Regulation of Human Innate Immune Cells by Inflammatory Lipids

Doctoral thesis by

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All other disciplines in medicine are supplementary to immunology.

Unknown
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I am deeply thankful to my supervisor, Professor Torill Berg. Your feedback and advice was essential towards the submission of this thesis. Thanks to Erik for stepping in at short notice as my co-supervisor.

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To my co-authors, I am grateful for your cooperation and help.

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Ane, my dear, I am grateful for you making my days matter.

Tynset, January 2016,

Johannes Rolin
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CSF1</td>
<td>Colony Stimulating Factor 1</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>CX3CR</td>
<td>CX3C chemokine receptor</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAM</td>
<td>DNAX Accessory Molecule</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>ED50</td>
<td>Effective Dose 50</td>
</tr>
<tr>
<td>Edg-1</td>
<td>Endothelial differentiation gene 1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibroblast Activating Protein</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fragment crystallizable type G receptor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>G2A</td>
<td>G2 Arrest</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage – Colony Stimulation Factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G Protein Coupled Receptor</td>
</tr>
<tr>
<td>GPR132</td>
<td>G-protein coupled receptor 132</td>
</tr>
<tr>
<td>Grz</td>
<td>Granzyme</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HODE</td>
<td>Hydroxyoctadecadienoic acid</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory Concentration 50</td>
</tr>
<tr>
<td>iDC</td>
<td>Immature Dendritic cell</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine-2,3-Dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin Growth Factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate Lymphoid Cell</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer cell Ig-like Receptor</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>LPA</td>
<td>Lyso phosphatidic acid</td>
</tr>
<tr>
<td>LPA1-6</td>
<td>Lyso phosphatidic acid receptor 1-6</td>
</tr>
<tr>
<td>LPC</td>
<td>Lyso phosphatidylcholine</td>
</tr>
<tr>
<td>LPL</td>
<td>Lyso phospholipid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTα</td>
<td>Lymphotoxin alpha</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein 1</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>mDC</td>
<td>Mature Dendritic cell</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-Derived Suppressor Cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MI</td>
<td>Migration Index</td>
</tr>
<tr>
<td>MICA</td>
<td>MHC class I chain-related protein</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory protein</td>
</tr>
<tr>
<td>NCR</td>
<td>Natural Cytotoxicity Receptor</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>NKG2</td>
<td>Natural Killer Group 2</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecule Pattern</td>
</tr>
<tr>
<td>PDC</td>
<td>Plasmacytoid Dendritic Cell</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
</tr>
<tr>
<td>PLA-A</td>
<td>Phospholipase A</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis Toxin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine 1-Phosphate</td>
</tr>
<tr>
<td>S1P1-5</td>
<td>Sphingosine 1-phosphate receptor 1-5</td>
</tr>
<tr>
<td>SphK1</td>
<td>Sphingosine Kinase 1</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGf-β</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Th1</td>
<td>T–helper cell type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T–helper cell type 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF receptor-associated factor 2</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF related apoptosis inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>TREM2</td>
<td>Triggering Receptor Expressed on Myeloid cells</td>
</tr>
</tbody>
</table>
Included papers

This thesis is based on the following papers, which will be referred to by their roman numerals:

I. Rolin, J.; Sand, K.L.; Knudsen, E.; Maghazachi, A.A.

FTY720 and SEW2871 reverse the inhibitory effect of S1P on natural killer cell mediated lysis of K562 tumor cells and dendritic cells but not on cytokine release, *Cancer Immunology and Immunotherapy* 2010;59:575-586

Johannes Rolin planned, prepared and executed most of the experiments of this work independently: Purification and culture of cells, cytotoxicity assay, flow cytometry, western blot, immune precipitation and ELISA. The data were analyzed by Azzam A. Maghazachi and Johannes Rolin. The paper was written by Johannes Rolin and Azzam A. Maghazachi.

II. Rolin, J.; Al-Jaderi, Z.; Maghazachi, A.A.


Johannes Rolin planned and performed the experiments on calcium mobilization, cytotoxicity, ELISA and immune phenotyping by flow cytometry. Zaidoon Al Jaderi performed cytotoxicity and flow cytometry experiments. Azzam A. Maghazachi performed the NK cell chemotaxis experiments. Johannes Rolin and Azzam A. Maghazachi wrote the paper.

III. Rolin, J.; Vego, H.; Maghazachi, A.A.

Oxidized lipids and lysophosphatidylcholine induce the chemotaxis, up-regulate the expression of CCR9 and CXCR4, and abrogate the release of IL-6 in human monocytes, *Toxins* 2014;6:2840-2856.

Johannes Rolin planned, performed and analyzed the experiments and wrote the manuscript. Heidi Vego helped with ELISA – experiments, and Azzam A. Maghazachi with the manuscript.
Introduction

Immunology is the discipline of science that aims to understand how organisms protect themselves against the challenges threatening their existence every day. This includes protection against invading microbes as well as clearance and regeneration of broken tissue. The complexity of the system devoted to these crucial tasks, the immune system, is related to the complexity of the organism that is to be protected. Our current understanding of the immune system is based on knowledge accumulated over more than two centuries of experimental research, a proud history dating back to Edward Jenner’s successful induction of protection against smallpox published in 1798. His observations were supported by more fundamental discoveries in the following century, and since the 1950s advances in techniques for cell and tissue culture, examination of DNA and proteins, histology, the development of genetically altered animals and many more have opened the possibility of molecular understanding of immunological phenomena. Inspired by these, a tremendous body of research in the field of immunology has fundamentally transformed our understanding of the immune system and by doing so provided the basis of modern medicine.
Basic aspects of immunology

A fundamental challenge to the immune system, from the simplest bacteria through vertebrates, is to identify invading microorganisms and discard them. More complex, multi-cellular organisms also need mechanisms to recognize and break down destroyed or altered cells while at the same time providing favorable conditions to others.

An example of the first, is the ability of bacteria and most archaea to specifically target and destroy invading DNA, through a system that samples foreign DNA from the environment in a resting situation, thus preparing for the potential occurrence of invaders.\(^1\)

Eukaryotes host a more elaborate system comprised as intra- and extracellular means of recognizing foreign material. Hence, they express intracellular receptors for bacteria\(^2\) as well as viral DNA.\(^3\) Further, these systems alert other specialized cells to launch a more comprehensive counterattack by secreting signaling molecules. And as the internal environment of multicellular organisms stretch beyond the limits of the single cells, there is also a need for receptors for sampling the intercellular milieu related to invaders and foreign material, which is achieved on the one hand by continuous sampling, and, on the other hand, by recognizing specific structures known to be associated with intruders such as bacteria or dying cells.

All these activities allow the organism to adapt to its environment and deal with changes therein. Then, in order to discern between self, non-self and altered self, these mechanisms combine in the immune response, by recognizing on the one hand structures such as pathogen associated molecular patterns (PAMPs) that prompt killing, and, in contrast, inhibition of killing upon recognition of self-molecules. Yet other mechanisms play a role in the absence of these two.

The adaptive immune system

The mammalian immune system is comprised of two cooperative branches, the innate and the adaptive immune system. Conceptually, the innate arm occupies itself with the recognition of common antigens such as components of dying cells and vital parts of common microorganisms. The adaptive system on the other hand is capable of genetic rearrangement of its receptors which gives the ability to recognize new and rare structures such as new strains of viruses, and it remembers them in order to adapt the individual to this new challenge.
The adaptive arm is comprised of B and T lymphocytes (B and T cells), that recognize antigens by use of immunoglobulins (Igs) or T cell receptors (TCR) respectively. B cells are through their Ig receptors able of recognizing antigens in unmodified form, followed by endocytosis and intracellular processing leading to presentation of peptide fragments on major histocompatibility complex class II (MHC class II). This leads to recognition of the B cells by Th cells which through their TCR identify the foreign peptides presented by the B cells thus activating the B cells. This forms the basis for T-B cell collaboration. Upon activation, B cells differentiate into antibody-producing plasma cells or memory B cells. This process takes place in the germinal centers of secondary lymphoid organs and leads to the differentiation of B cells with high affinity B cell receptors for antigen (memory B cells) or precursors of antibody producing plasma cells (the Ig subclass produced being dependent on the type of help they get from Th cells). Some B cells, however, are stimulated directly by the antigen, leading to IgM producing plasma cells.

T cells recognize antigens bound to MHC class I or class II depending on their co-expression of cluster of differentiation 8 (CD8) or CD4, respectively. Upon encountering malignant or infected cells, CD8 expressing (CD8⁺) T cells kill the cells with perforin and granzymes released from intracellular granules. Activated CD4⁺ cells take part in orchestrating the immune response by activating other immune cells including B cells and macrophages. Depending on the presence of certain soluble signaling proteins (cytokines) in their milieu during activation, CD4⁺ T cells may differentiate into distinct subsets such as T helper 1 (Th1), T helper 2 (Th2), T helper 17(Th17) or T regulatory cells (Treg) which all produce unique arrays of cytokines which influence the nature of the immune response (Table 1).4-6

Table 1. Overview of CD4+ T cell subgroups.

<table>
<thead>
<tr>
<th>Subset</th>
<th>Signature cytokines</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IFN-γ, IL-2, LTα</td>
<td>Intracellular pathogens, autoimmunity</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4, IL-5, IL-10, IL-13, IL-25, Amphiregulin</td>
<td>Extracellular pathogens, allergy and asthma</td>
</tr>
<tr>
<td>Th17</td>
<td>IL-17a, IL-17f, IL-21, IL-22</td>
<td>Extracellular bacteria, fungi, autoimmunity</td>
</tr>
<tr>
<td>Treg</td>
<td>IL-10, IL-35, TGF-β</td>
<td>Immune tolerance, lymphocyte homeostasis, immune response regulation</td>
</tr>
</tbody>
</table>
The innate immune system

The innate immune system is evolutionary more ancient than the adaptive immune system, and exists also in related forms in less complicated organisms such as insects and plants.\textsuperscript{7,8} The presence of homologs to mammalian receptors and signaling pathways reflects common ancient mechanisms for the recognition of antigens.\textsuperscript{9}

Innate immunity utilizes several strategies for immune recognition that are fundamentally different from the one based on genetic rearrangement used by the adaptive system. Firstly, recognition of PAMPs, or just pattern recognition, means recognition of molecules or parts of them that are common between several pathogens but are absent in the host. Their presence will thus be sufficient to evoke an immune response. Among the better known examples in this group are lipopolysaccharide (LPS) present in Gram-negative bacteria, lipoteichoic acids present in Gram-positive bacteria, virally derived double stranded RNA and CpG-motifs in microbial genomes. Secondly, the phenomenon denoted “missing self” in the NK cell literature\textsuperscript{10}, involves continuous negative signaling provided by ubiquitously expressed normal molecules in the host recognized by inhibitory receptors, and the initiation of an immune response in their absence. This is perhaps easiest envisioned as a change in normal cells upon diseases such as cancer or infection, leading to down regulation of molecules that would otherwise prevent disease development. An example of this is the recognition of unmodified “self” MHC class I (MHC-I) antigens on nucleated target cells of the body by Killer Immunoglobulin-like Receptors (KIRs) on human NK cells. Inhibitory KIR, in the presence of normal levels of MHC-I, inhibit NK cell cytotoxicity; however, downregulation or modification of MHC-I molecules by e.g. viral infection may lead to release of the inhibition and killing of the target. Notably, activating KIRs that are structural homologs of the inhibitory ones have also been identified. The interactions and implications of these receptors, however, are not as well understood as those of the inhibitory KIRs.

Stretching beyond the mere sorting of cells between good and bad, the regulation of many physiological processes on a daily basis is tightly associated with the innate immune system. An example of this is the endothelial cells, as they continuously contribute in regulating blood flow and pressure, as well as in the maintenance of vascular integrity when faced with minor damages. Further, granulocytes and macrophages are among other functions responsible for clearance of debris after tissue injury or the killing of bacteria.
An important feature of the innate immune system is the shaping of adaptive immune responses which was recently reviewed, emphasizing the crucial role of inflammatory cytokines in linking the innate and the adaptive immune system (Fig. 1).

![Diagram of the immune system](image)

**Figure 1.** Adapted from Iwasaki and Medzhitov with permission. Inflammatory cytokines in the regulation of adaptive immunity by the innate immune system.

According to the model presented by Iwasaki and Medzhitov, the recognition stimuli, such as pathogens or tissue damage, triggers the release of ‘level 1’ cytokines such as IL-1β and IL-6 in the case of a type 1 immune response, or another set of cytokines such as IL-1α, IL-25 or IL-33 in the case of a type 2 immune response. These cytokines act on a second category of immune cells such as lymphocytes and ILCs, which in response produce ‘level 2’ cytokines such as IFN-γ, IL-17 and IL-22 or IL-4, IL-5, IL-9 and IL-13. Finally, the level 2 cytokines act on the effectors of the immune system including neutrophils, B-cells, epithelial cells and others.

**Role of the innate immune system in diseases**

Two important examples of diseases, influenced by the immune system, are cancer and atherosclerosis.

**Cancer**

In the case of cancer, the immune system plays a role in the surveillance of spontaneous tumors. This is illustrated by the increased risk of cancer in immune compromised patients e.g. after organ transplantation. Observations in animal models further show us that this continuous effort results in shaping the tumor cell population, a phenomenon denoted ‘immuno-editing’ of developing tumors. This means that the process of destroying emerging transformed cells in order to protect the host constitutes an ‘immunological pressure’ leading to the selection of tumor cells able of withstanding immune defenses. Supporting this hypothesis, numerous reports show that tumors lacking antigen processing and presentation components, or the lack of inflammatory cytokines such as IFN-γ, are common. Efficient antitumor immunotherapies can therefore be
envisioned as a way to interfere with the host–tumor equilibrium by boosting immune defense and/or decreasing the immunosuppressive effect of tumors. Basic research into the interplay between host and developing cancer cells is a prerequisite for the development of such therapies and is currently an item of central interest in tumor immunology.

Atherosclerosis

Atherosclerosis is a chronic inflammation gone wrong. When endothelial cells are subjected to a milieu of shear stress and disturbed flow, they are damaged. They become permeable, fail to be aligned in the direction of flow and consequently increase their thrombogenicity, in addition to promoting oxidative stress and the release of proinflammatory cytokines. As a consequence of this, leukocytes and lipids accumulate. As the size of the plaque increases, oxygen levels decrease, leading at the same time to narrowing of artery lumen and development of central necrosis in the plaque. This further increases the risk of plaque rupture, leading to activation of the coagulation cascade with devastating consequences such as myocardial infarction.

The object of this work has been to investigate the regulatory impact of lipids on various functions of innate immune cells relevant to cancer and atherosclerosis. Therefore, the following will include an overview of the physiology of some of the innate immune cells. In addition, an overview will be given of the biology and the implications of the lipids that have been studied.
Natural killer (NK) cells

In the late 1960s, a discovery was reported regarding a population of lymphocytes capable of killing cancer cells without previous sensitization by antigens and, later, viral infections by distinct mechanisms of target cell recognition. The cells were named natural killer (NK) cells, and they perform several important functions. Among them is the regulation of the adaptive immune response by secreting cytokines such as IFN-γ and TNF-α as well as chemokines such as MIP-1α and MIP-1β, thus driving the T cell response towards Th1, and shaping the innate immune system by interacting with dendritic cells.

Originally NK cells were defined as a functional subset of lymphocytes exhibiting natural cytotoxicity and containing large azurophilic granules. With identification of receptors expressed on NK cells and the generation of antibodies towards them, NK cells have been defined phenotypically by surface molecules.

After the discovery of NK related cellular subsets in recent years, the concept of innate lymphoid cells (ILCs) has developed and prompted a tighter definition of what is to be considered NK cells. ILCs, of which NK cells comprise by far the best known and most widely studied subtype, are defined by three main features:

1. Absence of recombination activating gene-dependent rearranged antigen receptors.
2. Lack of some markers characteristic of myeloid cell and dendritic cells.
3. Lymphoid morphology.

As the need for a uniform nomenclature has risen due to the attribution of many different names to the same groups of cells, it was proposed to classify ILCs according to their corresponding T helper cell subsets, as groups among the ILCs have similar cytokine-secreting profiles as the T cells. A third group, however, ILC3, comprises cells dependent on the transcription factor RORγt for their development. These cells secrete IL-17A and/or IL-22 and possibly as well Th1 – and Th2 – related cytokines. Hence, the main categories, group 1, 2 – and 3 ILCs, were proposed and defined:

ILC1: Production of the Th1 cell-associated cytokine IFN-γ and inability to produce Th2 cell- and Th17 cell-associated cytokines.

ILC2: Production of Th2 cell-associated cytokines IL-4, IL-5 and IL-13.
ILC3: Capable of producing IL-17A and/or IL-22.

NK cells are considered to be a part of ILC group 1 due to their secretion of IFN-γ, but it is still a matter of debate if NK cells are at all ILCs. For example, the prediction of one common precursor for all ILCs led to the identification of an ILC precursor high in the transcription factor PLZF. PLZF was found in virtually all ILC subsets except classical NK cells. And while NK cells share several transcription factors with other subsets of the ILCs, only NK cells depended on E4BP4 for their development.

Human NK cells are defined as cells expressing CD56 but not CD3, though NKP46 is considered the best marker for human and murine NK cells. CD56 was suggested to play a role in NK cell adhesion to target cells, but the functional implications of this receptor is still unclear. Based on the expression levels of this protein and of the Fcγ receptor CD16, NK cells in the blood may be further divided in two major subsets; those that express CD56 but not CD16 (known as CD56brightCD16− or just CD56bright), and those that express CD16 and low CD56 (known as CD56dimCD16+ or just CD56dim). The CD56bright subset is regarded as the first functionally competent NK cell subset, and as they acquire CD16 and killer cell immunoglobulin-like receptors (KIRs), the inhibitory receptor NKG2A/CD94 is downregulated.

Several functional characteristics further contribute to the relevance of this division:

- The CD56brightCD16− subset is mostly regulatory. The cells secrete IFN-γ and other cytokines, but they possess low cytolytic activity. They lack perforin and they are KIRlow or natural cytotoxicity receptor (NCR) low, except low levels of NKP46. These cells express CC chemokine receptor 7 (CCR7), CXC chemokine receptor 4 (CXCR4), CXCR3 and CD62L, which implies that they are able to reach lymph nodes, whereas the CD56CD16+ subset is not able to enter secondary lymphoid compartments.
- The CD56dimCD16+ subset constitutes more than 95% of NK cells in peripheral blood and 85% in the spleen. They express perforin, the NCRs NKP30, NKP44 and NKP46, as well as KIRs. These cells are highly cytolytic, and upon a tumor cell encounter, they may secrete IFN-γ. They express the chemokine receptors CXCR1 and CX3CR1, which direct them towards inflamed tissues, as the chemokines specific for these receptors are produced during inflammatory events by macrophages, neutrophils, Dendritic cells (DCs) and endothelial cells.
Table 2. Functional and phenotypical subdivision of NK cells.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Killer receptors</th>
<th>Effector functions</th>
<th>Chemokine receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD56^{bright}/CD16^{dim/neg}</td>
<td>IFN-γ, IL-10, TNF-β, MIP-1α/β</td>
<td>+ KIR, +++CD94/NKG2A</td>
<td>+ADCC, +Natural cytotoxicity</td>
</tr>
<tr>
<td>CD56^{dim}/CD16^{bright}</td>
<td>Low production</td>
<td>+++KIR, +CD94/NKG2A</td>
<td>+++ADCC, +++Natural cytotoxicity</td>
</tr>
</tbody>
</table>

Maillard\(^{41}\) proposed that the regulatory effect of NK cells, which is observed upon stimulation by IL-18, might be due to the ability of the minor subset of CD56^{+}/CD16^{+} cells to migrate towards secondary lymphoid tissues, where they directly influence Th1 polarization. This notion is a preliminary perspective on the crosstalk between IL-18 secreting dendritic cells (DCs)\(^{42}\) and NK cells, which will be discussed later.

The different size of these two populations, the perforin negative CD56^{bright}/CD16^{−} cells in lymph nodes outnumbering the blood borne perforin positive CD56^{dim}/CD16^{+} cells, suggests the secondary lymphoid organs as a site of NK priming and differentiation\(^{34}\) and that the CD56^{bright} cells are precursors for the CD56^{dim} cells.\(^{32}\) Other important differences between these subtypes are the expression of adhesion molecules\(^{43,44}\) and chemokine receptors.\(^{45,46}\)

Recent evidence challenges this classical view of NK cells, as CD56 negative cells were shown to be more potent in the secretion of cytokines upon recognition of target cells.\(^{47}\)

This observation may relate to the proposed ‘split anergy’ (decrease in cytotoxicity and at the same time an increase in IFN-γ secretion) among NK cells, leading to the generation of a NK cell subset named NK regulatory cells (CD16^{dim}/CD56^{dim}/CD69^{+}) or just NKreg.\(^{48}\)

Indeed, this would be a CD56 negative subset that secretes cytokines. This theory is based on research through the last 20 years showing a tight relationship between the degree of maturation and NK cell cytolysis of target cells. Following killing of susceptible cells, NK cells are rendered less cytotoxic but highly potent in releasing cytokines.\(^{48}\) For example, the NK-sensitive tumor cell line K562, which is widely used in NK cell research, indeed caused loss of NK cell cytotoxicity and, at the same time, triggered secretion of TNF-α and IFN-γ. RAJI cells, on the other hand, were less susceptible to IL-2-activated NK cell cytotoxicity.\(^{49,50}\) The authors observed a change in the phenotype of NK cells that had been exposed to sensitive targets, in that CD16 was down-regulated, resulting in a CD56^{dim}/CD16^{−}/CD69^{+} phenotype. This phenotype has also been found in patients suffering from
cancer and HIV infection. Stimulation of NK cell receptors such as CD16, NKp46 and others, resulted in an active process, leading to changes in phenotype. In lieu of these observations, it was concluded that ‘anergized’ NK cells (i.e., efficient in cytokine production, but poor in cytotoxicity) are as important as the non-anergized NK cells.

**Opposing signals and their integration**

Human NK cells comprise about 10-15% of total blood lymphoid cells, and it was shown early that IL-2 is a potent stimulus for NK cell maturation. Maturation after IL-2-stimulation can be readily observed in the microscope for cells in culture. At the protein level, the cells up-regulated their natural cytotoxicity receptors, started to express perforin and acquired increased cytolytic activity for NK-sensitive target cells. It has therefore been hypothesized that NK cells are mobilized by IL-2 from secondary lymphoid tissues during the immune response.

Several classes of receptors are unique to NK cells, and these cells also express a unique combination of receptors also found on other cell types. The inhibitory NK cell receptors include KIR and NKG2A/CD94 heterodimers, among others. In humans, KIR can harbor two (KIR2D) or three (KIR3D) extracellular C-type Ig-like domains, which recognize motifs of HLA class I molecules, determined by the amino acids belonging to the C-terminal portion of the MHC class I α1 helix. Of note, though not as well described as their inhibitory counterparts, activating KIR exist and appear also to be important in NK cell education in order to secure tolerance.

As it was also reported that all mature NK cells express at least one self MHC class I inhibitory receptor, the ‘at least one rule’, ensuring NK tolerance to self, was proposed. This theory was tested, showing that 10-15% of NK cells expressed none of the MHC class I recognizing receptors. Those cells, which were tolerant to MHC class I-deficient lymphoblasts, showed impaired killing of tumor cells and reduced percentage of IFN-γ producing cells. This observation suggested that MHC I recognition is important for the functional maturation of NK cells. Thus, studies of NK cells from MHC class I-deficient individuals (e.g. patients with transporters associated with antigen presentation (TAP) deficiency) have shown an influence of MHC I on the acquisition of NK cell function, as cells from these patients exhibit an activation defect. Later, it was shown that this was not due to a lack of maturation, but a phenomenon comparable to anergy as is seen with B and T cells. NK cells are by default responsive, but upon continuous stimulation in the absence
of inhibitory signaling, they become anergized, or hyporesponsive, which is in line with a model of disarming.⁶¹ Indeed, the number of self MHC-specific receptors through a process called education or licensing, determines the degree of responsiveness at the level of the single NK cell.⁶²

Cells expressing a stress-induced MHC homolog are killed by NK cells via engagement of the receptor NKG2D,⁶³ providing an example of a complementary mechanism to the ‘missing self’, i.e. recognition of the changed self. NKG2D is an activating NK cell receptor (but does not structurally belong to the NKG2 family with NKG2A, -C and -E). It is a C-type lectin-like receptor that is activated by MHC class I chain-related protein 1 (MICA) and UL16 binding proteins. MICA is considered a stress-inducible danger signal,⁶⁴ that is expressed following for example, heat shock and UV-induced DNA damage.⁶⁵,⁶⁶ Further, the natural cytotoxicity receptors (NCRs; NKp46, NKp44 and NKp30) are linked to immuno receptor tyrosine-based activation motif (ITAM) bearing coreceptors.⁶⁷ The ligands for these receptors are still a matter of debate. A possibility for antibody dependent cell cytotoxicity even exists in the Fc receptor CD16.

NK cell activating receptors were cross-linked with activating antibodies, which revealed that CD16 was the only receptor capable of activating degranulation in primary resting human NK cells on its own.⁶⁸ As CD16 mediates antibody-dependent cell-mediated cytotoxicity (ADCC), this means that none of the tested receptors for natural cytotoxicity were sufficient to induce a functional NK cell response. In combination, however, several receptors were able in a synergistic manner to mount a response leading to cytolysis.⁶⁸,⁶⁹ Hence, the classical activating receptors NKG2D and the NCRs as well as receptors previously thought to be accessory or co-stimulating such as DNAM-1 and 2B4, are all ‘co-activation receptors’. The next question was then how signals from receptors with different signaling properties can converge to reach an activation threshold. It was shown that the synergism was at the level of the second messenger Vav-1, which was inhibited by the ubiquitin ligase c-Cbl; and this inhibition was overcome when two independent signals stimulated the phosphorylation of Vav-1.⁷⁰

Integration of inhibitory and stimulatory stimuli determines NK cell activity. A recent work contributes mechanistic data to how this integration is related to the immunological synapse. Using ligands for activating and inhibitory signaling in NK cells, it was shown that the signals were continuously integrated locally at the synapse level, so that large,
stable and symmetrical synapses were formed when activating signals dominated, whereas asymmetrical, migratory synapses were formed when inhibitory signals dominated.\textsuperscript{71}

Indeed, NK cells contact with DCs as well as target cells\textsuperscript{72,73} and the dynamics of such interactions were recently reviewed.\textsuperscript{74} Based on data from \textit{in vivo} imaging investigations, the authors described several important features of NK cell interactions:

1. In secondary lymphoid organs at steady state, NK cells are highly motile and establish successive short contacts of 1-3 minutes with DCs and stromal cells. It is suggested that in this process, NK cells are tuning their reactivity based on the presence of inhibitory and stimulatory ligands.

2. During priming, still in secondary lymphoid organs, NK cells remain motile and establish short contacts. In this way the cells are able to sense and integrate cytokine signals presented by several DCs.

3. Most of NK cell contacts with target cells are also short, lasting about 5-10 minutes. NK cells also remain motile during contact with target cells, which led the authors to suggest that the signals obtained from several contacts may be summed up in NK cells as part of their priming - or in target cells towards lysis. In a third scenario, only the few longer lasting cell contacts account for the killing.

**NK cell cytotoxicity**

A large prospective study suggested an association between increased cancer risk and low natural cytotoxicity in peripheral blood.\textsuperscript{75} The paradigm of applying IL-2 activated NK cells for targeted cytolysis is more than thirty years old,\textsuperscript{76} and has been applied both \textit{in vitro} and \textit{in vivo}.\textsuperscript{77-79}

NK cells share a common mechanism to target cell killing with cytotoxic T lymphocytes, relying on the secretion of granules containing cytolytic mediators such as perforin and granzymes.\textsuperscript{80} As one may expect, CD56\textsuperscript{dim} NK cells were shown to exert stronger spontaneous cytotoxicity against K562 erythroleukemic cells compared to CD56\textsuperscript{+} cells upon stimulation with IL-2 and/or IL-21.\textsuperscript{31,81} There were no differences in the expression of granzymes A or K, nor of perforin, but activated cells, particularly the CD56\textsuperscript{dim}, were better at forming conjugates with K562 tumor cells. Though conjugates are dependent on interaction of adhesion molecules and their ligands, the expression of CD11a/CD18 (Lymphocyte function-associated antigen 1, LFA-1), CD2 or CD58, all known to be
involved in conjugate formation, was not altered. It was therefore suggested that conformational changes in LFA-1 may cause an increased conjugate-forming ability.82,83 Also, observed up-regulation of CD69 on CD56dim NK cells might contribute to the target binding responsible for the observed increase in cytotoxicity.85

Exocytosis of granules containing proteins is the major mechanism used by NK cells for killing.86 The granules were characterized as a subtype of lysosomes, and contain proteins such as perforin and granzymes (Grz).87 The cells recognized by NK cells in many instances already have evaded mechanisms for apoptotic death. Still, NK cell-mediated death is rapid and powerful, largely due to their use of plural proteins engaging different ‘death pathways’. Through membrane pores formed by perforin, Grz may enter the target cell and evoke a variety of different pathways leading to cell death.88

Perforin is vital for NK- and T cell-mediated cytolysis,89 to the degree that loss of its function leads to devastating immunoregulatory disorders.90 Granzymes on the other hand come in many forms, and humans express five different subtypes; Grz A, B, H, K and M. Of significance, they are proteases, which activate caspases in order to initiate apoptosis of target cells, and they depend on perforin.89 In contrast to perforin, no congenital Grz deficiency has yet been identified, probably because loss of one single Grz would not be detrimental. Still, the combined deficiency of both Grz A and B rendered mice susceptible to viral infection similar to those deficient in perforin, highlighting the importance of these two granzymes.92

Though generally considered cytotoxic without prior activation, freshly isolated NK cells isolated from mice kept in a pathogen-free animal environment did not express perforin or Grz B, while Grz A was detected at a low level.93 In this study, the Grz and perforin genes were transcribed, but translation was regulated by activation. Frequencies of perforin- and Grz B-expressing NK cells peaked 2-4 days post infection, accompanying efficient viral clearance.93

The view that perforin penetrates the cellular membrane in order to allow access of granzymes has been debated over the last years. The classical view is that upon release of the constituents of the NK cell granules, perforin forms pores in the target cell through which Grz flow into the cytosol, and this view recently received support.94 Still, some observations support an alternative mechanism for the delivery of both granzymes and
perforin through endocytic vesicles into the target cells after which perforin lyses the vesicle from the inside.\textsuperscript{95}

- Granzymes and perforin are translocated into the cytosol of the target cells bound to the proteoglycan serglycin without producing detectable membrane pores.\textsuperscript{96}

- At least partly, perforin enters the cytosol due to its importance in disrupting endosomes.\textsuperscript{97}

- Granzymes accumulate within the nucleus of attacked cells by receptor-mediated endocytosis.\textsuperscript{98}

Other mechanisms for directed cytotoxicity by NK cells also exist. TRAIL, the TNF Related Apoptosis Inducing Ligand, is found in five different forms. Its expression on NK cells can be induced by IFN $\alpha$, $\beta$ and $\gamma$,\textsuperscript{99} thus increasing the cytotoxic capacity of NK cells.\textsuperscript{100} Activated NK cells also express the death receptor Fas ligand, which is used to kill cells expressing Fas. Intriguingly, by secreting IFN-$\gamma$, NK cells induced the expression of Fas on cancer cells after which they kill them by Fas ligand.\textsuperscript{101}

**Role of NK cells in cancer immunology**

As NK cells are proven to be highly efficient killers of various cancer cells *in vitro*, the next question is how the conditions in various *in vivo* settings affect this activity. This issue was recently reviewed from a clinical perspective, which led to the conclusion that in order to overcome the multiple inhibitory factors present in the tumor microenvironment, focus needs to be put on the different available activating cytokines for NK cell stimulation, as well as on the impact of drugs on NK cell functions.\textsuperscript{102} NK cells generally are not found in tumor growth sites, indicating that they do not home efficiently to malignant tissues, but when they do, a positive prognostic sign is achieved.\textsuperscript{103-105} In the clinic, NK cells are attractive alternatives to cytotoxic T cells in cellular anti-tumor therapy for several reasons. For one, there is no need for the selection and expansion of tumor-specific clones, and NK cells may be used as allogeneic transplants without risking graft versus host disease.\textsuperscript{106;107}

The homing of NK cells to tumor sites seems to be closely related to their activation stage, as a continuous rise in the numbers of NK cells within a tumor occurred up to 48 hours after the injection of IL-2.\textsuperscript{108} Activated NK cells are retained in lung tissue within minutes after injection, but are rapidly cleared. In lung tumors, however, they accumulate over time, leading to a more than 15 times increase in the concentration of activated NK cells
after 24 hours. Many of these cells extra-vasated and established membrane-contact with the malignant cells at tumor sites. How they reach the tumor is not completely understood, but it is believed that they partially arrive via the tumor microvasculature, and partially migrate from surrounding venules. Only few IL-2–activated NK cells were found in the circulation a short time after injection, and low numbers reached tumors downstream from the lungs, which may be related to the rigidity of the cells. The same is true for IL-2- and phytohaemagglutinin (PHA)-stimulated CD8+ T cells. Hence, stimulated lymphocytes may more easily be retained in the microvasculature.

Variations in the chemokine receptor repertoire of NK cells attract specific subsets to different tissues. Since treatment with IL-2 changes the chemokine repertoire of NK cells, they may be functionally redirected upon inflammation. Further, endothelial cells activated by pro-inflammatory cytokines express membrane-bound fractalkine to which the widely expressed NK cell receptor CX3CR1 binds. NK cells can therefore be attracted and activated locally upon inflammation. Recent observations documented NK cell localization within the stroma of colorectal carcinoma tissue, although not in contact with the cancer cells. This occurred despite high levels of the relevant cytokines and chemokines, and the suppression of melanoma by the chemoattractant chemerin suggested that chemokines alone may not be sufficient to recruit immune cells to the tumor cells.

In metastatic tissues of untreated patients, tumor-infiltrating NK cells were either not detectable or present in very small numbers. However, they were recruited to the tissues around and even inside the tumor nodules when treated with the cytokines IFN-α and IL-2. In the early nineties, clinical investigations were undertaken using activated NK cells. They could not prove such treatment to be superior to treatment by IL-2 alone or in combination with interferons; thus it was considered inefficient. Two important reasons for the lack of success of treatment by IL-2 or activated NK cells were the severe adverse effects of IL-2 and the failure of endogenous as well as adoptively transferred cells in reaching the sites of tumor growth.

The literature of clinical research on NK cells is dominated by investigations into the correlations between genetic alterations, their related NK cell functional defect and disease or correlations between NK cell numbers, phenotype, functions or distribution in pathophysiology. Recently, however, deeper understanding of the biology of the cells has
paved the road for directed therapies. These include the delivery of cytokines such as IL-2, IL-12, IL-15, IL-18, IL-21 and IFN-α in various combinations or in combination with antibodies or vaccines in vivo\textsuperscript{124,125} or injection of NK cells preactivated by such cytokines.\textsuperscript{126}

While hopes were high for the employment of NK cells in combating for instance autoimmunity, graft versus host disease, complications of pregnancy and viral infections, only some perspectives regarding the potential for cancer therapy will be discussed here.

Reviewing the most promising strategies for NK cell directed therapies in cancer, Terme et al concluded that ‘manipulating the balance between inhibitory and activating NK receptor signals, the sensitivity of target cells to NK cell-mediated apoptosis, and NK cell cross-talk with dendritic cells might hold therapeutic promise’.\textsuperscript{125} Efforts to modulate NK cell trafficking into inflamed tissues and/or lymph nodes, and to counteract NK cell suppressors, might also prove fruitful in the clinic. The authors described four strategies for the use of NK cell directed therapies in cancer:

1. Adoptive transfer of alloreactive NK cells.
2. Blocking of inhibitory NK signals.
3. Promotion of TRAIL expression.
4. Activation of ADCC towards tumors otherwise refractory to NK lysis.

Further, they identified three principle ways of enhancing these mechanisms, namely promotion of NK cell proliferation, inhibition of NK cell suppressors and finally vaccines that induce both NK and T cell activation and promote NK-mediated T cell polarization.

Each of the four strategies have been explored:

1. Transfer of alloreactive NK cells: While transfer of activated NK cells was not superior to treatment with IL-2 alone\textsuperscript{122}, four out of the five patients who received haploidentical infusions with NK cells, which showed KIR-ligand mismatch in the graft-versus-host direction, achieved complete remission from acute myeloid leukemia.\textsuperscript{127} This study suggested that several factors were crucial for the success of NK cell transfer, such as careful selection of donors (not autologous, in order to get a KIR - mismatch), identification and separation of the right subset, and the regimen for conditioning of the cells pre- and post-infusion.
2. Blocking of inhibitory NK signals: In line with the above mentioned investigation, which was based on observed inefficiency of autologous NK cell-transplantation due to lack of KIR-mismatch, blocking of KIR as an example of NK inhibitory receptors was recently done. In a KIR transgenic murine model of lymphoma, blocking of NK cell KIR receptors with antibodies augmented spontaneous NK cell cytotoxicity, and cytotoxicity was further enhanced by the combination of antibodies towards KIR and CD20 (Rituximab).128

3. Promotion of TRAIL expression: The TRAIL system complements other NK cell systems for the identification of target cells enabling circumvention of the KIR-tumor MHC class I interaction, and numerous agents such as IL-2 or IL-15,129 IFN-γ100 and IL-12130 enhance the expression of TRAIL in NK cells. Importantly, this mechanism worked in co-operation with Rituximab, the mAb towards CD20, to induce apoptosis in cancer cells.131 Furthermore, TRAIL worked to circumvent acquired resistance to Rituximab in the cancer cells in this study, providing a rationale for combination therapies.

4. Activation of ADCC: While other activating NK cell receptors require co-engagement in order to activate cytolysis, ADCC activation was initiated by activation of CD16 only.132 Seidel et al133 reviewed this topic, emphasizing that while a substantial role of ADCC mediated by NK cells has been demonstrated in vitro and in mouse tumor models, direct in vivo effects in human remains to be shown. Indeed, as mentioned above, antibodies such as Rituximab engaged NK cells. ADCC capacity was related to favorable outcome in a trial where patients received Trastuzumab, a Her2/neu-specific antibody.134 The study thus challenged the current practice of prescribing such therapies solely on the basis of clinical features. That is, ADCC as well as clinical outcome was related to Fc gamma receptor IIIa polymorphism, with the genotypes V/V and H/H predicting favorable clinical outcomes. Indeed, conflicting results regarding the relevance of such polymorphisms have been obtained,133 emphasizing the importance of taking the relevance of polymorphisms into account when evaluating the efficacy of such therapies.

In order to potentiate these mentioned therapeutic modalities and others, a number of factors that influence NK cell functions need to be taken into consideration. Some factors contributing to the priming and stimulation have been mentioned, and many more, for the most part inhibitory, are found in the tumor microenvironment.
A plethora of specific mediators that inhibit cellular functions of NK cells are secreted by cells in the tumor microenvironment, many of which at the same time facilitate tumor growth, angiogenesis, extracellular matrix degradation, metastasis and more. Kveberg et al. previously reported that resting as well as IL-2-activated NK cells express receptors for Sphingosine 1-phosphate (S1P), and that they move chemotactically toward the concentration gradients of S1P. Recently, it was also shown that S1P mediated inhibition of NK cell lysis of human melanoma cell line Hs294T and the Burkitt’s lymphoma cell line RAJI. Taken together, S1P acts on the one hand as a chemoattractant for NK cells, while at the other hand it inhibits their cytolytic activity. The impact of S1P on NK cell function was one of the topics for this work.
Monocytes and Dendritic Cells

Monocytes represent 5-10% of the blood leukocytes in mice and men. Blood monocytes are typically divided into two subsets based upon the presence of CD14, a co-receptor for the lipopolysaccharide receptor, and CD16, also known as FcγRIII or CD32. The CD14<sup>hi</sup>CD16<sup>lo</sup> cells count for some 85-95% of the cells in healthy humans, the remainder being CD14<sup>lo</sup>CD16<sup>hi</sup>.<sup>137</sup> Monocytes circulate in the blood and bone marrow during homeostasis<sup>138</sup> and migrate from blood to lymphoid or nonlymphoid tissues in response to signals caused by infection or tissue damage, upon which they differentiate into various cells such as DCs and macrophages.<sup>139-141</sup>

As an example of the many subsets of monocytes and their importance in health and disease, CD14<sup>hi</sup>CD16<sup>hi</sup> cells were more prevalent in patients with coronary artery disease than healthy donors.<sup>142</sup> Indeed, although not the very first step in the process, a prerequisite for the development of atherosclerosis is the adhesion of monocytes to the endothelial wall of the artery which is induced by only a few stimuli, including oxidized LDL.<sup>143</sup> The tight relation between monocytes and cardiovascular disease was realized, as these cells were shown to be the main cellular components of atherosclerotic lesions<sup>144</sup>, present already at the early stage of lipid accumulation in “fatty streaks” in the artery wall.<sup>145</sup> Evidence of their importance in the development of this disease came from investigations showing that depletion of monocytes from the circulation reduced plaque formation<sup>146</sup>, and that subsets among monocytes were critical in the development of atherosclerotic lesions.<sup>147</sup> Clues to the contribution of bioactive lipids in the process of monocyte recruitment and regulation came from studying ApoE<sup>−/−</sup> hypercholesterolemic mice.<sup>148</sup> In addition, there were no changes in the numbers of Gr1<sup>−</sup>Ly6<sup>lo</sup> (corresponds to the human CD14<sup>lo</sup>CD16<sup>hi</sup>) while Gr1<sup>+</sup>Ly6C<sup>hi</sup> (CD14<sup>hi</sup>CD16<sup>lo</sup>) were abundant and adhered to activated endothelium.

Dendritic cells (DCs) were first observed by Langerhans in 1868,<sup>149</sup> and their importance is reflected in that the Nobel Prize in Physiology or Medicine in 2011 was awarded to Ralph M. Steinman “for his discovery of the dendritic cell and their role in adaptive immunity”. In 1978 he published the paper 'Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice',<sup>150</sup> establishing the importance of DCs in launching an immune response. Later, DCs have been found to be present in nearly any tissue, continuously collecting and processing antigens.
There are multiple DC subsets, differing in phenotype, function, activation state and location. However, their relationship and developmental origins is a matter of debate.\textsuperscript{151-153}

In addition to the importance of various transcription factors, which affect lineage choice and DC development, important functions are attributed to an increasing number of compounds capable of driving DC development and differentiation. The wide variety of cytokines/growth factors and conditions that influence DC differentiation and activity further highlights the extreme heterogeneity of DC populations, reflecting the diverse phenotypes, functions and ontogeny displayed by these cells.

While constantly tolerizing the system for self-antigens and simultaneously prompting their own maturation once DCs encounter a potential pathogen, they are the front line as sentinels of the immune system. They are the link between the two main branches of immunity, namely innate and adaptive immunity, orchestrating the response by manipulating all co-players in the immune system.

Their multiple, central tasks in immunity, as summarized by Banchereau and Steinman\textsuperscript{154}:

- **Sentinels in vivo**: In situ distribution to optimize antigen capture and migration into lymphoid organs to optimize clonal selection of rare CD4+ and CD8+ T cells.
- **Initiators of immune responses**: Stimulation of quiescent, naïve and memory B and T lymphocytes.
- **Potency in stimulating T cells**: Capacity of small numbers of DCs and low levels of antigen to induce strong T cell responses.
- **Inducers of tolerance**: Deletion of self-reactive thymocytes and anergy of mature T cells.

DCs are found on virtually any figure drawn to explain ‘how the immune system works’; in any disease and at all anatomical or pathophysiological scenes. They are just as essential to the innate as to the adaptive immune defense, whether it be in response to cancer, auto-inflammation or microbiological agents. It is obvious that for a cell to be able to stand up to all these challenges, it needs to have an immense developmental potential. The relevance of autocrine cytokine production on DC development and functions has been poorly investigated.\textsuperscript{155} Factors released early after innate immunity cell interaction with pathogens represent good candidates as potential natural mediators of differentiation/maturation of DCs in vivo. Granulocyte/macrophage colony stimulating factor (GM-CSF), along with IL-4 or a type I IFN, have become the standard regimen for
stimulating dendritic cells in vitro from human peripheral blood monocytes by a single step procedure. IL-4 and GM-CSF and LPS induces the maturation of immature DCs (iDCs) into mature DCs (mDCs), which express molecules enhancing their functions as antigen capturers and selective receptors that would guide them to and from several sites in the body.

On the other hand, the generation of iDCs using pro-inflammatory cytokines to drive their differentiation from monocytes represents a paradox, arguing against their presence in physiological conditions. Still, several subtypes of DCs are present in normal mice, suggesting the existence of other factors capable of inducing their differentiation under physiological conditions.

As dendritic cells evolve from monocytes and become iDCs, they are able to capture antigens but unable of presenting them to T cells. This is due to relatively low levels of surface MHC class I and II as well as co-stimulatory molecules such as CD86. MHC class II molecules are synthesized, but diverted to endosomes and lysosomes. There, they may be loaded with antigens that are presented to T cells after activation. However, upon binding of pathogen material, maturation of DCs is correlated with phenotypic changes. They become extremely efficient in presenting peptides to T cells due to redistribution of MHC class II from intracellular compartments to the plasma membrane, parallel with up-regulation of co-stimulatory molecules CD80 and CD86 as well as T cell adhesion molecules such as CD48 and CD58.

The maturation of monocytes into dendritic cells is also reflected as sequential changes in the expression pattern of chemokine receptors. Maturation of monocytes into DCs was associated with up-regulation of CCR7, which directs the cells to secondary lymphoid organs. CCR6-positive monocyte-derived DCs were crucial for priming of CD8+ cytotoxic T lymphocytes.

When DCs express HLA class I molecules, they were spared from lysis by NK cells, whereas immature DCs were not, but became resistant upon maturation and expression of HLA I. The killing of iDCs was confined to NK cells lacking inhibitory KIRs for self HLA-class I alleles and expressing the HLA-E specific CD94-NKG2A inhibitory receptor. It was thus suggested that the reason may be that CD94-NKG2A, contrary to KIRs, were able to sense the sharp decrease in HLA-E levels in the iDC population.
contributing to the proposed “DC editing” by NK cells which is thought to remove the DCs that fail to mediate optimal antigen presentation and T cell priming.

Despite the general opinion that apoptotic or necrotic material engulfed and presented by DCs is generated by direct killing of tumor cells by NK cells and cytotoxic T lymphocytes, recent investigations pointed out several ways in which DCs themselves are capable of killing tumor cells, in addition to processing and presentation of tumor associated antigens to the other immune cells. While highly controversial, this role of different DC subsets in immune surveillance of cancers suggests that DCs may be able to deliver death signals.

Cross-talk among NK cells and DCs

Fifteen years ago, it was discovered in mice that not only T cells but also NK cells are stimulated through direct contact with activated DCs. Interactions between these subsets of immune cells are of great importance in health and diseases. NK cells induce the maturation of DCs by soluble mediators and direct cell-cell contact, hence bypassing the requirement of these cells to endocytosis of microbial material. DCs, on the other hand, induce IFN-γ production and the proliferation and cytotoxic potential of NK cells. Thus, NK cells may indirectly induce the antitumor T-cell response by maturing DCs, and DCs induce NK cell-mediated antiviral responses when they are activated through Toll-like receptors (TLRs). A number of studies have highlighted the importance of such interaction in the regulation of DC maturation as well as in NK cell activation and the capacity of both cell types in terms of immune regulation.

It was shown that transfer of DCs to mice with MHC class I negative tumors promoted NK cell dependent antitumor effects. Also, this work showed that resting NK cells in coculture with DCs increased their cytolytic activity and IFN-γ production in a cytokine as well as cell-cell contact dependent manner. Clinical investigations further showed that treatment of patients with DCs generated by Flt3-L, which induced DC expansion and the DC-mediated NK cell antitumor effect, was associated with prolonged survival. With regards to autoimmunity, DC-mediated recruitment of NK cells into the lymph nodes provided early IFN-γ secretion, which induced a strong DC-mediated IL-12 production, thus promoting the auto-immunogenic Th1 immune responses.

The importance of direct cell-cell contact was established already in the early studies of NK cell – DC crosstalk. These stimulatory synapses involved polarized secretion of IL-
12\textsuperscript{171} and IL-18\textsuperscript{172} from preassembled stores. Thus it has been proposed that abundance of cell-cell contact for the \textit{in vitro} activation of NK cells by DCs reflects:

1. Implication of membrane bound receptor/ligand pairs, and/or

2. Necessity for local delivery of cytokines in high concentrations.\textsuperscript{168}

Several cytokines known to trigger NK cell functions were secreted from DCs when they were stimulated by pathogens or other TLR ligands.\textsuperscript{173} For example, IL-12 derived from DCs induced IFN-\(\gamma\) production in NK cells in different \textit{in vitro} systems in both man\textsuperscript{171} and mouse.\textsuperscript{174} Hence, it has been proposed that NK cell-DC interaction in response to virus-induced tissue damage could be responsible for the rapid initiation of IFN-\(\gamma\) based antiviral responses.\textsuperscript{18} The cytotoxic potential of NK cells may also be enhanced by IL-12 when it is secreted together with IL-18 from DCs.\textsuperscript{175} A strictly regulatory subset termed NK helper cells was also induced by IL-18 stimulation.\textsuperscript{41} These cells, which displayed high migratory responsiveness towards lymph node associated chemokines, produced substantial amounts of IFN-\(\gamma\) when stimulated by DCs or T helper cells. Further, DCs from IL-12- and IL-18-deficient mice have been shown to induce IFN-\(\gamma\) secretion from NK cells through IL-2 – secretion and cell-cell contact.\textsuperscript{176} IL-15 secreted by IL-15R\(\alpha\)-positive DCs induced NK cell proliferation,\textsuperscript{177} and type I-Interferons IFN-\(\alpha\) or IFN-\(\beta\) from DCs induced NK cytotoxicity in both mouse\textsuperscript{174} and man.\textsuperscript{178}

NK cells also trigger maturation and activation of DCs. Cytokine release by DCs was induced in a manner dependent on soluble factors such as TNF-\(\alpha\) and IFN-\(\gamma\) as well as cell – cell contact, when immature, monocyte – derived DCs were cultivated at a low NK/DC ratio (1:5) with activated NK cells.\textsuperscript{179} NK cell produced IFN-\(\gamma\) can also assist in the polarization of Th1 responses by DCs.\textsuperscript{170} Also, IL-2-activated NK cells induced maturation of blood plasmacytoid DCs (pDCs) and myeloid DCs as well as synergized with concentrations of oligonucleotides which alone were suboptimal to promote strong IFN-\(\alpha\) and TNF-\(\alpha\) production by blood pDCs in a cell-cell contact dependent manner.\textsuperscript{178} This maturation depended on the expression of the natural cytotoxicity receptor NKp30 by NK cells, and was opposed by KIR and NKG2A inhibitory receptors.\textsuperscript{180} In mouse, triggering of TREM2 (Triggering Receptor Expressed on Myeloid cells 2)-signaling in GM-CSF/IL-4– derived bone marrow DCs by IL-2-activated NK cells resulted in up-regulation of the maturation marker CD86. IL-18-activated NK cells up-regulated CCR7, the homing
marker for secondary lymphoid tissue, and stimulated CCR7-expression and production of IL-12 from DCs.\textsuperscript{41,181}

The ability of NK cells to induce DC maturation in synergy with suboptimal levels of microbial signals, with subsequent induction of T-cell responses as a result, might prove important for understanding immunity in situations where inflammation is poor and NK-cell activation occurs as a result of direct target cell recognition, as is the situation with many tumors. The NK-DC interactions might function as an important control switch for amplifying or attenuating innate immune responses. They act at different stages of innate and adaptive immune responses, indicating that they have a role in controlling the links between innate and adaptive immunity.\textsuperscript{37} As DCs express receptors for S1P, they were included in the investigation into the impact of this lipid on innate immunity.
Inflammatory lipids in innate immunity

Complementing the multitude of cell–specific receptors for the recognition of pathogens or changed cells and cell–cell interaction, immune cells possess several receptors for soluble factors that impose important regulatory features on their function. Among these are receptors for eicosanoids, interleukins, chemokines, lysosphospholipids and many more. Features regulated by these receptors include secretion of inflammatory mediators, chemotaxis, cytotoxicity, proliferation and expression of receptors – all important for immunological function.

Sphingosine 1-phosphate (S1P)

Lysosphospholipids play essential and important roles in various cell types, and they are divided into lysoglycerophospholipids and lysosphingophospholipids. Members of the lysosphospholipids (LPLs) bind to heptahelical receptors, which are coupled to heterotrimeric G proteins. The most extensively studied are the glycerophospholipid Lysosphatidic Acid (LPA), and the sphingophospholipid Sphingosine 1-phosphate (S1P). Both LPA and S1P are secreted by platelets among other cells, and are abundant in serum and plasma. They perform multiple biological activities, and sometimes have opposite effects on the same cell type, which include neural, endothelial, hepatic and cardiovascular as well as tumor cells such as ovarian cancer or glioma cells and leukocytes.

Figure 4. Adapted from Rolin and Maghazachi. Overview of the multiple activities performed upon ligation between S1P and its receptors.
The biology of S1P has been extensively reviewed.\textsuperscript{192} It is a multifunctional lipid present in high concentrations up to the micro-molar range in serum, and it regulates many cell responses such as cell proliferation and apoptosis, cell differentiation and migration as well as immunological responses.\textsuperscript{185,193,194} S1P is generated from sphingolipids, which are essential plasma membrane lipids concentrated in liquid-ordered domains, commonly known as lipid rafts.\textsuperscript{192} Sphingolipids can be rapidly metabolized following stimulation of various plasma membrane receptors through the activation of an enzymatic cascade:

![Sphingomyelin cycle diagram](image)

**Figure 5.** Modified from Rolin and Maghazachi.\textsuperscript{191} Generation (on the right, in boxes) and reverse acting enzymes (on the left, without boxes) in the metabolism of S1P.

This pathway has been denoted as the sphingomyelin cycle, due to the fact that for all steps reverse reactions may take place, catalyzed by specific enzymes. Concerning the Sphingosine kinases (SphK), two subtypes (SphK1 and SphK2) have been described.\textsuperscript{195} The \textit{in vivo} differences between the effects of S1P produced by the two kinases are not clear as of yet, but they seem to have largely opposing effects.\textsuperscript{196} While SphK1 resides predominantly in the cytosol and can be translocated to the plasma membrane \textsuperscript{197}, SphK2 is thought to be localized in the ER, nucleus and mitochondria.\textsuperscript{198} Their regulation is largely unknown.

Even though S1P is synthesized in most cells, the levels of this metabolite in tissues, including lymphoid tissues, are small due to irreversible degradation by intracellular S1P lyase or dephosphorylation by S1P phosphatases.\textsuperscript{199-202} The exceptions will be blood, with low – micromolar range levels of S1P mainly originating from erythrocytes, and lymph,
where S1P levels are in the hundred – nanomolar range. Serum – protein partners might have a role in determining the uptake and intracellular degradation of S1P, as free S1P and S1P bound to serum albumin was more susceptible to degradation than was S1P bound to lipoproteins such as high-density lipoprotein (HDL). Also, inhibition of S1P-lyase activity resulted in an increase in the level of S1P in tissues, thus ablating the concentration gradient between blood and tissues.

S1P in the lymph is not derived from erythrocytes or other hematopoietic cells, but originates from a source resistant to radiation, probably the endothelium. It is reasonable to believe that the lymph S1P as well as the plasma S1P is regulated by the endothelium, as the physiological stimulus shear stress increased secretion of the lipid from these cells, whereas neither platelets nor mast cells seemed to play any role. Of note, while knock out both the SphK genes produced mice deficient in circulating S1P, loss of either of the isoforms altered the levels of circulating S1P without ablating the gradient between blood and tissues, suggesting their equal importance. In addition, two distant sources such as the endothelium and red blood cells, ‘inside-out’ signaling, in which S1P was released from a cell and able to act in an autocrine or paracrine manner, has been described. Such signaling is typically induced by production of S1P after Sphk had been activated in response to signaling molecules such as growth factors, cytokines or even S1P.

Plural mechanisms have been shown for the secretion of S1P. Still, the mechanism for the constitutive secretion of S1P from erythrocytes is unknown. Erythrocytes isolated from plasma or serum did not secrete S1P, indicating that one or more factors present in plasma might be required for S1P secretion from these cells. All together, this has led to speculation that involvement of any of the transport mechanisms might depend on its expression level in a cell, its localization relative to the site of S1P biosynthesis or whether a particular stimulus that leads to S1P biosynthesis may induce selective transporter activity.

The actions of S1P may be exerted via two different mechanisms, via the G protein coupled S1P receptors in the cell membrane or via recently described intracellular modes of action. Intracellularly, SphK1-derived S1P was necessary for TRAF2 ER ubiquitin ligase activity, which was necessary for mediation of TNF–effects. Further, S1P generated by SphK2 in the nucleus regulated epigenetic-mediated gene expression via its
inhibition of histone deacetylaces. The membrane bound receptors were the most important for S1P effects, however, and when they were cloned, they were first linked to the differentiation of endothelial cells. They were therefore named EDG (Endothelial Differentiation Gene) but renamed once it was realized that the ligand for the receptor family was S1P. Thus, they are now known as Edg-1/S1P1, Edg-5/S1P2, Edg-3/S1P3, Edg-6/S1P4, and Edg-8/S1P5. These receptors are expressed in different patterns throughout the immune system.

Kveberg reported that resting as well as IL-2-activated NK cells expressed several receptors for S1P (Table 3), corroborated with the ability of NK cells to move chemotactically towards the concentration gradients of these lipids. DCs expressed all five receptors for S1P, and human NK cells expressed mRNA for S1P1, S1P4 and S1P5, inconsistently for S1P2 but not at all for S1P3. All of these receptors may signal through G-protein coupled receptors, but differ in downstream effects.

The main function of many of the receptors was migration, and migration of NK cells toward a gradient of S1P relied on involvement of S1P5. Indeed, as NK cells mature, a switch from CXCR4 to S1P5 promoted NK cell egress from the bone marrow. S1P1 was required at a later stage for NK cell egress from the thymus and the peripheral lymphoid organs. S1P5 was required for NK cell homing in the steady state as well as for entering into inflamed tissues.

Murine mDCs, but not iDCs, migrated towards S1P in a pattern correlated with up-regulation of S1P1 and S1P3 during maturation. The S1P receptor agonist FTY720 did not trigger migration of DCs, leading to the proposition that part of the immune modulation accomplished by FTY720 may be caused by impaired DC migration.

Whereas low concentrations of S1P promoted chemotaxis in a S1P1-dependent manner, high concentrations seemed to be inhibitory. This inhibitory effect might partly be due to the down-regulation of S1P1 by high concentrations of S1P, a mechanism that could prove relevant in vivo during the transit of cells in blood, where the concentration of S1P

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<th>S1P receptor expression</th>
<th>NK cells</th>
<th>iDC</th>
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<tr>
<td>S1P1</td>
<td>+(^{135})</td>
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<tr>
<td>S1P2</td>
<td>-(^{135})</td>
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was high. In some immune cells, this concentration dependence was less evident, and in some cases the chemotactic response was associated with a particular stage of cell differentiation or cell activation, leading to changes in receptor expression. This was the case for DCs, as they mainly expressed S1P1 in the immature state but upon maturation up-regulated S1P3, which may mediate a chemotactic response to S1P.  

Two models might explain the regulation of immune cell trafficking by S1P, either an immune cell – or an endothelial cell – centered model. The immune cell–centered model claims that expression of S1P1 enables sensing of a gradient of S1P from lymph nodes towards blood, attracting cells out of lymph nodes while overriding CC-chemokine receptor 7 (CCR7) – mediated retention. The endothelium-centered model suggests that lymphocyte egress proceeds constitutively from lymphoid tissues under physiological S1P concentrations but is blocked by agonism of S1P1 on endothelial cells. This is consistent with the induced block of lymphocyte egress by S1P1 agonists such as FTY720, and the reported failure of S1P1 antagonism to induce an egress block. Support was given to the lymphocyte – centered model as it was shown that lack of S1P rendered mouse lymphocytes unable to egress into blood and lymph.

Still another important feature of S1P impact on the immune system is the differential cellular effects imposed on the different kinds of cells. Dendritic cells were subject to phenotypic programming when exposed to S1P, and the same was true for priming T – cells.

S1P is a target of research in autoimmune diseases and cancer, due to discovering of enhanced SphK1 mRNA expression in solid tumors compared to normal tissues, after the initial findings of overexpression of SphK1 in fibroblasts capable of developing into
tumors. Autocrine and paracrine actions of S1P secreted from cancer cells recently received attention, indicating multiple ways in which the lipid promotes cancer progression.

**FTY720**

FTY720, (fingolimod; Gilenya; 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride) is an immunosuppressive drug derived from myriocin, a fungal metabolite that resembles sphingosine and has high affinity for its receptors. It has been suggested that FTY720 is a prodrug, becoming active as an S1P receptor agonist after phosphorylation by the sphingosine kinases, especially SphK2, although no discriminatory effect has been observed between administration of the two forms. FTY720 binds to four of the five S1P receptors, namely S1P₁, S1P₃, S1P₄ and S1P₅, among them particularly S1P₁. Upon binding to S1P₁ FTY720 induced its internalization, hence inhibiting S1P activity. Although its mechanism of action through receptor binding is known, the effect of FTY720 as an agonist or an antagonist for S1P receptors is much debated. For example, in contrast to S1P, FTY720 has not been reported to inhibit T cell proliferation, and although it reduced the recirculation of CD8⁺ cells, it did not affect other T cell functions. FTY720 proved powerful in suppressing allograft rejections during kidney transplantation due to its ability to arrest lymphocytes. The arrest was related to internalization of the S1P receptors, rendering the lymphocytes unresponsive to the obligatory S1P – signal to egress from lymphoid organs. Yet, it was eventually withdrawn from clinical trials due to occurrence of macula degeneration. The mechanism resulting in this side-effect is still highly unclear, but a functional relation between S1P and vascular endothelial growth factor A, which is believed to play a central part in development of neovascular diseases of the eye, has been proposed. Recently, FTY720 was approved by the American Food and Drug Administration under the name Gilenya, as the first orally administered drug for the treatment of multiple sclerosis and with close monitoring of the eyes and an acknowledged risk of fatal viral infections.

A theory of an inverse relationship between the extracellular levels of S1P and the expression of the receptor S1P₁ has been supported in vivo. In double SphK1/2 knockout...
mice, S1P₁ was strongly up-regulated due to the absence of S1P. Surprisingly, egress of T lymphocytes was inhibited in these double knockouts in much the same way as seen upon treatment by FTY720. Thus, up-regulation as well as down-regulation of S1P₁ resulted in T cell homing to the lymph nodes. It was also suggested that in addition to inhibiting the egress of lymphocytes from lymph nodes, FTY720 reduced naïve lymphocyte release from the thymus and inhibited S1P-induced migration of DCs. Administration of FTY720 to kidney transplant patients did not affect the number of NK cells isolated from these patients. And, in an animal transplant model, only high but not low doses of FTY720 prevented NK cell infiltration into allogeneic corneal grafts. NK cells isolated from FTY720 treated mice also showed unaltered chemotaxis toward S1P gradient in vitro.

**SEW2871**

Another drug known as SEW2871 “5-(4-Phenyl-5-trifluoremethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-(1,2,4)-oxadiazole”, structurally unrelated to S1P, has been described. This drug was found to be selective for S1P₁ and is used to dissect the functions of S1P₁ versus S1P₃, which is not affected by SEW2871. SEW2871 was found to modulate the migration of DCs in vivo and in vitro. Like S1P, SEW2871 activates S1P₁ causing its internalization and recycling, but is unable to down-regulate the receptor expression level. FTY720, on the other hand, in addition to inducing internalization of the receptor, triggered receptor degradation.

SEW2871 also induced lymphopenia 5-6 h after injection into mice. A study of explanted mouse lymph nodes showed that treatment with SEW2871 caused reduced motility of T cells especially in the medullary region of the lymph node and reduced transmigration across the lymphatic endothelium. The hypothesis was thus put forward, that the inhibition of transendothelial migration induced by SEW2871 and other immunosuppressants was caused not by desensitization and down-regulation of S1P₁, but by agonism. According to this theory SEW2871 acts on stromal gates that are constitutively open during lymphocyte trafficking due to weak or absent agonist signal, but close in response to signaling mediated by S1P₁ receptors on sinus endothelial cells. SEW2871 has been employed in vivo in mice when researchers pursued to assess the
contribution of S1P\textsubscript{1} in vascular pathophysiology. For instance in mouse ischemic kidney failure, SEW2871 inhibited lymphocyte egress leading to greatly improved renal function.\textsuperscript{258}
LPC and the HODEs

Hydroxyoctadecadienoic acid (HODE) is a lipid derived from oxidation of linoleic acid (L.A). Linoleic acid is an essential, polyunsaturated fatty acid, which is abundantly found in vegetable oils, comprising more than half the weight of corn– and sunflower oils. In man, it is found in cell membranes, and it undergoes extensive metabolism leading amongst other lipids to the generation of arachidonic acid and, thus, some of the prostaglandins. The human body cannot produce linoleate, and a diet deficient in this lipid caused hair loss, mild skin scaling and poor wound healing in rat. Several isomers of HODE were formed from linoleate by an enzymatic process by either 15-lipooxygenase or cyclooxygenase in which a mixture of the R- and S-enantiomers of 13-HODE and the isomer 9-HODE were formed.

Low density lipoprotein (LDL) is a lipoprotein produced in the liver. It transports lipids, most notably cholesterol, in the aqueous environment around cells. The core of the LDL particles is surrounded by phospholipids and cholesterol, and contains largely linoleate. The LDL particles measure approximately 20 nm, which is in the same range as the 26 nm pores of endothelium. This was believed to explain the correlation between LDL levels and cardiovascular disease. In a non-enzymatic process, HODE was produced from LDL upon lipid peroxidation, which led to the suggestion that the levels of HODEs in blood may be used as a determinant of in vivo hydrogen-donor activity of antioxidants.

The HODEs are vasoactive, due to the ability of 13-HODE, and to a lesser extent 9-HODE, to induce the mobilization of intracellular calcium in cultured porcine aortic and pulmonary artery smooth muscle cells. Another lipid containing a free hydroxyl group, i.e. lysophosphatidylcholine (LPC) also robustly increased intracellular calcium in vascular smooth muscle cells in this study. The biochemical relation between these compounds was highlighted by the observation that 13-HODE was taken up by endothelial cells and incorporated into phosphatidylcholine, whereas most of the phosphatidylcholine is converted into LPC by phospholipase A activity during oxidation.
Hyperlipidemia is an important risk factor for cardiovascular disease, as shown first in the Framingham study and published by Thomas et al. This was recently highlighted in the guidelines for treatment of blood cholesterol published by the American College for Cardiology and the American Heart Association. They found the risk reduction for atherosclerotic disease upon statin treatment as outweighing the risk of adverse effects for patients with a baseline LDL cholesterol of ≥ 70 mg/dl within certain criteria while a level below 200 mg/dl was generally accepted as healthy.

The identity of the receptors responsible for various effects of the oxidized lipids as well as LPC has been a matter of much debate. Our group previously reported that NK cells express G2A (G2 Accumulation) and that they responded chemotactically to LPC. For LPC as well as for the different isotypes of HODE, several receptors seem to be targeted. G2A as well as peroxisome proliferator activated receptor-γ (PPAR-γ) were both established as important.

The HODEs accumulated in atherosclerotic lesions, and they were the most abundant oxidative products. Through the course of lesion development, the lipids imposed differential effects on its progression. Enzymatically produced 13-HODE predominantly acts on PPAR-γ, leading to the clearance of debris and largely protective effects, whereas the non-enzymatic oxidation of linoleic acid generated a mixture of 13-HODE and 9-HODE, which had a high affinity for G2A. Also the pro-inflammatory effects of 9-HODE and to a lesser extent 13-HODE were believed to be predominantly harmful. Indeed, genetic studies support a role for G2A in the pathogenesis of atherosclerosis and a knockout model of PPAR-γ resulted in increased atherosclerosis.

Most investigations of the effects of oxidized lipids in atherosclerosis or on immune cells have been done using oxidized LDL particles, rather than the single lipids. Therefore, investigating the implications of different HODE isotypes on NK cells and monocytes has been one of the goals in this work.
Aims of the thesis

Through binding to specific receptors, a number of bioactive lipids influence the function of the human innate immune system. Although they stimulate chemotaxis, the overall effect seems to be immunosuppressive. S1P is established as a pro-survival compound in the tumor microenvironment. Moreover, through a magnitude of effects on innate immune cells, LDL-related and -derived lipids contribute to the exacerbation of atherosclerosis. The aim of this work was to decipher the effect of some inflammatory lipids on NK cells, monocytes and DCs and to try to suggest mechanisms for their implications in cancer and atherosclerosis.

NK cells express receptors for S1P, and were therefore attracted to S1P, and increased their killing efficiency upon stimulation by S1P. It was not known, however, if NK cells were phenotypically altered upon stimulation with S1P, or if the NK cell production of cytokines was altered. Further, it was not known if the observations of NK cell killing of cancer cells were strictly a cancer-related phenomenon or which S1P receptors were responsible for the increased cytotoxic capacity. DCs also expressed receptors for S1P. However, it was not known, how they were affected upon stimulation by the lipid or if the killing of DCs by NK cells was altered.

The aims of paper I was to answer these questions:

1. Does S1P influence the ability of NK cells to kill cancer cells or DCs?
2. Are the NK- and DC phenotypes altered by stimulation with S1P?
3. Does S1P influence NK- and DC production of cytokines?
4. Are all S1P receptors equally important in mediating these effects?

Oxidized lipids such as the different isotypes of HODE and LPC were abundant in atherosclerotic plaques. NK cells were shown to be attracted to atherosclerotic lesions and to enhance the development of the disease. It was not known, however, if NK cells were attracted to HODEs. Further, the impact of HODEs and LPC on key functions of NK cells, cytotoxicity and cytokine production, has not been investigated.

The aims of paper II was to answer these questions:

1. Do the different isotypes of HODE or LPC induce NK cell chemotaxis?
2. Is NK cell cytotoxicity or cytokine production altered after stimulation with HODE isotypes or LPC?
3. What kind of signaling pathways are stimulated in NK cells by the lipids? Do all of the lipids employ the same signaling pathway when stimulating NK cells?

Monocytes constituted the main cellular component of atherosclerotic lesions, and they were present throughout the development of lesions. In the plaques, monocytes co-localized with oxidized lipids, and reports indicated that the lipids alter the function and maturation of monocytes. Therefore, we wanted to investigate if the same oxidized lipids examined in the case of NK cells (Paper II), also affected monocytes.

The aims of paper III was to answer these questions:

1. Do the different isotypes of HODE or LPC induce monocyte chemotaxis?
2. Is monocyte production of cytokines affected by stimulation with the different isotypes of HODE or LPC?
3. Is the monocyte chemokine receptor repertoire changed after stimulation with the different isotypes of HODE or LPC?
Summary of papers

Paper I: “FTY720 and SEW2871 reverse the inhibitory effect of S1P on natural killer cell mediated lysis of K562 tumor cells and dendritic cells but not on cytokine release”, Cancer Immunology and Immunotherapy 2010.

NK cells, i- and mDCs, as well as K562 cells were treated with S1P and agonists for S1P receptors. Then, NK cells were incubated with DCs and cancer cells as targets for cytotoxicity. These results showed that S1P protected both the tumor cells and iDCs from cytolysis by IL-2-activated NK cells. This protection was partially reversed by the selective S1P receptor agonists SEW2871 and FTY720, indicating that S1P1 is important for this effect of S1P. Further, we examined different phenotypic markers known to be important for the interaction among these cell types, as well as the secretion of relevant cytokines. We observed that secretion of inflammatory cytokines IL-17α and IFN-γ from activated NK cells was reduced by S1P in an S1P1-independent way. However, the expression of NKG2D, NKp30, NKp44 or CD158 on the surface of NK cells was not altered. Similarly, the compounds did not alter the expression of activation markers CD80, CD83 or CD86, but expression of classical HLA I and HLA E, ligands for NK cell inhibitory receptors, was increased in DCs, an activity that was reversed by FTY720 and SEW2871. This led us to conclude that S1P1 on the target cells was involved in the S1P mediated decrease of NK cell cytotoxicity, while other S1P receptors on NK cells were involved in the regulation of NK cell cytokine secretion.


First, we wanted to see whether the lipids would evoke a chemotactic response in NK cells and whether pertussis toxin (PTX) affected this response. Our results showed that 9S-HODE, 9R-HODE, 13R-HODE as well as LPC but not +/−13-HODE potently induced chemotaxis of NK cells. Pretreatment of the cells with PTX almost completely abolished the chemotaxis to 9S- and 13R-HODE, and there was a more than 50% inhibition of chemotaxis to 9R-HODE and LPC. When we examined mobilization of calcium intracellularly, all lipids except 13R-HODE induced calcium flux at different concentrations of the lipids. In order to address the question of whether all lipids utilized the same receptor, we performed desensitization experiments in which the cells were stimulated with one lipid prior to the other. Addition of 9S-HODE prior to LPC inhibited
more than 50% of the effect of LPC, whereas LPC completely inhibited the effect of 9S-HODE. Between LPC and 9R-HODE, there was a complete reciprocal inhibition of the calcium flux.

We then repeated our cytotoxicity protocol and examined the effects of culture supernatants from NK cells exposed to LPC and oxidized lipids for NK cell release of cytokines such as IFN-γ. The results showed that there was no change in cytolysis of K562 cells by NK cells, but 9R-HODE induced an increase in the numbers of IFN-γ expressing NK cells as well as the amount of IFN-γ released by these cells. Surprisingly, while we observed a dose–related 9R-HODE-induced increase in the amount of secreted IFN-γ, all the other lipids reduced the amount of this cytokine in the supernatants. We concluded that all of the lipids activated one or more G protein-coupled receptors in NK cells, thus inducing their migration. While 9S-HODE and 13R-HODE activated PTX-sensitive G proteins only, 9R-HODE and LPC activated both PTX sensitive and insensitive G proteins. However, we were not able to explain why only 9R-HODE had the ability to change the number of NK cells producing IFN-γ and to increase the production of this cytokine.

*Paper III: “Oxidized lipids and lysophosphatidylcholine induce the chemotaxis, up-regulate the expression of CCR9 and CXCR4, and abrogate the release of IL-6 in human monocytes”, Toxins 2014.*

Here, we examined the ability of LPC and oxidized lipids to induce chemotaxis in monocytes. LPC, 9R-, 9S- and 13R-HODE induced the migration of primary human monocytes. We then examined the cells for changes in chemokine receptor expression upon incubation with the lipids in concentrations representative for the milieu inside atherosclerotic plaques.

After 4 hours incubation with 20µM of lipid, LPC was the only lipid which induced the expression of CCR9, while all of the lipids had this effect after 24 hours. 9R-HODE, 13R-HODE and LPC, but not 9S-HODE, induced increased CXCR4 receptor expression after 4 hours, while only 9R- and 13R-HODE had this effect after 24 hours. The increased receptor expression was related to increased migration towards the CCR9 – ligand TECK and the CXCR4 – ligand SDF-1α/CXCL12. LPC was the only lipid that induced increased migration towards TECK after four hours, whereas 9S-, 9R- and 13R-HODE, but not LPC, had this effect after 24 hours. 9R- and 13R-HODE and LPC, but not 9S-HODE, induced increased migration towards SDF-1α/CXCL12 after 4 and 24 hours. We then wanted to examine if any of the lipids or chemokines induced an increase in intracellular calcium.
levels in monocytes. Surprisingly, only LPC and SDF-1α exerted such an effect. However, all of the lipids decreased the secretion of IL-6 from the monocytes.

On the basis of these observations, we proposed that the lipids might attract monocytes to inflammatory sites such as atherosclerotic plaques, and exert immune-modulatory and anti-inflammatory effects through alteration of homing and inhibition of the release of the pro-inflammatory cytokine IL-6 by recruited monocytes.
Methodological considerations

The results obtained in this work are obtained from experiments on primary, physiological cells obtained from healthy donors. Still, there are important limitations as to the implications of the observations made, due to the methods that have been used. In the following, these limitations will be presented and discussed.

Samples

DCs mature from monocytes in vitro by exposure to pro-inflammatory cytokine such as IL-4 and GM-CSF. Though our iDCs were generated from monocytes isolated using a cocktail of antibodies designed to extract a clean population of cells, subtle functional differences may exist between natural, in situ occurring and in vitro generated iDCs. Similarly, forced maturation of NK cells by IL-2, though a standard procedure used worldwide, may not yield physiologically completely representative cells.

These concerns could be met through investigations along several paths. Retrieval of phenotypically activated NK cells and DCs from blood by flow cytometric sorting, followed by the application of our experimental protocols, perhaps offers the most relevant cues to whether our protocol for maturation resulted in major artifacts. To test the relevance of exposing cells to S1P, one could isolate cancer cells, NK cells and DCs from solid cancers. Then, cytotoxicity assays with or without additional stimulation may serve as a test to whether S1P makes a functional difference to cells already acclimatized to the cancer microenvironment. Along the same lines, one may retrieve NK cells and monocytes from atherosclerotic plaques in order to test them functionally for the effect of incubation with oxidized lipids.

NK cells were always isolated using the RosetteSep negative selection human NK cell enrichment cocktail, which removes cells expressing CD3, CD4, CD19, CD36, CD66b and glycophorin A. This procedure yielded a purified population of NK cells, as determined by flow cytometry, though with minor contaminations. Indeed, contaminating T cells may have contributed to our results, as cultures contained ten percent CD3 positive cells (Paper II, Fig.1). However, resting peripheral blood T cells do not express neither the high nor the intermediate affinity IL-2R, whereas NK cells constitutively express the intermediate affinity IL-2R. Therefore, only NK cells but not T cells are expected to respond to IL-2 during culture, and so T cells are unlikely to influence the results of our experiments after 7 days in culture. Control experiments with NK cell lines would have provided a control
for this effect, though such results would not be representative for physiological cells like the ones used in this work.

In order to clean the cell population further, cultured cells could have been sorted through a flow cytometer with a cell sorter prior to experiments. Such treatment, however, may influence the properties of the cells.

**The use of cells derived from the blood bank**

In our experiments, we used samples from different, anonymous donors in the blood bank. When cells from different samples yielded similar results, it allowed us to generalize the observations to the common population. However, since we acquired our cells from healthy, anonymous donors rather than from cell lines, there will always be a certain degree of variation among the cells employed in the different experiments.

Due to the limited life span of cultured primary cells and the limited recovery of cells from a given blood sample, we were seldom able to use the cells from one donor for more than one experiment. It is unlikely that blood used to prepare cells came from the same donor more than once or twice. The experiments therefore included some biological variations.

**The use of K562 tumor cells**

The K562 cell line originated from a pleural effusion during a terminal blast crisis from a patient suffering from chronic myeloid leukemia in 1970. However, there was some controversy as to the lineage of the cell, since they are stripped of markers as well for normal and malignant granulocyte precursors. However, the presence of glycophorin, which is exclusively found in the erythroid line of cells, commits the cells to the erythrocyte lineage. This cell line has been shown to be an excellent target for lysis by NK cells. It has later become clear that this might be due to the down regulation of MHC I on these cells. The K562 erythroleukemic cells are an established reference system for NK cell cytotoxicity, which is why they were used in this work.

**Chemotaxis**

This method, though widely used for almost forty years, provides hypotheses as to how chemotaxis may be regulated in vivo. However, the functional derivate of observed changes in receptor expression and the like has been efficiently investigated – but the significance of the observations in vivo may be limited.
In chemotaxis experiments, counting of cells on the membranes is a highly subjective matter. In order to compensate for this, membranes were counted blinded by two people.

**Calcium mobilization**

Two different methods were used to study the level of intracellular calcium in immune cells. Both included the indicators that emit light upon binding with cytosolic calcium. The first method was based on the use of Fura-2 AM in a Perkin Elmer LS50, and the second Fluo-3 AM as indicator followed by detection by a flow cytometer. In both instances, changes in the intracellular level of calcium were quantified. The LS50 – protocol required ten million cells per sample in order to get a satisfying response. This obstacle was overcome with the conversion to a flow cytometer – based system. The possibility for subgroup analysis based on the simultaneous conventional use of flow cytometry is also promising for further investigations.

**Flow cytometry**

Flow cytometry is a powerful technique for analysis on the single cell level in a heterogeneous cell suspension. By passing thousands of cells in a single line past a laser beam, and then capturing light emerging from each cell, multiple characteristics may be examined. On the basis of relative size, granularity or internal complexity, in addition to fluorescence from fluorocrome-labelled antibodies attached to the cells, one may investigate several groups of cells.

In this work, proteins on the cell surface as well as intracellular stores of cytokines have been quantified by flow cytometry.

Though a high throughput and high resolution technique in which vast amounts of information may be obtained, the information collected from flow cytometric experiments is dependent on the preparations and analysis.

Unspecific antibodies of the same immunoglobulin isotype as the specific antibody were used as control in all experiments. During analysis, the number of cells labelled unspecifically by the control antibody was subtracted from the number of cells labelled by the specific antibody in order to control for unspecific binding. Specific markers for the different subsets of cells were used in order to identify the correct population of cells for analysis.
Detection of secreted cytokines by ELISA assay

In order to quantify the change in secretion of various proteins, supernatants from cells in culture were collected, frozen at -80°C and later analyzed by the ELISA method. Various kits were used, all of which were validated by the manufacturer, and the protocol provided by the manufacturer was always followed. In this method, antibodies bound to different antigens of interest were labeled with light-emitting chemicals, and the intensity of this light was quantification. The background staining/light is an issue in such assays, which was overcome by subtracting the amount of light emitted in the absence of samples. For reliable quantification, standards in which the concentration of the antigen had been determined by other methods, were included in every experiment.

Cytotoxicity assay

Cytotoxicity assays were used extensively in this project, involving NK cells and various target cells. While $^{51}$Cr release still is standard for the quantification of NK cell cytotoxicity in many laboratories, a new method has recently been developed\textsuperscript{292} and was used in this project. In this protocol, target cells were labelled with Calcein-AM prior to incubation with NK cells in 96-well flat-bottom plates. The acetomethoxy derivate of Calcein was transported into live cells, thus demonstrating cell viability – and, consequently, relative cell death. The acetomethoxy group was removed by esterases intracellularly, thus trapping the Calcein inside the cell and allowing for chelation with calcium, resulting in strong green fluorescence. The lack of esterases in dead cells rendered them unlabeled, and measurement of light intensity was therefore a measure of the number of viable cells:

\[
\text{% viability} = \left( \frac{F(E:T) - F(\text{Total Lysis})}{F(\text{Total viability}) - F(\text{Total Lysis})} \right) \times 100
\]

\[
\text{% cytotoxicity} = 100 - \text{% viability}
\]

Figure 10. Formula for calculation of NK cell cytotoxicity. F: fluorescence; E: effector; T: target; E:T means effector divided by target.

This method was safer than the one based on $^{51}$Cr since there is no radiation involved. It was also efficient, as the use of 96-well plates allowed many samples to be prepared and analyzed simultaneously. Comparisons were made, confirming the reproducibility of results yielded by the two methods.
**Statistics**

Significant group differences were determined using the two-tailed Student’s t-test. The test assumes a normal distribution of the data, which was assumed as cells for the experiments were sampled from several different donors and effect calculations were based on observations from several thousand cells per experiment.

In addition, IC\(_{50}\) values were calculated using one-site competition of nonlinear regression curves. One-way ANOVA corrected for multiple comparisons using Dunnell’s test, determined the effect on IL-6 secretion from monocytes.
Discussion

In this chapter, an overall discussion of the data obtained in this project will be presented in relation to relevant findings by other investigators. While the specific results have been interpreted and validated in the individual papers, the aim in this section is to interpret them in a broader context and to indicate future prospects.

Is S1P an inhibitor of innate immunity?

We asked how S1P affects the phenotype and function of innate immune cells, and how this will influence the effect of S1P in a cancer microenvironment.

We showed that S1P inhibited NK cell lysis of cells from the human myeloid leukemia cell line K562, compatible with observations made in other cancer cell lines. We observed the same effect on the ability of NK cells to lyse autologous or allogeneic immature DCs, and we showed that S1P exerted its effects on the target cells and not on the NK cells. MHC molecules have been shown to be important for the DC recognition by NK cells. Ferlazzo et al showed that an increase in HLA class I molecules, which was associated with DC maturation, was related to their resistance to NK cell cytolysis. Della Chieza et al reported that increased expression of HLA class I molecules, including HLA-E, rendered maturing dendritic cells resistant to killing by NK cells. In agreement with these observations, we found that up-regulation of classical HLA I and HLA E on the surface of DCs was associated with inhibition of NK-mediated cytotoxicity.

Since we did not observe any change in the maturation markers for DCs, i.e. CD80, CD83 and CD86, the up-regulation of classical HLA I and E may represent a very early stage of DC maturation, taking place before the changes of other maturation markers. K562 cells, on the other hand, lack expression of classical HLA-I and HLA-E. The reason why NK cells spare K562 cells after stimulation with S1P will have to be examined further.

We analyzed NK cells stimulated with S1P, FTY720 or SEW2871, for several markers known to be important for cytotoxicity and maturation, but there were no detectable phenotypic changes. However, we found that S1P inhibited the release of the inflammatory cytokines IL-17A and IFN-γ from NK cells. This reduction was not inhibited by SEW2871 or FTY720, implicating that other S1P receptors than S1P₁ were responsible for this effect.

Increased levels of IL-17 have been associated with increased angiogenesis and increased tumor size in cancer development, which seemed contradictory to our results. A
likely source of this IL-17, however are Th17 cells. Later experiments in our lab also showed that IL-17 was secreted only by a subtle subset among NK cells, suggesting that the decrease in cytokine secretion may not involve cancer-infiltrating NK cells. Further experiments may determine the individual effect of NK cell subsets. Of note, neither SEW2871 nor FTY720 ligates with the receptor S1P_2, opening the possibility that this receptor may be involved in regulating cytokine secretion. However, we did not see any expression of S1P_2 in the NK cells, compatible with earlier findings of the absence of mRNA for this receptor in NK cells.\\n
Our results indicated that S1P inhibited key functions of innate immune cells, suppressing the release of inflammatory cytokines while at the same time rendering NK cells unable to lyse target cells such as DCs, and consequently contributing to reduced inflammation. One consequence of this may be promotion of immune escape by tumor cells. Further investigations into the source of S1P in the cancer microenvironment are required in order to clarify the mechanisms behind the immune suppression. Several scenarios may explain our results:

- Given that the S1P at a given anatomical locus was secreted by tumor cells, our results showed that this will not simply be self-protective, but also shape the tumor environment by selectively increasing the viability of other cells, namely the iDCs. S1P is a chemo-attractant for both NK cells and DCs, and sparing certain iDCs might be a way of inhibiting the ‘quality inspection’ described by Moretta. Quality inspection is believed to be a way of eliminating DCs not capable of proper T cell stimulation. Sparing cells that otherwise would be removed for this reason, means practically energizing the adaptive immune response, eventually leading to immune escape. The simultaneous inhibition of pro-inflammatory cytokines IL-17 and IFN-γ secretion further amplifies the anti – inflammatory effect.

- S1P production by cancer cells may also be a result of an evolutionary pressure, allowing survival of cancer cells that produced S1P. Simultaneous sparing of iDCs may then simply be a collateral loss, as the iDCs might not have been capable of stimulating the T cells.

- Given that the S1P is derived from the endothelium, platelets or other physiological cells, the interpretation will be different. Now the inhibition of killing of iDCs may permit increased stimulation of T cells by DCs.
Finally, LPLs may down-regulate the cytolytic activity of NK cells, allowing them to activate rather than lyse DCs, hence potentiating the immune response.\textsuperscript{178}

While useful when seeking to dissect the effects of the different S1P receptors, \textit{in vitro} investigations on the effects of FTY720 and SEW2871 may also be viewed as pre-clinical research into the potential clinical effects of these compounds. The effects we observed may thus have been independent effects imposed by the drugs, and not merely receptor ligation/blocking. Indeed, S1P\textsubscript{1} agonists have been used to induce immune suppression after kidney transplantation and are currently used in the treatment of multiple sclerosis.\textsuperscript{249}

In a recent evaluation of the cancer risk in MS patients receiving immune modulatory drugs, it was concluded that dermatological monitoring should be performed when Fingolimod/Gilenya (FTY720) is used for these patients, due to cases of skin cancer.\textsuperscript{301}

Considering that it mimics the effect of S1P, a lipid that stimulates malignant transformation, cancer proliferation, vasculogenesis and resistance to apoptotic cell death, this should come as no surprise.\textsuperscript{302-305} Our results suggest that FTY720 should under no circumstance be considered simply as a lymphocyte – sequestering agent; the increased risk of skin cancer in patients treated with FTY720, may be due to S1P-like effects of this drug.

What remains to be clarified before we can fully understand the function of FTY720, is the regulation of the balance between its stimulatory and inhibitory effects. Indeed, FTY720 was shown to enhance neovascularization and vessel maintenance via S1P\textsubscript{1} and S1P\textsubscript{3},\textsuperscript{306} but in a different study it inhibited tumor angiogenesis via S1P\textsubscript{1}.\textsuperscript{307}

**Do oxidized lipids and LPC inhibit innate immunity?**

In paper I, we demonstrated that S1P imposed inhibitory effects on innate immunity, which was compatible with its role in promoting cancer. In papers II and III we investigated the impact upon innate immunity by LPC, a phospholipid-derived lipid, and several isoforms of HODE, derived from linoleic acid.

As a first step, we observed direct chemotactic responses of NK cells and monocytes towards the lipids, which was similar to S1P.\textsuperscript{135} This was expected, as several reports have documented this effect in other cell types.\textsuperscript{308,309} It was even suggested that the chemotactic response of NK cells to LPC was important for keeping NK cells within the circulation.\textsuperscript{271}

The chemotaxis assay is a test for the demonstration of a specific receptor. Directed chemotaxis of NK cells and monocytes towards the HODEs and LPC suggested that a G-
protein coupled receptor such as G2A was involved, since chemotaxis is induced through G-protein coupled receptors. Indeed, G2A was responsible for the chemotaxis of macrophages towards LPC. In the latter report, the researchers also showed that the Gαi protein was not required for LPC-induced chemotaxis towards the receptor, whereas coupling to Gq/11 and G12/13 was. To examine the dependence on G-proteins for chemotaxis in NK cells, the cells were pretreated with PTX prior to chemotaxis experiments. While the Gα subgroups Gαq, and Gαz are insensitive to bacterial toxins, Gαi, Gαo and Gαs are sensitive to such treatment. That is, Gαi and Gαo are ADP-ribosylated at the cysteine residue in the carboxy terminal of the α subunit by PTX. Our observations suggested that the different lipids employ different Gα subunits. After pretreatment of NK cells with PTX, we observed a significant decrease in migration towards all lipids examined. Hence, all of the four lipids may have utilized a G-protein coupled receptor such as G2A to induce their effects on NK cells. The effect was most pronounced for 9S-HODE and 13R-HODE, since the migration in these cases was reduced almost to the background level. This came as a surprise, as the effect of 9S-HODE was shown by Obinata et al. to be resistant to PTX inhibition. However, the latter observation was made in cells with forced expression of the receptor, and the difference between our observations and those of Obinata may therefore be due to the fact that we examined normal cells. In the case of 9R-HODE and LPC, we observed that the migration was reduced only by half. This was contrary to the observations of Yang et al. in macrophages, showing that pretreatment with PTX completely abolished the migration. However, it was compatible with earlier observations showing a 50% reduction of NK cell chemotaxis to LPC, leading us to conclude that these latter lipids signaled through Gα-proteins, insensitive and sensitive to PTX. G2A has been shown to bind to several G-proteins. We therefore proceeded to examine if the lipids signal through the same G-proteins in NK cells.

In line with our observations in chemotaxis, 9S-HODE only reduced the mobilization of intracellular calcium by half when administered prior to LPC in the calcium mobilization assay, indicating that LPC activated additional Gα-proteins to those activated by 9S-HODE. Further, there was a reciprocally complete inhibition of the mobilization of intracellular calcium between 9R-HODE and LPC, indicating that they may employ the same Gα proteins. 13R-HODE had no effect on the calcium mobilization in our experiments, which was contrary to that observed in vascular smooth muscle cells. However, it was in agreement with early evidence showing that 9-HODE potently induced...
calcium mobilization via the receptor G2A, whereas 13-HODE only had a weak ability to do so.\textsuperscript{276} This observation may also reflect differences between 9- and 13-HODE. The differences were reviewed, concluding that while 9-HODE has pro-inflammatory effects, 13-HODE also contributes to anti-inflammation.\textsuperscript{279} When we investigated the ability of the lipids to evoke an increase in intracellular calcium in monocytes, only LPC had this effect. This may relate to the fact that LPC, but none of the other lipids, binds to the G-protein coupled receptor GPR4.\textsuperscript{312}

We next examined whether the chemotaxis towards the lipids was associated with changes in the expression of chemokine receptors, since chemokines are highly important for atherosclerotic plaque development and cancer. Several reports of such effects have been published by others.\textsuperscript{313,314} We observed in monocytes that 9S-HODE, 9R-HODE, 13R-HODE and LPC increased the expression of CCR9, and that CXCR4 was up-regulated by 9R-HODE, 13R-HODE and LPC at high concentrations (Paper III). At lower concentrations, we observed no effect (Rolin J., unpublished results). Up-regulation of the receptors correlated with increased migration towards the ligands of these receptors, TECK/CCL25 and SDF-1α/CXCL12 (Paper III). These observations may have implications for atherosclerosis, since chemokines working through their specific receptors were important in a number of important steps in the development and resolution of atheromatosis.\textsuperscript{315}

CCR9 expression has been found in atherosclerotic plaques.\textsuperscript{316} CCR9 positive cells were abundant in the plaques in mice and also in samples from human, possibly derived from circulating immune cells. The number of CCR9-expressing cells was reduced after treatment with an angiotensin-converting enzyme inhibitor, and the TECK/CCL25-CCR9-axis was pro-atherogenic, as inhibition of CCR9 by RNA interference retarded the development of atherosclerosis in ApoE-deficient mice.\textsuperscript{316} It was therefore concluded that the receptor is important for the development of the disease. This conclusion was further supported by the identification of TECK in tissue samples. Levels of the chemokine were low in normal aortas, but increased in atherosclerotic vessels, and particularly in cells adjacent to the necrotic core of advanced plaques.\textsuperscript{316} Whether TECK is important for the recruitment of CCR9-expressing monocytes to the lesion, or merely to immobilize them upon up-regulation of the receptor, will require further research. A restricting effect on monocyte egress from plaques is suggested as we observed increased expression of CCR9
on monocytes when they were exposed to high but not low levels of lipids that are abundant in atherosclerotic plaques. Such a mechanism was forwarded by Han for CCR2, but the relative importance of the two mechanisms needs further investigation.

In a recent review on the chemokine field and the development of drugs targeting this system, the CCL25/CCR9 axis was high-lighted as an important mediator of inflammatory bowel diseases as well as melanoma metastasis in the small intestine. HODEs are abundant in the skin and so up-regulation of the receptor by these lipids calls for further investigation into similar effects in cells of the skin. The focus in clinical trials on CCR9 antagonists has been inflammatory bowel disease, and they were well tolerated. Although development was terminated due to lack of clinical effects in Crohn’s disease, it may still be worthwhile exploring its effect in other diseases. Increased CCR9-expression and activity in cells exposed to oxidized lipids as demonstrated in this work suggests that it may be worthwhile exploring the effects of such drugs in atherosclerosis and skin cancer metastasis.

We were not the first to investigate the ability of oxidized lipids in up-regulating CXCR4–expression. Monocytes, isolated by the same method that we used, were exposed to oxLDL. In accordance with our results, stimulation with oxLDL resulted in increased SDF-1α-elicited increase in intracellular calcium flux. There was also an increase in the expression of CXCR4, as measured by the level of mRNA, compared to LDL or unstimulated controls. Later, LPC was found to induce up-regulation of CXCR4 in CD4+ T cells. Increase in the SDF-1–stimulated production of the pro-inflammatory cytokines IL-2 and IFN-γ further suggested that the increase in CXCR4 expression led to increased inflammation. Finally, in the monocytic RAW 264.7 cells, oxLDL increased the expression of CXCR4, an effect which was counteracted by the addition of a hydrogen sulfide donor. Adding to these prior observations, our results suggested that in monocytes, 9R-HODE and 13R-HODE may be the main mediators of this oxLDL induced effect.

CXCR4 is regarded a homeostatic chemokine receptor, due to its importance in embryonic development and homeostatic migration of progenitor cells. Its importance in this respect was high-lighted by the number of CXCR4-targeting compounds under development for mobilization of stem cells to be used in stem cell transplantation. CXCR4 is associated with an unusually wide spectrum of activities, including migration, positioning, neovascularization, survival and growth. Accordingly, it is important also in
In atherosclerosis, the role of CXCR4/SDF-1 is still highly unclear, with observations in man and mouse indicating pro- as well as anti-atherogenic roles. While its ligand SDF-1 is expressed at a high level by several cell types in plaques, receptor as well as ligand expression was low in peripheral blood in angina pectoris patients. Furthermore, statins, the state of the art group of drugs for the treatment of lipid–induced vascular disease, seemed to increase the expression of CXCR4, since withdrawal from treatment lead to its down-regulation in classical monocytes. In addition to the increase in receptor expression that we observed upon stimulation with the lipids, we observed that the background expression of CXCR4 on cultured, fresh monocytes almost doubled overnight. This feature may reflect the importance of monocytes in preventing early atherosclerosis, as CXCR4 is accompanied by increased micropinocytosis upon ligation of the receptor. It was also suggested by Gupta et al., as they observed a similar increase during GM-CSF-induced maturation into macrophages, that the increase may contribute to the migration of intimal foam cells, subsequently leading to progression of the plaque area.

As mentioned in the introduction, there is consensus in the literature about the dual roles played by 13-HODE and later by both 9-HODE and 13-HODE on the development of atherosclerosis. Although examination of what receptors elicited the effects of the various oxidized lipids was not a topic in this work, a great body of literature points to the importance of at least two different receptor systems, i.e. nuclear receptor PPAR-γ and the G-protein coupled receptor G2A. In the early stages, enzymatically produced 13-HODE imposed anti-inflammatory effects through activation of PPAR-γ. This effect was overwhelmed by G2A-activation by both lipids at later stages, since the lipids were also produced through non-enzymatic oxidation of linoleic acid. Activation of PPAR-γ and G2A is not necessarily mutually exclusive, and different effects may simultaneously be induced by different receptors in the same cell. Our observations suggested that plural receptors were activated – or that plural signaling pathways were activated by the same receptor. Indeed, 9R-HODE and 13R-HODE acted similarly when they induced the expression of CXCR4, but only the 9R-HODE isomer induced calcium mobilization in NK cells. The recent identification of a surrogate agonist for the receptor G2A is promising for future research, as it will allow for selective stimulation of this signaling pathway in physiological cells in order to distinguish between the effects of the two receptors.
Finally, we examined the impact of the oxidized lipids on the production of cytokines by NK cells and monocytes. We found that 9R-HODE increased the total production and the number of NK cells producing IFN-γ (Paper II), a cytokine which was earlier shown to potentiate atherosclerosis in mice. On the contrary, all other lipids decreased the amount of IFN-γ in supernatants in our experiments. This corroborated the trend in the literature towards an overall protective effect of 13-HODE mediated largely by PPARγ at an early stage, with subsequent detrimental effects, mediated by the action of 9-HODE. The fact that 9R-HODE increased the secretion of IFN-γ and the number of cells producing it, suggested that the 9R-HODE isomer was responsible for the detrimental effects occurring during inflammation. It therefore came as a surprise when we observed a profound decrease in the secretion of IL-6 from monocytes exerted by all lipids (Paper III), as IL-6 exacerbated early atherosclerosis. On the other hand, PPARγ agonists were shown to inhibit the production of various cytokines including IL-6. Our group earlier observed differing pathways for the release of IL-6 upon stimulation of myeloid cells with inflammatory lipids that were related to the maturation stage of the cells, suggesting that the change in IL-6 release may be related to maturation of the cells as well as to direct ligation of a receptor.

Taken together, we observed activation of important cellular features upon stimulation of monocytes and NK cells with oxidized lipids, but an overall anti-inflammatory effect through inhibition of pro-inflammatory cytokine release.
Conclusions

1. S1P, largely through interaction with its receptor S1P₁, induced changes in DCs that allowed them to be spared from NK cell cytolysis. Similarly, S1P spared cancer cells from NK cell cytolysis. This effect may contribute to the dissemination of cancer.

2. The drugs FTY720 and SEW2871 may be seen as competitive agonists to the S1P receptors, as they inhibited the effect of S1P and at the same time mediated the same effects at a lower level. This should be taken into consideration when the drugs are considered for clinical applications.

3. Oxidized lipids attracted NK cells and monocytes, which may contribute to the accumulation of these cells in atherosclerotic lesions.

4. Oxidized lipids increased chemokine receptor expression and function in monocytes, which may contribute to the development of atherosclerosis.

5. S1P and oxidized lipids had the ability to alter, mostly decrease, NK cell and monocyte secretion of cytokines.
Future prospects

We showed that lipids commonly found in the cancer microenvironment, in atheroma and in blood can markedly influence NK cell and monocyte functions and the microenvironment of these cells. However, several questions remain unanswered, arising from observations made in this work.

- Future studies should investigate further the molecular effects on K562 cells by S1P in order to understand how they were protected from NK cell cytolysis.

- In the same way that was done by Pandya et al. concerning the observation of IL-17 secretion, it should be examined whether changes observed in monocytes and NK cells upon HODE/LPC stimulation, represent effects constituted by unique cell populations.

- The drugs FTY720 and SEW 2871 decreased the inhibition of NK cell cytotoxicity only, implying that they primarily work on target cells such as DCs and K562. An important question is whether this effect contributes to the clinical effects of the drugs.

- Several observations in this work suggested that plural receptors were responsible for the different effects that we report. It will be important to investigate closer the signaling of the lipids.

- We observed increased activity and expression of the chemokine receptors CCR9 and CXCR4 in a mixed population of human monocytes. Further investigation on monocyte subsets will be important. After all, only a minor subset of monocytes expressed CCR9, and a substantial number of cells did not express CXCR4. It would be interesting to investigate if any cells may up-regulate both.

- Monocytes treated with oxidized lipids or LPC did not mature towards DCs. Still, it would be interesting to investigate the maturation of cells that did or did not increase their chemokine receptor expression, in order to see whether they produce DCs with differing capacities. It would also be interesting to expose monocytes treated with oxidized lipids or LPC to NK cells, in order to investigate the cross-talk between these cells.
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