Natural killer cells and dendritic cells in multiple sclerosis – effect of drugs in vitro and in an experimental mouse model

Doctoral thesis by
Zaidoon Salim Kashkoul AL-Jaderi

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Division of Physiology & Anatomy
Department of Molecular Medicine
Institute of Basic Medical Sciences
University of Oslo
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1. PUBLICATIONS INCLUDED

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


III. Al-Jaderi Z, Maghazachi AA. Vitamin D(3) and Monomethyl Fumarate Enhance Natural Killer Cell Lysis of Dendritic Cells and Ameliorate the Clinical Score in Mice Suffering from Experimental Autoimmune Encephalomyelitis. Toxins (Basel) 2015; 7(11):4730-4744.
2. ABBREVIATION

1,25(OH)$_2$D  1,25-dihydroxyvitamin D
1,25(OH)$_2$D$_3$  Calcitriol
25(OH)D$_3$  Calcidiol
APC  Antigen presenting cell
BAT3  Protein HLA-B-associated transcript-3
BBB  Blood-brain barrier
CCL  Chemokine (C-C motif) ligand
CCR  CC chemokine receptor
CD  Cluster of differentiation
CD107a  Lysosomal-associated membrane protein-1
cDC  Conventional DC
CFA  Complete Freund’s adjuvant
CLP  Common lymphoid progenitor
Clr  C-type lectin-related molecule
CMP  Common myeloid progenitor
CNS  Central nervous system
CSF  Cerebrospinal fluid
CTL  Cytotoxic T lymphocyte
CTLA-4  Cytotoxic T-lymphocyte-associated protein 4
DC  Dendritic cell
DMF  Dimethyl fumarate
DNA  Deoxyribonucleic acid
dsRNA  Double-stranded RNA
EAE  Experimental autoimmune encephalomyelitis
EBV  Epstein-Barr virus
FasL  Fas ligand
Foxp3  Forkhead/winged helix transcription factor P3
FTY720  Fingolimod
G protein  Guanine nucleotide binding protein
GA  Glatiramer acetate
GM-CSF  Granulocyte macrophage-colony stimulating factor
GSH  Glutathione
HCMV  Human cytomegalovirus
HCS  Hematopoietic stem cells
HLA  Human leukocyte antigen
ICAM-1  Intercellular adhesion molecules 1
iDC  Immature dendritic cell
IFN  Interferon
Ig  Immunoglobulin
IL  Interleukin
iNK  Immature natural killer
iNKT  Invariant NKT
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthetase</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immune receptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immune receptor tyrosine-based inhibition motif</td>
</tr>
<tr>
<td>K562</td>
<td>Human myeloid leukemia cell line</td>
</tr>
<tr>
<td>Keap-1</td>
<td>Kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer cell immunoglobulin-like receptor</td>
</tr>
<tr>
<td>KLR</td>
<td>Killer cell lectin-like receptors</td>
</tr>
<tr>
<td>LAK</td>
<td>Lymphokine-activated killer</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRC</td>
<td>Leukocyte receptor gene complex</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MCMV</td>
<td>Murine cytomegalovirus</td>
</tr>
<tr>
<td>mDC</td>
<td>Mature dendritic cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MICA/B</td>
<td>MHC class I related chain A/B</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MLL5</td>
<td>Mixed-lineage leukemic protein 5</td>
</tr>
<tr>
<td>MMF</td>
<td>Monomethyl fumarate</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NCR</td>
<td>Natural cytotoxicity receptors</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular traps</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cell</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKG2</td>
<td>Natural killer group 2</td>
</tr>
<tr>
<td>NKR</td>
<td>Natural killer cell receptor</td>
</tr>
<tr>
<td>NKR-P1</td>
<td>Natural killer receptor protein 1</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NQO-1</td>
<td>Nicotinamide adenine dinucleotide phosphate quinone reductase 1</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor (erythroid-derived 2)-like 2</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid DC</td>
</tr>
<tr>
<td>PLP_{139–151}</td>
<td>Proteolipid protein_{139–151}</td>
</tr>
<tr>
<td>PML</td>
<td>Progressive multifocal leukoencephalopathy</td>
</tr>
<tr>
<td>RAE-1</td>
<td>Retinoic acid early-inducible protein 1</td>
</tr>
<tr>
<td>RAJI</td>
<td>Human lymphoma cell</td>
</tr>
<tr>
<td>RANTES</td>
<td>C-C motif chemokine ligand 5 or CCL5</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
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RORγt  RAR-related orphan receptor gamma t
ROS    Reactive oxygen species
RRMS   Relapsing-remitting multiple sclerosis
RXR    Retinoic acid X receptor
SHIP-1 SH2 domain-containing 5 inositol phosphatase 1
T-bet  T-box transcription factor
TCR    T cell receptor
TGF    Transforming growth factor
TGF-β1 Transforming growth factor beta 1
Th     T helper
TLR    Toll like receptor
TNF    Tumour necrosis factor
TRAIL  TNF-related apoptosis-inducing ligand
Treg   T regulatory
ULBP   UL16 binding protein
UVB    Ultraviolet B
VCAM-1 Vascular cell adhesion molecule 1
VDR    Vitamin D receptor
VDRE   Vitamin D₃ response elements
ZAP-70 ζ-chain associated protein kinase of 70kDa
    α    Alpha
    β    Beta
γδ T cell Gamma delta T cell
    δ    Delta
    ε    Epsilon
    ζ    Zeta
3. **INTRODUCTION**

3.1 **The immune system**

Mammals have developed a complicated professional immune system to defend against invading pathogens, and recognize neoplastic and allogeneic cells. The immune system in general can be divided into the innate and the adaptive immune system. The innate immune system represents the first line of defence, that responds immediately against invading pathogens and causes inflammation at sites of infection, before the adaptive immune system can mount efficient responses. The innate system is composed of granulocytes, mast cells, macrophages, monocytes, dendritic cells (DCs) and natural killer (NK) cells. These cells defend the infected tissues by eliminating pathogens through several mechanisms, like ingestion of bacteria or destruction of infected cells as well as secreting cytokines and chemokines to recruit and activate other cells. In contrast, the adaptive immune response is more specific against particular antigen and takes approximately one week to develop. This system consists of T and B cells. These cells express receptors that specifically recognize different antigens and can develop into effector and memory cells which are long lived and rapidly recognize and respond to the same pathogen. B cell receptor recognition of antigenic determinants (epitopes) leads to the activation of B cells, that differentiate to plasma cells that produce immunoglobulin (Ig) as well as memory cells. T cell receptor (TCR) recognizes peptide antigens presented by major histocompatibility complex (MHC) molecules (class I or II) on antigen presenting cells (APCs).

The central sites of the immune system in the body are the primary lymphoid organs (bone marrow and thymus) and the secondary lymphoid organs (lymph nodes, tonsils, Peyer’s patches, spleen and other mucosa-associated lymphatic tissues). All immune cells are generated in the primary lymphoid organs, from hematopoietic stem cells in the bone marrow through the process of hematopoiesis. The further maturation and activation of lymphocytes occurs in the secondary lymphoid organs.

3.2 **Hematopoiesis**

Hematopoiesis is the development of blood cellular components. It occurs in the bone marrow where hematopoietic stem cells (HSC) reside. These cells have the unique ability to self-renew and differentiate into multiple cell types [1] by a process called asymmetric cell division [2]. CD34 has been recognized as a surface marker for these cells in human [3], and CD122 in mouse [4]. HSCs can differentiate into common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) cells [5]. The CMP can further differentiate into megakaryocyte-erythrocyte progenitors and granulocyte-monocyte progenitors, which differentiate to erythrocytes, platelets, monocytes or
granulocytes, respectively. Similarly the CLP gives rise to T, B or NK cells [6;7]. Hematopoiesis is regulated by many factors including cell to cell interaction, the action of lineage-specific hematopoietic growth factors and cytokines [8]. For example, murine studies have shown that IL-15 is an NK-specific growth factor since IL-15 deficient mice show near absence of NK cells [9]. Similarly, mice with defective IL-2/IL-15 receptor β-subunit (CD122) expression [10], and mice with deficiency in the α-subunit for IL-15R [11], have decreased numbers of NK cells. IL-2/IL-15 provides survival signals for NK cell development. Signalling through the IL-2/IL-12 receptors leads to activation of JAK1/JAK3 and the transcription factors STAT3/STAT5b [12]. Deficiency in JAK3 or STAT5b also leads to deficiency in NK cell development and cytotoxicity [12]. It was believed that NK cell development occurs exclusively in the bone marrow [13], but recent studies show that NK cells can also develop in the thymus and secondary lymphoid tissue [14].

![Figure 1. A schematic view of hematopoiesis.](image)

NK cell development has been studied in both mice and human. In the human, the earliest stage 1 pro-NK cell is identified by the surface phenotype (CD34⁺CD45RA⁺integrinβ7⁺CD117⁻CD161⁻). Stage 2 pre-NK cells are (CD34⁺CD45RA⁺CD117⁺CD161⁺⁺). These cells may emerge from the bone marrow and traffic to secondary lymphoid tissues, where they subsequently differentiate to stage
3 immature NK (iNK) cells (CD34⁻CD117⁺CD161⁺CD94/NKG2A⁻NKp46⁻). Stage 3 iNK cells lack functionality. For example: these cells lack the ability to produce IFN-γ or kill tumor target cells. Stage 4 NK cells are (CD56brightCD117lowCD94/NKG2A⁺CD16⁺KIR⁺/⁻NKp46⁺). At this stage NK cells acquire the ability to produce IFN-γ in response to cytokine stimulation (IL-12 and IL-18) but have attenuated cytotoxicity. Stage 5 is the mature stage, and constitutes (CD56dimCD117⁺CD94/NKG2A⁺/⁻KIR⁺/⁻CD16NKp46⁺) NK cells. In stage 5 NK cells have potent cytotoxicity [15]. The NK cell development occurs under the influence of instructive cytokines, including IL-15.

**Figure 2.** Human lymphocyte development. The figure shows cytokines important to NK cell, T cell and B cell differentiation as well as the cytokines produced by mature cells.

In mouse the phenotypic markers are different. Stage 1 consists of NK precursors that are CD122⁺NK1.1⁻. Stage 2 consists of progenitors expressing NK1.1 (NKR-P1/CD161) and CD49/NKG2A. This is followed by gaining Ly49 at stage 3. NK cells at stage 4 undergo significant proliferation before reaching the final stage (stage 5), in which NK cells acquire CD11b and CD43. This stage is characterized by high cytotoxic ability and IFN-γ production.
NK cell maturation in the mouse can be described on the basis of surface expression of the TNF superfamily member CD27 and the integrin CD11b [16]. NK cell maturation starts at the double-negative stage (CD27$^{\text{low}}$CD11b$^{\text{low}}$). These NK cells progressively differentiate into immature (CD27$^{\text{high}}$CD11b$^{\text{low}}$), followed by transitional (CD27$^{\text{high}}$CD11b$^{\text{high}}$) and finally into mature NK cells (CD27$^{\text{low}}$CD11b$^{\text{high}}$). CD27$^+$ NK cells have the ability to migrate to lymphoid tissue and the ability to interact with dendritic cells (DCs), show high cytotoxicity and IFN-γ production in response to IL-2 or IL-18 stimulation, when comparing with CD27$^-$ NK cells [16].

**Figure 3.** Mouse NK cell development. The major classes of stem and NK progenitor cell described in the text are defined by cell surface phenotypes, which are listed under each population. Stage 1 common lymphoid progenitor, stage 2 Pre-NK precursor, stage 3 NK precursor, stage 4 iNK and stage 5 mature NK cells.

DCs can develop in the bone marrow from both the myeloid and the lymphoid progenitor. They migrate from the bone marrow to the blood and then to lymphoid and non-lymphoid tissues. The tow main types of DC are; conventional DCs (cDCs) and plasmacytoid DCs (pDCs). In the skin, immature conventional DCs are known as Langerhans cells.

In vitro DCs can also generated from monocytes after several days of culturing with different cytokines such as GM-CSF and IL-4 [17]. Adding LPS to the culture induces maturation of DCs [18].
3.3. Major histocompatibility complex

The major histocompatibility complex (MHC) is also known as HLA in human, H2 in mouse and RT1 in the rat. MHC is located on chromosome 6 in human, 17 in the mouse and 20 in the rat [19]. The MHC genetic structure is homologous between human and mouse. The mammalian MHC genes are usually subdivided into three classes (MHC class I, II and III) based on function. While the MHC class III region contains several genes involved in immunological function, such as heat shock proteins, TNF and complement factors, the MHC class I and II regions contain genes responsible for presentation of peptides to T cells. The MHC class I loci comprise classical (class Ia) and non-classical (class Ib) loci. MHC class Ia are highly polymorphic glycoproteins, comprising three major loci (HLA-A, -B and -C) in human, and two major loci (H2-K, -D) in mouse. MHC class I molecules are expressed on all nucleated cells and present peptides from inside the cell including viral peptides. These peptides are produced from proteins that are broken down in proteasomes. The non-classical MHC class Ib molecules comprise HLA-E, -F and -G in the human and H2-T, -Q and -M in the mouse. MHC class Ib molecules are less polymorphic, expressed at lower levels and exhibit a limited tissue distribution. The MHC class II molecules have a domain structure similar to MHC class I but consist of two polymorphic transmembrane peptides (α and β chains) equal in their size [20] and present antigenic polypeptides from outside the cell in the groove formed by the α and β chain [21]. MHC class II molecules are encoded by three loci in human (HLA-DP, -DQ and -DR) and two loci in mouse (H2-A and -E), and are expressed on APCs such as DCs, B cells and activated macrophages [19]. MHC class I molecules are composed of a transmembrane polypeptide chain (α-chain) which binds the peptide and a light chain (β₂ microglobulin) [22]. The main function of MHC class I is to present short intracellularly derived peptide (from the cytosol or nucleus) at the cell surface to be recognized by CD8⁺ T cells and NK cells [23]. MHC class II molecules present peptides from extracellular proteins taken up by endocytosis, phagocytosis and pinocytosis to be recognized by CD4⁺ T cells [24].

3.4. The innate immune system

This system consists of granulocytes, macrophages, monocytes, DCs, mast cells and NK cells. The innate immune system is ready to react immediately. This system distinguishes between self antigens and pathogen associated molecules using pattern recognition receptors such as Toll-like receptors (TLRs).

Neutrophil granulocytes are the most abundant type of the granulocytes (40% to 75%) of circulating leukocyte. They create the first line of defense against invading pathogens. They have a short half-life in circulation 7-14 hours then they undergo apoptosis and cleared by macrophages.
During an acute inflammatory response, particularly as a result of bacterial infection, neutrophils are the main cells that migrate from the intravascular compartment towards the site of infection. This migration is activated by chemotactic factors expressed by activated endothelium, mast cells, and macrophages. Neutrophils also release cytokines, which in turn recruits and activates other cells of the immune system.

Neutrophils employ three major strategies to combat bacteria: phagocytosis, degranulation and generation of neutrophil extracellular traps (NET). The latest one is formed via an alternative type of cell death called NETosis [25].

During phagocytosis, neutrophils engulf and take up microbes into specialized compartments known as phagosomes in a receptor-mediated, clathrin-independent process. Fusion of lysosomes with the phagosome results in the formation of a phagolysosome, allowing for the assembly of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex Nox2. The NADPH oxidase is a transmembrane multiprotein complex that transfers electrons to molecular oxygen, to generate superoxide anions into the lumen of the phagolysosome. This highly oxidative environment in combination with the exposure to antimicrobial factors leads to the inactivation and killing of ingested microbes. Degranulation involves the fusion of granules with the plasma membrane and the release of cytokines and antimicrobial contents into the extracellular space. These molecules help coordinate the immune response and control pathogens extracellularly.

In response to extracellular pathogens such as LPS (present in Gram-negative bacteria) or lipoteichoic acid (present in Gram-positive bacteria) or intracellular infection such as virus (dsRNA) the innate immune system releases a variety of cytokines enhancing B and T cells, as well as presenting antigens to the adaptive immune system. NK cells recognize target cells with intracellular infection. They also have a critical role in tumor surveillance and MHC mismatched haematopoietic transplantation [26]. NK cell activation leads to NK cell killing of infected cells as well as cytokine production. IFN-γ directs the adaptive immune system to a cellular Th1 response.

### 3.4.1. Monocytes and dendritic cells

Monocytes circulate in the blood and migrate into tissues where they give rise to DCs or macrophages [27]. Monocytes represent 5-10% of total blood leukocytes and are divided into several subsets based on the expression of CD14, CD16 (also known as FcγRIII) and CD32. DCs were first discovered by Langerhans in 1868. Human DCs are composed of two subpopulations: pDCs and cDCs [28]. The pDCs have a morphology resembling plasma cells and produce high levels of IFN-α/β [29] and are found in primary and secondary
lymphoid tissues as well as blood. The cDCs produce high levels of IL-12 and are found in most peripheral tissues in addition to primary and secondary lymphoid organ.

DCs represent a key link between the innate and adaptive immune system [30]. Not only T cells [31] but also NK cells are stimulated through direct contact with activated DCs [32]. DCs play a major role in regulating the immune response by releasing cytokines and expresses co-stimulatory molecules. DCs are capable of processing both exogenous and endogenous antigens and present them in the context of MHC class I or II molecules.

NK cell can also induce the activation of DCs by soluble mediators and direct cell to cell contact. Activation of DCs through TLR signalling leads to their maturation where they become efficient antigen-presenting cells [33]. This also leads to redistribution of MHC class I/II from intracellular compartments to the plasma membrane, up-regulation of co-stimulatory molecules CD80 and CD86 as well as adhesion molecules, and secretion of several cytokines [33]. CD80 and CD86 are ligands for CD28 and CTLA-4 [34]. Immature DCs capture antigens by phagocytosis, macropinocytosis and endocytosis. Chemokine responsiveness and chemokine receptor expression play essential roles in iDC migration to lymphvessals and draining lymph nodes. Immature DCs may express the chemokine receptors CCR1, CCR2, CCR5, CCR6 and CXCR1 [35]. iDCs are chemoattracted to the inflammatory sites primarily by MIP-3 alpha/CCL20, and also in response to RANTES/CCL5 or MIP-1 alpha/CCL3[36]. They can migrate to the lymph tissues where they complete the maturation process. Mature DCs express CCR7 [37] that promotes responsiveness to CCL19 and CCL21 [38]. The maturation of DCs decreases their ability to take up of antigen, and leads to up-regulation of MHC class I plus co-stimulatory molecules. Mature DCs presenting antigens to naïve T cells [31], regulate the immune responses by secreting cytokines and chemokines. The co-stimulatory molecules such as CD80, CD86 and CD40, which are expressed on the surface of mature dendritic cells, are critical for the adaptive immune response.

3.4.2. Natural killer cells

NK cells were independently discovered in 1975 by Kiessling [39] and Herberman [40], as a type of lymphocyte that spontaneously kills tumor cells in vitro. Comparing with T and B cells, NK cells have large kidney-shaped nuclei and contain azurophilic granules in their cytoplasm; therefore they were referred to as large granular lymphocytes [41]. NK cells do not express surface immunoglobulin (B cell receptors) or TCR/CD3 and do not require their target cells to express MHC class I. NK cells have the capacity to rapidly kill tumor cells [41], recognize and mediate cytolysis of virus-infected cells [42], as well as shaping the immune responses through secreting cytokines and chemokines [43], and by cell-to-cell cross-talk [44]. Human NK cells comprise about 10-15% of
blood lymphoid cells [45] and can be divided into CD56^{bright/+} and CD56^{dim/-} [46]. CD56 (NCAM/Neural cell adhesion molecule) was suggested to play a role in NK cell adhesion to target cells [47]. CD56^{bright/+} NK cells are CD16^+ and produce high amounts of cytokines such as IFN-γ making them immunoregulatory. They are perforin_{low}, and express low levels of NKp46 [48]. They express the chemokine receptors CCR7, CXCR3, CXCR4 and L-selectin (CD62L), which make them able to extravasate into tissues and lymph nodes [49]. In contrast, CD56^{dim/-} NK cells constitute about 85-90 % of peripheral blood NK cells and about 85% of spleen NK cells. They express CD16 and perforin, tend to be highly cytotoxic toward tumor cells [50] and may secrete IFN-γ [51]. They express NKp30, NKp46 and the killer cell immunoglobulin like receptor (KIR), as well as the chemokine receptors CXCR1 and CX3CR1 [52], which direct them towards inflamed tissues.

Mouse and rat NK cells are defined as CD3^−, NKR-P1^+, NKp46^+ [53]. NK1.1 (CD161), a member of the NKR-P1 receptor family is expressed by NK cells from C57BL/6, SJL and other strains but lacking in other strains like BALB/c [54].

NK cells can be generated in vitro from hematopoietic progenitor cells and/or activated by several cytokines including IL-2, IL-12, IL-15, IL-18 and IFN-α/β [55]. IL-2 activated NK cells are more cytotoxic. Activated NK cells have a dual role as cytolytic cells against the infection/tumor as well as regulatory cells by secretion of cytokines such as (IFN-γ, TNF-α and GM-CSF) and chemokines such as macrophage inflammatory protein-1 (MIP)-1α and (MIP)-1β [56].

NK cells kill target cells by exocytosis of granules containing perforin and granzymes [57]. This happens at the immunological synapse between NK cells and target cells. Perforin is a monomeric protein which has the ability to make channels in the target cell membranes by a calcium dependent polymerization process. The perforin channels allow granzymes to enter the target cells and induce apoptosis [58]. Another killing strategy employed by NK cells is the death receptor-mediated apoptosis. NK cells express several TNF superfamily death ligands such as FasL and TRAIL [59], which induce apoptosis in several tumor cells [60]. Fas is a transmembrane protein in the TNF family which upon binding to Fas ligand (FasL), induces apoptosis in target cells. Freshly isolated NK cells express FasL on the cell surface, and induce Fas-mediated cytotoxicity in Fas expressing target cells [61]. Fas is expressed on immature thymocytes, activated T cells and NKT cells, as well as some tumor cells and virally infected cells [61]. Furthermore, Fas and its ligand play a role in tissue injury in some diseases [62]. Moreover, the FasL on NK cells is regulated upon cytokine stimulation [63]. TNF-related apoptosis-inducing ligand (TRAIL) is a transmembrane protein expressed on immune cells, including NK cells, T cells, monocytes, dendritic cells and neutrophils and induces apoptosis in target cells. Human TRAIL induces apoptosis in target cells via DR4 and DR5 receptors [64]. TRAIL plays a role in tumor surveillance by NK cells and could be a target in cancer therapy [65]. Moreover, TRAIL may play a role in NK cell killing of dendritic cells [66]. Similar to FasL, TRAIL is increased after NK cell activation by cytokines [67].
3.4.2.1. Activation and inhibition of NK cells

NK cells have the capacity to recognize and kill tumor cells, infected cells, and MHC class I disparate hematopoietic cells. NK cell specificity is provided by an array of inhibitory and activating receptors co-expressed on the same cell. The balance between activating and inhibitory signals determines the NK cell response [68]. The activation of phosphatases by an inhibitory receptor leads to dephosphorylation of signaling proteins involved in the activation pathway, thereby blocking the activating signals.

The activating NK cell receptor signals are typically transmitted by small transmembrane adapter proteins which do not interact with ligands. Instead, these adaptor proteins have a glutamic or aspartic acid residue in their transmembrane domain which forms an ionic bond with a positively charged amino acid residue (lysine or arginine) in the transmembrane region of the associated receptor. In this way, the receptor and adaptor proteins are expressed together as a complex in the cell membrane [69].

Ligation of a receptor-DAP12 complex leads to tyrosine phosphorylation of the DAP12 ITAM by a Src-family kinase. Once the ITAM is phosphorylated it acts to recruit and activate the tyrosine kinases Syk or ZAP70 through their tandem SH2 domains. ZAP70 is only expressed by T cells and NK cells while Syk is expressed by myeloid cells, platelets, B cells, NK cells and subsets of T cells [70]. Downstream events after stimulation through ITAM-associated receptors, activates the nuclear factor of activated T cells (NFAT) and lead to increased cytotoxicity and cytokine secretion [165].

CD16, NKp46 and other receptors signal via the ITAM-containing adaptor molecules CD3ζ and FceRIy [71]. These two adaptors can be expressed as homo- or heterodimers. Again, phosphorylation of the ITAMs is mediated by a Src family kinase, thereby facilitating recruitment of Syk and ZAP70 tyrosine kinases.

NKG2D is associated with DAP10 in the human [72], while in the mouse a short NKG2D is associated with both DAP12 and DAP 10, whereas a long NKG2D is associated with DAP10 only [73]. The cytoplasmic region of DAP10 contains a YxxM motif, a SH2 domain binding-site that employs the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) as well as Grb2 [74].

The inhibitory receptors contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic regions. Upon interaction with target cell ligand, the ITIMs become phosphorylated, leading to recruitment and activation of the tyrosine phosphatase SHP-1, resulting in an inhibitory signal [75]. The prototypic ITIM sequence is V/IxYxxV/L. inhibitory NK cell receptors typically contain a pair of ITIMs spaced by 23–25 residues in the case of monomers, or a single ITIM in the case of homodimeric receptors [75]. Some ITIMs may partly exert their function through recruitment of SHP-2 or the inositol phosphatase SHIP-1 [75].
3.4.2.2. NK cell receptors

NK receptors belong to two different protein superfamilies: the C-type lectin superfamily and the immunoglobulin superfamily. Most NK cell receptors are encoded by two multigene complexes; the natural killer gene complex (NKC) (located on human chromosome 12) and the leukocyte receptor gene complex (LRC) (on human chromosome 19) [76]. The LRC contains the KIR superfamily, together with NKp46, CD89 and the LILR family [77]. The NKC contains the killer cell lectin-like receptor (KLR) families. These include Ly49 (KLRA), NKR-P1 (KLRB), NKG2A, -C and -E (KLRC), CD94 (KLRD), KLRF, -H, -I and -K.

Three of the Ig-like NK cell receptors are commonly termed natural cytotoxicity receptors (NCR), despite a lack of close sequence similarity and that only one of them are encoded from the LRC. These are NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3).

**Figure 4.** NK cell activity is regulated by a balance between activating and inhibitory signals derived from surface receptors that interact with MHC class I and other molecules on target cells. In the absence of ligands for activating receptors, the inhibitory signals dominate, and NK cell effector functions are not initiated. In the absence of inhibitory signal, activating signals dominate leading to lysis of target cells and cytokine production.
3.4.2.2.1. NKp30

NKp30 (CD337) is encoded by the NCR3 gene located in the MHC class III region. It associates with FcεRIγ alone or in heterodimeric form with TCRζ [78] and is expressed on both activating and resting NK cells [79]. NKp30 mediates NK cells killing of tumor cells as well as autologous immature dendritic cells (iDCs) [79]. B7-H6 which is expressed by several tumor cell has recently been identified as a ligand for human NKp30 [80]. Further, the intracellular protein HLA-B-associated transcript-3 (BAT3), recently re-termed BAG6, has been reported to be expressed on exosomes released from immature DC or from tumor cells and to serve as a ligand for NKp30 [81;82]. NKp30 is functional in the rat, but the mouse Ncr3 gene is a pseudogene [83]. IL-2 induces up-regulation of NKp30 [84], whileTGFβ1 down-regulates it, impairing NK cell cytotoxicity towards immature DCs [85]. Further, the HCMV protein pp65 has been reported to bind NKp30.

3.4.2.2.2. NKp44

NKp44 (CD336 or NCR2) is exclusively expressed in activated NK cells as well as γδT cells [86]. NKp44 associates with DAP12 and triggers NK cell lysis of tumor target cells [103] as well as virus infected cells. Studies have indicated that NKp44 may bind hemagglutinin of the influenza virus and hemagglutinin-neuraminidase of Sendai virus [87] or the intracellular protein MLL5 (mixed-lineage leukemic protein 5) that becomes expressed on the cell surface in a truncated form.

3.4.2.2.3. NKp46

NKp46 (CD335 or NCR1) is expressed by NK cells independent of activation status [88;89]. NKp46 is highly restricted to NK cells and is not normally expressed on CD3⁺ cells (T and NKT cells) or other hematopoietic cells [89], hence, it is proposed to be a relatively specific marker for NK cells [88;89]. NKp46 can also be expressed by a subset of the recently identified innate lymphoid cell subtypes ILC1 and ILC3. NKp46 has two extracellular Ig domains, and the TM domain contains the positively charged amino acid arginine [104] and associates with FcεRIγ alone or in heterodimeric form with TCRζ [78]. NKp46 triggers NK cell lysis of autologous, allogeneic and xenogeneic target cells [90]. The ligand for NKp46 remains somewhat controversial. Influenza has been reported to bind NKp46. Other studies implicated NKp46 in
bacterial infection, where NK cell killing of mycobacterium tuberculosis infected cells was blocked by anti-NKp46 [91].

NKp46 has also been implicated in controlling tumor metastasis in animals [92], where anti-NKp46 blocks NK cell lysis of tumor cells, demonstrating that NKp46 activated NK cells kill tumor cells and tumor cells expressing NKp46 ligands [89]. Other studies indicated that the absence of NKp46 impaired the eradication of lymphoma cells [93].

3.4.2.2.4. KIR

The human KIR family consists of 14 members, encoded by 13 loci, selectively expressed on NK cells and subsets of T cells. The first KIRs identified were inhibitory, and KIRs with activating function were isolated at a later stage [94]. The KIRs are denoted 2D or 3D dependent on the number of extracellular Ig domains, with the suffix S or L characterizing the short or long cytoplasmic tails, and the suffix P for pseudogene [95]. The inhibitory KIRs have long cytoplasmic domains and contain two ITIMs. The activating KIR has a truncated cytoplasmic region that lack ITIM, but instead associate with DAP12 through a positively charged amino acid residue in the transmembrane region. An exception is KIR2DL4 which carries both an ITIM and a positively charged transmembrane residue. Whereas inhibitory KIR bind to HLA-C (in the case of the KIR2DL), HLA-B (KIR3DL1) or HLA-A (KIR2DL2), ligands for the activating KIR have been difficult to identify. KIR2DS1 has affinity for some alleles of HLA-C, and KIR2DS4 may bind directly or indirectly to HLA-F. In contrast to the existence of KIR multigene families in primates and cattle, rat and mouse contain only one or two KIR, and their functions may diverge from KIR in primates.

3.4.2.2.5. CD94/NKG2

The CD94/NKG2 heterodimeric receptor is expressed on NK cells as well as subset of T cells. It is composed of CD94 (KLRD1) and either NKG2A, -C or –E (KLRC1, -2 or -3) [96]. CD94/NKG2A binds to HLA-E in the human and Qa-1 in the mouse [97]. Human CD94 has a short cytoplasmic region without any known signalling motifs, whereas human NKG2C and NKG2E are activating receptors associated with ITAM containing the adaptor protein DAP12 [98]. In the mouse and rat, these functions have been interchanged, so that CD94 binds the activating adaptors DAP12 and DAP10 and NKG2C and –E appear to lack signalling function. In all species, NKG2A contains two ITIMs and is inhibitory.
### 3.4.2.2.6. NKG2D

NKG2D (CD314 or KLRK1) is an activating homodimer expressed on NK cells, CD8⁺ T cells as well as NKT cells [99]. NKG2D binds to stress-inducible ligands expressed by e.g. virus infected or transformed cells. These include the MHC class I chain-related A and B (MICA and MICB) proteins, expressed on epithelial tissues and several tumor cell types [100], as well as the UL16 binding protein (ULBP) in human [101], the retinoic acid early inducible 1 (RAE-1) family and also H-60 as well as MULT1 in mouse [102]. NKG2D does not structurally belong to the NKG2 family and does not associate with CD94. Human NKG2D associates with DAP10, whereas mouse NKG2D associates with either DAP10 or DAP 12.

### 3.4.2.2.7. Ly49

The human genome encodes only one Ly49 member, the pseudogene Ly49L that lacks parts of the lectin-like domain and therefore is probably not functional [103]. In contrast, the rodent Ly49 families are large, counting close to 30 members in some rat strains. Ly49 are expressed as homodimers on NK cells and small subsets of T cells. The inhibitory Ly49 contain ITIMs in the cytoplasmic region and bind to allelic variants of classical MHC class I, and has been considered rodent functional equivalents of the structurally unrelated KIR in human [104].

The activating Ly4 lack ITIMs [105], but instead contain a positively charged arginine residue in the transmembrane region associated with the adaptor protein DAP10 or DAP12. The inhibitory receptor Ly49A recognizes H-2D^d, H-2D^k and H-2D^p on target cells [106]. The ligation of Ly49A with its ligands inhibits NK lysis of target cells [106]. The activating receptor Ly49D binds several H-2D ligands (H-2D^d, D^e and D^p2) with low affinity [105]. Ly-49H recognizes to the virus-encoded MHC class I-like “decoy” ligand m157, and promotes NK cell cytotoxicity against MCMV [107].

### 3.4.2.2.8. NKR-P1

The mouse and rat NKR-P1 families contain several inhibitory and activating members [108], but only one NKR-P1 exists in the human [109]. NKR-P1 is homodimers, and their expression is restricted to NK and NKT cells as well as granulocytes. NKR-P1 members bind to different members of the Clr family (C-type lectin related, also encoded from the NKC) [110]. The inhibitory NKR-P1 carry ITIM that mediate NK cell
inhibition [111]. Their role in monitoring Clr expression and their impact on NK cell receptor repertoires is subject to further investigation.

3.4.2.3. **Missing self hypothesis**

NK cells express inhibitory receptors that recognize target MHC class I. The missing self phenomenon implies that NK cells kill target cells that do not express sufficient levels of MHC class I, due to lack of inhibition. Tumor cells or virus-infected cells may down-regulate or lack MHC class I molecules to escape recognition by cytotoxic T cells but due to the missing self phenomenon they are instead recognized and eliminated by NK cells [112]. In contrast, healthy cells express normal levels of MHC class I molecules and thus the NK cells require a strong activating signal to overcome inhibition. In the same manner as MHC class I, some Clrs are expressed broadly on hematopoietic cells and may become downregulated on tumor cells, leading to missing-self recognition mediated by inhibitory NKR-P1 [113].

3.4.2.4. **NK cells and cancer**

NK cells were first described by their ability to spontaneously kill tumor cells in vitro. Further studies showed that NK cells interfere with tumor development in vivo [114], and low NK cell activity was associated with increased risk of cancer development [115]. Experiments in animals showed that NK cells migrate towards the lymph nodes after subcutaneous injection of melanoma cells, which crucial to control tumor formation in the draining LNs [116]. IL-2 activated NK cells are very effective in tumor killing. Clinical trials using adoptive transfer of IL-2 activated NK cells together with IL-2 in cancer patients show little benefit effect compared to treatment with IL-2 alone [117]. Activated NK cells produce IFN-γ [118], that promotes the development of Th1 responses associated with the generation of cytotoxic T cells and activation of macrophages which are found to be beneficial to the host response against tumors.

Several studies have shown a role of activating NK cell receptors in recognizing tumor ligands [100]. NKG2D knockout mice have a higher incidence to develop tumors than naïve mice [119]. NKG2D binds to MHC class I-related chains (MIC) A, B and ULBPs, which are up-regulated by cellular stress. In patients with a MIC expressing tumor, shedding of soluble MIC leads to tumor escape from NK cell and T cell lysis [120]. The UL16 binding proteins (ULBPs) are GPI-linked class I-like glycoproteins that are expressed by several tumor types [101]. In mice H60 and retinoic acid early inducible 1 (Rea1) family proteins have been identified as ligands for NKG2D. As another example, the activating receptor NKp30 also recognizes the tumor-specific surface antigen B7-H6 [121].
3.4.2.5. NK cells and infections

NK cells have a critical role in defence against viral infections such as cytomegalovirus, herpes simplex virus, influenza virus and poxvirus [122], and infection with bacteria [123], protozoa [124] and yeast [125]. Several studies have shown that NK cell deficiency leads to severe viral infection in animals [126] as well as in human [127]. Many viruses down-regulate MHC class I expression on infected cells to escape cytotoxic T cell recognition. MHC class I down-regulation or lack of it leads to the recognition and lysis by NK cells which clear the virus infection. Moreover, NK cells directly recognize viral infected cells by expressing activating receptors such as NKG2D in human and Ly49H in mice that detect viral infection. Ligand interaction induces proliferation, cytokine production and cytotoxicity. The human KIR3DS1 activation receptor has been implicated in resistance to HIV [128].

3.4.2.6. DC/NK cell interaction

DCs and NK cells can meet in cell to cell contact in tissue and lymphoid organs including spleen and lymph nodes.

DCs mediate NK cell activation through the release of soluble factors as well as cell-to-cell contacts. Pathogen activation signal via TLRs can induce DC maturation and secretion of cytokines, which can activate NK cells. Mature DCs produce IL-12 that stimulates NK cell secretion of IFN-γ. IL-18 can potentiate the effect of IL-12 by inducing the expression of IL-12R on NK cells. Furthermore, IL-18 synergizes with IL-12 for enhancing NK cell cytolytic activity [129]. Also pDCs by secretion of type I IFN activate NK cell cytotoxicity [130]. IL-15 produced by DC promotes NK cell development and survival. This cytokine can be presented by DCs via its binding to IL15Rα or as transmembrane protein[130].

NK–DC cross-talk may occur either in peripheral tissues or in lymph nodes, where, in both cases, NK cells can encounter distinct myeloid DC subsets. IL-12 secretion by DC in secondary lymph organs is favoring IFN-γ secretion and consequent Th1 polarization. Also, the interaction of CXC3CL1 expressed on DCs with CX3CR1 on NK cells results in IFN-γ release by NK cells [131] and it has been reported that influenza virus-infected DCs can support IFN-γ production by triggering the activating receptors NKp46 and NKG2D [132].

Moreover, activated NK cells can shape adaptive immune responses by causing DC activation and/or eliminating immature DC. NK cell activation can occur by triggering of activating receptors by target cells or by stimulation of soluble factors released by
accessory cells. Activated NK cell releases large amounts of TNF-α and IFN-γ, which affect DC maturation. TNF-α enhances the expression of costimulatory molecules on DCs and, synergizing with IFN-γ, leads to enhanced IL-12 expression by DC [133]. Moreover, exposure of NK cells to IL-12 and IL-18 produced by mDC can promote Th1 polarization [134]. INF-γ can also induce the expression of a membrane-bound form of IL-15 on DCs, supporting NK cell survival and activation [135].

Also, DCs can take up dying cells killed by NK cells and present their peptide fragments on MHC class I molecules to CD8+ T cells [136]. NK cells are believed to kill iDCs, but not mDCs [137]. DC maturation up-regulates surface expression of MHC class I molecules, which may protect mDC from NK cell lysis. Conversely, iDCs, expressing lower levels of MHC class I molecules, are more susceptible to NK cell killing [138].

**Figure 5.** DCs after antigen capturing provides cytokines can stimulate NK cell cytotoxicity. Conversely activated NK cells secrete IFN-γ, TNF-α and GM-CSF that provide maturation signals to DCs. Mature DCs migrate to draining lymph nodes where they present captured antigen to naïve CD4 T cells through MHC class II.
3.5. The central nervous system

The central nervous system (CNS) consists of the brain and spinal cord. The cerebrospinal fluid (CSF) floats in the subarachnoid space between the arachnoid and pial membranes. The CSF is a clear liquid produced from arterial blood by the ependymal cells of the choroid plexus in the ventricles of the brain. The brain lacks a conventional lymphatic system and the immune cells that enter the CNS must cross the blood-brain barrier (BBB). The BBB allows water, lipid-soluble molecules, some gases, as well as selected nutrients such as glucose and amino acids to pass through, while preventing neurotoxins from entering the CNS. Antibodies and other serum protein are too large to cross the blood–brain barrier, and only certain drugs are able to pass [139]. The BBB becomes more permeable during inflammation which allows leukocytes to access the brain parenchyma.

3.6. Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory autoimmune disease of the CNS in which the insulating myelin sheaths of nerve cell axons in the brain and spinal cord are attacked by the immune system [140]. The principal mechanism responsible for this disease is still incompletely understood. Activated T cells attack oligodendrocytes, leading to destruction of myelin sheaths (demyelination). Furthermore, the presence of inflammatory T cells in the CNS triggers recruitment of more T cells, as well as B cells, DC cells, microglia and NK cells.

The role of DC in MS is largely unknown. Normally, the role of DC is to migrate from peripheral tissue to lymph node where they present antigens to CD4+ T cells. The CNS lacks lymphatic vessels, and it is unclear where DCs may interact with T cells to prime encephalitogenic response. It has been observed that myeloid DCs migrate across the BBB and accumulate in the CNS during MS. These cells may differentiate within the CNS to induce both Th1 and Th17 differentiation.

Studies have shown that both CD4+ and CD8+ T cells which promote APCs, express IL-17 in MS lesions. Th17 cells may also produce GM-CSF initiating a positive feedback. Th17 cells may further increase permeability of the BBB by secretion IL-17 and IL-22 that interact with endothelium to allow further attraction of CD4+ subsets as well as other immune cells.

B cells may also contribute to the pathology of MS through antigen presentation, or production of immunoglobulins that react with oligodendrocytes. Memory B cells within the CNS may also play a central role by local activation of T cells.
Some studies have shown that the functional activity and number of blood NK cells in MS patient decreases during the active phases and is restored during remission [141]. As reduced numbers of NK cells were thought to be mediated by migration into tissues including the CNS, this could indicate a pathological role for NK cells. It could also be used as an indicator for new attacks. In support for that, treatment of MS patients with glatiramer acetate (GA) [142], resulted in decreased disease burden associated with increasing NK cell activity. Furthermore, NK cells express CXCR3 which may attract NK cells towards CXCL10, which is constitutively expressed in the CNS [143].

Nitric oxide (NO) is expressed by microglia in chronic MS patients [144]. NO reacts with superoxide anion to generate peroxynitrite, a highly reactive molecule capable of oxidizing proteins, lipids and DNA, resulting in toxicity to oligodendrocytes [144]. Moreover, NO can trigger immune responses that further enhance inflammatory-mediated CNS damage, whereas increased concentrations of NO can lead to enhanced expression of chemokine (C-C motif) ligand 2 (CCL2) on astrocytes, which in turn, leads to infiltration of CD11b cells and additional tissue damage [145]. The cytokine TNF-α is an important factor in the regulation of neuronal apoptotic cell death. TNF-α mRNA expression in blood mononuclear cells is correlated with disease activity in relapsing-remitting MS patients [146], and high IL-6 levels were detected in acute and chronic active MS plaques [147]. Transgenic mice overexpressing IL-6 develop acute neurodegenerative pathology, including ataxia, tremor and seizures suggestive of a profound effect of this cytokine on many components of the CNS [148]. Astrocytes may also produce cytokines that could contribute to the CNS damage [149]. The damage to myelin sheaths disrupts the ability of neurons to communicate, resulting in a wide range of physical and mental symptoms.

Viral infection, in particular Epstein-Barr virus (EBV), has been suggested as a risk factor [150]. In addition, genome association studies have identified the HLA class II genes DRB1*1501 and DQB1*0602 as strong risk factors for MS [151].

MS is a focal inflammatory disease that develops plaques in the white matter in the CNS which can be diagnosed by MRI. MS is three times more common in women than men [152]. The disease typically has its onset in early adult life (20–40). The etiology of the disease remains unknown. Several observations attribute the contribution of the environment [153], vitamin D deficiency [150], season of birth [154] and genetics as possible disease risk factors. MS occurs more frequently in countries with long winter and low exposure to sunlight [155]. Furthermore, MS patients have significantly lower bone mineral densities than healthy people [156] and lower vitamin D₃ levels during MS relapses [157]. In contrast high serum vitamin D₃ level is correlated with reduced relapse risk in MS patients [158].

The disease classified as relapsing-remitting multiple sclerosis shows acute episodes of neurological dysfunction (e.g. muscular weakness, diminished fine motor skills, visual and sensory impairment, gait instability, ataxia, and cognitive dysfunction) followed by periods of partial or complete remission.
3.7. MS animal models

An approach to study MS is to induce the disease in experimental animals and use these models to analyze how the disease develops and evaluate novel therapies. By using experimental animals in a controlled environment and with controlled genetic backgrounds, we can study biological effects and reduce the complexities of the human system.

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease characterized by an ascending paralysis resulting from lymphocytic infiltration of the CNS associated with macrophage and microglia activation [159]. EAE can be induced in mice by administration of peptides derived from myelin proteins together with Freund’s adjuvant and pertussis toxin. In SJL mice, EAE is commonly induced by immunizing with a peptide derived from proteolipid protein (PLP₁₃₉₋₁₅₁). This model induces a disease similar to the relapsing-remitting form of MS, which account for approximately 80% of MS patients [160]. An experimental model more similar to chronic progressive MS (approximately 20% of MS patients), can be induced in C57BL/6 mice by immunizing with peptide derived from myelin oligodendrocyte glycoprotein (MOG). Pathologically, both are associated with focal inflammatory demyelinating lesions in the white matter [160]. In Louis Pasteur’s 1885 rabies vaccination studies, sporadic cases of ascending paralysis were observed in patients who had been vaccinated with spinal cord preparations from rabies virus-infected rabbits [161]. This leads to the development of EAE model in mice.

The role of NK cells in EAE has not been extensively studied, and there are contradictory reports. It has been suggested that NK cells either ameliorate [162] or exacerbate the disease [163]. In some studies depletion of NK cells in EAE resulted in a severe relapsing disease, more pronounced CNS pathology and high production of cytokines [162]. Moreover, depletion of NK cells was associated with increased CD4⁺ T cell activity, suggesting that NK cells may have protective effects by killing these cells [164]. On the wither hand, stimulating NK cells can produce IFN-γ and may cause inflammation and promote Th1 cellular responses and exacerbating the lesion.

In EAE, the peptide antigen is injected subcutaneously leads to a local immune activation in the draining lymph node. There, DCs from the skin injection site meet and present peptide to CD4⁺ T cells that are believed to set off the disease.
3.8. Anti-inflammatory drugs

As yet, there are no available drugs that fully cure MS. Several anti-inflammatory drugs can minimize symptoms during MS exacerbations, slow the progression of disease and reduce the development of new brain lesions. In addition, they slow down the progression of MS, reducing future disability. Some of these drugs carry the risk of serious side effects. Immune modulation in many cases renders the patient susceptible to opportunistic infection. A particularly serious example is JC virus that may cause progressive multifocal leukoencephalopathy (PML), a serious and often lethal infection of the CNS.

Some important drugs used for treating relapsing-remitting MS are:

**Beta interferons**

β-interferon drugs (IFNβ1a: Rebif and Avonex; and IFNβ1b: Betaferon, Betaseron, Extavia) are the most commonly given medications to treat MS. They are injected under the skin or into muscle and can reduce the frequency and severity of relapses.

β-interferon affects the expression of pro- and anti-inflammatory cytokines, leading to a reduction in the number of inflammatory cells that cross the BBB. Neutralizing antibodies may develop, and one important side effect is increased susceptibility to infection.

**Glatiramer acetate**

Copaxone or GA is an immunomodulatory drug created from a copolymer of four amino acids (glutamic acid, lysine, alanine and tyrosine). The mechanism of action for glatiramer acetate is unknown. Some studies claimed that GA shifts T cells from Th1 to Th2.

**Dimethyl fumarate**

DMF is a new oral medication Tecfidera, formerly known as BG-12. The mechanism of action has not been completely understood and will be discussed later. DMF is used to treat psoriasis and can reduce MS relapses. PML has been reported as a very rare side effect of DMF.

**Fingolimod**

Gilenya is an orally administrated drug derived from a fungal metabolite. It is a sphingosine analogue that modulates the sphingosine-1-phosphate (S1P) receptors and prevents T cell migration from lymph nodes to the CNS, discussed in more detail later.
Teriflunomide

Aubagio is an oral medication. Teriflunomide is the active metabolite of leflunomide (Arava). Aubagio is an immunomodulatory drug that works through inhibition of an enzyme involved in de novo nucleotide synthesis. It therefore affects rapidly dividing sells such as activated B and T cells. Side effects include decreased neutrophil and lymphocyte numbers, leading to increased risk of infection and malignancy. It is harmful to the developing fetus.

Natalizumab

Tysabri is a humanized monoclonal antibody toward α4 integrin, and blocks the migration of immune cells from the bloodstream across the BBB to the CNS. It may be considered a first line treatment for some people with severe MS or as a second line treatment in others. It is also used to treat a Crohn's disease. One major side effect of Tysabri is progressive multifocal leukoencephalopathy.

Alemtuzumab

Lemtrada is a humanized monoclonal antibody against CD52. It is used in treatment of chronic lymphocytic leukemia, bone marrow, kidney transplantation and relapsing remitting MS.

The mechanism of action is not entirely clear. It is believed that binding to CD52 on mature T and B cells causes depletion.

Mitoxantrone

Novantrone is used to treat cancer and only rarely applied to treat severe, advanced MS. Mitoxantrone disrupts DNA synthesis and DNA repair in both healthy cells and cancer cells.

3.8.1. Vitamin D₃

The sun is main source of vitamin D₃. In humans, vitamin D₃ is created in the skin as a result of ultraviolet B (UVB) radiation exposure [165]. Pre-vitamin D₃ is photochemically produced in the dermis from 7-dehydrocholesterol [166]. Vitamin D is a lipid soluble vitamin and can be ingested from the diet. Animal vitamin D₃ (cholecalciferol) is found in milk, fat fish and egg, whereas plant vitamin D₂ (ergocalciferol) is found in fruits and vegetables.
Figure 6. Structure formulas of vitamin D$_2$ and vitamin D$_3$.

Vitamin D$_3$ is hydroxylated in the liver to calcidiol (25(OH)D$_3$), which is either stored or circulates in the blood by binding with serum proteins [167]. Vitamin 25(OH)D$_3$ is further hydroxylated in the renal proximal tubule to the biologically active form 1,25(OH)$_2$D$_3$ or calcitriol [168], that has a high affinity for vitamin D receptor (VDR). VDR belongs to the superfamily of the steroid/thyroid hormone nuclear receptors.

Figure 7. Vitamin D$_3$ metabolism.
Calcitriol binds to the VDR in the cytoplasm, and is then transported into the nucleus [169]. VDR heterodimerizes with retinoid X receptor (RXR) a nuclear gene transcription factor. The VDR/RXR/1,25(OH)₂D₃ complex binds to vitamin D₃ response elements (VDRE) in the promoter region of target genes and regulates gene transcription [170].

In spite of its name, vitamin D₃ is actually a steroid hormone. Vitamin D₃ maintains calcium homeostasis by regulating absorption of calcium from the gut and release of calcium from bone matrix. Vitamin D₃ deficiency can cause serious bone diseases such as rickets (in children) and osteomalacia (softening of the bones in adults). Recent evidence indicates that vitamin D₃ deficiency increases the possibility of getting diseases like asthma [171], cardiovascular disease [172], cancer [173] and MS [174].

Several effects of vitamin D₃ on the immune system have been reported.

Vitamin 1,25(OH)₂D₃ suppresses T cell proliferation and skews T cells towards Th2 through up-regulation of inhibitory ligand receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [175]. A reduced Th1 responses means a decrease in the production of TNF-α, IFN-γ, IL-2 and GM-CSF, and increase in IL-4 and IL-10 levels [176]. This enhanced development of Treg cells [177]. More recent studies have shown that 1,25(OH)₂D₃ inhibited proliferation of Th17 cells, and decreased IL-17 production by these cells [178]. VDR KO mice develop fewer invariant NKT (iNKT) cells and these iNKT cells express less IL-4 [179].

Vitamin 1,25(OH)₂D₃ can inhibit B cell proliferation by up-regulating the cell cycle checkpoint regulator p27, a Cdk inhibitor [180]. Consequently, it may prevent further differentiation into plasma cells and thus reduce antibody production [180].

Vitamin 1,25(OH)₂D₃ was found to inhibit DC differentiation and maturation leading to reduced expression of the co-stimulatory molecules CD80, CD83 and CD86 as well as MHC class II [181]. Further, the levels of the pro-inflammatory cytokines produced by DCs are reduced, while anti-inflammatory cytokine levels are increased [181]. Vitamin 1,25(OH)₂D₃ also reduced the expression of CCR7 on myeloid DC [182,183] and decreased the chemotaxis of Langerhans cells towards CCL21, probably due to an inhibitory effect on CCR7 expression [184]. These effects may decrease DC migration towards draining lymph nodes.

Vitamin 1,25(OH)₂D₃ triggers expression of the enzyme inducible nitric oxide synthetase (iNOS) in vitro in human macrophages [185], but decreases iNOS expression in rat EAE [186]. The iNOS enzyme is required for the production of NO by macrophages. The role of NO in MS and EAE is not yet fully clarified, but several studies have shown that NO production in the brain accelerates EAE [187], while others have shown that NO has an immune down-regulating effect in EAE [188].
The effects of vitamin 1,25(OH)₂D₃ on the maturation of NK cells are not well studied. Vitamin 1,25(OH)₂D₃ increased Fas expression on NK cells, resulting in enhancement of cytotoxicity.

Vitamin D₃ deficient mice show acceleration of EAE, while administration of vitamin 1,25(OH)₂D₃ to EAE mice reduced the white matter inflammation and accumulated lymphocytes in the central nervous system [189]. Additionally, low levels of vitamin D₃ have been associated with increased relapse rate in MS patients [190].

Further, high serum vitamin D₃ levels skew Th1 towards Th2 and increase the number of Treg cells [177], associated with less inflammatory lesions and less MS-activity as shown by Magnetic resonance imaging (MRI) [191].

The expression of both TLR7 and TLR8 was increased in the spinal cord in the early phase of the EAE [192]. Treatment of EAE mice with vitamin 1,25(OH)₂D₃ diminished the expression of TLR and inflammatory cytokines IL-1β and TNF-α [193]. Thus, a role for vitamin D₃ in MS is supported by several studies.

### 3.8.2. Calcipotriol

Calcipotriol is a synthetic analogue of calcitriol [194], used to treat psoriasis. Calcipotriol has a high binding affinity to the VDR and is equipotent to vitamin 1,25(OH)₂D₃.

![Figure 8. Structure formula of calcipotriol, a synthetic analogue of calcitriol.](image-url)
3.8.3. Dimethyl fumarate

Dimethyl fumarate (DMF) is a synthetic methyl ester of fumaric acid and is different from fumeric acid, which is naturally occurring in the body. DMF was first used to treat psoriasis.

DMF is marketed as the drug Tecfidera, which has recently been approved by the US Food and Drug Administration as an oral drug for MS patients [195]. The drug was shown to be safe during treatment of 257 patients who received high doses of DMF three times daily [196]. After oral intake, DMF is completely absorbed in the small intestine and only small amounts are excreted in the feces and urine [197]. After absorption DMF (half-life 12 min) [198] is hydrolysed by esterases to the most bioactive form monomethyl fumarate (MMF) [199]. MMF is further metabolized to carbon dioxide and water [200]. The mechanisms of action for MMF are not well known. One effect of MMF is an increased activity of the transcription factor Nrf2 (nuclear factor (erythroid-derived 2)-like 2), leading to increased cellular redox potential and increased levels of antioxidant metabolites including glutathione (GSH). Reactive oxygen species (ROS) play a role in several pathological conditions such as cancer and neurodegenerative diseases [201]. The effects of MMF on the cells in the immune system are not well understood, but the clinical effects of this drug in MS indicate a significant role.

![Dimethyl fumarate and Monomethyl fumarate](image)

**Figure 9.** Dimethylfumarate (DMF) and the metabolite monomethylfumarate (MMF).

The detoxification capabilities of DMF/MMF reduced the production and release of inflammatory molecules, such as TNF-α, IL-1β, and IL-6 and NO from microglia and astrocytes activated with LPS [202;203]. It also increases the production of detoxification enzymes such as nicotinamide adenine dinucleotide phosphate quinone reductase 1 (NQO-1), heme oxygenase-1 and/or cellular glutathione [204], and leads to inhibition of nuclear factor kappa B (NF-kB) translocation into the nucleus. Inhibiting this pathway results in decreased expression of NF-kB dependent genes that regulate the expression of a cascade of inflammatory cytokines, chemokines, and adhesion
molecules [205], which may reduce the damage to CNS cells by modulating the immune system. NQO-1 was also detected in the liver and CNS of DMF treated animals. Asadullah et al. [206] and Vandermeeren et al. [207] showed that DMF also inhibits TNF-α induced expression of ICAM-1, E-selectin, and the vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells [206]. This is correlated with activating Nrf2 [208], which is released from binding the kelch-like ECH-associated protein 1 (Keap-1) [209] via the activity of fumarates. The long-term effect of DMF in neuronal cells is most probably mediated via Nrf2.

DMF/MMF may induce apoptosis of CD4⁺ and CD8⁺ T cells [210], and switching the immune system towards a Th2 anti-inflammatory type response in MS and psoriasis [211]. This could be through inhibition of NF-κB and thereby inhibition of CD1a, CD40, CD80, CD86 and HLA-DR expression [212]. DMF also downregulated NF-kB in B cells, leading to inhibition the antiapoptotic protein Bcl-2 [201]. Moreover, DMF reduced macrophage-induced inflammation in the spinal cord in EAE [213]. It has been reported that MMF increased primary human NK cell lysis of K562 and RAJI tumor cells. Moreover, MMF up-regulated the expression of Nkp46 on the surface of NK cells and this increase was correlated with up-regulation of CD107a on the surface of CD56⁺ NK cells. CD107a (also known as lysosomal-associated membrane protein-1) is present in the membranes of cytolytic granules and expressed on the surface of CD8⁺ T cells and NK cells upon degranulation [214;215]. Furthermore, MMF did not affect killer cell immunoglobulin-like receptor CD158 expression on NK cells. These results suggest that MMF shifts the threshold towards activation rather than inhibition [216].

MMF inhibited the disease course in the EAE animal model [213]. Moreover, it inhibited macrophage infiltration in the spinal cord and increased expression of IL-10, with higher apoptosis rate of CD4⁺ T (Th1) cells in EAE [213]. Furthermore, the animal studies show that MMF crosses the BBB, and may act directly as a cytoprotective agent in the CNS [208].

### 3.8.4. Fingolimod

Fingolimod or FTY720 was the first oral MS therapy under the name Gilenya. Fingolimod is one of disease-modifying therapies, which is effective at reducing relapse rates, slowing disability progression, decreasing brain lesion accumulation and conversion of clinically isolated syndrome to relapsing remitting multiple sclerosis [217].
Figure 10. Fingolimod

FTY720 is composed of 2-amino-2-(2-(4-octylphenyl) ethyl)-propane-1,3-diol, a drug derived from myriocin, a fungal metabolite that resembles sphingosine. Its mechanism of action is related to binding four out of five S1P receptors, namely S1PR₁, S1PR₃, S1PR₄ and S1PR₅ (particularly S1PR₁) resulting in its internalization with a consequent inhibition of S1P activity [218]. The serum factor S1P is released by platelets, is found in serum and plasma [219] and induces multiple biological activities in lymphoid and non-lymphoid cells.

FTY720 inhibits S1P receptor function, and leads to reducing or eliminating the receptors from the cell surface. This makes lymphocytes unresponsive to S1P signals that normally allow them to egress from lymphoid tissues and recirculate to sites of inflammation. Studies have shown that FTY720 modulates lymphocyte egress but activation, proliferation and effector functions of T or B cells are not impaired by treatment with fingolimod.

DCs express all five receptors for S1P [220]. Mouse mDC migrate towards S1P whereas iDCs do not. This migration correlated with the up-regulation of S1PR₁ and S1PR₃ expression during maturation [221]. FTY720 is affects immune responses by impairing DCs migration. Further study shows that FTY720 did not affect the expression of CD80, CD83 or CD86 on the surface of DCs, where the expression of HLA-I and HLA-E was increased by FTY720 [222].

IL-2 activated NK cells also express S1PR₁, S1PR₃, S1PR₄ and S1PR₅, and S1P induced the in vitro chemotaxis of these cells [223]. It was also reported that S1P inhibited NK cell lysis of target cells including tumor cells and DCs [222], and that FTY720 reversed this inhibitory activity [222]. The study indicates that S1P is an anti-inflammatory molecule and it significantly reduced the release of IL-17A and IFN-γ from NK cells, but did not affect the expression of NK activation receptors [222].

FTY720 is able to cross the BBB, and S1P receptors are expressed on most neural lineages and resident CNS cells. By modulating the S1P receptors expressed on CNS cells, FTY720 may have a direct impact on neuropathological processes such as
neurodegeneration, gliosis and endogenous repair mechanisms, as well as neuronal function.

Treatment with fingolimod is associated with cardiac risks that require an electrocardiogram monitored during the first 6 hours after initiation and increase the risk of infections [224].

3.9. Lipopolysaccharide

LPS from Gram-negative bacteria is recognized by the innate immune system by TLR4 [225], expressed on the surface of macrophages, neutrophils, mast cells, dendritic cells and B cells, as well as epithelial and endothelial cells [226]. In vitro studies have shown that LPS induced chemotaxis and induced influx of intracellular Ca²⁺ in human NK cells [227].
4. **AIMS OF THE PRESENT STUDY**

NK cells possibly play important roles in MS. Human NK cells are classified into two major subsets; regulatory cells expressing CD56 but not CD16, and cytolytic cells expressing CD16 and little or no CD56. Based on cytokine release, NK cells have been classified into the subsets NK1, NK2 and NK22. We were interested in examining the effect of drugs used to treat MS on the in vitro activities of human NK cells DCs, as well as in vivo in an animal model of MS.

The aims of **paper I** was to answer these questions:

1- Do subsets of blood NK cells produce IL-17, in resting and IL-2-activated form?  
2- If so, what are the phenotypical features of these cells, in particular with respect to chemokine receptor expression?  
3- Can IL-17-producing NK cells be found in the CNS?

Anti-inflammatory drugs are used to treat autoimmune diseases, including MS. Moreover, vitamin D has been demonstrated to influence the immune system. More knowledge is needed about the detailed effects of anti-inflammatory therapies, including their effects on NK cells and DCs.

The aims of **paper II** was to answer these questions:

1- Is NK cell cytotoxicity against tumor cells and DCs altered after incubation with vitamin 1,25(OH)_2D_3, its analog calcipotriol or the anti-inflammatory drug FTY720?  
2- Do different concentrations of 1,25(OH)_2D_3, calcipotriol or FTY720 have the same effect on CD56^+ NK cells?  
3- Does vitamin 1,25(OH)_2D_3, calcipotriol or FTY720 influence CD56^+ NK cell receptor expression?  
4- Can vitamin 1,25(OH)_2D_3, calcipotriol or FTY720 influence the receptor expression by immature and mature DCs?  
5- Does 1,25(OH)_2D_3, calcipotriol or FTY720 influence NK cell chemotaxis?

The role of NK cells in EAE has not been extensively studied, and there are contradictory reports. It has been reported that NK cells either ameliorate or exacerbate the disease. Depletion of NK cells in EAE mice resulted in a severe relapsing EAE, and more pronounced CNS pathology. This depletion of NK cells was associated with increased CD4^+ T cells activity, all this suggest that NK cells may have protective effect against EAE by shaping the immune system. The biological role of vitamin D₃ in MS comes from the ability of vitamin D to modulate the human immune system. We wanted to investigate the effect of 25(OH)_2D_3, the active form of Vitamin D₃, and MMF on NK cells in the EAE model.
The aims of paper III was to answer the questions:

1- Does vitamin D$_3$ and/or MMF ameliorate EAE disease?
2- Does vitamin D$_3$ and/or MMF activate NK cells to kill dendritic cells?
3- Does vitamin D$_3$ and/or MMF influence surface expression receptors on immature and mature DCs?
5. SUMMARY OF PAPERS

Paper I
Identification of human NK17/NK1 cells

NK cells isolated from healthy donors were cultured with IL-2 for 7 days and investigated by flow cytometry for production of IL-17 and IFN-γ. A CD56⁺CCR4⁺ NK cell subset was stained by intracellular staining with antibodies towards IL-17 and IFN-γ. In contrast to the CCR4⁺ subset, no IL-17 staining was observed with the CCR6⁺, CCR7⁺, CCR9⁺ or CXCR4⁺ subsets. We named the CD56⁺CCR4⁺IL-17⁺IFN-γ⁺ subset NK17/NK1 cells. IL-17 and IFN-γ were detected in the supernatants of purified CD56⁺CCR4⁺ cells. CD56⁺CCR⁺ cells were obtained by two-step magnetic bead purification and analysed by flow cytometry. Our results showed that NK17/NK1 cells did not express IL-23R but expressed the transcription factors RORγt and T-bet as well as NK cell maturation markers. We double-sorted IL-2-activated NK cells, first into CD56⁺ and then into CCR4⁺ or CCR6⁺ cells by antibody conjugated beads. The results showed that the mature NK cell molecules NKp30, NKp44, NKp46, NKG2D, CD158 and CD161 were expressed by some cells in both subsets, whereas the immature cell marker CD127 (IL-7Rα) was not expressed. We observed expression of IL-23R by CCR6⁺ NK cells and not in NK17/NK1 (CD56⁺CCR4⁺) cells. Because NK17/NK1 cells expressed the ligand for CCR4, i.e. CCL22/MDC; we suggested that this chemokine may play a role in the maintenance and/or survival of these cells.

We examined the presence of NK17/NK1 cells in the blood of patients with MS. The results from five different patients showed that NK17/NK1 cells were not found in the blood, but could be generated from the peripheral blood of four patients upon activation with IL-2. We also examined CSF from MS patients. After isolation of non-activated CD56⁺ cells from the CSF of two MS patients, our results showed that isolated CCR4⁺ cells expressed both IL-17 and IFN-γ.

Paper II
Effects of vitamin D3, calcipotriol and FTY720 on the expression of surface molecules and cytolytic activities of human natural killer cells and dendritic cells

First we investigated whether 1,25(OH)₂ vitamin D₃, calcipotriol or FTY720 had any effect on CD56⁺ NK cell lysis of tumor cell or dendritic cell targets. Our results showed that 1,25(OH)₂ vitamin D₃, calcipotriol and FTY720 could to varying degrees, not in every case reaching statistical significance, enhance NK cell lysis of K562 or RAJI tumor target cells as well as immature and mature monocyte-derived DCs.

Moreover, we observed that 1,25(OH)₂ vitamin D₃, calcipotriol and FTY720 increased the fraction of NK cells expressing NKG2D, NKp30 and NKp44 on the surface after 4 h
incubation. NKp46 expression was not significantly altered. Interestingly, 1,25(OH)$_2$ vitamin D$_3$ and FTY720 reduced the staining with a monoclonal antibodies (mAb) towards the killer cell immunoglobulin-like receptors KIR2DL2/3, KIR2DS2 and KIR2DS4.

We sought to examine the effect of these drugs on NK17/NK1 cell activation. In vitro chemotaxis experiments showed that NK17/NK1 cells (CD56$^+$CCR4$^+$) migrated towards MDC/CCL22. We observed increased lysis of K562 cells by NK17/NK1 cells after incubation with 1,25(OH)$_2$ vitamin D$_3$ and calcipotriol.

Looking at how these drugs affect DCs, we found that 1,25(OH)$_2$ vitamin D$_3$, calcipotriol and FTY720 significantly up-regulated the expression of the co-stimulatory molecule CD80 on iDCs after 4h, but this effect was reduced after 24 h incubation. 1,25(OH)$_2$ vitamin D$_3$ and calcipotriol increased the percentages of CCR6$^+$ cells after 4 h incubation with iDCs, but after 24 h incubation the opposite effect was observed.

Next we investigated whether the drugs may modulate the expression of mDC surface molecules. The results showed no significant effects on the expression of HLA-I, HLA-E or CCR6. However, there was a tendency of up-regulating the chemokine receptor CCR7 after incubation with 1,25(OH)$_2$ vitamin D$_3$ or calcipotriol for 4 h.

**Paper III**

**Vitamin D$_3$ and monomethyl fumarate enhance natural killer cell lysis of dendritic cells and ameliorate the clinical score in mice suffering from experimental autoimmune encephalomyelitis**

In this study we found that in vivo treatment with vitamin D$_3$ or MMF ameliorated EAE and improved the clinical score in EAE mice, corroborating previous observations by other groups.

In this paper, we analysed the functional and phenotypic characteristics of freshly isolated NK cells from EAE mice treated with either 1,25(OH)$_2$ vitamin D$_3$, MMF or no drug. We also obtained immature and mature DCs from the same groups of mice by in vitro culture of bone marrow-derived monocytes. We observed that NK cells from 1,25(OH)$_2$ vitamin D$_3$-treated EAE mice lysed bone marrow monocyte-derived mDCs more efficiently than NK cells from control EAE mice.

Similar to 1,25(OH)$_2$ vitamin D$_3$-treated EAE mice, NK cell from MMF-treated EAE mice displayed enhanced lysis of bone marrow monocyte-derived DCs.

These results suggested that one important mechanism of action for MMF and 1,25(OH)$_2$ vitamin D$_3$ in reducing EAE clinical score was to enhance NK cell killing of DCs, reducing the numbers of DCs responsible for activating the disease-causing autoreactive T cells.
6. METHODOLOGICAL CONSIDERATIONS

6.1. Cell purification and culture

6.1.1. Human NK cells (papers I and II)

In papers I and II we isolated CD56+ NK cells from buffy coats from healthy volunteers (Ullevål Hospital, Oslo, Norway) and from blood or CSF obtained from MS patients at Oslo University Hospital. Buffy coat is the thin layer of cells located between the plasma and the red blood cells after density gradient centrifugation of peripheral blood, and mostly consists of leukocytes and platelets. In order to purify NK cells, we used RosetteSep NK Cell enrichment cocktail (StemCell Technologies) which depletes CD3, CD4, CD19, CD36, CD66b, CD123 and glycophorin A on erythrocytes leaving pure NK cells intact after centrifugation over histopaque gradients (Sigma-Aldrich). Human IL-2 activated NK cells were prepared from 20 to 60 million pure NK cells, and cultured in sterile flask at 1 with 200 U/mL human IL-2 (PeproTech) for 7 days. IL-2 was added to the culture on day 2, 4 and 6 days.

IL-2 activated NK cells were in some instances further sorted into CD56+ and CD56− by magnetic separation using EasySep Human CD56 Positive selection kit (StemCell Technologies). To isolate CD56+ cells, NK cells were re-suspended in RoboSep-buffer (phosphate buffered saline (PBS) without Ca2+ and Mg2+, 2% BSA and 1mM EDTA) (Sigma-Aldrich), at 1×10⁸ cells/mL and incubated in EasySep human CD56 positive selection kit. The magnetic nanoparticles were added and incubated at room temperature. After incubation the mixture was placed into the magnet. CD56+ cells were collected, counted and checked for viability.

CCR4+ cells were isolated from the CD56+ cells as a second step using immunomagnetic beads (Dynabeads) coated with anti-human CCR4 (R&D Systems). Recovered CCR4+ CD56+ cells were collected and examined.

6.1.2. Mouse NK cells (paper III)

NK cells were purified from spleens of SJL/J mice. Single cell suspensions were obtained by mechanical disruption of tissue followed by passage through 70 µm cell strainers (BD Biosciences), centrifugation on histopaque (Sigma–Aldrich) followed by negative selection (EasySep mouse NK cell enrichment kit, Stemcell Technologies). Following NK cell purification, cells were subjected to flow cytometry analysis with mAbs towards NKp46, NKG2D and NK1.1. The fraction of NKp46+ cells varied between experiments, in the range of 21 to 83 %, shown in fig. 6 below. Anti-NK1.1 yielded only weak staining, either as a result of a technical problem or possibly to low expression of this marker in the SJL strain.
Mouse NK cells are defined on the basis of surface markers. NKp46 is commonly regarded as the most specific marker for mouse and rat NK cells [228], as it is expressed on all conventional NK cells plus some subsets of innate lymphoid cells (ILC1 and ILC3). Another widely used marker is NK1.1 (NKR-P1), which is expressed on all NK cells in addition to NKT cells and some myeloid cells [53]. NKG2D is expressed on most or all NK cells, but also on cytotoxic T cells [229]. CD49b, recognized by the DX5 antibody, only stains 80-90% of NK cells plus a subset of T cells [230]. Other NK cell markers either fail to stain all NK cells (e.g. CD94, NKG2, Ly49) or stain substantial numbers of other cells [231].

In paper III, the variation in the fraction of NKp46$^+$ cells could reflect variable success in cell purification, yielding cell populations with between 21 and 82% NK cells. In support of this, the percentage of NKG2D$^+$ cells (when tested) closely followed the percentage of NKp46$^+$ cells (data not shown).

Alternatively, it is conceivable that, in SJL mice, NKp46 is only expressed on a subset of NK cells and varied between individuals as a result of external factors or state of the disease. The percentages of NKp46-expressing cells in normal untreated mice were significantly higher than vitamin D3-treated or untreated mice with EAE, but not different compared to MMF-treated EAE mice (Figure 6). This might be coincidental or reflect effects of the immunization process or the disease itself. Our observation that the fraction of NKp46$^+$ cells was not reduced in EAE mice treated with MMF could reflect a drug effect of MMF. Alternatively, that too could be related to the EAE disease process. The onset of clinically appreciable EAE was delayed by approximately 6-7 days in mice treated with MMF compared to vitamin D3-treated or untreated EAE mice.
The NK cells were sampled on day 15, in which case the untreated or vitamin D3-treated mice had been sick for approximately 10 days, whereas the MMF-treated mice had only presented clinical signs for 3-4 days. Thus, it could be argued that the percentage of NKp46+ cells correlated with the time since onset of clinically visible EAE.

Given the possibility that the purity of NK cells may have varied between experiments, we adjusted for this by dividing the cytotoxicity values by the fraction of NKp46+ effector cells and also excluding experiments with less than 20% NKp46+ cells (data not shown). With these adjusted numbers, similar differences between groups (MMF, vitamin D3) and killing of iDCs or mDCs were still observed.

We found that variation in NKp46 expression did not correlate strongly with the killing of iDC and mDC. Based on this finding, we concluded that the expression of NKp46 was not correlated with the cytolytic effects of NK cells against iDC and mDC.

6.1.3. Human monocytes and dendritic cells (paper II)

In paper II we isolated human monocytes from buffy coats using RosetteSep Human monocyte enrichment cocktail (Stem Cell Technologies) which depletes CD2, CD3, CD8, CD19, CD56, CD66b and glycophorin A on RBCs leaving monocytes intact. Monocytes were cultured in sterile Petri dishes at 1.5x10⁶/mL with 6 ng/mL human IL-4 and 25 ng/mL human GM-CSF (PeproTech) for 5 days to generate immature DCs. Mature dendritic cells were generated by adding 1 µg/mL LPS (Sigma-Aldrich) to the cultures of iDCs for additional 2 days, as described [232].

6.1.4. Mouse monocytes and dendritic cells (paper III)

Bone marrow cells were flushed from the tibia and femur of mice. After histopaque (Sigma-Aldrich) separation, the monocytes were purified using EasySep mouse monocyte Enrichment kit (StemCell Technologies). iDCs were generated by culturing of bone marrow-derived monocytes in the presence of GM-CSF and IL-4 for five days by a standard protocol [17]. On day six, LPS was added to the culture for 36-40 hours to induce mature DCs [233]. To examine the purity and maturation status of DC cell cultures, we stained with mAbs towards CD80, CD86, CD40, CD205 (DEC-205) and E-cadherin and analysed by flow cytometry. The relative expression of these markers varied between experiments, probably reflecting variations in culture purity as well as in maturation stages. Recent studies have shown that culturing bone marrow-derived monocytes in GM-CSF (without IL-4) results in a heterogeneous cell population that comprised at least two cell types, which both undergo maturation in response to LPS [234].
6.2. Induction of EAE in SJL mice

In paper III, female SJL/J (H-2k) mice were immunized subcutaneously with 200 µg of PLP₁₃₉₋₁₅₁ peptide (ABBIOTEC) emulsified in complete Freund’s adjuvant containing 1 mg Mycobacterium tuberculosis (Sigma-Aldrich) at four sites in the right and left flanks. Following each injection, 200 ng of Bordetella pertussis toxin (PTX) (EMD chemicals) was injected intraperitoneally after 0 and 48 h of immunization. The animals were independently observed and monitored daily, and the EAE clinical score was measured according to the following scoring scheme. 0 = no clinical disease, 1 = tail flaccidity, 2 = hind limb weakness, 3 = hind limb paralysis, 4 = forelimb paralysis and 5 = moribund or death. Female SJL/J mice at four to six weeks of age were obtained commercially and kept under conventional conditions in the in-house animal facility for a week before entering into experiments. The mice were euthanized with CO₂ before harvesting organs.

6.2.1. Drug treatment of EAE mice

SJL/J mice were divided into four groups and each group consisted of 10 mice. The first group was left as normal control, in the remaining three groups group EAE was induced. In group 2, mice were gavaged with vehicle as control. Mice in the third group were injected intraperitoneally with 100 ng 1α,25-Dihydroxyvitamin D₃ (Sigma-Aldrich) in 100 µL PBS every other day. The fourth group of mice was given 1 mg MMF (Sigma-Aldrich) in 100 µL PBS daily by oral gavage. On day 7 post immunization, bone marrow cells were collected for DC culture, and on day 15 spleens were isolated for NK cell isolation.

6.3. Cytotoxicity assay (papers I - III)

In papers II and III we used the Calcein-acetyoxymethyl method to measure cytotoxicity [235]. NK cells were prepared as previously described. Target cells were incubated with Calcein-AM (Sigma-Aldrich), washed and seeded in 96-well flat bottom plates with effector cells at various effector:target (E:T) cell ratios in triplicate for 4 h at 37°C in a 5% CO₂. To measure total killing, target cells were incubated with 0.5% Triton–X 100 (Sigma-Aldrich) for 30 min. Total lysis of target control cells was estimated by incubating target cells with media only. The fluorescence intensity of the calcein AM-loaded target cells was measured with A Bio-Tek FLX 800 plate reader (Bio-Tek Instruments Inc), using 485/528 nm fluorescence filters. The percentage of cytotoxicity was calculated according to the following formula: % viability = fluorescence units (FU) of targets incubated with NK cells (experimental), minus FU of targets incubated with detergent (total lysis), divided by FU of targets incubated in media only (total viability), minus FU of targets incubated with detergent (total lysis). Percent cytotoxicity was then calculated as 100% minus % viability.
In the calcein-AM cytotoxicity assay, the dye has a good retention in targets and low pH sensitivity. Further, there is no stain transfer among cells. The calcein acetoxyethyl ester is a lipid-soluble molecule that passively crosses the cell membrane and is then converted by intracellular esterases into a polar fluorescent product (calcein) which is retained by cells with intact membranes but released by damaged cells. Lytic activity could be determined by measuring the fluorescence intensity by plate-reading fluorimeter retained in living cells or by measuring the release of calcein in the supernatants recovered from cytotoxicity assay. The advantages of this method are; the ability to use a low number of effector cells, the ability to recover cells and supernatants after the assay for additional biological analyses such as FACS and ELISA and finally rapid data acquisition. The major disadvantage we have experienced in this method is the risk of losing cells during washing, resulting in overestimation of cytotoxicity results. The traditional chromium-51 (Cr⁵¹) release assay is accurate and reliable but relies on radioactive materials.

6.4. Flow Cytometry analysis

Cells were washed and re-suspended at 0.3× 10⁶ cells per well in FACS-buffer (PBS with 2% FBS and 10 mM NaN₃), then labelled with 2µg/mL of various antibodies in the dark for 45 min at 4°C. Marked cells were washed twice in FACS buffer, resuspended in PBS and analyzed in a flow cytometer (FACS Calibur or FACS Canto II, BD Biosciences).

For intracellular staining, cells were first labeled with antibody towards a surface molecule, then fixed with 4% paraformaldehyde for 20 min, and permeabilized by incubation in PBS, 0.1% saponin and 0.05% NaN₃. After that, the cells were stained with antibodies against intracellular molecules. Labeled cells were washed twice with FACS-buffer, resuspended in PBS then analyzed in a flow cytometer.

Two-step labelling was used with non-conjugated primary antibodies. The cells were first incubated with primary antibody in the dark for 45 min at 4°C, washed twice in FACS buffer and incubated in the dark for another 45 min at 4°C with secondary antibody (FITC-conjugated goat anti-mouse Ig, BD Biosciences). Stained cells were washed twice with FACS buffer, resuspended in PBS and then analyzed in a flow cytometer.

Data analysis was done by FlowJo software, and the number of positive cells was determined in comparison with isotype control experiments.

6.5. ELISA quantification of cytokine release

Cell culture supernatants were collected and kept at -70 °C until tested for the concentrations of IL-17 and IFN-γ were determined in triplicates with human
Quantikine ELISA Kit (R&D Systems) according to the manufacturer’s instructions, and examined at 450 nm (Power wave XS plate reader, Bio-Tek).

6.6. Chemotaxis assay (paper II)

CD56⁺ CCR4⁺ NK cells (5 × 10⁵) were placed in the upper wells of modified Boyden chambers, whereas MDC/CCL22 was placed in the lower chambers in 1000, 250, 100, 50 or 10 ng/mL concentrations. The cells were separated from the chemokine by 8 μm Nuclepore membranes (Whatman). After 2 h of incubation at 37 °C, the filters were removed, dehydrated, stained with 15% modified Giemsa stain for 7 min, and then mounted on glass slides and inspected in a light microscope with a 100x oil immersion lens. Each sample was counted as the sums of cells in ten fields of vision. Average values from triplicate experiments were used to calculate the migration index (as the number of cells migrating towards the concentration gradients of MDC/CCL22 divided by the number of cells migrating towards medium only).

7. ETHICAL CONSIDERATIONS

The ethical aspects of collecting blood and CSF samples from patients and healthy volunteers, was approved by the local REK committee (Ullevål Hospital and Oslo University Hospital). The patients and healthy volunteers were informed and had signed their consent prior to blood donation.

Animal procedures were approved by the Norwegian animal research authority (FOTS) and the Department of comparative medicine, Institute of basic medical sciences.
8. GENERAL DISCUSSION

8.1 NK17/NK1 cells

In paper I, we described the identification of a novel NK cell subset, NK17/NK1, characterized by expression of CD56, CCR4, IL-17 and IFN-γ. We found that this subset constitutes less than 2% of NK cells in the blood, but is substantially increased following IL-2 activation. Interestingly, NK17/NK1 cells were detected in high numbers in the CSF of MS patients, whereas their proportion of blood NK cells was normal. It is possible that CD56⁺CCR4⁺ (NK17/NK1) cells migrate from the periphery into the CSF guided by the ligand for CCR4 (i.e. CCL22) which is detected in the brain of MS patients and EAE mice [236]. Alternatively, NK17/NK1 cells arise locally in the CNS tissue, polarized by cytokines produced in the local inflammation. Of note, NK cells are only found in very low numbers in the normal brain [237]. Thus far, we have no information as to whether NK17/NK1 cells contribute to the autoimmune inflammation in MS, or whether they are non-important bystanders.

To my knowledge, NK17/NK1 cells have not yet been described in rodents. The increased proportion of NK17/NK1 cells in the CSF in MS suggested that this NK cell subset might be found also in other autoimmune diseases, in local areas of autoimmune inflammation. This warrants further investigations in both animal models and human clinical materials.

We also found that NK17/NK1 cells expressed the transcription factors RORγt (similar to Th17 cells) as well as T-bet, which is expressed by Th1 cells. These transcription factors are also important markers for several recently identified subsets of innate lymphoid cells (ILC). Although the field is still remain slightly unsettled, the level of consensus is increasing. Classical NK cells are closely related to ILCs, and are discriminated from ILC1 and ILC3 by expression of the transcription factor EOMES. ILC1 produce IFN-γ and may or may not express T-bet but do not express RORγt. ILC3, on the other hand, have been reported to express both RORγt and T-bet, and subsets of NCR3 cells produce IL-17 and IFN-γ. These cells do not express NKP46, however. Thus, in the light of recent evidence, it would be interesting to investigate the relationship between the NK17/NK1 cells that we have described and the NCR⁺ ILC3 and ILC1 subsets. Looking for EOMES expression in the NK17/NK1 cells may help to establish whether this subset is derived from classical NK cells or belongs to the ILC3 (or ILC1) groups.

8.2 Drug effects on NK cells

We have investigated the effects of three drugs that are either approved, or have the potential for treating multiple sclerosis (MS) patients, on the in vitro activities of human natural killer (NK) cells and dendritic cells (DCs).
We found that vitamin D₃ up-regulated the expression of NKp30 and NKp44 as well as NKG2D on the surface of NK cells (paper II) leading to increased NK lysis of tumor target cells. This finding is supported by another study showing that 1,25(OH)₂ vitamin D₃ increased NK cell-mediated killing of melanoma cell lines by up-regulating the expression of Fas on the surface of NK cells [238].

FTY720 is the first oral therapy for MS, acting through inhibition of S1P activity [218]. IL-2-activated NK cells express S1P₁, S1P₃, S1P₄ and S1P₅, and S1P induced the in vitro chemotaxis of these cells [223]. Our finding that FTY720 enhanced NK cell lysis is in accordance with a previous report showing that S1P inhibited NK cell lysis of target cells, including tumor cells and DCs [239]. Our group has also previously found that FTY720 reversed this inhibitory activity [222].

We correlated the cytolytic activity with the ability of these drugs to up-regulate the expression of activating NK cell receptors, and found that vitamin D₃, calcipotriol and FTY720 enhanced surface expression of NKp30, NKp44 and NKG2D. NKp30 induce NK cell killing by binding to the ligand B7-H6, expressed by tumor cells [80]. NKp30 has also been reported to be involved in NK cell lysis of iDCs by binding to an alternative ligand, BAG6, expressed on iDCs [81]. NKp44 has recently been found to bind to an alternatively spliced form of the intracellular protein MLL-5, frequently expressed by tumor cells [240]. A tumor target ligand recognized by NKp46 has not yet been described; it was suggested that glycosylation of tumor cells is required for recognition by NKp46 [241], but the role of NKp46 in NK-mediated killing of cancer cells is unclear. Finally, we found upregulation of the type II transmembrane protein NKG2D, which recognizes MICA and MICB as well as ULBP ligands expressed on stressed cells, including tumor cells [242]. Collectively, it can be suggested that one mechanism of action for vitamin D₃, calcipotriol and FTY720 in ameliorating disease is by up-regulate the expression of NKp30, NKp44 and/or NKG2D on the surface of NK cells. In addition, vitamin D₃ and FTY720 down-regulated KIR expression, but the antibody used did not discriminate between activating and inhibitory KIRs. Of course, down-regulation of inhibitory KIR may also increase NK cell cytolytic activity, as could several unknown drug effects not directly related to surface receptors.

8.3 Do vitamin D₃ and MMF affect NK cells in EAE mice?

Studies on the MS animal model EAE showed that vitamin D₃ deficiency accelerated the disease, while administration of calcitriol to EAE mice reduced the white matter inflammation and lymphocytes accumulation in the central nervous system [189]. Vitamin 1,25(OH)₂D₃ decreased the morbidity and mortality of EAE disease in the SJL mouse model [243] and completely prevented myelin basic protein-induced EAE as well as adoptive transfer of EAE in the B10.PL strain [244;245]. To confirm our in vitro observation of the effect of vitamin D₃ on NK cells and to compare the effect of vitamin
D₃ with that of MMF, we induced EAE in SJL/J mice. We observed that vitamin D₃ and MMF inhibited and/or prevented EAE in these mice, in line with the above.

Although we have found that vitamin D₃ augments NK cell cytotoxicity in vitro, the combined effects of this drug in vivo might have opposite effects. Merino et al. [246] found that vitamin 1,25(OH)₂D₃ affected NK cytotoxicity by decreasing IL-2 and IFN-γ produced by T cells, which negatively affected CD16⁺ NK cell generation from peripheral blood lymphocyte (PBL) cultures. IFN-γ is able to increase NK cells cytotoxicity [247], while IL-2 is a potent activator of NK cell proliferation and cytotoxicity [247]. Also, vitamin 1,25(OH)₂D₃ can inhibit proliferation of Th17 leading to a decrease IL-17 production [178]. NK cells also have several important roles in shaping the immune system through the secretion of cytokines and chemokines. The functional activities of NK cells are variable in individuals and was generally lower in MS patients than in healthy individuals [248].

It was previously reported that MMF affects the differentiation and polarization of DCs [249]. We observed no significant effects of MMF on the expression of CD80 in iDCs, or mDCs, after treating the mice with vitamin D₃ or MMF. No effect on the expression of CD205 was seen in iDCs, but vitamin D₃-treatment reduced the expression of this molecule on the surface of mDCs.

We showed that vitamin D₃ and MMF reduced the clinical score in mice suffering from EAE. Importantly, vitamin D₃ enhanced NK cell lysis of mDCs cultured from bone marrow monocytes isolated from similarly treated mice.

MMF enhanced NK cell lysis of DCs, and NK cells isolated from mice suffering from EAE significantly killed iDCs and mDCs isolated from similarly treated mice. These results suggested that one important mechanism of action for MMF in reducing the EAE clinical score is via removing the DCs responsible for activating disease-promoting T cells.

8.4 Effects of Vitamin D3, MMF and FTY720 on dendritic cells

DCs can express all five receptors for S1P [250]. FTY720 can affect the immune responses by impairing DC migration. In kidney transplantation, FTY720 proved powerful in suppressing allograft rejection, by inhibiting egress of lymphocytes from lymphoid tissue [251]. The number of NK cells was not affected in these patients [252].

We also observed that the three drugs down-regulated the expression of HLA-I after both 4 and 24 h of incubation with iDCs, suggesting that one effect of vitamin D₃, calcipotriol and FTY720 is to impede presentation of antigens to T cells. In contrast, no effect of any of the drugs was observed on mDCs. It was previously reported that generation of tolerogenic DCs from MS patients occurred after incubation with vitamin
These cells down-regulated the expression of CD83, CD86 and HLA-DR among other molecules after incubation with vitamin D₃. We also observed reduced expression of CCR6 in iDCs after 24 h incubation with vitamin D₃ or calcipotriol, thereby suggesting that vitamin D₃ or calcipotriol may encourage mDCs to migrate into secondary lymphoid tissues and, in particular, the peripheral lymph nodes.
9. CONCLUSIONS

We discovered a subset of CCR4+ NK cells which expressed IL-17 and IFN-γ after culture in IL-2, and named this subset NK17/NK1 cells.

NK17/NK1 cells were found in the CSF of patients with MS without activation with IL-2. In contrast, NK17/NK1 cells were not found in the blood of MS patients, but could be induced after culture on IL-2.

The biologically active metabolite of vitamin D₃, its analog calcipotriol and FTY720, drugs either approved or with the potential for treating autoimmune diseases, enhanced IL-2-activated NK cell lysis of K562 and RAJI tumor cell lines in vitro.

Vitamin D₃ and MMF reduced EAE clinical scores in the SJL mouse model.

In vivo treatment with vitamin D₃ or MMF enhanced the capacity of NK cells isolated from these mice to lyse immature and mature DCs, suggesting a mechanism that could partly explain the clinical effect of these drugs.
10. REFERENCES LIST


134. Agaugue S, Marcenaro E, Ferranti B, Moretta L, Moretta A. Human natural killer cells exposed to IL-2, IL-12, IL-18, or IL-4 differently modulate priming of naive T cells by monocyte-derived dendritic cells. Blood 2008; 112(5):1776-1783.


11. APPENDIX: PAPERS