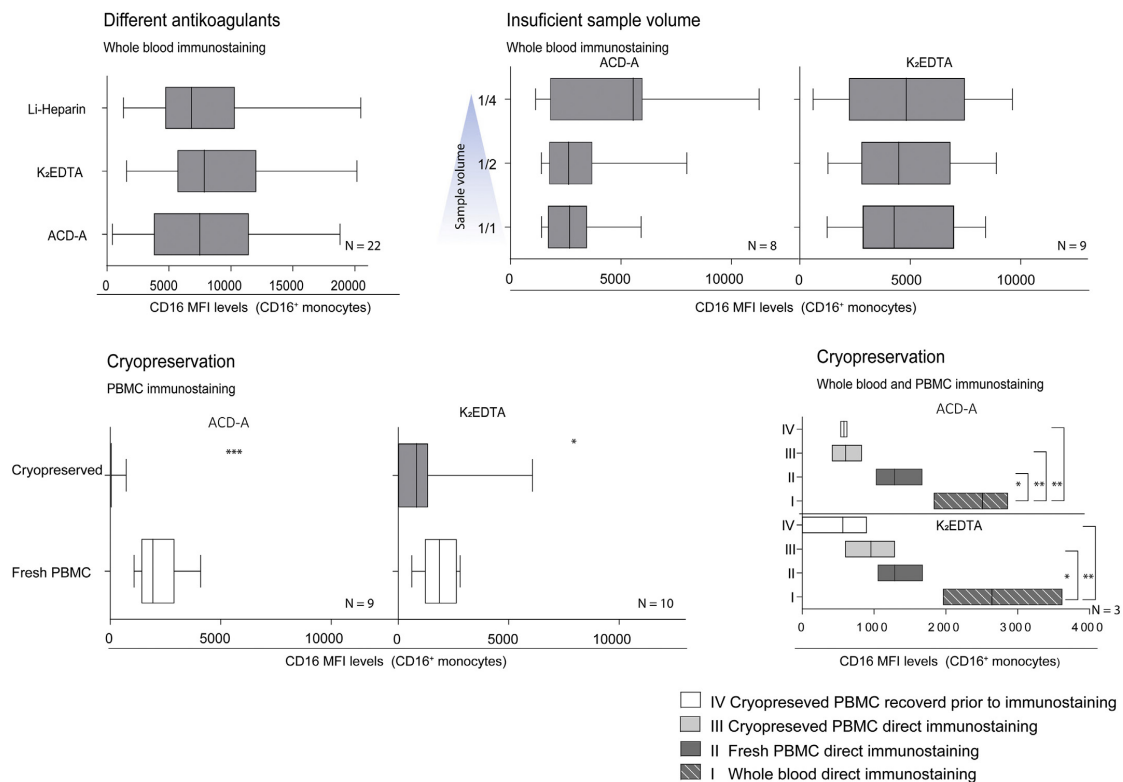


Fig. 3. MFI values of CD16⁺ monocytes derived from healthy individuals; a summary of the results from experiments comparing different anticoagulants, different sample volumes in the tubes and the effects of cryopreservation (see also Supplementary Fig. 1). The variables in each experiment are indicated to the left in each figure. All results are presented as the CD16 MFI levels of CD14⁺CD16⁺ monocytes. In Figs. A–C the results are presented as box plots with whiskers. Median values represent the vertical line in the box. The boxes represent the upper and lower quartiles (the interquartile range); the two lines outside the box (the whiskers) represent the variation range. (A) The anticoagulant experiments (N = 22); the results for Li-heparin, K₂-EDTA and ACD-A samples were compared. (B) Insufficient blood sample volumes of ACD-A (N = 8) and K₂-EDTA samples (N = 9); sample tubes were either filled with the correct volume (1/1) or with reduced volumes (1/2 or 1/4 of the correct volume). (C) PBMC isolated from ACD-A (N = 9) or K₂-EDTA samples (N = 10); the figure shows a comparison of samples analysed after cryopreservation/thawing (upper) and before the cryopreservation (lower). (D) Freshly isolated and cryopreserved PBMC (N = 3) a comparison of CD16 expression for monocytes after cryopreservation/thawing/recovery (IV), after only cryopreservation/thawing (III), staining of freshly isolated PBMC (II) and whole blood staining (I). Results are presented as box plots where the ends of the box extend to the lowest and highest observations, and median is marked by a vertical line inside the box. Statistical significant when $p < 0.05$ by One way-ANOVA with post hoc (panel A, B and D) or paired sample t-test (panel C): $p < 0.05$; *; $p < 0.01$; **, $p < 0.001$;***.

Lei et al., 2009). For the ACD-A blood the lower percent of monocytes relative to total leukocytes can possibly be explained by adherence of monocytes to the test tube, but this seems to be a random process that does not alter the relative levels of various monocyte subsets.

Minimizing blood collection volumes is definitely an advantage, especially in paediatric patients as well as seriously ill adults, however, insufficient sample volume is a common pre-analytical error (Kalra, 2004; Wallin et al., 2008; Plebani and Carraro, 1997; Carraro and Plebani, 2007; West et al., 2017; Xu et al., 2010). To the best of our knowledge, there are no previously published data on the effects of insufficiently filling of blood tubes on monocyte and monocyte subset distribution. We found that ¼ of correct sample volume in K₂EDTA tubes did not alter the total percentage of monocytes relative to total leukocytes or the relative distribution of monocyte subsets. This is in accordance with previous studies (Xu et al., 2010), where 1.0 mL of blood in a 4 mL K₂EDTA tube showed acceptable cell counts. However, our results suggest that the percentage of total monocytes is affected by insufficient sampling in ACD-A tubes. A likely explanation is that this is

due to the sample dilution. We did not observe any significant differences in the distribution of monocyte subsets in any of the incompletely filled samples. We therefore conclude that sample volumes can be reduced to ¼ of correct filling without any effects on the monocyte subsets percentages, in both K₂EDTA and ACD-A anticoagulated sample tubes.

Separation and cryopreservation of PBMC is a common strategy in longitudinal clinical studies. The methodological variations between analyses of different samples can then be reduced both when analysing several samples from the same patients and when comparing samples collected from different patients and different time points. Previous reports suggest that the gradient separation involved in production of PBMC can alter the relative percentage of monocyte subtypes (Mukherjee et al., 2015), their phenotype (Mukherjee et al., 2015; Nieto et al., 2012) and thus may also influence their function (Wong et al., 2012; Italiani and Boraschi, 2014). In our results, no significant differences were observed for CD16-MFI when comparing whole blood and PBMC, but the MFI of CD14 was doubled in PBMC compared to

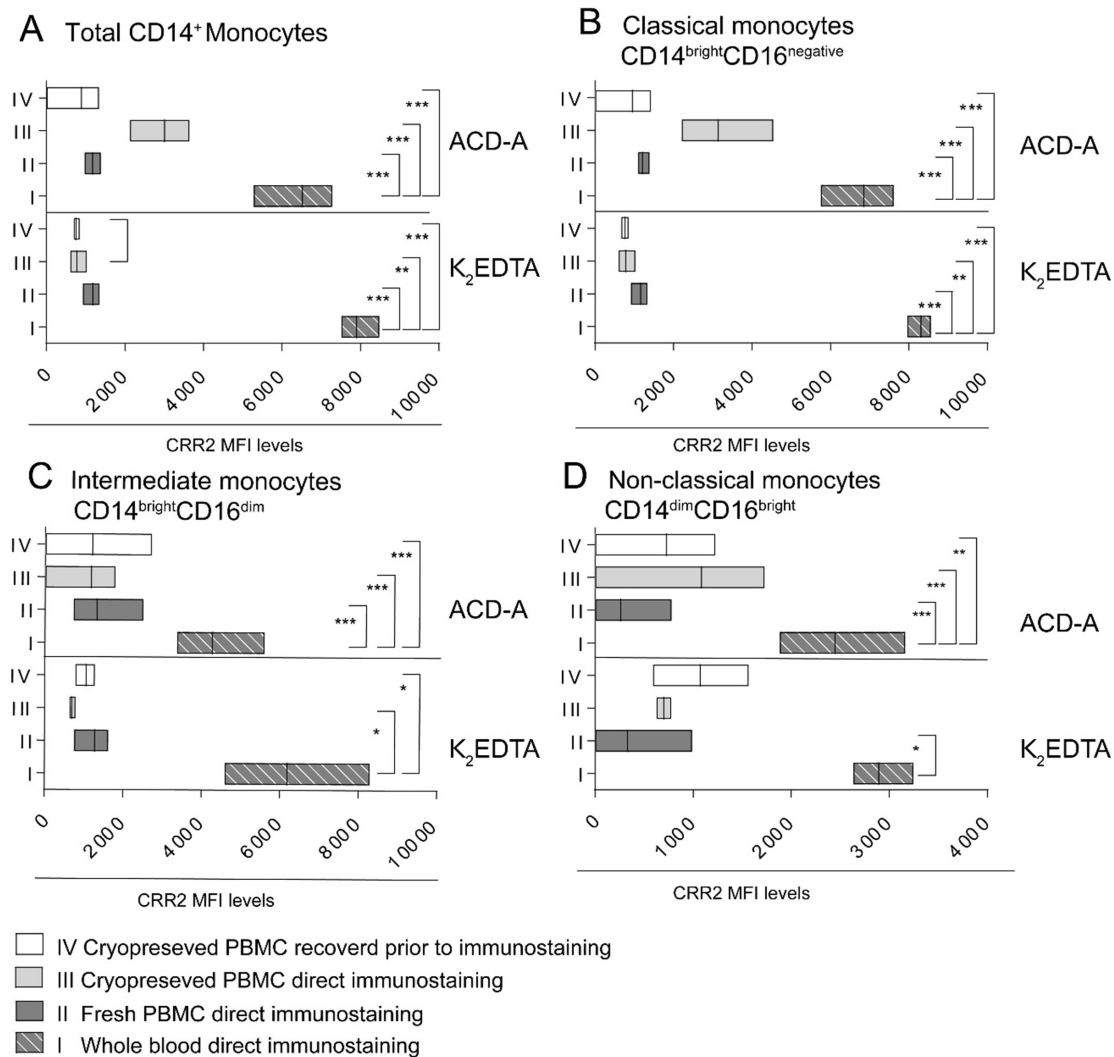


Fig. 4. CCR2 expression by monocytes derived from three healthy individuals; an analysis of the total CD14⁺ monocytes (A) and classical (B), intermediate (C) and non-classical (D) monocyte subsets. For each of the four analyses (A-D) we compared CCR2 expression of PBMC after cryopreservation/thawing/recovery (IV), after only cryopreservation/thawing (III), staining of freshly isolated PBMC (II) and whole blood staining (I). Results are presented as box plots where the ends of the box extend to the lowest and highest observations, and median is marked by a vertical line inside the box. For each donor immunofluorescences staining of K₂EDTA and ACD-A anticoagulated blood samples were examined. The results from statistical comparisons are indicated in the figure (One Way-ANOVA with post hoc): p < 0.05*; p < 0.01**; p < 0.001***.

whole blood for both K₂EDTA and ACD-A samples.

Cryopreservation caused a statistical significant increase in the amount of the CD14⁺CD16⁻ classical monocyte subset while intermediate- and non-classical monocytes were reduced in ACD-A samples. The increase in amount of CD14⁺ monocytes is probably a result of relative distribution secondary to a decrease in the amount of CD16⁺ monocytes after cryopreservation and thawing. In our data the CD14 expression was affected by the methodology as a statistically significant increase in its expression was observed after PBMC isolation compared with the observations in the whole blood analyses). However, this increased CD14 expression was not observed after cryopreservation, and

this was true for both anticoagulants investigated. The CCR2 chemokine receptor (CD192) is important for regulation of monocyte trafficking and has been used to aid classification of the three monocyte subsets (França et al., 2017). CCR2 is mainly expressed on the classical monocyte subset and its expression was significantly decreased when comparing whole blood samples to (i) freshly separated, (ii) cryopreserved and (iii) cryopreserved plus recovered PBMC. The CCR2 decrease was seen both for monocytes as described previously (Shantsila et al., 2011) and for all three monocyte subsets and the decrease was not dependent on the anticoagulant. The previous study by Nieto et al. (Nieto et al., 2012) suggests that separation-induced internalization is a

possible explanation for these difference.

We conclude that the detection and estimation of the classical, intermediate and non-classical monocyte subsets is not dependent on the type of anticoagulant, and insufficient filling of sample tubes down to ¼ of the correct volume does not alter the ratios between the various subsets. In contrast, preparation of PBMC before analysis reduces the total percentage of CD14⁺ monocytes while CD14 expression increases. The expression of CCR2 is reduced in PBMC compared to monocyte subsets analysed in whole blood, whereas cryopreservation mainly reduced CD16 expression and this was not reversed by post-thawing recovery. Our data thus indicate that cryopreservation alters the identification and hence the stoichiometry of the subsets. This alteration can be due to (i) decreased CD16-expression, (ii) increased CD14-expression, and/or (iii) a subset selection process due to the methodology used. It remains whether the non-classical monocytes are selectively lost during freezing or thawing, or that the expression of CD16 and/or CD14 is simply altered by the procedure. Our advice based upon our results are to investigate monocyte subsets in freshly drawn peripheral blood samples, thereby avoiding PBMC isolation and cryopreservation. Thus, a careful standardization of sample collection and handling as well as a detailed methodological description is necessary for studies of monocytes and monocyte subsets in peripheral blood.

Conflicts of interest

The authors declare there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jim.2018.06.003>.

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II

RESEARCH ARTICLE

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Circulating monocyte subsets in multiple myeloma patients receiving autologous stem cell transplantation – a study of the preconditioning status and the course until posttransplant reconstitution for a consecutive group of patients

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Abstract

Background: Induction therapy of multiple myeloma patients prior to autologous stem cell transplantation has changed from conventional chemotherapy to treatment based on proteasome inhibitors or immunomodulatory drugs. We used flow cytometry to analyze total monocyte and monocyte subset (classical, intermediate and non-classical monocytes) peripheral blood levels before and following auto-transplantation for a consecutive group of myeloma patients who had received the presently used induction therapy.

Results: The patients showed normal total monocyte concentrations after induction/stem cell mobilization, but the concentrations of classical monocytes were increased compared with healthy controls. Melphalan conditioning reduced the levels of total CD14⁺ as well as classical and non-classical monocytes, whereas intermediate monocytes were not affected. Thus, melphalan has a non-random effect on monocyte subsets. Melphalan had a stronger effect on total and classical monocyte concentrations for those patients who had received induction therapy including immunomodulatory drugs. Total monocytes and monocyte subset concentrations decreased during the period of pancytopenia, but monocyte reconstitution occurred before hematopoietic reconstitution. However, the fractions of various monocyte subsets varied considerably between patients.

Conclusions: The total level of circulating monocytes is normalized early after auto-transplantation for multiple myeloma, but pre- and post-transplant levels of various monocyte subsets show considerable variation between patients.

Keywords: Monocytes, Multiple myeloma, Autologous stem cell transplantation

Background

Multiple myeloma is usually characterized by proliferation of abnormal plasma cells in the bone marrow and secretion of monoclonal immunoglobulin [1–3]. Autologous stem cell transplantation is an established part of early myeloma-stabilizing treatment [4, 5], and the

patients usually develop a quantitative posttransplant CD4⁺ T cell defect that lasts for several months [6]. The posttransplant innate immune system is less well characterized, but early reconstitution of monocytes with reduced expression of HLA-DR and CD16 together with reduced cytokine production has been observed [7, 8], especially decreased release of proinflammatory cytokines (e.g. IL-6, TNF- α and IL-1 β) [8].

Monocytes constitute up to 10% of total circulating peripheral blood leukocytes in healthy individuals [9]; they can differentiate into macrophages or dendritic cells

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[10] and may also differentiate in endothelial direction [11]. Furthermore, immunomodulatory drugs (IMiDs, e.g. lenalidomide) can induce differentiation towards dendritic cells with modulation of the cytokine profile, the transcriptional regulation and the accessory cell functions [12]. Finally, based on the expression of CD14 (a cell surface co-receptor for lipopolysaccharide) and CD16 (the low affinity IgG receptor) monocytes are now divided into classical (CD14^{bright} CD16^{negative}), intermediate (CD14^{bright} CD16^{dim}) and non-classical (CD14^{dim} CD16^{bright}) monocytes [13–15]. Classical monocytes constitute 90% of the circulating monocytes in healthy individuals [13, 15, 16].

Monocytes seem to be involved in the development of myeloma bone disease [17–20] through the release of soluble mediators that stimulate osteoclastogenesis, and the presence of non-classical monocytes may be a potential marker for increased osteoclast precursors [18, 19]. However, monocytes are also important immunoregulatory cells, and they are important for the defense against complicating infections in myeloma patients [21–23]. Several new drugs have become available during the last decade for the treatment of multiple myeloma, and no previous studies have investigated the effects of these drugs on the levels of circulating monocyte subsets before and following auto-transplantation. In the present study, we therefore used a highly standardized methodology to characterize peripheral blood levels of monocyte subsets in auto-transplanted myeloma patients receiving pre-transplant induction treatment based on proteasome inhibitors and IMiDs.

Results

Myeloma patients show decreased concentrations of circulating total leukocytes prior to high-dose melphalan conditioning

We first compared the total leukocyte counts in peripheral blood for myeloma patients (Table 1, patients 2–18) and the healthy controls (12 males and 5 females, median age 51 years). The patients were tested immediately before high-dose melphalan conditioning, i.e. after initial induction treatment (see Table 1) followed by stem cell mobilization/collection based on cyclophosphamide plus G-CSF. At this time point they showed significantly decreased total leukocyte counts compared with the controls (Fig. 1a, $p = 0.004$), and patients receiving their first and second auto-transplantation showed a similar decrease. The decreased leukocyte counts were seen with both analytical methods (flow cytometry with counting beads, measurement by clinical hematology instrument), and the levels measured by these two methods were significantly correlated (Pearson correlation coefficient 0.963, p -value 0.0001). The total leukocyte levels prior to melphalan conditioning showed

no association with age, induction treatment (regimen, number of cycles), response to induction treatment, circulating CD34⁺ cell level at the day of harvesting or duration of posttransplant neutropenia/cytopenia (data not shown).

Myeloma patients show normal peripheral blood concentrations of total monocytes but decreased levels of classical monocytes prior to high-dose melphalan

The preconditioning peripheral blood concentrations of total CD14⁺ monocytes did not differ between the 17 myeloma patients (Table 1, patients 2–18) and 17 healthy controls (Fig. 1a). However, classical monocyte concentrations were then slightly increased (Fig. 1a, $p = 0.01$) whereas we could not detect any significant differences between patients and controls for intermediate and non-classical monocytes. The three patients admitted for their second auto-transplantation showed total monocyte and monocyte subset concentrations within the range for the patients admitted for their first transplantation (Fig. 1). Thus, the effect of mobilization/conditioning on circulating monocytes is a non-random effect mainly affecting the classical monocyte subset.

The total monocyte concentrations prior to the conditioning therapy showed no association with age, induction treatment (regimen, number of cycles), response to induction treatment, circulating CD34⁺ cell level at the first day of harvesting or the duration of posttransplant neutropenia/cytopenia (data not shown). The same was true for classical, intermediate and non-classical monocytes except that pre-harvesting CD34⁺ cell levels showed significant correlations to absolute and relative levels of intermediate ($r = 0.78/p = 0.001$ and $r = 0.75/p = 0.002$, respectively) and non-classical monocytes ($r = 0.63/p = 0.017$ and $r = 0.61/p = 0.047$, respectively).

Myeloma patients are heterogeneous with regard to the preconditioning monocyte subset distribution/percentage in peripheral blood

We first compared the relative levels of circulating total CD14⁺ monocytes (percentage of total leukocytes) and the various monocyte subsets (percentage of total monocytes) for 18 newly diagnosed myeloma patients (Table 1, patients 1–18) and the 17 healthy controls. The preconditioning percentage of CD14⁺ monocytes among total leukocytes was increased for the patients; this was expected since total leukocyte levels were decreased whereas the monocyte concentration was not significantly altered before melphalan conditioning (Fig. 1b, $p = 0.013$).

The preconditioning percentages of the classical, intermediate and non-classical monocyte subsets among CD14⁺ monocytes did not differ between patients and healthy controls (Fig. 1b). However, the variation range was wider for the patients both for the percentage of

Table 1 The characteristics of the myeloma patients included in the study

Patient	Age ^{d)}	M-component	Transplant ^{a)}	Conditioning therapy (drugs, number of cycles)		Effect of induction	CD34 count at the time of harvesting ^{b)}	Duration of cytopenia ^{c)}	
				Pretransplant treatment	Number of cycles			Neutrophils	Thrombocytes
1	60–70	IgG MM	Second	CVD	4	VGPR		5	2
2	60–70	IgG MM		CVD	4	VGPR	171,270	4	1
3	60–70	IgA MM	Second	CVD	4	VGPR		5	3
4	50–60	IgG MM		CVD	5	VGPR	32,180	5	5
5	50–60	IgG MM		CVD	5	PR	48,635	2	1
6	30–40	LCD		CVD (1), KRd (4)	5	VGPR	70,570	5	3
7	60–70	IgG MM		VTD (1), CVD (2)	3	VGPR	218,945	4	2
8	60–70	IgG MM		CVD	4	VGPR	90,930	4	4
9	60–70	IgA MM		CVD	5	PR	42,285	3	3
10	50–60	IgG MM	Second	PVD	6	PR		5	4
11	50–60	IgA MM		CVD	4	VGPR	32,125	5	4
12	60–70	IgA MM	Second	VRD	4	VGPR		5	2
13	60–70	LCD-L		CVD	4	VGPR	60,410	5	4
14	60–70	IgG MM		CVD, VTD, VD (3)	5	VGPR	123,190	6	1
15	60–70	IgG MM		VCD	4	PR	37,060	8	5
16	60–70	LCD-K		RD	4	VGPR	36,010	4	6
17	50–60	IgA-MM		CVD	4	VGPR	132,000	4	5
18	60–70	LCD-L		CVD (4 before and 2 after harvesting)	6	VGPR	27,690	5	5
19	50–60	IgG MM	Second	CVD	4	VGPR		5	3
20	50–60	Amyloid-MM IgG-L		CVD (3), VTD (1)	4		185,800	4	2
21	40–50	IgG MM		CVD (4 before harvesting), VTD (2 after), RD (2 after)	8	VGPR	56,380	4	6
22	50–60	LCD-L	Second	CVD	4	PR		7	5
23	50–60	IgG MM	Second	CVD, VTD, VD (4)	6	PR		5	5
24	50–60	LCD-L		CVD	4	VGPR	74,800	3	4
25	60–70	LCD-K	Second	CVD	4	PR		3	2

Abbreviations: CVD Cyclophosphamide, bortezomib (Velcade®), dexamethasone, LCD Light chain disease type lambda (L) or kappa (K), MM Multiple myeloma, PR Partial response, RD Lenalidomide (Revlimide®) plus dexamethasone, VGPR Very good partial response, VRD Bortezomib, lenalidomide (Revlimide®), dexamethasone, VTD Bortezomib (Velcade®), thalidomide, dexamethasone

^{a)} Patients undergoing their second auto-transplantation are indicated; the stem cell graft was the same as for the first transplantation for all these patients

^{b)} The peripheral blood concentration of CD34⁺ cells on the (first) day of harvesting is given; the level is expressed as $\times 10^3$ cells/mL

^{c)} Neutropenia was defined as the time from the first day of neutrophil peripheral blood concentration $\leq 0.2 \times 10^9/L$ until the first of three consecutive days with neutrophils exceeding $0.2 \times 10^9/L$ or alternatively the first day with neutrophil counts $> 10 \times 10^9/L$. The duration of thrombocytopenia was defined as the number of days from the first day of peripheral blood thrombocyte counts below $20 \times 10^9/L$ until the first day with thrombocyte count above $20 \times 10^9/L$ without thrombocyte transfusion

^{d)} The age of patients are grouped

total CD14⁺ monocytes and the three monocyte subsets. Firstly, classical monocytes constituted a majority of the CD14⁺ monocytes (corresponding to > 70%) both for the healthy controls and for all except five patients. We also observed wide variation ranges for the intermediate and non-classical monocyte subsets; exceptional patients showed intermediate monocyte levels exceeding 15% and non-classical monocytes levels up to 40% of the total CD14⁺ monocytes (Fig. 1b). Wide variations were observed both for patients admitted to their first auto-transplantation and for the four patients admitted to their second transplantation. The percentages of total

monocytes and monocyte subsets showed no significant associations with age, induction treatment, response to induction treatment, CD34⁺ cell level at the day of harvesting or time until posttransplant neutrophil/platelet reconstitution (data not shown).

The concentrations of circulating CD14⁺ monocytes decrease early after melphalan conditioning

We compared the peripheral blood levels of total leukocytes and CD14⁺ monocytes before the conditioning therapy (day -2) and 2 days later immediately before the autologous stem cell reinfusion (day 0). Ten patients

were available for this paired comparison (Table 1, patients 7–9, 11–17), and total leukocyte levels were not altered 2 days after the melphalan infusion (i.e. immediately before transplantation, see Fig. 2a). In contrast, the concentrations of circulating monocytes were significantly decreased 2 days after the conditioning, and a similar decrease was observed when total monocyte levels were analyzed by clinical hematology instrument(-data not shown) and when using whole-blood staining for flow cytometric analysis of CD14⁺ monocytes (Fig. 2b). A comparable decrease was seen for patients receiving their first and the second auto-transplantation, but neutrophil levels were increased for many patients (see below) so that total leukocyte levels were not significantly altered.

We finally compared the levels of circulating neutrophils, total lymphocytes and thrombocytes (estimated by clinical hematology instrument) immediately before and 2 days after melphalan conditioning (Table 1, patients 7–9, 11–17). The neutrophil levels 2 days after melphalan were slightly increased (median level 3.6 versus 4.9 × 10⁹/L, *p* = 0.06), whereas lymphocyte (median level 1.0 versus 0.2 × 10⁹/L, *p* = 0.002) and thrombocyte levels (median levels 225 versus 177 × 10⁹/L, *p* = 0.002) were significantly decreased similar to the monocyte levels.

Melphalan conditioning causes an early decrease in the percentages of circulating classical and non-classical monocytes whereas the levels of the intermediate subset are not altered

We compared the peripheral blood percentages of classical, intermediate and non-classical monocytes at day -2 pre-transplant (i.e. before conditioning) and day 0 (i.e. before stem cell transplantation) for 10 patients (Fig. 2c-e; Table 1 patients 7–9, 11–17). The concentrations of all three monocyte subsets were decreased for most patients 2 days after the conditioning, but the difference reached statistical significance only for the classical (Wilcoxon's test for paired samples; *p* = 0.002) and non-classical subsets (*p* = 0.0039). Thus, melphalan conditioning has a non-random early effect on circulating monocyte subsets. However, we observed a wide variation in the percentage of the various monocyte subsets among total CD14⁺ monocytes in preconditioning samples (Fig. 1), and wide variations persisted after conditioning both for the classical (variation range 41–93%), intermediate (2–48%) and non-classical (2–51%) monocyte subsets.

The early effect of melphalan conditioning on monocyte concentrations differs between patients receiving induction treatment with and without immunomodulatory drugs

IMiDs can alter the monocyte phenotype [24], and we therefore compared the effect of the melphalan conditioning for

patients receiving induction treatment with or without IMiDs (i.e. thalidomide, lenalidomide, pomalidomide). A total of 13 patients were included in this analysis. At day 0 (the day of transplantation) patients receiving induction treatment including IMiDs showed decreased concentrations of circulating total monocytes (median level 0.0023 × 10⁹/L with range 0.001–0.01 versus 0.0061 × 10⁹/L with range 0.003–0.03; *p* = 0.045) and classical monocytes (median 0.0022 × 10⁹/L with range 0.001–0.010 versus 0.0057 × 10⁹/L with range 0.0003–0.016; *p* = 0.046) compared with patients receiving induction treatment without these drugs). Furthermore, the absolute and relative levels of total monocytes and the various monocyte subsets 2 days after the conditioning therapy (i.e. on day 0, the day of transplantation) showed no association with age, response to induction treatment or levels of circulating CD34⁺ cells at the first day of harvesting (data not shown). Finally, the day 0 pretransplant levels of circulating neutrophils, total lymphocytes and thrombocytes did not differ between patients that had received induction therapy with and without IMiDs (data not shown). Thus, the IMiDs seem to have a non-random effect on the various monocyte subsets that becomes detectable after the melphalan infusion.

The peripheral blood concentrations of all three monocyte subsets show a further decrease during the period of severe neutropenia

We investigated the peripheral blood concentrations of the three monocyte subsets during the period of severe neutropenia for 8 myeloma patients (Table 1 patients 13, 14, 16, 17, 21–24); for five of these patients we could compare the levels immediately before stem cell reinfusion with the levels during severe cytopenia (patients 13, 14, 16, 17, 21). As expected the concentrations of all three monocyte subsets, especially the classical and intermediate subsets, decreased to low levels during cytopenia (Fig. 2). In contrast, the relative levels (i.e. percentage among total CD14⁺ monocytes) varied during pancytopenia when tested 6–8 days after stem cell reinfusion. All patients showed < 5% intermediate monocytes, whereas classical monocyte levels varied between 8 and 92% (median 62%) and non-classical monocytes also showed a considerable variation (median 18%, range 3–57%).

Auto-transplanted myeloma patients show expected early hematological reconstitution

The levels of circulating total leukocytes, neutrophils and thrombocytes were measured by clinical hematology instrument) for all our patients. Neutrophil reconstitution was defined as the first of 3 days with neutrophils above 0.2 × 10⁹/L. The median time from first day of

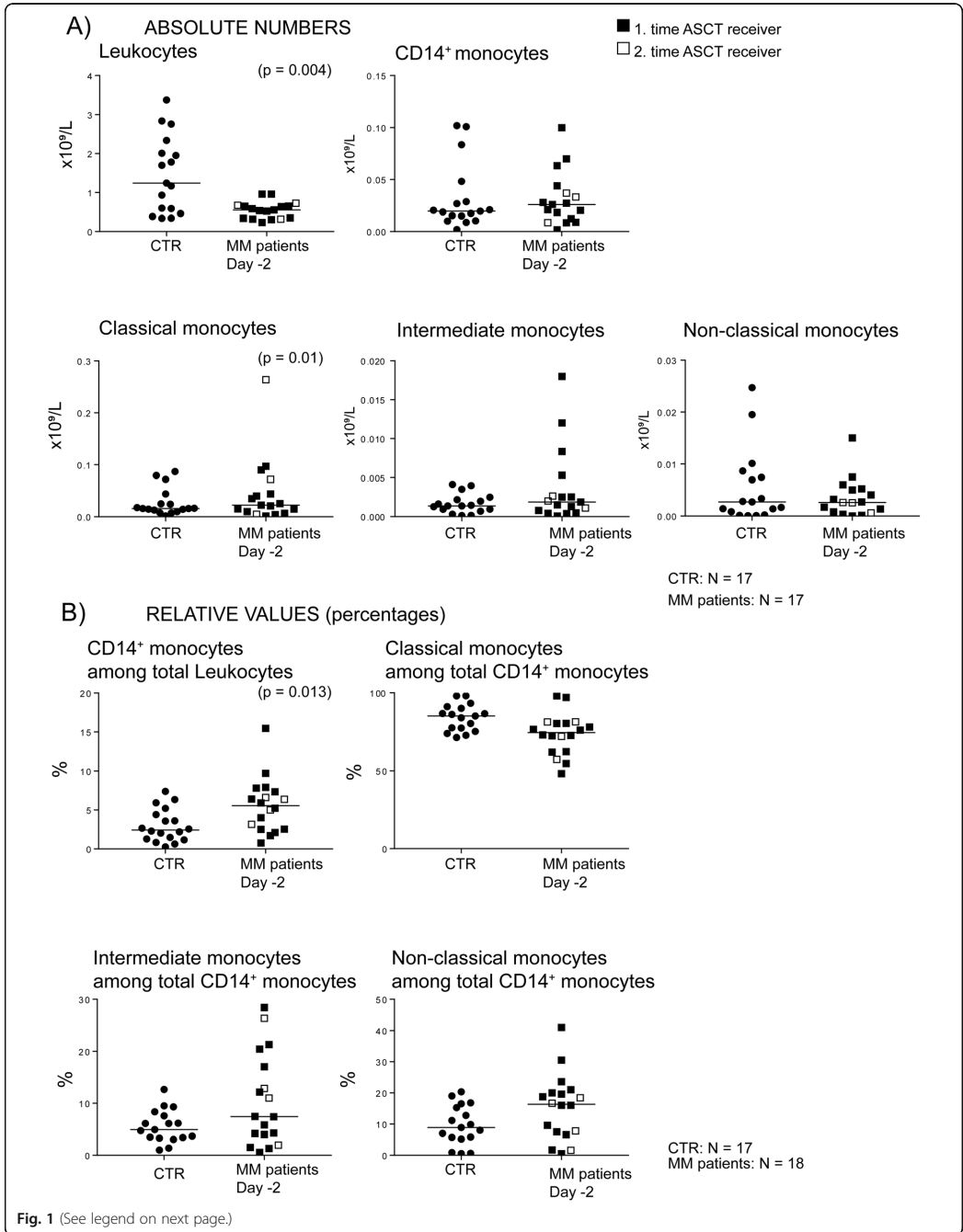


Fig. 1 (See legend on next page.)

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Fig. 1 The peripheral blood levels of total leukocytes, total monocytes and monocyte subsets; a comparison between healthy controls and multiple myeloma patients examined after the initial induction chemotherapy and stem cell harvesting with cyclophosphamide plus G-CSF, i.e. immediately before conditioning high-dose melphalan therapy (pre-transplant day - 2). **(a, UPPER FIGURES)** We used flow cytometry to estimate the concentrations of total circulating leukocytes, total CD14⁺ monocytes and classical, intermediate and non-classical monocyte subset. The results for 17 patients (Table 1, patients 2–18) were compared with 17 healthy controls (CTR) individuals. Three of the 17 patients received their second auto-transplantation. **(b, LOWER FIGURES)** The percentage of circulating CD14⁺ monocytes among total leukocytes and the percentages of classical, intermediate and non-classical monocytes among total CD14⁺ monocytes were estimated. The results for 18 patients (Table 1, patients 1–18) were compared with the 17 healthy individuals. Four patients received their second auto-transplantation. In all the figures, black symbols represent the levels for patients receiving their first auto-transplantation whereas open symbols represent levels for patients receiving their second transplantation. The Wilcoxon's test for paired samples was used for statistical analyses and the *p*-values for statistically significant differences are indicated in the figure

neutropenia (i.e. first day with neutrophils $\leq 0.2 \times 10^9/L$) until neutrophil reconstitution was 4 days (range 2–9 days). Furthermore, thrombocyte reconstitution was defined as the first out of three consecutive days with thrombocyte counts above $20 \times 10^9/L$ in transfusion-independent patients. The median duration of thrombocytopenia (i.e. thrombocyte levels below $20 \times 10^9/L$) was 4 days (range 1–6 days). Finally, time to neutrophil/thrombocyte reconstitution did not differ between patients receiving their first or second auto-transplantation and showed no significant associations with preconditioning (i.e. day - 2) or pre-transplant (i.e. day 0) total monocyte levels.

Auto-transplanted myeloma patients show early monocyte reconstitution

The absolute levels of total monocytes were followed daily during the period of early hematological reconstitution for 24 consecutive patients. The median time from transplantation until the monocyte levels exceeded the lower normal limit ($0.04 \times 10^9/L$) was 10 days; the median monocyte level was then $0.23 \times 10^9/L$ (range 0.05 – $0.78 \times 10^9/L$). The neutrophil levels at the first day of monocyte normalization were generally below the lower normal limit (median $0.5 \times 10^9/L$, range 0.1 – $3.8 \times 10^9/L$), i.e. for 18 patients the neutrophil levels were still below the lower normal limit. All patients still had severe thrombocytopenia (median $29 \times 10^9/L$, range 13 – $38 \times 10^9/L$) at the first day of monocyte normalization. Finally, there was no significant association between preconditioning or pre-transplant total monocyte levels and time to normalized circulating monocyte levels, and monocyte normalization did not differ for patients receiving induction treatment with or without IMiDs (data not shown).

We compared the absolute and relative levels of various monocyte subsets at day +10/+12 posttransplant with the corresponding preconditioning levels (day - 2); paired samples were then available only for eight patients (Fig. 3; Table 1 patients 14, 16, 17, 21–25). This posttransplant time point corresponds to the initial neutrophil reconstitution, but the neutrophil levels were still

below the lower normal limit for six of the eight patients (median level $0.7 \times 10^9/L$, range 0.2 – $6.8 \times 10^9/L$). The thrombocyte counts for all patients (median $30 \times 10^9/L$, range 20 – $52 \times 10^9/L$) were also below the lower normal limit. However, even at this early time point only 10–12 days post-transplant most patients showed normalized absolute (concentration) and relative (percentage) levels of total CD14⁺ monocytes as well as the three monocyte subsets within the pre-transplant variation range.

One of our collaborating local hospitals only investigated peripheral blood neutrophil but not monocyte counts at the out-patient evaluations; for this reason peripheral blood monocyte counts were only available at later time points for 15 patients. The total monocyte count (normal range 0.04 – $1.30 \times 10^9/l$) was tested early after neutrophil and platelet reconstitution, and at this time point (median time 14 days posttransplant, range 14–16) nine patients showed normal and six patients showed increased counts (median $0.95 \times 10^9/L$, range 0.40 – $2.1 \times 10^9/l$). The total monocyte counts tested at a later time point (median 30 days posttransplant, range 27–39 days) showed increased levels for a minority of four patients (median level $0.97 \times 10^9/L$, range 0.20 – $1.78 \times 10^9/L$).

Most patients shows disease stabilization lasting at least 2 years after the first transplantation

The posttransplant observation time for patients receiving their first auto-transplantation was 27–36 months (median 32 months). Five patients had disease progression less than 2 years post-transplant, one patient was lost from follow-up and all other patients remained in plateau phase during follow-up. Progression-free survival less than 2 years showed no association with preconditioning ($n = 16$) or pretransplant ($n = 18$) total monocyte or monocyte subset levels or with posttransplant time to normalized total monocyte levels ($n = 24$) (data not shown).

Discussion

Autologous stem cell transplantation is widely used in the treatment of younger myeloma patients up to 70

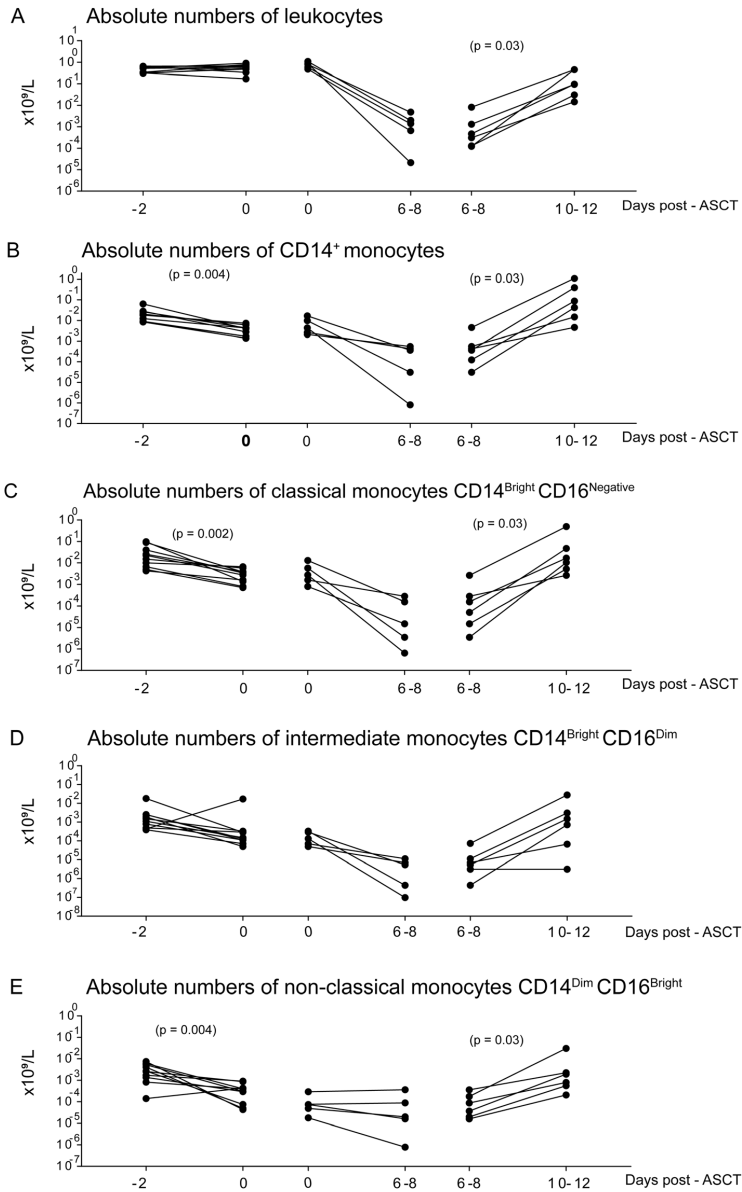


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Fig. 2 The peripheral blood concentrations of total leukocytes **(a)**, CD14⁺ monocytes **(b)**, classical **(c)**, intermediate **(d)** and non-classical **(e)** monocytes in auto-transplanted myeloma patients. We estimated the cell concentrations by flow cytometry. The left parts of each panel show a comparison of peripheral blood levels before versus 2 days after the conditioning melphalan therapy (day -2 versus day 0; Table 1, patients 7–9, 12–18). The middle parts show a comparison of levels at the time of transplantation (day 0) versus the levels during the period of severe posttransplant melphalan-induced pancytopenia with neutrophils $< 0.2 \times 10^9/L$ and thrombocyte transfusion dependency (tested 6–8 days posttransplant; Table 1, patients 14, 15, 17, 22, 24). The right panel show the comparison of peripheral blood levels during the severe pancytopenia versus the levels during initial hematopoietic and immunological reconstitution (tested 10–12 days posttransplant with increasing neutrophils $> 0.2 \times 10^9/L$ and thrombocyte transfusion independence; Table 1 patients 15, 17, 18, 22–24). The Wilcoxon's test for paired samples was used for statistical analyses and significant p-values are indicated in the figure (ASCT, autologous stem cell transplantation)

years of age [25]. The pre-transplant conditioning therapy has direct anti-leukemic effects, but previous studies suggest that immune-mediated anti-myeloma activity may also contribute to the effect of this therapy [26–28]. The lymphoid reconstitution has been investigated in previous studies [29], but the monocytes are less well characterized and for many of the previous studies the induction treatment included conventional cytotoxic drugs and not proteasome inhibitors or IMiDs. In our present study, we investigated the levels of circulating total monocytes and monocyte subsets in auto-transplanted myeloma patients. Although our study is relatively small, we observed that the pre-transplant induction and stem cell mobilization by cyclophosphamide plus G-CSF seemed to have only a minor effect on the preconditioning monocyte levels even though the concentration of total circulating leukocytes was decreased compared with the healthy controls.

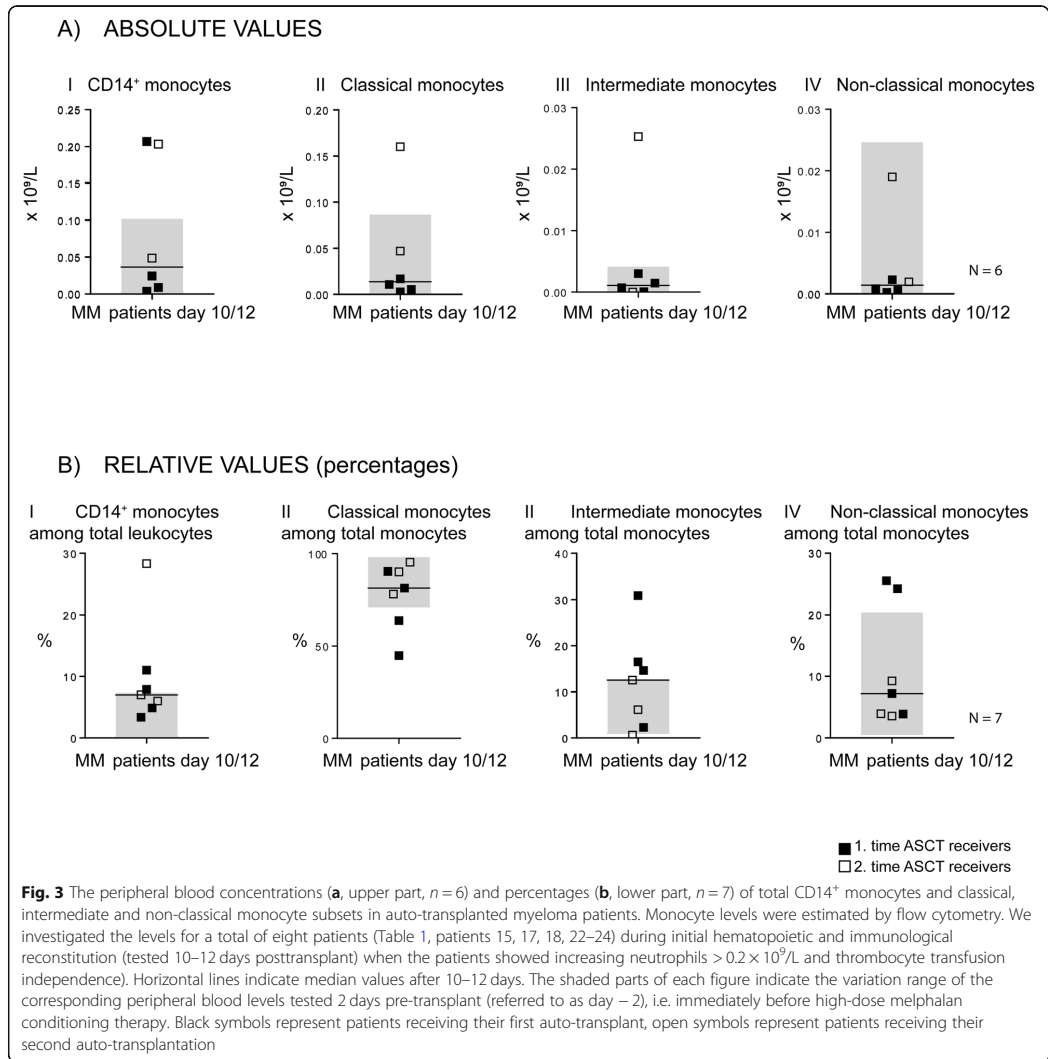
Our present observations will probably not only depend on the use of IMiDs and proteasome inhibitors in the induction treatment but on the overall clinical and biological context of our patients. One should emphasize that the use of cyclophosphamide in stem cell mobilization will probably influence our results. The same may be true for our use of posttransplant G-CSF therapy, e.g. through its effects on systemic metabolic regulation that influence the metabolic environment of regenerating hematopoietic and immunocompetent cells [30]. Furthermore, studies in healthy donors show that G-CSF has a mobilizing effect on many different immunocompetent cells, including monocytes [31, 32]. These effects differ between healthy donors; they will also influence the levels of immunocompetent cells in the stem cell grafts and possibly also outcome in allotransplant recipients. To the best of our knowledge it is not known whether similar differences exist for auto-transplanted myeloma patients, and unfortunately we do not have information about graft levels of various monocyte subsets for our patients.

We analyzed the total number of monocytes by two different methodological approaches, i.e. by using a hemocytometer and by using flow cytometry to estimate the levels of CD14⁺ total monocytes. Both these analyses showed that the preconditioning patient levels did not differ from healthy controls, whereas the levels 2 days

after the conditioning (i.e. immediately before stem cell reinfusion) were decreased compared with the preconditioning levels. However, the levels of CD14⁺ monocytes were lower than the monocyte levels estimated in the alternative assays, and this difference is probably due to a random loss of cells during the washing steps.

Our studies included all except one patient from a defined geographic area and during a defined time period; for this reason it should be regarded as a population-based study. We could not investigate all patients at every time point during the treatment. However, we would emphasize that this was due to practical reasons such as transfer of patients to their local hospital or long traveling distance from their home to the transplantation center; it was not because of the disease, the treatment or development of complications. Leukocyte levels show diurnal variations [33–36], and for this reason we sampled the patients only in the morning, and shipment of samples or analysis of cryopreserved cells was not possible due to our standardized methods for handling of the samples [37].

Our present study showed that the preconditioning patient levels did not differ from healthy controls, i.e. the myeloma disease itself, the induction treatment and the stem cell mobilization by cyclophosphamide plus G-CSF have only minor effects on monocytes except for a slight increase of classical monocytes. In contrast, the melphalan conditioning seemed to have a nonrandom effect of the monocyte subsets before an early reconstitution of all three subsets was observed. However, it should be emphasized that there is a wide variation between patients with regard to the effects of the conditioning therapy. A short duration of this monocytopenia is also suggested by previous studies [7, 8], but our study is the first to suggest that this is true also for patients receiving IMiD- or proteasome inhibitor-based induction therapy and for different monocyte subsets. Furthermore, the studies by Callander et al. [38] suggest that even though monocyte levels are normalized at day 100 posttransplant, the levels of total CD14⁺ and CD14⁺CD16^{low/negative} classical monocytes are then associated with prolonged progression-free survival after auto-transplantation. Thus, taken together these studies show that monocyte reconstitution occurs early (according to our study very early) after auto-transplantation, but



despite this normalization there is still a relatively wide variation between patients and this heterogeneity in monocyte (subset) levels seems to persist until day 100 posttransplant and may even have a prognostic impact. The antimyeloma effect of posttransplant monocyte targeting may therefore vary between patients and depend on the monocyte subset profile. IL6 is regarded as a possible target in multiple myeloma [39]; monocytes constitute a subset of the bone marrow stromal cells that are regarded as important regulators of both normal and malignant hematopoietic cells [16, 40]. IL6 is released by monocytes, especially classical monocytes, in response to ligation of various Toll-like receptors,

and therapeutic targeting of IL6/monocytes may therefore be most effective for those patients with high levels of classical monocytes.

Most of our patients received only 3 or 4 induction cycles before stem cell transplantation, whereas 6 cycles are now often recommended, especially for patients who have not received a complete remission [25, 41, 42]. Alternative induction cycles have also been used in other studies [33, 41, 42], and future studies have to clarify whether our present results are representative also for patients receiving additional cycles or alternative induction treatment.

Our comparison of induction treatments with and without IMiDs suggests that the post-conditioning monocyte concentrations are influenced by the previous use of immunomodulatory drugs in the induction therapy, whereas the capacity of stem cell mobilization and response to the induction therapy are less important. However, G-CSF responsiveness (i.e. CD34⁺ cell mobilization) was associated with the levels of intermediate and non-classical monocytes before conditioning therapy.

Previous studies have shown that early posttransplant lymphoid reconstitution is associated with a favorable prognosis of auto-transplanted myeloma patients [26–29]. More recent studies suggest that this effect may be due to early NK cell reconstitution [29]. Even though monocytes have important immunoregulatory functions, the previous studies have not investigated whether the early monocytic reconstitution is required for the prognostic impact of early lymphoid reconstitution. A recent study of allotransplant recipients suggests that monocytes can mediate anti-myeloma effects [43], and monocytes derived from auto-transplanted patients may even be used for immunotherapy due to their presentation of myeloma-associated peptides to the adaptive immune system [44]. Our present studies thus suggest that a close to normal monocyte system is present in myeloma patients even early after auto-transplantation and may then be an immunotherapeutic target.

Monocytes and macrophages are important members of the bone marrow stem cell niches that support both normal and malignant hematopoiesis [40]. IMiDs can alter the differentiation of monocytes [24], and we therefore investigated whether the use of such drugs for induction therapy was associated with an altered balance between monocyte subsets later during treatment or with other differences in hematopoietic reconstitution between patients. Our present study showed that the type of induction therapy actually has an influence on monocytes/monocyte subsets, but this difference was only detected after additional melphalan therapy in pre-transplantation (Day 0) samples.

The observation time for our patients was relatively short (27–35 months) for our patients that received their first auto-transplantation and a majority of them were still in a plateau phase. We could not detect any associations between time to progression (i.e. progression before 2 years posttransplant) and monocyte subset levels/reconstitution. However, these data should be interpreted with great care because the patient cohort is relatively small for such analyses and the observation time is short and patients with early relapse are few.

Conclusions

Although our study is relatively small, we observed that the total level of circulating monocytes is normalized

early after auto-transplantation for multiple myeloma. However, the levels of various monocyte subsets show considerable variation between patients. Clinical studies including larger number of patients and a longer observation time are needed to clarify whether these differences are associated with overall survival, time to relapse and/or frequencies of severe infections.

Methods

Aim, design, characteristics of myeloma patients and healthy controls

Proteasomal inhibitors and IMiDs are now commonly used in induction treatment of young and fit myeloma patients prior to stem cell harvesting and auto-transplantation. These drugs may thereby influence the pretransplant immunological status of the patients and the immunocompetent cells in the stem cell graft. The aim of our present study was therefore to investigate the preconditioning status of the monocyte system in myeloma patients treated with induction chemotherapy based on IMiDs or proteasomal inhibitors, and to characterize the monocyte subset levels in autotransplanted patients during the early post-transplant period until hematological reconstitution.

Our hospital is the only center for stem cell transplantation in a defined geographical area of Norway (Health Region III), and our patients represent all myeloma patients except one receiving autologous stem cell transplantation in this area during an 8 months period. Our study should therefore be regarded as a population-based study of unselected patients.

The diagnosis of multiple myeloma was based on generally accepted criteria [1, 45], and induction therapy was initiated in accordance with generally accepted international guidelines [45, 46]. The patient characteristics are presented in Table 1. All patients received premobilization therapy including either a proteasome inhibitor or an IMiD; this induction treatment was followed by stem cell mobilization using cyclophosphamide plus G-CSF [47, 48]. The stem cell grafts were cryopreserved in 5% dimethyl sulfoxide and stored in liquid nitrogen until reinfusion [49, 50]. All patients were transplanted with at least 6×10^6 CD34⁺ cells per kilo body weight, and the median time from start of induction to transplantation was 16 weeks (range 13–28 weeks). For patients receiving their first auto-transplantation the grafts had been stored for 3–5 weeks, whereas for those patients receiving their second transplantation the grafts were stored for at least 2 years. All patients received conditioning therapy with melphalan (Fresenius Kabi, Oslo, Norway), 200 mg/m² administered as an intravenous infusion 2 days before stem cell reinfusion. They received G-CSF 5 µg/kg from day + 4 post-transplant until stable neutrophil recovery, i.e. peripheral

blood neutrophil levels above $0.2 \times 10^9/L$ for three consecutive days or exceeding $10 \times 10^9/L$.

The normal controls were healthy blood donors; in accordance with the approved routines at the Blood Bank, Haukeland University Hospital peripheral venous blood samples for medical research were donated after written informed consent.

Blood sampling

Blood samples were drawn in ACD-A (9 mL, #248368, BD Vacutainer, San Jose, CA, USA) blood sampling vacuum tubes. We collected the first patient sample immediately before melphalan conditioning (day - 2). The second sample was collected 2 days later immediately before reinfusion of the peripheral blood stem cell graft (day 0). The third sample was collected on day + 6 post-transplant when patients had severe neutropenia (peripheral blood neutrophil counts below $< 0.2 \times 10^9/L$) and thrombocytopenia. The last sample was collected on the first or second day with peripheral blood neutrophil counts exceeding $0.2 \times 10^9/L$ (10–12 days posttransplant). The control samples were derived from 17 healthy blood donors (5 females and 12 males, median age 51 years with range 22–82 years). All samples were processed at room temperature within 120 min. All samples were collected between 08:00 and 10:00 am. It was not possible to get samples from all patients at all four time points; this was due to either transfer to their local hospitals after stem cell reinfusion or the patient was not available for sampling at the indicated time in the morning.

Flow cytometric analysis

Four mL of ACD-A (9, #248368, BD Vacutainer) anticoagulated whole blood and 46 mL of lysing buffer (# 55589, BD Biosciences) were mixed and incubated for 15 min at room temperature. Subsequently, leukocytes were collected by centrifugation (400×g, 5 min, room temperature) and thereafter washed in phosphate-buffered saline with 1% Bovine Serum Albumin (BSA, Bovine Serum Albumin Fraction V #10735086001, Sigma-Aldrich/Merc KGaA, Darmstadt, Germany). The cells were reconstituted in 200 µL 1% BSA/PBS with 10% immunoglobulin solution (Octagam 100 mg/mL, Octapharma, Lachen, Switzerland). The following mouse anti-human antibodies were included in the antibody panel (all from BD Biosciences, San Jose, CA, US): CD14 Alexa 488 (Clone M5E2), CD56 Alexa 647 (Clone B159), CD16 PerCpCy™5–5 (Clone 3G8), CD45 V500 (Clone HI30), CD11b V540 (Clone ICRF44 (44)) and HLA-DR PE (Clone G46–6). The staining procedure and gating strategy for identification of monocytes and monocyte subsets has been described in detail in a previous methodological article [37].

All samples were analyzed by a 10-parameter BD FACS Verse flow cytometer equipped with 404, 488 and 640 nm lasers. We used BD FACSuite™ CS&T Research Beads (#650621, BD Biosciences, San Jose, CA, USA) for regular quality control of the instrument, single-stained compensation bead samples (#552843, BD Biosciences) for compensation and unstained samples as gating controls. At least 5000 monocytes were analyzed for each sample (based on SSC/FSC properties). We used counting beads (Count Bright Absolute Counting beads™, # C36950, Invitrogen™, Thermo Fischer Scientific, Waltham, MA USA) when estimating the concentrations of monocytes/monocyte subsets. FlowJo software (Tree Star, Inc., OR, USA) was used for analysis of the results.

Analysis of total leukocytes, neutrophils, monocytes and thrombocytes in peripheral blood

Analyses of peripheral blood levels of total leukocytes and total monocytes were performed by using accredited clinical hematology instrument (Laboratory for Clinical Biochemistry and Hematology at Haukeland University Hospital).

Statistical analyses

We applied IBM SSP statistics 23 for all statistical analyses. The Wilcoxon's rank sum test and the Wilcoxon's test for paired samples were used for comparison of different groups and for comparison of paired observations, respectively. The Pearson's test was used for correlation analyses. Differences were regarded as statistically significant when *p*-values were below 0.05.

Abbreviation

IMiDs: Immune modulatory drugs

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Authors' contributions

IMR performed the experiments. ABA and ØB recruited patients and provided clinical data. IMR, AR, EE and ØB designed the study. IMR, AR, EE, ABA and ØB wrote the article. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets used and analyzed during the study are available from the corresponding author on request.

Ethics approval and consent to participate

The study was conducted according to the Declaration of Helsinki. Collection of patient samples and the use of these samples in the present study were approved by the Regional Ethics Committee. Samples were collected and

stored after written informed consent (REK Vest 2015/1759), and the use of biological material and the present project was also approved by the committee (REF Vest 2017/305, 2013/102). The normal controls were healthy blood donors; in accordance with the approved routines at the Blood Bank, Haukeland University Hospital, peripheral venous blood samples for medical research were donated after written informed consent.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Article

A Pilot Study of Circulating Monocyte Subsets in Patients Treated with Stem Cell Transplantation for High-Risk Hematological Malignancies

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Abstract: *Background and Objectives:* Autologous and allogeneic stem cell transplantation is used in the treatment of high-risk hematological malignancies, and monocytes are probably involved in hematological reconstitution as well as posttransplant immunoregulation. The aim of our study was to investigate the levels of circulating monocyte subsets in allotransplant recipients. *Materials and Methods:* The levels of the classical, intermediate, and nonclassical monocyte subsets were determined by flow cytometry. Sixteen patients and 18 healthy controls were included, and the levels were analyzed during pretransplant remission ($n = 13$), early posttransplant during cytopenia ($n = 9$), and early reconstitution ($n = 9$). *Results:* Most patients in remission showed a majority of classical monocytes. The patients showed severe early posttransplant monocytopenia, but the total peripheral blood monocyte counts normalized very early on, and before neutrophil and platelet counts. During the first 7–10 days posttransplant (i.e., during cytopenia) a majority of the circulating monocytes showed a nonclassical phenotype, but later (i.e., 12–28 days posttransplant) the majority showed a classical phenotype. However, the variation range of classical monocytes was wider for patients in remission and during regeneration than for healthy controls. *Conclusions:* The total peripheral blood monocyte levels normalize at the very early stages and before neutrophil reconstitution after stem cell transplantation, and a dominance of classical monocytes is reached within 2–4 weeks posttransplant.

Keywords: monocytes; leukemia; stem cell transplantation; flow cytometry; hematology

1. Introduction

Acute myeloid (AML) and acute lymphoblastic leukemia are both aggressive malignancies characterized by the accumulation of immature malignant cells in the bone marrow [1,2]. However, several less aggressive hematological malignancies are also regarded to have unfavorable prognoses with short expected survival, e.g., the high-risk myelodysplastic syndromes [3] and certain polymorphocytic leukemia variants [4]. The only or the best possibility for cure for all these malignancies is intensive conventional chemotherapy, possibly combined with allogeneic or autologous stem cell transplantation [1]. However, several new and promising therapeutic approaches are now considered and/or are available for high-risk hematological malignancies, including T-cell targeting immunotherapy [5,6], new monoclonal antibodies for the treatment of acute lymphoblastic leukemia [7], modulation of apoptotic regulation with increased proapoptotic activity, or [8] inhibition of intracellular

signaling, including metabolic targeting [9,10]. These new therapeutic strategies may be used as a part of the initial treatment to reduce the risk of later relapse and thereby reduce the need for high-toxicity antileukemic therapy (e.g., allogeneic stem cell transplantation), or they can be used to reduce the risk of posttransplant relapse [9,11].

The median age for the time of diagnosis is 60–70 years for most hematological malignancies [1], and the most intensive therapeutic strategies can only be used for relatively young patients without severe comorbidity [12]. Conventional intensive chemotherapy is usually considered for patients up to 75–80 years of age [13], whereas stem cell transplantation is usually considered for patients up to 70–75 years of age [12,14]. However, the use of these most intensive therapeutic strategies in elderly patients will not only depend on the age, clinical evaluation, and comorbidity score of the individual patient, but will also differ between institutions. For most patients, stem cell transplantation is therefore used as a consolidation therapy after they have reached complete hematological remission (i.e., disease control without morphological signs of leukemia) in response to the initial induction chemotherapy [1,14]. The strong antileukemic effect of stem cell transplantation is caused by the intensive conditioning therapy and, especially for allotransplant recipients, antileukemic immune reactivity mediated by graft immunocompetent cells [15].

Monocytes constitute up to 10% of peripheral blood leukocytes in healthy individuals [16]. They can be differentiated into macrophages and dendritic cells [17], in the endothelial direction [18] and possibly also in the direction of monocytic myeloid-derived suppressor cells [19,20]. Based on their expression of the two cell surface receptors CD14 and CD16, monocytes are divided into classical (CD14^{bright}CD16^{negative}), intermediate (CD14^{bright}CD16^{dim}), and nonclassical (CD14^{dim}CD16^{bright}) subsets [21–23]. CD16 is a low-affinity IgG receptor that can initiate intracellular signaling, and it is thereby important for regulation of monocyte cytotoxicity [24]. CD14 is a pattern recognition receptor; it functions as a co-receptor for Toll-like receptors but has also several functions independent of these receptors, including transport of inflammatory lipids to induce phagocytosis [25]. Thus, the functional heterogeneity of various monocyte subsets is reflected by the two molecular markers used for identification of the three monocyte subsets, and both markers are involved in the regulation of important phenotypic characteristics through their modulation of intracellular signaling. Classical monocytes often constitute at least 90% of circulating monocytes in healthy individuals [21,23,26].

The monocyte subset levels during the first 4 weeks posttransplant have not previously been characterized in detail, but a few studies have investigated later monocyte reconstitution, from day +28 until day +100 posttransplant. Firstly, both monocytes and neutrophils show early posttransplant reconstitution [27–29], and total monocyte reconstitution occurs early, both after myeloablative and reduced intensity conditioning [28]. Total monocyte reconstitution also seems to occur early in patients receiving haploidentical transplantation for nonmalignant bone marrow disorders [30]. Secondly, the levels of circulating CD14⁺CD16⁺ monocytes (i.e., intermediate and nonclassical monocytes) after day +30 posttransplant are associated with decreased incidence of chronic graft versus host disease (GVHD) after allotransplantation [20]. Thirdly, another study described an association between relatively high levels of classical monocyte (i.e., CD14⁺CD16⁻) levels early posttransplant, as well as improved survival, relapse risk, and transplant-related mortality. These associations seem to be maintained during the first 100 days posttransplant [31]. The patients included in this last study were heterogeneous and half of them received umbilical cord blood grafts [31].

The peripheral blood levels of several immunocompetent cells are altered after stem cell transplantation, and the posttransplant CD4⁺ T-cell defect can last for months [27]. Our hypothesis was that the levels of the various circulating monocyte subsets are also altered, especially during the early (i.e., first 4 weeks) posttransplant period, even though the total monocyte levels normalize at the early (i.e., before the neutrophil and platelet counts) stages of this period. The immunoregulatory events during the first weeks posttransplant are important for outcome after stem cell transplantation (for detailed discussion and additional references see [15]), and our aim was therefore to characterize circulating monocyte subset levels during this period. We used a highly standardized methodology to

characterize peripheral blood levels of monocyte subsets for a relatively homogeneous and unselected group of patients (i.e., all having hematological malignancies with adverse prognosis) receiving peripheral blood stem cell transplantation. All patients were transplanted after reaching complete hematological remission, and our studies of circulating monocyte subsets included pretransplant levels (i.e., after reaching remission), early levels during severe posttransplant pancytopenia, and levels during hematological reconstitution.

2. Materials and Methods

2.1. Patients and Healthy Controls

The study was conducted according to the Declaration of Helsinki. All samples were collected after written informed consent (Regional Ethics Committee REK Vest 2015/1759), and the use of biological material in the present project was also approved (REF Vest 2017/305, 2013/102). Control samples were derived from 18 healthy blood donors (7 females and 11 males, median age 53 years with range 21–73 years). In accordance with the approved routines at the Blood Bank, Haukeland University Hospital, peripheral venous blood samples for medical research were donated after written informed consent. Our hospital is the only center providing intensive antileukemic treatment in a defined geographical area of Norway, and our patients represent a consecutive group of patients receiving intensive antileukemic treatment/stem cell transplantation during an eight month period. Our study should therefore be regarded as population-based.

All our patients received initial chemotherapy to achieve disease control (Table 1, Supplementary Table S1). Thus, we investigated peripheral blood levels of monocyte subsets for stem cell recipients who had achieved complete hematological remission, i.e., normal levels of immature/abnormal cells in the bone marrow judged by light microscopy, peripheral blood neutrophils $> 1.0 \times 10^9/L$ and peripheral blood platelet counts $> 100 \times 10^9/L$ [1]. One myelodysplastic syndrome (MDS) patient reached complete remission with incomplete peripheral blood normalization. In addition, patients with lymphoproliferative disease did not show flow cytometric evidence of minimal residual bone marrow disease, and imaging studies did not show evidence of residual disease immediately before stem cell transplantation.

Sixteen patients were included in the various parts of our study (see Table 1, right part), but only 11 of these patients were treated with stem cell transplantation. They were all treated at Haukeland University Hospital during the period June 2016–June 2017. Patients were transplanted after reaching complete hematological remission, and they received G-CSF mobilized peripheral blood stem cell grafts. All our allotransplant recipients received grafts from HLA (human leukocyte antigen)-identical sibling donors, the same GVHD prophylaxis (cyclosporine plus methotrexate) [32], VOD prophylaxis (i.e., ursodeoxycholic acid) [32] and pretransplant trimethoprim–sulfamethoxazole treatment, but no fungal prophylaxis. Five patients were not allotransplanted for the following reasons: because they had a favorable karyotype (one patient), were unfit for stem cell transplantation (two patients), had a second relapse before transplantation could be performed (one patient), or had been transplanted at another hospital (one patient).

Table 1. Clinical characteristics of the patients included in the study and the use of blood samples in the various parts of the study. Age is given in years. The time when tested is indicated in the right part of the table (CC, cytopenia after conventional chemotherapy; REM, remission after chemotherapy; CSCT, cytopenia after stem cell transplantation; REC, reconstitution).

Id	AGE	SEX	Diagnosis	Treatment	Treatment			
					CCC	REM	CSCT	REC
Conventional Induction and Consolidation Followed by Stem Cell Transplantation								
1	29	M	ALL	Remission induction followed by allotransplantation				
2	61	F	MDS-HR2	Remission induction followed by allotransplantation	Conditioning with busulfan plus cyclophosphamide	+	+	+
3	67	M	PLL-T	Remission induction followed by allotransplantation	Conditioning with fludarabine and treosulfan	+	+	+
4	64	M	MDS-HR2	Remission induction followed by allotransplantation	Conditioning with fludarabine and treosulfan			
5	54	F	AML-MDS	Remission induction followed by allotransplantation	Conditioning with busulfan plus cyclophosphamide	+	+	+
6	72	M	AML de novo	Remission induction followed by allotransplantation	Conditioning with fludarabine and treosulfan	+	+	+
7	53	F	AML de novo	Remission induction followed by allotransplantation	Conditioning with busulfan plus cyclophosphamide	+	++	+
8	57	M	AML de novo	Remission induction followed by allotransplantation	Conditioning with fludarabine and treosulfan	+	+	+
9	51	M	AML de novo	Remission induction followed by autotransplantation	Conditioning with busulfan plus cyclophosphamide			
10	43	M	APL relapse	Remission induction followed by autotransplantation	Conditioning with busulfan plus cyclophosphamide	+	+	+
11	34	M	AML de novo	Remission induction followed by autotransplantation	Conditioning with busulfan plus cyclophosphamide	+	+	+
Conventional Induction and/or Consolidation Chemotherapy								
12	65	F	AML de novo	Induction chemotherapy, regeneration to remission	Induction with daunorubicin plus cytarabine. First consolidation: mitoxantrome, cytarabine.	+	++	
13	63	F	AML relapse	Conventional induction, regeneration to remission	Second consolidation: etoposide, amsacrine and cytarabine	+		
14	68	F	AML de novo	Conventional induction leading to remission; later consolidation chemotherapy	Induction with etoposide, amsacrine and cytarabine Induction: daunorubicin, cytarabine.	+	+	
15	18	F	AML de novo	Induction chemotherapy, regeneration to remission	First consolidation: mitoxantrome, cytarabine. Daunorubicin plus cytarabine	+		
16	64	M	AML relapse	Conventional induction leading to remission; later consolidation chemotherapy	Induction: daunorubicin, cytarabine. First consolidation: mitoxantrome, cytarabine.	+	+	

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; F, female; M, male; MDS-HR2, myelodysplastic syndrome, high-risk class 2; PLL-T prolymphocyte leukemia T cell type.

2.2. Blood Sampling, Flow-Cytometric Analysis of Monocyte Subsets and Analysis of Peripheral Blood Leukocytes

Peripheral venous blood was drawn in ACD-A blood sampling tubes (#248368, BD Biosciences, San Jose, CA, USA). All samples were collected between 08:00 and 10:00 a.m. and were processed at room temperature within 120 min. Sampling was conducted at the same standardized time interval because total leukocytes show diurnal variations [33–36]. Four milliliters of the anticoagulated blood and 46 mL of lysis buffer (#55589, BD Biosciences) were mixed and incubated for 15 min at room temperature. Subsequently, leukocytes were collected by centrifugation ($400\times g$, 5 min, room temperature) and thereafter washed in phosphate-buffered saline with 1% bovine serum albumin (BSA, Bovine serum albumin fraction V, #10735086001, Sigma-Aldrich/Merc KGaA, Darmstadt, Germany). The cells were reconstituted in 200 μ L 1% BSA/PBS with 10% immunoglobulin solution (Octagam 100 mg/mL, Octapharma, Lachen, Switzerland). The following mouse anti-human antibodies were used (all from BD Biosciences): CD14 Alexa 488 (Clone M5E2), CD56 Alexa 647 (Clone B159), CD16 PerCpCyTM5-5 (Clone 3G8), CD45 V500 (Clone HI30), CD11b V540 (Clone ICRF44 (44)) and HLA-DR PE (Clone G46-6). The staining procedure and gating strategy for identification of monocytes and monocyte subsets has been previously described in detail [29,37], and all samples were analyzed using a 10-parameter BD FACS Verse flow cytometer equipped with 404, 488, and 640 nm lasers.

Comparison of peripheral blood levels of neutrophils and total monocytes was based on analyses using an accredited clinical hemocytometer (Laboratory for Clinical Biochemistry, Section for Hematology, Haukeland University Hospital).

2.3. Statistical Analyses

We applied IBM SSP statistics 23 for all analyses. The Kruskal–Wallis test was used for comparison when several groups were included in the analysis. The Wilcoxon’s rank sum test was used for comparison of differences between two different groups of individuals, whereas the Wilcoxon’s test for paired samples was used for statistical comparison of two different observations in the same patients. *p*-values below 0.05 were regarded as statistically significant.

3. Results

3.1. The Pretransplant Status of Our Patients with Hematological Malignancies; Monocyte Subsets for Patients in Complete Hematological Remission after Conventional Chemotherapy

Patients are usually treated with initial conventional chemotherapy to achieve disease control (see Section 2.1) [1]. We analyzed the monocyte subset levels after 13 different chemotherapy cycles in 11 patients (Table 1) who fulfilled the criteria of disease control/complete remission at the time of sampling, and in addition they all had peripheral blood total monocyte counts within the normal range. The results are presented in Figure 1. The monocyte subset levels showed a wider variation in the patients than in the healthy controls, but for 11 of the 13 samples, the majority of circulating monocytes (>80%) belonged to the classical monocyte subset. For the last two patients, classical monocytes constituted <20% of total monocytes. When comparing the overall results, the percentages of classical, intermediate, and nonclassical monocytes for the remission patients did not differ significantly from the healthy controls. The two exceptional patients were patients 1 and 16 (Table 1). For five of the patients, we also estimated the peripheral blood concentrations of the various monocyte subsets, and both classical ($p = 0.002$), intermediate ($p = 0.002$), and nonclassical monocyte ($p = 0.006$) concentrations were significantly decreased compared with the corresponding concentrations in the healthy controls.

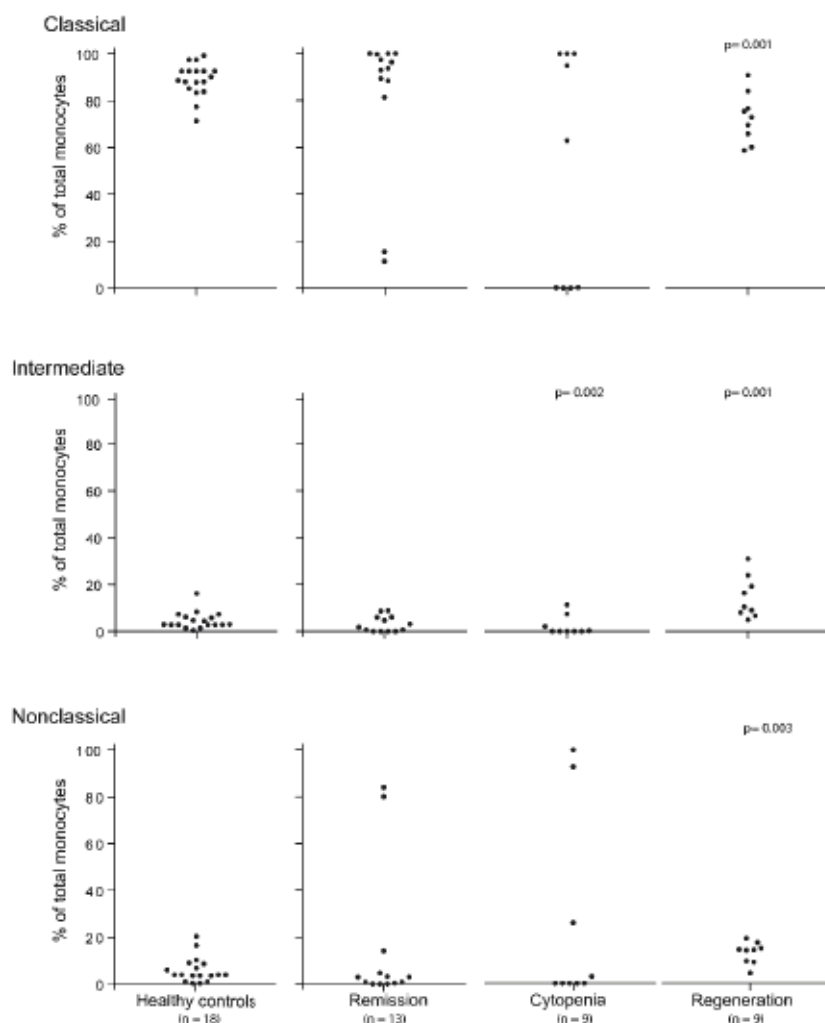


Figure 1. Peripheral blood levels of the classical, intermediate, and nonclassical monocyte subsets in patients receiving intensive chemotherapy for high-risk hematological malignancies. The figure presents the levels for healthy controls (left) and leukemia patients in stable complete hematological remission (i.e., no morphological signs of leukemia, neutrophils $> 1.0 \times 10^9/L$, and independence of platelet transfusions; all patients, in addition, had normal total monocyte counts) (middle left), patients with severe posttransplant pancytopenia (neutrophils $< 0.2 \times 10^9/L$, dependency of platelet transfusions, total monocytes $< 0.2 \times 10^9/L$; middle right), and during posttransplant regeneration (i.e., normal total monocyte counts, increasing neutrophil counts $> 0.5 \times 10^9/L$; right). Those two patients without detectable monocyte levels during cytopenia are marked among the lowest percentages for all three monocyte subsets. Statistical analysis using the Kruskal–Wallis test showed significant variations for classical ($p = 0.029$), intermediate ($p = 0.001$), and nonclassical monocytes ($n = 0.03$). The p -values for statistically significant differences between healthy controls and individual patient/monocyte subset combinations (Wilcoxon’s test for paired samples) are indicated at the top of each of these figures. The results are presented as the percent among total monocytes in peripheral venous blood.

3.2. The Peripheral Blood Levels of Various Monocyte Subsets Show Wide Variation during the Period of Severe Posttransplant Pancytopenia

The levels of circulating monocyte subsets during the period of severe posttransplant pancytopenia were investigated for nine transplant recipients (Figure 1). Severe pancytopenia was then defined as total peripheral blood leukocytes $\leq 0.3 \times 10^9/L$, neutrophils $< 0.2 \times 10^9/L$, total monocytes $< 0.1 \times 10^9/L$, and dependency of regular platelet transfusions to keep the platelet count above $10\text{--}20 \times 10^9/L$. Two of these patients had overly low peripheral blood leukocyte counts to allow for reliable estimation of monocyte subset distribution (Table 1, patients 2 and 11). The two exceptional patients with detectable but low (i.e., $<3\%$) levels of classical monocytes were the two allotransplant recipients, patients 1 and 8. The other seven patients showed a distribution within the variation ranges of the normal controls. Thus, the transplant recipients show a wider variation than the healthy controls in venous blood monocyte subset levels during the period of severe posttransplant pancytopenia. The percentage of intermediate monocytes was even significantly decreased compared with healthy controls (only the seven patients with detectable levels included, $p = 0.002$), even though healthy controls also show levels for this subset. By contrast, the percentages of classical and nonclassical monocytes did not differ significantly from the healthy controls.

For two of the patients, we estimated the venous blood concentrations of the three monocyte subsets and, as expected, both classical, intermediate, and nonclassical monocytes for these two patients showed peripheral blood concentrations outside the corresponding variation ranges of the healthy controls.

We investigated the peripheral blood levels of the three monocyte subsets for patients with severe pancytopenia, as defined above, after conventional intensive antileukemic chemotherapy (seven patients examined). These patients also showed a wide variation in their peripheral blood levels of both classical (range $< 0.01\text{--}100\%$), intermediate (range $< 0.01\text{--}42\%$), and nonclassical monocytes (range $< 0.01\text{--}22\%$). Thus, a wide variation in peripheral blood levels during treatment-induced cytopenia is observed not only for stem cell transplant recipients, but also for patients receiving conventional intensive antileukemic chemotherapy.

3.3. Stem Cell Transplant Recipients Show Early Posttransplant Total Monocyte Reconstitution

Previous studies have demonstrated that stem cell recipients show early reconstitution of total monocytes, and this is true for both allotransplant [18,19] and autotransplant recipients [20]. All our stem cell transplant recipients showed early reconstitution with normalized levels of circulating monocytes before the normalization of the neutrophil counts (Figure 2, Supplementary Table S2). Furthermore, many of the recipients showed a posttransplant period of increased levels of circulating monocytes; this could be observed for eight of our 12 stem cell transplanted patients, including six out of the nine allotransplant recipients. The maximal total monocyte levels for our patients were reached between day +21 and +35 posttransplant, increased levels were seen both for patients receiving autologous and allogeneic stem cell transplantation, and the maximal levels varied between $1.41 \times 10^9/L$ and $5.85 \times 10^9/L$ (normal range $0.04\text{--}1.30 \times 10^9/L$).

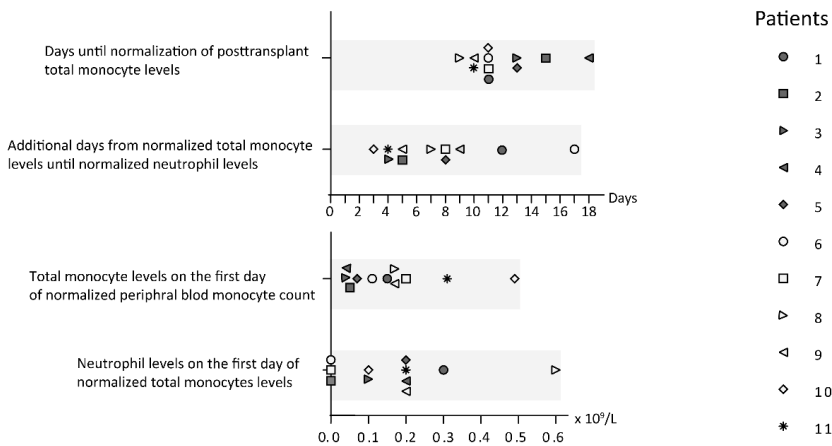


Figure 2. Monocyte and neutrophil reconstitution after conditioning therapy followed by stem cell transplantation. All 11 stem cell transplant recipients (patients 9–11 received an autologous stem cell graft) were included in this part of the study; the symbols for each of the patients are indicated to the right in the figure. The upper part of the figure shows the first posttransplant day with normal total monocyte cell counts (normal level $0.4\text{--}1.3 \times 10^9/\text{L}$; day 0 being the day of stem cell infusion) and the number of additional days until normalized peripheral blood neutrophil counts (lower normal limit of neutrophils $1.7 \times 10^9/\text{L}$). The lower part of the figure presents the peripheral blood concentrations of total monocytes and neutrophils on the first day with normalized total monocyte counts in the blood. All samples were collected between 07:00 and 08:30 a.m.

3.4. Early Posttransplant Monocyte Subset Regeneration after Stem Cell Transplantation

We investigated the posttransplant peripheral blood levels of the three monocyte subsets during early regeneration when the total monocyte counts had reached normal levels (normal range $0.04\text{--}1.3 \times 10^9/\text{L}$). Nine patients were tested 12–28 days posttransplant when monocyte counts had normalized (median concentration $0.74 \times 10^9/\text{L}$, range $0.45\text{--}1.66 \times 10^9/\text{L}$). At the time of testing, five of the patients were still neutropenic; the patients had median neutrophil levels of $1.6 \times 10^9/\text{L}$ with a variation range of $0.5\text{--}2.8 \times 10^9/\text{L}$ (lower normal limit $1.7 \times 10^9/\text{L}$). The levels of the three monocyte subsets are presented in Figure 1. The majority of circulating monocytes early after posttransplant reconstitution belonged to the classical monocyte subset for all patients (median 76%, range 58–100%), but the variation range was wider for the regenerating patients than for the healthy controls. The levels of both intermediate and nonclassical monocytes were below 20%. The percentages of classical (decreased, $p = 0.001$), intermediate (increased, $p = 0.001$), and nonclassical monocytes (increased, $p = 0.003$) for patients showing hematological regeneration differed significantly from the healthy controls. However, the variation between patients was smaller during regeneration than for patients in pretransplant remission.

We investigated the levels of circulating monocyte subsets at various time points both during and soon after the period of severe posttransplant cytopenia for nine stem cell transplant recipients. The results for three patients are presented in Figure 3. It can be seen that the percentage of classical monocytes was initially decreased, and could be very low before it increased after 2–4 weeks. It was also observed that the intermediate monocytes constituted a minority during the whole period but showed a wider variation range than the healthy controls, whereas the nonclassical monocytes showed high/increased levels during the first week, but after 4 weeks, they were a small minority. All nine patients showed a similar pattern for the classical monocytes with initially low/decreased levels with a later increase, until after 4 weeks, they constituted a majority of the circulating monocytes.

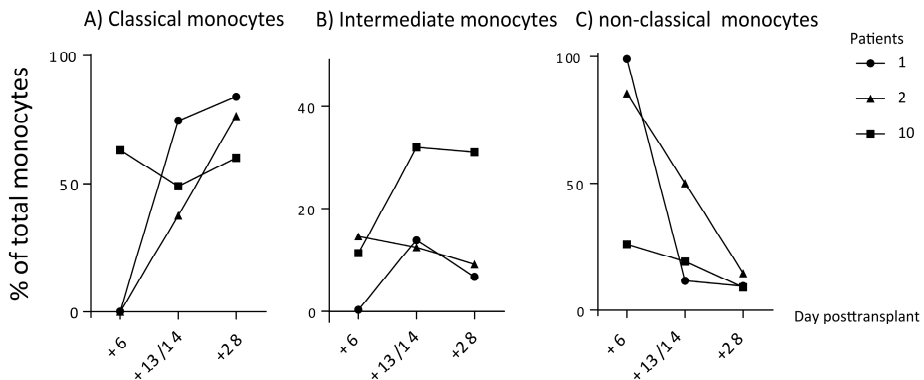


Figure 3. Posttransplant peripheral blood levels of classical (A), intermediate (B), and nonclassical monocytes (C) in three leukemia patients. The figure presents the levels for days +6, +13/+14, and day +28 posttransplant for patients 1, 2, and 10 (see Table 1). These three time points after the stem cell infusion correspond to severe cytopenia and early posttransplant hematological regeneration with normalization of monocyte counts and, finally, also normalized neutrophil counts. The results are presented as the percentage of each monocyte subset among total monocytes. The day of testing is indicated on the x-axis; day 0 is the day of stem cell infusion.

For four of the patients, we estimated the peripheral blood concentrations of the three monocyte subsets, and classical ($p = 0.002$), intermediate ($p = 0.008$), and nonclassical monocyte ($p = 0.006$) concentrations differed significantly from the levels in the 18 healthy controls.

4. Discussion

Several observations suggest that monocytes are important for outcome after allogeneic stem cell transplantation. Firstly, differences in the amounts of graft monocytes between younger and older stem cell donors may contribute to the adverse prognosis when using older stem cell donors [38,39]. Secondly, monocytes seem important for the development of posttransplant tolerance [40]. Finally, the monocyte-lymphocyte ratio seems to have a prognostic impact at least in haploidentical transplantation [41]. In our present study, we therefore investigated the balance between classical, intermediate, and nonclassical monocytes after stem cell transplantation in patients with high-risk leukemia or MDS. To the best of our knowledge, this is the first study of early monocyte subset reconstitution after stem cell transplantation for hematological malignancies. We observed very early reconstitution of total monocytes, and also the dominating classical monocyte subset after intensive conditioning therapy followed by stem cell transplantation.

In our present study, we focused on the early posttransplant period, i.e., the first 4 weeks after stem cell reinfusion. This period is important for outcome after transplantation; this is illustrated by the observations that both the type of conditioning therapy as well as the use of hematopoietic growth factors during this period influence the risk of severe posttransplant complications [15]. We investigated a group of consecutive patients with high-risk hematological malignancies admitted to our institution for intensive chemotherapy. All patients had hematological malignancies with adverse prognoses, and received intensive chemotherapy, including stem cell transplantation for 11 of them. We included all admitted patients without any selection, and for this reason, we investigated patients with different diagnoses who had received different forms of pretransplant conditioning therapy. However, all patients received peripheral blood mobilized stem cell grafts; most of them received allografts (all from HLA-matched siblings) but a minority of our patients received autologous stem cell grafts. The pattern of reconstitution seemed to be similar for allotransplant and autotransplant

recipients, which is not surprising as autotransplanted patients received the same conditioning chemotherapy as allotransplanted patients.

Our present study included a small number of patients, but our patients are relatively homogeneous. We would also emphasize that our patient cohort represents a consecutive group of patients from a defined geographical area during a defined time period. Thus, our patients should also be regarded as unselected.

All transplant recipients included in our study had severe hematological malignancies, and they initially received intensive conventional chemotherapy with the intention to induce complete hematological remission. They all reached stable complete remission prior to transplantation. Patients receiving such intensive conventional chemotherapy showed a similar wide variation in monocyte subset levels during treatment-induced cytopenia to the patients with posttransplant cytopenia, even though they had received repeated cycles of intensive consolidation treatment with relatively short intervals after remission induction [1].

Previous studies have demonstrated that monocyte reconstitution (i.e., normalization of peripheral blood monocyte levels) occurs relatively early after allogeneic stem cell transplantation [37], and this was also true for all our allotransplant and autotransplant recipients. Normalization of the circulating monocyte levels usually occurred before normalization of the neutrophil levels. Thus, even though our study included relatively few patients, they should be regarded as representative because they show the expected early normalization of both total monocyte and neutrophil peripheral blood levels.

We have previously investigated the levels of circulating monocyte subsets in myeloma patients receiving autologous stem cell transplantation [29]. These myeloma patients differ from the patients included in our present study as (i) they received different and less intensive pretransplant treatment without remission induction; (ii) the intention of the transplantation is stabilization but not cure; and (iii) they received less intensive conditioning therapy as well as growth factor treatment posttransplant. The myeloma patients also showed early monocyte reconstitution, but displayed wider variations in the monocyte subset levels both pretransplant and immediately after transplantation during cytopenia. However, despite these differences, both patient groups showed early monocyte reconstitution, and a majority of circulating monocytes show classical phenotypes within 4 weeks, both for the majority of myeloma patients and all the present leukemia patients.

The CD14 and CD16 markers used for identification of monocyte subsets reflect the functional heterogeneity of the three subsets because both these surface molecules initiate downstream intracellular signaling that is involved in the regulation of important functional characteristics, including proinflammatory and phagocytic activity [24,25]. Thus, our present results show that the balance between functionally different monocyte subsets can be altered not only immediately before, but also during the early posttransplant period (i.e., the first 4 weeks) when compared with later after transplantation.

Several previous observations suggest that immunological events during the early posttransplant period are important for outcomes after stem cell transplantation, especially allogeneic transplantation. The effect of G-CSF therapy on outcome after allogeneic stem cell transplantation has been investigated in two large studies. In the European study, G-CSF therapy was associated with decreased overall survival due to increased non-relapse mortality (i.e., severe GVHD) [42], whereas in another study, G-CSF therapy did not influence survival [43]. A major difference between the two studies was the increased use of total body irradiation in the European study. An experimental study has previously shown that total body irradiation is associated with an increased activation of dendritic cells, which can also be differentiated from monocytes, and thereby increased proinflammatory alloreactivity [44]. Furthermore, the intensification of GVHD prophylaxis during the first one or two posttransplant weeks with methotrexate or cyclophosphamide also demonstrates that immunological events early posttransplant are important for outcome [45–47], and that this intensification may not only affect the T-cells, but also directly influence the monocytes [48,49]. Taken together, all these observations suggest that the altered balance between various monocyte subsets early after allotransplantation may

be important for the later outcome. Other studies suggest that this may also be true for autologous stem cell transplantation [50].

Our present study shows that patients with hematological malignancies have a wider variation in the relative levels (i.e., percentage) of circulating monocyte subsets than healthy individuals during periods of antileukemic therapy. However, this variation is reduced after stem cell transplantation due to rapid posttransplant reconstitution of total and classic monocytes, although there is still a wider variation between patients after transplantation. Previous studies have shown that later posttransplant variations in monocyte subset are associated with outcome after transplantation [20,31]. Other investigators have observed that clinical events during the very early period after stem cell transplantation are important for outcome/survival [15]. Future studies should therefore try to clarify whether the early variations described in our present study are also associated with outcome after transplantation, and if so, whether these early differences between patients may be used as clinically relevant biomarkers and a basis for early interventions to improve outcome. These studies should possibly also investigate the balance between remaining recipient and donor monocytes in allotransplant recipients [44]. Finally, previous studies have demonstrated that the role of monocytes in inflammation may be influenced by other leukocyte subsets, and the interactions between monocytes and other normal leukocytes during the early posttransplant period also need to be investigated [51,52].

5. Conclusions

Our present data show that monocytes reconstitute early after stem cell transplantation for high-risk leukemia and MDS patients. Our data also show a greater variation in the distribution of monocyte subpopulations among patients during both pretransplant stable remission and posttransplant pancytopenia, whereas the variation is less 4 weeks posttransplant, although still larger than for healthy controls. Thus, our present studies show an expected normalization of total monocyte counts early after stem cell transplantation, but our results also show that normalized total monocyte counts do not mean a normalization of monocyte functions (i.e., normalization of the balance between functionally different monocyte subsets).

Supplementary Materials: The following are available online at <http://www.mdpi.com/1010-660X/56/1/36/s1>. Table S1: Detailed clinical and biological characteristics of the patients included in the study. Table S2: Monocyte and neutrophil reconstitution after conditioning therapy followed by stem cell transplantation.

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Article

Immunomodulatory Drugs Alter the Metabolism and the Extracellular Release of Soluble Mediators by Normal Monocytes

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Abstract: Immunomodulatory drugs (IMiDs) are used in the treatment of hematological malignancies, especially multiple myeloma. IMiDs have direct anticancer effects but also indirect effects via cancer-supporting stromal cells. Monocytes are a stromal cell subset whose metabolism is modulated by the microenvironment, and they communicate with neighboring cells through extracellular release of soluble mediators. Toll-like receptor 4 (TLR4) is then a common regulator of monocyte metabolism and mediator release. Our aim was to investigate IMiD effects on these two monocyte functions. We compared effects of thalidomide, lenalidomide, and pomalidomide on in vitro cultured normal monocytes. Cells were cultured in medium alone or activated by lipopolysaccharide (LPS), a TLR4 agonist. Metabolism was analyzed by the Seahorse XF 96 cell analyzer. Mediator release was measured as culture supernatant levels. TLR4 was a regulator of both monocyte metabolism and mediator release. All three IMiDs altered monocyte metabolism especially when cells were cultured with LPS; this effect was strongest for lenalidomide that increased glycolysis. Monocytes showed a broad soluble mediator release profile. IMiDs decreased TLR4-induced mediator release; this effect was stronger for pomalidomide than for lenalidomide and especially thalidomide. To conclude, IMiDs can alter the metabolism and cell–cell communication of normal monocytes, and despite their common molecular target these effects differ among various IMiDs.

Keywords: monocytes; immunomodulatory drugs; cell metabolism; cytokines

1. Introduction

The immunomodulatory drugs (IMiDs) are widely used in the treatment of multiple myeloma and are also considered for the treatment of other hematological malignancies [1]. Cereblon is a common molecular target for IMiDs, and the drugs can have direct anticancer effects on malignant cells or indirect effects mediated via cancer-supporting nonmalignant cells (e.g., antiangiogenic effects) [2]. Monocytes are important both for immunoregulation and for regulation of normal and malignant hematopoiesis [3,4], and IMiD effects on monocytes may therefore be important both for their efficiency and toxicity in anticancer treatment.

Monocytes undergo morphological, phenotypic, and functional changes in response to their metabolic microenvironment [5]. Most circulating monocytes have a classical phenotype and their metabolism can be altered by ligation of Toll-like receptor 4 (TLR4), e.g., by lipopolysaccharide (LPS) or

certain metabolites (e.g., oleic acid, palmitic acid), and the downstream NF κ B activation following TLR4 ligation [6]. However, very high levels of TLR4 ligands may instead induce tolerance [7]. Cholesterol, triglyceride-rich lipoproteins, low density lipoproteins (LDL), very low density lipoproteins (VLDL) and high density lipoprotein (HDL) can also modulate the monocyte phenotype through non-TLR4 mechanisms and thereby alter the balance between pro- and anti-inflammatory effects [5–8]. Finally, even dietary intake seems to influence the phenotypic characteristics of monocytes, including the balance between pro- and anti-inflammatory effects [8]. Thus, monocyte functions are modulated by the monocyte's metabolic status/environment.

TLR4 receptors can be activated not only by microbial products but also by endogenous ligands as described above [9,10]. TLR4 ligation can stimulate monocyte release of several cytokines [11,12] and at the same time also activate a metabolic switch towards glycolysis leading to production of metabolites that are important for the pentose–phosphate pathway, fatty acid synthesis, and amino acid metabolism [8]. This relative block in the citric acid cycle will increase the availability of citric acid and succinate with further modulation of phospholipid and cholesterol synthesis [13,14]. Thus, the balance between glycolysis and oxidative phosphorylation is not only modulated by TLR4 ligands but also by the metabolomic profile of the extracellular microenvironment [15], e.g., glutamine that both feeds into the tricarboxylic acid cycle and also acts as a regulator of TLR4 responses [13,16]. These examples further illustrate the complex overlap/crosstalk between TLR4 signaling, metabolic regulation, and extracellular mediator release.

Monocytes can influence the development of various malignancies through direct and indirect effects [17], possibly also multiple myeloma. First, the balance between various leukocyte subsets in peripheral blood seems to have a prognostic impact, and high neutrophil- or monocyte-to-lymphocyte ratios at the time of diagnosis are associated with unfavorable clinicobiological features [18]. Secondly, monocytes can modulate the cell surface molecular profile of the myeloma cells [19]. Third, activated monocytes release cytokines that directly stimulate myeloma cell proliferation or indirectly facilitate disease progression, e.g., through increased angiogenesis [20,21]. Finally, monocyte-derived dendritic cells can present myeloma-associated antigens [22], whereas monocyte-derived myeloid-derived suppressor cells have immunosuppressive effects [23]. Thus, monocytes may influence myeloma development/progression and susceptibility to chemotherapy, but the final effects are difficult to predict.

The immunomodulatory drugs (IMiDs) thalidomide, lenalidomide, and pomalidomide are widely used in myeloma treatment [24] and can inhibit TLR4/LPS-induced secretion of inflammatory cytokines [25]. Cereblon is a common molecular target for these drugs, but their cereblon binding as well as their clinical effects in myeloma treatment differ [2]. As described above there may be a crosstalk between TLR4 signaling, metabolic modulation, and capacity of cytokine release in normal monocytes. In this context we have compared the effects of these IMiDs on monocyte metabolism and release of soluble mediators by normal monocytes.

As described above monocytes seem to support disease development in multiple myeloma [17], but because monocytes are a part of the bone marrow stem cell niches they may also influence the development and chemosensitivity of other hematological malignancies, e.g., through their release of leukemia-supporting cytokines [4]. IMiDs are therefore considered for the treatment of other hematological malignancies [2]. The aim of the present study was to use standardized *in vitro* models to investigate how various IMiDs influence important phenotypic characteristics of normal monocytes and whether these effects differ among various IMiDs that have cereblon as a common molecular target [26]. Thus, monocytes are important in physiological immunoregulation as well as in carcinogenesis, and our goal was to investigate IMiD effects on metabolic regulation and cytokine release; two functional characteristics that seem to be important are the roles of normal monocytes in both immunoregulation and carcinogenesis [8,17].

2. Results

2.1. TLR4/LPS Activation Alters the Balance between Glycolysis and Oxidative Phosphorylation in the Direction of Glycolysis

Monocytes derived from 10 healthy blood donors (4 males and 6 females; median age 33 years with range 25–71 years) were incubated for four hours in medium with and without LPS; the cells were thereafter incubated for one hour without CO₂ before cell metabolism was analyzed. We first investigated the basal mitochondrial metabolism (see Supplementary Figure S1 upper part) of the monocytes; the balance between oxidative phosphorylation (oxygen consumption rate, OCR) and glycolysis (i.e., extracellular acidification rate, ECAR) is presented as the OCR:ECAR ratio. The results from a representative experiment are presented in Supplementary Figure S1 (lower part).

Preincubation for four hours with LPS 1 ng/mL decreased the OCR:ECAR ratio significantly, compared with the medium control (Figures 1a and 2A). A similar effect on the OCR:ECAR ratio was also seen when analyzing the maximal respiration period where LPS also decreased this ratio significantly (Figures 1b and 2D). Thus, LPS altered the balance between glycolysis and oxidative phosphorylation in the direction of glycolysis, but LPS did not alter the coupling efficiency (i.e., the efficiency of mitochondrial ATP production) or spare respiratory capacity (i.e., showing how much of the respiratory capacity was being used) (Figure 1c).

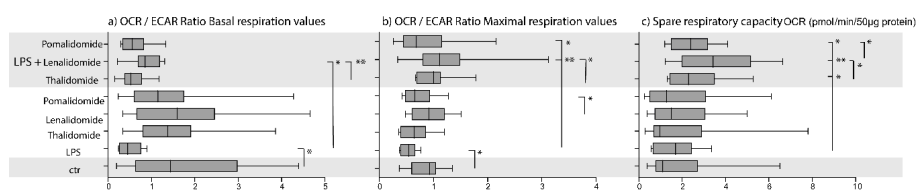


Figure 1. Analysis of monocyte metabolism using the XF Mito Stress Test assay and the Seahorse XF 96 cell analyzer; a summary of the overall results. Normal monocytes were cultured in medium alone or in the presence of LPS (lipopolysaccharide) 1 ng/mL, or IMiDs (immunomodulatory drugs) 5 µg/mL for four hours before the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined. Each of the figures presents the OCR:ECAR ratio (diagrams (a,b)) or the spare respiratory capacity (diagram (c)) for cultures prepared in (from the bottom to the top of each diagram) (i) medium alone (ctr), (ii) LPS control, (iii) each of the IMiDs—thalidomide, lenalidomide, or pomalidomide—in medium alone without LPS; and (iv) LPS in combination with either thalidomide, lenalidomide or pomalidomide. The figures show the results for (a) the OCR:ECAR ratio during the initial period of basal incubation; (b) the period of maximal respiration after addition of FCCP (Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazine; an uncoupling agent); and (c) the calculated spare respiratory capacity. The figure summarizes the results for 10 independent experiments testing monocytes from 10 healthy blood donors. The results are presented as median, box (i.e., the 25–75 percentiles) and whiskers (the 5–95 percentiles). The Wilcoxon test for paired samples was used for the statistical comparisons, and *p*-values < 0.05 were regarded as statistically significant. The significant comparisons are indicated in the figure (* *p* < 0.05, ** *p* < 0.01).

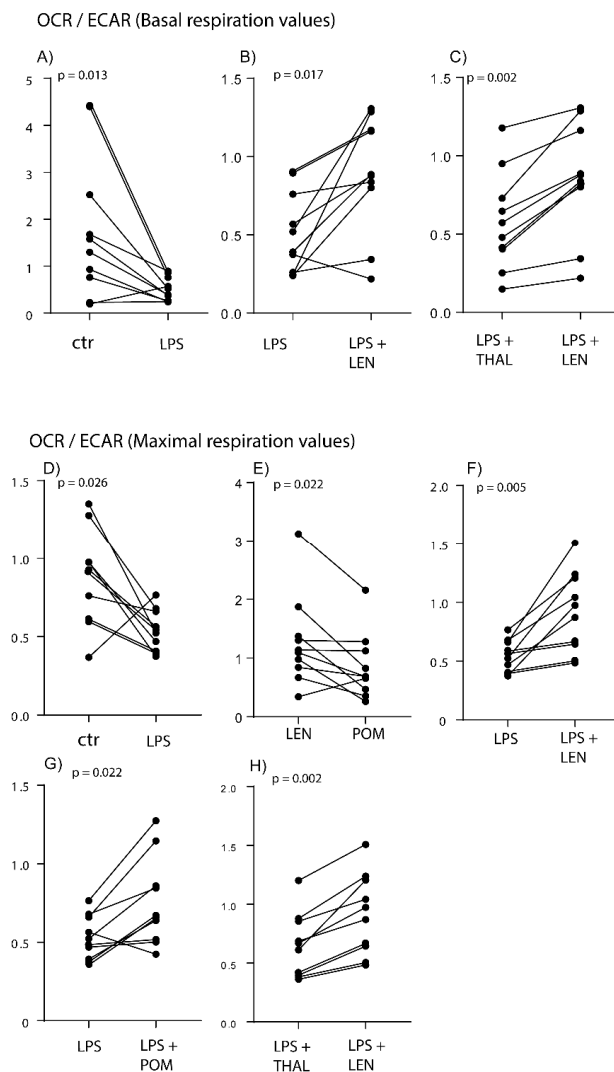


Figure 2. The effects of IMiDs on the metabolism of normal monocytes; a presentation of statistically significant effects of LPS and IMiDs on the OCR:ECAR ratio, (A–C) is OCR:ECAR ratio at basal levels and (D–H) at maximal respiration. Normal monocytes derived from 10 different healthy individuals were cultured with and without LPS 1 ng/mL and/or with and without IMiDs 5 μ g/mL in the presence of LPS. Metabolism was analyzed using the XF Mito Stress Test assay and the Seahorse XF 96 cell analyzer. The figure presents the results for all statistically significant comparisons. The culture conditions that were compared in each of the statistical analyses/diagrams are indicated on the x-axis, the OCR:ECAR ratio is indicated on the y-axis and the corresponding *p*-values from the statistical analyses (Wilcoxon test for paired samples) are indicated at the top of each diagram. We present the results from analysis of basal (A–C) and maximal respiration (D–H). Each diagram compares the results for (i) cultures prepared with medium alone versus LPS (A,D); (ii) cultures prepared with LPS without and with an IMiD (B,F,G), and (iii) cultures prepared with two different IMiDs but otherwise with similar culture conditions (E,H).

2.2. IMiDs Differ in Their Effects on the Balance between Glycolysis and Oxidative Phosphorylation; Especially Lenalidomide Increases Oxidative Phosphorylation

We first investigated whether thalidomide, lenalidomide, and pomalidomide altered monocyte metabolism when cells from 10 healthy individuals were cultured in medium alone without TLR4/LPS stimulation. The OCR:ECAR ratio was not significantly altered by any of the IMiDs when analyzing the initial basal metabolism or the maximal respiration (Figure 1a,b).

We then investigated the effects of the three IMiDs on the OCR:ECAR ratio in the presence of LPS/TLR4 ligation, i.e., an intervention that alters the metabolic balance in the direction of glycolysis (see above). Lenalidomide caused a significant increase in this ratio when testing the cells under basal conditions (Supplementary Figure S1 lower part, Figures 1a and 2B), but for 8 out of the 10 tested individuals the ratio was still lower for the lenalidomide cultures than for the medium controls. The lenalidomide levels were also significantly different from the thalidomide values (Figure 2C) but did not differ significantly from pomalidomide (data not shown). Thus, lenalidomide was the only IMiD that significantly altered monocyte metabolism and decreased the relative importance of glycolysis (i.e., increased the OCR:ECAR ratio) in the presence of TLR4/LPS activation.

We also investigated the OCR:ECAR ratio during maximal respiration (see Figures 1–3). The ratio was significantly higher for lenalidomide than for pomalidomide when testing normal monocytes incubated in medium alone without LPS (Figure 2E). In the presence of LPS the IMiDs had different effects on the OCR:ECAR ratio; lenalidomide caused a highly significant increase (Figure 2F) whereas pomalidomide caused an increase of borderline significance (Figure 2G). The ratio was significantly higher for lenalidomide than for thalidomide (Figure 2H) in the presence of LPS.

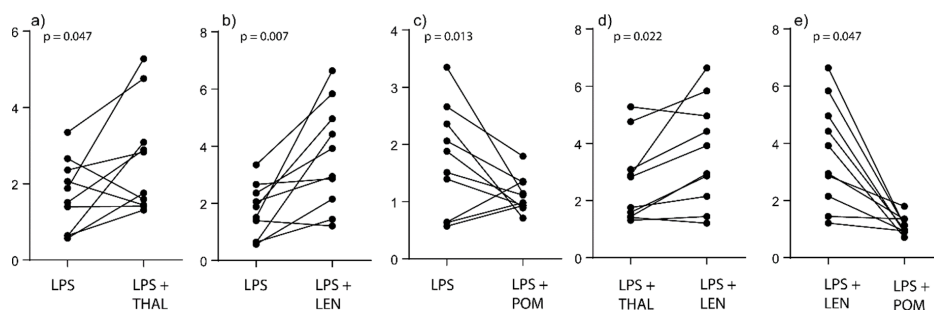


Figure 3. The effect of IMiDs on the metabolism of normal monocytes; analysis of the spare respiratory capacity. Normal monocytes derived from 10 healthy individuals were cultured with LPS 1 ng/mL alone or in combination with an IMiD (5 µg/mL). Monocyte metabolism was analyzed using the XF Mito Stress Test assay and the Seahorse XF 96 cell analyzer. The figure presents the results for all statistically significant comparisons. The cultures compared in each of the statistical analyses/diagrams are indicated on the x-axis, the spare respiratory capacity is indicated on the y-axis (pmol/min/50 µg protein) and the p-value for the statistical analysis (Wilcoxon test for paired samples) is indicated at the top of each diagram. We present the results for LPS stimulated cultures with and without an IMiD (a–d) and with two different IMiDs (e).

None of the IMiDs had significant effects on the coupling efficiency (i.e., the efficiency of mitochondrial ATP production, see Supplementary Figure S1) (data not shown).

2.3. IMiDs Have Different Effects on the Spare Respiratory Capacity of Normal Monocytes

We first investigated the effects of the three IMiDs on the spare respiratory capacity in the absence of TLR4/LPS stimulation; none of the drugs then had any significant effect (Figure 1). We also investigated the effects of the three IMiDs on the spare respiratory capacity in the presence of TLR4/LPS stimulation (Figures 1 and 3). All three IMiDs altered this capacity significantly compared

with the LPS control cultures (Figure 3a–c). Lenalidomide then caused a highly significant increase (Figure 3b, $p = 0.007$), and this lenalidomide effect was significantly stronger than the increase caused by thalidomide (Figure 3d) and the decrease caused by pomalidomide (Figure 3e). Thus, the differences among IMiDs with regard to modulation of monocyte metabolism are not only reflected in OCR:ECAR ratio but also in the spare respiratory capacity.

2.4. Healthy Individuals Differ in Their Spontaneous and TLR4/LPS-Induced Mediator Release

We investigated the spontaneous release of 14 soluble mediators for normal monocytes derived from 15 healthy individuals (7 males and 8 females, median age 48 years with range 23–71 years). Monocytes showed a spontaneous mediator release, but these levels were relatively low and varied between patients (Supplementary Table S1, Figure 4).

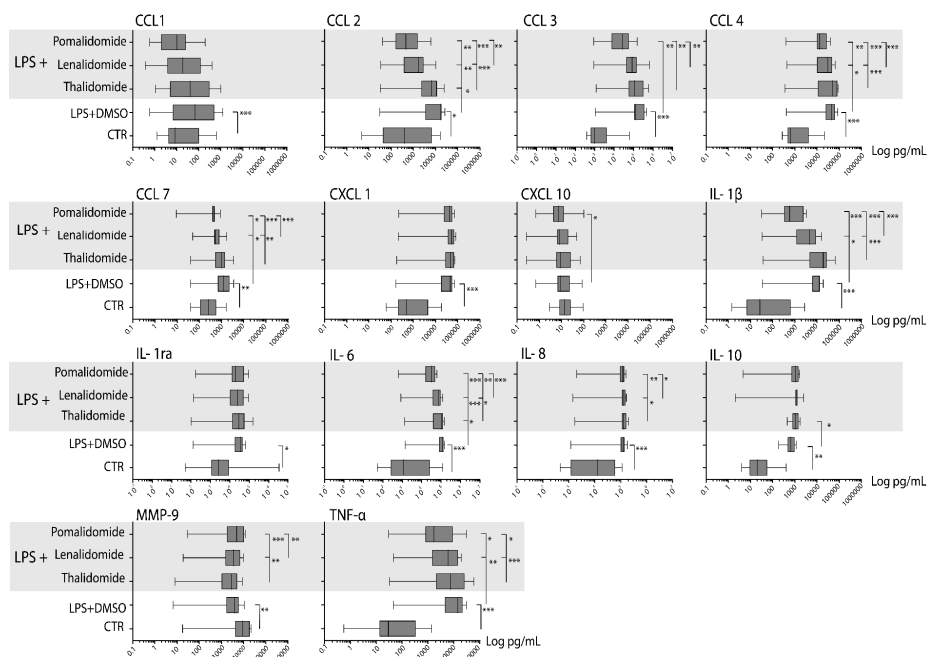


Figure 4. Analysis of monocyte cytokine release during in vitro culture; a summary of the overall results. Normal monocytes were cultured in medium alone or in the presence of LPS 1 ng/mL, or IMiD 5 μ g/mL for 24 h before supernatants were harvested and the supernatant levels of the 14 soluble mediators determined. Each of the diagrams/figures present the level for cultures prepared in (from the bottom to the top of the figure) (i) medium alone (ctr), (ii) LPS + DMSO alone, (iii) each of the IMiDs—thalidomide, lenalidomide or pomalidomide—in combination with either thalidomide, lenalidomide, or pomalidomide. The diagrams show the results for each individual soluble mediator. The results are presented as the median, box (i.e., 25–75 percentiles), and whiskers (5–95 percentiles). The Wilcoxon test for paired samples was used for all statistical comparisons, and p -values < 0.05 were regarded as statistically significant. All statistically significant comparisons are indicated in the figure (* $0.01 < p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

For seven of these individuals we also investigated the release in cultures prepared in medium with LPS 1 ng/mL. As expected we observed a LPS-induced increased in soluble mediator release, and the levels did not differ significantly when we compared cultures with LPS alone and cultures with LPS + DMSO (i.e., the control cultures for the IMiD experiments) (data not shown). Thus, the

TLR4/LPS-induced mediator response was maintained in the presence of the DMSO concentration used in our IMiD experiments, and the LPS response is reflected in the difference between the spontaneous levels presented in Supplementary Table S1 and the levels in the DMSO containing control cultures presented in Table 1 (see also the overview in Supplementary Figure S2). Finally, LPS significantly increased the levels for all soluble mediators except for CCL1 and CXCL10, and there were no significant correlations between the spontaneous release for cultures prepared in medium alone and the LPS cultures, except for IL10 (r -value 0.821, p -value 0.001) and MMP9 (r -value 0.621, p -value 0.018).

We did a hierarchical clustering analysis of the TLR4/LPS-induced mediator release for the 15 healthy individuals (Supplementary Figure S3). These results are presented as the relative responses, i.e., the levels in LPS containing cultures versus the levels in control cultures prepared in medium alone. The TLR4/LPS responsiveness differed among individuals, and a strong response was seen especially for the lower six individuals (2, 10, 6, 7, 3, 1) that clustered together. We performed similar analyses for thalidomide/lenalidomide/pomalidomide containing cultures, and these analyses showed that similar variations among individuals in TLR4/LPS responsiveness (i.e., variations in absolute mediator levels) persisted also in the presence of IMiDs (data not shown).

Table 1. The effects of thalidomide, lenalidomide, and pomalidomide on TLR (Toll like receptor) 4-induced release of soluble mediators by normal monocytes derived from healthy individuals. The data are presented as the median and range of the levels detected in culture supernatants. Fifteen healthy individuals were examined. The Wilcoxon test for paired samples was used for the statistical analyses (n.s., not significant). Unless otherwise stated all concentrations are given as pg/mL.

Mediator Classification	TLR4-Induced Release in Control Cultures	TLR4-Induced Release in the Presence of Thalidomide	p-Value	TLR4-Induced Release in the Presence of Lenalidomide	p-Value	TLR4-Induced Release in the Presence of Pomalidomide	p-Value
Chemokines							
CCL1	63.8 (0.6–1229)	40.7 (1.1–994)	n.s.	22.2 (0.4–406)	n.s.	10.4 (0.6–191)	n.s.
CCL2	11,479 (30.3–26,405)	6751 (32.4–24,724)	0.036	1816 (34.5–10,349)	0.003	461 (40.8–6173)	0.001
CCL3	973 (350–62,216)	198,871 (1175–566,384)	n.s.	84,338 (919–652,373)	n.s.	26,298 (878–150,656)	n.s.
CCL4	43,275 (409–86,040)	48,452 (359–90,664)	n.s.	30,375 (437–66,560)	0.031	13,308 (394–31,896)	0.003
CCL7	1274 (39.7–3571)	1088 (39.8–3473)	n.s.	524 (50.7–1724)	0.011	410 (9.1–485)	0.003
CXCL1	46,239 (161–70,248)	47,546 (177–76,381)	n.s.	56,508 (214–83,477)	n.s.	43,350 (218–71,720)	n.s.
CXCL10	8.3 (<0.1–86.3)	9.3 (<0.1–69.7)	n.s.	8.2 (<0.1–49.1)	n.s.	7.5 (<0.1–102)	0.019
Interleukins							
IL1 β	13,578 (34.9–19,091)	20,310 (37.4–66,422)	n.s.	6363 (33.4–15,877)	0.02	580 (31.8–2442)	0.001
IL1RA	97,396 (130–66,232)	29,043 (102–158,380)	n.s.	23,839 (137–91,545)	n.s.	19,870 (168–93,618)	n.s.
IL6	117,615 (2928–104,657)	117,722 (1324–60,904)	n.s.	77,344 (865–125,094)	0.0125	32,080 (615–61,886)	0.0007
IL8/CXCL8	123,383 (2692–176,743)	134,328 (1697–199,724)	n.s.	135,616 (1428–165,045)	n.s.	129,439 (1988–161,864)	n.s.
IL10	931 (<2.1–2500)	1255 (<2.1–1743)	0.038	1082 (<2.1–1573)	n.s.	1092 (<2.1–1573)	n.s.
Other mediators							
MMP9	3943 (50–10,680)	2862 (8.2–8639)	n.s.	2895 (18.5–9801)	n.s.	4856 (28.3–11,681)	n.s.
TNF α	17,983 (46–21,758)	7366 (63–60,688)	0.0018	5997 (46–19,516)	n.s.	1709 (30–31,550)	0.0268

2.5. Thalidomide Shows a Weak, Lenalidomide an Intermediate, and Pomalidomide a Strong Inhibitory Effect on the TLR4/LPS-Induced Release of Soluble Mediators by Normal Monocytes

The supernatant levels of 14 soluble mediators were compared for monocyte cultures prepared in medium with DMSO (i.e., control cultures) and cultures prepared in medium with DMSO and 5 $\mu\text{g}/\text{mL}$ of either thalidomide, lenalidomide, or pomalidomide (Figure 5), Supplementary Figure S2, Table 1). All three IMiDs modulated TLR4/LPS-induced soluble mediator release by normal monocytes, but the effects differed among the drugs. First, thalidomide caused a significant reduction for only three mediators (CCL2, IL10, MMP9) and a highly significant effect was only seen for MMP9. Second, lenalidomide caused a significant reduction for five mediators, but a highly significant effect was only seen for CCL2. Third, pomalidomide caused a significant reduction for seven mediators and the effect was generally stronger (i.e., lower p -values) for pomalidomide than for the other two IMiDs (Table 1, Supplementary Figure S2). A generally stronger effect (i.e., lower concentrations) by pomalidomide was also observed when comparing the absolute levels for cultures with LPS + pomalidomide versus LPS + thalidomide/lenalidomide (Supplementary Table S2).

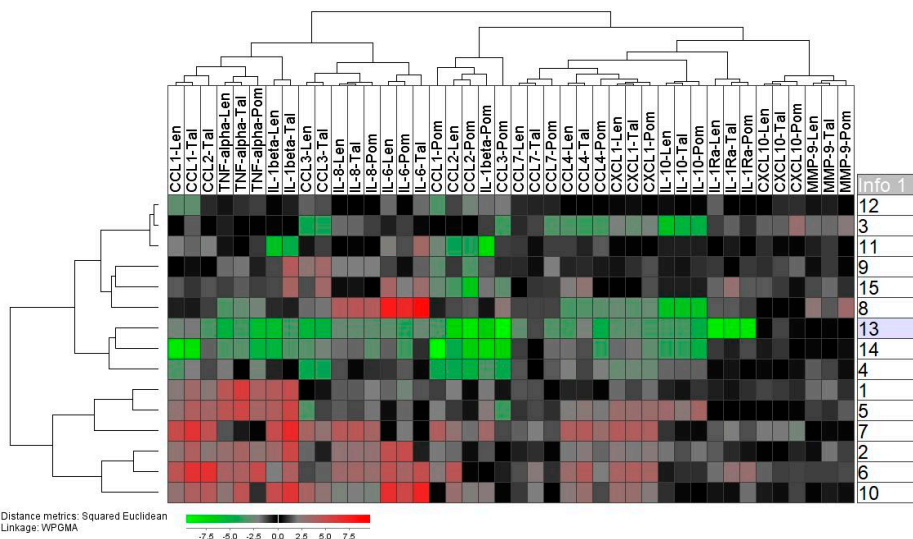


Figure 5. The effects of IMiDs on TLR4/LPS-stimulated cytokine release by normal monocytes; a hierarchical clustering analysis of the overall IMiD effects. The analysis was based on the relative mediator level, i.e., level in IMiD-containing LPS cultures relative to the level in the corresponding LPS stimulated IMiD-free control. Before the clustering analysis all relative responses for each mediator were normalized to the median relative response for all three IMiDs, i.e., they were normalized to the median relative response of all 45 IMiD responses for each mediator (3 IMiDs, 15 individuals). Thus, green color thus means a relatively strong inhibitory effect. It can be seen that even for mediators showing highly significant effects of IMiDs there was a considerable variation among individuals with regard to the effects of IMiDs.

We also did a hierarchical clustering analysis including all the drugs and all the mediators (Figure 5). This analysis was based on the relative mediator levels in drug-containing cultures, i.e., levels in IMiD cultures relative to the level in the corresponding LPS control culture. Each of these relative responses for a mediator was next normalized to the median relative response for all three IMiDs, i.e., the median relative response of the 45 IMiD responses for each soluble mediator (3 drugs times 15 individuals). It can be seen that even for mediators showing highly significant overall effects of IMiDs there was a considerable variation among individuals with regard to the effect.

Furthermore, weaker responses were often seen for the lower cluster including six patients, and all these patients showed relatively strong TLR4/LPS responses (see Supplementary Figure S3). Thus, the effect of IMiDs in the presence of TLR4/LPS stimulation seemed to depend on the LPS responsiveness, and the IMiD effects were stronger for patients with a relatively weak TLR4/LPS responsiveness.

2.6. Lenalidomide and Pomalidomide Reduce TLR4/LPS-Induced Mediator Release by Normal Monocytes Also at Concentrations Corresponding to Their Therapeutic Serum Levels

Thalidomide was tested at a concentration corresponding to its therapeutic serum level (i.e., 5 µg/mL) in the previous experiments, whereas lenalidomide and pomalidomide *in vivo* levels are lower than this. We therefore tested the effect of lenalidomide 500 ng/mL and pomalidomide 100 ng/mL on the TLR4/LPS-induced mediator release for five healthy donors, and both these IMiDs could inhibit soluble mediator release even when tested at the lower concentrations corresponding to their serum levels (uncorrected *p*-value of 0.031, Wilcoxon test for paired samples).

2.7. Pomalidomide Inhibits Soluble Mediator Release also in the Presence of Bortezomib

The effect of the proteasomal inhibitor bortezomib was tested for five healthy blood donors (two males and three females, median age 63 years with range 21–66 years) when monocytes were cultured in medium alone or in the presence of LPS. Bortezomib had minor and divergent effects when testing monocytes cultured in medium alone (data not shown), whereas it caused an inhibition of CCL1 and CXCL10 for all five individuals when tested in the presence of LPS (Figure 6). Furthermore, we tested whether the inhibitory effects of lenalidomide and pomalidomide on TLR4/LPS-induced release by normal monocytes was maintained in the presence of bortezomib. An inhibitory effect of pomalidomide was observed for a limited number of mediators also in the presence of bortezomib, whereas lenalidomide had generally weaker and divergent effect (Figure 6).

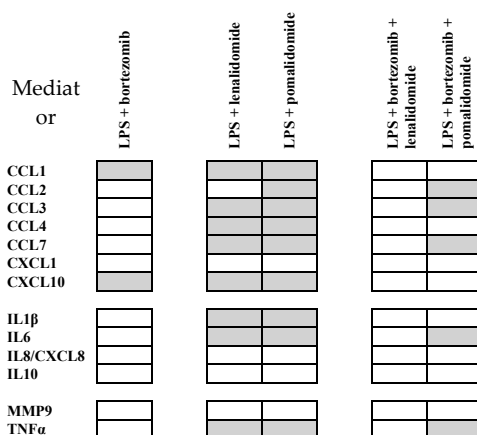


Figure 6. The effects of TLR4/LPS, bortezomib, and IMiDs on the release of soluble mediators by normal monocytes; a summary of the overall results for five healthy blood donors. We compared the mediator levels for (i) cultures prepared in medium with and without LPS 1 ng/mL; and (ii) DMSO containing control cultures with LPS alone versus cultures with LPS together with either lenalidomide 500 ng/mL or pomalidomide 100 ng/mL (i.e., concentrations corresponding to the serum levels reached during myeloma treatment); and (iii) cultures with LPS + bortezomib versus cultures with LPS and bortezomib together with either lenalidomide or pomalidomide. The comparisons showing a similar decrease (i.e., at least 25% reduction) in the soluble mediator level for all five individuals included in the comparisons are indicated in grey; a decrease for all five individuals corresponds to an uncorrected *p*-value of 0.031 when using the Wilcoxon test for paired samples.

3. Discussion

Normal monocytes seem important both in immunoregulation and possibly also for carcinogenesis/leukemogenesis [1,17]. The aim of the present study was therefore to investigate whether monocyte functions are altered by IMiDs, a class of pharmacological agents that have cereblon as a common intracellular molecular target [2] and are considered for the treatment of various cancers, especially hematological malignancies [1,27,28]. Our main conclusion based on the present study is that IMiDs can alter both monocyte metabolism as well as cytokine release, and despite their common molecular target these pharmacological effects differ among IMiDs.

Thalidomide and its derivatives lenalidomide and pomalidomide are used in the treatment of multiple myeloma [29–34]. The antimyeloma effect is probably due to both direct and indirect effects on the myeloma cells, including immunomodulation as well as anti-angiogenic, anti-inflammatory and direct antiproliferative effects [31,32,35]. These drugs are often combined with steroids and/or proteasome inhibitors [34] and can be used in frontline therapy [34]. The E3 ligase protein cereblon is a well-characterized molecular target of IMiDs [36,37], but animal studies suggest that IMiDs also have other intracellular targets [37]. The binding to cereblon modulates the stability of cereblon-interacting molecules [36] and thereby promotes apoptosis by activating caspase-8 [38]. However, the clinical evidence suggests that there are important pharmacological differences among IMiDs, and resistance against one IMiD does not exclude a response to another IMiD [39]. In the present study we investigated how IMiDs differ in their effects on normal monocytes.

TLR4 is expressed by human monocytes [11,12,40]. LPS is a TLR4 ligand that initiates the activation of several downstream intracellular pathways in monocytes, including NF κ B, extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun N-terminal kinase (JNK) and p38 pathways that activate several transcription factors [11,12]. The signaling leads to metabolic modulation and a well-characterized cytokine release response including chemokines, TNF α , IL8/CXCL8, and members of the IL1 family [41]. Furthermore, TLR4 binds microbial molecules as well as several host-derived ligands, e.g., molecules derived from degradation of extracellular matrix molecules (e.g., hyaluronic acid, heparin sulphate), heat shock proteins, fibrinogen, lipoproteins, and amyloid [9,10]. Thus, TLR4 is both a cellular sensor of the extracellular microenvironment and a regulator of cellular communication. In this context we investigated the effects of IMiDs on monocyte metabolism and cytokine release in the presence of TLR4 activation.

We used highly standardized *in vitro* models for our comparison of pharmacological effects. The same medium was used in all studies. This medium was supplemented with inactivated fetal calf serum, and the monocytes were thereby exposed to lipids during culture. Stimulation of TLR4 by lipids or lipoproteins may at least partly explain why monocytes show spontaneous release of several soluble mediators during culture, even in the absence of TLR4 stimulation [42].

Our monocyte populations had a purity exceeding 83%, and a major part of the contaminating cells were small lymphocytes. Furthermore, we cultured the cell for only 24 h, whereas lymphocyte activation will often require a longer incubation time for cytokine release, even during mitogenic stimulation [43]. For these reasons we regard our cytokine responses to be monocyte responses.

Thalidomide (molecular weight 358), lenalidomide (359), and pomalidomide (372) were tested at a concentration of 5.0 μ g/mL, and due to these minor differences in molecular weight their molar levels in the cultures should in our opinion be regarded as comparable. However, despite comparable molar levels the IMiDs differed in their pharmacological effects; lenalidomide showed a stronger effect on monocyte metabolism, whereas pomalidomide had the most significant effect on soluble mediator release. This last difference in their effects on cytokine release is probably not caused by different effects on monocyte viability; if so, one would expect all mediators to be affected and not only certain mediators, as we observed.

The concentration of 5 μ g/mL was chosen because this is the systemic level reached during thalidomide treatment of myeloma patients [44]. However, the systemic levels of lenalidomide and pomalidomide are usually lower, and for this reason we also tested lenalidomide and pomalidomide

at levels corresponding to their systemic levels reached during myeloma treatment (i.e., 500 and 100 ng/mL, respectively) [45–47]. These experiments showed that both lenalidomide and pomalidomide reduced monocyte release of soluble mediators also when tested at these lower concentrations.

Our studies showed that LPS as expected altered monocyte metabolism in the direction of glycolysis [41]. IMiDs could further modulate the metabolism of normal monocytes in the presence of TLR/LPS stimulation, and lenalidomide had the strongest effects, with a significant increase in the OCR:ECAR ratio (i.e., increased importance of glycolysis) and an increased spare respiratory capacity. These observations were only made in the presence of TLR4/LPS stimulation but not when cells were cultured in medium alone, an observation demonstrating that the differences between IMiDs with regard to metabolic modulation depend on the biological context. Finally, even though lenalidomide seems to have the strongest effect on metabolism, pomalidomide had the strongest effect on the extracellular release of soluble mediators. Thus, the IMiD effects on these phenotypic characteristics are probably mediated at least partly through different molecular mechanisms.

There was a considerable variation among individuals with regard to the levels of the various soluble mediators, and a possible explanation for this could be immunogenetic differences between cell donors. Single nucleotide polymorphisms (SNPs) exist both for NF κ B and TLR4 signaling [48–53], and certain SNPs seem to be clinically relevant and are associated with differences in regulation of monocyte activation. First, certain polymorphisms have been associated with cancer risk, and this is possibly due to a genotypic influence on intracellular signaling [50,52]. Second, the risk and/or severity of infections have also been associated with such genetic variants [49,53]. Finally, immunogenetic characteristics may be important for the role of monocytes in the development of inflammations [51,53]. Both clinical and experimental studies suggest that differences in monocyte cytokine responsiveness are important for the associations between immunogenetic differences and severity of infections [53], but differences in TLR4 expression levels may also contribute [48]. In this context it is not surprising that we detect a considerable variation among healthy individuals in the cytokine responsiveness of normal monocytes to TLR4 ligation.

Proteasome inhibitors are also widely used in the treatment of multiple myeloma [27]. These drugs inhibit the NF κ B pathway, one of the pathways also activated by TLR4 [9], and may thereby be able to reduce the release of NF κ B-regulated soluble mediators [54]. Proteasome inhibitors and IMiDs can be combined in myeloma treatment [27]. The proteasome inhibitor bortezomib had relatively weak and divergent effects on the mediator release by normal monocytes and inhibited TLR4/LPS-induced release only for a few mediators. However, bortezomib seemed to modulate the inhibitory effects of IMiDs on the mediator release, and this was true especially for lenalidomide, whereas several effects of pomalidomide were maintained also in the presence of bortezomib.

Infections are a major cause of morbidity and mortality in myeloma patients, and immunosuppressive effects of IMiDs may contribute to the risk of infections [55]. Advanced myeloma is associated with dysregulation of several immunocompetent cells [29], including monocytes [18,56–62]. The effects of IMiDs on monocyte mediator release will influence communication between immunocompetent cells and possibly contribute to the risk of infections. Myeloma patients receiving antimyeloma therapy seem to have a quantitative monocyte defect [63] and our present results suggest that these patient also have a qualitative effect, but in our opinion the myeloma-associated B-cell defect is probably most important for the increased risk of infections [64].

Our present *in vitro* studies suggest that various IMiDs alter the metabolic regulation and the immunomodulatory functions of normal monocytes, but for several reasons our results should be interpreted with great care. First, additional clinical studies are needed to clarify whether these effects are relevant *in vivo*. Second, normal monocytes consist of different subsets with different immunoregulatory functions [17,65], and it is not known whether IMiDs have similar effects on different monocyte subsets. Third, it is not known whether circulating normal monocytes derived from cancer patients, especially allotransplant recipients [63,65], show similar pharmacological effects compared with monocytes from healthy individuals, or whether the IMiD effects are similar for

young/middle-aged/elderly patients. Finally, we have only investigated IMiDs in combination with the proteasome inhibitor bortezomib; additional combinations used for treatment of multiple myeloma [27] as well as for other hematological malignancies [28] also need to be investigated.

Based on our present experimental studies we conclude that IMiDs modulate metabolism and communication of normal monocytes, and despite their common molecular target [2] these effects differ among IMiDs. These pharmacological in vivo effects may be relevant for immunoregulatory as well as cancer-supporting effects of normal monocytes [1,17], and future clinical studies should therefore try to clarify whether these effects are also important in vivo.

4. Materials and Methods

4.1. Cell Donors

Normal monocytes were derived from healthy blood donors. In accordance with the approved routines at the Blood Bank, Haukeland University Hospital peripheral venous blood samples were donated after written informed consent. The project was approved by the Regional Ethics Committee (REK VEST 2013/635, 2017/305).

4.2. Reagents

A stock solution of lipopolysaccharide (LPS) from *Escherichia coli* (#L2654-1MG; Merck KGaA, Darmstadt, Germany) was dissolved in medium (1 mg/mL) and stored at -80°C . LPS was used at a concentration of 1 ng/mL based on titration experiments using monocytes in the Seahorse assay. Bortezomib (#5043140001; Merck KGaA) was dissolved in medium and used at a final concentration of 25 nM; this concentration can inhibit in vitro constitutive chemokine release by myeloid cells [54]. Stock solutions of thalidomide 12 $\mu\text{g/mL}$ (#14610), lenalidomide 16 $\mu\text{g/mL}$ (#14643), and pomalidomide 15 $\mu\text{g/mL}$ (#19877; all from Cayman Chemicals, Ann Arbor, MI) were prepared in DMSO (D2650-5X5ML, Merck KGaA), aliquoted, and stored at -80°C . DMSO reached a final concentration of 0.55 mg/mL (corresponding to 0.055%) in the experiments. The molecular weights for the IMiDs are thalidomide (C₁₃H₁₀N₂O₄) 258, lenalidomide (C₁₃H₁₃N₃O₃) 259, and pomalidomide (C₁₃H₁₁N₃O₄) 273 (see Supplementary Figure S4). The IMiDs were used at a final concentration of 5 $\mu\text{g/mL}$; this concentration corresponds to the systemic levels reached in vivo during thalidomide treatment of myeloma patients [44] (for molar concentrations, see Supplementary Figure S4).

4.3. Preparation of Enriched Normal Monocytes

Monocytes were isolated from buffy coats that were diluted 1:1 with phosphate-buffered saline (PBS), and peripheral blood mononuclear cells (PBMC) were then isolated by density gradient separation (Lymphoprep™, NycoMed, Oslo Norway; density 1.077 g/mL; centrifugation 800 G/30 min). The PBMC were washed twice in PBS, resuspended in 5 mL RPMI 1640 medium (#R7509, Merck KGaA) and overlaid 4 mL Percoll solution (P4937, Merck KGaA) [66]. After centrifugation (500 G, 30 min, room temperature) monocytes were harvested, washed, resuspended in 30 mL PBS and counted by a TC20™ Automated Cell Counter (BIO-RAD, Oslo, Norway).

The cells were thereafter centrifuged and resuspended (10⁷ cells/40 μL in the recommended buffer for the Pan Human Monocyte Isolation Kit (#130-096-537; MACS Miltenyi Biotec, Bergisch Gladbach, Germany) and the separation procedure performed strictly according to the manufacturer's recommendations. Briefly, the LS column (#130-042-401, MACS Miltenyi Biotec), and the 30 μm pre-separation filter (#130-041-407, MACS Miltenyi Biotec) were prepared according to the instructions. Cells were incubated with the FcR-block and Pan Monocyte Biotin–Antibody Cocktail for 5 min at a concentration of 10 μL per 10⁷ cells; an additional 40 μL of buffer per 10⁷ cells was thereafter added, followed by 20 μL Anti-Biotin MicroBeads per 10⁷ cells. The cell suspension was thereafter incubated for 10 min before buffer was added, the cells were centrifuged and resuspended in buffer at a concentration of 10⁸ cells/0.5 mL. Thereafter 0.5 mL of the cell suspension was added to the pre-filter

and the suspension was run through the column and collected. The column was subsequently washed with 3 mL buffer that was collected together with the monocytes. The collected cells were centrifuged and reconstituted in 50 mL PBS before counting. Flow cytometric analysis of isolated cell populations showed at least 83% monocytes (median 89.5%, range 83.4–95.3%); the majority of contaminating cells being small lymphocytes. The monocytes included a median of 84% classical (range 76–90%), 5% (range 4.1–9.3%) intermediate, and 9% (range 4.8–18.4%) nonclassical monocytes. The monocytes were centrifuged and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (1.25×10^6 cells/mL), and 200 μ L were distributed to each culture well. After 30 min of preincubation at 37 °C the cells were used in the experiments.

4.4. Analysis of Monocyte Metabolism

In vitro culture of enriched monocytes. Monocytes were incubated for four hours in medium with and without IMiDs/LPS before the medium was changed. Thereafter the cells were incubated for one hour without CO₂ before analysis by the Seahorse XF 96 cell analyzer. No significant difference was detected for control cultures (with or without LPS) with and without DMSO. Pilot experiments compared the effects of IMiDs after 2, 4, and 24 h of *in vitro* culture; the effects of IMiDs were strongest after four hours and this incubation time was used for all experiments.

Extracellular flux assays. The XF Mito Stress Test Kit (#103,325-100 and #103,015-100; Agilent Technologies, Inc., CA) was used strictly according to the manufacturer's instructions. Briefly, assay medium was prepared by supplementing XF Base Medium minimal DMED (#102,353-100; Agilent Technologies) with glucose 10 mM (#103,577-100, Agilent Technologies), pyruvate 1 mM (S8,636-100 mL, Merck KGaA), and glutamine at 2 mM (#103,575, Agilent Technologies); the pH was adjusted to 7.4 with NaOH. Cells were washed twice in assay medium before being resuspended in 180 μ L medium and incubated (humidified atmosphere, 37 °C, without CO₂) for 60 min. The cartridges that had been hydrated according to manufacturer instructions one day earlier, were prepared with the drugs for injections, i.e., oligomycin 2 μ M, carbonyl cyanide-p-trifluoromethoxy-yphenyl-hydrazon (FFCP, a protonophore) 1 μ M, and rotenone/antimycin A 0.5 μ M (all solutions prepared in assay medium). The extracellular flux assays were performed; subsequently the assay medium was discarded and the plate stored at –80 °C for at least 24 h before the amount of protein was measured (see below). All assays were prepared with 6–8 parallels.

The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were estimated by a Seahorse XF 96 cell analyzer. The spare reservoir capacity and coupling efficiency was calculated by XF Cell Mito Stress Test Excel Template. The definitions of the parameters used in the metabolic analyses can be seen in Supplementary Figure S1 (upper part).

Estimation of total cell proteins. The Pierce™ BCA Protein Assay Kit (#23,225; Thermo Scientific™, Waltham, MA, USA) was used for normalization of data and performed strictly accordingly to manufacturer's instructions. A total of 10 μ L of standard samples was added to each well together with 200 μ L of working reagent. After 30 s on a plate shaker and 30 min incubation 150 μ L was transferred to a new plate and read at 595 nm by a iMark™ Microplate Absorbance Reader (Bio-Rad laboratories, Oslo, Norway).

4.5. Analysis of TLR4/LPS Induced Cytokine Release

Enriched normal monocytes were cultured in RPMI 1640 medium (#R8758-1L; Merck KGaA) supplemented with 10% inactivated fetal calf serum (#S181B-500; Biowest, Nuaillé, France) and penicillin-streptomycin (#MS00AO100H; Biowest). Cultures were prepared in Agilent Seahorse XF95 cell culture microtiter (96-well) plates with each well containing 250,000 monocytes in 200 μ L medium. LPS 1 ng/mL was added together with the drugs, cultures were thereafter incubated (37 °C, humidified atmosphere, 5% CO₂) for 24 h before supernatants were harvested. The supernatants were stored frozen at –20 °C until analyzed.

Supernatant levels of soluble mediators were determined by Luminex high performance assays (Biotechne, Abingdon, UK) and included CCL1, CCL7, CXCL10, IL-8, MMP-9, CCL2, CCL4, CXCL1, IL-1 β , and IL-10. ELISA assays (Biotechne) were used to determine levels of CCL3, IL1RA, IL6, and TNF- α . All assays were performed strictly according to the manufacturer's instructions; the assays were performed in duplicates and differences between duplicates were generally <10%.

4.6. Statistical Analysis

The Wilcoxon rank sum test and the Wilcoxon test for paired samples were used for statistical analyses and *p*-values < 0.05 were regarded as statistically significant.

Supplementary Materials: The following are available online, Table S1: The spontaneous release of soluble mediators, an overview; Table S2: The effects of thalidomide, lenalidomide, and pomalidomide on TLR4/LPS-induced release of soluble mediators by normal monocytes derived from healthy individuals; a comparison of the effects of the three pharmacological agents; Figure S1: Analysis of monocyte metabolism using The XF Mito Stress Test assay and the Seahorse XF 96 cell analyzer; an overview of the Seahorse assay (upper part) and the results from a representative experiment (lower part); Figure S2: The effects of TLR4/LPS activation and IMiDs on the release of soluble mediators by normal monocytes; a summary of the results for 15 healthy blood donors presented in Table 1 in the article; and Figure S3: A hierarchical clustering analysis of the TLR4/LPS-induced stimulation of soluble mediator release by normal monocytes. Figure S4: The chemical structure of the three IMiDs investigated (thalidomide, lenalidomide, and pomalidomide), their molecular weights, and the molar concentrations corresponding to 5 μ g/mL.

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Sample Availability: Samples of the compounds are not available from the authors.



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