The Role of Polymorphonuclear Neutrophils in Inflammatory Condition

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Zusammenfassung

Neutrophile bilden die größte Population an Leukozyten im Blut und sind eine der ersten Immunzellen am Ort von Infektionen. Für deren Bekämpfung sind sie mit einer Vielzahl an Mechanismen ausgestattet. Sie eliminieren Mikroorganismen: (I) durch Phagozytose und intrazellulärer Degradation, (II) durch Freisetzen der Effektormoleküle in den extrazellulären Raum, (III) durch die Entlassung von Strukturen, die aufgebaut sind aus Chromatin assoziiert mit Zytoplasma-, Granula-, Kernproteinen und als NETs bezeichnet werden. NETs sind eine sehr effiziente Waffe gegen Pathogene, aber aberrante NETs-Bildung scheint auch assoziiert zu sein mit Autoimmun- und nicht-infektiösen Erkrankungen.

Im ersten Teil dieser Arbeit wurden Serumproben von Patienten untersucht, die an Multipler Sklerose (MS) erkrankt sind. Damit sollte die Frage beantwortet werden, ob NETs auch in der Pathogenese von MS eine Rolle spielen. Es konnte dabei eine signifikant höhere Anzahl an NETs Strukturen in Serumproben von RR-MS Patienten im Vergleich zu gesunden Kontrollen gemessen werden. Weiterhin wurden in den Serumproben dieser Patienten eine erhöhte Konzentration an C-reaktiven Protein, IL-8, eine höhere Anzahl an Monozyten im Blut und eine erhöhte Anzahl an Mikropartikel von aktivierten Thrombozyten gemessen, was auf eine mögliche Entzündung oder Infektion hindeutet.

Nicht nur bei der MS sondern auch bei einer Vielzahl weiterer Autoimmunerkrankungen spielen T-Lymphozyten pathogenetisch eine bedeutende Rolle. Deshalb wurde im zweiten Teil der Arbeit der Effekt von NETs auf T-Lymphozyten im Allgemeinen untersucht. Dabei konnte gezeigt werden, dass der direkte Kontakt von NETs mit isolierten $CD4^+$ T-Lymphozyten zur Ausbildung von Clustern, der Hochregulierung von CD25 und CD69 und der Phosphorylierung von ZAP-70 führt, aber nicht ausreicht um die Zellen zu aktieren. Jedoch führt eine anschließende Stimulation mit einem suboptimalen Stimulus oder einer niedrigen Antigenkonzentration zur Aktivierung, charkterisiert durch die Produktion von proinflammatorischen Zytokinen und Zellproliferation. Während die Inhibierung der T-Zellrezeptorsignalkaskade mittels Herbimycin A zur Aufhebung des NET Effektes führte, hatte die Inhibierung vom TLR 9 durch Chloroquine keinen Einfluss auf die Aktivierung.

Zusamenfassend konnte in dieser Arbeit eine Subgruppe von RR-MS Patienten identifiziert werden, die wahrscheinlich als Folge einer Entzündung oder Infektion eine erhöhte Anzahl an NET Strukturen im Serum aufweisen. Außerdem konnte in dieser Arbeit zum ersten Mal gezeigt werden, dass NETs T-Lymphocyten primen können, indem sie die zur Aktivierung notwendige Schwelle reduzieren und dadurch ermöglichen, dass T-Zellen bereits mit einer

niedrigeren Antigenkonzentration oder sogar mit einem suboptimalen Stimulus aktiviert werden können. Der Mechanismus ist TLR9 unabhängig, erfordert aber direkten NETs-Zellkontakt und den Signalweg über den T-Zellrezeptor. Mit der NETs-vermittelten Aktivierung von T-Lymphozyten konnte im Rahmen dieser Arbeit neben der direkten Eliminierung von Pathogen eine neue Funktion von PMNs entdeckt werden, welche gleichzeitig eine Brücke bildet zwischen angeborenen und erworbenen Immunsystem.

Abstract

PMNs are the most abundant circulating leukocyte and one of the first defenders against invading microorganisms. They are equipped with a variety of defense mechanisms such as antimicrobial proteins or radical oxygen species and kill pathogens: either by engulfment and intracellular degradation, by releasing noxious molecules or by releasing NETs - chromatin fibers composed with nuclear, granule and cytoplasmic proteins. NETs are most likely the most efficient defense mechanism, nevertheless, aberrant formation might also be associated with non-infectious and autoimmune diseases.

In this study, serum samples from MS patients were analyzed to answer the question as to whether NETs are involved in the pathogenesis. The level of circulating NETs was significantly higher in a subgroup of RR-MS patients compared to healthy controls. Significantly higher CRP concentration, increased IL-8 concentration, higher frequency of monocytes in the blood and enhanced amounts of activation- induced platelet-derived microparticles were measured and hint to infections and inflammation as putative triggers.

Since MS is considered a $CD4^+$ T cell-mediated inflammatory and demyelinating disease of the CNS, the putative effect of NETs on T cells was investigated. This study revealed that direct NETs contact with purified T cells leads to cluster formation, increases CD25 and CD69 surface expression and mediates phosphorylation of ZAP-70 but was not sufficient for full activation as characterized by production of pro-inflammatory cytokines such as IFN-γ and IL-17 and T cell proliferation. However, NET-primed T cells could be activated upon subsequent stimulation with a sub-optimal stimulus or with lower concentrations of specific antigen. T cell activation was completely blocked by the inhibition of the TCR-signaling using herbimycin A whereas the toll-like receptor 9 inhibitor, chloroquine, did not show any effect on cell activation.

In summary, this study identified a subgroup of MS patients with circulating NETs in the periphery. Inflammatory mediators, present in serum, are probably responsible for this phenotype. Furthermore, this study`s data demonstrates that NETs can prime T cells directly by reducing their activation threshold. NETs-mediated priming increases T cell responses to specific antigens and even responses to sub-optimal stimuli, which would not induce a response in resting T cells. This mechanism is TLR9-independent but requires NETs-cell contact and TCR signaling. NETs-mediated T cell activation adds to the list of PMN functions and demonstrates a novel link between innate and adaptive immune responses.

Content

1 INTRODUCTION

The human body constantly encounters a variety of pathogenic mircroorganisms such as bacteria, virus, fungi and parasites. Invasive and unwelcome organisms have to be recognized efficiently and eliminated in order to protect against dangerous infection. Therefore, higher vertebrates such as humans developed a complex and sophisticated immune system. Rapid responses, as well as specific targeting of invading pathogens, are carried out by two different parts of the immune system – the innate and the adaptive immune response.

Both types of immune responses have diverse effector cells, specificity, receptor diversity and memory. In case of an infection, cells of the innate immune response (monocytes /macrophages, granulocytes, dendritic cells, natural killer cells and mast cells) recognize a broad range of pathogenic structures, pathogen-associated molecular patterns, by a small number of germ-line encoded pattern recognition receptors (PRRs), e. g. toll like receptors (TLRs). Conversely, cells of the adaptive immune response, B and T cells express a unique B cell receptor (BCR) and T cell receptor (TCR) that comprise a much wider range of different receptors and are much more specific than PRRs expressed by cells of the innate immune system. Another main difference in both types of immune responses is the immunological memory. Whilst the second exposure to a pathogen provokes the same character of innate immune response as the first exposure, B and T cells additionally form antigen-specific memory cells that respond faster and stronger after repeated infection with the same pathogen[1,2].

1.1 Polymorphonuclear neutrophils – a major effector cell of the innate immune response

Polymorphonuclear neutrophils (PMNs) are the most abundant circulating leukocytes and one of the first defenders against invading microorganisms. They derive from hematopoietic stem cells and leave the bone marrow terminally differentiated[3], meaning that PMNs are fully equipped with the whole arsenal of antimicrobial molecules and enzymes which are stored in granules[4]. Therefore, it has been assumed for a long time that mature PMNs have low or no capacity of *de novo* protein synthesis but it has been shown that isolated human PMNs can express a variety of effector molecules, e. g. cytokines and chemokines either constitutively or under inflammatory conditions [5-7].

PMNs have a tightly regulated life cycle and show a rapid homeostatic turnover in the periphery. Once released from the bone marrow, they circulate through the periphery for up to 10 hours[8,9] and undergo spontaneous apoptosis in the absence of any infection. Apoptotic PMNs are mainly cleared by Kuppfer cells in the liver[10] but also by macrophages in the bone marrow and the spleen, as demonstrated in mice[11]. Under inflammatory conditions, the life-span of PMNs can be prolonged to several days[12] and apoptotic cells are eliminated by macrophages at the site of inflammation. After sensing an infection PMNs emigrate from the blood vessels into extravascular tissue and use different effector mechanisms to eliminate microbes. One of their main functions is oxidative burst, a process resulting in the production of reactive oxygen species (ROS) by non-mitochondrial reduction of oxygen[3]. Additionally, they contain anti-microbial molecules that are stored in granules. PMNs have two options-either to engulf microorganisms by phagocytosis and kill microbes intracellularly in phagolysosoms filled with ROS and anti-microbial proteins/peptides or extracellularly by disgorging noxious molecules into the extracellular space. In the latter case, oxidative burst and degranulation destroy the pathogens but can also harm the surrounding tissue. Due to this reason, release and clearance of PMNs are tightly regulated[13,14]. Granulocyte-colony stimulating factor (G-CSF) is the major cytokine governing the production and survival of PMNs. Thus, it has been reported that deficiency of G-CSF or its receptor causes severe neutrophenia in humans[15]. The production of G-CSF can be influenced by PMNs themselves. At inflammatory sites, apoptotic PMNs are engulfed by macrophages. The clearance of the dead PMNs indirectly suppresses G-CSF production. Activated macrophages release interleukin (IL)-23 that triggers IL-17 production by T cells[16] and this in turn stimulates G-CSF production in bone marrow stromal cells. Uptake of apoptotic PMNs by macrophages suppresses the IL-23 production[17] that leads to decreased G-CSF production. This feedback loop[18], which has been demonstrated in mice, represents one mechanism that tightly regulates granulopoiesis and may limit unimpaired PMN recruitment and tissue breakdown.

1.1.1 Neutrophil extracellular traps

Recently, the formation of neutrophil extracellular traps (NETs) was discovered as a new effector mechanism. NETs are extracellular fibres composed of chromatin, granule proteins and other PMN proteins that are able to trap and kill microbes[19] (Figure 1). These structures can bind gram-positive[19,20], gram-negative bacteria[19,21] as well as fungi in yeast and hyphal form[21].

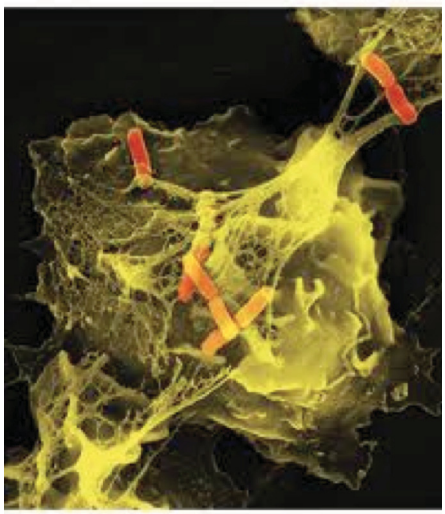

Figure 1 PMNs have trapped Shigella bacteria by Neutrophil extracellular traps. Source: http://stke.sciencemag.org/content/vol2007/issue379/

NETs were observed at inflammatory sites of spontaneous appendicitis[19], periodontitis[22], cystic fibrosis[23] and sepsis[24] but also in non-infectious diseases such as pre-eclampsia[25] and autoimmune diseases such as small vessel vasculitis (SSV)[26], system lupus erythematodes (SLE)[27] and psoriasis[28,29].

So far, different stimuli could be identified as NET inducers, e. g. microbes, fungi, IL-8, phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS), activated platelets[19,24] and placental microparticles[25]. Fuchs et al. first described the NET-forming process termed NETosis as a cell death process, which is clearly distinct from apoptosis and necrosis. Stimulation of PMNs with a potent NET inducer facilitates disappearance of the membrane of PMN granules and disintegration of the nuclear envelope into vesicles. Nuclear, cytoplasmic and granule components merge when the cell membrane is still intact. Finally, NET structures are spilled out after disruption of the outer cell membrane. The NET-forming process is dependent on ROS production by NADPH oxidase as PMNs isolated from patients suffering from chronic granulomatosus disease (CGD) cannot produce NETs upon stimulation *in vitro*[30,31]. CGD patients have a deficient NADPH oxidase that leads to severe bacterial (e. g. *Staphylococcus aureus* and *Salmonella* species) and fungal infections (e. g. *Candida* and *Aspergillus* species). Furthermore, NET formation requires chromatin decondensation and histone citrullination that is mediated by the peptidylarginine deiminase 4 (PAD4)[32-35], which is constitutively expressed in mature PMNs[36]. Most recently, Papayannopoulos et al. have demonstrated that the granule proteins neutrophil elastase (Ela2) and myeloperoxidase (MPO) also mediate decondensation of chromation that enable NET formation[37].

The formation and release of extracellular traps (ETs) might not be restricted to PMN granulocytes. Yousefi et al. could show that interleukin (IL)-5/interferon-γ (IFN-γ)-primed eosinophils are able to spill out their mitochondrial DNA upon LPS stimulation *in vitro* and could observe such ETs in patients with Crohn's diseases[38]. Koeckritz-Blickwede et al. demonstrated that mast cells, both human and murine, release ETs after stimulation with PMA or bacterial pathogens such as *Streptococcus pyogenes*[39]. Furthermore, monocytes and

macrophages have been reported to be able to release ETs upon stimulation with statins *in vitro* [40,41].

1.2 T cells – an effector cell of the adaptive immune system

T cells derive from hematopoietic stem cells and mature in the thymus. They are characterized by expression of a highly diverse antigen receptor, the TCR[42]. Unlike the B cell receptor antigen, TCR antigen recognition requires peptide presentation by human leukocyte antigen (HLA) molecules. The repertoire of TCRs is generated by somatic recombination at DNA level of TCR-encoding gene segments during T cell development[1]. Since gene rearrangement occurs randomly, T cells that either express "nonsense" TCR or a TCR with high affinity to self-antigens have to be selected. T cells expressing TCRs with intermediate affinity for self-peptide MHC complexes are poitively selected, while those with very low affinity die by neglect and those with high affinity are eliminated by apoptotic cell death (negative selection). Both positive and negative selection of immature T cells takes place in the thymus and are considered mechanims of central tolerance[43,44]. Mature autoreactive T cells that escaped clonal deletion in the thymus can be inactivated in the periphery by different mechanisms, e. g. clonal deletion, anergy and Tregs (peripheral tolerance)[45,46], although these peripheral control mechanisms are as yet poorly understood.

Mature T cells express the pan-T cell marker CD3 and one of two co-receptors CD4 or CD8. Depending on the latter they are denoted as $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells exhibit cytoxic properties and therefore are also termed cytotoxic T cells. They are responsible for killing of virally infected cells and tumor cells by releasing cytotoxins such as perforin and granzyme[47,48]. They recognize the cognate antigen associated with HLA class I molecules. HLA class I is expressed by all nucleated cells in the body[49].

$CD4^+$ T cells are also known as T helper (Th) cells and mainly contribute to elimination of infections by providing signals such as cytokines to regulate the immune response. They are able to activate macrophages[50,51], to provide B cell help[52] as well as growth factors, i. e. help to $CD8^+$ T cells[53]. Naïve T cells differentiate into different subsets of T helper cells, which secrete different cytokines and fulfill distinct effector function[54,55]. In contrast to $CD8^+$ T cells $CD4^+$ T cells bind to the cognate antigen associated with HLA class II molecule. HLA class II is mainly expressed in professional antigen-presenting cells (APCs), e. g. dendritic cells (DCs), monocytes, macrophages and B cells[1,2], but under inflammatory conditions other cell types such as endothelial cells can also express IFN-γ-induced HLA class II molecules[56-58].

1.2.1 CD4$^+$ T cell activation

The antigen-specific activation of T cells requires three signals. The first one is provided by direct TCR-peptide/HLA class II contact[59], the second signal by interaction of co-stimulatory molecules[60] and the third signal is delivered by cytokines[2].

CD4$^+$ T cells scan the environment of the local lymph node for the presence of professional APCs presenting antigens. After encounter with cognate antigen, both cells form a structure termed the "immunological synapse"[61]. The immunological synapse is a well organized structure within the T cell-APC contact area composed of accumulated TCR-peptide/HLA complexes stabilized by adhesion molecules, e. g. lymphocyte-function associated antigen-1 (LFA-1) and inter-cellular adhesion molecule-1 (ICAM-1) binding[62-64]. CD3 and co-receptors such as CD4 that are important for intracellular TCR signaling mediate the specific binding of peptide/HLA class II complexes[65,66]. Co-stimulatory molecules (e. g. CD28:CD80/CD86 or cytotoxic T-lymphocyte antigen 4 (CTLA-4): CD80/CD86)[60,67-71] are assembled in the immunological synapse to contribute to signal transduction and subsequent T cell activation. The formation of the immunological synapse results in phosphorylation of certain intracellular domains of the CD3- and TCR molecules and leads to further signaling events such as calcium-influx and gene expression (transcriptionfactor, e. g. NFAT and NF-κB and subsequent secretion of e. g. cytokines such as IL-2).

The magnitude of TCR stimulation is defined by the TCR-peptide/HLA binding strength (affinity), the sum of interactions of all costimulatory and adhesion molecules and co-receptors within the immunological synapse (avidity) and the duration of antigenic stimulation[72,73,74]. The threshold of activation depends both on the affinities of these interactions and their timing[75]. The threshold of activtion can be further modulated e. g. by change of the TCR-signaling pathways as shown in memory CD4$^+$ T cells[76]. They respond faster and stronger than naïve CD4$^+$ T cells in response to a stimulus[77-79].

CD4$^+$ T cells can also be activated by superantigens in an antigen-unspecific manner (e. g. staphylococcal enterotoxin B). Superantigens are often derived from bacteria and are able to cross-link the HLA-class II molecule and the TCR promoting T cell proliferation and cytokine production[80,81].

1.2.2 CD4$^+$ T helper cell subsets and their functions

Due to the enormous variety of pathogens that enter the human body, the immune system has to provide T effector cells that elicit appropriate and successful immune responses. The

differentiation of activated naïve T cells into distinct helper cell subsets is dependent on the cytokine milieu present at the time of activation and on the type of pathogen. The environment regulates the expression of characteristic transcription factors that drive the differentiation of naïve T cells into various T helper subsets termed Th1, Th2, Th17 or inducible regulatory T cells (iTreg) (Figure 2).

Macrophages produce IL-12[82] that drives expression of signal transducer and activator of transcription (STAT)1/STAT4[83] and T-bet (human homolog: TBX21)[82,84] in response to intracellular pathogens. These are necessary for Th1 cell differentiation and the production of its major effector cytokine IFN-γ. Th1 cells are required for activation of macrophages[50,51] and cytotoxic $CD8^+$ T cells[53].

Infections with extracellular pathogens and parasites are controlled by Th2 cells and their effector cytokines IL-4, IL-5 and IL-13[85]. Their differentiation is driven by IL-4-mediated[86] STAT6[87] expression that in turn induces GATA3 expression[88]. Recently, different groups could demonstrate that not only professional APCs but also basophils can function as APC in the context of Th2 differntiation. They co-express major histocompatibility complex class II and CD80/CD86 and are able to induce Th2 differntiation in an OVA-induced allergen mouse model and in mice infected with *Trichuris muris*, respectively[89-91].

TGF-β and IL-6[92] mediate Th17 cell differentiation. They can be found at sites of infections with extracellular pathogens such as fungi. Th17 cells are also potent inducers of PMNs[18]. The expression of RORγt[93] after activation of STAT3[94] governs Th17 differentiation and the production of IL-17A and IL-17F.

Th1 and Th17 cells are involved in the development of organ-specific autoimmune diseases[95], and Th2 cell can be associated with allergy[96]. Besides these a fourth helper subset has been defined, i. e. regulatory T cells (Tregs). They have immunomodulatory potential and are important for regulation of T cell responses e. g. in the context of maintaining tolerance to self-antigens[97-99]. Tregs are either produced in the thymus as mature T cell subpopulation, also term natural Tregs, or they can be induced from naïve $CD4^+$ T cells[98]. The differentiation of naïve $CD4^+$ T cells in inducible regulatory T cells (iTregs) in the periphery is mediated by TGF-β[100]. Both, nTreg and iTreg, express the transcription factor Foxp3[101] and produce cytokines such as TGF-β, IL-10 and IL-35[102] in order to regulate ongoing immune responses. Previously published date have demonstrated that both types of Tregs are necessary for maintaining peripheral tolerance[103].

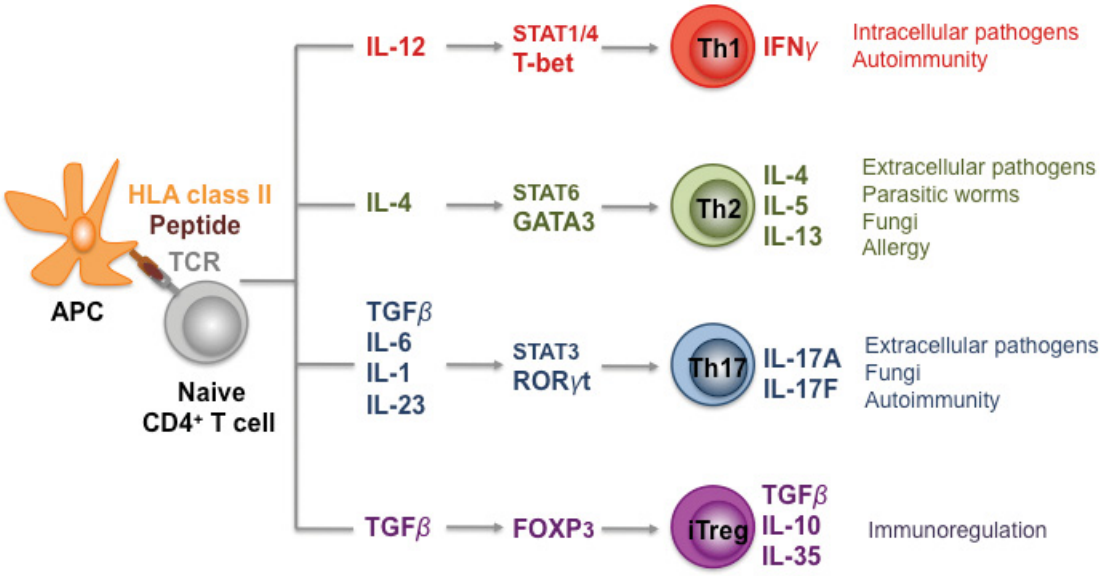

Figure 2 Differentiation of CD4$^+$ T cell subsets and their functions.
Activated naïve CD4$^+$ T cells differentiate into Th1, Th2, Th17 or iTregs depending on the antigen and cytokine microenviroment.

1.3 Crosstalk between polymorphonuclear neutrophils and other immune cells

The function of PMNs is not only restricted to the detection and elimination of microbial pathogens. PMNs also contribute to the activation of immune cells of the innate and the adaptive immune system[3]. They release effector molecules that attract monocytes, immature DCs and plasmacytoid DCs (pDCs)[104-106] or support the differentiation and activation of DCs and tissue-resident macrophages[105,107]. PMNs also contribute to the maturation, proliferation, survival and cytotoxic activity of natural killer cells *in vitro*[108109]. In addition to their effect on cells of the innate immune system, PMNs are able to orchestrate cells of the adaptive immune system. PMNs can support the maturation, proliferation and survival of B cell by release of B-lymphocyte stimulator (BLyS) and a proliferation inducing ligand (APRIL)[110,111]. They also facilitate T cell response (see next part 1.3.1).

PMNs release low amounts of cytokines and effector molecules compared with other cells. However, since they are very abundant at sites of inflammation, the overall release of mediators can be sufficient to influence the immune respose[3].

1.3.1 Polymorphonuclear neutrophils and T cells

Alongside their prominent role in the first line of defense against pathogenic invaders, PMNs have been shown to influence T cell responses in several ways. They can have pro-

inflammatory as well as anti-inflammatory effects depending on the milieu at infected site. PMNs can secrete cytokines that modulate $CD4^+$ T cell differentiation into various effector T helper subsets. PMN-derived IL-12 can drive Th1 differentiation in a murine model of Pneumonia induced by *Legionella pneumophila*[112] and in other murine models for infectious diseases[112,113]. They are also able to induce the differentiation of human $CD4^+$ naïve T cells into Th17 cells in *vitro*[114]. It is not only T cell differentiation that can be affected by PMN-derived cytokines. PMNs produce chemokines that recruit T cells to sites of infection. Activated PMNs can secrete CCL2/CXCL10 and CCL2/CCL20 that attract T_h1 and T_h17 cells *in vitro*, respectively[115]. PMNs granule proteins such as cathepsin G, azurocidine and α-defensin have also chemoattractive properties for T cells [104,116]. Furthermore, the serine proteases Cathepsin G and PMN-derived Elastase are also able to increase T cell proliferation *in vitro* in mouse and human[117,118]. Additionally, several *in vitro* studies show that PMNs can function as APC. Upon IFN-γ and GM-CSF exposure, purified human PMNs are able to upregulate MHC class II expression[119,120] and induce superantigen-mediated T cell activation[119,121] or present peptide antigens to T cells[120,122].

The PMN-T cell communication also occurs the other way around. Cytokines produced by T cells support granulopoiesis both directly and indirectly. Furthermore, they also support the recruitment and activation of PMNs through induction or secretion of CXCL1, CXCL8, tumor necrosis factor, granulocyte-macrophage colony-stimulating factor and G-CSF[115,123]. Finally, T cell-derived IFN-γ prolonges the life-span of PMNs at inflammatory sites[12].

In addition to their pro-inflammatory effects, PMNs constitutively express and store high amounts of arginase I in their granules. T cell activation can be suppressed by the release of arginase I *in vitro* by activated PMNs, which causes depletion of L-arginine, a crucial molecule for T cell activation[124]. Additionally, PMN-derived radicals such as nitric oxid species, can act as T cell suppressor[125]. Interestingly, the cytokine milieu as well as direct cell-cell interaction seem to influence the properties of PMNs. Thewissen et al. have demonstrated that the suppressive effect on T cells depends on direct cell-cell contact. When both cell types are separated by a transwell the release of soluble factors results in an enhanced T cell response[114].

1.4 Multiple sclerosis

Multiple sclerosis (MS) is an inflammatory and demyelinating disease affecting the central nervous system in young adults[126]. MS is considered a $CD4^+$ T cell-mediated autoimmune

disease that appears in genetically predisposed individuals but may require additional environmental factors. The strongest genetic risk factor in MS are the HLA class II molecules HLA- DRB1*15:01, -DRB5*01:01 and –DQw6 (corresponding genotypes: DQA1*01:02, DQB1*06:02)[127]. While the HLA-DR15 haplotype confers by far the most of the genetic risk, more than 50 quantitative trait loci are also involved albeit with much lower contribution at the level of the individual gene[128,129].

The Role of environmental factors as possible triggers of the disease is indicated by geographic variations in the incidence and prevalence of MS. Different environmental factors are proposed such as low sunlight exposure, vitamin D deficiency, diet, air pollution or cigrattes smoke[130]. Furthermore, infectious agents seem to be involved in the pathogenesis of MS as several studies with MS patients show that disease exacerbations (relapses) often follow viral infections[131-133]. Furthermore, late infections with measles, mumps, rubella and Epstein-Barr virus in young adults can increase the risk of developing MS[134,135]. Two mechanisms are hypothesized as to how viral infections can contribute to the pathogenesis of MS: molecular mimicry[136,137] and bystander activation. When self and viral peptide share sequence similarities, cross-reactive recognition of viral and self epitope can occur and may result in activation of autoreactive T cells, e. g. Lang and colleagues have identified a T cell receptor from a MS patient which recognize both a DRB1*1501-restricted myelin basic protein (MBP) and DRB5*0101-restricted Epstein-Barr virus peptide[138] which could support the concept of molecular mimicry in MS. Other studies in mice and with human T cell clones also revealed a variety of virus and bacterial peptides which share immunological epitopes with MBP[139-142]. Interestingly, Hemmer et al. discovered that peptides, different in all amino acid positions, were able to stimulate the TCR of the same HLA-class II restricted autoreactive $CD4^+$ T cells clone[143] pointing to that sequence homology might not mandatory necessary to activate autoreactive $CD4^+$ T cells.

Bystander activation can result in activation of autoreactive T cells during viral infection due to the local inflammatory cytokine milieu. Killing of virally infected cells can also cause damage of the surrounding tissue. During this process self-antigens are released that can be taken up and subsequently presented by local professional APCs to autoreactive T cells[126]. Furthermore, microbial-derived superantigens can evoke T cell activation independent of the specific antigen by cross-linking of HLA class II molecules with the TCR[80]. Brocke and et al. demonstrated that staphylococcal enterotoxin B may induce relapses in experimental autoimmune encephalomyelitis (EAE), the murine model of MS[144]. Bystander activation can

also be induced by pathogen-pattern recognition independent from the antigen e. g. LPS induced EAE [145].

1.4.1 Pathogenesis

MS is considered a $CD4^+$ T cell mediated autoimmune disease of the CNS, although other cells also contribute to the pathogenesis[126]. Potential autoantigens are myelin proteins (myelin basic protein (MBP)[138], myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP)[146], neurofilament M[147], α-B crystalline[148] and oligodendrocyte-specific glycoprotein [149], although the data is most solid for the three mylin proteins MBP, MOG and PLP.

Acute inflammatory immune response in the CNS is evoked by autoreactive $CD4^+$ T cells. They become activated in the periphery and express adhesion molecules that facilitate migration through the blood-brain-barrier (BBB). Transmigrated cells become locally reactivated by CNS resident APCs[150]. Subsequent release of proinflammatory cytokines and chemokines attracts other immune cells from the peripheral blood ($CD8^+$ T cells, B cells, monocytes and mast cells) and activate CNS-resident cells such as microglia and astrocytes[126]. The accumulation of activated cells and the release of proinflammatory molecules/mediators, proteases, oxygen and nitrogen radicals results in formation of inflammatory lesions characterized by damage of myelin sheaths, oligodendrocytes and axons. The resolution of inflammation is facilitated by secretion of anti-inflammatory cytokines and secretion of various growth factors by resident CNS cells and T cells[151-153]. Additionally, activated oligodendrocyte precursors have the capability to remyelinate axons to a certain extent[154]. Over a period of time, repeated acute neurological episodes and incomplete recovery can progress into a neurodegenerative phase caused by chronic activation of microglia and characterized by oligodendrocyte depletion, neuronal and axonal degeneration.

Lucchinetti and colleagues have identified four different pathologic MS subtypes (Pattern I-IV) based on cell compositions of lesions[155]. This can provide an explanation for the high heterogeneity of disease courses of MS patients. Pattern I shows areas of active demyelination associated with T cell and macrophage infiltration. Patter II typically shows lesions with antibody (specific for MBP and MOG) and complement deposition. Injury in Pattern III is mainly associated with either inflammation-induced vascular damage or macrophage-derived toxins. Pattern IV is characterized by nonapoptotic oligodendrocyte degeneration that could be linked to a genetic defect, which results in susceptibility of oligodendrocytes to injury.

MS can be subdivided into two main subtypes (Figure 3). 85-90 % of patients show a relapsing-remitting MS (RR-MS) disease type that typically starts between 20 and 40 years of age and shows higher incidence in women than in men (2:1)[126]. RR-MS patients suffer from occasionally occurring inflammatory events (relapses) with full or incomplete recovery (remission). The clinical definite diagnose of RR-MS is today based on magnetic resonance imaging (MRI) criteria termed "McDonald Criteria"[156]. Criteria are fulfilled if lesions are disseminated in time and space within the CNS (brain and spinal cord). Furthermore, 95 % of patients show intrathecal immunoglobulin synthesis indicated by the presence of oligoclonal bands in the cerebrospinal fluid[157]. Over time, the disease course is characterized by incomplete recovery and accumulation of neurological symptoms/deficits, so that about 65 % of the patients develop secondary progressive MS (SP-MS)[158]. SP-MS is characterized by progressive neurological worsening without or only rarely occurring relapses. Between 10-15 % of patients suffer from a progressive disease course from the onset, termed primary progressive MS (PP-MS). In comparison to RR-MS, the disease begins later in life (40 years) and is characterized by no distinct relapses and minor or no improvement. Both genders are equally affected[126].

Patients wih a first acute neurological episode are termed clinically isolated syndrome (CIS) patients.

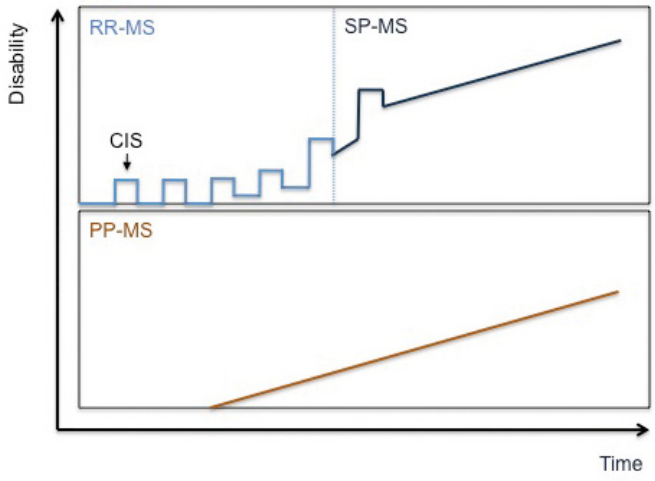

Figure 3 Clinical courses of multiple sclerosis.

1.4.2 Polymorphonuclear neutrophils and MS

The relevance of PMNs in the pathology of MS has been controversial to date, as MS patients` lesions are mainly characterized by infiltrations of activated macrophages, microglia

and lymphocytes[159]. PMNs are only observed in MS-like diseases such as neuromyelitis optica (NMO), also known as Devic's disease, optical spinal MS or in the more aggressive Marburg's variant of MS[160-163].

Interestingly, PMNs seem to enter the CNS during the preclinical and acute phase of disease in several models of EAE[164-166]. The development of an acute phenotype of EAE is completely abrogated upon depletion of $Gr1^+$ cells (Gr1 is mainly expressed by PMNs)[167] or upon the blocking of the relevant chemokine receptor CXCR2[164,168]. Blocking of CXCR2 also seems to prevent the disruption of the BBB[164], as BBB breakdown can be initiated by PMN proteases[169,170]. PMN-attracting chemokines like CXCL1/2(mice)/CXCL8 (human) are expressed by CNS resident cells in EAE as well as in peripheral blood in MS[162,164,171-173]. IL-17 is a crucial inducer of such chemokines and is also elevated on mRNA level in blood, mononuclear cells isolated from cerebrospinal fluid and brain tissue of MS patients[174,175]. Furthermore, it is essential for the development of EAE[176]. Kroenke and colleagues recently identified two different subsets of T helper cells as initiators of clinically identical EAE. IL-12-mediated Th1-induced EAE is characterized by elevated pro-inflammatory chemokines such as CXCL9/10/11 and subsequently CNS infiltration of monocytes and lymphocytes. Conversely, Th17 cell-induced EAE displayed high levels of PMN-attracting chemokines in the brain and spinal cord leading to PMN infiltration in the CNS[177]. So far the mechanisms as to how PMNs contribute to the pathology of EAE as well as the role of PMNs in MS remain in need of clarification, as findings from studies with PMNs from MS patients are quite conflicting. Some groups have found significantly higher neutral protease activity in the purified PMNs of active MS patients compared to inactive MS[178,179] and Aoki et al. could demonstrate that the activity of medullasin, a serine protease, is increased in relapse several days before onset of exacerbation and diminished in remission[180]. Further studies show a primed (pre-activated) phenotype of peripheral PMNs in MS patients that is characterized by altered effector functions, e. g. oxidative burst, altered expression of surface molecules and delayed apoptosis[181,182]. In contrast, some studies show unaltered PMNs properties or even reduced chemoattractive, phagocytic and bactericidal activity in peripheral blood of MS patients[183-185].

2 AIMS

Polymorphonuclear neutrophils (PMNs) appear to contribute to the pathogenesis of experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis (MS). In contrast, the relevance of PMNs in the pathogenesis of MS is quite controversial to date, as MS patients` lesions are mainly characterized by infiltrations of activated macrophages, microglia and lymphocytes. The aim of this study was the functional characterization of PMNs in inflammatory conditions and to determine a putative role in the pathogenesis of MS.

Specific aims:

Investigation of PMN effector functions

In order to identify potential differences of PMN effector functions in MS patients and corresponding controls, both, purified PMNs and whole blood were analyzed by ELISA and FACS assays to investigate oxidative burst, degranulation, phagocytosis and NET formation.

Identification of potential triggers of NET formation in MS

Potential triggers of NET formation were analyzed in order to identify putative mechanisms that could lead to NET formation in MS patients. Different assays were used to measure soluble factors in serum samples by ELISA and cell populations/microparticles form MS patients by flow cytometry.

Analysis of NET-mediated T cell responses

Due to the central role of T cells in autoimmune diseases and the previously revealed contribution of NETs in the development and perpetuation of autoimmune diseases, the participation of NETs in T cells responses was examined more generally by *in vitro* cell culture experiments. Activation markers, cytokine production and proliferation were measured to elucitate whether NETs potentially could regulate T cell activation.

3 MATERIAL AND METHODS

3.1 Materials

3.1.1 Blood donors

Healthy donors were recruited from the Blood Bank at the University Medical Centre Hamburg-Eppendorf (UKE). All patients were recruited from the inims outpatient clinic and day hospital at the UKE. RR-MS diagnosis was based on the revised McDonald criteria[156]. Patients, who had not received steroids at least four weeks prior to enrollment or any immunomodulatory or immunosuppressive agent during the last three months, were considered untreated and included in the study. Patients were classified into two groups, patients with the first manifestation of multiple sclerosis, but not yet definitive diagnosis termed CIS and RR-MS. The CIS and RR-MS groups included patients that were either neurologically stable for at least 30 days before sampling (remission) or exhibited an acute episode of neurological worsening lasting for more than 24 hours at the time of sampling (relapse). Patients did not show clinical signs or symptoms of acute infection. The group of control patients included patients suffering from inflammatory neurological diseases other than MS (OIND), patients with non-inflammatory neurological diseases (OND) and patients with neuromyelitis optica (NMO). The characteristics of healthy controls (HC), MS and control patients are listed under each experiment.

This study was approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg), and written informed consent was obtained from all patients and controls before blood was drawn.

3.1.2 Reagents

Media and Supplements

	Company	**Cat #**
HBSS-	Invitrogen	1417503
HBSS+	Invitrogen	14025050
Hepes	Invitrogen	15630-080

Interleukin-2, recombinant	Roche, Tecin	
Lymphocyte Separation Medium (Ficoll LSM 1077)	PAA	I15-004
Phosphate Buffered Saline (PBS)	PAA	H15-011
X-vivo 15, serum free medium	Lonza	BE 04-418F

Chemicals and others

	Company	**Cat #**
Bovine serum albumin (BSA) Reagent diluent	R&D	841380
Chlorquine	Sigma Aldrich	C6628
Dextran 200	Roth	8946.1
Dihydrorhodamine (DHR)	Sigma-Aldrich	D1054
Dimethylsulfoxid (DMSO)	AppliChem	A3672
Diphenyleneiodonium chloride	Calbiochem	300260
Fluoresbrite™microspheres 1 µm	Polysciences Inc.	1572
Fluoromount-G™	Southern Biotech	0100-01
Herbimycin A	Sigma-Aldrich	H6649
^{3}H-Thymidine	Hartmann Analytic	MT6038E
Hoechst 3325	Sigma-Aldrich	B1155
Micrococcal Nuclease (MNase)	Wothington Biochemical	4797
N-formyl- Methionyl-Lencyl-Penylalanine (fMLP)	Sigma-Aldrich	47729
Paraformaldehyde	Roth	0335
Phorbol myristyl acetate (PMA)	Sigma-Aldrich	P1585
Poly-L-lysine	Sigma-Aldrich	P4707
Sphero™Rainbow Fluorescent Particles	BD	556291

Triton X 100	Roth	3051
Tryptan blue solution, 0.4 %	Sigma-Aldrich	T8154
Tween 20	Sigma-Aldrich	P1379

Antibodies

	Clone	**Company**	**Cat #**
Anti-CD1c APC	AD5-8E7	Miltenyi Biotec	130-090-692
Anti-CD3 Pe-Cy7	UCHT1	eBioscience	25-0038-42
Anti-CD4 APC	RPA-T4	eBioscience	17-0049-42
Anti-CD4 FITC	RPA-T4	eBioscience	11-0049-42
Anti-CD8 PB	DK25	Dako	PB984
Anti-CD11c PerCP/Cy5.5	3.9	BioLegend	301624
Anti-CD14 PB	MϕP9	BD Biosciences	560349
Anti-CD16 APC-Cy7	3G8	BioLegend	302018
Anti-CD16b FITC	1D3	Beckman Coulter	IM2353
Anti-CD19 PB	HIB19	BD	560353
Anti-CD31 PE	WM-59	eBioscience	12-0319-42
Anti-CD25 PE	BC96	eBioscience	12-0259-42
Anti-CD40 PE	HB14	Miltenyi Biotec	130-094-135
Anti-CD42a FITC	GRP-P	AbD Serotec	MCA1227F
Anti-CD45 PE-Cy7	HI30	eBioscience	25-0459-73
Anti-CD62P APC	AK-4	BD	550888
Anti-CD69 FITC	FN50	BD	555530
Anti-CD80 PE	2D10	BioLegend	305208
Anti-CD83 APC	HB13e	BD	551073

Anti-CD86 FITC	BU63	Dako	F7205
Anti-CD123 PE-Cy7	6H6	BioLegend	306010
Anti-CD141 APC	AD5-14H12	Miltenyi Biotec	130-090-907
Anti-CD303 APC	AC144	Miltenyi Biotec	130-090-905
Anti-elastase (Ela2)	39A	AbD Serotec	4050-0096
Anti-HLA-DR/DP/DQ FITC	TU39	BD	555558
Anti-myeloperoxidas (MPO)	MEM-56	Invitrogen	MHCD45RA18
Anti-ZAP70	17a/P-ZAP70	BD	557818
mIgG1 APC	P3.6.2.81	eBioscience	17-4714-42
mIgG1 APC-Cy7	X40	BD	348812
mIgG1 FITC	MOPC-31C	BD	550616
mIgG1 PB		Dako	X0987
mIgG2b PE		eBioscience	12-4031-82
mIgG1 PE	MOPC-31C	BD	550617
mIgG1 PE-Cy7	P3.6.2.8.1	eBioscience	25-4714-42
mIgG2a (isotype for MHCII block)	MOPC-173	BioLegend	400224

Kits

	Company	**Cat #**
Assserchrom®Human β-thromboglobulin Elisa	Roche	11875370011
Blood Dendritic Cell Isolation Kit II	Miltenyi Biotec	130-091-379
CD4 T lymphocyte enrichment set, human	BD	558131
Naïve CD4 T cell enrichment set, human	BD	558521
Memory CD4 T cell enrichment set, human	BD	558520
CD8 T lymphocyte enrichment set, human	BD	557941

Cell Death Detection ELISA^{PLUS}	Roche	11774425001
Click EdU Flow Cytometry Assay Kit	Invitrogen	A10202
Human PMN-Elastase Platinum ELISA	eBioscience	BMS269
Human IFN-β ELISA	BioLegend	430102
Human IL-2 ELISA	R&D	DY202
Human IL-4 Antibody Pair	Invitrogen	CHC1283
Human IL-8 Antibody Pair	Invitrogen	CHC1303
Human IL-10 Antibody Pair	Invitrogen	CHC1323
Human IL-17A ELISA	eBioscience	88-7176
RayBio® Human PF4 ELISA	RayBiotech, Inc.	ELH-PFA-001
Human sCD144 (sVE-cadherin) ELISA	PromoCell GmbH	PK-EL-60266
Quant-iT™PicoGreen® dsDNA Assay Kit	Invitrogen	P7581

3.1.3 Buffers and Solutions

	Company	**Cat #**
BD Cytofix™	BD	554655
BD FACS clean	BD	340345
BD FACS Flow	BD	342003
BD FACS™lysing solution	BD	349202
BD™Phosflow PermBuffer III	BD	558050
BD FACS rinse	BD	340246

Blocking Buffer	5 % donkey serum
	0.06 % Triton X 100
	in PBS

Coating Buffer	15 mM Na_2CO_3
	35 mM $NaHCO_3$
	2 mM NaN_3
	in H_2O
ELISA Wash Buffer	0.05% Tween20
	1x PBS
FACS Buffer	0.1% BSA
	0.02% NaN3
	in 1x PBS
MACS/Sorting Buffer	0.5% human Serum
	2mM EDTA
	1x PBS

3.1.4 Equipment

Beta counter, 1450 Microbeta	Perkin-Elmer
Centrifuges	Eppendorf and Hereaus
Filtermat Cassettes	Perkin-Elmer
Freezers	Liebherr and Sanyo
Freezing Container, Nalgene Cryo 1°C	Roth
Fridges	Liebherr
Gamma irradiator, Cs-137, 49.2 TBq, Biobeam 2000	Eckert & Ziegler
Harvester 96 MACH III M	Tomtec
Heat Sealer 1295-012	Wallac
Incubator, Hera Cell 240	Thermo Scientific
LSRII FACS analyser	BD Bioscience

MACS MultiStand	Miltenyi Biotech
Magnet, IMag	BD
Magnet, MACS Mini and Midi	Miltenyi Biotech
Microscope Confocal F1000	Olympus
Microscope Axio Imager M1	Zeiss
Nitrogen tank	tec-lab
Pipets	Greiner, eppendorf
Pipette help, accu jet	Hassa
Racks	Roth
Sterile bank, MSC-Advantage	Thermo Scientific
Thermomixer	Eppendorf
Wallac Victor 1420 multilabel plate reader	PerkinElmar

3.1.5 Consumables

Cell culture plates	Greiner, Sarstedt
Columns for magnetic cell isolation	Miltenyi Biotec
Cover slips (12 mm)	Menzel
ELISA 96 plates, flat bottom, certified high binding	Costar
Eppendorf tubes	eppendorf
FACS tubes	Sarstedt
Falcon tubes, 15 and 20 ml	BD
Filtermat A (GF/C)	Perkin-Elmer 1450-421
Filtermat bag	Perkin-Elmer 1450-432
Glass microscope slides HistoBond®	Marienfeld Laboratory Glassware
Liquid reservoir for mutlichannel pipettes	Roth

Pipette tips	Sarstedt
Transwell®plates (24 well, 6.5 mm inserts, 0.4 µM polycarbonate membrane)	Costar

1.1.5 Software

AxioVision 4.6	Zeiss
CorelDraw Graphics Suite 12	Corel
FACSDiVa analysis software	BD
FlowJo FACS analysis software	TreeStar Inc.
PRISM Graphpad V5.02	Graphpad Software Inc.

3.2 Methods

3.2.1 Isolation of polymorphonuclear neutrophils

PMNs were purified from fresh-drawn EDTA blood or buffy coats using Dextran-Ficoll method[186]. Briefly, blood was mixed with freshly prepared 3 % Dextran 200/HBSS- medium (Roth/Invitrogen) at the ratio 2:1 by inverting the tube two times. Subsequently, the lid was changed to avoid contamination with erythrocytes. All erythrocytes had sedimented after 30 minutes incubation at room temperature. Afterwards, the top phase (containing all blood leukocytes) was collected and the top phase was layered onto Ficoll (PAA). Peripheral blood mononuclear cells (PBMCs) were separated from PMNs using density gradient centrifugation without brake at 860 g for 30 minutes at room temperature. The whole supernatant including PBMC phase was discarded. The pellet containing PMNs and remaining erythrocytes was resuspended in 500 µl fresh HBSS- medium and transferred into a new tube to avoid PBMC contamination during the following washing step with medium at 480 g for 5 minutes at room temperature. The cell pellet was resuspended in 500 µl medium. In order to eliminate remaining erythrocytes, 5 ml cold sterilized and deionized water was added for 20 seconds. After adding an equal amount of fresh medium, cells were centrifuged again. PMNs were resuspended in HBSS+ medium (Invitrogen) containing 10 mM Hepes (Invitrogen) for subsequent stimulation experiments and cells were counted.

Fcγ receptor IIIB (CD16b) is only expressed on PMNs and is involved in their activation. The purity of isolated PMNs was checked by flow cytometry using anti-CD16b antibody (Beckman Coulter) (Figure 4).

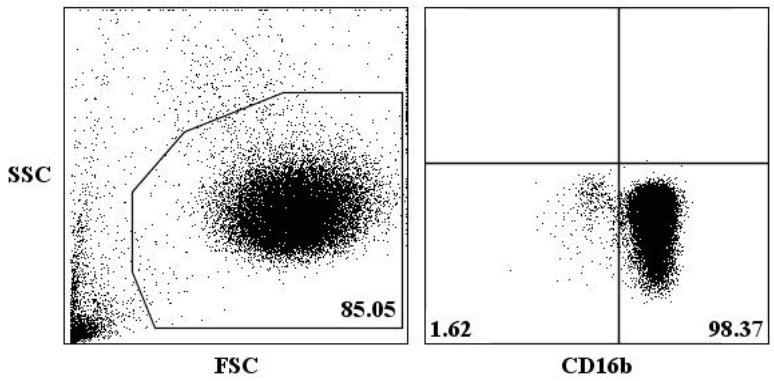

Figure 4 Representative FACS blot of purified PMNs.

3.2.2 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were purified from fresh drawn EDTA blood or buffy coats. Blood or buffy coats were mixed with PBS (PAA), carefully layered onto Ficoll solution (PAA) and centrifuged without brake at 860 g for 30 minutes at room temperature. After density gradient centrifugation the PBMC band was collected into a new tube and washed two times with cold PBS. Subsequently, the cell pellet was resuspended in x-vivo medium (Invitrogen) or MACS buffer and cell counts were determined.

3.2.3 Isolation of $CD4^+$ T cell, $CD8^+$ T cells and DCs

$CD4^+$ T cells, $CD4^+$ naïve T cells, $CD4^+$ memory T cells, $CD8^+$ T cells and DCs were negatively selected from freshly isolated PBMCs using *Magnetic* Activated *Cell Sorting* MACS kits from BD or Miltenyi Biotech. All isolation procedures were performed as described in the manufacturer's instructions.

T cell clone TCC36 was kindly provided by my thesis mentor Mireia Sospedra, PhD. TCC36 was established from CSF of an untreated MS patient by limiting dilution. Stimulatory peptides were identified using positional scanning combinatorial peptide libraries[187].

3.2.4 Phagocytosis assay

For phagocytosis assay 100 µl fresh-drawn heparin blood was incubated without or with 100 nM N-formyl-methionine-leucine-phenylalanine (fMLP) (Sigma Aldrich) for 15 minutes at 37 °C and 5 % CO_2. FITC-labelled latex beads (∅ 1 µm, PMN/bead ratio 1:10) (Polysiences Inc.) were added into tubes and incubated for further 30 minutes. As control, tubes with stimulated and unstimulated PMNs plus beads were incubated at 4 °C to rule out unspecific binding of the beads on the cell surface. One tube was left without beads to take into account autofluorescence of PMNs. Afterwards 2 ml cold PBS (PAA) were added into each tube and centrifuged at 480 g for 5 minutes. After a second washing step, erythrocytes were lysed for 10 minutes using 1 ml/tube lysing solution (BD) in the dark. Cells were washed two more times with PBS, resuspended in 250 µl PBS and transferred into a FACS tube. The percentage of cells, which have engulfed fluorescence labelled latex beads, were analysed by FACS.

Table 1 Demographic characteristics of patients and healthy controls (HC) for phagocytosis assay. R* in relapse

donor	sex	number	mean age
HC	female	15	29 ± 6
	male	9	29 ± 7
RR-MS	female	8	37 ± 8
	male	9	38 ± 6
RR-MS /R*	female	9	32 ± 8
	male	5	39 ± 6

3.2.5 Oxidative burst assay

100 µl freshly drawn heparin blood was incubated without or with 100 nM fMLP (Sigma Aldrich) for 15 minutes at 37 °C and 5 % CO_2. Afterwards 10 µM dihydrorhodamine 123 (DHR123) (Sigma Aldrich) was added and blood was incubated for further 5 minutes. After incubation erythrocytes were lysed for 10 minutes using 1 ml/tube lysing solution (BD) in the dark and remaining cells were washed twice with cold PBS (PAA). Cells were resuspended in 250 µl PBS and transferred into a FACS tube. DHR 123 is converted to rhodamine in the presence of radical oxygen species and can be detected by flow cytometry. The median fluorescence intensity (MedFI) of PMNs was analysed by FACS.

Table 2 Demographic characteristics of patients and healthy controls (HC) examined for oxidative burst assay. R* relapse

donor	sex	number	mean age
HC	female	10	31 ± 7
	male	5	32 ± 7
RR-MS	female	11	37 ± 7
	male	9	37 ± 8
RR-MS /R*	female	3	28 ± 3
	male	1	37

3.2.6 Degranulation assay

$1x10^6$ purified PMNs were stimulated with 100 nM fMLP (Sigma Aldrich) or left unstimulated in HBSS+ medium (Invitrogen). 500 µl of supernatant were taken after 30 minutes and stored at -80 °C. All supernatants were analyzed at one time point. The amount of released PMN elastase was examined by ELISA following manufacturer's instructions (R&D).

Table 3 Demographic characteristics of patients and healthy controls (HC) examined for degranulation assay. R* relapse

donor	sex	number	mean age
HC	female	16	37 ± 11
	male	4	39 ± 13
RR-MS	female	13	39 ± 11
	male	3	40 ± 12
RR-MS /R*	female	2	39.5
	male	1	33

3.2.7 Induction and isolation of neutrophil extracellular traps (NETs)

$1x10^6$ purified PMNs/ml were seeded in a culture plate and stimulated with 25 nM phorbol-12-myristate-13-acetate (PMA) (Sigma Aldrich) for 3 hours at 37 °C and 5 % CO_2. Subsequently, wells were washed very carefully three times with pre-warmed X-vivo medium

(Invitrogen) and incubated with new medium for 10 minutes at 37 °C and 5 % CO_2. NETs released by activated PMNs were digested with 10 U/ml micrococcal nuclease (Worthington Biochemical Corp.) as previously described[21]. Briefly, medium was removed and 500 µl fresh pre-warmed medium containing 10 U/ml micrococcal nuclease were added and incubated for further 15 minutes at 37 °C and 5 % CO_2. Supernatant was collected into new reaction tubes. Finally, NET supernatant was heated for 5 minutes at 70 °C to inactivate nuclease. EDTA could not be used as inhibitor since iones like Ca^{2+} and Mg^{2+} are important for maintaining the function of leukocytes in culture experiments.

3.2.8 Immunofluorescence

For immunofluoescence analysis, $1x10^6$ PMNs/ml were seeded on poly-l-lysine coated coverslips (Menzel Gläser GmbH) and were either left unstimulated or stimulated with 25 nM PMA (Sigma Aldrich) for three hours at 37 °C and 5 % CO_2. Afterwards, coverslips were very carefully washed two times with PBS (PAA) and subsequently fixed with 4 % paraformaldhehyde (PFA) (Roth) for 20 minutes at 37 °C. After fixation and two more washing steps, 300 µl blocking buffer were added and coverslips were stored at 4° C overnight. Coverslips were washed four times with PBS and incubated with 1 µg/ml anti-human MPO antibody (AbD Serotec) for 1 hour at room temperature. This step was followed by the incubation with Cy3-labelled anti-mouse IgG secondary antibody (Jackson ImmunoResearch Labratories, Inc.) and Hoechst 33258 (Sigma Aldrich) for 30 minutes at room temperature. Stained coverslips were washed four times again. Finally, they were covered with Fluoromount G (Southern Biotech) on glass slides and analysed with a confocal microscope (F1000 Olympus).

3.2.9 Quantification of NETs

NETs are composed of chromatin fibres and PMN granular proteins. In order to quantify NETs in serum, cerebrospinal fluid samples and cell supernatants, a previously described ELISA assay[26] detecting myeloperoxidase-DNA (MPO-DNA) complexes was used. Briefly, 96-well ELISA plate was coated with 75 µl/well of 5 µg/ml anti-MPO antibody (AbD Serotec) diluted in PBS overnight at 4 °C. For avoiding unspecific binding on the surface of the plate, 300 µl/well of 3 % BSA/PBS solution (R&D/PAA) were added after three washing steps with PBS/0.05% Tween20 (Sigma). After 2 hours, 40 µl/well of serum or liquor were added and subsequently mixed with 80 µl/well incubation reagent containing secondary

antibody anti-DNA-POD and incubation buffer (component 2+4 of the cell death detection ELISA kit from Roche). ELISA plates (Costar) were incubated for 2 hours with continuous shaking followed by seven washing steps with PBS/0.05 % Tween20. ELISA was developed with 100 µl/well ABTS solution (component 7 cell death detection ELISA) for 1 hour and stopped with 100 µl /well with stop solution (component 8 cell death detection ELISA, Roche). The adsorption/well was measured at a wavelength of 405 nm using a µQuant microplate reader. Many other granule proteins are associated with NET structures. To clarify that NET structures are detected using this ELISA, a further NET-associated protein, elastase associated with chromatin (Ela2-DNA complexes) was analyzed. For the detection of Ela2-DNA complexes, plates were coated with 75 µl/well of 5 µg/ml anti-Ela2 antibody (AbD Serotec) diluted in PBS.

Table 4 Demographic characteristics of patients, healthy controls (HC) and control patients examined for circulating NET serum levels, R* relapse

donor	sex	number	mean age
HC	female	20	34 ± 7
	male	20	31 ± 8
CIS	female	43	37 ± 11
	male	29	37 ± 8
CIS/R*	female	30	30 ± 7
	male	15	36 ± 9
RR-MS	female	41	37 ± 8
	male	26	37 ± 8
RR-MS/R*	female	38	35 ± 9
	male	18	36 ± 8
OIND	female	3	56 ± 19
	male	2	35
OND	female	5	39 ± 12
	male	5	49 ± 13
NMO	female	16	39 ± 14
	male	4	45 ± 5

Table 5 Demographic characteristics of patients, healthy controls (HC) and control patients examined for circulating NET cerebrospinal fluid levels, R* relapse

donor	sex	number	mean age
CIS	female	4	24 ± 5
	male	6	32 ±15
CIS/R*	female	6	33 ± 7
	male	6	29 ± 7
RR-MS	female	6	39 ± 13
	male	5	39 ± 3
RR-MS/R*	female	4	32 ± 8
	male	4	41 ± 11
OIND	female	5	48 ± 18
	male	4	31 ± 8
OND	female	6	36 ± 12
	male	5	49 ± 13

Table 6 Diagnosis of patients with other inflammatory neurological diseases (OIND)

Patient ID	sex	Diagnosis
0556WI	f	Hashimoto´s thyroiditis and Vitamin B6 deficiency
0593KA	f	Idiopathic trigeminal neuralgia (left-sided)
0732BR	f	Neurosarcoidosis
0780IN	f	Neuroborreliosis
0856RE	f	Paraneoplastic mixed axonal-demyelinating sensory-motor neuropathy
0557KE	m	(Autoimmune) uveomeningeal syndrome
0570SI	m	Viral meningoencephalitis
0840FR	m	Neuro-Behçet´s disease
0924SI	m	Bacterial meningitis

Table 7 Diagnosis of patients with other neurological diseases (OND)

Patient ID	sex	Diagnosis
0534SU	f	Shoulder pain of unknown etiology
0549Do	f	Optical atrophy
0594CA	f	Chronic musculoskeletal pain of unknown etiology
0844CE	f	Vasculopathy
0898MI	f	Distal paraesthesia of unknown etiology
0925AY	f	Migraine with aura
0747TH	m	Muscle atrophy of unknown etiology
0839JE	m	Hypokinetic-rigid idiopathic parkinson´s diseasse
0863JA	m	Tinnitus of unknown etiology
0865WO	m	Monoparesis of the right hand, most likely due to cerebral ischemia
0866KU	m	Suspected myoclonic epilepsy with ragged red fiber syndrome

3.2.10 Quantification of total DNA

For the quantification of total double-stranded (ds) DNA in serum samples, the *Quant*-iT™ *PicoGreen*® dsDNA Assay *Kits (Invitrogen)* were used[30]. PicoGreen has the property to intercalate in ds DNA. The assay was performed as described in the manufacturer's instructions. Sytox Green, the DNA staining reagent of *the Quant*-iT™ *PicoGreen*® dsDNA Assay *Kits*, has the property to intercalate in ds DNA.

3.2.11 Quantification of proteins, cytokines and antibody titer

Serum concentration of C-reactive protein (crp) and IL-6 were determined at the central laboratory of the University Medical Center Hamburg-Eppendorf. Serum concentration of IL-8 (Invitrogen), platelet factor 4 (PF4) (RayBiotech, Inc.), ß-thromboglobulin (Asserachrom®ß-TG) (Roche), soluble VE-cadherin (PromoCell GmbH) and concentrations of IFNγ (BioLegend), IL-2 (R&D), IL-10, IL-4 (Invitrogen), IL-17 (eBioscience) in cell culture supernatants were measured using commercial ELISA kits. Assays were performed as described in manufacturer's instructions.

Anti-nuclear antibodies (ANAs), anti-neutrophil cytoplasmic antibodies (ANCAs) and anti-ds DNA titer in serum samples were analysed by Euroimmun (Luebeck) in cooperation with PD Klaus-Peter Wandinger.

3.2.12 Isolation and analysis of platelets and microparticles

Platelet-rich plasma was obtained from sodium-citrate blood after centrifugation at 240 g for 20 minutes at room temperature. Subsequently, platelet-rich plasma was centrifuge at 2000 g for 2 minutes at room temperature and platelet-poor plasma was collected. For the analysis of microparticles (MP), 50 µl of platelet-poor plasma were stained with anti-CD31, anti-CD45 (eBioscience), anti-CD42a and anti-CD62P. Platelets MP were measured by flow cytometry after washing with FACS buffer. For the analysis, 1 defined drop of fluorescence labeled particles (BD) was added into the FACS tube. For the comparison of different donors, measured at different time points, 10 000 beads were recorded for the analysis of MP.

3.2.13 Measurement of whole blood cell counts

Cell counts were determined using an AcT diff Coulter Counter, standardized with Coulter 4C-ES Cell Control.

3.2.14 Analysis of monocytes and dendritic cells

PBMCs were thawed in X-vivo medium, centrifuged at 310 g for 7 minutes. Subsequently, cell pellet was washed with FACS buffer at 1500 rpm, for 5 minutes at room temperature. $1x10^6$ PMBCs were used per condition. Cells were resuspended in 100 µl FACS buffer and stained with anti-CD14 PB (BD), anti-CD16 APC-Cy7 (Biozol), anti-CD19 PB (BD) and anti-CD40 PE (Miltenyi Biotec) for the characterization of monocytes and with anti-CD14, anti-CD19, anti-CD40, anti-CD86 FITC (DAKO), anti-CD141 APC (Miltenyi Biotec), anti-CD303 APC (Miltenyi Biotec) or anti-CD1c APC (Miltenyi Biotec) for the characterization of dendritic cells. Corresponding isotype controls/cell type were also measured. Samples were analysed by flow cytometry.

3.2.15 Preparation of glass coverslips

Sterile glass coverslips (∅ 12 mm) (Menzel Gläser GmbH) were placed into a 24 well plate. A 70 µl drop of 0.01% Poly-l-lysine solution (Sigma Aldrich) was placed on each coverslip, and plates were incubated at 37 °C and 5 % CO_2 for 2 hours. Poly-l-lysine coated coverslips

were ready for cell culture after washing with sterile PBS (PAA) three times and stored at 37 °C until use.

3.2.16 Cell culture of polymorphonuclear neutrophils

For cell culture experiments, $1x10^6$ PMNs/ml were seeded into tissue culture plates on poly-l-lysine coated coverslips. PMNs were left unstimulated or stimulated as follows: with 25 nM PMA (Sigma Aldrich) for 15 minutes or 3 hours (PMN-NETs), with 100 nM fMLP for 3 hours, pre-incubated with 100 µM Diphenyleneiodonium chloride (DPI) (Sigma Aldrich) for 30 minutes and subsequently stimulated with 25 nM PMA for 3 hours. Also, unstimulated or PMA-stimulated PMNs were fixed with 4% PFA (Roth) at 37 °C for 20 minutes. For the induction of apoptosis and secondary necrosis, PMNs were exposed to UV light for 60 minutes and subsequently incubated at 37 °C and 5 % CO2 for further 16 hours before co-culture experiments were performed.

3.2.17 Co-culture experiments

Co-culture of resting or NET-activated DCs with unprimed or NET-primed $CD4^+$ T cells

Both, purified $2x10^6$ $CD4^+$ /$CD4^+$ naïve/ $CD4^+$ memory T cells and purified $4x10^5$ DCs were separately stimulated either with unstimulated PMNs or NET supernatant at 37 °C and 5 % CO_2 overnight. $CD4^+$ T cells and DCs were washed with fresh pre-warmed X-vivo (Lonza) medium and co-cultured for further 48 hours. When indicated, cells were physically separated by a transwell polycarbonate permeable membrane (0.4 mm pore size, Costar). Also when indicated 2.5 µM ofchloroquine (Sigma), 3µM of herbimycin A (Sigma), 60 µg/ml of anti-HLA DR-blocking antibody (kindly provided by Dr. Rammensee, Department of immunology, Tübingen) or 60 µg/ml of the corresponding isotype control (BioLegend) were added into the culture. After 24 hours supernatant was collected and after 63 hours cells were harvested for further analysis.

Co-culture of $CD4^+$ T cells / TCC36 with unstimulated PMNs or PMN-NETs and subsequent stimulation with suboptimal stimulus or specific peptide

$CD4^+$ T cells were pre-incubated with either unstimulated PMNs or PMN-NETs for 24 hours. Afterwards, cells were washed, respuspended in fresh pre-warmed X-vivo medium (Lonza) and stimulated or not with 0.025 µg/ml of soluble OKT3 or IL-2 for 48 hours.

TCC36 was pre-incubated with either unstimulated PMNs or PMN-NETs for 24 hours. After washing, $2.5x10^4$ cells/well of TCC36 were seeded in quadruplicate in 96 well-plate together with $1x10^5$ cells/well of autologous, at 3 000 rad irradiated, PBMC with or without different concentrations of the specific stimulatory peptide for 72 hours.

Co-culture of resting DCs with unstimulated PMNs or PMN-NETs and $CD4^+$ and $CD8^+$ T cells

For co-culture experiments, $4x10^5$ purified DCs were incubated with unstimulated PMNs, adherend on coverslips, or NET supernatants for 24 hours. DCs were collected and washed with pre-warmed X-vivo medium (Lonza). Subsequently, $2x10^5$ resting DCs were co-cultured with unprimed or NET-primed $CD4^+$ or $CD8^+$ T lymphocytes for 48 hours, at 37 °C and 5 % CO_2. When indicated, cells were physically separated by a transwell (polycarbonate permeable membrane, 0.4 mm pore size, Costar). After 24 hours supernatant was collected, and after 63 hours cells were harvested for further analysis.

Co-culture of unstimulated or stimulated PMNs with PBMCs

For co-culture experiments, $1x10^6$ PMNs were seeded on poly-L-lysine coated glass coverslips and stimulated with different stimuli or left unstimulated (see 3.2.16 Cell culture of PMNs). After 3 hours coverslips were very carefully washed three times and transferred into a new 24 well plate and $1.5x10^6$/ml PBMCs were added into each well. After 24 hours supernatant was collected and after 63 hours cells were harvested for further analysis.

3.2.18 Transmission light microscopy

PBMCs and T cells formed cell clusters after stimulation with PMN-NETs or NET supernatant. To visualize the cluster formation, cells were seeded on poly-l-lysine coated coverslips, carefully washed and fixed with 4 % PFA (Roth) for 20 minutes at 37°C. Coverslips were embedded on Fluoromount G medium (Southern Biotech) on glass slides and analyzed in transmission light by using AXIO Imager.M1 from Zeiss.

3.2.19 Analysis of the expression of surface molecules

Expression of CD25 and CD69 on T cells

Cells were washed with FACS buffer at 480 g for 5 minutes and resuspended in 100 µl FACS buffer containing anti-CD3 PE-Cy7, anti-CD8 PB (DAKO), anti-CD4 APC (eBioscience), anti-CD25 PE (eBioscience) and anti-CD69 FITC (BD) antibodies. Tubes were incubated for 30 minutes, at room temperature in the dark. Afterwards cells were washed with FACS buffer, resuspended in 250 µl FACS buffer and analysed by flow cytometry. Corresponding isotype controls were also stained and measured.

Expression of CD40, CD80, CD83, CD86 and MHC class II on DCs

For surface marker expression analysis, $4x10^5$ purified DCs were incubated with unstimulated PMNs, adherend on coverslips, or NET supernatants for 24 hours. Subsequently, DCs were collected in FACS tubes and washed with FACS buffer at 1500 rpm for 5 min. After blocking with human IgG, DCs were stained with anti-CD40, anti-CD80, anti-CD83, anti-CD86 and anti-MHC class II FITC (BD) antibodies for 30 minutes, at room temperature in the dark. DCs were washed and anaylzed by flow cytometry. Corresponding isotype controls were also aquired.

3.2.20 Proliferation assays

EdU proliferation assay

The proliferation of T cells was measured using a Click-i TM EdU Flow Cytometry Assay Kit (Invitrogen). All steps were performed as described in the manufacturer's instructions.

Afterwards, EdU-labelled cells were resuspended in 100 µl FACS buffer containing anti-human CD3 PE-Cy7 (eBioscience), anti-human CD4 FITC (eBioscience) and anti-human CD8 PB (DAKO) and incubated for 30 minutes at room temperature. Cells were washed with FACS buffer. Subsequently, 250 µl FACS buffer were added and cells were analysed by flow cytometry. Figure 5 depictes the gating strategy of proliferating $CD4^+$ T lymphocytes.

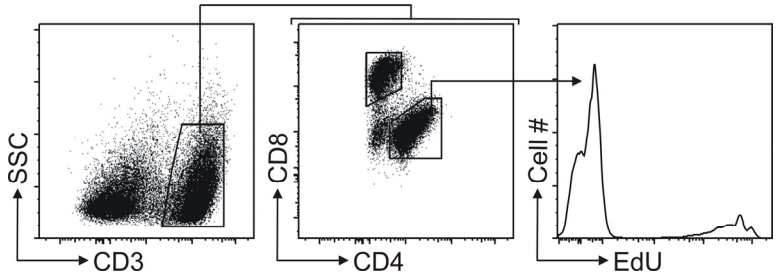

Figure 5 Representative FACS blots of EdU proliferation assay.

^{3}H-thymidine incorporation

Cells were pulsed with 1 µ Curie ^{3}H-thymidine (Hartman Analytic) and incubated for 15 hours at 37 °C, 5 % CO_2. Afterwards cells were harvested on a filter mat (Perkin-Elmer). The dried mat was wetted with scintillation liquid (PerkinElmer) and shrink-wrapped in a plastic bag. ^{3}H-thymidine incorporation was measured by using a scintillation beta counter.

3.2.21 Analysis of phosphorylation of ZAP70

CD4 lymphocytes were left unstimulated in X-vivo medium at 37 °C and 5 % CO_2 overnight. Next day, $CD4^{+}$ lymphocytes were incubated with either unstimulated PMNs, PMN-NETs or NET supernatant for 10 minutes at 37 °C and 5 % CO_2. Subsequently, cells were fixed with an equal volume of fixbuffer (BD) for 15 minutes at 37 °C and 5 % CO_2. Cell pellet was resuspended in 1 ml ice-cold phospho-perm buffer (BD) for 30 min on ice after washing with PBS (PAA). After two washing steps with FACS buffer and blocking of unspecific binding sites with human IgG (Jackson ImmunoResearch Labratories, Inc.), CD4 lymphocytes were stained with anti-human ZAP70 (pY319) PE (BD) or isotype control for 30 minutes, at room temperature in the dark. Phosphorylation of ZAP70 (pY319) of CD4 lymphocytes were analysed by flow cytometry after washing with FACS buffer.

3.2.22 Statistical analysis

Statistical analyses were performed with GraphPad Prism 5. Descriptive statistics are reported as median ± SEM. Parametric tests were applied for two-group comparisons using unpaired t-tests with two-tailed p-values. Comparisons of three groups and more were assessed by one-way ANOVA with Bonferroni's correction for multiple comparisons. P-values < 0.05 were considered as statistically significant.

4 RESULTS

4.1 PMNs from MS patients are characterized by an enhanced oxidative burst and an increased degranulation upon stimulation

Whole heparin blood or purified PMNs were stimulated with N-formylmethionyl-leucyl-phenylalanine (fMLP), a potent activator of PMNs produced by certain bacteria, in order to analyze the phagocytic capacity, the oxidative burst and the degranulation of PMNs from RR-MS patients. Fluorescence-labeled latex beads were added to whole blood during fMLP stimulation and samples were analyzed by flow cytometry to measure phagocytosis. As control, blood was incubated with beads at 4 °C to take into account unspecific binding of the beads to the cell surface. No differences could be found in the phagocytic capacity of PMNs from patients (n=31) when compared to HC (n=24) (Figure 6A). Dihydrorhodamine 123 (DHR123) was added to whole blood upon fMLP simulation to measure oxidative burst. DHR123 is converted to rhodamine by ROS and can be used for the detection of intracellular ROS by flow cytometry. The difference (Δ) in mean fluorescence intensity, i. e. in superoxide production, between stimulated and unstimulated PMNs is shown in Figure 6B. PMNs from RR-MS patients showed a significantly higher oxidative burst in response to fMLP (Δ MFI 145.1 ± 24) than PMNs from healthy controls (HC) (Δ MFI 77.4 ± 19). Elastase released by purified stimulated and unstimulated PMNs from HC (n=20) and RR-MS patients (n=20) was quantified by ELISA in order to measure degranulation. The difference in elastase release between stimulated and unstimulated PMNs is shown in Figure 6C. PMNs from RR-MS patients (Δ pg/ml 121 ± 16) release significantly more elastase after stimulation than PMNs from HC (Δ pg/ml 70 ± 11). In conclusion, the data strongly suggest altered PMN effector functions, more precisely an enhanced oxidative burst and an increased degranulation in MS patients.

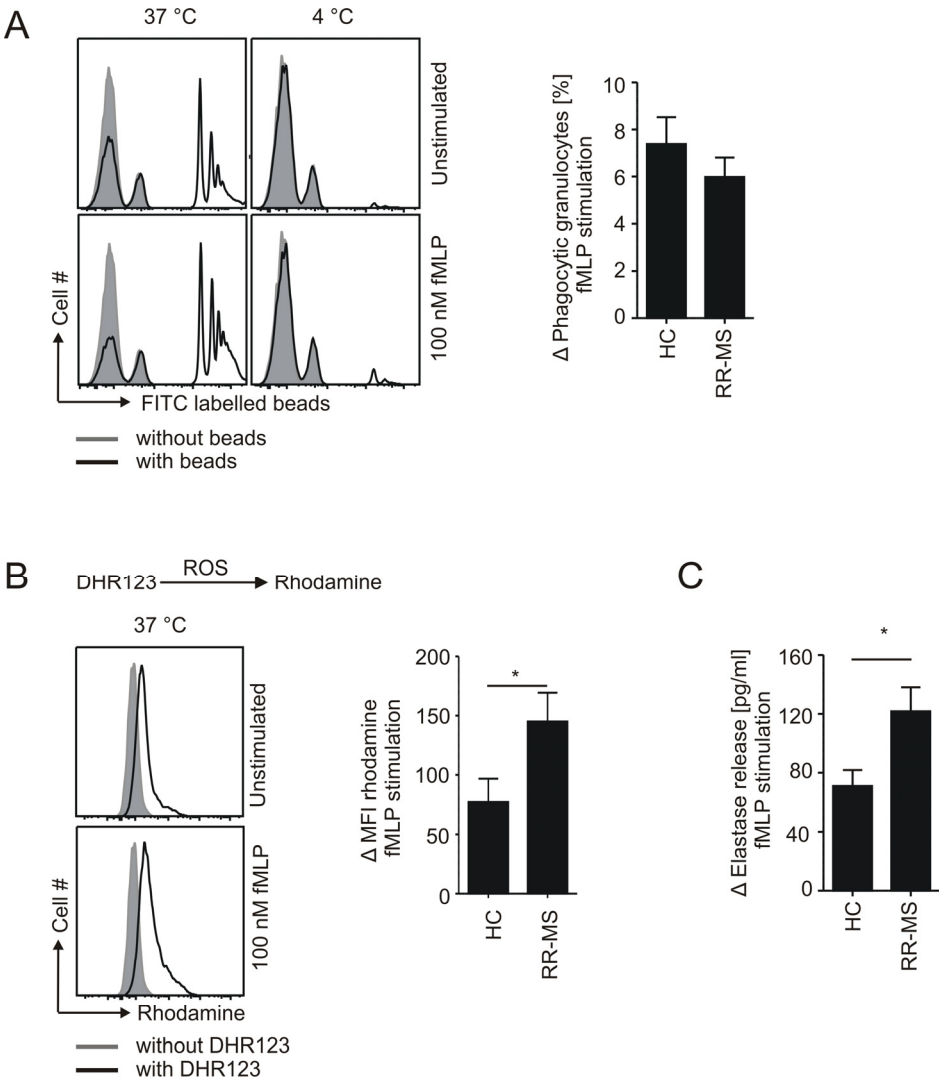

Figure 6 Altered oxidative burst and degranulation of PMNs from RR-MS patients.

A) Phagocytic capacitiy of PMNs from RR-MS patients (n=31) compared to healthy controls (HC) (n=24) after stimulation with 100 nM fMLP. Histograms show representative FACS blots. Whole blood was stimulated with fMLP (lower two graphs) or left unstimulated (upper two graphs) and incubated with fluorescence-labeled latex beads (black line) or not (grey curve) at 37 °C (two left graphs, histogram) and at 4 °C (two right graphs, histogram) to rule out unspecific binding on the cell surface. Graph represents Δ % ± SEM of phagocytic granulocytes. **B)** ROS production of PMNs from RR-MS patients (n=24) compared to HC (n=15). Histograms show representative FACS blots. Whole blood was stimulated with fMLP (lower graph) or left unstimulated (upper graph) and incubated with (black line) or without DHR123 (grey curve). Graph represents Δ MFI ± SEM of produced rhodamine. **C)** Degranulation of PMNs from RR-MS patients (n=20) compared to HC (n=20). Purified PMNs were incubated with fMLP. The concentration of elastase was measured in cell culture supernatants using an ELISA. Graph represents Δ pg/ml ± SEM of elastase release. *p<0.05

4.2 Higher circulating NET levels in sera from RR-MS patients

In order to examine the most recently discovered effector function of PMNs, the ability to kill invaders by neutrophil extracellular traps (NETs), serum and cerebrospinal fluid (CSF) samples from patients with CIS (serum n= 118; CSF n=22) and the RR-MS (serum, n=126; CSF n=19) disease type were analyzed using a myeloperoxidase (MPO)-DNA complex ELISA. Samples from HC (serum, n=40), patients with neuromyelitis optica (NMO, n=20), patients suffering from other inflammatory neurological diseases (OIND, serum n=5, CSF n=9) and other neurological diseases (OND, serum n=11, CSF n=11) were used as control. Significantly higher circulating NET levels were found in serum samples from RR-MS patients compared to HC (Figure 7A). In order to examine whether MPO-DNA complexes in serum were related with disease activity, serum samples from CIS and RR-MS patients in remission and relapse were compared. No significant differences in MPO-DNA complex amounts were observed between patients in remission and relapse (Figure 7B). Furthermore, MS patients with high MPO-DNA complexes (OD > 0.3, n=17) and patients with low MPO-DNA complexes (OD < 0.2, n=19) were selected from whom MRI had been obtained within two weeks prior or after serum sampling. The number of T1 gadolinium-enhancing (Gd^{+}) lesions was examined in these patients in order to determine whether the amount of MPO-DNA complexes correlated with the number of lesions. No significant differences in the number of T1 gadolinium-enhancing lesions were observed between patients with low and high MPO-DNA complex amount (Figure 7C).

It was also analyzed whether MPO-DNA complexes were present in CSF samples from CIS (n=20), RR-MS (n=21), OIND (5) and OND (10) patients. No MPO-DNA complexes were detectable in CSF (Figure 7D).

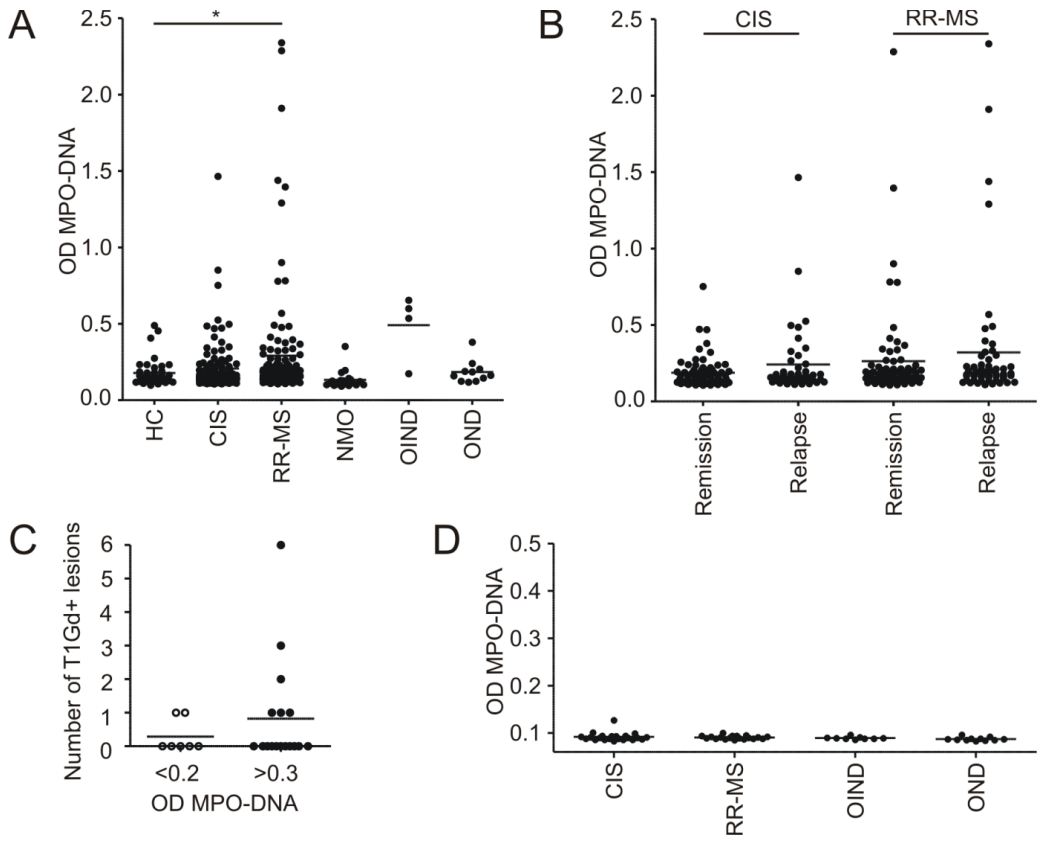

Figure 7 Higher amounts of neutrophil extracellular traps in serum samples from MS patients.

A) ODs of MPO-DNA complexes in serum samples from healthy controls (HC, n=40), patients with clinically isolated syndrome (CIS, in remission and relapse, n=118), relapsing-remitting patients (RR-MS, n=126), patients with neuromyelitis optica (NMO, n=20), donors with other inflammatory neurological diseases (OIND, n=5) and donors with other neurological diseases (OND, n=11). MPO-DNA complexes were measured using ELISA. **B)** ODs of MPO-DNA complexes in serum samples from CIS patients (n= 73 in remission and n=45 in relapse) and RR-MS patients (n=70 in remission and n=56 in relapse). MPO-DNA complexes were measured using ELISA. **C)** Number of gadolinium-enhancing (Gd+) lesions in T1 weighted MRI scans from MS patients with low MPO-DNA OD (<0.2, n= 7) and high MPO-DNA OD (>0.3, n=11). **D)** ODs of MPO-DNA complexes in CSF of CIS patients (n=22), RR-MS patients (n=19), OIND patients (n=9) and OND patients (n=11). MPO-DNA complexes were measured using ELISA. *p<0.05

In order to confirm that MPO-DNA ELISA allows the quantification of nucleosomes derived from NETosis and to exclude these from apoptosis and necrosis, sera with low (OD<0.2) and high (OD>0.3) MPO-DNA complexes were selected (Figure 8, first graph). Nucleosomes associated with neutrophil elastase (Ela2) (Figure 8, second graph), another granule compound, were measured in these samples. Elevated levels for Ela2-DNA were detected in serum samples with high MPO-DNA levels. Total DNA was also measured in both groups with *Quant-iT™ PicoGreen®* dsDNA assay to to rule out the possibility that patients with higher NET OD may have a general DNaseI defect. DNaseI is described as an enzyme responsible for NET degradation[188]. No significant differences were found in dsDNA between the two groups (Figure 8 third graph), confirming that MPO-DNA ELISA mainly quantifies nucleosomes derived from NETosis.

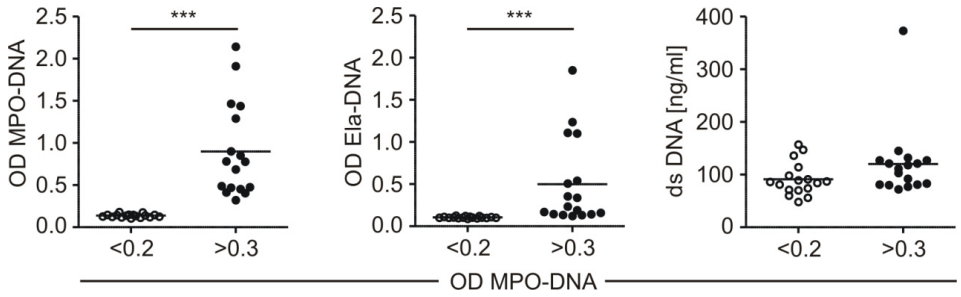

Figure 8 NETs are characterized by MPO-DNA and Ela2-DNA complexes.

ODs of MPO-DNA complexes, ODs of Ela-DNA complexes and concentration of ds DNA in serum samples from donors low (<0.2, n=17) and high (>0.3, n=17) NET amount were assessed. MPO-DNA complexes were measured by ELISA and concentration of ds DNA by *Quant-iT™ PicoGreen®* dsDNA. ***p<0.001

4.3 Infections and inflammation as putative triggers of NET formation in RR-MS patients

As an ongoing infection could activate granulocytes and increase circulating NET levels in serum samples, the question of whether the high amount of MPO-DNA complexes in the sera from a sub-group of MS patients could be explained by infections was addressed. C-reactive protein (CRP) and IL-6 levels were measured in serum samples from RR-MS patients with high (OD > 0.3, n=32) and low (OD < 0.2, n=32) MPO-DNA complexes in serum (Figure 9A). CRP, an acute-phase protein, the levels of which rise in response to inflammation, was significantly elevated in the group of patients with high MPO-DNA OD (7.84 ± 1.47) compared to the group with low ODs (5.28 ± 0.28) (Figure 9A, first graph), however no correlation between CRP and NET levels was found. IL-6, an important mediator of acute-phase responses, was not increased in patients with higher serum levels of NETs (Figure 9A, second graph).

Recent evidence suggests that NETs can be induced in various clinical settings in absence of microbial infection. The chronic inflammatory processes in MS could result in NET stimulation. IL-8 appears to be able to induce NET formation[19,25] and is increased in serum samples from MS patients[189]. It could, therefore, be a good candidate as mediator of NET induction in MS. IL-8 was measured in serum samples from RR-MS patients with high (OD > 3, n=18) and low (OD < 0.2, n=18) MPO-DNA complexes in serum to examine whether this cytokine is related to the high level of NETs in some MS patients. Indeed, higher IL-8 levels were found in RR-MS patients with high MPO-DNA complexes although differences were not significant (Figure 9A third graph).

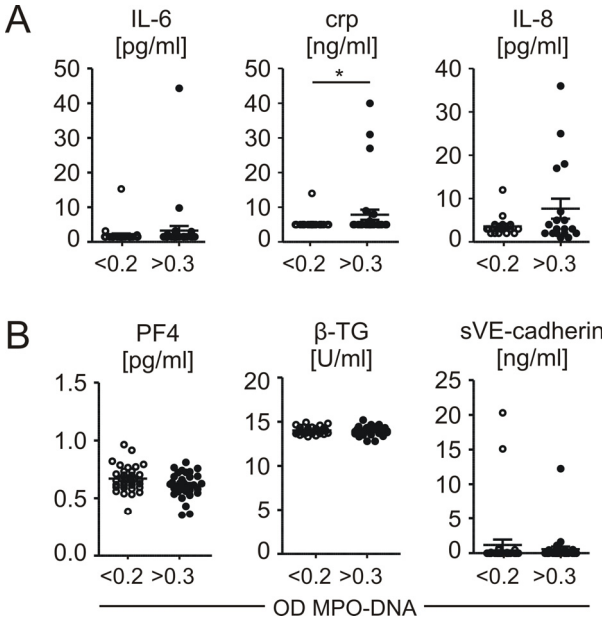

Figure 9 Higher frequency of monocytes is associated with higher amounts of NETs.

A-B) Concentration of IL-6, C-reactive protein (CRP), IL-8, platelet-factor 4 (PF4), β-thromboglobulin (β-TG) and sCD144 (soluble vascular endothelial cadherin=sVE-cadherin) of serum samples from HC (n=2) CIS patients (n=12) and RR-MS patients (n=18) for both, low (<0.2) and high (>0.3) NET amounts. Commercial ELISA kits were used. *p<0.05.

4.4 Endothelial and platelet activation as putative triggers of NET formation in RR-MS patients

It has been shown that PMNs are susceptible to be activated by platelets via TLR4, when they adhere to activate endothelium. This activation leads to robust NET formation[24,190]. In addition, activated endothelial cells are able to induce NET formation and are susceptible to NET-mediated damage[191]. Since both cell types are reported to be activated in MS patients[190,192], it was examined whether platelet and/or endothelium activation underlies the high MPO-DNA complexes in a subset of RR-MS patients. The level of platelet activation factor (PF4) and soluble β-thromboglobulin (β-TG) as markers of platelet activation[193,194] and soluble CD144 or sVE cadherin as marker of endothelial activation and damage[195] were measured. These factors were analyzed in samples from RR-MS patients with high (OD > 3, n=18) and low (OD < 0.2, n=18) MPO-DNA complexes in serum (Figure 9). No significant differences in PAF, β-TG or sVE cadherin levels between both groups of patients were found (Figure 9B).

Activated platelets as well as apoptotic and activated endothelial cells release membrane-derived microparticles[196,197], and elevated plasma platelet-derived (PMP)[192] and endothelial-derived (EMP) microparticles[198] have been found in MS patients. In pre-eclampsia, a severe

pregnancy-related disorder, it has been shown that microparticles released by syncytiotrophoblasts are able to activate PMNs and induce NET formation[25]. Based on these observations, it was examined whether PMP or EMP could have caused the high MPO-DNA complexes found in a subset of RR-MS patients. PMP and EMP derived from activation in plasma samples from RR-MS patients with high (OD > 3, n=10) and low (OD < 0.2, n=12) MPO-DNA complexes in serum were examined by flow cytometry. No significant differences in EMP levels between both groups of patients were found (Figure 10). However, higher PMP levels were observed in RR-MS patients with high MPO-DNA complexes (% 55.86 ± 2.4) compared to those with low MPO-DNA complexes (% 47.00 ± 2.7) (Figure 10). The right FACS blots in Figure 10 shows the gating stategy of MP in plasma samples.

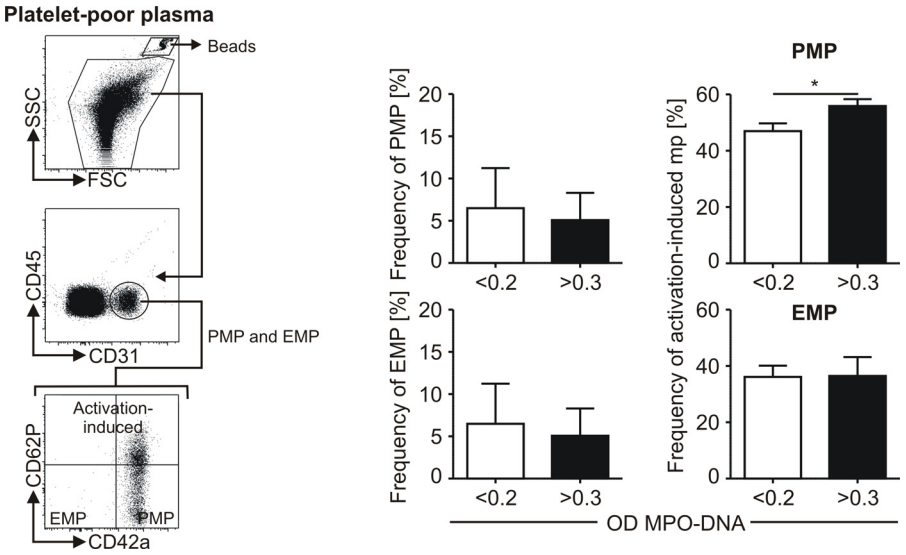

Figure 10 Elevated activation induced platelet-derived microparticles in plasma samples from donors with higher amounts of NETs in serum samples.

Characterization of microparticles of plasma samples from MS patients (n=12, for low (<0.2) and (n=10) for high (>0.3) NET amounts. CD42a and CD62P expression were analyzed by flow cytometry. *p<0.05, PMP = platelet-derived microparticles, EMP = endothelial-derived microparticles

4.5 Autoantibodies in RR-MS patients with NETs in serum

SLE pathogenesis is characterized by the presence of anti-self antibodies. It has been shown that anti-self antibodies are able to activate PMNs and to induce NET formation in this disease[199]. These NETs contain the targeted autoantigens, such as DNA, MPO or histones, and may facilitate the generation of autoantibodies. Anti-neutrophil cytoplasmic antibodies were found in patients with the optic-spinal form of MS in Japan[200]. Therefore, serum samples from patients with low (n=15) and high (n=15) NET levels were analyzed for autoantibodies, more precisely anti-nuclear antibodies (ANAs), anti-neutrophil cytoplasmic

antibodies (c and pANCAs) and antibodies against ds DNA with the company Euroimmun (Luebeck, Germany). They could not detect any differences between groups (Figure 11).

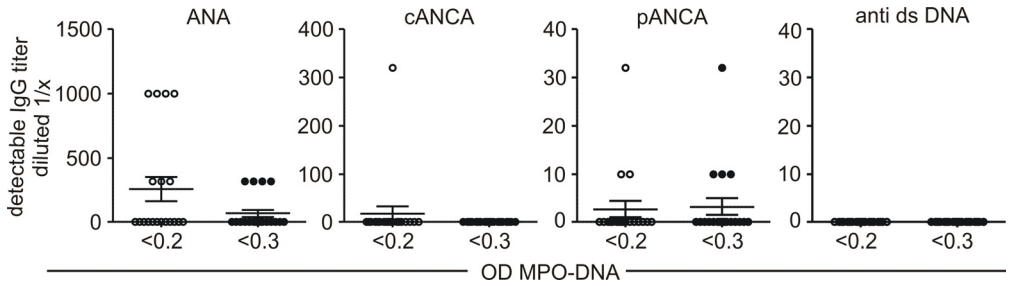

Figure 11 Titer of ANAs, ANCAs and anti-ds DNA antibodies.

Titer of cytoplasmatic anti-neutrophil cytoplasmatic antibodies (cANCAs), peri-nuclear anti-neutrophil cytoplasmic antibodies (pANCAs) and of antibodies against ds DNA were analysed in serum samples from of HC (n=2), CIS patients (n=2 in remission, n=4 in relapse) and RR-MS patients (n=5 in remission, n=4 in relapse) for both, low (<0.2) and high (>0.3) NET amounts using ELISAs. *p<0.05.

4.6 Characterization of monocytes from RR-MS patients with NETs in serum

As a next step, it was examined whether high MPO-DNA complexes in serum are related to variations in the main peripheral blood cell populations. Absolute granulocyte, monocyte and leukocyte counts were measured in whole blood from RR-MS patients with high (OD > 3, n=18) and low (OD < 0.2, n=18) MPO-DNA complexes in serum. No significant differences in absolute granulocyte and lymphocyte counts between both groups of patients were found (Figure 12A). However, higher monocyte counts were detected in patients with higher MPO-DNA complexes in serum (Figure 12A). Percentages of non-classical ($CD14^{low}CD16^{+}$), classical ($CD14^{high}CD16^{-}$) and intermediate ($CD14^{high}CD16^{+}$) monocytes were analyzed in both groups of patients as well as their expression of CD40 in order to characterize the monocyte population expanded in patients with high MPO-DNA complexes. The three subtypes did not differ, neither in their frequency nor CD40 expression between donors with low circulating NET levels and the donors with high NET levels (Figure 12B). The upper scheme shows the gating strategy (Figure 12B).

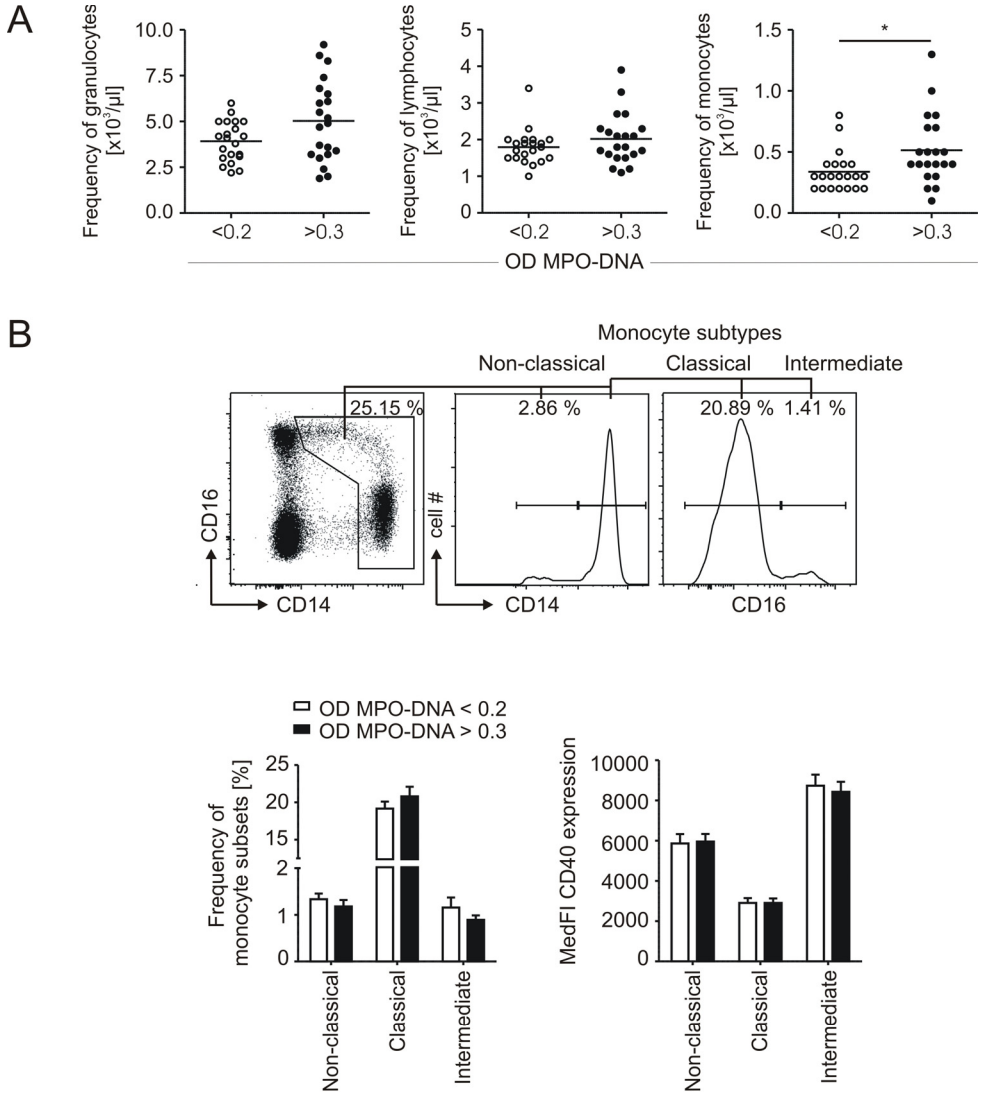

Figure 12 Characterization of monocytes from MS patients with NETs in serum.

A) Absolute cell number of granulocytes, lymphocytes and monocytes were measured with automatic cell counter. **B)** Frequency and CD40 expression of non-classical (CD14low CD16^{+}), classical (CD14high CD16^{-}) and intermediate (CD14high CD16^{+}) monocyte subsets. Frozen PBMCs from donor (n=27), for both low (<0.2) and high (>0.3) NETs amounts in serum, were analyzed by flow cytometry. Graph represents MedFI ± SEM (CD40 expression) and mean % ± SEM. *p<0.05

4.7 Characterization of dendritic cells from RR-MS patients with NETs in serum

It has been demonstrated that NETs can activate plasmacytoid dendritic cells (pDC) via TLR9[199,201]. In order to clarify whether the subset of RR-MS patients with high MPO-DNA complexes in serum have expanded or more activated pDCs, percentages and activation state of pDCs in RR-MS patients with high (OD > 3, n=18) and low (OD < 0.2, n=18) MPO-DNA complexes in serum were examined. Plasmacytoid DCs (pDCs, CD303^{+}) and myeloid DCs (mDCs, CD1c^{+} and CD141^{+}) did not vary in their frequency and expressed the same levels of

activation markers CD40 and CD86 in both groups of patients (Figure 13). The upper scheme depicts the gating strategy for human DCs (Figure 13).

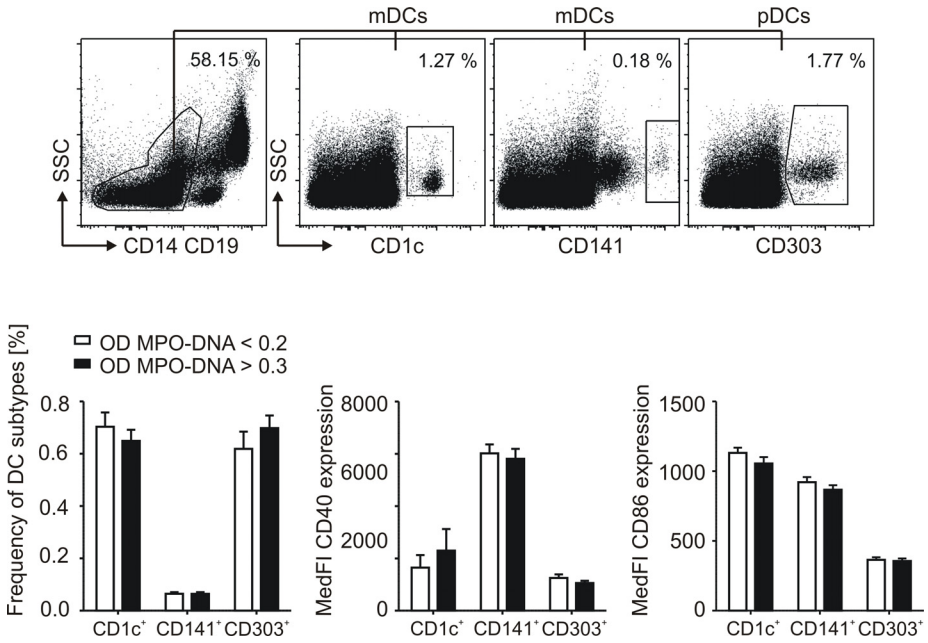

Figure 13 No alteration of dendritic cell in PBMC samples from donors with high NET amounts in serum samples.

Frequency and expression of activation molecules CD40 and CD86 of myeloid dendritic cells (mDCs, $CD1c^+$ and $CD141^+$) and plasmacytoid dendritic cells (pDCs, $CD303^+$). Frozen PBMCs from donors (n=27), for both low (<0.2) and high (>0.3) amounts of NETs, were analyzed by flow cytometry. Graph represents MedFI ± SEM (CD40/CD80 expression) and mean % ± SEM.

4.8 $CD4^+$ T cell priming induced by NETs

In order to examine NETs-mediated effects on T cells, purified human $CD4^+$ T cells were co-cultured with PMNs releasing NETs (PMN-NETs) and unstimulated PMNs. NET formation was induced by incubating highly pure PMNs with 25 nM Phorbol 12-myristate 13-acetate (PMA) for 3 h (Figure 14A). To assure that the role of NETs is examined and not of other PMA-induced mediators, $CD4^+$ T cells were also incubated with PMNs pre-treated with diphenyleneiodonium (DPI), a NADPH inhibitor of NET formation, before stimulation with PMA. Furthermore, NETs were digested from stimulated PMNs with MNase and purified $CD4^+$ T cells were incubated with these cell-free supernatants containing NETs (NETs-supernatant). $CD4^+$ T cells co-cultured with PMN-NETs formed clusters indicative of cell activation or proliferation that were absent, when cells were co-cultured with unstimulated PMNs (Figure 14B). As a further sign of activation, CD25 (NETs-supernatant, MedFI 644 ± 108) and CD69 (NETs, MedFI 3671 ± 458; NETs-supernatant, MedFI 6424 ± 640) expression were upregulated on purified $CD4^+$ T cells co-cultured with PMN-NETs and NETs-supernatant, but not on T cells co-cultured with unstimulated PMNs or DPI-PMNs (Figure 14C). To examine whether these effects of NETs on $CD4^+$ T cells involved TLR9 and TCR signaling, $CD4^+$ T cells were co-cultured with NETs-supernatant in the presence or absence of the TLR9 inhibitor chloroquine, which blocks TLR9-DNA interactions in endosomes[202] or the antibiotic herbimycin A that inhibits TCR-mediated signaling[203]. The upregulation of CD25 and CD69 on $CD4^+$ T cells co-cultured with NETs-supernatant remained unchanged in the presence of chloroquine, while it was reduced by herbimycin A (CD25, MedFI 271 ± 15; CD69, MedFI 2155 ± 153), suggesting that the effect of NETs is independent of TLR9, but involves TCR signaling (Figure 14D).

To investigate further the TCR-mediated effect of NETs, they were analyzed to see whether NETs induced recruitment of the TCR-proximal tyrosine kinase ZAP-70, which plays a critical role in early steps of TCR-mediated signaling. Purified $CD4^+$ T cells were co-cultured for 10 min with unstimulated PMNs, PMNs-NETs or NETs-supernatant, and ZAP-70 (pY319) phosphorylation was measured by flow cytometry. ZAP-70 phosphorylation was significangly upregulated in $CD4^+$ T cells co-cultured with NETs-supernatant (MedFI 123.22 ± 2.4) compared with unstimulated PMNs (MedFI 64 ± 19) (Figure 14E).

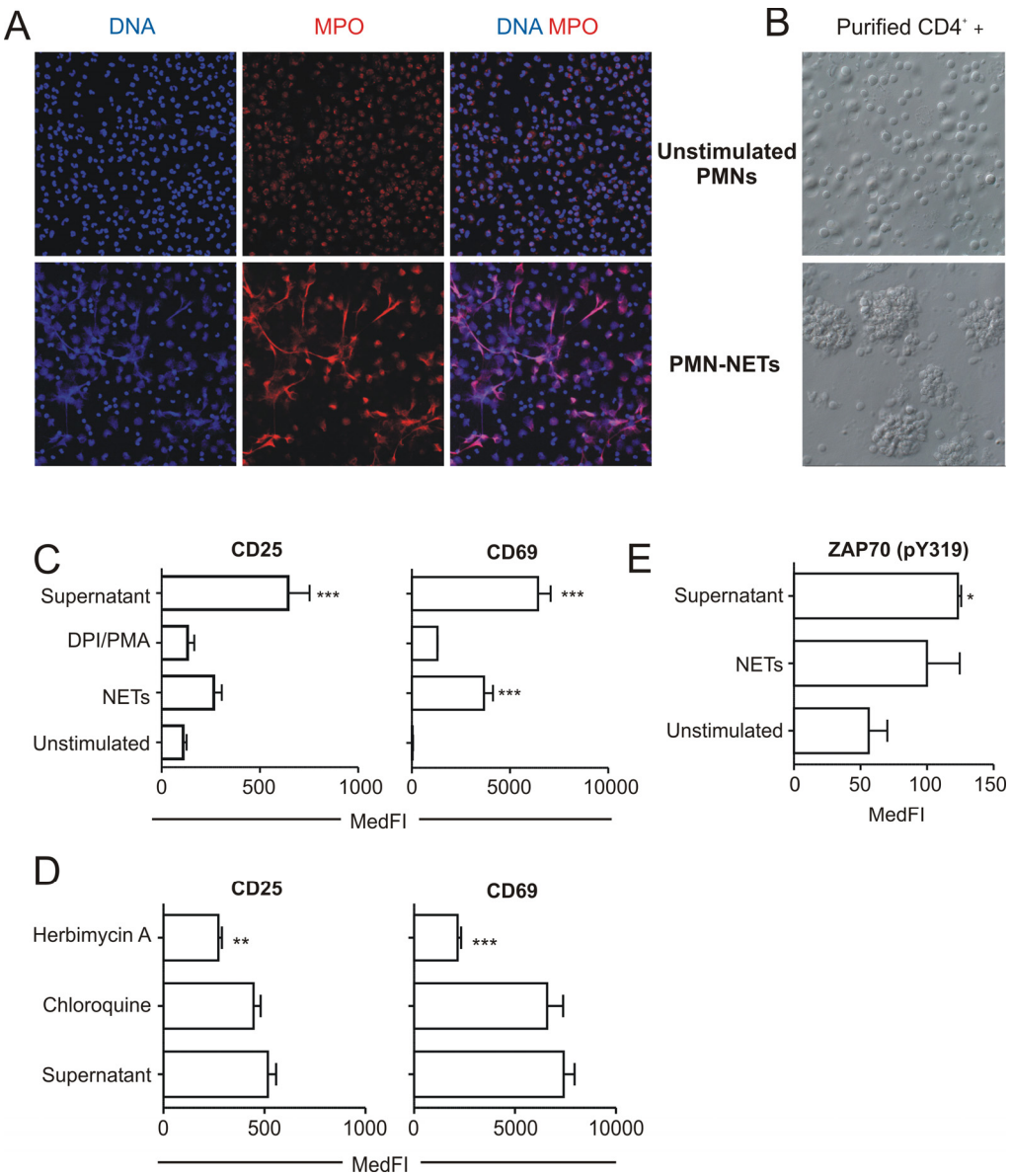

Figure 14 CD4$^+$ T cell priming induced by NETs.

A) Representative images of unstimulated PMNs and NETting-PMNs visualized using fluorescence microscopy. DNA in blue (hoechst 33258) and MPO in red. **B)** Transmission light images of cluster formation of purified CD4$^+$ T cells co-cultured with unstimulated PMNs and NETting-PMNs (NETs). **C-D)** CD25 and CD69 surface expression on purified CD4$^+$ T cells co-cultured 24 h with unstimulated PMNs, NETting- PMNs, DPI-PMNs and NETs-supernatant with or without chloroquine or herbimycin A. Graphs represent MedFI ± SEM from 5 independent experiments. *p<0.05 and ***p<0.001. **E)** Flow cytometry analysis of ZAP-70 (pY319) phosphorylation in CD4+ T cells co-cultured for 10 min with unstimulated PMNs, NETting-PMNs and NETs-supernatant. Graph represents MedFI ± SEM from 3 independent experiments. *p<0.05. *p<0.05, **p<0.01, ***p<0.001, Significance refers to unstimulated (Figure C and E) or supernatant (Figure C) condition.

CD8$^+$ T cells were also purified and co-cultured with unstimulated PMNs and NETting PMNs (PMN-NETs). They show the same behavior like CD4$^+$ T cells. They formed clusters (Figure 15A) and they significantly upregulated CD25 and CD69 after stimulation with PMN-NETs (CD69, MedFI 3643 ± 805) and NETs-supernatant (CD25, MedFI 592 ± 140; CD69, MedFI

3643 ± 805). (Figure 15B). For further experiments the focus was set on $CD4^+$ T cells since $CD8^+$ T cells survive the incubation with NETs only with very low cell numbers.

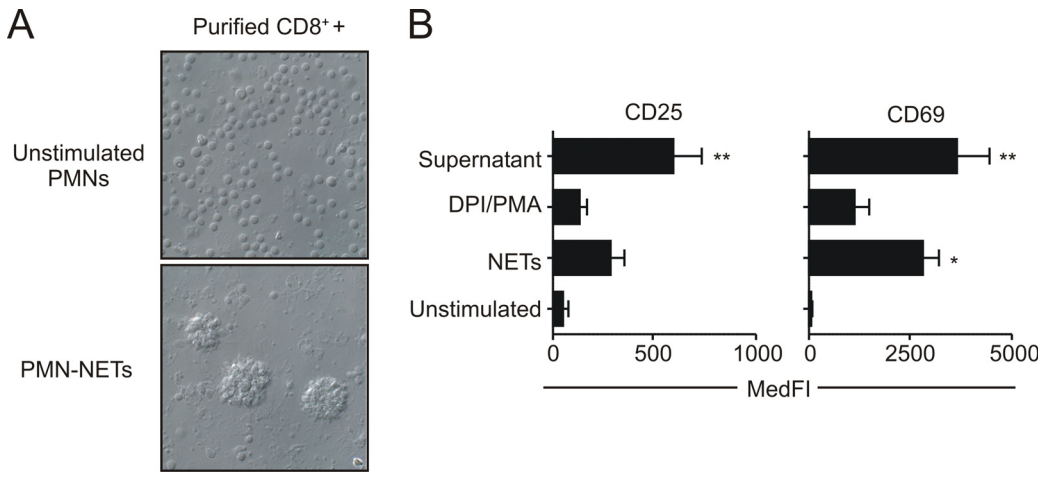

Figure 15 $CD8^+$ T cell priming induced by NETs.

A) Transmission light images of cluster formation of purified $CD8^+$ T cells co-cultured with unstimulated PMNs or PMN-NETs. B) CD25 and CD69 expression of $CD8^+$ T cells co-cultured with unstimulated, PMN-NETs (NETs), DPI pre-treated and PMA stimulated PMNs (DPI) and NETs-supernatant (supernatant). Graphs represent MedFI ± SEM of 3 independent experiments. *p<0.05, **p<0.01, Significance refers to unstimulated condition.

4.9 T cell priming by NETs lowers the activation threshold

The changes that NETs exerted on $CD4^+$ T cells suggested that they lead to a state of preactivation or priming. Therefore it was examined whether interaction with NETs reduces the activation threshold of $CD4^+$ T cells. Firstly, the effect of NETs were studied on antigen-specific responses using a previously well characterized CD4+ T cell clone (TCC36)[187]. TCC36 was pre-cultured for 24 h with unstimulated PMNs or NETs-supernatant and then seeded with autologous irradiated PBMCs pulsed or not with its specific target peptide. TCC36 primed by NETs proliferated significantly more vigorously in response to PBMCs loaded with 10 and 1 µg/ml of specific peptide (10 µg/ml, 24438 ± 335 cpm; 1 µg/ml, 2733 ± 44 cpm) when compared to the unprimed TCC36, i.e. pre-cultured with unstimulated PMNs (10 µg/ml, 11457 ± 766 cpm; 1 µg/ml, 623 ± 64 cpm) (Figure 16A).

Then, the question was addressed whether priming by NETs reduces the activation threshold of $CD4^+$ T cells and renders them capable to be activated by sub-optimal stimuli. Purified $CD4^+$ T cells were pre-cultured with unstimulated PMNs, NETting-PMNs, DPI-PMNs or NETs-supernatant for 24 h. Next, T cells were carefully washed and cultured for another 48 h with low concentrations of soluble anti-CD3 antibody (OKT3) or IL-2 (signal 1) in the

absence of antigen presenting cells (APCs, signal 2), a sub-optimal stimulus, which alone is not able to induce proliferation of resting T cells. Purified $CD4^+$ T cells pre- cultured with unstimulated PMNs or with DPI-PMNs failed to proliferate (Figure 11B). In contrast, 13.96 ± 0.79 % of $CD4^+$ T cells pre-cultured with NETting-PMNs and 6.70 ± 1.94 % pre-cultured with NETs-supernatant proliferated in response to anti-CD3 antibody (OKT3) (Figure 16B).

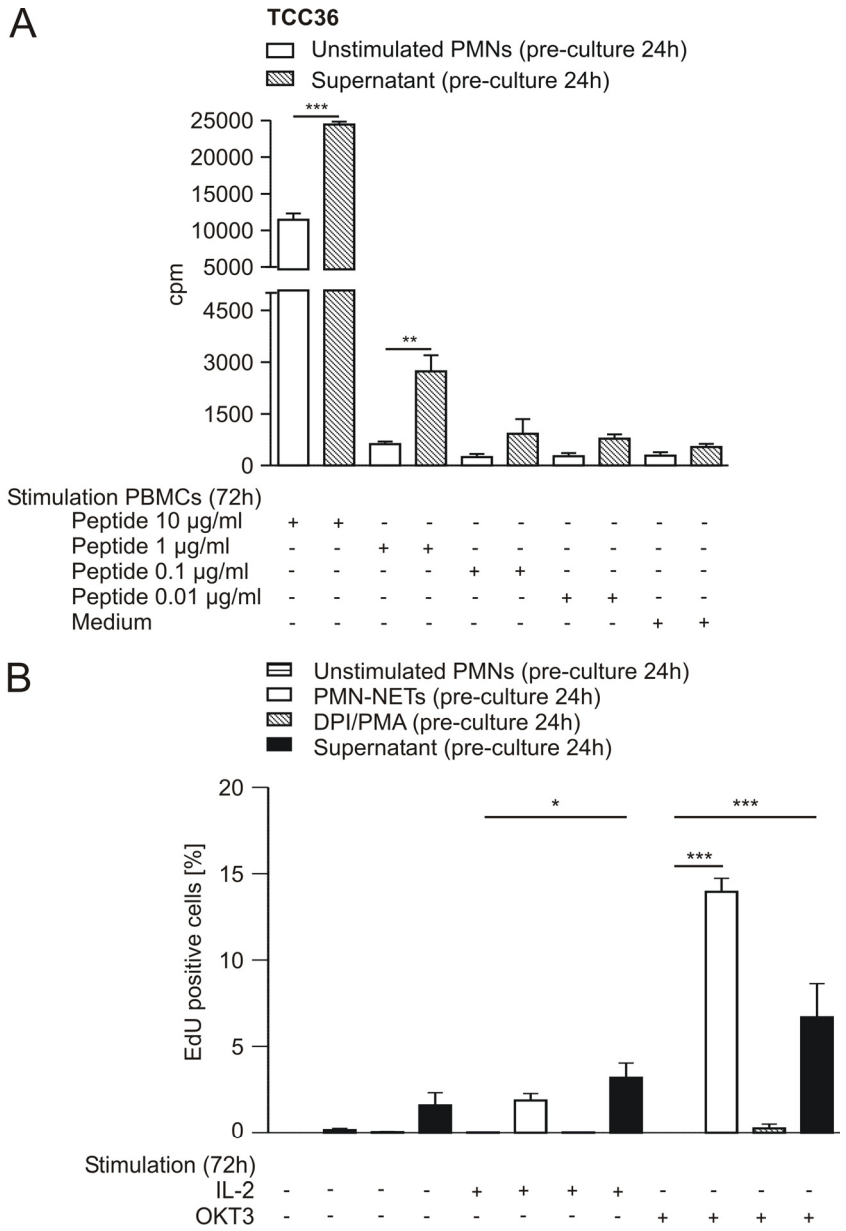

Figure 16 T cell priming by NETs lowers the activation threshold.

A) T cell proliferation assessed by thymidine incorporation in TCC36 pre-cultured with unstimulated PMNs or NETs-supernatant and stimulated with autologous irradiated PBMCs pulsed with a specific peptide. Graph represents cpms (mean values ± SEM) from ≥ 3 independent experiments. **B)** T cell proliferation assessed by EdU incorporation in purified CD4+ T cells pre- cultured with unstimulated PMNs, NETting-PMNs, DPI-PMNs or NETs- supernatant, and stimulated or not with low concentrations of anti-CD3 antibody (OKT3) or with IL-2. Graph represents the % of EdU positive cells (mean values ± SEM) from ≥ 3 independent experiments. *p<0.05, **p<0.01 and ***p<0.001

4.10 Resting dendritic cells are able to activate NETs-primed CD4+ T cells in the absence of specific antigen

Next it was examined whether a more physiologic, but sub-optimal stimulus such as the interaction of T cells with resting DCs in the absence of specific antigen, which alone does not induce proliferation of resting T cells, stimulates a response of NETs-primed $CD4^+$ T cells. $CD4^+$ T cells were pre-cultured for 24 h with unstimulated PMNs (unprimed CD4) or with NETs-supernatant (NET-primed CD4) for 24 h. Simultaneously, purified DCs were pre-cultured with unstimulated PMNs (resting DCs). After this period, both cell types were carefully washed and co-cultured for additional 48 h. 8.7 ± 1.3 % of NET-primed purified $CD4^+$ T cells proliferated and released 2092 ± 459 pg/ml of IFN-γ, while no activation was observed in un-primed $CD4^+$ T cells (Figure 17A). In order to confirm that NETs-priming is TLR9 independent, but involves TCR signaling, CD4+ T cells were primed with NETs-supernatant in the presence or absence of the TLR9 inhibitor chloroquine or herbimycin A. Proliferation was not affected in $CD4^+$ T cells primed in the presence of chloroquine, however, herbimycin A clearly reduced proliferation (1.72 ± 1.42 %) (Figure 17B), confirming that T cell priming by NETs is independent of TLR9, but involves TCR signaling.
In order to understand better the interaction between NETs-primed CD4+ T cells and resting DCs, NETs-primed purified CD4+ T cells and resting DCs were co-cultured in the presence or absence of a blocking anti-HLA DR antibody, the corresponding isotype control, or herbimycin A. The presence of an anti-HLA DR antibody reduced the proliferation of primed CD4+ T cells (3.76 ± 1.05 %) suggesting that class II molecules are involved in this activation (Figure 17C). The fact that blocking of HLA DR did not abolish T cell proliferation completely suggests that class II molecules other than DR expressed on DCs, i.e. HLA DQ and DP molecules, could still provide an activation signal. The presence of herbimycin A completely abrogated the proliferation of primed CD4+ T cells indicating that activation of primed T cells by DCs requires TCR signaling (Figure 17C).

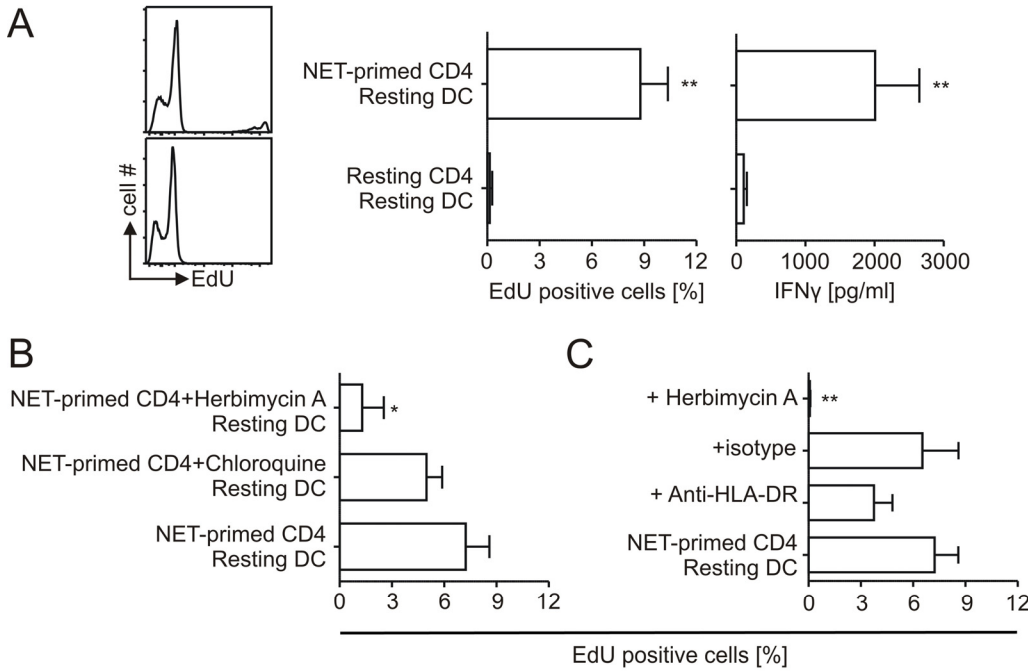

Figure 17 Resting DCs are able to activate NETs-primed CD4$^+$ T cells in the absence of specific antigen. (**A-C)** CD4$^+$ T cell proliferation assessed by EdU incorporation. Histograms represent the proliferation from a representative experiment. Graph represents the % of EdU positive cells and pg/ml of IFN-γ (mean values ± SEM) from ≥ 3 independent experiments. **A)** Purified CD4$^+$ T cells pre-cultured with unstimulated PMNs (unprimed) or with NETs-supernatant (NETs-primed) were co-cultured with DCs previously pre- cultured with unstimulated PMNs (resting). **B)** CD4+ T cells pre-cultured with NETs-supernatant in presence or absence of TLR9 inhibitor chloroquine or TCR-signaling inhibitor herbimycin A, and then co- cultured with resting DCs. **C)** NETs-primed CD4$^+$ T cells co-cultured with resting DCs in presence or absence of a HLA DR blocking antibody, the corresponding isotype control or herbimycin A. *p<0.05 , **p<0.01

4.11 NET-activated pDCs do not exert an effect on un-primed CD4$^+$ T cells

Next, it was also examined whether DCs, particularly pDCs, pre-cultured with NETs-supernatant (NETs-activated DCs) were able to activate CD4$^+$ T cells. Following purification, approximately 40% of DCs were pDCs and 60% myeloid DCs (mDCs). After 24 h of co-culture with NETs, all mDCs died, while pDCs survived and upregulated some maturation and activation markers such as CD40, CD80, CD83 and CD86, but not HLA class II (Figure 18A). Unprimed CD4$^+$ T cells co-cultured with NETs-activated pDCs did not proliferate nor did they produce IFN-γ (Figure 18B). NETs-activated pDCs were able to induce proliferation (3.83 ± 1.00 %) and IFN-γ release (1000 ± 475 pg/ml) only in NETs-primed CD4$^+$ T cells (Figure 18B), suggesting that the NETs-mediated activation of pDCs in our in vitro setting does not exert an effect on T cell activation. NETs-activation of pDCs seems to play a role in some autoimmune diseases and it has been suggested that the large amounts of IFN-α produced by NETs-activated pDCs can activate mDCs and increase antigen presentation to T

cells[199,201204-206]. The loss of mDCs after culture with NETs most likely prevented this indirect effect of NETs-activated pDCs on T cells in our *in vitro* system.

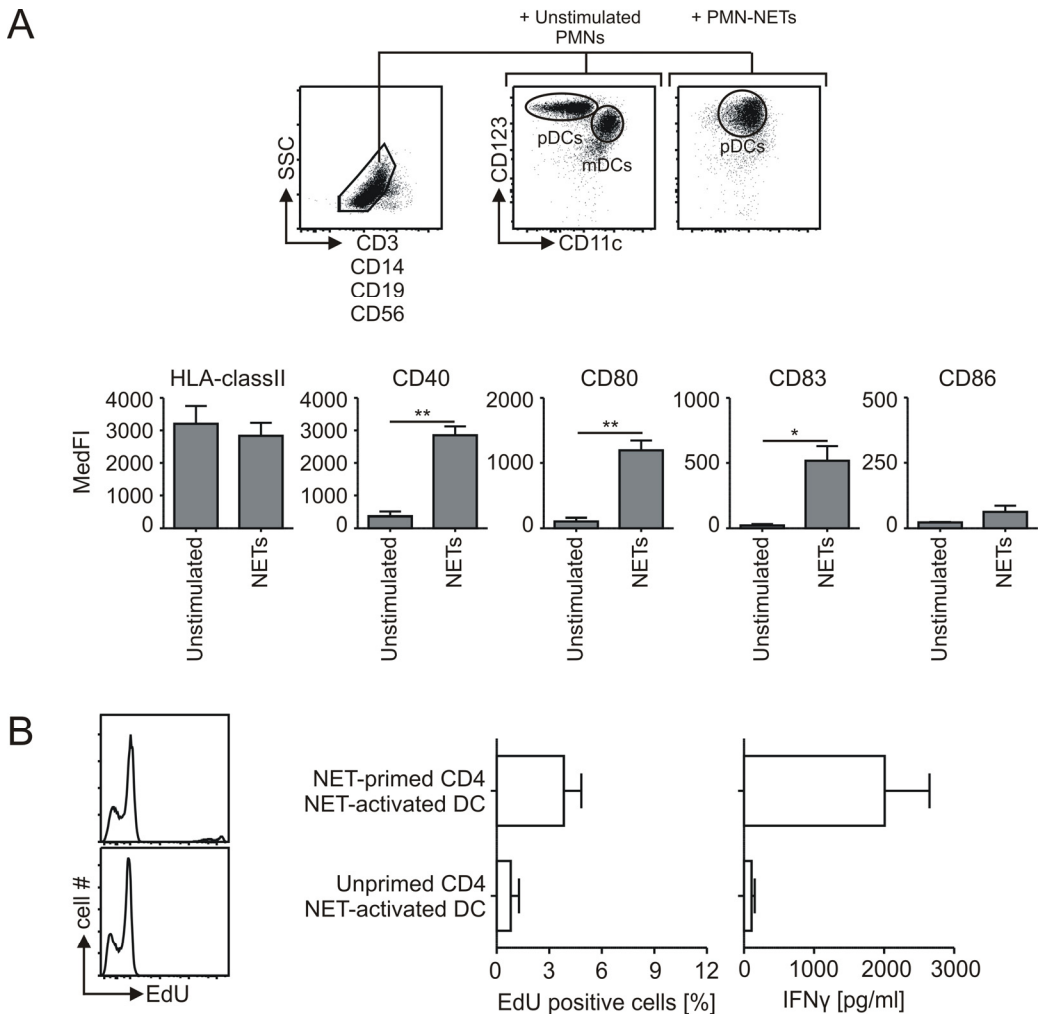

Figure 18 NETs-activated pDCs do not exert an effect on unprimed CD4$^+$ T cells.

A) HLA class II, CD40, CD80, CD83 and CD86 expression on gated NETs-activated pDCs. Values show MedFI ± SEM. **B)** Purified CD4$^+$ T cells pre-cultured with unstimulated PMNs (unprimed) or with NETs-supernatant (NETs-primed) were co-cultured with DCs previously pre-cultured with NETs-supernatant (NETs-activated). CD4$^+$ T cell proliferation was assessed by EdU incorporation. Histograms represent the proliferation from a representative experiment. Graph represents the % of EdU positive cells and pg/ml of IFN-γ (mean values ± SEM) from ≥ 3 independent experiments. *p<0.05 , **p<0.01

4.12 Co-culture of NETting PMNs, DCs and T cells results in T cell activation

Then the question was asked whether co-culture of T cells, DCs and NETting-PMNs results in T cell activation and whether NETs exert the same effect on $CD4^+$ and $CD8^+$ T cells. NETting-PMNs were used instead of NETs-supernatant as more physiologic condition. Purified $CD4^+$ or $CD8^+$ T cells were co-cultured with unstimulated or NETting-PMNs in the absence or presence of purified DCs. Proliferation and secretion of IFN-γ, IL-17A, IL-4, IL-10 and IL-2 were measured as indicators of T cell activation (Figure 19). Purified $CD4^+$ and $CD8^+$ T cells co-cultured with unstimulated PMNs alone or in the presence of purified DCs did not proliferate nor produce cytokines (Figure 19A+B). Similarly, no proliferation or cytokine release was observed upon co-culture with NETting-PMNs in the absence of DCs (Figure 19A+B). In contrast, both proliferation and cytokine release was observed when purified DCs were added to the co-cultures. 13.44 ± 2.2 % of $CD4^+$ and 8.15 ± 1.1 % of $CD8^+$ T cells proliferated (Figure 19A+B). Regarding cytokine production purified $CD4^+$ T cells co-cultured with NETting-PMNs and DCs secreted 901 ± 233 pg/ml of IFN-γ and 289 ± 99 of IL-2. Purified $CD8^+$ T cells secreted only IFN-γ (981.6 ± 293 pg/ml) (Figure 19B).

Direct contact of T cells with DCs and NETting-PMNs was required for T cell activation. When $CD4^+$ or $CD8^+$ T cells were physically separated from DCs and NETting-PMNs by a transwell, no activation was detected (Figure 19A+B). The proliferation of $CD4^+$ T cells co-cultured with NETting-PMNs and DCs remained unchanged if $CD8^+$ T cells were added (19 ± 5.3 %), however conversely, the proliferation of purified $CD8^+$ T cells co-cultured with NETting- PMNs and DCs increased if purified $CD4^+$ cells were added into the co-culture (30.4 ± 0.4 %) (Figure 19A).

It was also addressed whether the ability of NETs to induce T cell proliferation was comparable between naïve and memory $CD4^+$ T cells. Purified naïve or memory CD4+ T cells were co-cultured with unstimulated PMNs or NETting- PMNs in the presence of purified DCs. 10.0 ± 4.0 % of naïve and 7.5 ± 3.3 % of memory $CD4^+$ T cells co-cultured with NETting-PMNs and purified DCs proliferated, while neither naïve nor memory $CD4^+$ T cells proliferated upon co-culture with unstimulated PMNs and DCs (Figure 19C).

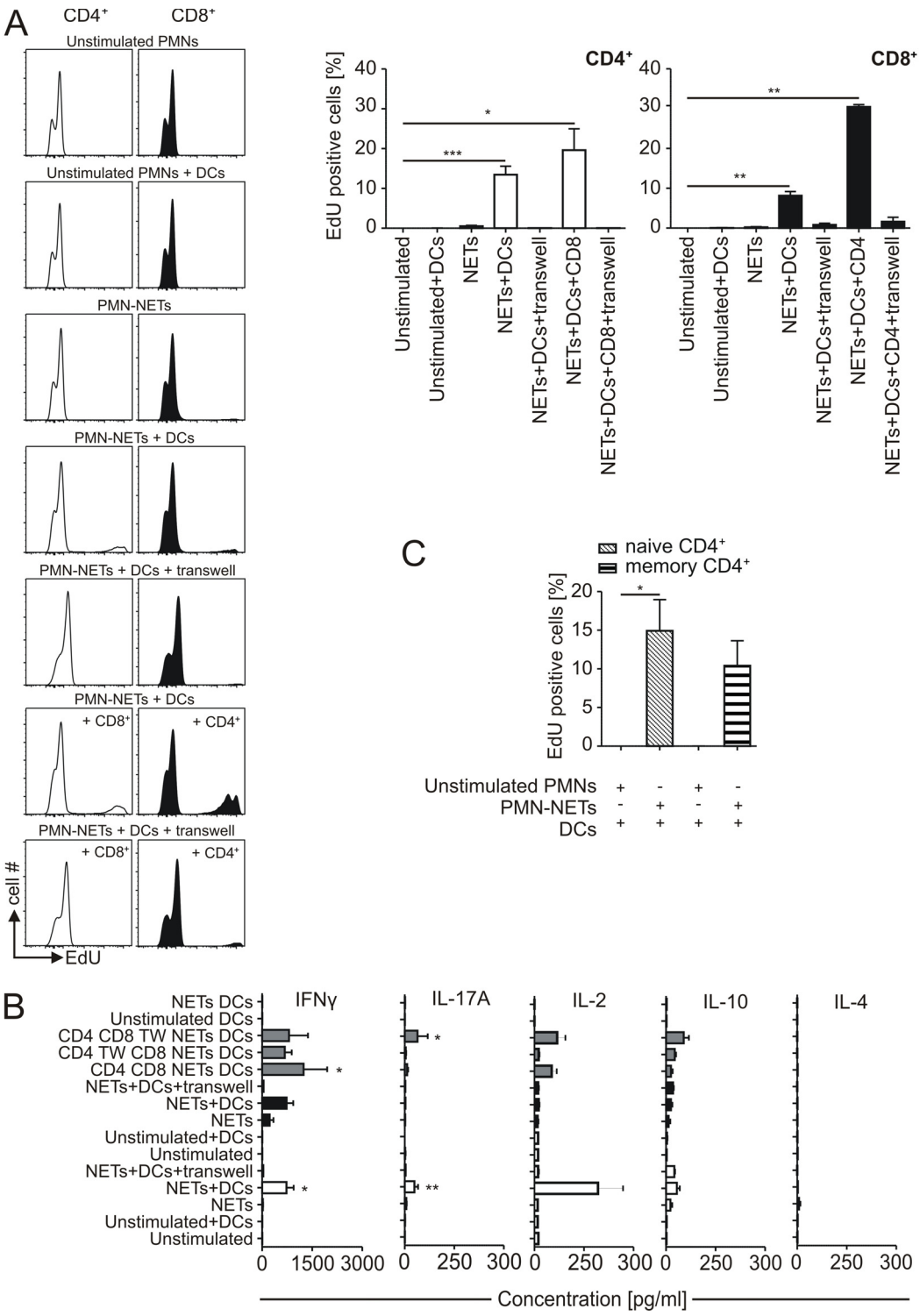

Figure 19 Co-culture of T cells with NETting-PMNs and DCs results in T cell activation.

A) T cell proliferation assessed by EdU incorporation in purified $CD4^+$ (white) and purified $CD8^+$ (black) T cells co-cultured with unstimulated PMNs in the presence or absence of DCs, NETting-PMNs (NETs) in the presence or absence of DCs and separated or not by a transwell, and in the presence or absence of additional purified $CD8^+$ or $CD4^+$ T cells respectively. Histograms represent the proliferation from a representative experiment. Graphs represent the % of EdU positive cells (mean values ± SEM) from ≥ 3 independent experiments. **B)** IFN-γ, IL-17A, IL-4, IL-10 and IL-2 produced by purified $CD4^+$ (white), $CD8^+$ (black) or both T cells together (in grey) co-cultured as indicated in **(A).** Values show mean pg/ml ± SEM. **C)** T cell proliferation assessed by EdU incorporation in purified naïve (diagonally striped bars) and memory (straight striped bars) $CD4^+$ T cells co-cultured with unstimulated PMNs or NETting-PMNs in presence of DCs. Graph represents the % of

EdU positive cells (mean values ± SEM) from 3 independent experiments. *p<0.5, **p<0.01 and ***p<0.001. Significance refers to unstimulated condition.

4.13 NETs mediate T cell activation of peripheral blood mononuclear cells

Finally, the activation of $CD4^+$ and $CD8^+$ T cells mediated by NETs was characterized in bulk PBMCs, a condition that reflects better the circumstances, under which T cells will encounter NETting PMNs *in vivo*. PBMCs include in addition to T cells and DCs also monocytes and B cells that could both influence T cell activation mediated by NETs. PBMCs co-cultured with NETting-PMNs formed large clusters that were absent when cells were co-cultured with unstimulated PMNs (Figure 20A) and strongly up-regulated CD25 and CD69 expression on both $CD4^+$ and $CD8^+$ T cells (Figure 20B). In addition, within the PBMCs 10.5 ± 1.0 % of $CD4^+$ and 26.73 ± 5.2 % of $CD8^+$ proliferated after co-culture with NETting-PMNs, while no proliferation was observed when PBMCs were co- cultured with unstimulated PMNs (Figure 20C). The ability of NETting-PMNs to mediate proliferation in $CD4^+$ and $CD8^+$ T cells showed great inter-individual variability (Figure 20D). PBMCs co-cultured with NETting PMNs also secreted 1777 ± 272 pg/ml of IFN-γ and 93.5 ± 25 pg/ml of IL-17A, but neither IL-4 nor IL-10 (Figure 20E). No cytokine secretion was detected when PBMCs were co-cultured with unstimulated PMNs.

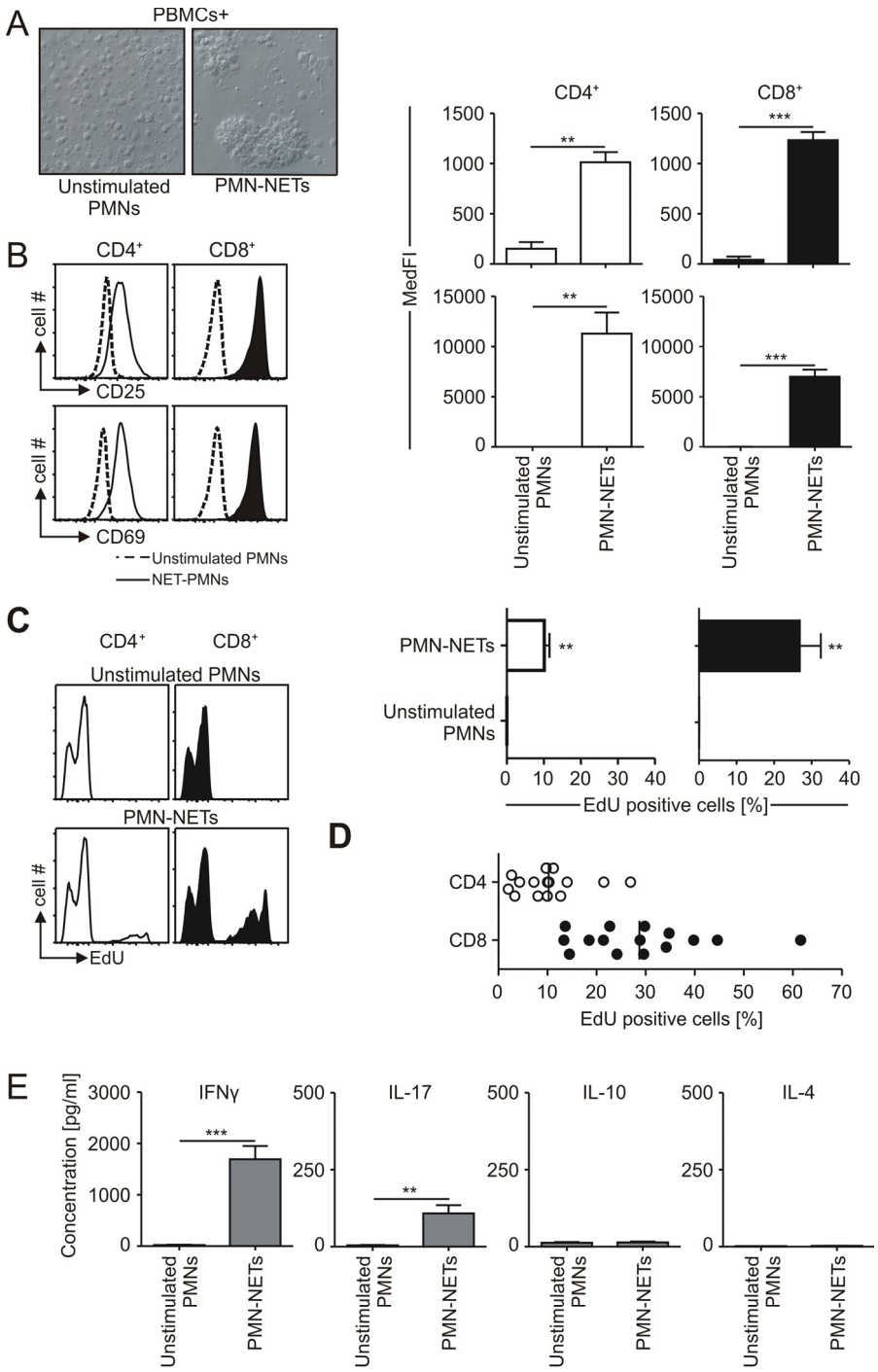

Figure 20 NETs-mediated T cell activation within PBMCs.

A) Transmission light images of cluster formation of PBMCs co-cultured with unstimulated PMNs and NETting-PMNs. **B)** CD25 and CD69 surface expression after gating on $CD4^+$ (white) and $CD8^+$ (black) T cells. Histograms represent the expression from a representative experiment. Dotted line (PBMCs co-cultured with unstimulated PMNs), solid line (PBMCs co-cultured with NETting-PMNs (NETs)). Graphs represent mean fluorescence intensity (MedFI) ± SEM from five independent experiments. **C)** T cell proliferation in PBMCs co-cultured with unstimulated PMNs and NETting-PMNs. T cell proliferation was assessed by EdU incorporation after gating on $CD4^+$ (white) and $CD8^+$ (black) T cells. Graphs represent the % of EdU positive cells (mean values ± SEM) from ≥ 5 independent experiments. Histograms represent the proliferation from a representative experiment. **D)** Scatter plot showing T cell proliferation, in which each dot represents one individual donor. **E)** IFN-γ, IL-17A, IL-4 and IL-10 produced by PBMCs co-cultured with unstimulated PMNs and NETting-PMNs (NETs). Values show mean pg/ml ± SEM from 5 independent experiments. **p<0.01 and ***p<0.001.

4.14 NETs but no other factors mediate T cell activation

In order to confirm that NETs and not other factors mediated the substantial T cell activation in bulk PBMCs, a broad range of controls were included. First, in order to discard a possible effect of contaminating PMA on T cell activation, PBMCs were co-cultured with PMNs treated with PMA for only 15 min, a period too short to induce substantial NET production. No up-regulation of CD25 and CD69 expression (Figure 21A), T cell proliferation (Figure 21B) nor production of cytokines (Figure 21C) were observed under this condition. PMNs activated with formyl-methionyl-leucyl-phenylalanine (fMLP), a stimulus less efficient than PMA in inducing NETs, also failed to mediate T cell activation. NETting PMNs, i.e. PMNs undergoing NETosis, are dying cells. To exclude an effect of dead cells on T cell activation, PBMCs were co-cultured with PMNs dying by other mechanisms than NETosis. Apoptosis in PMNs was induced by exposure to UV light for 60 min. Cells were subsequently incubated for additional 16 h in order to increase the number of dying cells and then co-cultured with PBMCs. No activation was detected under these co-culture conditions (Figure 21A+B+C).

In order to exclude that PMA-induced mediators other than NETs were responsible for T cell activation, PMNs were stimulated with PMA for 3 h and then fixed with paraformaldehyde (PFA) prior to co-culture with PBMCs. After fixation, PMNs are not able to produce any mediators, and only NETs remained on cells. PBMCs co-cultured with NETting, PFA-fixed-PMNs showed up-regulation of CD25 and CD69 expression (Figure 21A), T cell proliferation (Figure 21B) and cytokine release (Figure 21C) similar to those observed with unfixed cells. As an additional control, PBMCs were co-cultured with NETting PMNs, but physically separated by a transwell to avoid cell-cell or NETs-cell contact. No up-regulation of CD25 expression, T cell proliferation or release of cytokines (Figure 21A+B+C) was detected under this condition excluding that soluble mediators produced by PMNs are responsible for T cell activation. Contact between NETs and PBMCs was required for T cell activation as already observed with purified cells (Figure 21). Interestingly, some up-regulation of CD69 was observed in this condition suggesting that the expression of this molecule is partially mediated by PMA-induced soluble factors (Figure 21A). Finally, as expected, the co-culture of PBMCs with DPI-PMNs did not induce T cell activation, while the co-culture with NETs-supernatants did (Figure 21A+B+C).

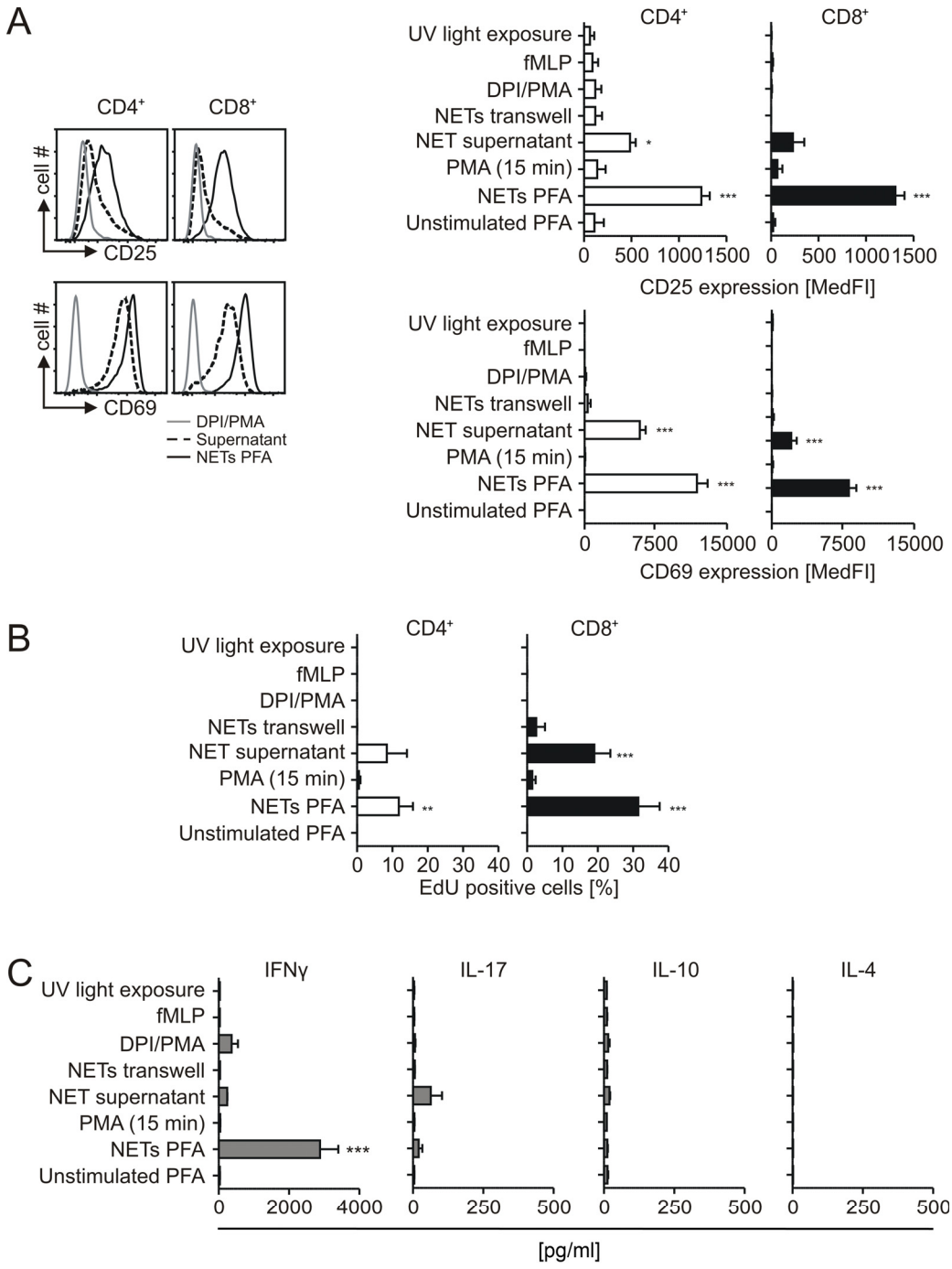

Figure 21 NETs but not other factors mediate T cell activation.

A) CD25 and CD69 surface expression after gating on $CD4^+$ (white) and $CD8^+$ (black) T cells in PBMCs co-cultured with unstimulated PMNs, NETting-PMNs (NETs), PMNs stimulated with PMA 15 min, PMNs stimulated with fMLP, dying PMNs exposed to UV light, NETting-PMNs fixed with PFA, NETting-PMNs physically separated by a transwell, DPI-PMNs and NETs-supernatant. Graphs represent mean fluorescence intensity (MedFI) ± SEM from five independent experiments. **B)** T cell proliferation assessed by EdU incorporation after gating on $CD4^+$ (white) and $CD8^+$ (black) T cells in PBMCs co-cultured as indicated in **(A)**. Graphs represent the % of EdU positive cells (mean values ± SEM) from five independent experiments. **C)** IFN-γ, IL-17A, IL-4 and IL-10 produced by PBMCs co-cultured as indicated in **(A)**. Values show mean pg/ml ± SEM from five independent experiments. *p<0.5, **p<0.01 and ***p<0.001. Significance refers to unstimulated PFA.

5 DISCUSSION

5.1 PMN functions are altered in MS patients

MS is considered a $CD4^+$ T cell-mediated autoimmune disease of the central nervous system[126]. Several studies have reported the role of PMNs in the development of EAE, the murine model for MS. PMNs are able to enter the CNS during the pre-clinical and acute phase and contribute to the development of an acute phenotype of EAE, and this acute phenotype is completely abrogated upon depletion of $Gr1^+$ cells or the blocking of the relevant chemokine receptor CXCR2[164,167,168]. In contrast to EAE findings the role of PMNs in the pathogenesis of MS is very controversial, since lesions of MS patients are mainly characterized by infiltrations of activated macrophages, microglia and lymphocytes[159]. So far published studies about PMNs in MS are contradictory. Some groups did not find any alterations in neutrophil[183,184] phenotype and effector functions, e. g. oxidative burst, whereas other reported altered protease activity in purified PMNs of active MS patients compared to inactive MS, a changed phenotype and effector functions of PMNs in MS[178-182]. The data of the present dissertation shows a significant increase of intracellular ROS upon stimulation with fMLP in PMNs from RR-MS patients. Additionally, degranulation was altered in PMNs from RR-MS patients after stimulation as PMNs from RR-MS patients also released more elastase, which is stored in their azuphilic granules, in comparison to controls. In contrast, the phagocytic capacitity of PMNs from RR-MS patients was not different. Our group has recently found that PMNs from RR-MS patients express increased levels of formyl peptide receptor 1, the receptor for fMLP[182] that could explain the increased degranulation and oxidative burst upon stimulation with fMLP in PMNs from RR-MS patients.

In addition to phagocytosis, degranulation and oxidative burst, the release of NETs by PMNs has recently been described by Brinkman et al., as a new effector mechanism[207]. NETs are fibres of chromatin decorated with granule, cytoplasmic and nuclear proteins able to trap and kill foreign microorganisms very efficiently and serve, at the same time, as physiological barrier preventing microbial spread and diffusion of proteases into the surrounding tissue. However, NETs are also associated with pathophysiological conditions, such as autoimmunity and complex systemic inflammation[21,26,188,199,201]. In this thesis a subgroup of RR-MS patients could be identified that has higher level of circulating NETs in serum samples compared with healthy controls. These results contradict previous data reported by

Kessenbrock et al.[26], who could not detect any circulating NETs in serum samples from MS patients. This discrepancy can most likely be explained by the low number of MS patients studied by Kessenbrock et al.. The high inter-individual variability observed in our RR-MS group did not correlate with disease activity. Unexpectedly, no circulating NETs were detectable in patients with NMO, a MS-related autoimmune inflammatory disorder of the central nervous system affecting primary the optical nerve and the spinal cord, in which PMNs play an important role[160]. As expected, CSF samples from MS patients and all control groups were negative for NETs, in agreement with the fact that PMNs have been not found in brain lesions from MS patients[155,159].

Bacterial and fungal infections are described as potential triggers of NET formation[21,207]. Therefore, serum concentrations of CRP, an acute-phase protein released by the liver in response to infections, and IL-6, another pro-inflammatory cytokine, were analyzed. A higher concentration of CRP was detected in samples of patients with high serum NET levels suggesting that an infection might induce NETs release. However, the concentration of CRP was very low overall, as only one donor reached the concentration of 40 mg/l indicative of mild inflammation or viral infection and no patient reaching values higher than this. CRP concentrations of about 200 mg/ml are associated with active inflammation and bacterial infections[208]. Therefore, the question remains unanswered as to whether the high NETs serum level in some RR-MS patients are triggered by bacterial and fungal infections.

In addition to bacterial or fungal infections, studies have described that NETs can also be induced in the absence of any infection. IL-8 has been identified as an efficient NET inducer[25,207] and elevated serum levels of IL-8 have been shown in serum from MS patients[189]. Higher IL-8 levels have been measured in RR-MS patients with high numbers of MPO-DNA complexes. Unfortunately, the difference was not significant between both groups. Amongst other cells, endothelial cells store IL-8 in intracellular vesicles and release the cytokine upon activation. Furthermore, activated endothelial cells themselves have the property to induce NETosis in PMNs[25]. Additionally, PMNs adherend to activated endothelial cell are more susceptible for platelet-mediated NET induction[24]. As increased levels of activated platelets[192] and endothelial cells[190] are also associated with MS, serum level of β-thromboglobulin and platelet factor 4 as marker for platelet activation[193] and serum levels of soluble vascular endothelial cadherin as marker for endothelial cell activation were measured. No correlation between these markers and higher circulating NET serum levels in a subgroup of RR-MS patients could be found.

Both endothelial cells and platelets are able to shed small membrane vesicles in response to cell activation[196]. These small vesicles that contain cytosol, receptor and surface molecules from their mother cell, are called microparticles. They circulate in the blood stream of healthy individuals[209,210], but they are elevated under certain pathological conditions, e.g. cardiovascular disorders[209], pre-eclampsia[25,211] and rheumatoid arthritis[212]. Endothelial-derived microparicles were revealed as inducer of NETosis in PMNs as well as their corresponding microparticles in the case of pre-eclampsia[25]. Elevated plasma platelet-derived[192] and endothelial-derived microparticles[198,213] have been found in MS patients. In order to investigate whether microparticles are responsible for the higher NET amount in a subgroup of MS patients, both types of microparticles were analyzed. Whilst no correlation with endothelial-derived microparticles induced by activation could be observed, a significantly elevated percentage of platelet-derived microparticles induced by activation in patients with high serum levels of circulating NETs could be found, suggesting that these microparticles could be involved in NET induction in MS patients. It might be worth investigating platelet-derived microparticles in the NETs and MS context in more detail.

NETs are immunogenic structures made up of PMN proteins and DNA as potential autoantigens can be exposed to the immune system under pathological conditions. In some autoimmune disease such as systemic lupus erythematodes (SLE) patients have high serum titers of autoantibodies against PMN-derived proteins or anti-DNA. These autoantibodies are able to induce NETosis in PMNs by themselves[26,199,201]. Anti-neutrophil cytoplasmic antibodies (ANCAs), anti-ds DNA antibodies and anti-nuclear antibodies (ANAs) are not characteristic for patients suffering MS, and indeed serum samples of donors with high levels of circulating NETs did not contained increased titer of ANAs, ANCAs and anti-ds DNA antibodies when compared with donors with low levels.

For patients suffering from SLE or psoriasis, another autoimmune disease, it has been described that NET-DNA, which is associated with the antimicrobial peptide LL-37, participates in the NET-induced activation of plasmacytoid DCs (pDCs) via TLR9 resulting in a high amount of IFNα secretion[199,201]. Therefore, the frequency of DCs and their activation status in donors with high and low circulating NET serum levels were measured in this thesis. Furthermore, the frequency and activation status of monocytes were analyzed, as different groups were able to demonstrate that monocytes can differentiate in fully mature DCs, able to present antigens to T cells[214-216]. No differences could be found between both groups. Additionally, the frequency of monoctytes, the frequency of granulocytes as well as

the frequency of lymphocytes were determined in whole blood samples of patients with high serum NET levels. Interestingly, monocytes were the only cell type that was significantly elevated in patients with high NETs. However, as previously mentioned, this result could not be confirmed by FACS analysis. Neither an increase in frequency nor a more active phenotype of monocytes could be demonstrated. It is possible that the freezing process influences the DC and monocyte frequency and their activation.

In conclusion, recent data about altered effector functions of PMNs in MS could be confirmed in this thesis, as increased oxidative burst and degranulation could be shown. Additionally, a subgroup of RR-MS patients could be identified which shows elevated circulating NET levels. However, despite intensive search, no putative causes could be demonstrated. Higher CRP and IL-8 concentration, a higher frequency of monocytes measured in whole blood and activation-induced platelet-derived microparticles hint at an inflammation or infection as triggers for NET formation in this subgroup.

5.2 NETs participate in increasing responsiveness of the adaptive immune response

The main function of the innate immune system during infection is to rapidly sense microbial pathogens, limit their spread and eliminate them with minimum collateral tissue damage. The composition of NETs, fibers of chromatin decorated with antimicrobial proteins, turn them into optimal structures to perform this task[217]. The main advantages of NETs are the following: (i) NET fibers trap pathogens and act as physical barriers preventing microbial spread, (ii) NETs render antimicrobial proteins more efficient by concentrating them on the DNA/protein fibers, which keeps them together and allows them to act synergistically, (iii) the collateral tissue damage is reduced since proteases do not diffuse into the tissue, and, in addition, binding to NETs reduces the toxic activity of some of these proteases[218], and (iv) NETs also allow co-localization of adjuvants and danger signals that can modulate inflammation, for example of self-DNA and LL-37, which are able to activate pDC. Here, a novel function of NETs is described, being able to directly prime T cells by reducing their activation threshold and in consequence mediate T cell activation. This previously unknown property of NETs demonstrates that their role is not limited to innate immune mechanisms, but also involved in activating the adaptive immune system.

$CD4^+$ T cell activation mediated by NETs unfolds as a two-step process. In the first, NETs prime $CD4^+$ T cells by direct contact, which reduces their threshold of activation. $CD4^+$ T

cells primed by NETs showed increased antigen-specific responses and can be activated by sub-optimal stimuli such as soluble anti-CD3 antibody (signal 1) in the absence of APCs (signal 2) or resting DCs in the absence of specific antigen, which are both not sufficient to induce a response of resting $CD4^+$ T cells. It was observed that NETs-T cell contact induced the formation of cell clusters, up-regulation of the activation markers CD25 and CD69, as well as some phosphorylation of ZAP-70 in $CD4^+$ T cells. These changes were insufficient to fully activate T cells and support T cell proliferation, but lowered their activation threshold. Unexpectedly, TLR9 is not involved in this NETs-mediated T cell priming since the TLR9 inhibitor chloroquine had no effect. If other TLR9-independent activation pathways that could be induced by DNA, such as those induced by CpG in monocytes[219] or nucleosomes in neutrophils[220], are involved in NETs-mediated T cell priming requires further investigation. While TLR9 does not play a role, T cell priming by NETs apparently involves TCR engagement and signaling since herbimycin A, a well-known inhibitor of TCR-mediated signal transduction, strongly reduced T cell priming. Further studies need to dissect, which of the many components of NETs engages the TCR and induces TCR signaling.

The lower activation threshold of NETs-primed T cells has been demonstrated only for CD4+ T cells. The survival of $CD8^+$ T cells pre-cultured with NETs and subsequently incubated with DCs was very low and prevented us from performing priming experiments with $CD8^+$ T cells. It has been reported that the antimicrobial peptide LL-37, which is present in NETs, induces granzyme-mediated apoptosis of cytotoxic T lymphocytes[221], which could explain the lower survival of $CD8^+$ T cells pre-exposed to NETs, but this possibility was not addressed here. $CD8^+$ T cells primed with NETs showed cluster formation and up-regulation of CD25 and CD69 expression comparable to that observed in NETs-primed $CD4^+$ T cells, which suggests a similar behavior of both cell types after NETs priming. Furthermore, only minor differences were found in proliferation and release of IFN-γ between $CD8^+$ and $CD4^+$ T cells co-cultured with NETting-PMNs and DCs. However, higher proliferation and IFN-γ release were found in $CD8^+$ T cells co-cultured with NETs and DCs in the presence of $CD4^+$ T cells. These data suggest that, although $CD8^+$ T cells were activated by NETs, $CD4^+$ T cells most likely enhanced $CD8^+$ T cell activation via IL-2 production when both cell types were present. Interestingly, NETs mediated the activation of both $CD4^+$ naïve and memory T cells. It is well known that memory $CD4^+$ T cells have less stringent activation requirements than naïve $CD4^+$ cells and are able to respond faster and stronger to lower doses of antigen and to lower levels of co-stimulation than naïve $CD4^+$ T cells[77-79]. In this context, the results suggest that priming

of naïve $CD4^+$ T cells by NETs may induce changes in these cells similar to those that accompany memory T cell generation.

Despite the well documented importance of NETs as effective antimicrobial first line defense mechanism, there is increasing evidence that NETs occur in various clinical settings in the absence of microbial infections and that they are probably also associated with pathophysiological conditions[222]. Abnormally high production and/or low degradation of NETs can lead to tissue damage, and the activation of pDCs and simultaneous exposure of the immune system to autoantigens may result in the generation of anti-self antibodies, which in addition can stimulate NET formation creating a vicious cycle. Aberrant NETs formation can therefore not only participate in the organ damage observed in chronic inflammatory disorders[24,25,30,191], but furthermore contribute to the development and perpetuation of autoimmune diseases such as systemic lupus erythematosus[188,199,201,204,223], small-vessel vasculitis[26] and psoriasis[204]. Due to the central role of T cells in most of these disorders, the finding of this study that NETs are able to mediate T cell proliferation and secretion of Th1 pro-inflammatory cytokines represents a novel mechanism how NETs may contribute to T cell-mediated autoimmune diseases or certain aspects of their pathogenesis.

In conclusion, the results of the study show for the first time the ability of NETs to directly prime T cells by reducing their activation threshold and consequently to enhance adaptive immune responses. This novel feature adds to the list of interesting properties of NETs and the functions of neutrophils in general. NETs as a highly specialized structure of PMNs are according to the observations of this study not only important for the first-line innate immune defense, but also participate in rendering adaptive immune responses more efficient. Data of the study on the involvement of NETs in adaptive immunity still leaves many important questions, and, among them, it will be very interesting to examine the role of NETs in pathological immune responses and autoimmune diseases and explore if a better understanding may lead to new therapeutic strategies.

Conclusion

The schemes depicted in Figure 22 and 23 summarize proposed mechanisms as to how NETs contribute to T cell activation.

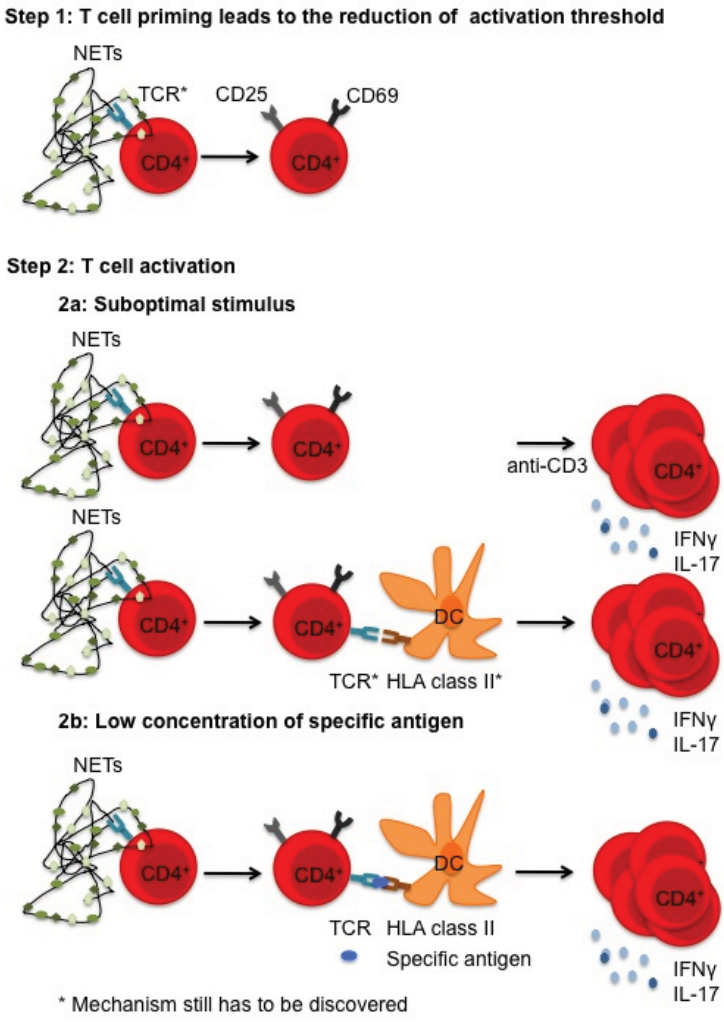

Figure 22 Proposed mechanism how NETs influence adaptive immune response.

The induction of T cell proliferation and cytokine secretion by NETs is a two step mechanism. First, T cells are primed by direct NET contact via TCR signalling resulting in upregulation of CD25 and CD69 (Step 1). NET priming reduces the activation threshold of T cells. Suboptimal stimuli like low concentration of anti-CD3 in absence of APCs or the presence of HLA class molcules without specific peptide are enough to induce T cell proliferation and cytokine secretion (Step 2a). After NET priming of T cells, low concentration of antigen are sufficient to activate T cells (Step 2b). The mechanisms how NETs interfere with TCR for T cell priming and T cell activation still has to be discovered.

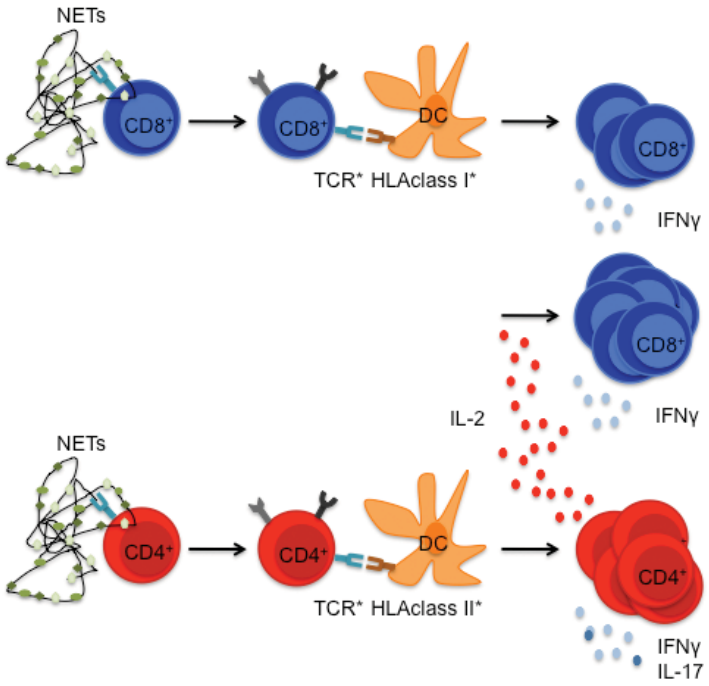

Figure 23 Proposed mechanism how NET stimulated CD4$^+$ T cells support CD8$^+$ T cell proliferation.
CD8$^+$ T cell proliferation and cytokine secretion is inducible by NET-DC contact. The activation of CD8$^+$ T cells is enhanced in presence of CD4$^+$ T cells. NET primed and activated CD4$^+$ T cells secrete, besides IFNγ and IL-17, IL-2 that might support CD8$^+$ T cell proliferation.

5.3 OUTLOOK

Further studies need to be carried out in order to investigate the mechanisms that underlie T cell priming and activation by NETs in more detail. The next step could be to produce artificial NETs to examine which NET component is relevant for the priming effect on T cells und their subsequent activation. One could also perform a gene expression analysis of CD4$^+$ T cells cultured with unstimulated PMNs vs. NETs by microarray to study the changes in T cells during the NET-priming process. Another interesting step would be to examine whether CD4$^+$ T cells from patients suffering from autoimmune diseases like systemic lupus erythematodes, small vessel vasculitis and rheumatoid arthritis have lower activation thresholds to disease-specific antigen or a suboptimal stimulus and if this could be connected to NETs. In the case of MS, Bielekova et al. have already demonstrated that MS patients have autoantigen-specifiic T cells that recognize myelin peptides with higher avidity[146]. It would be particularly interesting in the case of MS to investigate whether patients with high circulating NET levels in serum have a DNaseI defect or whether additionally associated proteins such as antibodies prevents the degradation of NETs. This has been already shown for a subgroup of SLE patients and small vessel vasculitis[26,188].

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Appendix

List of abbreviations

ANCAs	Anti-neutrophil cytoplasmic antibodies
ANAs	Anti-nuclear antibodies
APC	Antigen-presenting cell
APC	Allophycocyanine
APRIL	a proliferation inducing ligand
BBB	blood-brain barrier
BCR	B cell receptor
BLyS	B-lymphocyte stimulator
CD	Cluster of differntiation
CGD	Chronic granulomatous disease
CIS	Clinically isolated syndrom
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
APC	Antigen-presenting cell
CTL	Cytotoxic lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCL	C-X-C motif chemokine
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium chlorid
ds	Double stranded
EAE	Experimental autoimmune encephalomyelitis
EdU	5-ethynyl-2`-deoxyuridine
Ela2	Neutrophil elastase
ELISA	Enzyme-linked immunosorbent assay
EMP	Endothelial-derived microparticle
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony-stimulating factor
Gd^+	Gadolinium-enhancing
HC	Healthy control
HLA	Human Leukocyte Antigen
ICAM	Inter-cellular adhesion molecule 1
IL	Interleukin
IFN	Interferon
LFA	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide

MedFI Median fluorescence intensity

MFI Mean fluorescence intensity

MBP Myelin basic protein

mDC Myeloid DC

MOG Myelin oligodentrocyte glycoprotein

MPO Myeloperoxidase

MRI Magnetic resonance imaging

MS Multiple sclerosis

NADPH Nicotinamide adenine dinucleotide phosphate

NETs Neutrophil extracellular traps

NFAT Nuclear factor of activated T cells

NFκB Nuclear factor'kappa-light chain enhancer' of activated B cells

NMO Neuromyelitis optica

OD Optical density

PAD4 Peptidylarginine deiminase 4

PB Pacific blue

PBMC Peripheral blood mononuclear cells

PBS Phosphate-buffered saline

pDC Plasmacytoid DC

PFA Paraformaldehyde

PE Phycoerythrin

PE-Cy7 PE cyanine 7

PMN Polymorphonuclear neutrophils

PMP Platelet-derived microparticles

PP-MS Primary-progessive MS

ROS Radical oxygen species

RR-MS Relapsing-remitting MS

SEM Standard error of mean

SLE Systemic lupus erythematosus

SP-MS Secondary-progessive MS

SSV Small vessels vasculitis

STAT Signal transducers and activators of transcription

TCC T cell clone

TCR T cell receptor

TGF-β T cell growth factor β

Th T helper

TLR Toll like receptor

Treg Regulatory T cells

UV Ultraviolet light

ZAP-70 Zeta-chain-associated protein kinase 70

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