Autoimmune inflammation in systemic lupus erythematosus and Alzheimer's disease

Autoimmuninflammation im systemischen Lupus erythematodes und Morbus Alzheimer

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Contents

Contents

Preface

This dissertation is divided into two different, but interactive aspects, the study of inflammatory mechanisms in systemic lupus erythematosus (SLE) and Alzheimer's disease (AD). Thus, it will focus on the characterization of the immune response in autoimmune and neurodegenerative disorders. The premise is that understanding inflammation in the context of the disease may lead to a better understanding of immunopathogenesis and regulation. For the sake of clarity, the information contained in this work has been organized as follows:

At the beginning a short summary provides the audience with the main findings of the present studies. The first chapter is intended to be an immunological framework conceptually useful to understand the key regulatory steps of inflammation and how misjudgement during this process can lead to autoimmunity. Chapter II will focus on the interaction within the immune system which leads to a loss of self-tolerance to SLE autoantigens (autoAg) that explains the onset and development of this prototype of systemic autoimmunity. Chapter III provides special information about cells of innate immunity and their role in chromatin-mediated pathogenesis in SLE. The accumulation of the self-peptide amyloid-beta (Aβ) plays a causal role in the neuropathology of AD. As the assembly of misfolded proteins is accompanied by inflammation, the fourth chapter gives attention to autoimmunity in the central nervous system (CNS). The intention of this general introduction is not to give a complete review of the different aspects but rather comprehensive background knowledge based on key publications.

Chapters V to VII contain the main achievements of this dissertation based on already published results and submitted manuscripts as indicated. Every section is divided into an introduction, material and methods, results, discussion and references. This structure will enable the audience to find all relevant information within an accurate format for the presentation of scientific results which also guarantees this work to be comprehensible for international readers. These chapters represent the state-of-the-art when the respective project was prepared for publication and have been supplemented with the most relevant recent findings. Importantly in all sections that contain work from my colleagues, I have specified the extent of my contribution. Subsequently, the research results are recapitulated thoroughly followed by a final conclusion.

Preface

In addition, aspects of current research as well as future directions are mentioned, which should encourage other individuals to proceed with these experiments. I am looking forward to hearing from successes of the next generation of students towards realizing the full potential of these projects. I hope that the information contained within this dissertation will provide you with the knowledge to promote your investigations in a productive environment and friendly fashion. Abbreviations are listed at the end.

The projects mentioned here include my research efforts under the guidance of Prof. Hans-Georg Rammensee from March 2008 till March 2010 at the Department of Immunology, Institute for Cell Biology, University of Tübingen. This work is my own intellectual property and has been written autonomously. Wherever further resources have been used, this has been indicated. Enjoy reading.

Dennis Lindau

Summary

Autoimmune inflammation potentially arises through aberrant reactions of innate or adaptive immunity. This results in cellular destruction and the failure to heal the inflamed tissue, complications which are seen in SLE and AD. The present study has as overarching theme the understanding of inflammatory mechanisms in both diseases. One major autoAg in SLE is the nucleosome. (1) We report here that nucleosomes from SLE patients' plasmas induce the secretion of inflammatory mediators in neutrophils (PMN, polymorphonuclear leukocytes). Nucleosome-induced activation occurred independently of endotoxins, endosomal acidification, unmethylated cytosine-phosphate-guanosine (CpG)-motifs and Toll-like receptor (TLR) 9. Thus, the signaling pathway used is different from the classical pathway of unmethylated CpG-oligonucleotides (ODN). TLR9 might play different roles in nucleosome-induced innate immunity and anti-nucleosome autoimmunity. (2) We followed initial results by Viktoria Rönnefarth and report TLR9 expression at the cell surface of primary PMN. Cell surface TLR9 recognizes CpG-ODN and is functional, leading to PMN activation. Thus, we describe a novel pathway for DNA-recognition and PMN activation. The assembly of misfolded Aβ in AD is accompanied by microgliosis. (3) Close examination of infiltrating and CNS-resident immune cells lead to the identification of dendritic-like microglia. Characterization in transgenic (tg) AD mice as well as in human brain material, demonstrated that these microglia possess a high activity of phagocytosing Aβ ex vivo. However, this mechanism is highly regulated in vivo by the expression of several inhibitory signals. We were not able to restore the phagocytosing activity of dendritic-like microglia by simply ablating the gene for Programmed Death (PD)-1, which was highly and exclusively expressed on dendritic-like microglia. Our data solidify the interpretation that microglial activation is more complex than a simple all-or-nothing process. Depending on the stimulus and biochemical environment, a wide range of qualitatively different immune responses may be elicited in vivo. The next important step is to develop a thorough understanding of how all these tightly regulated effector mechanisms of PMN and microglia can be modulated to produce qualitatively desired outcomes in SLE and AD. This knowledge is likely to advance our understanding of immune dysregulation and hyperactivation, which not only provides novel insight in pathomechanisms but also has implications for our current understanding of autoimmune inflammation.

Zusammenfassung

Autoimmuninflammation kann durch einen Irrtum der angeborenen oder adaptiven Immunantwort entstehen. Daraus resultiert Zellzerstörung und der Verlust entzündetes Gewebe zu heilen. Diese Komplikationen sind im systemischen Lupus erythematodes (SLE) und in der Alzheimer-Krankheit (AK) anzutreffen. Ziel dieser Dissertation war es in beiden Krankheiten zu einem besseren Verständnis inflammatorischer Mechanismen beizutragen. Im SLE stellt das Nukleosom ein primäres Autoantigen dar. (1) Wir zeigen, dass Nukleosomen im Plasma von SLE-Patienten die Sekretion pro-inflammatorischer Mediatoren in Neutrophilen (PMN, polymorphonukleäre Leukozyten) induzieren. Die Nukleosomen-induzierte Aktivierung war unabhängig von Endotoxinen, endosomaler Azidifizierung, unmethylierten Cytosin-phosphatidyl-Guanosin (CpG)-Motiven und dem Toll-ähnlichen Rezeptor (TLR) 9. Der hier verwendete Signalweg scheint im Gegensatz zu nicht methylierter CpG-ODN Unterschiede aufzuweisen. Demnach spielt TLR9 bezüglich des Nukleosoms eine andere Rolle in der angeborenen Immunität oder vermittelten Autoimmunität. (2) Außerdem berichten wir die Expression des TLR9 auf der Zelloberfläche von primären PMN. Dieser TLR9 erkennt CpG-Oligonukleotide (ODN), ist funktional und führt zur Aktivierung der PMN. Somit beschreiben wir einen neuen Signalweg für CpG-ODN und die Aktivierung von PMN. Im Morbus Alzheimer wird aggregiertes Aβ durch eine Mikrogliose begleitet. (3) Die Untersuchung von infiltrierenden und im Gehirn vorhandenen Immunzellen führte zur Identifizierung dendritisch-ähnlicher Mikroglia. Die Charakterisierung in transgenen (tg) Alzheimer Mausmodellen sowie in humanen Gehirnen, zeigte, dass diese Zellen über eine gesteigerte Aktivität zur Phagozytose von Aβ ex vivo verfügen. Diese Fähigkeit wird jedoch durch die Expression verschiedener inhibitorischer Signale in vivo reguliert. Es war nicht möglich das Potential zur Phagozytose in vivo durch die genetische Deletion von Programmed Death (PD)-1 in dendritisch-ähnlichen Mikroglia wiederherzustellen. Unsere Daten festigen die Annahme, dass die Aktivierung von Mikroglia komplexer ist als ein einfaches Alles-oder-Nichts Prinzip. Abhängig vom Stimulus und von der biochemischen Umgebung zeichnet sich eine Reihe von qualitativ unterschiedlichen Immunantworten in vivo ab, die die Möglichkeit zur therapeutischen Intervention signalisieren. Der nächste wichtige Schritt ist es, die Modulation der hoch regulierten Effektormechanismen von PMN und Mikroglia-Zellen besser zu verstehen, um qualitativ gewünschte Resultate im SLE und in der AK zu erzielen. Dadurch ergeben sich nicht nur neue Einblicke in Pathomechanismen sondern auch Konsequenzen für unser Wissen über die Autoimmuninflammation.

Chapter I

The origin and mediators of inflammation, autoimmunity and self-tolerance

Inflammation

Inflammation can be defined as the result of provocations by invading pathogens or even autologous components in the body, thus representing an immune response to these noxious stimuli. The hallmarks of inflammation (i.e. redness, heat, swelling and pain) were already described by the Roman encyclopedist Celsus around AD40. At present it is well known that they are initiated by the innate immune system. The latter has evolved under the constant selective pressure of microbial pathogens and uses a limited number of non-clonal receptors with fixed specificities to unmask them [1]. The benefit of this strategy is speed. The cost is that the system is critically dependent on the selection of appropriate microbial targets. If the target is easily mutated, then the immune response fails to hit the target. In response to the virtue of mutational and other alterations of pathogens, the adaptive immune response has developed. It recognizes pathogens which have escaped innate immunity by utilizing millions of clonally expressed receptors [2, 3]. Thus, this specific immune response is rather slow but has the unique ability to provide memory and protection against re-infection [4]. Despite these distinct properties, both arms of immunity are designed on the principle of distinguishing self from non-self and eliminating the foreign one.

The ideal inflammatory response is rapid, yet specific and self-limiting [5]. However, if the target is not unique to pathogens, then the mediators of acute inflammation may attack the host, leading to maladaptive chronic inflammation. When this homeostatic imbalance in one or several physiological systems occurs they are not any more functionally related to host defense or tissue repair. The result is collateral damage seen in many autoimmune disorders. The emergence of autoimmunity is often connected with aging, and is suggested to be linked to activation of the innate immune system in individuals suffering from chronic infections. However, the mechanisms underlying the efficient activation of innate and adaptive immunity with prevention of

reactivity to autologous tissues remain elusive [6]. Thus, studying the balance between the immune system and the trigger of chronic inflammatory disorders will reveal the check points in self and non-self discrimination with fundamental implications for our understanding of autoimmunity.

Inducers and sensors of inflammation – TLR sound the alarm

The innate immune system comprises multiple cell types, receptors, signaling systems and soluble mediators equipped with germ line encoded pattern recognition receptors (PRR). TLR form one major group of PRR which recognize common pathogen-associated molecular patterns (PAMP) on foreign organisms [1]. PAMP are nucleic acids, lipids, lipoproteins, carbohydrates or peptidoglycans from bacteria, fungi or protozoa. These microbial markers are recognized by a family of structurally related type I transmembrane (TM) receptors which received their name due to their similarity to the gene Toll originally discovered in Drosophila melanogaster [7, 8]. The mammalian homologues possess multiple copies of leucine at the extracellular N-terminus and a C-terminal intracellular Toll/interleukin (IL1)-1 receptor (TIR) domain [9]. TLR-dependent pathways of PAMP recognition play a central role in the innate immunity. To date the TLR family consists of at least 10 well characterized members in humans and 13 in mice [10]. TLR involved in the recognition of non-nucleic acids and components from bacteria and fungi (TLR1, TLR2, TLR4, TLR5 and TLR6) are located in the plasma membrane and can be recruited to phagosomes. By contrast, TLR which sense viral and bacterial nucleic acids (TLR3, TLR7/8 and TLR9) are normally found intracellularly. The ligands for TLR10, TLR12 and TLR13 remain elusive. TLR8 lacks functionality in mice and TLR10 is only expressed in human but not in murine tissue. TLR11, which mediates recognition of profilin, is together with TLR12 and TLR13 only found in mice but not in humans. TLR2 either builds a heterodimer with TLR1 or TLR6 to distinguish between bacterial- and mycoplasma-derived peptidoglycans [11, 12]. Double stranded (ds) ribonucleic acid (RNA) produced during viral replication can be detected by TLR3 located in the endoplasmatic reticulum (ER) [13]. Lipopolysaccharide (LPS) is the ligand of TLR4 [14] and TLR5 recognizes bacterial flagellin [15]. Single stranded (ss) RNA and imidazoquinolines such as resiquimod (R848) are recognized in endosomes by TLR7/8 [16]. Most closely related to TLR7/8 is TLR9. Gene knockout (KO) experiments in mice suggested that this receptor binds to and signals in the presence of bacterial deoxyribonucleic acid (DNA) as well as synthetic ODN that contain unmethylated dinucleotides in a specific sequence context (CpG) [17, 18]. In

contrast, mammalian DNA contains only a small number of CpG. Cytosine of these dinucleotides is usually methylated at position 5 so that they lack immunostimulatory effects [19]. However, free circulating [20] and immune complex (IC)-associated [21] mammalian nucleic acids have been shown to stimulate PRR under particular conditions and they have been linked to the development of autoimmune diseases. Ligand binding results in TLR dimerisation and conformational changes of their extra- and intracellular TIR domains. TIR recruits specific adapter proteins which are shared by many TLR such as TRIF (TIR-domain-containing adapter-inducing IFN-β), TRAM (TIR-containing adaptor molecule), TIRAP (TIR domain containing adaptor protein) and myeloid differentiation primary response gene (MyD) 88 in order to propagate activation of downstream signaling events [1]. The signal itself is amplified by transcription factors including activator protein-1, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), interferon regulatory factors (IRF) 3, 5 and 7 which are required for the release of pro-inflammatory mediators. Despite TLR-associated pathways, it is well known that TLR-independent routes exist, which effectively mediate recognition of PAMP. Viral RNA can also be detected by intracellular DExD/H box RNA helicases, referred to as retinoic acid inducible gene-I or melanoma differentiation associated antigen-5 [22, 23]. DNA-recognition does not seem to be only via TLR9 [24]. Recent studies identified intracellular AIM (absent in melanoma)-2 as a cytoplasmic DNA-sensor for the inflammasome [25]. Another putative DNA-sensor is ZBP (Z-DNA binding protein)-1, or DAI (DNA-dependent activator of IRF) [26]. Interestingly, it is now apparent that other receptors than TLR9 might exist at the cell surface to recognize DNA, the mechanisms of action of which remain to be elucidated [27].

Mediators and effectors of inflammation

Recruitment of immune cells to the site of inflammation

Recognition of PAMP by TLR mediates the generation of numerous inflammatory mediators which in turn alter the functionality of many tissues and immune cells [28]. Activated through their PRR, tissue-resident macrophages (MΦ) start to produce a variety of inflammatory molecules including chemokines, products of proteolytic cascades and cytokines such as IL-1, 6, 8 and tumor necrosis factor (TNF). Perivascular mast cells release histamines, eicosanoids and tryptases causing vasodilatation (responsible for the heat and redness) and extravasation of fluid (the

cause of swelling) [29]. The main effect of these pro-inflammatory stimuli is to induce the formation of an exudate, in which leukocytes become tissue-migratory and gain access to the side of inflammation [5, 6]. These areas show massive infiltrations of PMN, the prototypic inflammatory immune cells. PMN form the most abundant group of leukocytes in the peripheral blood and play a central role in host defense [30]. They belong to the myeloid family and possess a range of surface membrane proteins [31], which commit adhesion, signal transduction and initiation of PMN effector function [32]. The cluster of differentiation (CD) 66b is expressed at the external plasma membrane, and represents a marker for the fully differentiated PMN [33]. Granulocytes in murine tissues lack CD66b expression but the Gr-1 epitope present on the two receptors Ly-6G and Ly-6C has been useful in their identification [34]. However, it has been shown that Ly-6G is almost uniquely expressed by neutrophils [35]. PMN trafficking to sites of inflammation follows a defined temporal pattern in which endothelial-cell selectins and integrins and chemokine receptors mediate capture, tethering and rolling [36]. Interestingly, TLR9 signaling promotes adherence in this process [37] and IL-8 arrests moving on endothelium [38]. When PMN reach the afflicted tissue, either the recognition of antibody (Ab) secreted by B (plasma) cells, complement opsonized particles, heat shock proteins or the direct contact of bacterial products, triggers neutrophil effector functions [39, 40]. Activated PMN produce and release pro-inflammatory cytokines like IL-6 and IL-8 which allow them to attract additional PMN. In addition, through cell-cell contact or secretion of macrophage-inflammatory proteins (MIP) and TNF, PMN can actively recruit players of adaptive immunity. Vice versa products of monocytes, T cells and dendritic cells (DC) can activate PMN in a bidirectional feedback loop [32]. PMN attempt to engulf microorganisms which are killed intracellularly when their antibacterial granules fuse with the phagosome [41]. They also degranulate thereby releasing antimicrobial factors into the extracellular space. However, incomplete closure of antibacterial granules or aberrant release of reactive oxidants species (ROS) [42] and proteases to the surrounding microenvironment may be responsible for organ damage and fungal sepsis, promoting their role as double-edged swords in autoimmune issues [43]. This example is fairly illustrated by the fact that TNF promotes the release of elastase, cathepsin-G and proteinase 3 even if PMN do not directly encounter any pathogen [32]. Thus, apoptotic PMN are normally removed by MΦ to prevent the release of ROS and other neutrophilic proteins [44]. Likewise, many autoimmune disorders such as SLE are linked to defective clearance mechanisms of apoptotic material and loss-of-function mutations of receptors can lead to spontaneous inflammation, as most clearly shown by complement component 1q (C1q) deficiency [45]. Migration, degranulation [46],

phagocytosis and respiratory burst [47] are provoked by ligation of CD11b/CD18 (Mac-1). Although vital to normal homeostasis of PMN, Mac-1 has been implicated in the pathogenesis of inflammatory tissue damage. Elevated levels of CD11b and activation status correlate positively with indicators of disease severity [48, 49]. Increased PMN CD11b levels may also predict adverse outcomes and provide an early diagnostic marker for neonatal infection [50]. Recently, Zychlinsky and colleagues proposed an additional mechanism for extracellular killing of microorganisms. The authors demonstrated that activated PMN possess the ability to release granule proteins and chromatin which unite to form extracellular matrices. These structures were herein after referred to as neutrophil extracellular traps (NET). These web-like DNA matrices contain proteolytic activities, i.e. elastase, cathepsin-G, myeloperoxidase, lactoferrin, and gelatinase that are able to entrap pathogens and disarm their surface virulence factors [51]. However, all options for PMN end in the same eventual fate – their death by apoptosis and clearance by MΦ [5, 32].

Resolution of inflammation

If the acute inflammatory response successfully leads to the elimination of the pathogen, then the overall response is shifted towards anti-inflammatory signals. In particular, lipopoxins, protectins and resolvins send anti-inflammatory signals that actively promote the resolution of inflammation and a phase of repair [5, 29]. These anti-inflammatory mediators are induced by MΦ that have engulfed apoptotic PMN [44]. Lipopoxins stop the accumulation of PMN into sites of infection or tissue injury, mediate the uptake of apoptotic cells by MΦ, and recruit additional monocytes to participate in the removal of dead cells and tissue debris [5, 29]. Additionally, several innate immune cells produce secretory leukocyte protease inhibitor (SLPI) [52]. SLPI inactivates the contents released from PMN granules and promotes the acute inflammatory response towards resolution [29, 53].

Persisting inflammation

If the acute inflammatory response fails to eradicate the infectious agent or the autologous stimulus, inflammation persists and adopts new characteristics. PMN are replaced by professional antigen presenting cells (APC), mainly MΦ and DC. Both cell types sample their environment by virtue of their high endocytic activity. When these

cells detect pathogens with their various PRR, they become activated and undergo profound phenotypic changes. They start expressing high levels of major histocompatibility complexes (MHC), called human leukocyte antigen (HLA) in humans, and co-stimulatory molecules (CD40, CD80, CD86). Together they stimulate T cells in a process termed antigen (Ag)-presentation. Ag derived from endogenous sources (i.e. those synthesized by the cells themselves) are presented on their MHC class I molecules for the stimulation of $CD8^+$ T cells (CTL, cytotoxic T cells) [54]. CTL are essential for the control of viral infections. They are armed with an arsenal of cellular weapons to destroy and lyse infected cells [55]. CTL produce cytokines such as interferon (IFN)-gamma (γ) and TNF, and kill their target either by releasing perforins or granzymes that create holes in the target cell membrane or by triggering Fas-induced apoptosis [56-58]. Peptides from exogenous sources (i.e. generated by an extracellular source) are presented on MHC class II molecules and recognized by $CD4^+$ T cells (T_H, T helper cells) [59]. However, substantial crosstalk between these two pathways has been observed (cross-presentation). As the synonym implies, T_H predominantly possess helper activity supporting other cells of the innate and the adaptive immune response. Due to different cytokine patterns, T_H can be further divided into T_H1 and T_H2 cells [60]. T_H1 cells produce cytokines such as IFN-γ, IL-2 or TNF which contribute to the activation of MΦ and CTL. In contrast, T_H2 cells support the activation of Ab-secreting plasma cells by generating mainly IL-4, IL-5 and IL-13. Each T_H subset promotes its own development and inhibits the proliferation of the other cell type taking advantage of its unique cytokine spectrum [61]. The induction of one type of response suppresses the induction of the other [62]. T_H17 cells represent the third group of T_H cells and have been discovered upon studies on the IL-17 family [63]. The function of T_H17 cells is currently receiving considerable attention and it seems that they are critical mediators in human autoimmune disorders, allergy and antimicrobial defense [64]. T cell reactions can also be inhibited or down regulated by another type of $CD4^+$ lymphocytes, known as $CD25^{high}$ regulatory T cells (T_{REG}) [65].

Chronic inflammation

If the combined efforts of these effectors are still insufficient, a chronic inflammatory state ensues. The characteristics of this inflammatory state can dramatically differ in the effector type of T cells, the magnitude of inflammatory mediators and the class of APC that are present. For example increasing quantities of type I IFN (alpha [α]/β) not only promote normal but also autoimmune responses by a multitude of effects. One

major effect is DC activation, leading to efficient self-Ag presentation to previously quiescent low-affinity autoreactive T cells. IFN-α activated DC further produce cytokines, which promote B cell survival, differentiation and isotype switching, characteristics which are connected to SLE [66]. The main producers of IFN-α are plasmacytoid DC (pDC) [67]. They are most likely playing an important role in recognizing viruses and their nucleic acids through TLR7/8 and TLR 9 [68]. Although pDC represent only 0.2-0.8 % of leukocytes in the peripheral blood, their tissue number increases under pathological conditions. They are present in psoriatic skin [69] and they are believed to be an important source of IFN-α in skin lesions of patients with SLE [70]. Moreover, IFN-α inhibits the generation of naïve B and T cells [71] thereby limiting the dilution of expanded autoreactive clones. Indeed, IFN-α has strong immunostimulatory effects illustrated at its best by inducing systemic autoimmunity with increased titers of autoantibodies (autoAb) and arthritis during the course of IFN-α therapy in patients with carcinoid tumors [72]. Subsequent studies have shown similar results and obviously raised the question whether IFN-α could be involved in the pathogenesis of SLE.

In addition to persistent infection, chronic inflammation can result from other causes of tissue damage such as the persistence to autoAg or undegradable foreign bodies as seen in many neurodegenerative diseases such as AD, both leading to autoimmune issues. Unsuccessful attempts by MΦ to engulf and destroy undegradable foreign bodies can lead to the formation of granulomas. The latter is characterized as an organized collection of MΦ which wall off the autoAg [6], a clinical feature reported in SLE patients [73]. However, regardless of whether a foreign body is too large to be phagocytosed or disrupts the plasma membrane, when MΦ encounter foreign bodies, the NALP (NACHT-, LRR- and pyrin domain-containing protein) 3-inflammasome is activated [6]. The inflammasome is one of the first sensors of cell stress [74]. The danger signal theory proposes that in addition to foreign objects, the immune system senses and responds to damaged self [75]. Undegradable inclusion bodies and abnormally folded proteins may be endogenous signals derived from damaged or dead neurons in neurodegenerative disorders that are sensed by the NALP3-inflammasome. Indeed, granulomas and activation of the NALP3-inflammasome have been identified during the course of AD when studying microglia, described as the CNS-resident counterpart of MΦ, and the innate immune response to Aβ [76, 77]. To this end, several mechanisms, which are definitively not exclusive of one another, probably account for the loss of self-tolerance in different tissues.

Autoimmunity – the predisposition for self-destruction

Inflammation may result in disharmony between the players of the immune system and non-affected tissue which results in collateral damage of the surroundings [78]. Therefore, in some cases the immune system can be directed towards self-tissue, causing inflammatory injury. This phenomenon is termed autoimmunity and was first described as horror autotoxicus by Paul Ehrlich in 1902 [79, 80]. Autoimmune diseases are complex processes, in which genetic susceptibility interacts with some triggers that sets the progress in motion [81]. In general, such disorders are classified by autoreactive lymphocytes directed against autoAg and/or autoAb [82]. Autoreactivity can be triggered by several identified mechanisms. Sequence similarity between exogenous and self-Ag (molecular mimicry) is thought to activate and enable cross-reactive T cells to cause autoimmune diseases [83]. Tight association with infection and autoimmune diseases is reasonable and illustrated by several examples such as arthritis, multiple sclerosis (MS), diabetes, atherosclerosis and myocarditis [84-88]. The first confrontation between host and pathogen is mainly mediated by PRR. Therefore, after receptor-ligand interaction the subsequental signaling pathway can influence secretion of pro-inflammatory cytokines and chemokine expression by APC [89]. In this way the production of cytokines and chemokines depends strongly on the type of PRR, which results in an optimized pathogen-host balance but may also result in autoimmunity [90, 91]. Especially, excessive activation of TLR9 through recognition of IC-associated self-DNA can lead to chronic inflammation and amplify autoimmune disorders [92, 93]. Other scenarios which can break peripheral tolerance and initiate autoimmunity include spreading of epitopes, excessive autoAg, modified autoAb or bystander activation [94-96]. APC activated by pro-inflammatory stimuli can induce T cell responses to self-Ag through co-stimulatory signaling [97-99]. Consequently, a substantial number of clones from mature self-reactive T cells can specifically proliferate in response to their cognate self-Ag, and differentiate into effector cells in the context of inflammatory stimuli [100-102]. Most prominent examples are SLE [103] and rheumatoid arthritis [104]. Both of them belong to the group of systemic autoimmune diseases, because especially in the case of SLE virtually every organ can be affected, e.g. skin, joints, hematopoietic system, kidney, CNS and heart. By contrast, in organ-specific autoimmune diseases like MS and its rodent model, experimental autoimmune encephalitis (EAE), T cells reactive to the myelin basic protein contribute to CNS pathology [105]. AD is rather defined as a neurodegenerative than an autoimmune disorder, but there is evidence that effectors of immunity directed against the self-Ag Aβ play a considerable role during the course of the disease [106].

Self-tolerance

The immune system has developed a set of control mechanisms which contribute to the maintenance of self tolerance. Several strategies are used to provide central and peripheral tolerance. Central tolerance takes place during B and T cell development, e.g. through deletion or receptor editing [107]. This selective mechanism ensures that potentially self-reactive lymphocytes are eliminated. However this process is incomplete. Recently, it has been illustrated that nearly 40 % of autoreactive T cells can escape negative selection, even in the presence of the tolerance-inducing ligand in the thymus [108]. The repertoire of mature lymphocytes is strongly biased towards self-reactivity [109]. This indicates that the selection process rather shapes the peripheral lymphocyte repertoire and not simply deletes potentially destructive self-reactive cells [110]. This phenomenon is known as the immunological homunculus [111]. To deal with pathogenic autoreactive lymphocytes which have escaped the cellular checkpoints regulating self-reactive receptors, the organism provides additionally peripheral tolerance [112]. These mechanisms include intrinsic as well as extrinsic features, e.g. anergy, biochemical tuning or regulation of self-reactive receptors. It is well documented that B and T cells with the ability to recognize self-Ag can become impaired by over-stimulation (anergy) [113]. Permanent confrontation with the autoAg can cause a progressive loss of function. This might begin with the inability to produce certain cytokines and ends in apoptosis (AICD, Ag-induced cell death) [114]. Self-reactivity may be prevented because of abortive receptor signals for self-Ag, lack of T cell co-stimulation from APC or even by simple anatomical seclusion of autoreactive lymphocytes from their target autoAg [76, 115]. Despite these passive mechanisms, regulatory DC, B and T cells can also actively suppress the activation and proliferation of self-reactive lymphocytes [65, 116, 117]. Additionally, it has been proposed that PMN participate in the preservation of peripheral tolerance. The production of several pro-inflammatory cytokines [32], the possibility of Ag-presentation and the expression of the co-stimulatory molecules CD80 and CD86 [118, 119] provides strong evidence for communications and interactions between PMN and T cells. Moreover, PMN may not only have an impact on the induction but also on the quality of T cell responses. PMN are equipped with effector systems that can mediate T cell suppression. The two L-arginine metabolizing enzymes arginase and nitric oxide synthase, as well as the phagocytosis-associated oxidases that generate ROS can inhibit T cell activation [120].

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Chapter II

Systemic lupus erythematosus – the prototype of systemic autoimmunity

The origin of the name lupus

SLE was first recognized and described as 'wolf bite' because of the redness across the cheeks in the 13th century. However, the term lupus érythémateux was first coined by the French physician Pierre Cazénave in 1851 to differentiate the non-infectious forms of lupus from cutaneous tuberculosis (lupus vulgaris) [1]. Lupus erythematosus derives from the Latin meaning of wolf and the Greek word of redness (ἐρυθήμα, erythema). Further investigations into the heterogeneous manifestations of lupus erythematosus by Moritz Kaposi recognized the relationship between discoid lupus erythematosus and SLE manifestations. To this end he extensively described the facial cutaneous signs of SLE also known as the butterfly erythema (Fig. 1).

In 1872, M. Kaposi (1837-1902) described SLE as an acute febrile eruption with pronounced painful joint involvement and a characteristic facial erythema that he termed erysipelas persistans faciei. At the end of the 19th century, reports by Hutchinson, Goeckerman and Osler further emphasized the different expressions and variations of this disorder with respect to cutaneous and systemic manifestations. Thus, the heterogeneity of SLE was first characterized and evaluated by its cutaneous manifestations before the analysis and study of its systemic manifestations established the multifactorial nature of SLE (as reviewed in [2]).

Figure 1. Historical illustrations of SLE. This classical drawing shows a woman with the characteristic butterfly-like rash over the cheeks and further skin manifestations on the hands (Picture is adapted from [3]).

SLE is an autoimmune disorder with multifactorial pathology

SLE is a chronic relapsing-remitting autoimmune disorder of unknown etiology and characterized by widespread organ involvement (Fig. 2). Although it is believed that susceptibility to SLE is largely dependent on genetic predisposition [4], a multifactorial combining effect of genetic, environmental and hormonal factors is most likely. The prevalence in European countries is 25 in 100,000 individuals [5]. SLE primarily affects women during their childbearing age (women/men ratio of 8/1 among affected individuals). Furthermore it is more prevalent in the non-Caucasian than Caucasian populations. SLE is characterized as an IC-mediated (also referred to as type III hypersensitivity) systemic autoimmune disorder which becomes manifested in the central and peripheral nervous system (Fig. 2A, B), skin (Fig. 2C, D), by inflamed joins leading to arthritis (Fig. 2E) as well as kidney dysfunctions (Fig. 2F). The latter is known as glomerulonephritis (GN) and characterized, as the other manifestations, by the widespread of autoAb, causing inflammation and tissue destruction upon deposition of IC.
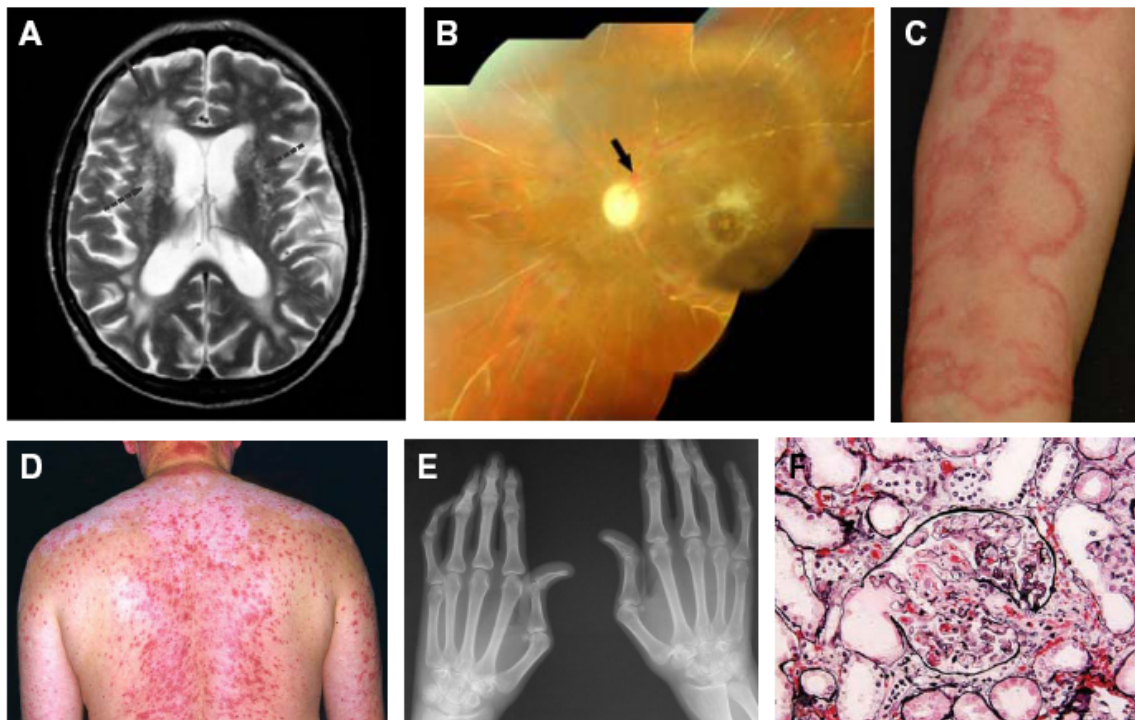
Figure 2. SLE is a highly heterogeneous, multiorgan disorder. (A) The solid arrow indicates a previous hemorrhage in the right frontal lobe of the brain from an individual suffering from SLE, supported by a high-signal change within the periventricular white matter which could resemble cerebral vasculitis. **(B)** The classical finding in lupus retinopathy is the cotton-wool-spot (indicated by the arrow), leading to complete blindness. The spot shows often deposits of IC with the complement factors C1q and C3. **(C, D)** Patients are suffering from discoid lupus erythematosus. **(E)** Inflamed joints leading to arthritis. **(F)** Depicted is a glomerulus of the kidney which shows severe thrombosis, swelling of the endothelial cell layer, necrosis and the massive infiltration of PMN indicated as black dots (Pictures are adapted from [6-11]).

To diagnose SLE at least four of the following criteria are serially or simultaneously required: facial and/or skin rash, sensitivity to sun light, mouth aphtha, fluid reposition around the lungs, heart and joints, immunological disorders, low white blood cell and/or platelet counts, anemia, arthritis, CNS and/or kidney malfunctions as well as the presence of anti-nuclear autoAb (ANA) [12]. Circulating autoAb that are specific for autoAg such as DNA and nuclear proteins are the hallmark of SLE and will be further discussed below.

Circulating nucleosomes in systemic lupus erythematosus: not only passive targets for autoantibodies

The following text is derived from an article by Viktoria Rönnefarth, Annika Erbacher, **Dennis Lindau** and Patrice Decker (in preparation). The author of this thesis classified the knowledge about TLR and the pathogenesis of circulating nucleosomes in SLE, designed figure 3 and was instrumental in correcting the manuscript.

The production of huge amounts of circulating autoAb directed against nuclear and cytoplasmic autoAg is characteristic for lupus. SLE is typified by the presence of ANA and in particular, autoAb directed against the nucleosome and its individual components, dsDNA and histones [13]. Among these autoAg, the nucleosome is a main autoAg in SLE. It is the fundamental packing unit of DNA in chromatin within the nucleus and is composed of 180 base pairs (bp) of dsDNA and histones. Two copies of each histone protein H2A, H2B, H3 and H4 are organized into an octamer which has 146 bp of DNA wrapped around it to form the nucleosome core. This structure is stabilized by the linker histone H1. A nucleosome has a molecular weight of 262 kD with a histone/DNA ratio of 1. The X-ray crystal structure of the nucleosome core particle was resolved at high resolution [14]. Circulating (oligo)-nucleosomes are found in the blood of SLE patients and lupus mice [15]. The levels of these circulating nucleosomes are raised in SLE patients with renal and CNS manifestations [16]. Originally, it has been thought that circulating DNA and not nucleosomes plays a role in tissue damage in SLE through the formation and deposition of DNA-anti-DNA-IC [17]. Since naked DNA is poorly immunogenic and because the DNA detected in the circulation of SLE patients is complexed in nucleosomes, nucleosomes are probably the triggering Ag for anti-dsDNA, anti-histone and anti-nucleosome Ab. Although, anti-dsDNA Ab are a hallmark of SLE [18, 19], nucleosome-restricted autoAb have been described in SLE patients [20]. Importantly, anti-nucleosomal autoAb are present in patients with active and inactive SLE and their level positively correlates with disease activity suggesting that anti-nucleosome Ab reactivity may be a more sensitive marker than anti-dsDNA [21, 22]. Particularly, increased immunoglobulin (Ig) G3 anti-nucleosome levels were shown to be associated with active nephritis [23]. Besides, it was demonstrated in several lupus-prone mice that anti-nucleosome Ab emerge early in life and are potentially pathogenic [24, 25]. It is thought that the nucleosomes are also a major Ag for the pathogenic autoAb-inducing T_H cells which arise spontaneously

in SLE [26]. Thus the presence of nucleosome-specific T_H cells in lupus-prone mice is necessary for the production of pathogenic autoAb and the development or acceleration of nephritis. Likewise, nucleosome-specific T_H cells have been detected and characterized in SLE patients [27]. In addition, it was demonstrated that a single lupus nucleosome-specific T_H clone can provide help to dsDNA-, histone- or nucleosome-specific B lymphocytes [26]. These nucleosome-restricted Ab only react with the whole nucleosome complex but not with its individual components (dsDNA and histones), and anti-dsDNA and anti-histone Ab are only minor components of the IgG anti-nucleosome response. It is believed that nucleosomes that are complexed with anti-nucleosome Ab are involved in the development of GN. Thus it was demonstrated that nucleosome-specific monoclonal Ab (mAb) complexed to nucleosomes bind to rat glomerular basement membrane in vivo, partly via the heparan sulfate, and activate the complement, whereas nucleosome-specific Ab alone do not [28]. Anti-nucleosome Ab are also associated with haematuria, malar rash, arthritis and oral ulcers [29].

Dysfunctional removal of autoantigens in SLE patients

To avoid an immune response to nuclear contents like nucleosomes, such components rarely appear in the circulation of healthy organisms. Very efficient removal strategies avoid their presence into the surrounding tissues. Single nucleosome fragments are a product of the programmed cell death called apoptosis. Within hours (h) the cell morphology changes drastically and during this process a caspase cascade is initiated which results in the activation of the caspase activatable DNase (deoxyribonuclease) [30]. This DNase is responsible for the characteristic cleavage of the DNA, degrading the chromatin in its basic units, the nucleosome. An early event during apoptosis is the relocation of the phospholipid phoshatidylserine from the inner to the outer leaflet of the plasma membrane without loosing the membrane integrity. The exposed phoshatidylserine is then recognized by phagocytes via a variety of receptors, for example the phosphtidylserine receptor [31] and opsonins like the milk fat globule EGF-factor 8 (MFGE8) [32]. The so identified apoptotic cells are internalized and cleared before any release of immunogenic molecules can take place. The elimination of cells undergoing apoptosis is therefore believed to be an anti-inflammatory process due to the involved receptors on monocytes and MΦ, and their secreted immunosuppressive cytokines like IL-10 and the transforming growth factor (TGF)-β [33]. Apoptotic cells are also characterized by cell shrinkage and bleb formation on the cell surface. These blebs accumulate cytoplasmic and nuclear material, which are

known to serve as autoAg in SLE [34]. Late apoptotic cells can also become cleared via backup mechanisms that involve opsonins like C1q [35] or the short pentraxins C-reactive protein (CRP) [36] and serum amyloid P (SAP) [37]. The highlighted cells are then recognized by a set of phagocyte receptors like MFGE8 that differ from the former by rather binding to the opsonins than directly to the cells. When apoptotic cells escape these clearance systems they undergo secondary necrosis in the circulation, in which they loose their membrane integrity and release their noxious contents. Unlike apoptosis, secondary necrosis is able to evoke immune reactions involving pro-inflammatory cytokines like TNF, IL-1β and IL-12 [38]. The efficiency of the removal mechanisms are demonstrated specifically in tissues with high cell turnover like the thymus [39]. Indeed, due to the failed positive or negative selection of lymphocytes more than 95 % of them undergo apoptosis. Accumulation of apoptotic bodies at the site of immune development like the thymus (but also in lymph nodes, bone marrow (BM) and the spleen) might lead to a rapid development of immune reactions against released intracellular contents. Human SLE is associated with an impaired clearance of apoptotic cells and moreover with an augmented apoptosis rate [40, 41]. Monocyte-derived as well as tingible body MΦ of SLE patients are affected in their phagocytic function [40, 42]. High amounts of non-ingested apoptotic cells were observed in germinal centers and cell culture supernatants, and additionally only few MΦ contained engulfed cell debris in these experiments. The impaired phagocytosis of apoptotic cells by MΦ is thought to be partly associated with the membrane tyrosine kinase c-mer. Mice lacking the intracellular domain of c-mer show a delayed clearance of apoptotic cells, and develop additionally lupus-like manifestations [43]. Those results are striking due to the fact that MΦ are the most potent phagocytes for apoptotic and necrotic material. Their disability allows other phagocytes, for example DC which are specialized APC, to engulf apoptotic bodies or free material and present autoAg to developing lymphocytes. Other groups published studies in which immune cells of SLE patients, like lymphocytes and PMN, seem to be more susceptible to apoptosis in vitro compared to cells from healthy controls or patients with other chronic inflammatory diseases [41, 44]. Unfortunately the experiments could not determine if the high amount of circulating apoptotic cells in vivo results from impaired clearance mechanisms or increased apoptosis rates. Contrasting effects were reported concerning the clearance and frequency of apoptotic keratinocytes in patients with lupus compared to controls. About 30 % to 50 % of the patients are photosensitive and ultraviolet (UV) B light irradiation is known to induce the typical skin lesions. Moreover, UVB is a very effective inducer of apoptosis. But neither an increased apoptosis rate nor severe clearance defects have been observed in the skin of SLE patients after UVB

irradiation [45]. However, these findings do not exclude each other. They rather place emphasis on the theory. The presence of circulating nucleosomes in sera of SLE patients is thought to be a result of the enhanced rate and inefficient clearance of apoptotic cells, which results in the generation and release of nucleosomes in the circulation. It was shown in vitro that nucleosomes release from dying cells occurs about 24 to 48 h after apoptosis induction in the absence of phagocytes [46]. This indicates that removal of apoptotic cells from the circulation takes approximately 12 hours. Mouse in vivo experiments reported equal results. Serum nucleosome levels peaked 24 to 48 h after induction of apoptosis in MΦ of healthy mice [47]. In addition, the same study showed that MΦ play a major role in the generation of circulating nucleosomes from apoptotic and necrotic cells in vivo and the lack of nucleosomes in their absence. There are several effector molecules suggested to be involved in the clearance of chromatin, once it is released into the circulation. Amongst them is the DNase1, an endonuclease present in body fluids like serum and urine. It is known that its activity is decreased during the active disease in SLE patients [48]. DNase1-deficient mice display lupus-like symptoms, including ANA-production [49]. Interestingly, only mice with a lupus-prone background, for example mice with a mixed (129xC57BL/6)F2 genetic background, show symptoms of SLE, illustrating that DNase1 deficiency is not the only triggering factor for developing lupus but amplifies the severity of the disease. DNase1, in combination with plasminogen degrades the DNA of necrotic cells in an internucleosomal manner [50]. It has also been shown that the ability of DNase1 to degrade chromatin from necrotic cells is facilitated and increased in the presence of C1q [51]. As mentioned above, C1q is known to promote the clearance of apoptotic cells by binding to the surface blebs of apoptotic cells [35, 52]. A deficiency of C1q predisposes to the development of SLE, shown by the fact that over 90 % of the reported individuals with a complete absence of this recognition molecule develop SLE [53, 54]. Besides this, the level of circulating C1q is decreased in SLE patients with active disease, probably due to an enhanced consumption of complement components by anti-C1q autoAb [55]. C1q-deficient mice only develop symptoms on a lupus prone genetic background, accentuating once more the multifactorial origin of the disease. Interestingly, the binding of C1q to DNA was also reported [56], suggesting a role of C1q in the clearance of free chromatin. The recognition molecule of the lectin pathway of the complement system, the mannose-binding-lectin (MBL), shares functional and structural similarities with C1q. It binds, like C1q, to apoptotic cells and facilitates their uptake by MΦ [57]. Likewise, a binding to DNA is also observed indicating its possible involvement in chromatin removal [58]. But, compared to DNase1- and C1q-KO mouse models, MBL null mice do not develop

spontaneous autoimmunity, even on a lupus-prone background [59]. Another group of serum opsonins, which share functional and structural similarities to MBL and C1q, are Ficolins. Two members of this family, Ficolin-2 and Ficolin-3 (also known as Hakata Ag) are known to bind to dying cells. Ficolin-3 contributes to the uptake of late apoptotic cells by phagocytes via receptors like the Calreticulin-CD91 complex [60]. Ficolin-2, in contrast to Ficolin-3, is additionally known to bind DNA [61]. However, it is not known if their activity is decreased in SLE patients. Pentraxins are serum proteins of the acute phase response and serve additionally as opsonins for apoptotic cells and are known to enhance their phagocytosis [36, 37, 62, 63]. CRP and SAP are also thought to bind to circulating chromatin and considered to be involved in its clearance [64, 65]. SAP-KO mice spontaneously develop autoimmunity and GN. Nevertheless, it was recently reported that SAP levels are not decreased in SLE patients [66]. But a study by Sørensen et al. [67] could show a negative correlation between the ability of SAP to form complexes with DNA and high anti-DNA titers in SLE patients. Likewise, a low acute phase CRP response has been observed in SLE patients during the active phase of the disease and this deficit is now known to be associated with a polymorphism in the CRP locus [68]. The Pentraxin 3 (PTX 3) shares structural homology with CRP and SAP, and is also known to bind to dying cells [69]. But unlike these proteins, the binding of PTX 3 rather inhibits the uptake of apoptotic cells by DC. By this, PTX 3 regulates the maturation of the DC and thus restrains the activation of autoreactive lymphocytes [70].

The role of nucleic acid-sensing TLR in SLE

Recent studies strongly suggest that microbial and even endogenous nucleic acids can induce systemic autoimmune disease as well as subsequent clinical flares via the engagement of TLR7/8 and TLR9 [71]. These sensors and their molecular signaling pathways are sequestered intracellularly which normally excludes recognition of extracellular host-derived nucleic acids [72]. Furthermore, methylation of DNA motifs [73] and the subsequent degradation of nucleic acids by nucleases are thought to prevent autoimmunity. However, the immunostimulatory CpG-motifs in SLE autoAg reveal higher contents of unmethylated sequences [74]. Additionally SLE-patients show defects in DNA methylation [75] and degradation [54]. Defects in mechanisms of clearing apoptotic cells or IC, such as complement, Fc-receptor (FcR) or phagocytotic defects, serve to elevate the absolute concentration of available autoAg. In humans TLR9 maps to chromosome 3p21.3 and some polymorphisms predispose to SLE in

Japanese [76, 77]. Furthermore, variants of the transcription factor IRF5 downstream of TLR7/8 and TLR9 have been identified as an important susceptibility factor in humans [78, 79]. TLR can sense circulating autoAg released from apoptotic cells and such self-recognition may be crucial in the induction and perpetuation of SLE (Fig. 3). The accessibility to TLR in the endolysosomal compartments by self-nucleic acids is thought to be mediated by the complexing of these sensors with SLE-associated autoAb with corresponding specificities. These IC activate autoreactive B cells and pDC through a mechanism dependent on dual engagement of TLR9 and the B cell receptor (BCR) or FcRγIIa (CD32), respectively [80-82]. Moreover, free DNA activates DNA-specific lymphocytes via the BCR and TLR9 [81]. CD32 mediates uptake by pDC of apoptotic material complexed with serum IgG autoAb, leading to the secretion of IFN-α, the major pro-inflammatory cytokine found in SLE [83]. Interestingly, predisposing autoreactive B cells to IFN-α lowers the BCR activation threshold and relaxes the selectivity for SLE-autoAg [84]. Likewise, in vitro activation of B cells by RNA-containing IC depends on TLR7 [85, 86]. It is thought that fragments of apoptotic DNA have a greater stimulatory capacity than genomic DNA, which may be due to the release of proteins such as high motility group box (HMGB)-1 from necrotic cells. HMGB-1 was identified as a CpG-binding protein which accelerates CpG-TLR9-complex formation and enhances TLR9-dependent cytokine response such as IL-6, IL-12 and TNF [87]. Recent studies have shown that aberrant expression of TLR7 and TLR9 is a key player in the production of pathogenic autoAb and/or in development of clinical features in mouse models of SLE [88]. Rheumatoid factor B cells from lupus-prone MRL (Murphy Roths Large) lpr/lpr mice are activated by CpG through cross-linking of BCR by IgG2a-IC [89]. Thus, TLR9 expression has a specific requirement in promoting autoAb formation in vivo, although the results highly depend on the genetic background of the mouse models. MRL lpr/lpr mice deficient in TLR9 show an inhibition of anti-dsDNA and anti-chromatin autoAb, although no clinical effect was seen. By contrast, the ablation of TLR3 did not inhibit the formation of autoAb to dsRNA- or dsDNA-containing Ag [90]. Similarly, C57BL/6 mice deficient in TLR9 show significantly smaller autoAb IC deposits in the glomeruli and survive longer than their TLR9-sufficient litter mates. In this case, B cells do not undergo IgG class switching to the pathogenic isotypes IgG2a and b [91]. TLR7 deficiency can also influence autoAb production. TLR7-deficient MRL lpr/lpr mice do not produce autoAb that react with RNA-associated autoAg and do not have exacerbated clinical disease. Therefore, either TLR7 or TLR9 deficiency in MRL lpr/lpr mice indicated that TLR9 is required for the secretion of anti-DNA Ab but not for the development of nephritis whereas TLR7 promotes the disease [92]. Furthermore, TLR7 gene duplication in mice induces SLE

General introduction

[93]. These observations led to the ideal to eliminate TLR3, TLR7, and TLR9 in lupus-prone mice at once by inactivating the Unc93b1 gene essential for transporting these TLR to the endolysosome to function. Compared to lupus-prone mice with a functional Unc93b1 gene, the mice with the Unc93b1 mutation produced fewer ANA and had fewer and less severe symptoms of SLE. This result suggests that the three endosomal TLR, or UNC-93B itself, might be good targets for therapy [94].
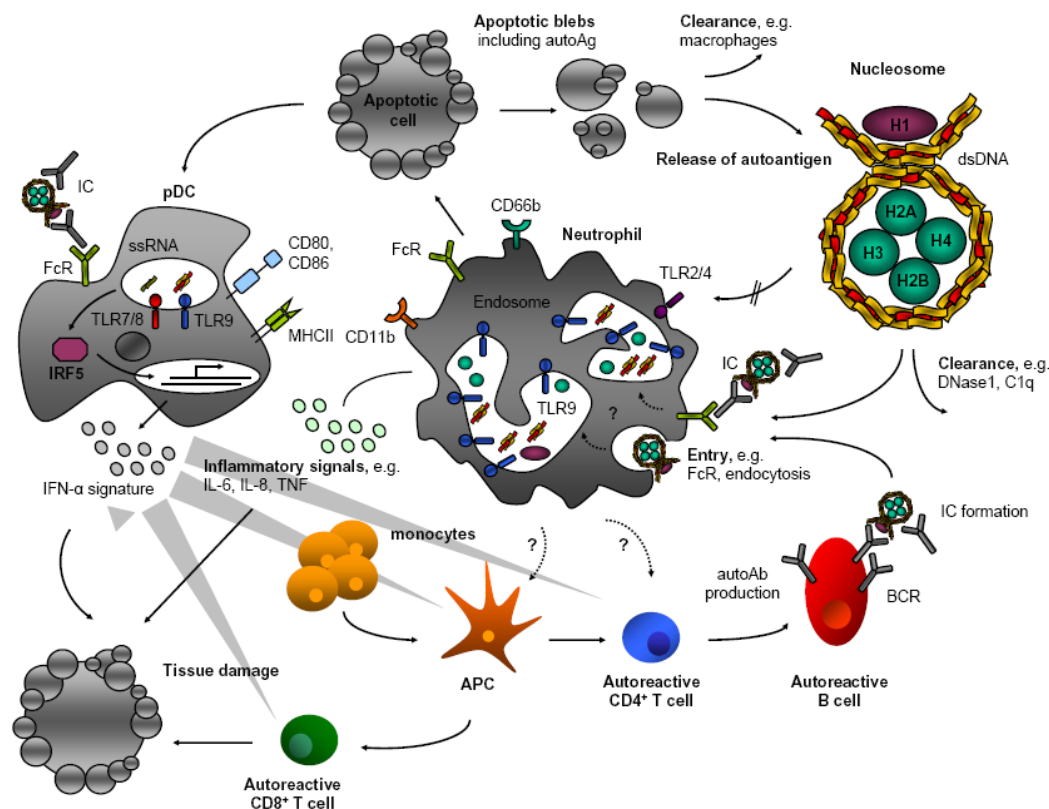
Figure 3. AutoAg trigger chronic inflammation in SLE. Host-derived autoAg are released from apoptotic cells. Clearance checkpoints to get rid of autoAg such as nucleosomes are indicated, but may fail under certain circumstances leading to the uptake of autoAg by DC, B cells and neutrophils. The exact mechanism for internalizing free nucleosomes by PMN is unknown. However, nucleosome-induced PMN activation is independent of TLR2/4 and TLR9, and results in the release of pro-inflammatory cytokines. Neutrophils are discussed to interact with DC and T cells. FcR-captured nucleosomal IC lead to TLR signaling in pDC, in which IRF5 serves as transcription factor. In genetically predisposed individuals, IRF5 is expressed at higher levels and/or functionally more potent in pDC, leading to the up-regulated secretion of IFN-α. The latter induces a IFN-α signature (grey arrows) in various immune cells and has pleiotropic effects, such as enhanced survival and proliferation, cross-presentation, up-regulation of MHC molecules and isotype switching. One key principle of IFN-α is the rapid induction and amplification of the

signaling pathway, which generates a feed-forward loop of IFN-α production. Although such feed-forward pathways are highly adaptive when it comes to rapid and effective eradication of intruders, this amplification can be maladaptive in immune responses directed against host tissues. Monocytes mature in the presence of apoptotic blebs and IFN-α into APC which present autoAg and activate autoreactive T cells. CD4^{+} T cells help autoreactive B cells to produce autoAb, which lead to IC formation. In total, these events accumulate and contribute to tissue collateral damage. IC formation and nucleosomes from damaged tissue, in turn, may work on immune effectors to accelerate autoreactive process or the attraction of other immune cells to the site of inflammation. Thus, systemic inflammation leads to the creation of autoamplification feedback loops and chronicity of these syndromes.

Pathogenicity of free circulating nucleosomes

The immunogenic effect of IC-associated nuclear autoAg has been demonstrated several times in SLE but the role of free circulating autoAg such as the nucleosome is still a field of extensive research. Nucleosomes are detected at higher concentrations in the blood of SLE patients. Circulating nucleosomes directly bind [95] and act on diverse cell types [96]. They bind to the surface of mesangial cells [97], a specialized cell type organizing the glomerular capillaries in the kidneys, and enhance their proliferation which may contribute to GN. Natural killer (NK) cells prevent autoimmunity, but nucleosomes have been shown to inhibit the lysis of target cells by NK cells [98]. Although the direct effect of nucleosomes on the activation of NK cells remains elusive, enhancing NK cell activity in SLE may be an attractive therapeutic intervention. Nucleosome-indcued activation of DC [99] and PMN [100] leads to the secretion of several pro-inflammatory mediators (Fig. 3) which may result in further recruitment of cells to the site of inflammation but also to collateral damage of the surrounding tissue. Interestingly, free circulating nucleosomes activate PMN in a pathway independent of TLR2/4 [100] and TLR9 (present work, see chapter V). Highly purified nucleosomes from calf thymus induce necrosis-like cell death of lymphocytes [101]. Nucleosomes further inhibit the removal of apoptotic cells by MΦ [102], stimulate B cells [103] and induce IL-6 secretion in splenocytes [96]. Thus, in combination with the dysfunctional removal of apoptotic blebs the nucleosome promotes its own generation and release into the circulation. In summary the nucleosome-induced activation of the innate immunity network may partly explain why the peripheral tolerance is broken in SLE.

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Chapter III

Neutrophils, dendritic cells and chromatin-mediated pathogenesis in systemic lupus erythematosus

Abstract

Nucleosomes are key lupus autoAg and have been shown to activate several types of immune cells as well as the complement system, resulting in inflammation. The elevated concentrations and the immunostimulatory capacities of circulating nucleosomes may favor the break of peripheral tolerance in genetically predisposed individuals. Nevertheless, in most cases the signaling pathways involved are not elucidated yet, although Tir8/Sigirr (single Ig IL-1R-related molecule) deficiency has been recently shown to enhance cell activation upon exposure to nucleosome-containing immune IC [1].

Introduction

Persisting inflammation may result in disharmony between cells of the immune system and the surrounding healthy tissue which results in collateral damage of the latter. SLE is a rheumatic autoimmune disease characterized by the loss of tolerance against ubiquitous autoAg leading to serious tissue injury. SLE is a multifactorial and inflammatory disorder classified as an immune IC-mediated disease. The continuous production of autoAb against autoAg results in IC deposition in multiple organs, e.g. the kidneys.

Nucleosomes and defective clearance of apoptotic cells in SLE

The autoimmune pathogenesis in SLE is associated with increased serum levels of IgG autoAb against self-components. Among these, the nucleosome and its constituents (histones and dsDNA) seem to play a central role as pathogenic mediators [2]. In the eukaryotic cell nucleus, DNA is associated with a set of specialized proteins (histones), resulting in a structure called chromatin. The fundamental repeating unit of chromatin fibers is the nucleosome. The core particle consists of 8 core histone proteins (2 molecules of each of H2A, H2B, H3 and H4) around which 146 bp of DNA is wrapped [3]. In addition, histone H1 binds to DNA outside the core particle to form the mononucleosome, which contains 180 bp of DNA. In higher-order chromatin structures, nucleosomes are connected through varying lengths of linker DNA. Although, under normal conditions these constituents are not released from the inner cell mass, patients with SLE show increased serum levels of circulating nucleosomes [4]. The latter are most likely released upon increased apoptosis in combination with impaired uptake of apoptotic cells [5], leading to secondary necrosis. Indeed, apoptotic cells represent a source of lupus autoAg. Thus, defects in the clearance of apoptotic cells or apoptotic cell-derived material may favor development of SLE. For example, it has been shown that the specific deletion of the genes encoding DNase 1 [6], SAP [7] or C1q [8] leads to the development of anti-nuclear Ab and a lupus-like disease in mice.

The nucleosome is a major lupus autoantigen

Autoreactive B and T_H lymphocytes specific for nucleosome autoepitopes are detected in patients with SLE. The latter help nucleosome-specific B cells to produce potentially pathogenic chromatin-reactive autoAb [9]. Thus, the amounts of circulating anti-histone H1, ANA and anti-dsDNA autoAb correlate with disease activity, and the latter are used as a disease marker. Particularly, nucleosome-restricted autoAb, i.e. Ab that recognize the whole nucleosome particle but not its individual components have been reported [10]. ANA are detected before anti-dsDNA and are potentially nephritogenic in lupus mice. Importantly, ANA of the IgG3 isotype are not only increased in SLE patients but also correlate with disease activity [11].

Polymorphonuclear leukocytes and dendritic cells in SLE

DC play an important role in the regulation of immune responses. SLE DC have been shown to express higher levels of activation markers and to secrete higher amounts of the pro-inflammatory cytokine IL-8 [12], a phenotype characterizing activated DC and thus favoring T cell activation. We observed that DC derived from patients with SLE spontaneously overexpress CD86, another DC activation marker [13]. PMN are the first cells recruited at sites of inflammation where they are activated. Neutrophils from patients with SLE with more active disease express higher levels of activation markers and expression levels correlated with disease activity [14]. Thus, both PMN and DC have altered functions or phenotypes in SLE. Importantly, PMN have been suggested to link innate and adaptive immunity, e.g. by attracting DC, pointing at a potentially interesting crosstalk in the pathogenesis of SLE.

Nucleosomes and inflammation

Several reports have shown that extracellular chromatin fragments may influence cell behavior. Whereas, most studies focused on the effects of chromatin containing IC in the context of SLE, showing that they activate rheumatoid factor-positive B cells as well as DC, some groups investigated the direct effects of free nucleosomes. Thus, chromatin activates directly chromatin-reactive B cells [15]. Likewise, nucleosomes induce IL-6 secretion by mouse spleen cells [16]. Consistently, we have shown that circulating nucleosomes activate DC [17] and PMN [18] resulting in high cytokine secretion, especially IL-8. Thus, upon endocytosis of nucleosomes, DC are activated and then potentially process and present HLA associated nucleosome-derived peptides to autoreactive T cells. During activation DC secrete IL-8, leading to PMN recruitment. Likewise, nucleosome-activated PMN secrete IL-8, amplifying PMN recruitment, and activate and recruit other cells. Those mechanisms support the inflammation and tissue damages observed in SLE patients. Moreover, we have observed that circulating nucleosomes directly activate the complement system upon binding to C1q, leading to an increased C3a generation (Erbacher AE, in preparation). Interestingly, C1q is secreted by DC whereas the anaphylatoxin C3a stimulates PMN. Furthermore, C3a has been recently shown to interact with DC and naive T_H cells, favoring DC maturation and T cell activation [19].

Nucleosome-mediated pathogenicity

As explained above, SLE is an IC-mediated inflammatory disease. IC deposit into tissues and activate the complement system, causing inflammation and tissue damages in the form of a type III hypersensitivity reaction. Renal disease is a major complication in SLE. Particularly, the deposition of nucleosome/anti-nucleosome IC in the glomerular basement membrane in the kidney is believed to be involved in the development of GN. Free nucleosomes have also been suggested to be directly pathogenic by affecting mesangial cells [20]. Our findings on the C1q-nucleosome interaction suggest that tissue-deposited nucleosomes may participate locally in complement activation and C3a production, e.g. in the kidney where the C3a receptor is highly expressed in SLE patients with GN. During the inflammatory process, PMN and MΦ are recruited and release their granule contents and reactive oxygen species, the latter being involved in tissue damage but also in the alteration of autoAg, prone to modify their immunogenicity. Consistently, we have observed that nucleosome-induced activation enhances oxidative burst in PMN (unpublished). Finally, nucleosomal Ag have also been found in the epidermal basement membrane of non-lesional skin in patients with SLE and GN, suggesting that nucleosome-mediated binding of autoAb may occur at sites other than the glomerulus [21].

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General introduction

Chapter IV

Alzheimer's disease

Historical background

In 1901 Auguste Deter was admitted to the state asylum in Frankfurt (Fig. 1A). She was suffering from progressive dementia, characterized by memory loss and disordered cognitive functions. In the hospital she was examined by the medical doctor Alois Alzheimer (1864-1915, Fig. 1B). In 1903 Alzheimer joined Emil Kraepelin's group in München. Three years later Auguste Deter died and her brain was analyzed by Alois Alzheimer with then newly identified histological methods. For the first time, Alois Alzheimer described extracellular miliary foci and intracellular dense bundle of fibrils as pathological characteristics of this disease [1]. In 1906 A. Alzheimer presented his observations at a psychiatry meeting in Tübingen. The results of this study were published in 1907 [2]. In 1910 Emil Kraepelin honored the study of A. Alzheimer by naming the disease after him. Until now both findings remain still the diagnostic hallmarks of AD post mortem (Fig. 1C). They are now referred to as senile plaques and neurofibrillary tangles (NFT). However, the composition of the plaques remained unknown until 1985, when the components were identified as Aβ [3].
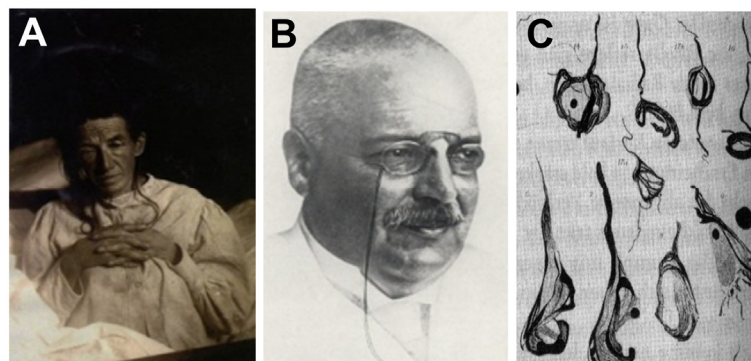
Figure 1. Historical aspects of AD. Pictures show the first documented AD patient Auguste Deter **(A)** by the German psychiatrist Alois Alzheimer **(B)**. **(C)** NFT drawn by Alois Alzheimer (adapted from [4]).

Neuropathology

The most widely accepted criteria for the clinical definition of AD have been introduced by studies of the National Institute for Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association [5]. Definite AD requires the clinical finding of dementia as determined by the Mini-Mental Status Exam or other standardized neuropsychological testings to specify cognitive changes over time, and imaging studies. The exam must demonstrate deficits in two or more areas of cognition with progressive memory loss in the absence of delirium. Probable AD is supported by the findings of impaired activities of daily living, loss of specific cognitive functions such as language and motor skills, and a familial history. However, a definitive diagnosis can only be established at autopsy. Over time the brain shrinks (Fig. 2A) and the histological findings that Alois Alzheimer reported nearly 100 years ago become manifested: extracellular Aβ plaques and intracellular NFT composed of the hyperphosphorylated microtubule-associated tau-protein (Fig. 2B and C).

Figure 2. Imaging atrophy and aggregates in AD. (A) Decreased brain volume of an AD patient (left) as compared to a healthy control person (right), visualized by positron emission tomography imaging [Courtesy of Alfred Pasieka, Science Photo Library, Photo Researchers, Inc.] **(B)** Stainings of Aβ plaques in the cortex of an AD patient (left). At higher magnification the plaque core appeared built-up by amyloid fibrils and accompanied by dystrophic neurons (right). **(C)** Within the cell body of dystrophic neurons, NFT are visualized with silver-staining (left). Pictures taken by electron microscopy (EM) reveal that NFT are composed of tau-protein that is assembled into paired helical filaments (right, pictures are adapted from [6]).
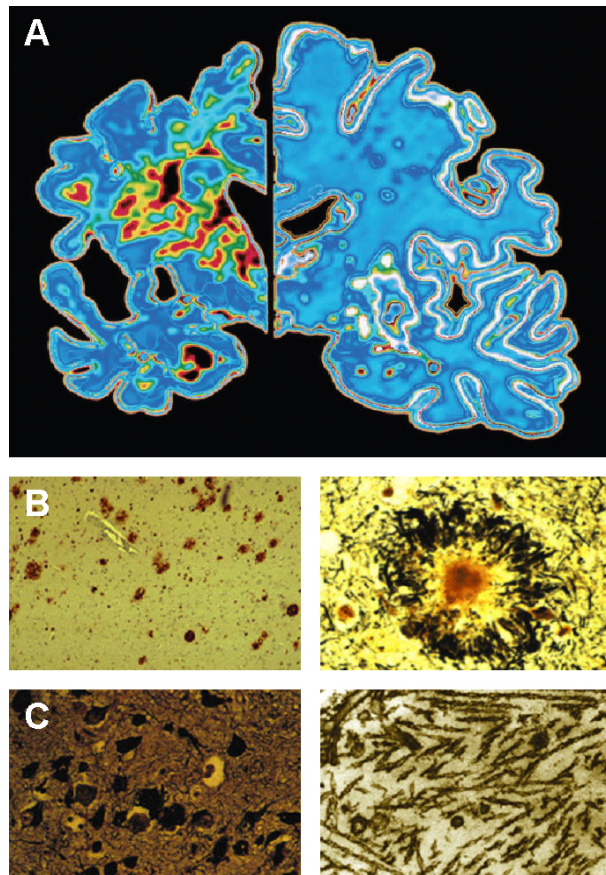
Small cleavage products of the amyloid precursor protein (APP) are the main components of Aβ plaques. However, neuropathologcial lesions that involve the extra- or intracellular cytoplasmic deposition of abnormally folded proteins are not restricted to AD. They are shared by most neurodegenerative diseases, e.g. Parkinson disease (PD) or Huntington's disease [7]. These diseases are therefore summarized as proteopathies. Controlled aggregation of protein species may serve as cytoprotective functions, such as isolating misfolded forms [8] and it may also promote their clearance [9]. Therefore, imbalances in the build-up and clearance of Aβ aggregates in the CNS are linked to the initiation of AD. This observation led to the amyloid cascade theory [10]. The prevailing view is that excessive generation of Aβ is the primary cause of neuronal dysfunction. An initiating factor occurs when Aβ monomers undergo a conformational change and transform into oligomers, protofibrils or fibrils. Indeed, Aβ oligomers block hippocampal long-term potentiation and spatial memory in an AD mouse model [11, 12]. The accumulation of multimeric Aβ is concentration-dependent and confers toxicity. Metabolism of Aβ by normal physiology is poorly understood. Interestingly, a recent study identified the cellular prion protein (PrP^C) but not the infectious PrP^{Sc} conformation as a possible high-affinity cell-surface receptor for Aβ on neurons [13]. Thus, the following chapter will focus on the molecular pathways leading to Aβ production.

APP and the molecular pathways to the self-peptide Aβ

APP is ubiquitously expressed, suggesting a fundamental requirement for its signaling in many tissues [14]. Even though its exact function could not be identified yet, APP plays a role in blood clotting, transmembrane signal transduction, calcium regulation, neurite outgrowth, cell proliferation as well as cell adhesion [15-19]. Furthermore, APP may be involved in the migration of newly differentiated neurons into the developing cortex [20]. Recently, APP was identified as a ligand for death receptor 6 which triggers neuronal death through caspase 6 [21]. APP belongs to a family of type I membrane proteins containing a larger extracellular region, a TM domain and a shorter cytoplasmatic tail (Fig. 3). After synthesis in the ER, the glycoprotein is transported to the cell surface through the secretory pathway. At this point, the interaction of three different secretases leads to either an amyloidogenic (Fig. 3, lower part) or a non-amyloidogenic processing (Fig. 3, upper part).
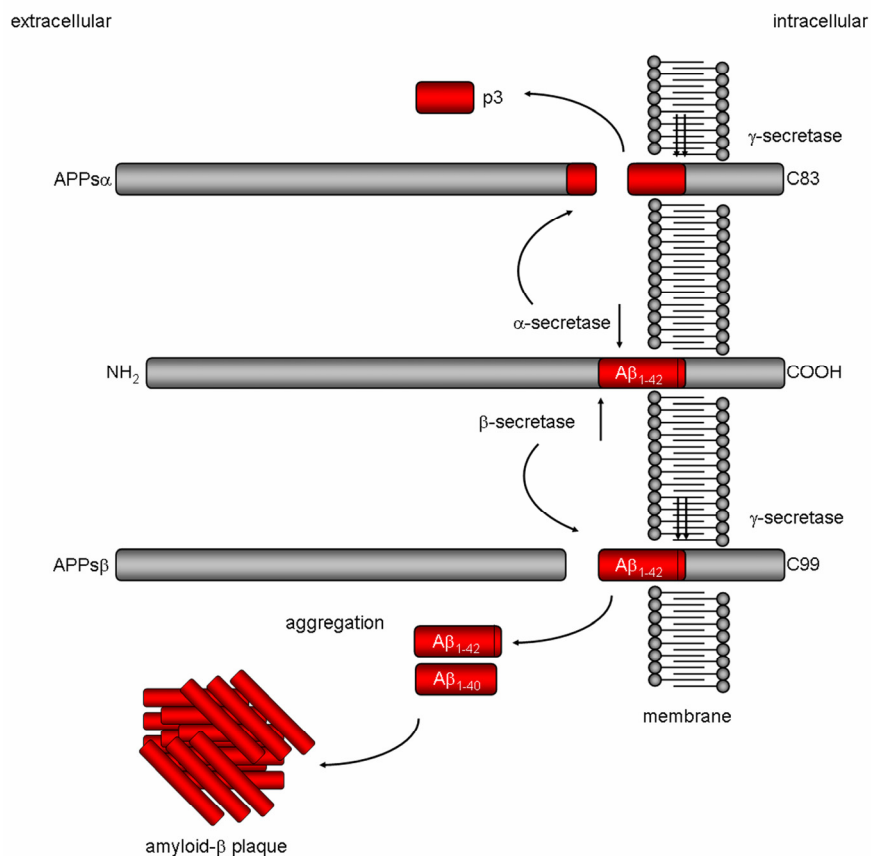
Figure 3. Proteolytic processing and resulting fragments of APP. Schematic structure of APP is shown. The region encoding Aβ is highlighted in red. The main part of APP is processed in the non-amyloidogenic pathway (upper part). Note that α-secretase cleaves APP within the Aβ domain which precludes the formation of Aβ plaques. In contrast the amyloidogenic pathway (lower part) results mainly in the generation of $Aβ_{40}$ or $Aβ_{42}$. Both tend to aggregate and deposit as senile plaques. Cleavage sides of secretases are indicated by straight black arrows.

In the Golgi compartment as well as during reinternalization into endosomes, APP (amino acids [aa] 1-770) is cleaved by β-secretase (BACE1, β-site cleaving secretase). This cleavage results in the release of APPsβ (N-terminus) and in the membrane bound C99 (C-terminus) [22-24]. The latter is subsequently cleaved by the protein complex γ-secretase, liberating Aβ fragments into the extracellular space [25]. The tetrameric enzyme complex γ-secretase is composed of the presenilins (PS) 1 and 2, anterior pharynx-defective phenotype-1 and nicastrin [26]. Several available therapeutic strategies aim to target γ-secretase activity with to some extent detrimental side effects [27]. New achievements identified G protein-coupled receptor 3 as

potential target because its expression levels regulate the localization of γ-secretase, thereby affecting the regulated intramembrane proteolysis (RIP) of APP [28]. RIP of APP results in the generation of different Aβ species, e.g. Aβ_{40} (aa711), Aβ_{42} (aa713), Aβ_{43} (aa714) and Aβ_{49} (aa720). Aβ_{40} and Aβ_{42} are the most common forms. Aβ_{40} has been found to be less neurotoxic as the slightly longer Aβ_{42} fragment and is by itself not able to form senile plaques [29]. By contrast, Aβ_{42} is more prone to aggregate and most probably forms the seed of senile plaque formation [30, 31]. Due to its fibrilization properties Aβ_{42} is the dominant species in amyloid-β plaques [32, 33]. The non-amyloidogenic pathway is committed by α-secretase, which is active in late Golgi, transport vesicles and at the cell surface [34-36]. Although α- and β-secretase compete for the APP substrate [37], α-secretase generally cleaves APP earlier in cell compartments than β-secretase [35]. The α-secretase (ADAM, a disentegrin and metalloproteinase 10) cleaves APP into the ectodomain N-terminal APPsα and the membrane-associated C83 [38]. Thereby, the sequence of Aβ is destroyed [39]. C83 is further processed by γ-secretase, which results in the release of the small p3 peptide. The p3 fragment is non-amyloidogenic, due to its smaller size than the larger Aβ fragment starting at position 17 of Aβ_{42}.

Epidemiology and genetics

The social impact and economic burden of AD is growing rapidly due to increasing lifespan and a decreasing ratio of working to retired population in the western societies [40]. Indeed, the prevalence is partially due to the fact that aging represents a significant risk factor for developing AD [41]. Thus, there is a compelling need for treatment and prevention of AD, although a cure is realistically not within sight due to the multi-factorial nature of the disease. Despite the normal aging process it is well established that genetic risk factors play a major role in the pathogenesis of AD. Two different forms of AD are distinguished based on their genetic cause [42]. The first form is sporadic AD (SAD) which probably results from the synergistic interactions of disease-related genes with an individuals' lifestyle and additional environmental factors. Up to now the apoE4 (apolipoprotein E4) allele is the only identified genetic risk-factor for developing SAD [43]. This protein is encoded on chromosome 19 and has functional properties in the lipid metabolism [44]. It has been shown that several copies of the apoE4 allele exist in SAD patients and may possibly affect Aβ deposition and NFT formation [45]. Mutations in three genes that are inherited in an autosomal

dominant fashion have been linked to the second, rare familial form of AD (FAD). These genes include the information encoding APP (chromosome 21), PS1 (chromosome 14) and PS2 (chromosome 1). Variability of their expression contributes to the risk of developing AD before 60 years of age (early-onset AD). Several missense mutations have been discovered in APP (Fig. 4) which affect the β- and γ-secretase cleavage. However, less than 3 % of these mutations account for early-onset forms of AD [46]. The majority of FAD mutations are found in the genes encoding PS1 and PS2. More than 175 mutations have been identified. Mutations in PS1 account for 70 % and PS2 mutations for 20 % of all FAD cases [47-49]. Individuals with mutations in PS1 have been shown to develop the most severe forms of AD with a mean age of onset that varies between 30-60 years [50]. There are no pathological and clinical symptoms that distinguish SAD from FAD [51]. In summary, all of the genetic risk factors of SAD and the autosomal dominant mutations of FAD ultimately lead to an overproduction of Aβ and its deposition in the CNS. To date the multifactorial etiology of AD makes it impossible to predict the disease onset or the severity of dementia.
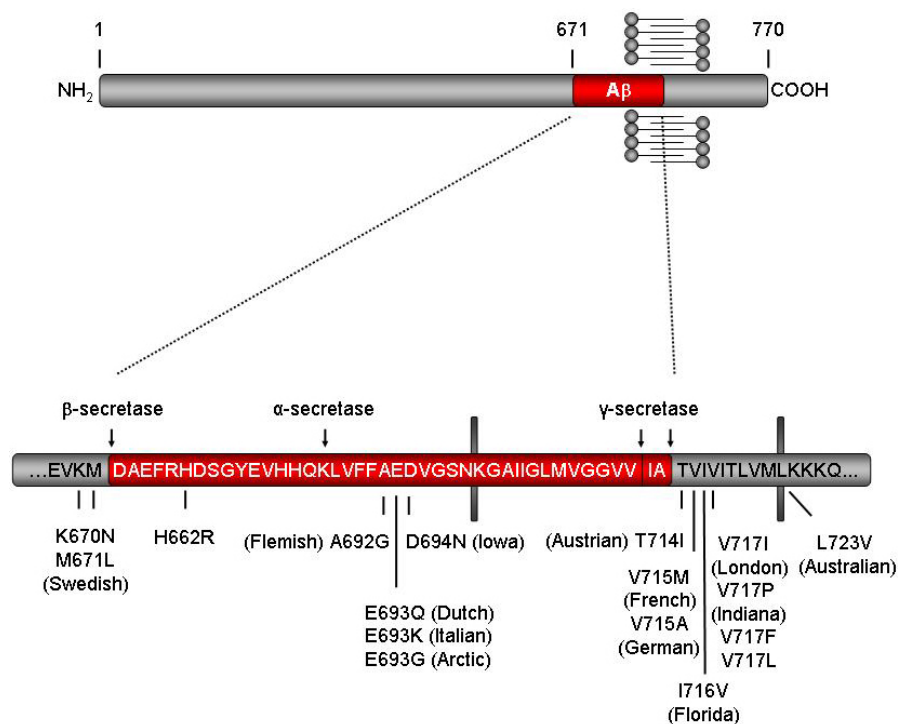
Figure 4. FAD-linked mutations in APP. Schematic drawing of APP (grey) and Aβ (red). The aa sequence of Aβ (straight line divides $A\beta_{40}$ and $A\beta_{42}$) is indicated and expanded to its flanking sides. The arrows represent cleavage sides of α-, β- and γ-secretase. The TM domain is depicted with grey lines (lower part). Several mutations cluster close to the cleavage sides of the secretases and the most important aa substitutions are indicated.

APP-transgenic mice are only partial models of AD

The rationale for studying AD in a model system is mainly based on the discovery of FAD-linked mutations seen in APP, PS1 and PS2. These mutations subsequently opened the door for the design of tg mice. Most of them are generated in agreement with the amyloid cascade theory. Thus, the overproduction of Aβ triggers a cascade which eventually results in neuronal loss, dystrophic neuritis, inflammatory responses, learning impairments and deficits of synaptic interactions [10]. The strength of rodent models is that they develop AD-like pathology sharing many similarities with humans [52, 53]. However, it also points out some weaknesses. Rodent models show only features of rare, early-onset FAD and do not resemble the more common late-onset form of SAD. Moreover, there are notable differences in the AD pathology seen in humans and APP-tg mouse models. Human AD brains exhibit further processing of Aβ plaques into a more insoluble state [54, 55] and reveal a higher degree of inflammation which is mainly related to the role of the complement system [56]. Since mouse C1q poorly recognizes human Aβ deposits, complement activation in tg mice is minimal [57]. In human AD, there is full activation of the complement system resulting in neuronal destruction by the membrane attack complex [58]. None of the APP-tg strains do show neuronal loss and most of them do not exhibit NFT as seen in human AD brains. Tg mouse models of tauopathies exist but also mimic only parts of AD neurodegeneration [59]. Despite the fact that a large number of AD models have independently produced similar data, technical criticisms announce the possibility of artifacts due to the overexpresssion of APP with physiologically CNS-irrelevant promoters, the possibility of multifaceted transgene silencing and the use of cDNA rather than genomic DNA in these mice [60]. Thus, each mouse model has its own specific strengths and weaknesses for studying different aspects of neurodegeneration. In 1995 the first APP-tg strain was developed by Games and colleagues. This model shows extracellular Aβ deposits due to V717F mutated APP expression under control of a platelet derived growth factor-promoter [61]. Deficits in memory and learning have not yet been reported for this model. In Tg2576 mice AD-like pathology is triggered by expressing high amounts of K670N/M671L mutated APP in many cell types using the hamster prion protein promoter [62]. Besides Congo red positive Aβ plaques which are characteristic of amyloid structures [63], aging Tg2576 mice also develop correlative cognitive deficits [64]. APP23 and APPPS1 mice develop amyloidosis due to the same APP mutation as used in Tg2576 mice but in both models the expression is under control of a neuron-specific Thy 1 promoter element [65, 66]. APPPS1 mice show an earlier onset of disease in comparison to the APP23 model due to the coexpression of

General introduction

L166P mutated PS1. The latter provokes first cognitive deficits at the age of 24 years in humans [67]. Despite the age-related accumulation of Aβ and gliosis, both models also show cerebral amyloid angiopathy (CAA). It develops from soluble Aβ that drains from the CNS along the cerebral and leptomeningeal microvasculature predisposing blood vessels to failure, thus increasing the risk of a hemorrhagic stroke [68]. Recent studies even presented models with multiple genetic manipulations. For example, the Tg-SwDI triple tg mice express low levels of double Swedish mutations (KM670/671NL), the Dutch (E693Q), and the Iowa (D694N) mutations in neurons and therefore shows vasculotropic mutant Aβ peptides and microvascular deposits [69]. Even the generation of a triple-tg model of AD by introducing KM670/671NL mutated APP and P301L mutated tau into the germline of the mutant PS1 knock-in mouse does not recapitulate all features of human AD [70]. In summary, AD mice develop pathogenesis to a varying degree, depending on the mutation used, the promoter element, the expression levels and gender differences. In addition, the integration site or even the murine genetic background may influence neuropathology. These differences should be taken into account when using mice as preclinical models of human AD. To study the effect of immune effector molecules on amyloid-β pathology several immune transgenes or immune gene knock-outs were generated in APP-tg mice [71]. It seems that the lack of CD4$^+$ T cells retards amyloidosis, whereas the lack of autoAb-producing B cells has no effect on the extent of Aβ deposition [71, Lindau et al. unpublished]. Following these results some studies addressed the role of IL-12 family members in AD, because these cytokines are known to control T cell differentiation and activation. This family of cytokines compromises several heterodimeric proteins with pleiotropic activities including IL-12 (p35 + p40) and IL-23 (p19 + p40). IL-12 is produced by APC and has been shown to play a major role in T_H1 differentiation. Interestingly, a decrease in T_H1 immunity caused by ablation of IL-12 seems to affect pathology in AD mice, which is currently investigated in detail [Burkhard Becher, University of Zurich, personal communication]. Indeed, a detrimental role for T_H1 cells was already claimed in AD immunotherapy [71]. These results argue for T cell involvement and against a role of autoAb in the development of cerebral amyloidosis, but it also suggests caution in interpreting results obtained from immunodeficient APP-tg mice. The immune and the nervous system utilize many of the same molecules during development (MHCI and its subunits, CD3Zeta (ζ), Transporter associated with Ag-processing 1), communication (TLR3, TLR7), and genomic organization (recombination activating gene 1). Thus, both systems share similar principles of action to achieve specific functions such as signaling, migration and homeostasis (as highlighted by a special co-issue from Immunity and Neuron [72]).

Inflammation in Alzheimer's disease

Critically, the dialogue between the immune system and the CNS during the course of AD is poorly understood. It is well established, that soluble mediators of immunity such as chemokines or complement proteins are up-regulated both peripherally and in the brain of AD patients [71]. The generation of a pro-inflammatory cytokine milieu at sites of Aβ aggregates potentially suppresses neurogenesis thus favoring the cognitive decline seen in AD patients [56]. The CNS shows only limited expression of MHC on neurons and microglia [73] and is shielded from Ab by the blood-brain barrier (BBB) [74]. The capillaries of the CNS form a permeability barrier and are structurally different from other tissues. They are lined with a special layer of endothelial cells that lack fenestrations and are sealed with tight junctions. This architecture results in a very high transendothelial electrical resistance of 1500-2000 Ω x cm^2 in the CNS compared with 3-33 Ω x cm^2 in other tissues. Furthermore, astrocyte foot processes surround the capillaries, forming a second barrier of the BBB. Thus, only lipophilic molecules that can freely diffuse through the capillary endothelial membrane may passively cross the BBB. This barrier makes the CNS practically inaccessible to lipophobic, small polar molecules or any larger protein. Consequently, a specialized local innate immune response has developed to effectively fight infectious agents [75]. In vivo imaging, in vitro and post mortem studies have shown that microglia and astrocytes are activated in the presence of misfolded proteins [76-78]. Furthermore, the BBB is easily penetrated by activated T cells and MΦ. To this end, inflammation is definitively present in AD, but little is known about its pathological relevance.

Star shaped astrocytes

Under normal conditions astrocytes contribute to CNS homeostasis and provide neurons with energy and substrates for neurotransmission. They act as physical barriers between the synaptic connections of neighboring neurons, and remove excess neurotransmitter molecules from the extracellular space, allowing discrete and precise encoding of synaptic signals and neurotransmission. Even more exiting, they seem to be involved in the formation of synapses and in modulating synaptic function through bidirectional communication with neurons [79]. Surprisingly, they are largely ignored by the scientific AD community in contrast to the intensively studied microglia. Not much is known about their role during the course of neurodegeneration which is remarkable because astrocytes are one of the most numerous cell types in the CNS [80].

Histologically, astrocytes are characterized by expressing glial fibrillary acidic protein (GFAP) and, similar to microglia, they are located at sites of Aβ aggregates. Astrocytes secrete a set of chemokines such as monocyte chemoattractant protein-1 thus contributing to neuronal damage by recruiting phagocytes to Aβ deposits with the concomitant release of neurotoxins [81]. Furthermore some studies have shown that astrocytes can also degrade amyloid-β plaques [82]. Caspase activity has also been seen in astrocytes which leads to cellular degeneration in vitro and may indirectly play a role in AD progression [83].

Microglia

Microglia cells have been implicated in the pathogenesis of AD and generally are regarded as key players during several different proteopathies [84]. These immune cells represent 10 % of the cells in the adult CNS [71] and are most commonly characterized by the ionized calcium binding adaptor molecule (Iba)-1 which is unfortunately not directly related to function and phenotype [85]. Their differentiation and activation into different phenotypes is mainly seen by their morphological appearance [86]. Parenchymal microglia cells have been classified as BM-derived myeloid progenitors which enter the CNS from the peripheral blood circulation early during development and become resident APC [87]. These resident phagocytes survey the brain for damage and infection, engulfing apoptotic cells and debris [88]. Therefore, Aβ may be recognized as Ag which needs to be cleared and thus provokes activation of microglia [89]. If microglia fail to clear the Ag, their activation status becomes chronic and toxic. This concept is also known as the microglia dysfunction hypothesis [90]. Using gene expression profiling from microglia at different time points, it has been shown that these cells may fight against the build-up of Aβ plaques at the beginning of AD pathogenesis [91]. However, they lose their phagocytotic activity during the course of the disease and instead show increased expression of pro-inflammatory cytokines. These cytokines compromise IL-1, IL-6, IFN-γ and TNF which have been shown to mediate neurotoxicity in vitro [92-94]. In addition, microglia cells are able to secrete neurotoxic substances such as ROS and proteases [95]. Thus, the possibility is given that microglia may even participate in CAA and neuronal loss. Indeed, EM studies actually suggest that early microglial activation is one key factor in promoting progression of CAA [96]. Despite, these detrimental properties several studies also addressed beneficial functions of microglia. They have been implicated in synaptic remodeling during the development of the CNS, removing inappropriate synaptic

connections through the process of phagocytosis [97]. It has been shown that microglia can express neurotrophic factors like nerve growth factor and brain-derived neurotrophic factor. Both of which have been shown to promote neurogenesis in rodent and primate models of AD [98]. There is also evidence that even the above mentioned pro-inflammatory cytokines possess beneficial brain repairing properties [97]. These two opposing functions of microglia dominate lively discussions whether these cells may be therapeutically utilized to fight AD. Although, in vitro experiments showed an uptake of aggregated Aβ species [99] the histopathology of human brains does not support a role for microglia in Aβ clearance [96]. Moreover, evidence that microglia in brains of AD mouse model [100] as well as human patients [101] are able to degrade and harbor Aβ in their endolysosomal compartments is still missing. To this end, intensive research in AD mouse models has been focused on the peripheral counterparts of microglia, namely monocytes and CNS-exogenous MΦ. Peripheral myelopoesis is faster and more efficient as compared to the generation of brain endogenous microglia [102, 103]. Even more interestingly, it has been reported that CNS-exogenous MΦ are competent Aβ phagocytes [104]. Blood-derived monocytes are able to pass the BBB, settle close to cerebral vessels and Aβ deposits. Genetic ablation of these cells increases parenchymal Aβ plaque load in mouse models of AD [105, 106]. Furthermore, it has been shown that ablation of TGF-β signaling in CD11c$^+$ cells results in accumulation of MΦ in cerebral vessels, CNS penetration of these cells, and clearance of Aβ deposits in mice [107]. Data from different proteopathies highlights the fact that monocytic phagocytes undergo profound changes in protein synthesis permanently, including surface receptors and pro-inflammatory cytokines, especially when activated under conditions of acute injury. Thus, microglial activation may be understood as a set of responses ranging from a productive (pro-phagocytic and anti-inflammatory) to a deleterious (anti-phagocytic and pro-inflammatory) phenotype [108]. Further support of beneficial microglial phenotypes comes from a study which showed that immunization of an AD rodent model with glatiramer acetate (GA, Copaxone) induced Aβ clearance, neurogenesis, improved memory and learning [109]. The investigators found in GA-treated mice microglia expressing the DC markers MHCII and CD11c which produced neuroregenerative insulin-like growth factor-1. This revealed a phenotypic shift in contrast to the activated CD11b$^+$ microglia present in the PBS-injected control animals. The MHCII$^+$CD11c$^+$ phenotype suggests the ability of Ag-presentation and indeed significantly more T cells were associated with Aβ deposits and microglia in GA-injected mice than in the untreated state.

Aβ-peptide autoimmunity – the role of T cells in AD

The role of lymphocytes, which is observed by autoAg-specific T cells in the CNS pathology of MS is not present in AD. However, several studies demonstrate T cell entry into the brain of APP-tg mice [109, 110]. Interestingly, T cell surveillance of the CNS has been described in the elderly and in AD patients [111, 112]. This is line with early reports of T cell brain-tissue entry irrespective of their Ag-specificity [113], but only T cells capable of reacting with a CNS-derived Ag seem to remain there [114, 115]. Within the brain microglia or MΦ may under some conditions react with lymphocytes because they are able to present HLA-associated Ag [116] and continuously survey the CNS [77]. In addition considerable data exist supporting the ability of DC to enter and leave the brain, presenting CNS-derived Ag to prime T cells in the periphery [117, 118]. These observations collectively demonstrate that the CNS does not escape immune surveillance but induces a tight and selective communication with adaptive immunity, being accessible to small numbers of T lymphocytes on a continuing basis [119]. During a pathological insult such as neurodegeneration or neuroinflammation the data exchange between both systems may be disturbed. The overall permeability of the BBB is increased and traffic signals for cell migration are altered [120]. Additionally, the expression of adhesion molecules and set of chemokine receptors permit the enhanced invasion of lymphocytes in the brain parenchyma [121]. Recent results suggested that intracerebral Aβ interacts with RAGE (Receptor for advanced glycation endproducts) at the BBB thus up-regulating endothelial expression of the chemokine receptor CCR5, thereby causing increased T cell infiltration [122]. In general, RAGE is expressed at increased levels in brains of patients with AD. Although a pathogenic role for Aβ-specific T cells has not been established in humans or mice, the invasion of T cells with unknown specificity in AN1792 (synthetic Aβ$_{42}$)-immunized AD patients illustrates potential consequences [123, 124 and see below]. Analysis of brain-infiltrating T cells as well as the peripheral repertoire resembles the memory phenotype in vitro and in vivo [125, 126]. Generally memory T cells predominate at inflammation sites and respond briskly to recall Ag [127, 128]. Interestingly, individuals with AD show an increased expression of the co-stimulatory receptor CD28 in the total T cell population [129, Lindau et al. unpublished]. Signaling via CD28 and the T cell receptor results mainly in cytokine production to facilitate proliferation and survival but also in the prevention of anergy induction [130]. In vitro Aβ-specific T cell responses are present in virtually all individuals. The frequency of circulating Aβ-specific T$_H$ cells is increased in the elderly and individuals with AD and the allele HLA-DRB1*1501 seems to play a highly immunogenic role [131, 132]. This is in contrast to studies that

generally showed reduced T cell reactivity upon aging [133]. Whether this phenomenon has an etiological contribution to the disease or is simply a marker of the CNS damage present in AD remains elusive. Recently, it was suggested that homozygosity but not heterozygosity for HLA-A2*0201 is associated with an increased risk of SAD development [134]. One post mortem case report even demonstrated an increased presence of CTL in the brain [135] and was interestingly typed HLA-A2*0201[+] [Lindau et al. unpublished]. The increased amount of CTL in the brain of AD patients is also commonly seen in other CNS-inflammatory diseases [136-138]. Aβ-derived CTL epitopes were identified and human Aβ-specific CTL visualized ex vivo showed neurotoxicity in vitro [Lindau et al. unpublished]. However, detrimental properties of autoreactive Aβ-specific T cells might be silenced in vivo due to an increased activity of T_{REG} observed in AD patients [139]. It has been shown that neurons possess immune-regulatory properties by mediating T cell apoptosis [140] or even activate T_{REG} in response to inflammation to suppress autoreactive T lymphocytes which mediate neurodegeneration [141]. In addition, one has to take into account that reactions may change over time and switch from damaging properties to a mode that promotes tissue repair [142-144]. T cells can play a role in neuroregeneration [145]. Studies using models of optic nerve or spinal cord crush have shown that CNS-Ag-specific T cells are involved in reorganization and regeneration after CNS trauma [146]. Furthermore, T cells are known to produce neurotrophic factors that could prevent CNS tissue from secondary degeneration [147]. Whether a comparable regenerative potential also holds true for AD is unknown. However, first studies suggested a possible beneficial role for CNS-infiltrating T cells because nasal Aβ-peptide and GA-administration into APP-tg mice prevented Aβ deposition [148].

Immunotherapeutical strategies in AD

Studies in APP-tg mouse models have placed attention on the clearance effect associated with Aβ vaccination as being a possible disease-modifying strategy. Immunization has been shown to reduce amyloid burden in AD mouse models both actively and passively, using synthetic $A\beta_{42}$ or small fragments of it and through the peripheral administration of anti-Aβ Ab, respectively [149, 150]. Some data also suggest that stimulation of innate immunity using CpG-ODN is highly effective at reducing the Aβ plaque burden [151]. However, it has been shown that microglia expressing TLR9 and responding to CpG-motifs in vitro mediate neuronal injury [152]. Thus, the amyloid clearing effect associated with this strategy has been confirmed by several experiments, but the exact mechanism remains speculative. Three mechanisms have been postulated to explain how Aβ deposits are reduced in the CNS: (1) One hypothesis suggests that the Ab may bind directly to Aβ. This effect could lead to FcR-mediated phagocytosis of Aβ by microglia. However, successful reduction of Aβ in APP-tg mice deficient in FcR argues that other mechanisms besides FcR-mediated phagocytosis exist. (2) In this regard, some scientists favor the idea that Ab themselves react as Aβ disaggregating agents by preventing the formation of plaques. Consistent with these thoughts, directly injected Ab into the CNS decrease Aβ deposits. (3) In the last scenario, Ab are thought more directly to initiate a peripheral sequestration of Aβ into the plasma (peripheral sink hypothesis). These proposed mechanisms are not mutually exclusive; the Aβ clearance is likely to involve a combination of processes [153-155]. The finding that active Aβ immunization markedly reduced the amyloid plaque load in an animal model without any obvious side effects led to a randomized placebo-controlled double-blinded Phase II clinical trial [156]. In 2002 Elan Pharmaceuticals, Inc. halted the AN1792 trial prematurely because 6 % of the treated subjects developed encephalitis (Fig. 5A). The cause of inflammation remains controversial but was most likely induced by the massive infiltration of T cells into the CNS (Fig. 5B-D) [123, 135]. Although the trail was interrupted, cognitive analyses and biomarker testing revealed that Aβ immunotherapy had a positive clinical impact on AD [124]. Post mortem analysis revealed a reduction in the amyloid plaque load associated with gliosis, which might be connected to clearing mechanisms, but the NFT remained unchanged [123]. Investigations into the cause of the meningoencephalitis support the view that the full-length $A\beta_{42}$ that was used in the trial induced the generation of a T cell response. However, it has also been suggested that the increased Ab levels, found in immunized subjects, induced damage to the microvasculature and consequently led to

the massive T cell influx from the periphery [157]. In support of this, Ab administration can lead to an autoAb response. This could facilitate the formation of serum IC which generates vasculitis and microhemorrhage upon deposition at the blood vessel wall as observed in other chronic disorders such as SLE. Nevertheless, the anti-Aβ vaccination trial seems to have promise at clearing Aβ and behavioral stabilization [123, 124]. To circumvent possible side-effects alternative immunization strategies using the immunomodulatory drug GA (Teva Pharmaceutical Industries Ltd.) are currently being investigated [148]. But how and whether immunization will influence the functions of already destroyed neuronal circuits remains to be elucidated. The most optimistic view is that it will stop disease progression, and that brain plasticity mechanisms will allow some recovery over time.
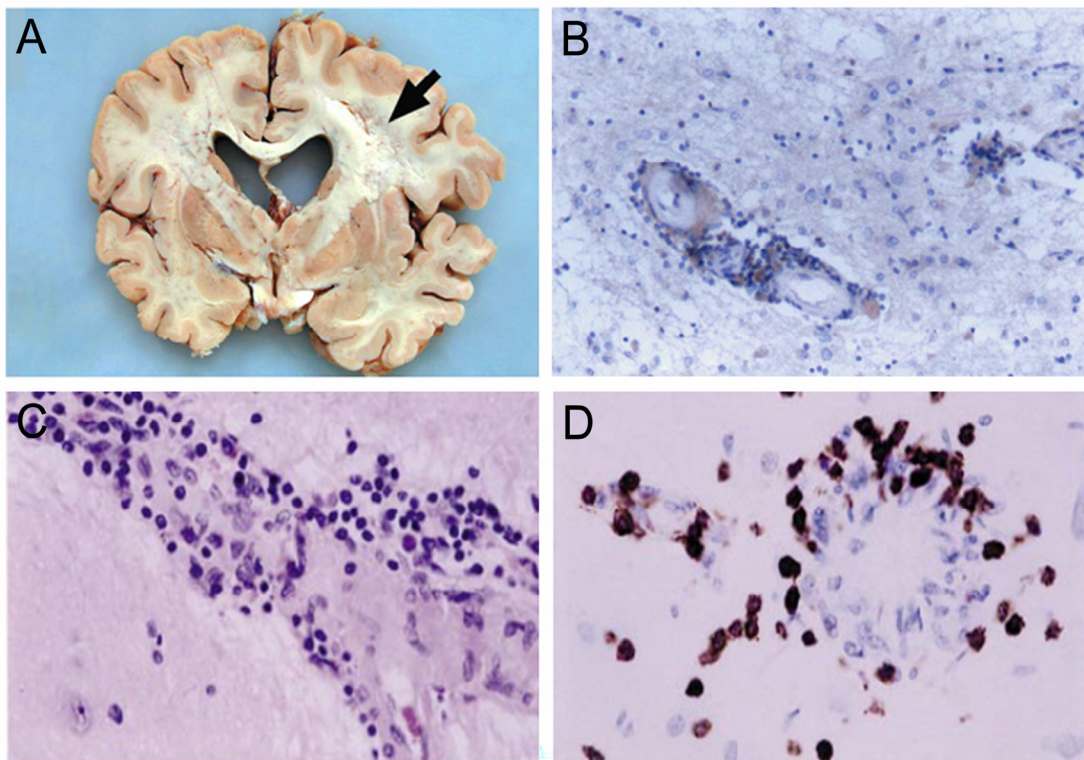
Figure 5. Post mortem analysis of encephalitis in AN1792 treated AD patients. (A) Meningoencephalitis is indicated by the black arrow in the white matter. **(B)** T cell infiltrates showed a mixed population of CD4$^+$ and CD8$^+$ T cells in one subject, whereas **(C)** a different patient revealed mainly CTL infiltrates into the brain. **(D)** CTL surrounding a protein aggregate within the CNS (Pictures are adapted from [122] and [135]).

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Chapter V

Nucleosome-induced neutrophil activation occurs independently of Toll-like receptor 9 and endosomal acidification: implications for systemic lupus erythematosus

This chapter is derived from an article submitted for publication by **Dennis Lindau**, Viktoria Rönnefarth, Annika Erbacher, Hans-Georg Rammensee and Patrice Decker.

The author of this thesis has performed the experiments leading to figure 1, 2, 3, 4, 5, S1 and S2. Furthermore, he wrote the manuscript together with HGR and PD.

Abstract

The nucleosome is a major autoAg known to activate PMN in SLE. TLR9 recognizes bacterial and even mammalian DNA under certain circumstances. Nevertheless, the role of TLR9 in SLE development is still unclear. Since nucleosomes are partly composed of DNA, we investigated whether TLR9 is required for nucleosome-induced PMN activation. Neutrophils were isolated from human blood and mouse BM. Cells were cultured with purified nucleosomes, lupus plasmas and other stimuli in the presence/absence of various inhibitors. Cell activation was estimated by flow cytometry and ELISA. TLR9 expression and nucleosome endocytosis were measured by flow cytometry and confocal microscopy. We found that nucleosomes circulating in lupus plasmas induce the secretion of pro-inflammatory cytokines by PMN. Nucleosomes activate human PMN independently of the presence of unmethylated CpG sequences in nucleosomal DNA, leading to IL-8, IL-6 and TNF secretion and CD11b up-regulation. Nucleosomes accumulate in the cytoplasm of PMN upon endocytosis but do not reach the nucleus. Nucleosome-induced activation was not inhibited by polymyxin B (PB), chloroquine (CQ), ammonium chloride (AC) or a TLR9 antagonist. Moreover, both PMN isolated from wild-type (WT) and TLR9-deficient mice were activated by nucleosomes, as detected by MIP-2 secretion and CD11b up-regulation. Activation occurred thus independently of endotoxins, endosomal acidification and TLR9. Although TLR9 is not required for nucleosome-mediated PMN activation, nucleosomes induced the up-regulation of TLR9 in part of the donors. TLR9 may thus be differently required in the triggering of nucleosome-induced innate immunity and anti-nucleosome B-cell autoimmunity.

Introduction

SLE is a rheumatic autoimmune disease. It is a systemic inflammatory disease characterized by the production of numerous autoAb. Upon binding to their target autoAg, the resulting IC deposit into tissues causing inflammation and damages. Nevertheless, the etiology of the disease is still unknown. The nucleosome is a major lupus autoAg composed of 180 bp DNA, one molecule of histone H1 and two copies of histones H2A, H2B, H3 and H4. Circulating nucleosomes are present in the blood of lupus patients [1] and increased concentrations are associated with the development of nervous and renal manifestations [2]. Nucleosome-restricted autoAb [3] and nucleosome-specific autoreactive T_H lymphocytes [4] are also observed in SLE patients and the former strongly correlate with disease activity scores [5]. It is therefore believed that circulating nucleosomes trigger the production of anti-nucleosome, anti-histone and anti-dsDNA autoAb (a disease marker in SLE). Nucleosomes are thus a key lupus autoAg, although the exact mechanisms leading to anti-nucleosome autoimmunity are not completely elucidated. PMN are the first cells recruited at inflammation sites. Upon inappropriate activation, PMN-derived reactive species may induce tissue damages and may alter self-Ag, transforming a harmless self-Ag in a potent autoAg. PMN are activated in SLE patients [6, 7], especially during active disease, and secrete IL-8, the concentration of which correlates to disease activity [8]. Importantly, PMN have been suggested to link the innate and adaptive immunity by influencing e.g. DC [9-11]. PMN activation may thus favor adaptive immunity. Beside the expected activation of nucleosome-specific autoreactive B cells via BCR engagement, free nucleosomes induce IL-6 secretion by mouse spleen cells [12], affect glomerular mesangial cell function [13], induce necrosis-like cell death in lymphocytes [14] and trigger DC activation [15]. Those results suggest that circulating nucleosomes are not only passive targets for autoAb but might be pathogenic. Nevertheless, the impact of TLR 9 in those situations is not known. TLR9 is activated in endosomal/lysosomal compartments by unmethylated CpG motifs present in pathogen-derived DNA [16]. Nevertheless, vertebrate DNA can also trigger TLR9 under certain circumstances although TLR9-independent pathways are triggered in addition [17]. Moreover, natural phosphodiester ODN lacking CpG motifs activate TLR9 upon enforced endosomal translocation [18]. Thus, intracellular localization of TLR9 is important to prevent activation by host-derived DNA [19]. Nevertheless, it is unknown whether TLR9 recognizes free nucleosomes and whether nucleosome-induced cell activation requires TLR9. The role played by TLR9 during SLE development is still not elucidated. TLR9 is required for anti-nucleosome autoAb production whereas titers of autoAb against dsDNA are

Results and discussion

increased in TLR9-deficient animals [20]. Moreover, TLR9 is necessary for the class switching to pathogenic autoAb isotypes in lupus mouse models [21]. On the contrary, disease induction was enhanced in different TLR9-deficient mouse models and TLR9 was suggested to protect against lupus [22-24]. DNA-reactive B cells are stimulated by CpG-rich DNA fragments and the stimulation is abolished by inhibitors of the TLR9 signaling pathway, whereas non-DNA-reactive autoimmune B cells are not activated by those fragments [25]. The data indicate that both TLR9 and the BCR are required and this mechanism might apply to B lymphocytes specific for DNA-binding proteins. However, the recognition of free nucleosomes by TLR9 has not been investigated in detail. On the contrary, some groups focused on nucleosome-containing IC but did not analyze PMN. In contrast, we analyzed the direct effects of free nucleosomes and found that nucleosomes activate DC in a partly MyD88-independent manner [15], suggesting the existence of a TLR9-independent pathway without excluding a second TLR9-dependent pathway. Interestingly, the accumulation of endogenous apoptotic DNA escaping lysosomal degradation activates a TLR9-independent innate immune response in vivo [26]. Although the contribution of TLR9 in SLE development has been investigated, the impact of TLR9 triggering in PMN in the context of SLE is unknown. PMN express TLR9 and respond to TLR9 ligands [27] but not to mammalian DNA [28]. We have shown that circulating nucleosomes activate and recruit PMN in vitro/in vivo in a HMGB1-independent manner in the absence of anti-chromatin Ab [29]. Since nucleosomes are partly composed of DNA and because TLR9 is able to recognize mammalian DNA and even self-DNA when DNA has access to TLR9 [19], we investigated whether TLR9 is required for chromatin-induced PMN activation in SLE. Indeed, TLR9 recognition of DNA in nucleosomes may differ from that of isolated DNA. Likewise, TLR9 might be differentially required for nucleosome-induced activation in B lymphocytes and PMN. We show here that nucleosomes activate PMN independently of TLR9 and endosomal acidification. Our results highlight the importance of the innate immune system and TLR9-independent pathways in SLE development.

Materials and Methods

Mice. C57BL/6 mice were purchased from Charles River. C57BL/6-TLR9-KO mice (described in [16]) were obtained from Prof. H. Wagner (Munich, Germany). Experiments were approved by the local animal ethics committee (Regierungspräsidium Tübingen, § 4 Abs. 3).

Human samples. Heparin-blood from random, healthy individuals and SLE patients were used. Lupus blood was kindly provided by Prof. I. Kötter (Tübingen, Germany) after receiving the informed consent of the donors. Patients fulfilled the American College of Rheumatology criteria. The patient characteristics are presented in Table I. Human experiments were approved by the local ethics committee (reference 146/2001V).

Nucleosome purification. Mononucleosomes were purified from calf thymus under sterile conditions by ultracentrifugation onto sucrose gradients as described previously [30]. As a control, an empty gradient (not loaded with chromatin) was prepared. DNA and protein contents were verified. We could previously demonstrate that purified nucleosomes do not contain detectable amounts of HMGB1 and that nucleosome-induced PMN activation occurs in a HMGB1-independent manner [29]. For cell surface binding/endocytosis analysis, nucleosomes were dialyzed against phosphate buffered saline (PBS) and conjugated to fluorescein isothiocyanate (FITC) as previously described [14]. Importantly, histones are among the most conserved proteins. For instance, there is no difference in protein sequence between histone H4 from calf, human and mouse. The level of endotoxins was low (10-50 IU/mg nucleosomes) as determined using a Limulus Amebocyte Lysate assay (BioWhittaker). The same endotoxin concentration (in IU/ml) was measured in purified nucleosomes and in the purification buffer (empty gradient, Gdt) which was used as a negative control. Moreover, nucleosome preparations were always first tested using PMN cultured with and without the LPS inhibitor PB and only preparations which activate PMN without being inhibited by PB were used.

Results and discussion

Cell isolation and culture. Human PMN were isolated from heparinized peripheral blood by density centrifugation as described previously [29]. Mouse neutrophils were isolated from BM cells obtained from adult WT and TLR9-KO mice. Neutrophils were enriched by positive selection for Ly-6G$^+$ cells using magnetic beads (Miltenyi Biotec). Purity of the cell preparations was analyzed by flow cytometry. Human (defined as CD3$^-$, CD19$^-$, CD56$^-$, CD11b$^+$, CD66b$^+$ cells, purity > 85 %) and mouse PMN (defined as SSC^{high}, F4/80$^-$, CD3$^-$, B220$^-$, CD11b$^+$, Ly-6G^{high} cells, purity > 90 %) were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (BioWhittaker) supplemented with 10 % fetal calf serum (FCS, from PAA), 100 U/ml penicillin, 100 mg/ml streptomycin (both from Gibco) and 50 µM (β-mercaptoethanol (Roth). Granulocyte colony-stimulating factor (50 ng/ml, ImmunoTools) was added to mouse PMN. Cells (1-1.5 x 10^6 cells/ml) were incubated in medium alone or activated with purified nucleosomes, the purification buffer as negative control (Gdt), R848 (Alexis), LPS (from Salmonella typhimurium or Escherichia coli, Sigma), CpG-motif-containing ODN (CpG-ODN, 2006 or 1826, Metabion) and GpC-ODN (2006, Metabion). Human PMN were pre-incubated in the presence/absence of PB (25 µg/ml, Fluka), CQ (5 µg/ml, Sigma), AC (10 mM, Roth) or a TLR9 antagonist (an ODN containing a G5 motif (G-ODN), 4 µM, Metabion). After 16 h, cell culture supernatants were harvested and cells were analyzed by flow cytometry. In some experiments, human PMN were cultured in the presence of 25 % plasma. In parallel, the experiment was performed in the absence of PMN. In a second set of experiments, human plasmas were first depleted from nucleosomes. Thus, cell culture plates were coated with 5 µg/ml of a nucleosome-binding mAb (Chemicon) or a mouse IgG1 or PBS only overnight at 4 °C (in 100 µl/well). Plates were then washed three times with PBS and incubated with 50 µl/well undiluted plasma for 4 h at room temperature (RT, plate 1). Freshly isolated PMN were cultured in another cell culture plate in 150 µl/well medium and the 50 µl plasma from plate 1 were transferred and added to PMN to reach a medium containing 25 % plasma. After 16 h, cell activation was estimated by measuring IL-8 secretion. Peripheral blood mononuclear cells (PBMC) were prepared from buffy-coats (blood bank, Tübingen, Germany) by density centrifugation through Ficoll (PAA). Human monocyte-derived DC were obtained from plastic-adherent PBMC cultured in the presence of granulocyte/macrophage colony-stimulating factor (Sandoz) and IL-4 (R&D) for 7 days as described previously [31].

Flow cytometry. PMN were first incubated with PBS/5 % heat-inactivated human serum or PBS/2 % heat-inactivated mouse serum and then stained with specific mAb or the corresponding isotype controls. mAb used were anti-human Peridinin Chlorophyll Protein Complex (PercP)-conjugated CD3, Phycoerythrin (PE)-conjugated CD11b, Allophycocyanin (APC)-conjugated CD19, CD56-PE and CD66b-FITC (all from BD Pharmingen) and anti-mouse Ly-6G-FITC, CD11b-PE, B220-PE (all from BD Pharmingen), F4/80-APC (eBioscience) and CD3-FITC (SouthernBiotech). Dead cells were excluded by 7-amino-actinomycin D (Pharmingen) staining. For intracellular TLR9 staining, PMN were first stained for surface markers, permeabilized with Cytofix/Cytoperm (BD Pharmingen) and incubated with anti-TLR9-FITC (Abcam) and then anti-FITC-Alexa Fluor 488 (Invitrogen) in saponin-buffer (0.1 %, Sigma). Cells were finally fixed (1 % paraformaldehyde) and analyzed on a FACSCalibur apparatus with CELLQuest software (Becton Dickinson). To analyze nucleosome cell surface binding and endocytosis, 10^6 cells/ml were incubated with or without 10 µM FITC-conjugated nucleosomes (FITC-nucleosomes) on ice (to detect cell surface binding) or at 37 °C (to measure binding and endocytosis) in 10 % FCS-containing PBS for 30 minutes (min). Cells were then washed, incubated in the presence/absence of 0.1 % (PMN) or 0.4 % trypan blue to quench the extracellular FITC fluorescence or propidium iodide to exclude dead cells and immediately analyzed by flow cytometry. To confirm the inhibition of extracellular fluorescence, cells were stained with an anti-CD66b-FITC (PMN) or an anti-HLA-DR-FITC (BD Pharmingen) mAb. Monocytes were gated upon staining with an anti-CD14-PE mAb (BD Pharmingen).

Confocal microscopy. To analyze nucleosome endocytosis, 200,000 cells were incubated with 30 µM FITC-nucleosomes together with another fluorophore-labelled reagent. Cells were incubated at RT and live cell imaging was performed immediately using an inverted LSM510 confocal laser scanning microscope (Carl Zeiss) fitted with a Plan-Apochromat 63x/1.4 OIL DIC objective. The pinhole was set to scan layers of 1 µm. To study macropinocytosis, 10 µM Alexa Fluor 647-conjugated dextran (Molecular Probes) was used as a marker. Phagocytosis was estimated using crimson red fluorescent carboxylate-modified microspheres (1/2,500,000, 0.02 µm in diameter, Molecular Probes). To stain the nucleus, cells were first incubated with FITC-nucleosomes and then fixed, permeabilized and incubated with TO-PRO-3 (1/1,000, Molecular Probes) for 15 minutes (min) at RT. For the simultaneous detection of FITC-nucleosomes and fluorophore-conjugated markers, the 488 nm line of an argon ion laser and the light of a 633 nm helium neon laser were used. The fluorescence was

Results and discussion

detected using a BP505-530 band pass filter for the FITC detection and LP650 long pass filter for the detection of fluorescent markers.

ELISA. Cytokine secretion by PMN was analyzed by sandwich ELISA using mAb pairs and streptavidin-peroxidase conjugate from BD Pharmingen or R&D Systems according to the manufacturer's instructions. Cytokine concentrations in supernatants are depicted as mean ± standard deviation of triplicates.

Statistical analysis. The mean IL-8 concentrations induced in PMN by either SLE or normal plasmas were compared using a Mann-Whitney rank sum test. Correlations between the capacity of SLE plasmas to induce IL-8 secretion in normal PMN and the doses of therapeutic agents received by lupus patients were determined using Spearman correlation test. The Spearman correlation coefficient (R) and the probability (p) are reported. The SigmaStat software was used in all cases. P values < 0.05 were considered significant.

Results

Plasmas from SLE patients activate PMN in a nucleosome-dependent manner

We have previously shown that purified nucleosomes activate human PMN, leading to IL-8 secretion and CD11b up-regulation [29]. Activation occurs independently of HMGB1 and independently of autoAb, indicating that HMGB1-nucleosome complexes or nucleosome-containing immune complexes are not required. Sera from SLE patients with active disease have been shown to activate PMN [32]. To support a role of nucleosomes in neutrophil activation in patients, PMN isolated from healthy individuals were cultured in the presence/absence of plasmas prepared from healthy individuals or SLE patients. IL-8 secretion was measured by ELISA in order to screen for lupus plasmas having a PMN stimulatory activity. Among the eleven lupus plasmas tested, nine significantly stimulated PMN (Fig. 1A) whereas autologous plasmas or plasmas from unrelated healthy donors did not (mean IL-8 concentration = 0.46 ng/ml [SLE plasmas] and 0.04 ng/ml [normal plasmas]; p = 0.005). LPS was used as a positive control for PMN activation. Importantly, IL-8 was specifically secreted upon incubation of PMN with SLE plasmas as IL-8 was not detected when plasmas were incubated in the absence of PMN. To prove that PMN activation was mediated by circulating nucleosomes, we next measured PMN activation by SLE plasmas which were first depleted from circulating nucleosomes using a nucleosome-specific mAb or the corresponding isotype control bound onto a solid phase. Using the most potent stimulatory plasmas, IL-8 induction was not affected when plasmas were first treated with mouse IgG1 (Fig. 1B). On the contrary, upon depletion of nucleosomes from SLE plasmas IL-8 induction was completely inhibited in three samples and partially inhibited in two samples. Those results indicate that nucleosomes in SLE plasmas were involved in PMN activation in all the strong stimulatory samples tested. Circulating nucleosomes were either the main stimulatory activity in some patients or one of the IL-8 inducers among others. No IL-8 secretion was detected with autologous plasma, the mAb alone or the isotype control alone. Interestingly, a statistically significant negative correlation was observed in lupus patients between the capacity of SLE plasmas to induce IL-8 secretion in normal PMN and the doses of therapeutic agents received by those patients (Fig. S1). All those results suggest that circulating nucleosomes activate PMN in vivo in patients and that untreated patients display a high stimulatory capacity which is down-regulated by the therapy.
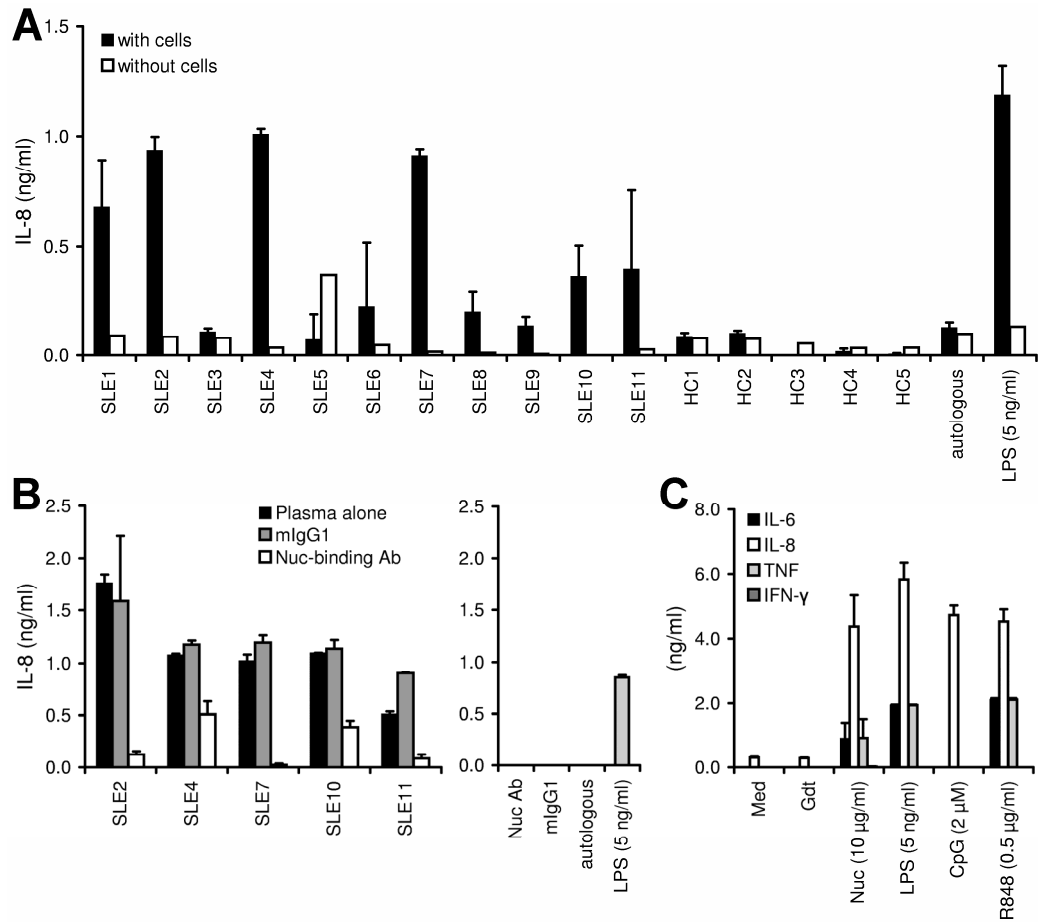
Figure 1. Nucleosomes in SLE plasmas induce the secretion of pro-inflammatory cytokines by human neutrophils. (A) Normal PMN were cultured in the presence of plasma (autologous or prepared from healthy donors (HC, n = 5) or SLE patients (n = 11)). Each number represents an independent blood donor. IL-8 concentrations were determined by ELISA. To exclude that the IL-8 detected comes from the plasmas and not from PMN activated in the cell culture, the experiment was performed in the absence of PMN (without cells). **(B)** Lupus plasmas were first depleted from circulating nucleosomes using a nucleosome-binding mAb or mouse IgG1 as a control, and then used to activate PMN as described in A. The absence of side effects from the mAb or the corresponding isotype control in the absence of plasma was also verified. **(C)** PMN were cultured in the presence of purified nucleosomes or TLR ligands and the concentrations of IL-6, IL-8, TNF and IFN-γ in the cell culture supernatants were measured. The data are from three independent experiments using PMN isolated from three independent donors. Med, medium; Gdt, buffer used during the nucleosome purification; Nuc, nucleosomes. LPS was from Salmonella typhimurium. The CpG-ODN 2006 was used. Standard deviations are shown.

Nucleosomes stimulate PMN to secrete IL-8, IL-6 and TNF

We have previously described that PMN isolated from healthy donors as well as SLE patients are activated by nucleosomes [29]. We then extended those results and analyzed the cytokine secretion profile of human PMN activated by nucleosomes in more detail. Nucleosomes not only induce IL-8 secretion by PMN but also IL-6 and TNF secretion (Fig. 1C), although to a lesser extent. Nucleosome-induced PMN activation did not lead to IFN-γ secretion and the lack of IFN-γ secretion upon activation was observed in response to all stimuli tested. Interestingly, IL-6 and TNF secretion was usually stronger upon stimulation with nucleosomes than with CpG-ODN. Thus, nucleosomes induced the secretion of several pro-inflammatory cytokines known to be present at higher concentrations in patient sera as compared to healthy individuals.

Nucleosome-induced PMN activation is not inhibited by a TLR9 antagonist

To determine whether TLR9 was required in human PMN for nucleosome-induced activation, we first measured the level of PMN activation in the presence/absence of a TLR9 antagonist or PB as a control to exclude the presence of endotoxins (Fig. 2A and B). Nucleosomes induced a clear concentration-dependent CD11b up-regulation starting at 2.5 µg/ml nucleosomes (PMN cultured in medium only) which was not inhibited by PB or G-ODN. No activation was observed with the nucleosome purification buffer alone. As expected, LPS-induced CD11b up-regulation was inhibited by PB but not G-ODN, excluding the presence of endotoxins in the nucleosome preparation. As a control, activation with the TLR7/8 ligand R848 was not inhibited by PB or G-ODN. Importantly, CpG-ODN induced PMN activation, which was completely inhibited in the presence of G-ODN but not PB. Moreover, the same ODN with GpC motifs instead of CpG was unable to activate PMN. Our results indicate that CD11b up-regulation by ODN 2006 is CpG-motif-dependent and probably requires TLR9. On the contrary, those results indicate that the presence of CpG motifs in nucleosomes is not necessary for PMN activation and suggest that nucleosome-induced activation occurs in a TLR9-independent manner. Staining with the isotype control was not modified upon cell activation, excluding unspecific binding of the mAb. PMN activation was confirmed by measuring IL-8 secretion. Again, nucleosome-induced IL-8 secretion was not inhibited by PB or G-ODN whereas CpG-ODN-induced activation was completely inhibited by G-ODN and GpC-ODN did not activate PMN (Fig. 2C), supporting the involvement of a CpG-independent, TLR9-independent pathway in nucleosome-

Results and discussion

induced PMN activation. Similar results were obtained when IL-6 and TNF secretion was measured (Fig. 2D), although higher concentrations of nucleosomes were usually required to stimulate the production of those cytokines. Moreover, nucleosomes were often a more potent inducer of those two cytokines compared to CpG-ODN, which suggests that nucleosomes and CpG-ODN are recognized by different mechanisms.
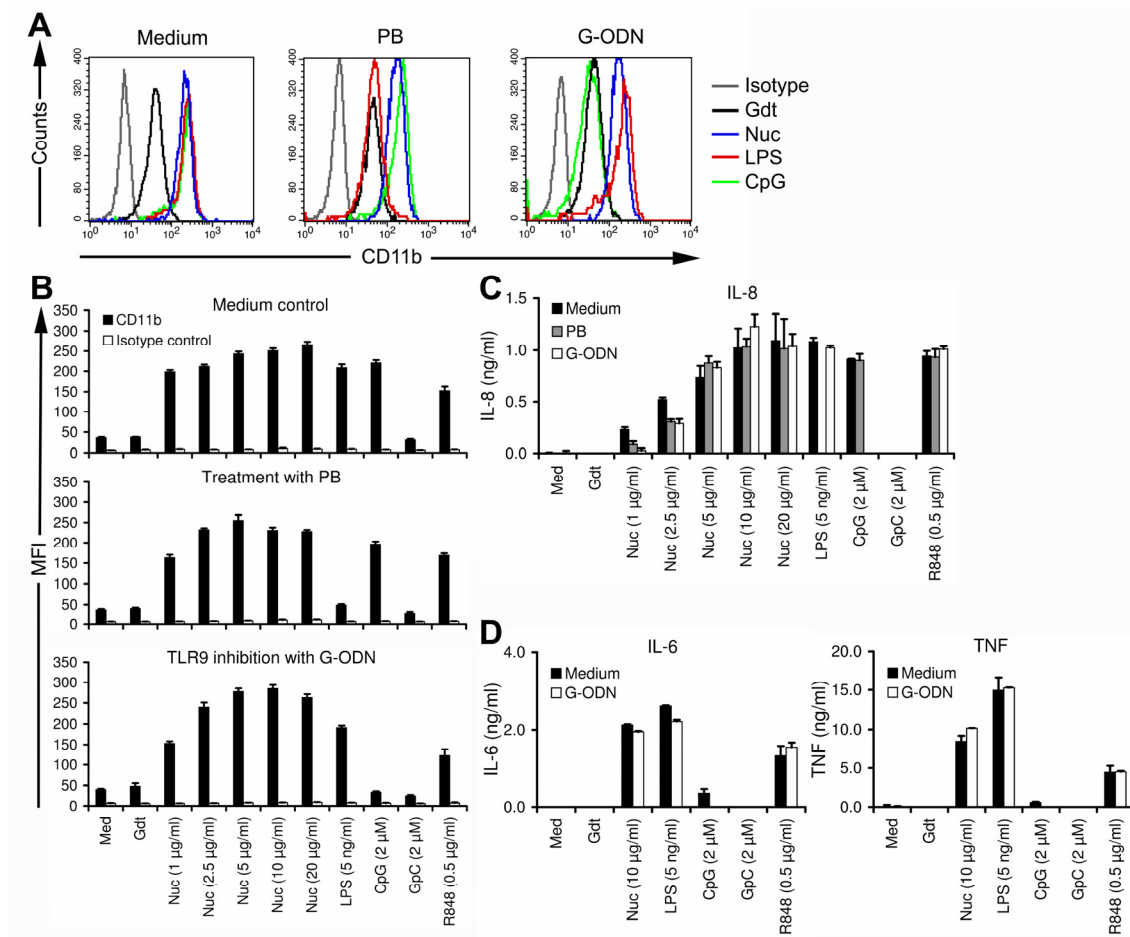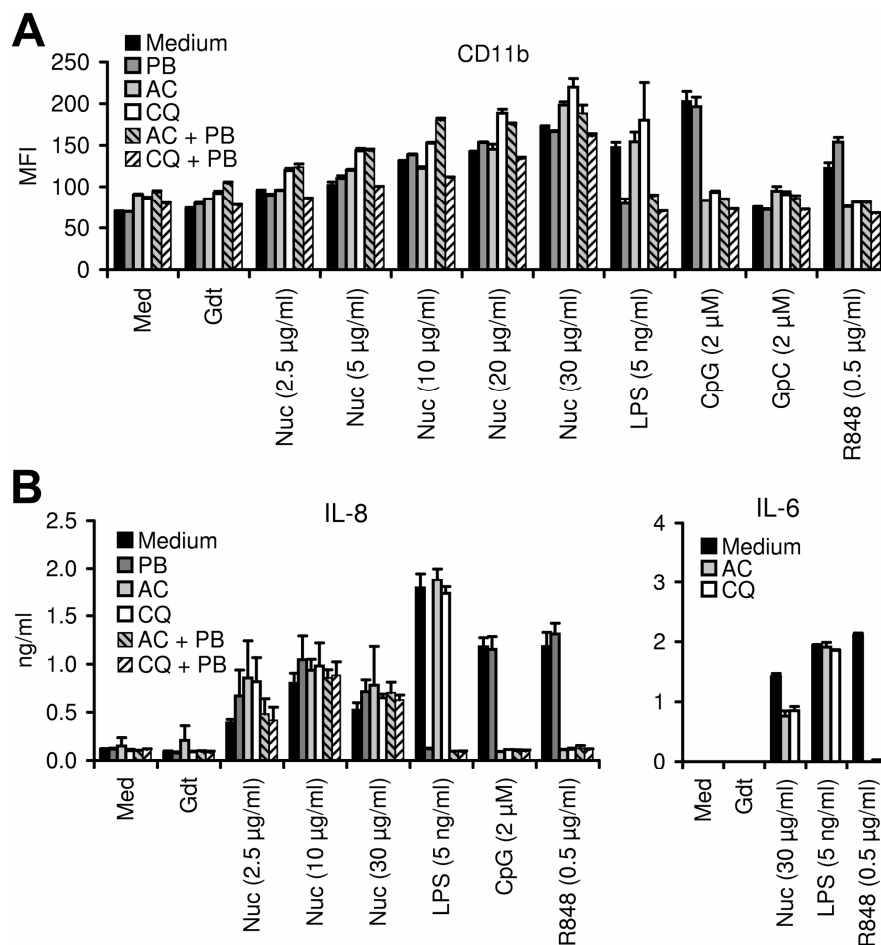

Figure 2. Nucleosome-induced PMN activation is not inhibited by a TLR9 antagonist. Freshly isolated PMN were pre-incubated in the absence (medium) or presence of polymyxin B (PB) or a TLR9 antagonist (G-ODN) and were then cultured for 16 h with purified nucleosomes or other stimuli. Cell culture supernatants were harvested and cells were analyzed by flow cytometry for CD11b expression (A, B). **(A)** Representative flow cytometry histograms showing CD11b expression and isotype control staining are depicted. **(B)** CD11b expression and unspecific staining for all the stimuli tested are presented. Cell activation was also estimated by measuring the secretion of IL-8 **(C)** as well as IL-6 or TNF **(D)** by ELISA. Med, medium; Gdt, buffer used during the nucleosome purification; Nuc, nucleosomes; MFI, mean fluorescence intensity. Data presented are representative of at least five independent experiments.

Nucleosome-induced PMN activation occurs independently of endosomal acidification

To support a TLR9-independent pathway in PMN activation, we then investigated whether endosomal acidification was required during activation with nucleosomes. PMN were cultured in the presence/absence of PB, AC, CQ, or both PB and AC or PB and CQ and cell activation was determined. AC and CQ (another anti-malarial drug used in SLE therapy) are indeed known to inhibit endosomal acidification, which is required to trigger TLR9.

Figure 3. Nucleosome-induced PMN activation occurs independently of endosomal acidification. PMN were pre-incubated in the absence (Medium) or presence of polymyxin B (PB), ammonium chloride (AC), chloroquine (CQ), PB and AC, PB and CQ. Cells were then incubated for 16 h with purified nucleosomes or other stimuli and PMN activation was estimated by flow cytometry for CD11b expression **(A)** and by ELISA to estimate cytokine secretion **(B)**. Data are representative of at least five independent experiments. See figure 2 for abbreviations.

Results and discussion

Nucleosome-induced CD11b up-regulation (medium control) was not inhibited in the presence of PB, AC, CQ, PB with AC or PB with CQ (Fig. 3A). PMN activation with CpG-ODN and R848 was abolished in the presence of AC or CQ. As a control, LPS was inhibited by PB and GpC-ODN did not activate. Importantly, all the stimuli except nucleosomes were inhibited by PB with AC and PB with CQ, indicating that nucleosome-induced PMN activation did not require endosomal acidification. Those results were confirmed by measuring cytokine secretion upon activation. Nucleosomes induced IL-8 secretion by PMN (medium control), which was not decreased by PB or inhibitors of endosomal acidification, in combination or not with PB (Fig. 3B, right). In contrast, activation with CpG-ODN or R848 was completely inhibited by AC or CQ. Likewise, LPS was completely inhibited by PB. Again, all the stimuli except nucleosomes were inhibited by AC plus PB or CQ plus PB, confirming that endosomal acidification was not required for nucleosome-induced activation. Similar results were obtained when IL-6 secretion was measured (Fig. 3B, left). Cell activation with R848, but not with nucleosomes, was completely inhibited in the presence of AC or CQ.

TLR9 is not required for nucleosome-induced PMN activation

To confirm the existence of a TLR9-independent pathway in nucleosome-induced PMN activation, neutrophils were isolated by positive selection from the BM of WT or TLR9-KO mice and compared. The online supplemental figure S2 shows the yield of enrichment, a typical flow cytometry analysis and a representative activation of WT PMN. Cell activation was estimated by CD11b expression (by Ly-6G^{high}SSC^{high} cells, i.e. PMN) and MIP-2 secretion. Nucleosomes as well as LPS induced CD11b up-regulation and MIP-2 secretion in both WT and TLR9-deficient PMN (Fig. 4). On the contrary, CpG-ODN only activated WT PMN, confirming that nucleosome-induced PMN activation occurs in a TLR9-independent manner and that nucleosomes and CpG-ODN trigger different pathways in PMN. The fact that TLR9 is not required in nucleosome-induced PMN activation was confirmed in a human system using 293T cells transfected with a vector coding for human TLR9. 293T-TLR9 transfectants were activated by CpG-ODN 2006 but not by nucleosomes, whereas 293T-mock transfectants were unresponsive to stimulation (Fig. S3, left panel and data not shown). As a control, mouse DC were activated using the same nucleosome preparation, as estimated by IL-12 secretion (Fig. S3, right panel).

Figure 4. TLR9 is not required for nucleosome-induced PMN activation. BM neutrophils were prepared from WT and TLR9-KO mice and their sensitivity towards activation with purified nucleosomes or TLR ligands was compared. PMN activation was estimated by flow cytometry (CD11b expression, upper panel) and ELISA (MIP-2 secretion, lower panel). Two mice were pooled per group and per experiment. Data are from four independent experiments. See figure 2 for abbreviations. LPS was from Escherichia coli. The CpG-ODN 1826 was used.
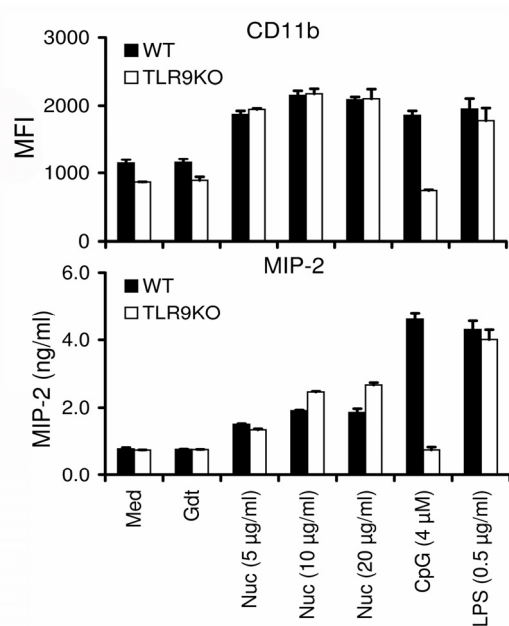
PMN activation with nucleosomes induces TLR9 up-regulation in part of the donors

Although TLR9 was not required in PMN for nucleosome-mediated activation, we analyzed whether nucleosomes may modulate TLR9 expression upon cell activation. PMN were isolated from healthy donors and untouched cells were stained for CD66b and CD11b to estimate the quality of the preparation by flow cytometry. Figure 5A (upper panel) shows the phenotype of PMN. All the cells gated according to FSC/SSC parameters were homogeneously and strongly CD66b-positive, excluding the presence of contaminating cells, and expressed moderate CD11b levels, confirming that the gated cells were non-activated PMN. Cells were then incubated with different stimuli, stained for CD11b surface expression and intracellular TLR9 expression and analyzed by flow cytometry. PMN cultured in medium alone express TLR9 (Fig. 5A, middle panel) and TLR9 was up-regulated upon activation by CpG-ODN as well as nucleosomes, cell activation being confirmed by the concomitant CD11b up-regulation (Fig. 5A, lower panel). Activation with LPS or R848 also led to TLR9 up-regulation, confirming that non-TLR9 ligands may also modulate TLR9 expression (Fig. 5B). As expected, TLR9 up-regulation was not observed in the presence of GpC-ODN. We then analyzed TLR9 expression by PMN from several healthy donors upon activation by nucleosomes in more detail. Interestingly, some donors up-regulated TLR9 in a dose-dependent manner upon nucleosome-induced activation (Fig. 5C, upper panel), whereas in a second group of donors nucleosome-induced PMN activation did not lead to TLR9 modulation (Fig. 5C, lower panel).
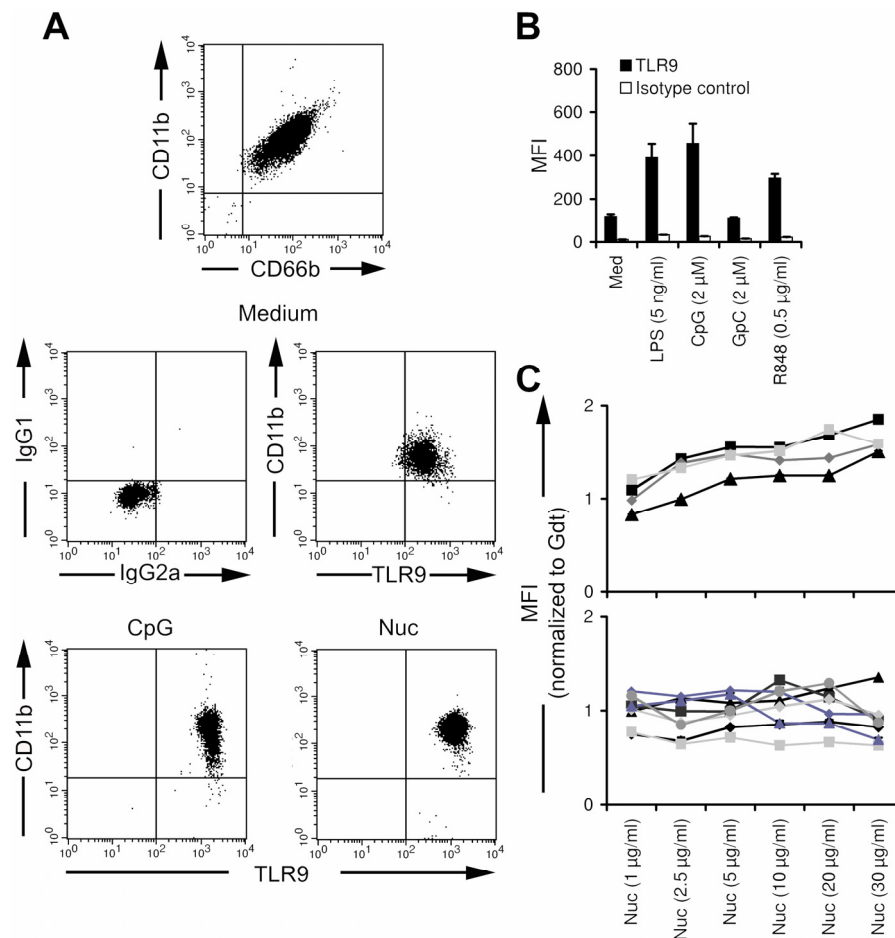
Figure 5. Nucleosome-activated PMN up-regulate TLR9. (A) Freshly isolated normal PMN were first analyzed by flow cytometry for CD66b and CD11b expression to exclude the presence of contaminating cells (upper panel). Cells were then cultured for 16 h in medium alone (middle panel) or medium supplemented with CpG-ODN or purified nucleosomes (lower panel). PMN were harvested, stained for surface CD11b and intracellular TLR9 expression with specific mAb or the corresponding isotype controls and analyzed by flow cytometry. **(B)** PMN were cultured with different TLR ligands and analyzed by flow cytometry for intracellular TLR9 expression as described in (A). **(C)** PMN were isolated from several healthy donors, activated with purified nucleosomes and analyzed as described in (A). Intracellular TLR9 expression was normalized to TLR9 expression by non-stimulated cells (Gdt) in order to show the TLR9 up-regulation (fold increase) upon activation. The upper and lower panels present two groups of donors whose PMN up-regulate TLR9 or not, respectively. Each line represents an independent donor (n = 12). Data are representative of at least six independent experiments. See figure 1 and 2 for abbreviations.

Free nucleosomes accumulate in the cytoplasm of PMN upon endocytosis

We have previously shown that circulating nucleosomes are endocytosed by PMN [29]. To gain more insight we first compared different human leukocyte populations for their ability to bind or endocytose free nucleosomes. Cells were incubated on ice (to detect cell surface binding) or at 37 °C (to measure binding and endocytosis) in the presence/absence of FITC-nucleosomes and cells were analyzed by flow cytometry with or without trypan blue in order to quench the extracellular FITC fluorescence. As a control, cells were stained with FITC-conjugated mAb specific for surface markers. Nucleosomes slightly bind to the cell surface of PMN or DC (Fig. 6A). Nevertheless, PMN and DC strongly endocytose nucleosomes as demonstrated by the signal observed at 37 °C, which was not inhibited by trypan blue (in contrast to the control Ab staining on ice). Likewise, monocytes strongly engulf nucleosomes. However, in contrast to previous cell types, nucleosomes also strongly bind to the monocyte cell surface. On the contrary, lymphocytes only slightly bind and do not endocytose nucleosomes. Interestingly, only PMN and DC but not monocytes or lymphocytes are activated by free nucleosomes [15, 29]. We then analyzed whether macropinocytosis was involved in nucleosome endocytosis. Cells were incubated with FITC-nucleosomes in the presence of Alexa Fluor 647-conjugated dextran and analyzed by confocal microscopy. In contrast to DC, PMN do not endocytose dextran, whereas FITC-Nucleosomes were taken up by both cell types (Fig. 6B). Even in DC, engulfed nucleosomes do not co-localize with dextran, indicating that free nucleosomes are not ingested via macropinocytosis in PMN or DC. We also investigated whether nucleosome endocytosis occurred in PMN via phagocytosis by using FITC-nucleosomes and fluorescent microspheres. Microspheres and nucleosomes were rapidly taken up by PMN but did not co-localize inside cells (Fig. 6C), suggesting that nucleosomes are not internalized via phagocytosis in PMN. Nevertheless, because primary PMN are sensitive and short-lived cells, we did not gain more and unambiguous insight regarding the endocytic pathway (clathrin-mediated or caveolae-mediated endocytosis) and the intracellular destination of nucleosomes using confocal microscopy. Such studies are actually often performed with cell lines in order to achieve optimal conditions for confocal microscopy. However, using FITC-nucleosomes and the DNA dye TO-PRO-3, we could observe that free nucleosomes are not translocated into the nucleus of PMN upon endocytosis (Fig. 6D).
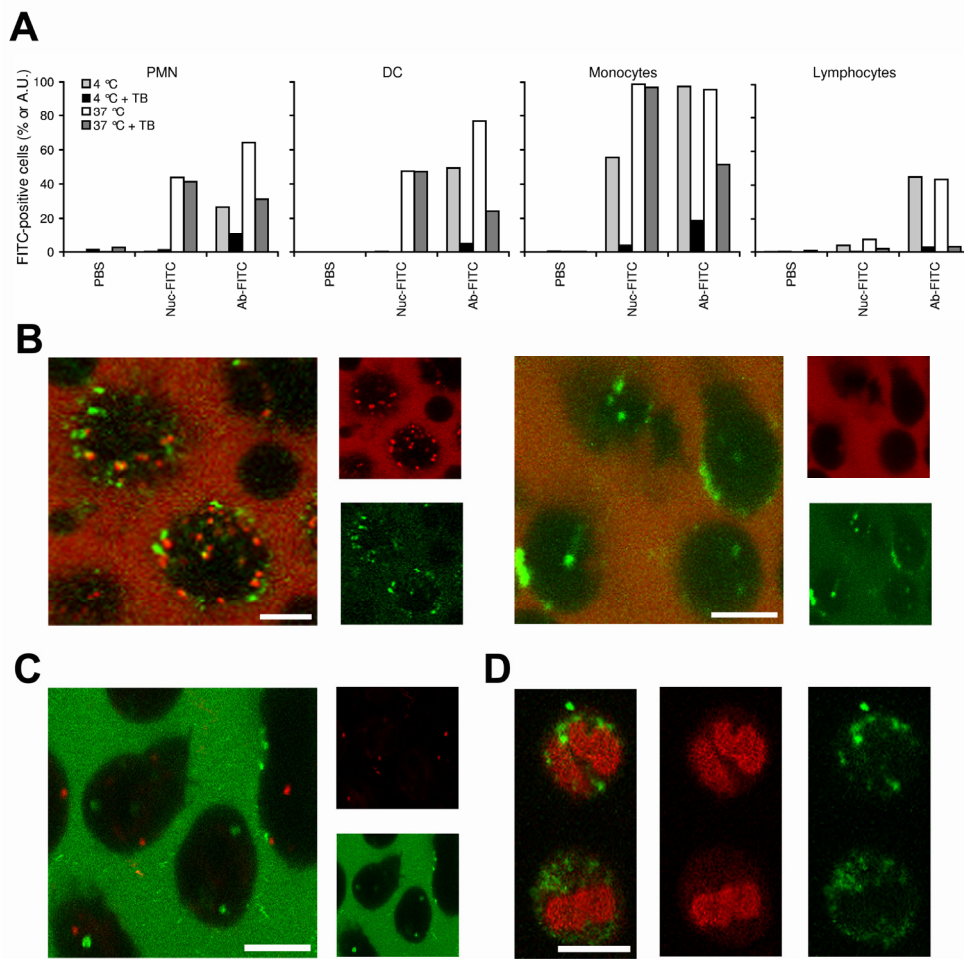
Figure 6. Endocytosed circulating nucleosomes accumulate in the cytoplasm of PMN. (A) PMN, DC, monocytes and lymphocytes were incubated on ice or at 37 °C with PBS or FITC-nucleosomes in the presence/absence of trypan blue (TB) to quench the extracellular FITC fluorescence. The different cell types were analyzed by flow cytometry to compare their ability to bind and endocytose nucleosomes. As a control, cells were stained for cell surface molecules with FITC-conjugated mAb to verify the activity of TB. **(B-D)** Endocytosis of FITC-nucleosomes (green) by PMN and DC was investigated by confocal microscopy. **(B)** Macropinocytosis by DC (left panel) or PMN (right panel) was analyzed using Alexa Fluor 647-conjugated dextran (red). **(C)** Phagocytosis of FITC-nucleosomes by PMN was estimated using fluorescent microspheres (red). **(D)** The translocation of FITC-nucleosomes into the nucleus (red) of PMN upon endocytosis was followed in the presence of the DNA dye TO-PRO-3. Confocal images show individual fluorescence channels for FITC-nucleosomes or the endocytic markers (right) and the corresponding overlays (left pictures). A.U., arbitrary units (only for Ab staining of PMN); Ab-FITC, FITC-conjugated mAb specific for cell surface molecules; TB, trypan blue. The data are from three independent experiments using cells isolated from independent donors. Bars in B-D, 5 µm.

Discussion

We have clearly shown that in the absence of autoAb free nucleosomes activate PMN independently of CpG motifs, endosomal acidification and TLR9, leading to the secretion of pro-inflammatory cytokines. The pathway involved in the recognition of circulating nucleosomes is therefore different from the classical pathway involved in the recognition of CpG-ODN. Importantly, similar results were obtained when we activated normal PMN with SLE plasma whereas nucleosome-depleted plasmas were not stimulatory, showing that the activation was specifically mediated by circulating nucleosomes. The elevated concentrations of circulating nucleosomes observed in part of the patients may be responsible for the PMN activation reported in SLE. Our results highlight the influence of the innate immunity and TLR9-independent pathways in SLE development. Those observations do not rule out that TLR9 might be required in other processes. TLR9 might be differently required during nucleosome-induced PMN activation and Ag-specific stimulation of nucleosome-specific autoreactive B lymphocytes. Our results are in agreement with an in vivo role of circulating nucleosomes in PMN activation, even if other factors might contribute to this process. Continuous exposure to nucleosomes in vivo may favor PMN exhaustion and may partly explain the low PMN counts and the inflammatory status observed in patients. It may also explain the apparent low activity of lupus PMN reported in other studies, although lupus PMN might also have an endogenous alteration rendering them less sensitive to activation. The role of TLR9 in SLE development is still a matter of debate. The involvement of TLR9 in nucleosome recognition has often been analyzed using chromatin-containing IC and not free nucleosomes, or upon enforced uptake of free mammalian DNA, and not nucleosomes, upon transfection. However, DNA is not circulating as a free molecule in SLE patients but in the form of nucleosomes [1]. In the latter system, intracellular mammalian DNA stimulates myeloid DC to produce type I IFN through a TLR9-independent pathway [33]. Likewise, vertebrate and even mammalian DNA trigger TLR9-independent pathways [17, 26]. Consistently, non-CpG dsDNA (phosphodiester) aggravates the disease in a TLR-independent manner in lupus mice [34]. All those results support a TLR9-independent mechanism in nucleosome-induced PMN activation. Other receptors have been suggested to recognize DNA and/or nucleosomes. A nucleosome-specific receptor might be expressed at the cell surface [35]. Oligonucleosomes bind to glomerular mesangial cells in a receptor-mediated fashion [36]. Recently, cytosolic receptors able to recognize different types of DNA have been reported, leading to type I IFN production [37], innate immune responses [38, 39], or activation of the inflammasome and cell

Results and discussion

death [40-43]. Nevertheless, it is unknown whether those pathways are triggered by free nucleosomes. We have observed that circulating nucleosomes are endocytosed in PMN as well as in DC and monocytes. In PMN, nucleosomes slightly bind to the cell surface and are not translocated to the nucleus upon endocytosis, which could have been expected for a nuclear autoAg. Our results suggest that nucleosomes are not taken up via macropinocytosis or phagocytosis in PMN. Unfortunately, we could not identify the endocytic pathway (clathrin-mediated or caveolae-mediated endocytosis) used. Nevertheless, such experiments are usually performed with cell lines for technical reasons, whereas we focused on primary short-lived cells. Likewise, using FITC-nucleosomes together with a fluorescent lysosome/endosome dye, we could not determine by confocal microscopy whether nucleosomes reach the lysosome upon endocytosis. However, we could clearly show that lysosomal acidification is not required to activate PMN with nucleosomes, suggesting that if nucleosomes reach the lysosome to activate a receptor, nucleosome denaturation is not required. Our results also indicate that if nucleosomes are transported to lysosomes, TLR9 is not triggered since nucleosome-induced activation is TLR9-independent in PMN. Interestingly, we observed that nucleosome-induced PMN activation leads to TLR9 up-regulation in part of the donors. Nucleosomes may thus pre-activate PMN in SLE, leading to TLR9 up-regulation and render them more sensitive to a concomitant or a further activation with TLR9 ligands. TLR9 up-regulation due to circulating nucleosomes in patients with bacterial infection might lead to a synergistic response, leading to an uncontrolled inflammatory response which may favor SLE development. Indeed, PMN link innate and adaptive immunity, which may lead in part of the patients to the break of peripheral tolerance and anti-nucleosome autoimmunity upon continuous exposure to circulating nucleosomes. Since TLR9 has been reported to be protective against lupus, the fact that the innate immunity stimulated by the nucleosome, a major lupus autoAg, occurs in a TLR9-independent manner supports the major role of this autoAg in disease development. Nucleosomes are pro-inflammatory and trigger innate immune responses. Now SLE is an inflammatory disorder. Nucleosomes are thus not only passive targets for autoAb but may play an active role in lupus pathogenesis.

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Supplemental material
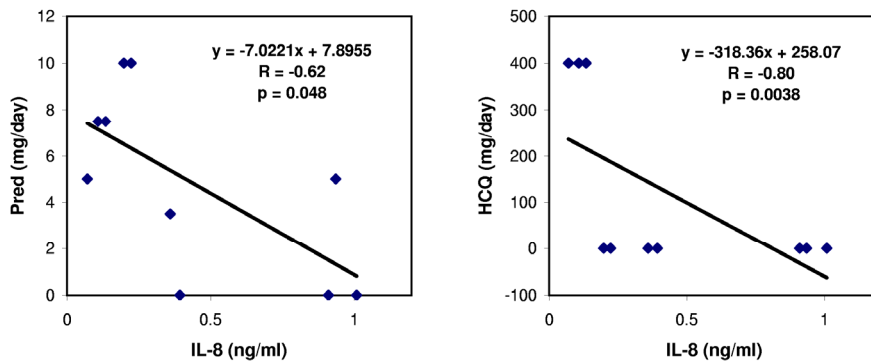
Figure S1. We analyzed whether prednisolone (Pred) or hydroxychloroquine (HCQ), two drugs used to treat SLE patients and having anti-inflammatory and anti-malarial activity, respectively, may influence IL-8 induction. We observed a negative correlation between the doses of therapeutic agents applied in SLE patients (n = 10) and the capacity of the SLE plasmas to induce IL-8 secretion in normal PMN (R = -0.62 and p = 0.048 for Pred; R = -0.80 and p = 0.0038 for HCQ). R, Spearman correlation coefficient; p, probability; each dot represents an independent lupus patient.
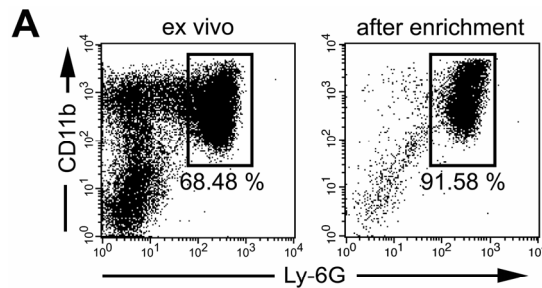
Neutrophil purification.

	Purity	CD11b	Ly-6G	CD3	B220	F4/80
WT	89.6	96.0	91.4	0	0	0
KO	93.7	92.1	92.1	0	0.3	2.5
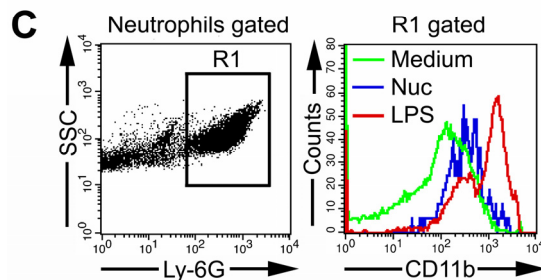
Figure S2. Characteristics of neutrophils isolated from WT or TLR9-KO mice. BM neutrophils were enriched by positive selection using Ly-6G magnetic beads. Cells were first analyzed by flow cytometry for Ly-6G and CD11b expression before and after enrichment gating on large and granular cells according to FSC/SSC parameters. As shown in figure S2A, the percentage of neutrophils (defined as Ly-6G^{high}CD11b^{+} cells) was strongly increased upon positive selection as compared to BM cells analyzed ex vivo, yielding one homogenous cell population. Neutrophils isolated from WT and TLR9-deficient mice had the same phenotype and the cell populations showed similar purity, as determined by flow cytometry (Fig. S2B; percentages are shown). We first tested whether neutrophils isolated from WT mice were also sensitive to nucleosome-induced activation by measuring CD11b up-regulation on Ly-6G^{high}SSC^{high} cells (also defined as neutrophils) using flow cytometry. A representative flow cytometry analysis of WT neutrophils showing the gating strategy and CD11b expression upon activation is depicted. Cells incubated in medium alone showed a moderate CD11b expression which was clearly up-regulated in the presence of nucleosomes or LPS (Fig. S2C).
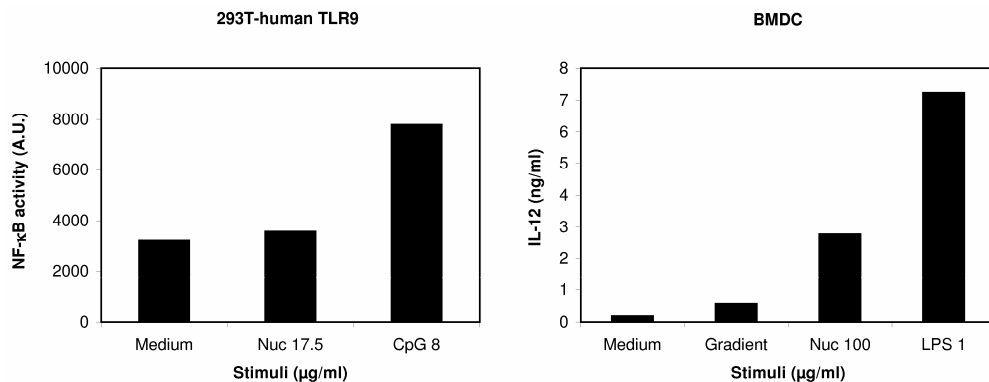
Figure S3 shows that TLR9 is not required in nucleosome-induced activation of 293T cells transfected with a vector coding for human TLR9 and a vector coding for a NF- B luciferase reporter. Cell lysis and measurement of luciferase activity in extracts were performed with the Luciferase Assay System Kit from Promega according to manufacturer's recommendations. 293T-TLR9 transfectants were activated by CpG-ODN 2006 (CpG) but not by nucleosomes (Nuc; left panel; A.U., arbitrary units). 293T-mock transfectants were unresponsive to stimulation. As a control, the same nucleosome preparation was able to activate mouse BM-derived DC (BMDC, obtained after a 7 day culture with Granulocyte macrophage colony-stimulating factor, as shown by an enhanced IL-12 secretion after stimulation with nucleosomes (Nuc, right panel). Gradient is the buffer used for the nucleosome purification and serves as a negative control. LPS was used as a positive control. Numbers indicate the concentrations of stimuli. This experiment was performed only once.

Results and discussion

Table 1. SLE patient characteristics.

Patient	Gender	Age	SLEDAI	PRED	AZA	CSA	HCQ	MMF	MTX
		yr		mg/day	mg/day	mg/day	mg/day	g/day	mg/day
1	W	43	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.
2	W	30	8	5	-	150	-	-	-
3	W	32	2	7.5	-	230	400	-	-
4	W	26	0	-	-	-	-	-	-
5	M	50	2	5	-	-	400	-	-
6	W	25	2	10	20	-	-	-	-
7	W	26	0	-	-	-	-	-	-
8	W	42	4	10	-	-	-	2	15
9	W	63	2	7.5	-	-	400	-	-
10	W	61	2	3.5	50	-	-	-	-
11	M	57	6	-	-	-	-	-	-

PRED, prednisolone; AZA, azathioprine; CSA, cyclosporine A; HCQ, hydroxychloroquine; MMF, mycophenolate mofetil; MTX, methotrexate; n.k., not known; SLEDAI, SLE disease activity scores; yr, year.

Chapter VI

Primary blood neutrophils express a functional cell surface Toll-like receptor 9

This chapter is derived from an article submitted for publication by **Dennis Lindau**, Britta Janina Wagner, Viktoria Rönnefarth, Ivan Jelcic, Hans-Georg Rammensee and Patrice Decker.

The author of this thesis was involved in all experiments except the one leading to figure 4. Furthermore, he wrote the manuscript together with HGR and PD.

Abstract

PMN represent one of the first lines of defense against pathogens. TLR9 is normally expressed in endosomes/lysosomes where it is activated by pathogen-derived DNA. Here we show by flow cytometry and confocal microscopy that human and mouse neutrophils express TLR9 at the cell surface. The signal is TLR9-specific since it is only observed with neutrophils isolated from WT but not TLR9-KO mice. Moreover, surface TLR9 expression is up-regulated upon activation of neutrophils with different stimuli and not only TLR9 ligands. Importantly, surface TLR9 is active and functional. TLR9 ligands, ODN with unmethylated CpG motifs (CpG-ODN), indeed bind to human surface TLR9 and binding was only observed at the cell surface of WT but not TLR9-deficient mouse neutrophils. Finally, CpG-ODN cross-linked onto a solid phase and having no access to intracellular TLR9 induces neutrophil activation, leading to IL-8 secretion. As a control, only WT and not TLR9-deficient mouse neutrophils are activated by CpG-ODN. This is the first demonstration of a functional TLR9 expressed at the cell surface of human primary cells. This pathway may be triggered when pathogen-derived TLR9 ligands can not reach the endosome, offering a rescue mechanism for PMN activation.

Introduction

PMN are the first cells recruited at inflammation sites. They play a central role in host defense and their accumulation into sites of acute infection or tissue injury is a critical component of inflammation [1]. Activated PMN are directly mobilized to drive pro-inflammatory reactions and to eradicate pathogens. However, upon inappropriate activation, PMN-derived products may induce tissue damages and may alter self-Ag, transforming a harmless self-Ag in a potent autoAg. Importantly, PMN have been suggested to link innate and adaptive immunity [2-4] by influencing e.g. DC. PMN activation may thus favor adaptive immunity but may represent double-edged swords leading to tissue injury or autoimmunity. TLR9 is activated in endosomal/lysosomal compartments by unmethylated CpG motifs present in pathogen-derived DNA [5] as well as in synthetic ODN. Nevertheless, vertebrate DNA can also trigger TLR9 under certain circumstances although TLR9-independent pathways are triggered in addition [6]. Moreover, natural phosphodiester ODN lacking CpG motifs activate TLR9 upon enforced endosomal translocation [7]. All those results refer however to intracellular TLR9. Since nucleic acids have to be released from microbial particles, concentrated or modified in endosomal-lysosomal compartments [8, 9] before receptor-ligand interactions can take place with cleaved TLR9 [10, 11] an emerging paradigm in innate immunity is the intracellular expression of TLR9 [12]. It has been suggested that TLR9 is sequestered intracellularly to exclude recognition of extracellular host-derived nucleic acids, thus preventing autoimmune responses [13]. Endosomal compartmentalization of TLR9 may thus reflect an evolutionary strategy to avoid TLR9 activation by self-DNA and localization of the nucleic acid-sensing TLR is critical in discriminating between self and non-self nucleic acid. PMN recognize PAMP on foreign organisms by expressing TLR1-10, except TLR3 [14]. Particularly, PMN express TLR9 and respond to TLR9 ligands, leading to cytokine secretion and CD11b up-regulation, although the subcellular localization of TLR9 in PMN has not been investigated. A few studies have demonstrated that specialized human primary immune cells such as B lymphocytes [15-18], tonsil cells [19] and monocytes [20] as well as intestinal epithelial cells [21], might express TLR9 at the cell surface although the functionality of the human surface TLR9 was not demonstrated. APC represent therefore the major cell populations expressing cell surface TLR9. Nevertheless, human primary PMN have not been studied so far. Only studies using engineered cell lines and expressing a chimeric cell surface TLR9 could clearly show that surface TLR9 responds to its ligand and even to mammalian DNA [13]. Such a demonstration is not available for primary cells.

Results and discussion

We therefore investigated whether human and mouse primary PMN express a cell surface TLR9 and whether this receptor can trigger an activation signal upon binding and recognition of its ligand in the extracellular milieu. Using an approach combining different immunological and microscopic techniques, we investigated the cellular distribution of TLR9 in lymphoid and myeloid cell types. We demonstrate that surface TLR9 interacts with CpG-ODN, leading to the activation of human PMN, and that surface expression of TLR9 is enhanced upon PMN activation.

Material and methods

Mice. TLR9-KO mice [5] on a C57BL/6 genetic background were obtained from Prof. H. Wagner (Munich, Germany). WT C57BL/6 control mice were purchased from Charles River. Experiments were approved by the local animal ethics committee (Regierungspräsidium Tübingen, § 4 Abs. 3).

Human samples. Heparin-blood from random, healthy individuals was used. Human experiments were approved by the local ethics committee (reference 386/2006V).

Nucleosome purification. Mononucleosomes (major lupus autoAg known to induce PMN activation [22]) were purified from calf thymus under sterile conditions by ultracentrifugation onto sucrose gradients as described previously [23]. As a control, an empty gradient (not loaded with chromatin) was prepared. DNA and protein contents were verified. The preparations contain low endotoxin levels (10-50 IU/mg nucleosome) as determined using a Limulus Amebocyte Lysate assay (BioWhittaker). A similar concentration (in IU/ml) was measured in purified nucleosomes and in the purification buffer (Gdt) which was used as a negative control. Moreover, nucleosome preparations were always first tested using PMN cultured with and without the LPS inhibitor PB (Fluka) and only preparations which activate PMN without being inhibited by PB were used.

Cell isolation, culture and activation. Human PBMC and PMN were isolated from heparinized peripheral blood by density centrifugation as described previously [22]. Contaminating red blood cells were lysed using a hypotonic buffer. Mouse neutrophils were isolated from BM cells obtained from adult WT and TLR9-KO mice (sex-and age-matched, 8-12 weeks old). Neutrophils were enriched by positive selection for Ly-6G$^+$ cells using magnetic beads (Miltenyi Biotec) according to the manufacturer's recommendations. Contaminating red blood cells were lysed. Human (defined as CD3$^-$, CD19$^-$, CD56$^-$, CD11b$^+$, CD66b$^+$ cells, purity > 85 %) and mouse PMN (defined as F4/80$^-$, CD3$^-$, B220$^-$, CD11b$^+$, Ly-6G^{high} cells, purity > 90 %) were cultured in RPMI 1640 medium (BioWhittaker) supplemented with 10 % FCS (PAA), 100 U/ml penicillin, 100 mg/ml streptomycin (both from Gibco) and 50 µM β-mercaptoethanol (Roth). For cell activation studies, human and mouse PMN were plated onto 96-well plates at a density of 1 or 1.5 x 10^6 cells/ml, respectively. Granulocyte colony-stimulating factor (50 ng/ml, ImmunoTools) was added to mouse PMN. Cells were either incubated in medium alone or activated with purified nucleosomes, the purification buffer as negative control (Gdt), R848 (Alexis), LPS (from Salmonella typhimurium or Escherichia coli, Sigma), CpG-ODN (2006 and 1826, Metabion), GpC-ODN (2006, Metabion) and FITC-CpG-ODN (2006, Invivogen). All CpG-ODN and the GpC-ODN were phosphorothioate ODN. Experiments were conducted in the presence/absence of PB (25 µg/ml) or a TLR9 antagonist (an ODN containing a G5 motif (G-ODN), 4 µM, Metabion). After 16 h, cell culture supernatants were harvested and cells were analyzed by flow cytometry.

Antibodies and flow cytometry. All Ab were purchased from BD Biosciences, if not stated otherwise. The following mAb conjugated with different fluorochromes were used: anti-human CD3-PerCP (clone SK7), CD11b-PE (ICRF44), CD19-APC (HIB19), CD56-PE (B159), CD66b-FITC (G10F5), anti-human/mouse TLR9-FITC (5G5, Abcam) and anti-mouse CD11b-PE (M1/70), Ly-6G-FITC (1A8), F4/80-APC (BM8, eBioscience), CD3-FITC (145-2C11, SouthernBiotech), B220-PE (RA3-6B2). Human PMN and PMBC were first incubated with PBS/5 % heat-inactivated human serum, whereas PBS/2 % heat-inactivated autologous mouse serum was used for mouse PMN. After washing, cells were incubated with the indicated mAb or the corresponding isotype controls in PBS/0.01 % sodium azide to reduce the unspecific uptake of mAb. For surface TLR9 staining, cells were incubated with FITC-CpG-ODN 2006 or additionally washed and incubated with anti-TLR9-FITC (or the corresponding isotype control) and then with anti-FITC-Alexa Fluor 488 (Invitrogen). For intracellular staining, cells were first stained for surface markers, permeabilized with Cytofix/Cytoperm (BD

Results and discussion

Biosciences) and incubated with anti-TLR9-FITC (or the corresponding isotype control), washed and then with anti-FITC-Alexa Fluor 488 or directly incubated with FITC-CpG-ODN 2006 in PBS/0.1 % Saponin (Sigma). All incubations were carried out at 4 °C for 30 min. Cells were finally fixed with 1 % paraformaldehyde and analyzed on a four-color FACSCalibur apparatus (Becton Dickinson). Data were evaluated with CELLQuest software (Becton Dickinson).

Confocal microscopy. To demonstrate the cell surface expression of TLR9 in mouse and human PMN, cells were incubated with FITC-CpG-ODN 2006 or were consecutively incubated with anti-TLR9-FITC (or the corresponding isotype control) followed by anti-FITC-Alexa Fluor 488 in PBS/0.01 % sodium azide to reduce the unspecific uptake of fluorochrome-conjugated molecules (30 min, 4 °C for all steps). To stain the cell surface, cells were incubated with Alexa Fluor 555-conjugated cholera toxin subunit B (CTB, 20 μg/ml, Molecular Probes) and fixed. For intracellular TLR9 staining, cells were permeabilized and incubated with anti-TLR9-FITC (or the corresponding isotype control) and then with anti-FITC-Alexa Fluor 488 or directly incubated with FITC-CpG-ODN 2006. To stain the nucleus, cells were then incubated with TO-PRO-3 (1/1,000, Molecular Probes). Finally, cells were mounted on Lab-Tek Chambered 1.0 borosilicate coverglass systems (Nunc) and kept in the dark at 4 °C. Confocal microscopy was conducted using an inverted LSM510 confocal laser scanning microscope (Carl Zeiss) fitted with a Plan-Apochromat 63 x/1.4 OIL DIC objectives, and the pinhole was set to scan layers of 1 μm. FITC and Alexa 488 were excited at 488 nm with an argon-ion laser. Alexa 555 and TO-PRO-3 were excited at 543 and 633 nm, respectively, with helium-neon lasers. Images were acquired by single-track measurement and scales are indicated.

ELISA. Detection of IL-8 and MIP-2 secretion by human and mouse PMN was analyzed by sandwich ELISA using OptEIA set or mAb pairs and streptavidin-peroxidase conjugate from BD Biosciences or R&D Systems, respectively, and according to the manufacturer's instructions. Cytokine concentrations in cell culture supernatants are depicted as mean ± standard deviation of triplicates.

Cell surface TLR9 functional assay. Nitrocellulose plates (Millipore, MSHA S4510) were activated by UV irradiation at 254 nm (UV Stratalinker 1800, Stratagene, 20J/cm^2) and coated with PBS or FITC-CpG-ODN 2006 in PBS (1.5-2.5 μM) for 4 h at RT. The presence of the FITC-CpG-ODN 2006 was confirmed by measuring the FITC fluorescence using a SPECTRA Fluor fluorometer (TECAN). Plates were then washed three times with PBS and the last wash was measured with the fluorometer to confirm the absence or free (unbound) FITC-CpG-ODN. The plate was filled with PBS and the FITC fluorescence was measured in order to estimate the CpG-ODN binding efficiency. Plates were then emptied and used for cell culture using human PMN (1.5 x 10^6 cells/ml). LPS and free FITC-CpG-ODN 2006 were used as controls. After 16 h, the FITC fluorescence in the plate was measured. Cell culture supernatants were then transferred, the FITC fluorescence was measured and the IL-8 secretion was estimated by ELISA. Simultaneously, PMN were harvested and analyzed by flow cytometry for the FITC fluorescence.

Statistical analysis. The mean FITC fluorescences measured by flow cytometry in the presence/absence of ammonium chloride (10 mM, Roth) were compared using a Student's t-test or a Mann-Whitney rank sum test. The mean IL-8 concentrations induced in PMN by either the medium alone or the coated CpG-ODN were compared using a Student's t-test after normalization of the different experiments to the actual concentration of the coated CpG-ODN. The SigmaStat software was used and a p value < 0.05 was considered significant.

Results and discussion

Results

Human PMN spontaneously express TLR9 at the cell surface

To investigate the ex-vivo expression of TLR9 at the surface of non-stimulated cells, we first analyzed simultaneously all the human leukocyte populations in several independent healthy donors. Cells were prepared by dextran centrifugation and the two layers containing PBMC or PMN were harvested and mixed, yielding a leukocyte population depleted from erythrocytes and platelets. Cells were then analyzed by polychromatic flow cytometry in the presence of sodium azide to reduce the uptake of fluorochrome-conjugated Ab and thus to minimize unspecific staining. As shown in figure 1, the R1 gate contained only PMN (CD66b-positive cells), whereas the R2 gate contained essentially T lymphocytes, B lymphocytes, NK/NKT cells and a few monocytes. Cells were then stained for cell surface or intracellular TLR9 using a specific mAb recognizing both human and mouse TLR9. Focusing on gate R1, we identified cell surface expression of TLR9 on PMN (Fig. 1B), all the cells being double-positive for CD66b and CD11b. Gating on R2, and as already reported, B lymphocytes also express TLR9 at the cell surface (Fig. 1C, middle panel). Likewise, gating on CD3⁻ CD19⁻ cells, we confirmed that part of the monocytes also express cell surface TLR9 (Fig. 1C, lower panel). Interestingly, no cell surface TLR9 was detected on T lymphocytes (Fig. 1C, upper panel), NK cells or NKT cells (Fig. 1D, lower and upper panels, respectively). However, all the cell types analyzed expressed TLR9 intracellularly and expression was stronger than surface expression. No binding of the isotype control was observed, indicating that the staining was TLR9-specific. The specificity of the TLR9 staining was confirmed by comparing BM cells prepared from WT and TLR9-KO mice. Indeed, the 5G5 anti-TLR9 mAb used in the present study recognizes both human and mouse TLR9 [8]. We gated on Ly-6G^{high} cells (most of them are CD11b$^+$ cells and thus granulocytes) which are known to express intracellular TLR9. WT PMN express intracellular TLR9 whereas no expression was observed in TLR9-KO PMN, as expected (Fig. 1E). Those results indicate that the signal measured at the surface of human PMN corresponds to TLR9. More importantly, only the cells expressing TLR9 at the cell surface (namely PMN, B lymphocytes and monocytes) also bind the TLR9 ligand (FITC-CpG-ODN 2006) at the cell surface (Fig. 1B-C), whereas all the cell types were stained positively with that ligand upon fixation/permeabilization, suggesting that the CpG-ODN also binds to its intracellular receptor.

This observation confirms the presence of TLR9 at the cell surface and the specific interaction with its ligand suggests that the surface TLR9 is active.
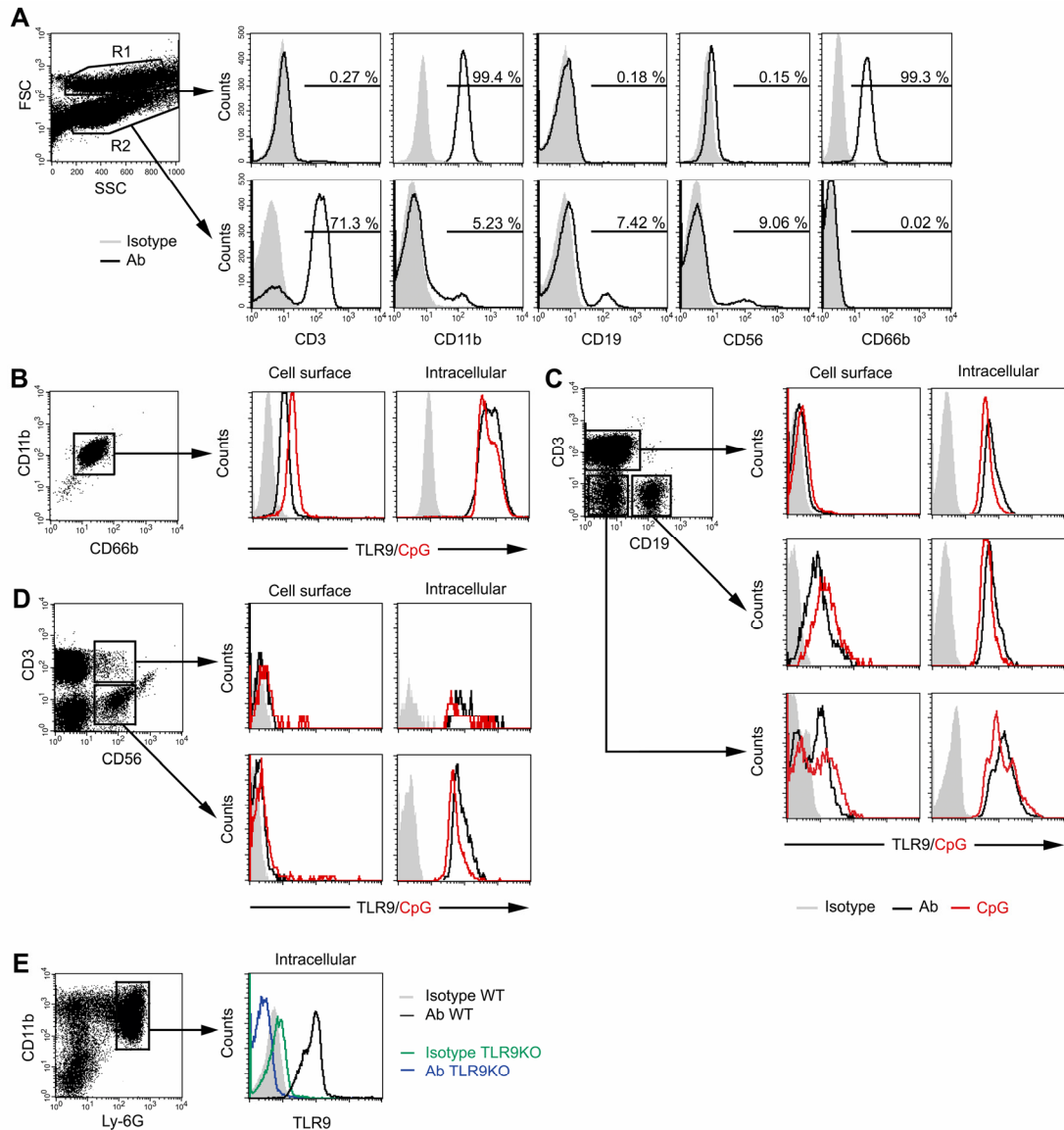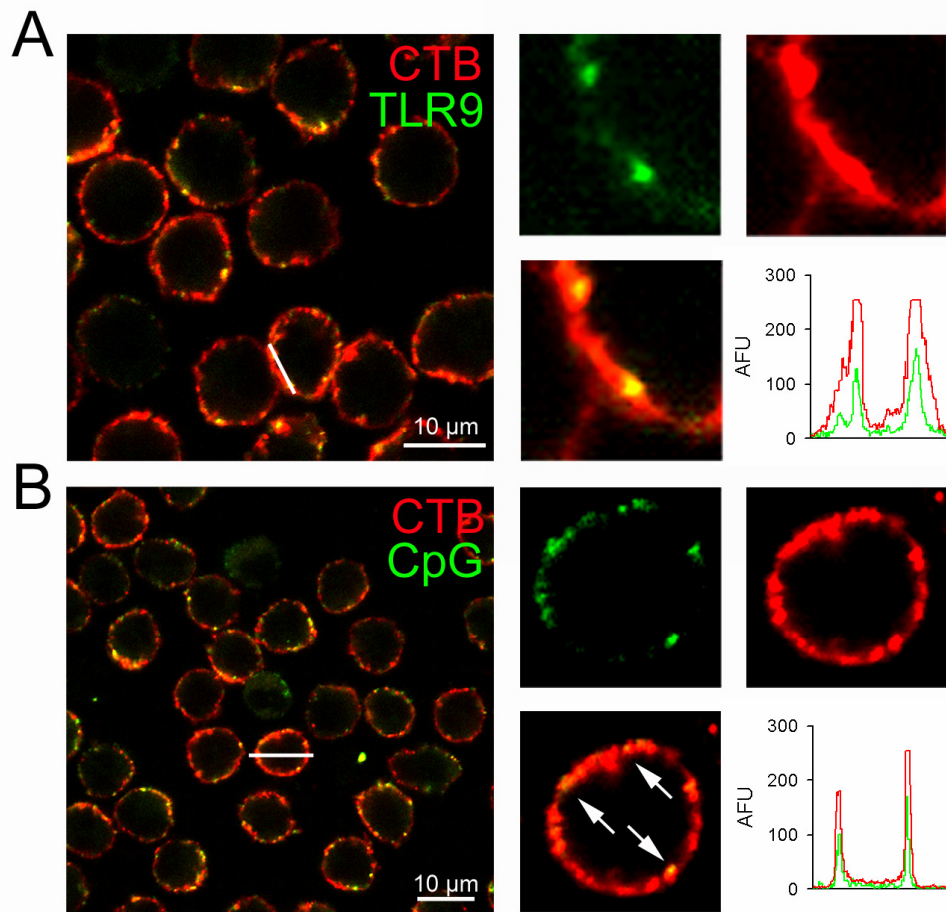

Figure 1. Human primary PMN express TLR9 at the cell surface. PBMC and PMN were freshly isolated from the peripheral blood of healthy individuals by dextran centrifugation and mixed. Upon Ab staining, cells were analyzed by multicolor flow cytometry using different gates. **(A)** Cells contained in gates R1 and R2 were characterized using anti-human (CD3, CD11b, CD19, CD56, CD66b) mAb. The percentages of gated cells positive for the marker of interest are indicated. **(B)** Cells from gate R1, which are PMN according to the staining for CD66b and CD11b (left), were stained for cell surface (without permeabilization) and intracellular (after permeabilization) TLR9 using an anti-TLR9-FITC mAb in combination with an anti-FITC-Alexa Fluor 488 Ab. Alternatively, cells were stained

Results and discussion

with a FITC-CpG-ODN (a TLR9 ligand). **(C, D)** Cells from gate R2 were stained for CD3 and CD19 (C) or CD3 and CD56 (D) and analyzed for surface and intracellular TLR9 expression as in (B). Cells were also stained with the FITC-CpG-ODN as described in (B). **(E)** BM cells were isolated from WT and TLR9-KO mice and stained for Ly-6G and CD11b or the corresponding isotype controls. Ly-6G-positive cells were then analyzed for intracellular TLR9 expression as in (B). Ab, antibody; CpG, FITC-CpG-ODN 2006.

To confirm those results, human PMN were isolated from fresh blood and analyzed by confocal microscopy. We used from now on highly purified PMN, yielding only the cell population corresponding to gate R1 and PMN were not mixed with PBMC. Cells were stained for TLR9 expression (green) at 4 °C in the presence of CTB (red) to stain the cell surface. As shown in figure 2A, a TLR9-specific signal was observed at the cell surface of PMN, as demonstrated by the merged signal with CTB staining. The histogram depicting the intensity of both signals (measured along the white bar) nicely shows that both signals overlay along the cell membrane. PMN were then incubated with FITC-CpG-ODN 2006 (green) in the presence of CTB and analyzed (Fig. 2B). The TLR9-ligand bound to the cell surface of PMN (merged signal), in line with the cell surface expression of TLR9. When analyzing both signals across the cells (white bar), we only detected the FITC-CpG-ODN at the cell surface, as depicted by the overlay in the histogram showing the intensity of both signals. The binding of the CpG-ODN at the cell surface suggests that TLR9 expressed at the surface of PMN is active.

Figure 2. Human primary PMN express a cell surface TLR9 and bind to TLR9 ligands. PMN were purified from fresh blood and analyzed by confocal microscopy in the absence of PBMC. **(A)** PMN were stained consecutively with anti-TLR9-FITC and anti-FITC-Alexa Fluor 488 (without permeabilization) at 4 °C in the presence of Alexa Fluor 555-CTB. The individual fluorescence channels and the merged signal are shown (right). The intensity of both signals (measured along the white bar) is also depicted. **(B)** PMN were then incubated with FITC-CpG-ODN 2006 at 4 °C in the presence of Alexa Fluor 555-CTB and analyzed. The individual channels, the merged signal (arrows) and the intensity of both signals across the cells (white bar) are depicted. All incubations were performed in PBS/0.01 % sodium azide. Confocal microscopy was conducted using an inverted LSM510 confocal laser scanning microscope (Carl Zeiss) fitted with a Plan-Apochromat 63 x/1.4 OIL DIC objectives, and the pinhole was set to scan layers of 1 µm. The scale in (A) and (B) represents 10 µm. CpG, FITC-CpG-ODN 2006; AFU, arbitrary fluorescence units.

Results and discussion

To confirm the specificity of the TLR9 staining and to show that the CpG-ODN specifically binds to the cell surface TLR9 and not to an as yet unknown DNA sensor as proposed [24], PMN were isolated from WT and TLR9-KO mice by magnetic cell sorting. Mouse PMN were stained in the presence of CTB and analyzed by confocal microscopy. WT PMN showed a nice cell surface TLR9 signal upon staining with the 5G5 mAb, as demonstrated by the co-localization with the CTB signal depicted in the histogram, which was not detected upon staining with the corresponding isotype control (Fig. 3A). More importantly, the TLR9 staining was not observed with TLR9-KO PMN, indicating that the cell surface signal measured with WT PMN was TLR9-specific. Likewise, the TLR9-ligand CpG-ODN only bound to the cell surface of WT PMN, whereas no binding was detected with TLR9-KO PMN, confirming that the binding of the CpG-ODN to the cell surface of PMN is mediated by TLR9. The same experiment was then reproduced with fixed and permabilized cells (Fig. 3B). Cells were stained with the DNA dye TO-PRO-3 instead of CTB in order to stain the nucleus. Only WT, but not TLR9-KO, PMN showed a TLR9 signal, whereas no signal was observed upon staining with the isotype control, confirming that the mAb used was TLR9-specific and that the control mice used where TLR9-KO. Similarly, the CpG-ODN only bound to the intracellular TLR9 present in WT PMN. The surface expression of TLR9 by those purified PMN was confirmed by flow cytometry. Non-permeabilized PMN were stained at 4 °C in the presence of sodium azide. WT PMN show a strong expression of surface TLR9, which was not observed with TLR9-deficient PMN (Fig. 3C). To confirm that the FITC-CpG-ODN 2006 used is an active TLR9 ligand for mouse PMN, cells were cultured with different stimuli and cell activation was estimated. Indeed, CpG-ODN are often cell type-specific and the conjugation to a FITC molecule may interfere with cell activation. WT PMN were equally activated by CpG-ODN 2006 and FITC-CpG-ODN 2006, as shown by CD11b up-regulation and MIP-2 secretion (Fig. 3D), whereas activation was not observed with TLR9-deficient PMN. Likewise, only WT PMN were activated by the mouse-optimized CpG-ODN 1826. As a control, WT and TLR9-deficient PMN were activated by LPS, indicating that all the CpG-ODN used (conjugated to FITC or not) are TLR9 ligands and are not contaminated by endotoxins (which is an important control for the functional assay of surface TLR9 described below). All those results confirmed the specificity of the TLR9 staining and the presence of TLR9 at the cell surface of PMN. The binding of the CpG-ODN at the cell surface of WT but not KO PMN suggests that the surface TLR9 recognizes its ligand.
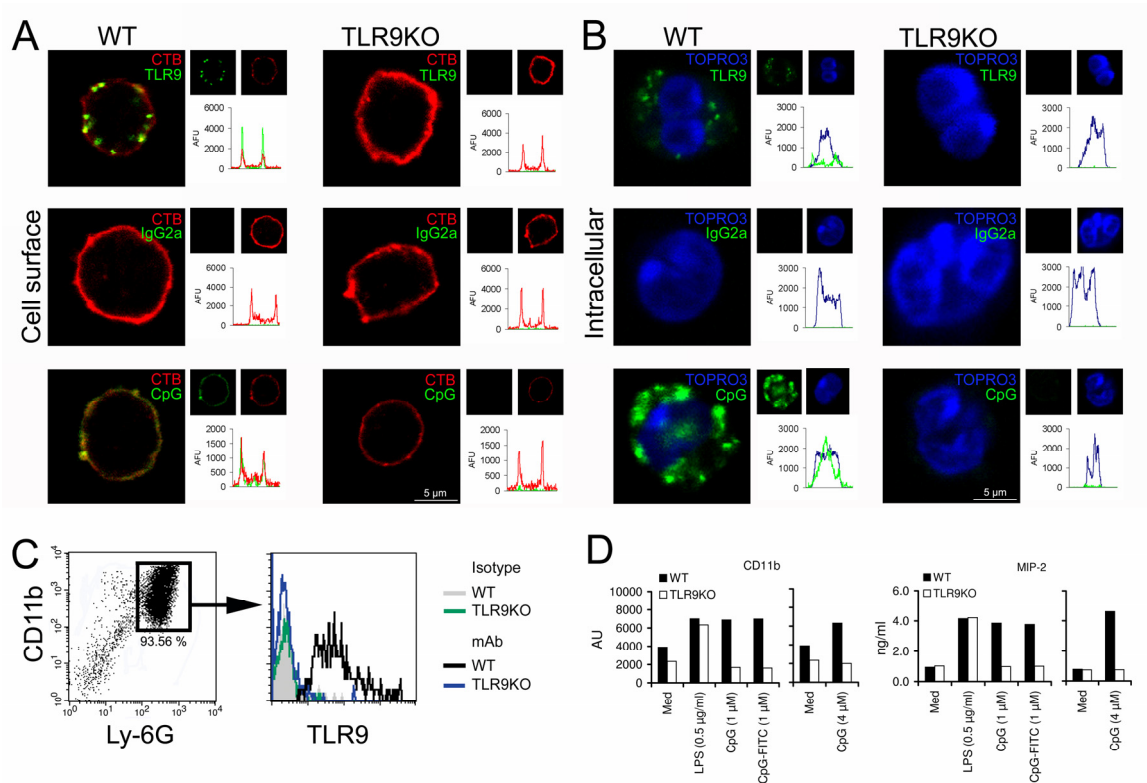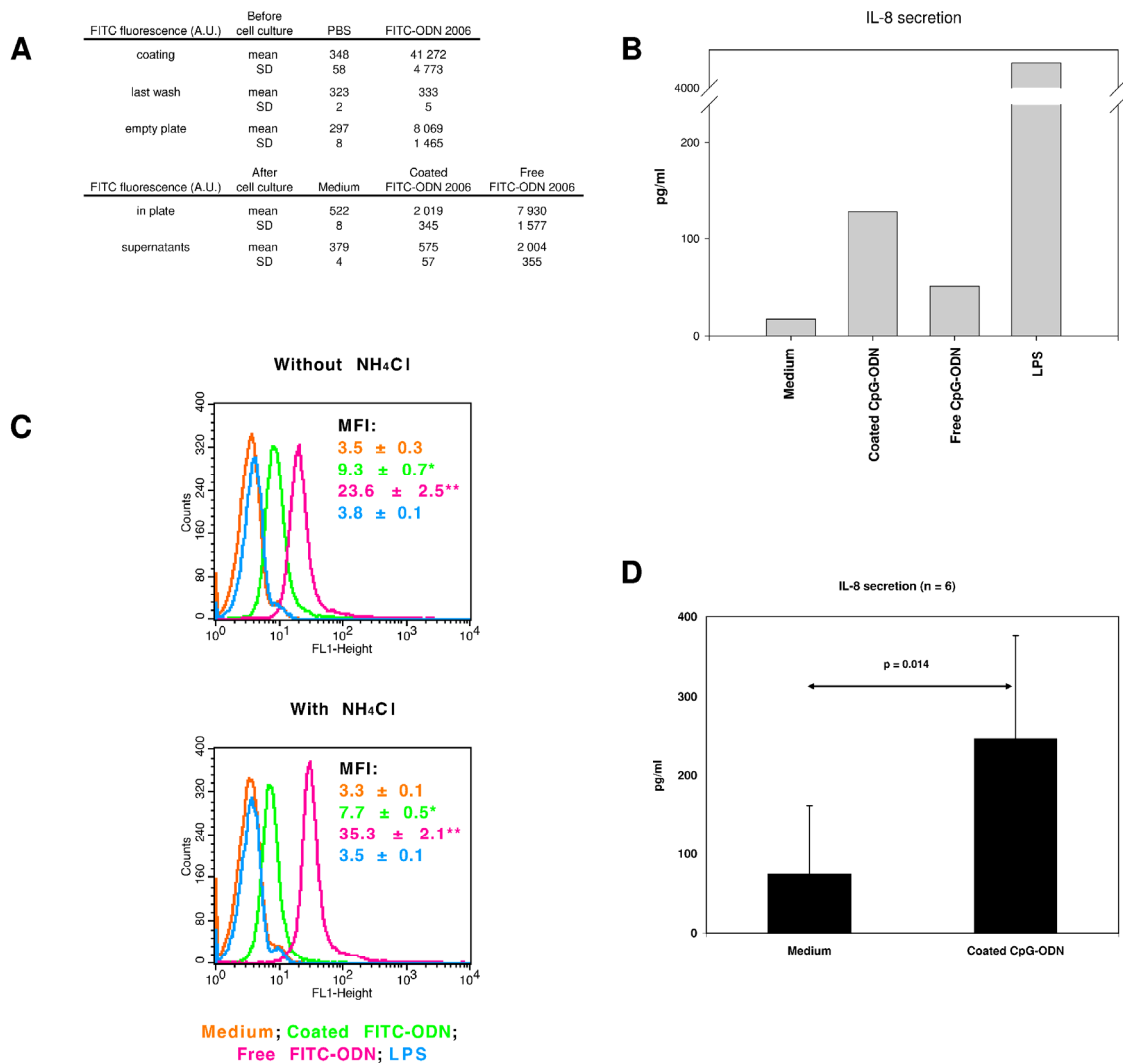
Figure 3. Mouse PMN express also a surface TLR9 and the staining is specific. PMN were purified by Ly-6G positive selection from the BM of WT and TLR9-KO mice. **(A)** Cells were stained consecutively with anti-TLR9-FITC (upper panel) or the corresponding isotype control (middle panel) and anti-FITC-Alexa Fluor 488 or were directly incubated with FITC-CpG-ODN 2006 (lower panel) at 4 °C in the presence of Alexa Fluor 555-CTB (without permeabilization). PMN were then analyzed by confocal microscopy as described in figure 2. The individual channels, the merged signal and the intensity of both signals across the cells are depicted. The scale in (A) and (B) represents 5 µm. **(B)** The same staining was performed using permeabilized cells to detect intracellular TLR9 expression and CpG-FITC binding, except that PMN were stained with TO-PRO-3 instead of CTB. **(C)** Purified PMN isolated from WT and TLR9-KO mice were analyzed by flow cytometry. The phenotype of the isolated cells was verified by Ly-6G and CD11b staining. Non-permeabilized Ly-6G-positive cells (all of them are CD11b-positive) were analyzed for cell surface TLR9 expression as described in figure 1. **(D)** The mouse PMN isolated in (C) were cultured with LPS, CpG-ODN 2006, FITC-CpG-ODN 2006 or CpG-ODN 1826 (right histograms) for 16 h. Cell activation was then estimated by measuring CD11b up-regulation (flow cytometry) and MIP-2 secretion (ELISA). CpG, FITC-CpG-ODN 2006; AFU, arbitrary fluorescence units; Med, cell culture medium.

Results and discussion

TLR9 expressed at the cell surface of primary neutrophils is functional

The results described above clearly show that the TLR9 ligand binds to the cell surface of primary PMN and that the binding is specifically mediated by TLR9. It therefore indicates that this receptor is at least partially functional since it recognizes this ligand. We then analyzed whether ligation of FITC-CpG-ODN 2006 to the cell surface TLR9 triggers activation of primary human PMN. In order to specifically stimulate the cell surface TLR9, the FITC-CpG-ODN 2006 was cross-linked by UV irradiation onto a sterile nitrocellulose filter-containing cell culture plate. The efficacy was verified by measuring the FITC fluorescence using a fluorometer. Wells incubated with the FITC-ODN displayed a strong fluorescence, as expected (Fig. 4A, upper part, "coating"). The plate was washed three times to eliminate unbound CpG-ODN and the fluorescence in the last wash was measured. No FITC fluorescence was detected in the last wash (Fig. 4A, upper part), confirming that all free CpG-ODN were removed. The empty plate was then measured to estimate the amount of CpG-ODN remaining in the wells (Fig. 4A, upper part), showing that the ODN was covalently bound to the wells. Nevertheless, only 25.6 ± 9.8 % of the incubated ODN was efficiently cross-linked to the plate. Freshly isolated PMN were then cultured in the CpG-ODN-coated plate. As negative and positive controls, PMN were cultured in medium alone or supplemented with LPS, respectively. As a comparison, PMN were cultured with the FITC-CpG-ODN 2006 free in solution at a sub-optimal concentration. The concentration of the free ODN used was either the same than that of the cross-linked ODN (as estimated by measuring the FITC fluorescence) or slightly higher. Indeed, UV irradiation did not allow the cross-linking of an optimal amount of ODN. After overnight cell culture, the cross-linked ODN was still detectable (Fig. 4A, lower part, "in plate") and the fluorescence was much stronger in the presence of the free ODN, indicating that the latter was present at a higher concentration. The cell culture supernatants were then harvested and measured. Only a slight signal was detected for the cross-linked ODN (Fig. 4A, lower part), suggesting that only part of the ODN is released and that most of it is nitrocellulose-bound. A stronger signal was detected for the free ODN, indicating that part of it was still in solution. Importantly, the cross-linked ODN induced the activation of PMN, as shown by the IL-8 secretion (Fig. 4B), indicating that the cell surface TLR9 is functional. The activation was even stronger than the activation measured with the same ODN in solution, whereas the latter was used at a stronger concentration.

A

FITC fluorescence (A.U.)	Before cell culture	PBS	FITC-ODN 2006	
coating	mean	348	41 272	
	SD	58	4 773	
last wash	mean	323	333	
	SD	2	5	
empty plate	mean	297	8 069	
	SD	8	1 465	

FITC fluorescence (A.U.)	After cell culture	Medium	Coated FITC-ODN 2006	Free FITC-ODN 2006
in plate	mean	522	2 019	7 930
	SD	8	345	1 577
supernatants	mean	379	575	2 004
	SD	4	57	355

B

IL-8 secretion

C

Without NH₄Cl

MFI:
3.5 ± 0.3
9.3 ± 0.7*
23.6 ± 2.5**
3.8 ± 0.1

With NH₄Cl

MFI:
3.3 ± 0.1
7.7 ± 0.5*
35.3 ± 2.1**
3.5 ± 0.1

Medium; Coated FITC-ODN;
Free FITC-ODN; LPS

D

IL-8 secretion (n = 6)

p = 0.014

Figure 4. Human primary PMN express a functional surface TLR9. (A) UV-treated nitrocellulose plates were incubated with PBS or FITC-CpG-ODN 2006 in PBS (upper panel, before cell culture). The efficiency of the ODN cross-linking was verified by measuring the FITC fluorescence in the plate (coating), in the last wash and in the empty plate after washing using a fluorometer. Human PMN were isolated from healthy individuals and cultured in the cross-linked plate (lower panel, after cell culture) for 16 h. As a control, cells were activated with free (non-cross-linked) FITC-CpG-ODN 2006 or LPS (5 ng/ml). The FITC fluorescence was measured in the plate and in the cell culture supernatants. The fluorescence background was the same in medium and in medium supplemented with LPS (data not shown). The actual concentrations were 0.48 and 1 µM for the cross-linked and the free ODN, respectively. Shown is one representative experiment. **(B)** The level of PMN activation was estimated by measuring IL-8 secretion by ELISA. **(C)** Cultured PMN were also analyzed by flow cytometry for the FITC fluorescence in the absence (upper panel) or presence (lower panel) of AC (NH₄Cl). The impact of AC on the FITC fluorescence was

Results and discussion

estimated; * p = 0.029, ** p < 0.001. MFI, mean fluorescence intensity (mean ± standard deviation). **(D)** PMN were isolated from six independent healthy individuals. The capacity of the cross-linked ODN to trigger surface TLR9-mediated cell activation was tested in six independent experiments. IL-8 secretion was determined by ELISA. The cumulative results are shown and the probability is indicated. Experiments were normalized to the actual concentration of the cross-linked ODN.

Upon cell culture, the FITC fluorescence was also measured by flow cytometry. As expected, a strong signal was detected in PMN incubated with the free FITC-ODN (Fig. 4C, upper panel). Surprisingly, PMN incubated with the cross-linked ODN showed an intermediate signal, suggesting that PMN are able to detach part of the FITC molecules or part of the cross-linked ODN from the nitrocellulose filter. To determine whether the signal corresponds to a cell surface signal only, the same cells were measured in the presence of AC, an inhibitor of endosomal acidification. The FITC fluorescence is indeed inhibited at low pH values and AC protects the endosomal FITC fluorescence. As shown in figure 4C (lower panel), whereas the FITC fluorescence of the free ODN was significantly increased in the presence of ammonium chloride (p < 0.001), the fluorescence of the cross-linked ODN was slightly but significantly decreased (p = 0.029), indicating that the ODN has different properties when cross-linked. Compared to the expected behavior of the free ODN, the results confirm that the cross-linked ODN bound only to the PMN surface (i.e. is cell-bound but remains external) and did not reach the endosomal TLR9. The activation observed with the cross-linked ODN is therefore triggered by the cell surface TLR9. Primary PMN were then isolated from six independent healthy donors and cell activation by the cross-linked ODN was determined in six independent experiments. The coated ODN induced a significant IL-8 secretion (p = 0.014), showing clearly that the TLR9 expressed at the cell surface of PMN recognized its ligand and is functional (Fig. 4D).

TLR9 surface expression is up-regulated in response to some stimuli

We next investigated whether the expression of the cell surface TLR9 is modulated upon PMN activation. Cells from healthy individuals were incubated with different TLR ligands or purified nucleosomes, an autoAg which activates PMN in a TLR9-independent manner (see chapter V). Interestingly, CpG-ODN-mediated PMN activation led to an up-regulation of the cell surface TLR9 in part of the donors, which correlated to either the down-regulation or the up-regulation of the intracellular TLR9 (Fig. 5A). As expected, no significant effect was observed when PMN were cultured with the control GpC-ODN. Activation with R848, a TLR7/8 ligand in humans, also led to surface TLR9 up-regulation in some donors, whereas the TLR4 ligand LPS did not induce up-regulation of TLR9 at the cell surface but rather intracellularly, suggesting that PMN activation with TLR4 ligands is associated with the up-regulation of endosomal TLR9. Importantly, nucleosome-induced PMN activation was associated with a concentration-dependent up-regulation of the cell surface TLR9 in most of the donors (Fig. 5A), which correlated to a down-regulation of the intracellular TLR9. Modulation of the TLR9 expression is therefore not restricted to TLR9 ligands. PMN activation was verified by measuring the CD11b up-regulation (Fig. 5B) and IL-8 secretion (Fig. 5C). Nucleosomes trigger a concentration-dependent PMN activation which was not inhibited by the LPS inhibitor PB or the TLR9 antagonist G-ODN. As a control, LPS and CpG induced also PMN activation but were inhibited by PB or G-ODN, respectively, whereas GpC-ODN did not activate. R848 activated PMN and was not inhibited by PB or G-ODN. Thus, all the potential stimuli led to PMN activation, indicating that the modulation of endosomal/cell surface TLR9 expression is correlated to cell activation. Only LPS-induced PMN activation did not lead to a significant surface TLR9 up-regulation. The results indicate also that the nucleosome-mediated PMN activation and the surface TLR9 up-regulation occurred independently of TLR9 triggering and are not influenced by endotoxins.
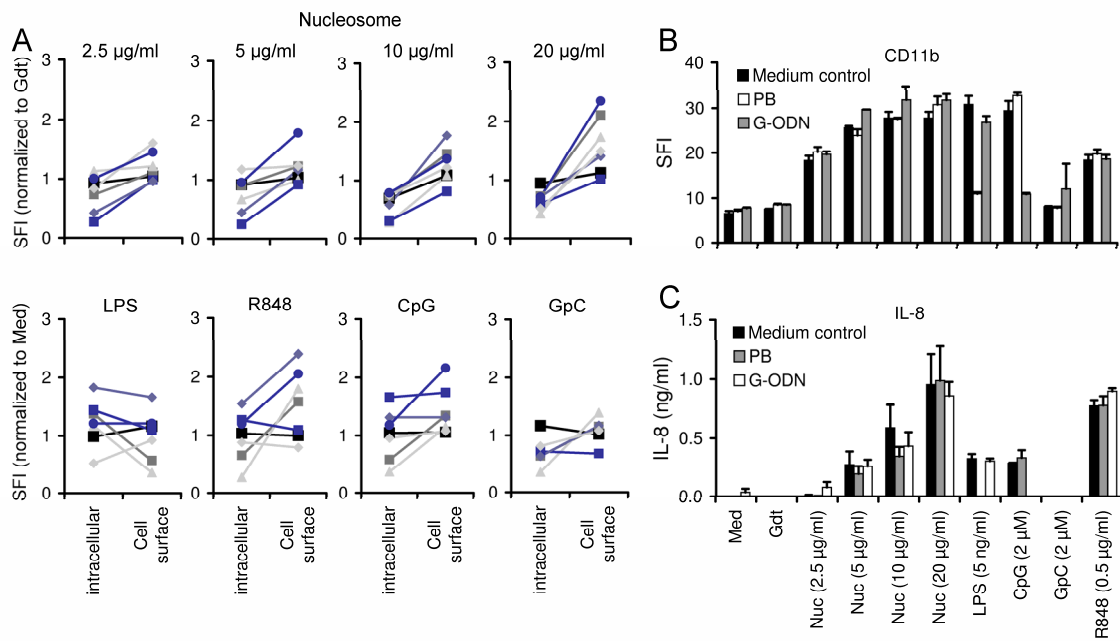
Figure 5. The surface TLR9 is up-regulated in human PMN upon cell activation. PMN were isolated from seven independent healthy donors and cultured with different concentrations of nucleosomes, LPS (5 ng/ml), R848 (0.5 µg/ml), CpG-ODN (2 µM) or GpC-ODN (2 µM). **(A)** After 16 h, cells were stained for surface and intracellular TLR9 and analyzed by flow cytometry as described in figure 1. The specific fluorescence indexes (SFI, ratios between the specific TLR9 staining and the isotype control staining) were determined and normalized to that of the gradient or medium controls in order to visualize the modulation (fold increase) of TLR9 expression induced by either nucleosomes or the TLR ligands, respectively. Shown are all the donors tested; each line represents a donor. The level of PMN activation was verified by measuring the CD11b up-regulation by flow cytometry **(B)** and IL-8 secretion by ELISA **(C)**. Shown is one representative donor. PB, polymyxin B; G-ODN, TLR9 antagonist; Med, medium; Gdt, nucleosome purification buffer; Nuc, nucleosomes.

Discussion

We have shown for the first time that primary human and mouse PMN express TLR9 at the cell surface. This is also the first demonstration that a functional TLR9 is expressed at the cell surface of human primary cells. Previous studies have already reported that monocytes and B lymphocytes express a cell surface TLR9 [15-18, 20] but PMN have not been described. Moreover, the functionality of the cell surface TLR9 has not been addressed so far. Recently, bacterial DNA has been shown to bind to the cell surface of human PMN but the role of TLR9 in this process has not been analyzed [24]. Although activation by the cross-linked ODN was relatively low, IL-8 secretion was significantly increased. It therefore suggests that the surface TLR9 is less efficient than the endosomal TLR9. Nevertheless, other causes, essentially technical reasons, may explain the apparent low activity of the surface TLR9. We observed that the nitrocellulose plates used are less adequate for PMN activation. Thus, when PMN were cultured with CpG-ODN 2006 in solution in classical cell culture plates and nitrocellulose plates in parallel, cell activation was much stronger in classical plates (data not shown). Moreover, UV irradiation only allowed the cross-linking of low amounts of CpG-ODN (0.51 ± 0.20 µM, final concentration in nitrocellulose plates) whereas we found that 2 µM of the same ODN is required for optimal PMN activation in classical plates. We can also not exclude that UV irradiation (and the induced ODN alterations) partially affects the capacity of the ODN to activate PMN. Likewise it is possible that ODN free in solution are simply more efficient in activating PMN than cross-linked ODN. By measuring the FITC fluorescence at all steps of the cross-linking procedure, we have clearly shown that the cross-linked ODN did not reach the endosomal TLR9. Activation could therefore only be achieved through the triggering of surface TLR9. Similarly to what we explained above, nitrocellulose and classical cell culture plates gave slightly different fluorescence values when measured with the fluorometer, explaining why values in the cell cultures and in the wash/supernatants are not perfectly additive. However, this approach evidenced that the cross-linked ODN was not taken up by PMN. The flow cytometric analysis of the FITC fluorescence confirmed that the free CpG-ODN reached the endosome, as evidenced by the increased signal intensity in the presence of ammonium chloride. On the contrary, a decrease in signal intensity was observed with the cross-linked ODN in the presence of ammonium chloride, indicating that this ODN only bound to the cell surface. Altogether, we have confirmed that the CpG-ODN used in the present study triggers only TLR9 and that this cross-linked ODN is cell-bound. Therefore, the signal triggered at the cell surface of human PMN by the cross-linked ODN is TLR9-mediated.

Results and discussion

We observed that intracellular TLR9 is up-regulated in some donors upon activation. TLR9 up-regulation occurred not only in PMN activated with CpG-ODN but also in the presence of LPS or R848. Consistently, previous studies have reported that intracellular TLR9 is up-regulated at the messenger RNA and protein expression levels in activated human immune cells [25] and in LPS-activated mouse MΦ [26]. On the contrary, nucleosome-stimulated PMN rather down-regulated intracellular TLR9. We also found that cell surface TLR9 was up-regulated in PMN activated with CpG-ODN or R848, although not in all donors. Up-regulation of cell surface TLR9 was less pronounced in LPS-activated PMN and was only observed in a few donors. In contrast, nucleosomes were the best inducers of surface TLR9 up-regulation. At 20 µg/ml nucleosomes, PMN strongly up-regulated surface TLR9 in most of the donors, which was associated with intracellular TLR9 down-regulation. Moreover, when surface TLR9 was not up-regulated, intracellular TLR9 was not down-regulated. Those results suggest that upon PMN activation, especially with nucleosomes, intracellular TLR9 may be translocated to the cell surface. In conclusion, CpG-ODN do not need to be accumulated in subcellular compartments for TLR9 binding and signaling in PMN. We suggest that surface TLR9 expression works as a rescue mechanism for PMN activation when pathogen-derived TLR9 ligands have no access to endosomal TLR9, e.g. when the ligand is membrane-bound and the pathogen can not be endocytosed, or when intracellular TLR9 is not activable. Alternatively, surface TLR9 may also be involved in the transport of extracellular TLR9 ligands to endosomes. Finally, the cell surface TLR9 may recognize ligands that normally do not activate intracellular TLR9 as previously reported for transfected cell lines [13]. Increased cell surface expression of TLR9 may be a pro-inflammatory activation marker during infectious or autoimmune diseases. Thus, identification of TLR9 cell surface expression in PMN is critical for our further understanding of the triggering and the regulation of inflammation, and might lead to the discovery of so far unknown TLR9 ligands specific for the cell surface receptor.

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Chapter VII

PD-1 expression is not sufficient to induce exhaustion of dendritic-like microglia in models of protein misfolding diseases

This chapter is derived from an article submitted for publication by Christian Thetard, Jens Schittenhelm, **Dennis Lindau**, Tasuku Honjo, Phillip Kahle, Thomas Gasser and Lars Stoltze.

The author of this thesis generated BM chimera leading to figure 3. He performed pilot experiments on the isolation of immune cells from the CNS and initiated the collaboration with the Neuropathology, Institute for Brain Research at the University of Tübingen. At the end he contributed to finalize the manuscript.

Abstract

Microglia cells are thought to play a pivotal role in cellular inflammation of protein misfolding diseases such as Alzheimer's and Parkinson's disease. Close examination of microglia subtypes during the development of α-synuclein (α-syn) as well as Aβ protein deposits revealed the parallel appearance of a dendritic-like microglia population, characterized by CD11c expression in mouse and human CNS. This cell type surrounds amyloid deposits and is also found at neighboring neurons containing α-syn, tau or transactive response DNA binding protein (TDP)-43 deposits. Furthermore, cells were characterized by expression of several regulatory molecules and the exclusive expression of PD-1 in the parenchymal microglia/MΦ CNS population. Regulatory surface molecules have been shown to control cellular inflammatory responses with PD-1 as major regulator for T cell responses in chronic infections. The emergence of such molecules on a protein misfolding-specific microglia population, suggests a tight regulation of inflammation in response to aggregation. Manipulating such regulation would be a promising target to influence these diseases. However, the single elimination of PD-1 in mice, as prime suspect for regulation, did not result in obvious changes of inflammation or dendritic-like microglia function. Thus, multiple regulatory molecules control the response of this protein misfolding-specific inflammatory cell type.

Introduction

Cellular inflammatory responses are usually stringently regulated to avoid overreaction, which may result in bystander damage or autoimmunity. Especially surface molecules reacting with specific ligands have been shown to regulate a cellular response by inhibitory or activating signals. Regulating signals may result from an active interaction or from the lack of this interaction between receptor and ligand [1]. In some instances activating and inhibiting signals balance the response of a certain cell type and may combine from several receptors, such that the cellular reaction depends on the relative strength of various signals. Classical examples for regulation by these mechanisms are NK or T cells, which are regulated by many different molecules [2, 3] and regulatory receptors include surface molecules containing intracellular ITIM (Immunoreceptor Tyrosine-based Inhibitory Motifs). One such ITIM-bearing molecule is PD-1, for which expression has been described on T cells, B cells, monocytes and DC in humans and T, B and stem cells in mice [4]. PD-1 interaction with its ligands PD-L1 and PD-L2 is highly important to maintain peripheral tolerance [5] and PD-1 expression during persistence of foreign antigens leads to immune exhaustion in T cells [6, 7]. This has been observed in many chronic viral infections such as HIV (Human immunodeficiency virus) and hepatitis C in humans [8, 9] or LCMV (Lymphocytic Choriomeningitis Virus) in mice [10]. The extent of cellular exhaustion may however, depend on the expression of further inhibitory molecules [11]. CNS immunity is more stringently regulated than in other organs to prevent damage to the sensitive environment, which is described as immune-privileged [12]. Still, in many CNS diseases inflammation is an important part in the course of disease. For example, CNS parenchymal protein misfolding leads to protein deposition and neuronal impairment. In most cases this aggregation is a slow progressive process with increasing pathology over years and neuroinflammation is associated with disease progression. This inflammation during neurodegeneration is characterized by strong microglia activation as well as astrocytosis [13]. The cause and effects of this inflammatory reaction is still under debate [14]. In the case of externally triggered immune responses in different animal models of various protein misfolding diseases, microglia and other immune effector mechanism showed effects against CNS-localized protein aggregation [15-18]. On the other hand, other data support the view that microglia and associated immune mechanisms contribute to neurodegeneration [19-21]. Due to the slow progression of CNS-protein misfolding the immune system might be challenged in a similar constant way as with chronic infection by foreign pathogens. To address the issue of microglia immune status during protein misfolding we characterized their reaction in mouse models to Aβ peptide and α-syn

aggregation, the most common misfolded proteins in AD and PD, respectively. This resulted in the identification of a new microglia subtype, appearing in parallel to the development of protein aggregate pathology, which expressed several regulatory molecules including the inhibitory regulator PD-1. The newly appearing microglia subtype was verified in four different human CNS-protein misfolding diseases, underlining the importance of the cell type. The effect of deleting the inhibitory PD-1 molecule was subsequently analyzed in a mouse model of Aβ aggregation.

Materials and methods

Mice. All animals used were housed in the animal facility of the Hertie-Institute for Clinical Brain Research under SPF conditions and experiments approved by the Regierungspräsidium Tübingen. Tg6799 [22], enhanced cyan fluorescent protein (eCFP)-tg [23] and C57BL/6 mice were obtained from The Jackson Laboratory, Bar Harbor, USA. (thy1)-h[A30P]SYN (i.e. the amplified human (WT)α-SYN-coding sequence with A30P mutation under control of a neuron specific Thy 1 promoter element) mice [24] were bread in-house and C57BL/6-PD-1$^{-/-}$ mice [25] were kindly provided by Tasuku Honjo (Kyoto University, Kyoto, Japan) and obtained from the RIKEN BioResource Center (Koyadai, Japan). Tg6799 mice were backcrossed to C57BL/6 mice, at N3 intercrossed with C57BL/6-PD-1$^{-/-}$ and analyzed at N5. Mice were genotyped by tail biopsy and polymerase chain reaction.

Patients. Human autopsy brains from histopathologically confirmed AD, PD, multiple system atrophy (MSA) and Frontotemporal lobar degeneration (FTLD) as well as age-related controls were obtained from the archives of the Neuropathology at the University Hospital Tübingen, which serves as local BrainBank Center for the German BrainNet. Use of the tissue specimen was approved by the local ethics committee. Autopsy was performed in a standardized fashion for relevant CNS structures according to the BrainNet Europe protocol for neurodegenerative diseases [26]. Detailed clinicopathological data of the cases are given in supplemental table S1.

Antibodies. All Ab used for flow cytometry and immunohistology are listed in table S2. Matthias Mack (University of Regensburg, Regensburg, Germany) and Howard Katz (Harvard Medical School, Boston, USA) kindly provided the Ab recognizing chemokine (C-C motif) receptor 2 (CCR2) and gp49B1 respectively. Anti-CCR2 was FITC (Molecular Probes) labeled by standard procedure.

CNS cell preparation and flow cytometry. CNS-cells were purified as previously described [27] with the following modifications. Mice were anesthetized by a mixture of Ketamin/Xylazin and perfused with ice-cold PBS. Removed tissue was mechanically dissociated by a razor blade, digested by collagenase/DNase and a single cell suspension generated by a 40 µm cell strainer (BD Bioscience). Immune cells were separated by Percoll purification and analyzed by flow cytometry in FCS/2 % BSA (bovine serum albumin). For flow cytometry Ethidium monoazide (EMA) (Invitrogen) was used for dead cell exclusion and blocking of FcR as well as Ab staining followed at 4 °C. Cells were measured on a CyAn™ ADP (Dako) and analyzed with Summit 4.4 software.

Immunohistochemistry. Methanol/2 % paraformaldehyde fixed 10 µm cryoconserved mouse tissue slices were incubated with primary Ab overnight at 4 °C in PBS/10 % FBS. After washing with Tween20 slices were incubated with secondary Ab for 90 min at 4 °C and developed with Vectastain ABC kits and Vector SG (Vector Laboratories) according to manufacturer's instruction. Congo red and Methyl Green staining was performed according to standard practice using 10 % (w/v) filtered Congo red dye cleared with alkaline alcohol or 0.5 % (w/v) Methyl Green dye dehydrated with ethanol. For double staining of human tissue formalin-fixed, paraffin-embedded tissue was immunostained with the Benchmark immunohistochemistry system (Ventana, Tucson, AZ) using a standardized protocol with slight optimizations for each Ab pair. In short, after heat pre-treatment and blocking of endogenous peroxidase, slides were incubated with rabbit anti-CD11c for 30 min, washed and stained by secondary swine anti-rabbit Ab (DakoCytomation). For visualization in brown, sections were incubated with I-View Streptavidin-horseradish peroxidase followed by I-View diaminobenzidine (Ventana). Slides were then again heat-pretreated, incubated with the second primary Ab (anti-Aβ(DR-1), anti-α-syn (KM51), 30 min; rabbit anti-tau, 20 min, rabbit anti-TDP-43, 20 min and methanol pre-treatment), washed and stained by a standard secondary Ab kit (Ventana). After signal amplification slides were developed with the FastRed staining kit (Ventana) according to manufacturers' recommendations before counterstaining with hemalaun.

Proteinase K (PK)-digested paraffin-embedded tissue (PET) blot. PK-PET-Blots were performed as previously described [24]. Briefly, 5-µm paraffin-embedded tissue sections were dried onto nitrocellulose membrane (Bio-Rad Laboratories Inc.), deparaffinized with xylol, 1:1 xylol/2-propanol and rehydrated in descending 2-propanol. Membranes were wetted in 0.1 % Tween-20/H20 and digested with 50 µg/ml

Results and discussion

PK (Roche, Germany). After Tris-buffered saline Tween-20) TBS-T (10 mM Tris-HCl, pH 7.8; 100 mM NaCl; 0.1 % Tween-20) wash and denaturation with 4 M guanidine isothiocyanate, membranes were blocked (0.2 % casein in TBS-T) and PK-resistant α-syn detected by 15G7 incubation o/n at 4 °C. 15G7 was detected by bridging rabbit anti-rat-IgG and affinity purified goat anti-rabbit-IgG for 1 h each at RT and signals developed by NTM/BCIP reaction (Roche). Membranes were embedded in Sylgard 184 Silicone Elastomer and evaluated by light microscope.

BM reconstitution. Newborn mice were conditioned with sub lethal (2 x 2 Gy) whole-body irradiation. Intrahepatic reconstitution with eCFP-tg BM in 25 μl PBS followed 2 h after last irradiation with a 30G syringe (Hamilton; Switzerland). Reconstitution in individual mice ranged from 26 to 70 %.

Ex vivo assays. FITC-β-Ala-Aβ (1-42) (Bachem, Switzerland) was aliquoted on ice to 400 μM in 0.1 % ammonium hydroxide, relyophilised and stored at -20 °C. For pre-aggregation one aliquot was diluted to 200 μM and incubate under shacking at 37 °C for 24 h (fAβ). For soluble Aβ one aliquot was thawed and dissolved prior to cell addition (sAβ). For Aβ uptake Percoll-purified cells from one hemisphere were EMA-stained and plated in a 24 well plate for 1.5 h in RPMI 1640 (2 % FCS, 1 % glutamine, 1 % penicillin/streptomycin) at 37 °C and from the second hemisphere for 1 h at 37 °C and pre-cooled for 30 min on ice. fAβ and sAβ were added at 1 μM and incubated for 20 min. The reaction was terminated by addition of ice-cold RPMI 1640 and directly centrifuged at 4 °C in 7 times the volume ice-cold RPMI 1640. Cells were transferred to 96 well plates, washed twice with PBS/2 % FBS and stained for flow cytometry. TNF production was determined with Percoll-purified cells from one hemisphere. Cells were incubated for 5 h in RPMI 1640 (10 % FBS, 1 % glutamine, 1 % Pen/Strep, 50 μM β-mercaptoethanol) at 37 °C in the presence of 3 μg/ml Brefeldin A. Cells were stained for surface molecules and permeabilized with fix/perm-buffer (eBioscience) for 1 h 4 °C. After washing and blocking with 2 % rat serum, cells were incubated with rat anti-TNF Ab and analyzed by flow cytometry. The second hemisphere was used for isotype staining.

Statistics. P values were calculated by the nonparametric Mann-Whitney-U-Test using GraphPad Prism5 software.

Results

Protein misfolding induces dendritic-like microglia

Tg6799 mice, which express APP with three FAD mutations (Swedish, Florida and London) and PS1 with two FAD mutations (M146L and L286V), develop an age-dependent gliosis which parallels Aβ deposition [22]. Detailed analysis of marker expression by flow cytometry (for gating scheme see figure S1) revealed a new appearing microglia type, which paralleled aggregate deposition (Fig. 1A-C). This population is characterized by being CD11b/CD11c double positive (Fig. 1A). CD45 expression is only intermediate on these cells (CD45^{int}), allowing further differentiation from standard MΦ (CD45^{high}) and conventional microglia (CD45^{low}) (Fig. 1B). Due to their expression of CD11c, a peripheral pan-DC marker, we termed this subpopulation dendritic-like microglia. When amyloid deposition has reached later stages CD11c$^+$CD45^{int} dendritic-like microglia represent more then 20 % of the parenchymal microglia/macrophage CNS population (defined as CD45$^+$CD11b$^+$) and is only marginally present in non-tg (ntg) controls (Fig. 1B). The appearance of dendritic-like microglia is not restricted to Aβ aggregation but is also found in the CNS of (thy1)-h[A30P]SYN mice (Fig. 1D), an established model for α-syn protein aggregation [24]. Similar to the tg6799 mice, dendritic-like microglia showed intermediate CD45 expression and increasing numbers in parallel to α-syn aggregation (Fig. 1E, F). After development of prominent pathology the increase in dendritic-like microglia was highly significant in both aggregation models for all mice analyzed (Fig. 1G, H).
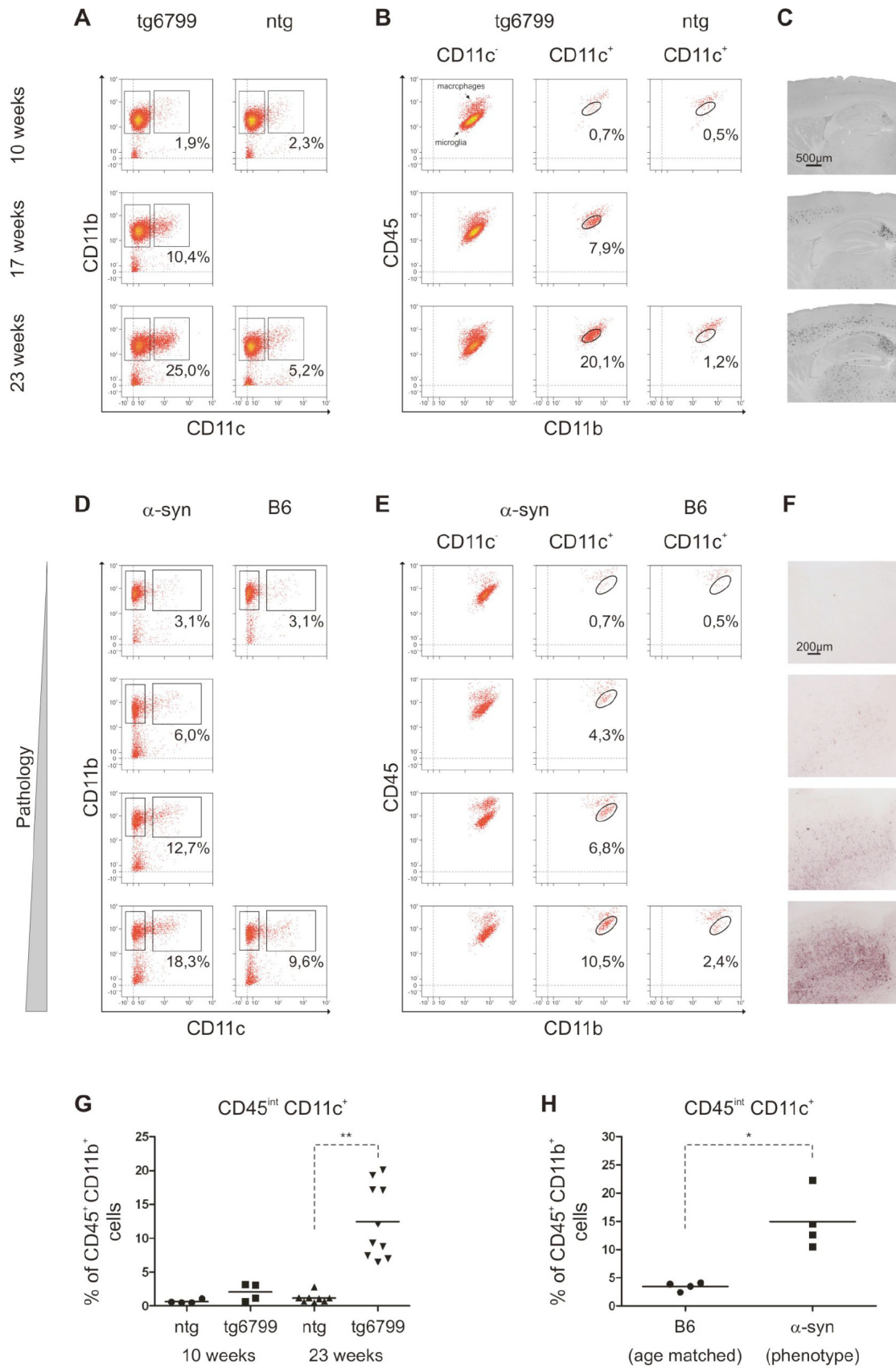
Figure 1. The appearance of dendritic-like microglia parallels protein aggregation. CNS-derived cells of the left brain hemisphere from 10, 17 and 23 week-old tg6799 and ntg littermate controls were analyzed for dendritic-like microglia. **(A)** The percentage within CD45$^+$CD11b$^+$ cells was determined for CD11b$^+$CD11c$^+$ cells and **(B)** CD45^{int}CD11b$^+$ CD11c$^+$ cells. Arrows indicate conventional microglia and MΦ. **(C)** In parallel the increasing Aβ aggregation was analyzed by DR-1 staining in the right brain hemisphere of the tg animals. CNS-derived cells of the left brain hemisphere from (thy1)-h[A30P]SYN mice and age-matched C57BL/6 mice were analyzed with increasing α-syn pathology for dendritic-like microglia. **(D)** The percentage within CD45$^+$CD11b$^+$ cells was determined for CD11b$^+$CD11c$^+$ cells and **(E)** CD45^{int}CD11b$^+$CD11c$^+$ cells, in comparison to **(F)** α-syn pathology determined in the inferior colliculs of the right brain hemisphere by PK-PET blot of the tg animals. One representative experiment of 3 (tg6799) and 2 (α-syn) time series is shown. **(G)** For 23 week-old tg6799 mice dendritic-like microglia increased highly significantly in comparison to ntg littermates for all mice analyzed. 10 week-old mice, n = 4 each and 23 week-old mice, n = 8 ntg and n = 10 for tg6799 mice. **(H)** All phenotypic (thy1)-h[A30P]SYN mice analyzed revealed also significantly more dendritic-like microglia compared to C57BL/6 age-matched controls. Both groups n = 4. Solid lines in G, H represent mean values, ** P < 0.01, * P < 0.05.

Dendritic-like microglia surround amyloid deposits and are CNS- derived

To obtain a first functional indication for dendritic-like microglia in protein misfolding, we determined their localization in the CNS parenchyma of tg6799 mice by immunohistochemistry (Fig. 2). CD11c$^+$ cells were only found in areas with amyloid deposition (Fig. 2A, B). Thereby, most if not all CD11c$^+$ cells were attached to deposits, but not all deposits had attached CD11c$^+$ cells (Fig. 2B). Thus, dendritic-like microglia seem to be attracted by CNS protein aggregates and they attempt to surround or shield these aggregates (Fig. 2C). Ntg mice and isotype controls showed no detectable signal for CD11c (data not shown).
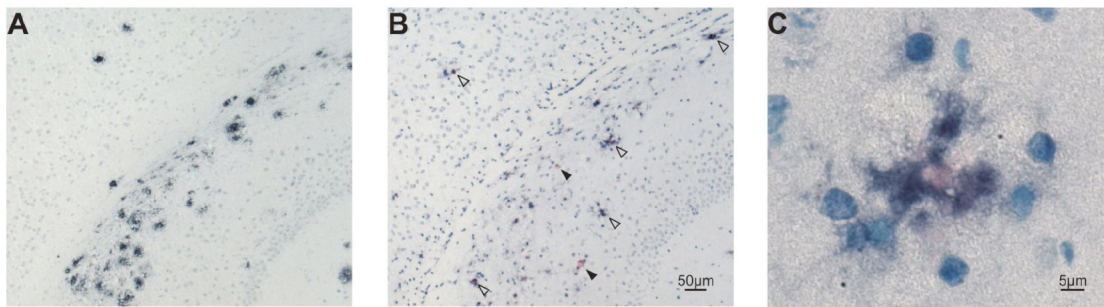
Figure 2. Enclosure of Aβ deposits by dendritic-like microglia. (A) Aβ aggregates were visualized by DR-1 staining on cryoconserved slices of a 23 week-old tg6799 mouse. **(B)** The adjacent slice depicts CD11c staining (black) and Congo red-stained amyloid, with cell nuclei counterstained by Methyl Green. Filled arrows indicate aggregates without CD11c staining and open arrows CD11c cells attached to aggregates. **(C)** An enlargement from B highlights CD11c cells surrounding a Congo red-stained aggregate. One representative mouse of 4 is shown.

Since MΦ/microglia from the periphery have been implicated in the removal of Aβ deposits [28], we addressed the issue of peripheral origin for the dendritic-like microglia by eCFP-tg BM transfer in newborn tg6799 mice. Our protocol of intrahepatic engraftment resulted in 19 week-old mice in a reconstitution level of 70 % for PBMC (Fig. 3A). CD8$^+$ T cells found in the CNS parenchyma showed the identical percentage of reconstitution with eCFP$^+$ cells (Fig. 3B) and the majority (at least 50 %) of the MΦ present in the CNS was of peripheral origin (Fig. 3C, D). As expected, conventional microglia remained eCFP negative and the dendritic-like microglia population entirely lacked eCFP expression in all mice investigated (Fig. 3E, F). Therefore, most if not all dendritic-like microglia are CNS-derived and not recruited from the periphery.
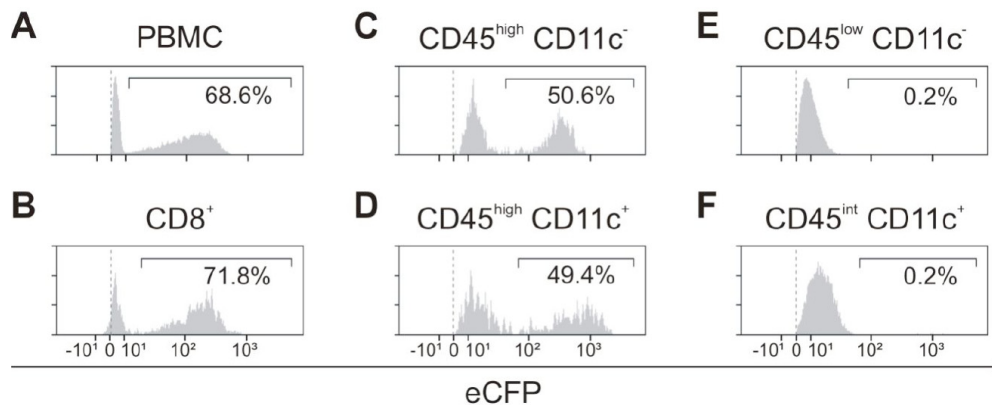
Figure 3. Dendritic-like microglia are not recruited from the periphery. Newborn tg6799 mice were sub lethally irradiated and intrahepatically reconstituted with eCFP-tg BM. 19 week-old mice were analyzed and depicted are the percentage of eCFP$^+$ cells for (A) PBMC, (B) CNS-derived CD8+ T cells as well as CD11b$^+$ cells which were (C) CD45^{high} CD11c$^-$ MΦ, (D) CD45^{high}CD11c$^+$ MΦ/DC, (E) CD45^{low}CD11c$^-$ conventional microglia and (F) CD45^{int}CD11c$^+$ dendritic-like microglia. Data are from one representative mouse. The experiment was performed twice independently with similar results per tg mouse and two litters, each with total n = 14 and 17.

Dendritic-like microglia are present in human protein misfolding diseases

To verify the relevance of dendritic-like microglia in human protein misfolding diseases, we analyzed CD11c expression in post-mortem human CNS tissue from four different neurodegenerative diseases (Fig. 4). For Alzheimer's patients, identical results were obtained as in the mouse model. All dendritic-like microglia in the neocortex were attached to Aβ deposits but not all deposits were surrounded by dendritic-like microglia (Fig. 4A, B). In humans infiltration of Aβ deposits by CD11c expressing cells was even more prominent than in mice (Fig. 4B). No Aβ staining and only occasionally single CD11c$^+$ cells were visible in neocortex of age related controls (Fig. 4C). Staining for tau protein and CD11c also revealed co-localization as well as tau positive NFT without the presence of CD11c$^+$ cells (Fig. 4D). CD11c$^+$ dendritic-like microglia were also visible in the substantia nigra of Parkinson's patients in close proximity to Lewy bodies (Fig. 4E), but absent in the substantia nigra of controls (Fig. 4F). However, co-localization of α-syn positive Lewy bodies and CD11c was not evident. CD11c appearance in the

Results and discussion

neocortex and especially cerebellum of MSA patients was more prominent than in PD (Fig. 4G, H). CD11c staining was always in close proximity of α-syn staining and direct contact was seen more often than in Parkinson but less than in Alzheimer patients. In FTLD a large number of CD11c^{+} cells appeared in close vicinity of TDP-43 positive inclusions in the hippocampus (Fig. 4I). In cases with associated motor neuron affection (FTLD-MND), CD11c^{+} cells directly contacted motor neurons with TDP-43 positive inclusions (Fig. 4J).
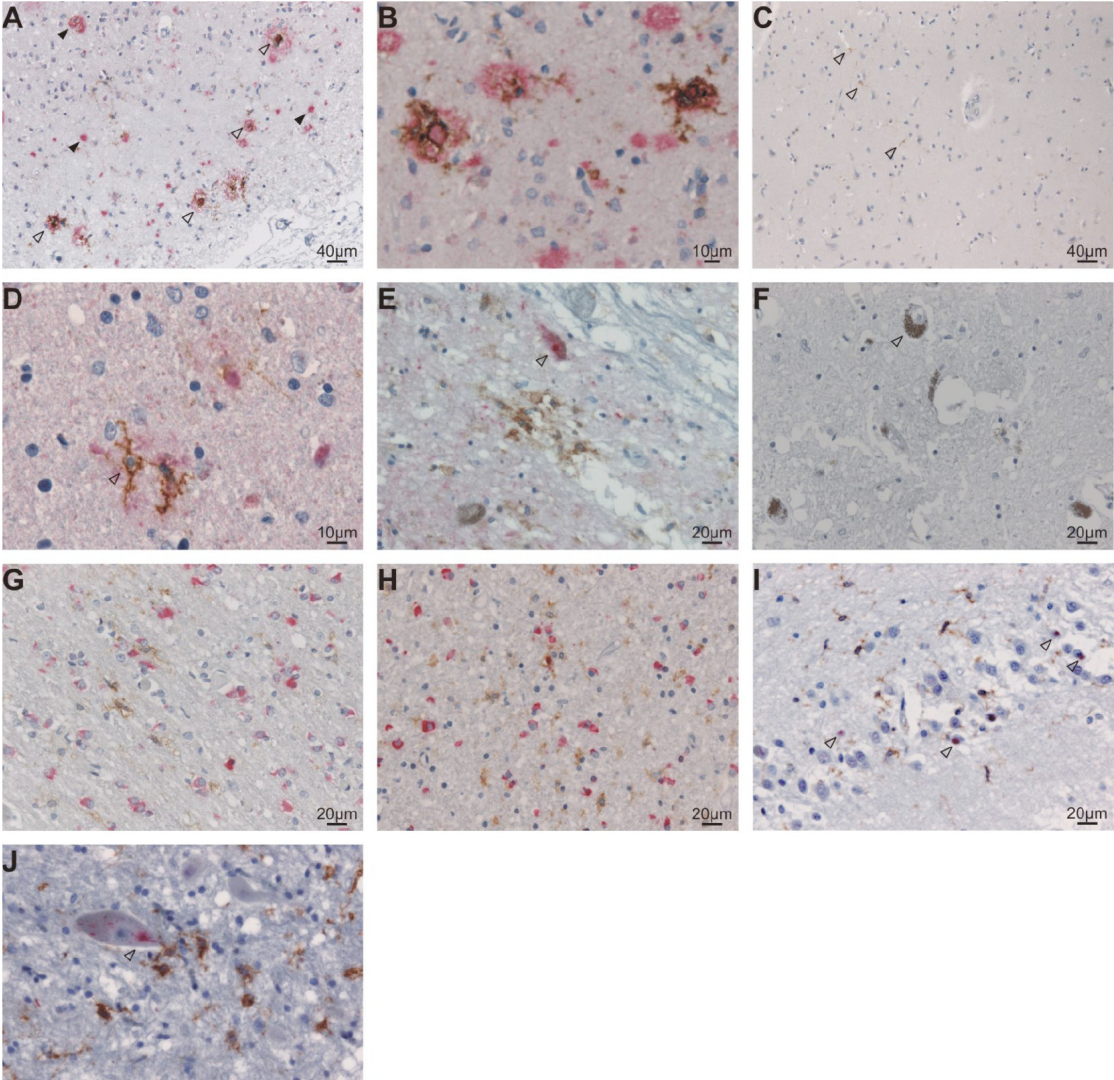
Figure 4. Dendritic-like microglia frequently exist in human protein misfolding diseases. Immunohistological staining of paraffin embedded human tissue identified CD11c$^+$ cells (brown) in four different neurodegenerative diseases. **(A)** In the cortex of Alzheimer's patients, amyloid deposits (red) without CD11c cells exist (filled arrows) but nearly all CD11c cells were attached to amyloid deposits (open arrows). **(B)** Infiltration of amyloid by CD11c cells was highly prominent in Alzheimer's patients. **(C)** In control cortex only few cells with faint CD11c signals could be detected (open arrows). **(D)** CD11c$^+$ cells were also found close to tau aggregates (red) in the cortex of Alzheimer's patients (open arrow). **(E)** α-syn positive Lewy bodies (red, open arrow) in the substantia nigra of Parkinson patients had CD11c$^+$ cells in their vicinity but rarely directly attached. **(F)** Control substantia nigra tissue revealed no CD11c positive cells in or around dopaminergic neurons (arrow). **(G)** The cortex and **(H)** cerebellum of MSA patients showed many CD11c$^+$ cells, in close proximity or directly attached to numerous α-syn positive glial inclusions (red). **(I)** In the granule cell layer of the dentate gyrus in the hippocampus of FTLD patients the majority of neurons with cytoplasmic TDP-43 positive inclusions (red) had CD11c$^+$ cells in close vicinity but not directly attached (open arrows). **(J)** A FTLD patient with additional motor neuron degeneration in the spinal cord (FTLD-MND) showed direct contact of CD11c$^+$ cells to the degenerated motor neuron with TDP-43 positive inclusions (open arrow). Tissue from representative cases are shown and similar results were obtained for n = 6 control, n = 10 Alzheimer, n = 5 Parkinson, n = 6 MSA, n = 3 FTLD patients.

Dendritic-like microglia exhibit high activity despite expression of multiple inhibitory surface markers

Phenotypical analysis of dendritic-like microglia in tg6799 mice revealed a more activated state compared to conventional microglia (Fig. 5). MHC class I and TLR2 were markedly increased, whereas CD86 and CCR2 (more pronounced in (thy1)-h[A30P]SYN mice, Fig. S2A) showed only a small increase in expression (Fig. 5A). No significant change was seen for CD80. MHC class II, Dec205 and CD40 were neither expressed on dendritic-like microglia nor conventional microglia. Because of their localization and reports of positive effects from CD11c$^+$ cells in models of protein misfolding [29, 30], we assumed that the dendritic-like microglia try to prevent aggregate growth. Obviously clearance is not sufficiently achieved and we hypothesized a restrained activation state for dendritic-like microglia. To verify this hypothesis the expression of several regulatory receptors with inhibitory potential for cellular immune responses was measured [31-35]. No signal on either cell type was obtained for CTLA-4 (Cytotoxic T-Lymphocyte Ag-4), BTLA (B and T lymphocyte

Results and discussion

attenuator), 2B4 or SiglecE (Fig. 5B). Expression of Tim-3 (T-cell immunoglobulin domain and mucin domain-3) increased on dendritic-like microglia and gp49, more specifically the inhibitory gp49B1, was only expressed on dendritic-like microglia (more pronounced in (thy1)-h[A30P]SYN mice, Fig. S2B). Most remarkable was the high expression of PD-1, which was completely absent on all other cell types analyzed (for phenotype of MΦ/DC populations see Fig. S3). Expression of PD-1 appeared to be constitutive with only a small proportion PD-1 negative at the beginning of dendritic-like microglia appearance. This constellation did not change over the time frame of aggregate development (Fig. S4). PD-1 appearance was further complemented by the expression of its ligand PD-L1 on half of the dendritic-like microglia (Fig. 5C, D) and some MΦ/DC (Fig. S3C), whereas all cell types were negative for PD-L2. Dendritic-like microglia induced by α-syn aggregation in (thy1)-h[A30P]SYN mice revealed an identical phenotype (Fig. S2). The expression of three receptors with inhibitory potential on dendritic-like microglia strengthened the notion of tightly regulated activity. Therefore, their ability to endocytose different aggregation states of FITC-labeled Aβ was tested in ex vivo assays. Surprisingly they were much more efficient than conventional microglia in the uptake of pre-aggregated Aβ at 37 °C in comparison to no internalization at 4 °C (Fig. 6A). In line with previously published data a freshly prepared solution of soluble Aβ was less efficiently internalized than pre-aggregated Aβ [36] but dendritic-like microglia again had a higher internalization rate compared to conventional microglia (Fig. 6A, B). Cytokine production was likewise more pronounced in dendritic-like microglia compared to conventional microglia as determined by TNF ex vivo staining (Fig. 6C). Again no difference was seen for dendritic-like microglia from (thy1)-h[A30P]SYN mice (data not shown).
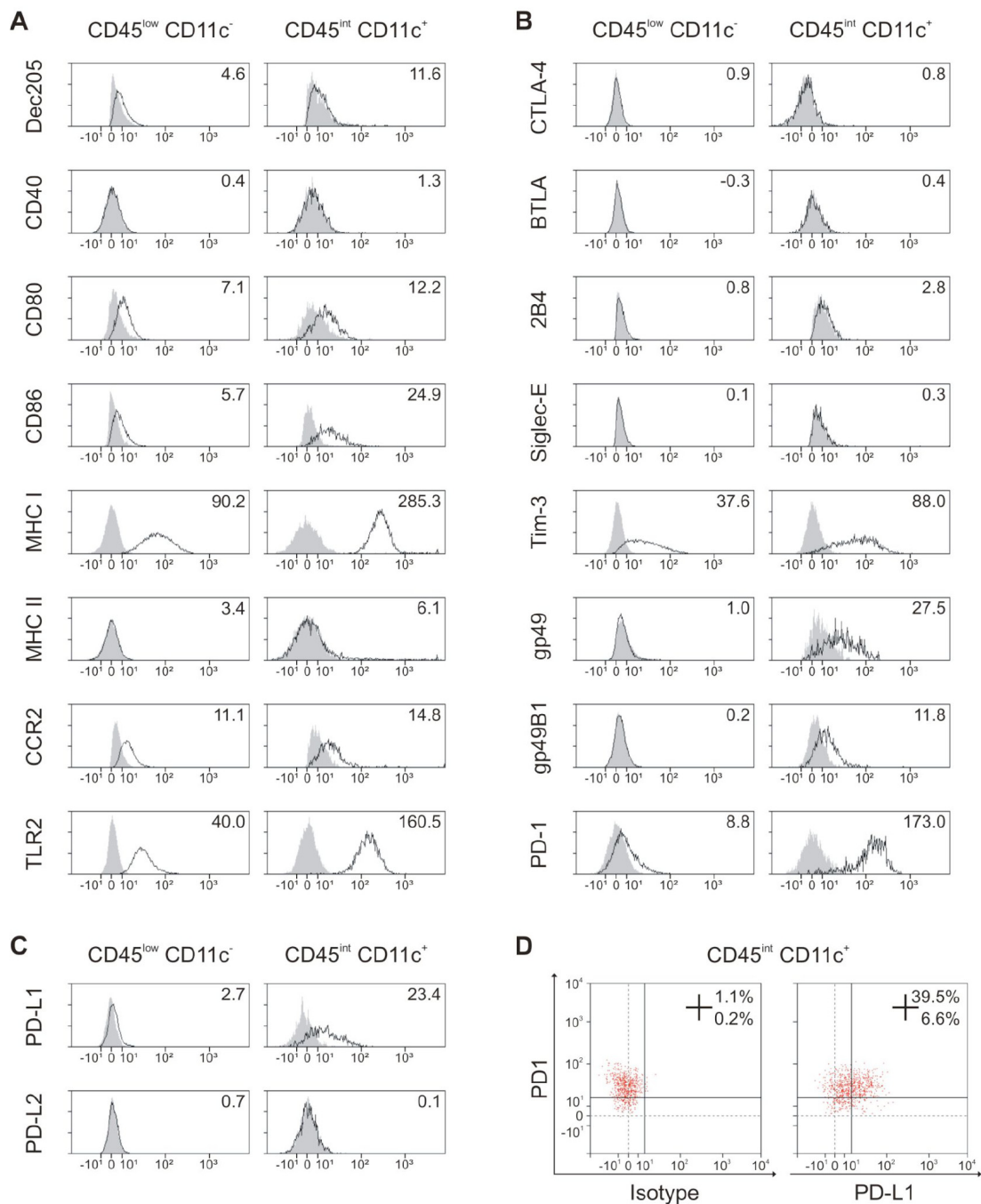
Figure 5. Activated phenotype and inhibitory markers on dendritic-like microglia. CD11b$^+$ CNS-derived cells of tg6799 mice were analyzed as CD45^{low}CD11c$^-$ conventional microglia and CD45^{int}CD11c$^+$ dendritic-like microglia. **(A)** Expression of indicated functional and **(B)** inhibitory surface marker, as well as **(C)** ligands for PD-1 and **(D)** the distribution of PD-L1 on CD45^{int}CD11c$^+$PD-1$^+$ dendritic-like microglia were examined. Numbers in A-C represent Δ (delta) MFI of isotype (gray area) and test signal (open area). Markers were measured at least twice.
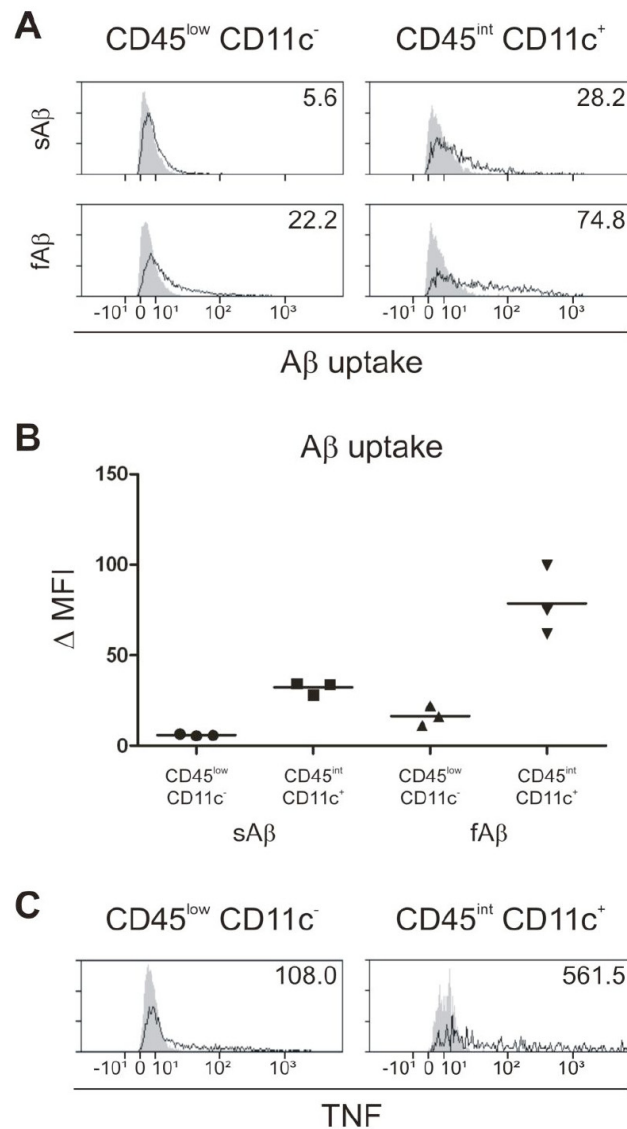
Figure 6. High activity of dendritic-like microglia. Aβ uptake and cytokine production activity was tested ex vivo on CD11b^{+} CNS-derived cells of tg6799 mice. Depicted are data for CD45^{low}CD11c^{-} conventional microglia and CD45^{int}CD11c^{+} dendritic-like microglia. (A) Uptake of FITC-labeled Aβ was measured for soluble (sAβ) and preaggregated fibrillar (fAβ) material. Numbers represent Δ MFI of 4 °C control (gray area) and uptake signal at 37 °C (open area). (B) Identical results were obtained in three independent experiments for sAβ and fAβ each. Solid lines indicate mean values. (C) Ex vivo TNF production was also higher in dendritic-like microglia compared to conventional microglia. Numbers represent Δ MFI of isotype (gray area) and TNF signal (open area). One representative mouse is shown from two independent experiments with similar results.

The sole elimination of PD-1 expression does not influence dendritic-like microglia function

Since PD-1 was the inhibitory marker expressed exclusively and very strongly by dendritic-like microglia, the effect of PD-1 deficiency on their functionality was analyzed. Tg6799 mice were crossed to PD-1-deficient mice and corresponding littermates analyzed. PD-1-deficient mice showed a clear increase in CNS-localized CD8$^+$ T cells, which was further enhanced by the presence of aggregating Aβ (Fig. 7A). All CD8$^+$ cells were exclusively CD3$^+$ (data not shown). In spite of this expected effect on T cells, the lack of PD-1 influenced neither the percentage nor the localization of dendritic-like microglia (Fig. 7B, C).
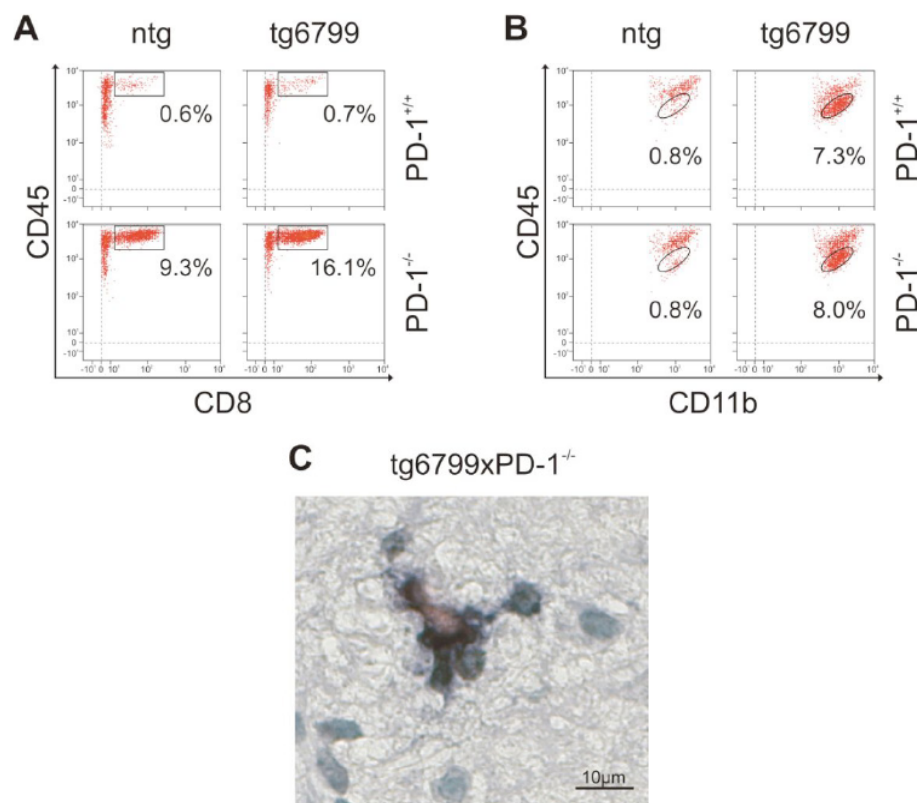
Figure 7. Dendritic-like microglia in PD-1-deficient mice. Tg6799 mice were intercrossed to PD-1-deficient mice and 23 week-old littermates analyzed. (A) CNS localized CD8$^+$ T cells increased in PD-1-deficient mice. This effect was further enhanced in tg6799xPD-1$^{-/-}$ mice. The percentage of CD45$^+$ cells is shown. (B) PD-1 deficiency did not influence dendritic-like microglia numbers in tg6799 mice. The percentage of CD45$^{low/int}$CD11b$^+$ cells is shown. (C) Their location around amyloid deposits in immunohistological staining was also not influenced. Black: CD11c staining, red: Congo red-stained Aβ.

Results and discussion

The kinetics and total amount of amyloid deposit development was also not obviously changed (Fig. 8A). This is in line with the observation that in terms of uptake for pre-aggregated Aβ or the production of TNF their functional activity was equally unaffected by lack of PD-1 (Fig. 8, B - D). Pre-aggregated Aβ uptake was independent of PD-1 genotype and was again much higher in dendritic-like microglia (Fig. 8C). TNF production was similarly more intense compared to conventional microglia (Fig. 8D).
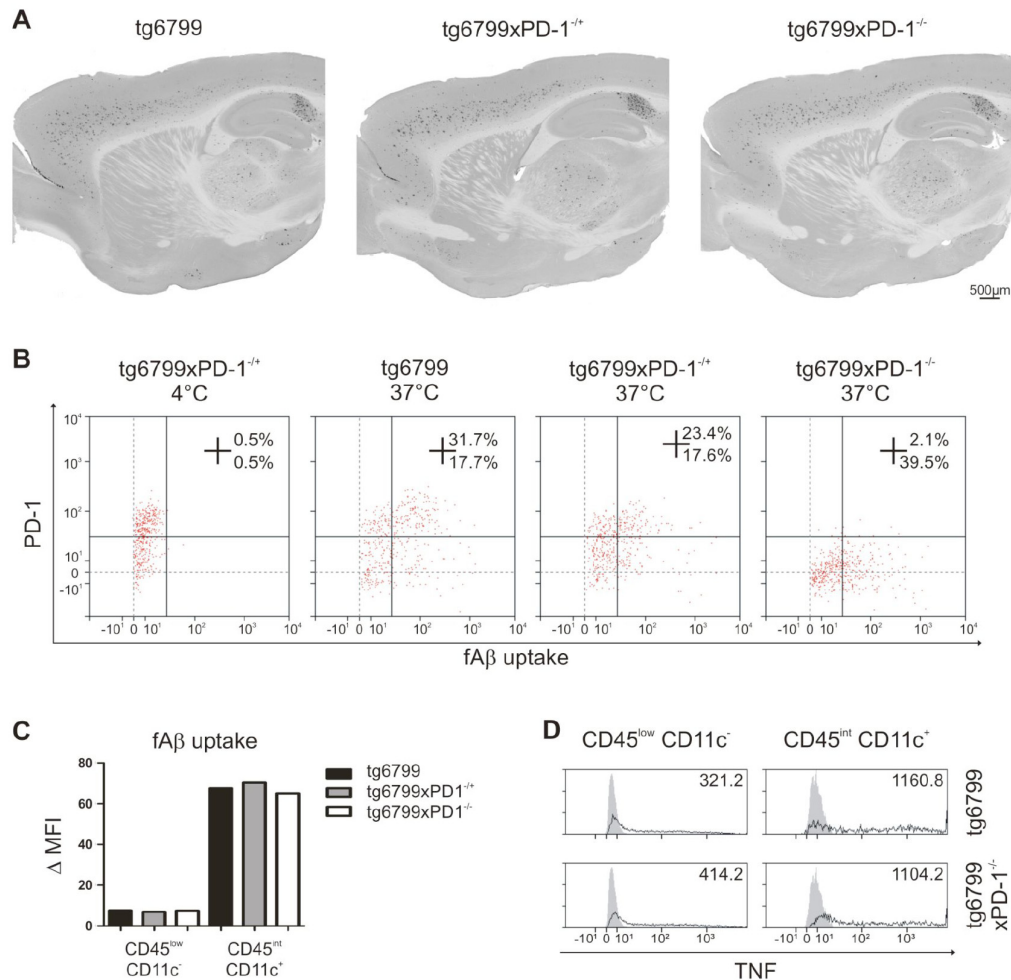
Figure 8. Activity of dendritic-like microglia in PD-1 deficient mice. Tg6799 mice were bred to PD-1 deficient mice and 23 week-old littermates analyzed. (A) The amount of DR-1-stained amyloid in the cortex and hippocampus of tg6799 mice was independent of PD-1 genotype status. (B) The percentage of dendritic-like microglia internalizing FITC-labeled fAβ in ex vivo assays was similar in tg6799 mice with different PD-1 genotypes. (C) Dendritic-like microglia (CD45^{int}CD11c$^+$) internalized also in tg6799xPD-1$^{-/-}$ mice more fAβ and (D) produce more ex vivo measured TNF compared to conventional microglia (CD45^{low}CD11c$^-$). Values in C and numbers in D represent Δ MFI of 4 °C control/isotype (gray area) and test signal (open area).

Discussion

Immune responses and inflammatory reactions are tightly regulated to avoid unspecific side effects on the organism. On the downside inhibitory regulative signals may leave existing immune effector mechanisms unable to attack and lead to the persistence of chronic infections [7, 37, 38] or prevent efficient cancer immunity [39]. Protein misfolding diseases of the CNS are slowly progressing in terms of pathology and inflammation development. Permanent exposure of new structures (aggregates) as well as self-Ag (cell damage) to immune cells shows similarity to chronic infection. The accompanying CNS-inflammation has demonstrated beneficial effects by external modification [15-18], which argues for a restrained state of the existing spontaneous immune response. These circumstances lead to the induction of the here described new microglia subtype, which expresses CD11c and several regulatory immune receptors. CD11c positive cells in the CNS have been described in mice and humans in response to infection, autoimmunity, injury or degeneration [40]. CNS-localized CD11c^{+} cells have been primarily attributed to peripheral origin [28, 30, 41]. This is in line with the observation, that in a model of multiple sclerosis (EAE) only minor numbers of the here described dendritic-like microglia (CD11c^{+}CD45^{int}) were found, with the majority of CD11c^{+} cells being CD45^{high} (data not shown). In contrast the dendritic-like microglia described here are clearly CNS-derived similar to CD11c^{+} microglia in a drug induced demyelination model [42]. This suggests that these cells develop either from CNS-resident microglia, which have the potential to up-regulate dendritic cell markers [43-45] or from a CD11c parenchymal CNS population, which is in normal adult mice difficult to detect [46, 47] and is present only in low numbers in normal aged human brain. The emergence and upregulation of dendritic-like microglia may contribute to protein profile changes of the total CNS CD11b population in $A\beta$ accumulating mice [48], but appears to be a response to general protein misfolding. The aggregating protein or its anatomic location in the CNS seems to be of minor importance, because pathology in α-syn-transgenic mice starts in the spinal cord and spreads further to the brain stem and midbrain, whereas in tg6799 mice protein aggregates are predominantly found in the hippocampus and the cortical areas. In previous studies CD11c^{+} cells were undetectable in untreated/unmodified $A\beta$ accumulating mice [29, 30], which might be due to the use of different mouse models. In spite of this, the identification of CD11c^{+} dendritic-like microglia in four human CNS-protein misfolding diseases and two different mouse models underlines the important relevance of these cells. Especially, these cells appear in response to different protein aggregates ($A\beta$, α-syn, tau or TDP-43) in diversely affected CNS regions. Dendritic-like

Results and discussion

microglia were always located in close proximity to protein aggregates suggesting direct attraction by aggregates or their local effects on neighboring cells. Their extent of direct aggregate penetration depends on the aggregating protein, which is very strong for Aβ but less frequent for more intracellular aggregating α-syn, tau or TDP-43. Here the cellular location of aggregates or their poly- or oligomeric state might be of importance for the dendritic-like microglia response. The functional state and number of CD11c$^+$ cells strongly influences CNS-inflammation [49-52]. According to their surface marker profile, dendritic-like microglia represent a more activated state compared to conventional microglia. MHC class I and CD86 can be used to communicate with CNS-infiltrating CTL, which increase later in pathology of tg6799 and (thy1)-h[A30P]SYN mice (data not shown) and which has also been suggested for CD11c$^+$ cells in a model of amyotrophic lateral sclerosis (ALS) [53]. CCR2 deficiency has been shown to affect amyloid pathology due to cellular mobilization impairment [54] and the slight increase of CCR2 on dendritic-like microglia may indicate movement to surround existing amyloid. MΦ seem to rely more on CCR2 (Fig. S3A), which is in agreement with their migratory behavior from the periphery. Likewise, it has been shown that TLR2 deficiency affects Aβ levels in mice, because TLR2 seems to be a receptor for Aβ clearance or Aβ-mediated microglia activation [55-58]. The high TLR2 level on dendritic-like microglia may thus account for their increased capability of Aβ uptake. CD40 ligand influences amyloidosis as well [59] but here all microglia appeared CD40-negative arguing for a different molecular or cellular target for CD40L. The high activity of dendritic-like microglia in terms of endocytosis and cytokine production stands in contrast to the expression of at least three inhibitory receptors. Especially PD-1 with its exclusive expression on dendritic-like microglia was expected to induce a negative signal. This was anticipated, because prolonged PD-1 expression induces functional T cells exhaustion [60, 61] prevents naïve T cell priming [62] and inhibits B cell signaling [63]. However, PD-1 deficiency seems not to affect dendritic-like microglia or the phenotype of tg6799 mice. Even though we have to restrict this conclusion to the activities and pathologic markers tested, several explanations exist for this observation. A first possibility would be no function at all for PD-1 on dendritic-like microglia. Due to the specific and rare expression of PD-1 described up to now, this seems to be highly unlikely. A second option could be that activating signals overcome the negative PD-1 signal, which would result in no phenotype for PD-1 deficiency. This situation would resemble the PD-1 expression on acute effector T cells [61] but one has to consider that dendritic-like microglia never down-regulate PD-1. A third option would be a defective signaling in the WT situation, so that a lack of PD-1 changes actually nothing. A fourth possibility takes into account the additional influence of other cell types

affected by PD-1 deficiency, such as CD8^{+} T cells. Their increasing numbers may directly affect dendritic-like microglia, due to their high MHC class I expression. We have also to consider that the model of tg6799 mice represents a very fast kinetic for amyloid development. Diminishing inhibitory signals for dendritic-like microglia may still not be sufficient to cope with this aggressive model. With the current knowledge of immune regulation, we favor the notion of complex control for dendritic-like microglia activation due to the additional expression of Tim-3 and gp49B1. Complex activity regulation represents an additional cellular control mechanism and exhausted T cells for example, for which PD-1 has a strong effect, express at least one or more additional regulatory molecules allowing for different exhaustion stages [11, 64]. Activating signals by TLR2 and other surface molecules may outbalance dendritic-like cell activation with regulatory signals of PD-1, Tim-3, gp49B1 and further here not tested molecules [65-67]. Tim-3 has been described as marker for exhausted T cells [68] and terminator of T cell immunity [69], trigger of MΦ [70] and DC [69] and implicated to mediate phagocytosis [71]. Whether Tim-3 contributes to TLR2 signaling on dendritic-like microglia or to restrained activity will be interesting to see. On the other hand the ITIM-containing gp49B1 (also termed Leukocyte Ig-like receptor B4 (LILRB4) or Ig-like transcript 3 (ILT3)) has been shown to negatively regulate the cytokine response of NK and T cells [72] as well as DC function [73]. The human counterpart is predominantly expressed on APC [74], decreases pro-inflammatory cytokine production in DC [75] and renders human monocytes and DC tolerogenic through CD8^{+} T cells [76]. Thus, it may act more synergistically with PD-1. Furthermore, a complexly regulated, restrained activation state of dendritic-like microglia would be in line with the observation, that microglia reduction has no obvious pathology-changing effect in another mouse model of Aβ aggregation [77]. Therefore, only the first steps have been initiated in understanding the complex regulatory mechanisms behind inflammation in protein misfolding diseases [30, 78] and more experiments are clearly necessary. Regulating such mechanisms, in either beneficial or detrimental function, will be an interesting objective for therapeutic intervention with the newly identified dendritic-like microglia as an attractive cellular target for protein misfolding diseases.

Results and discussion

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Online supplemental material

Supplemental Legends
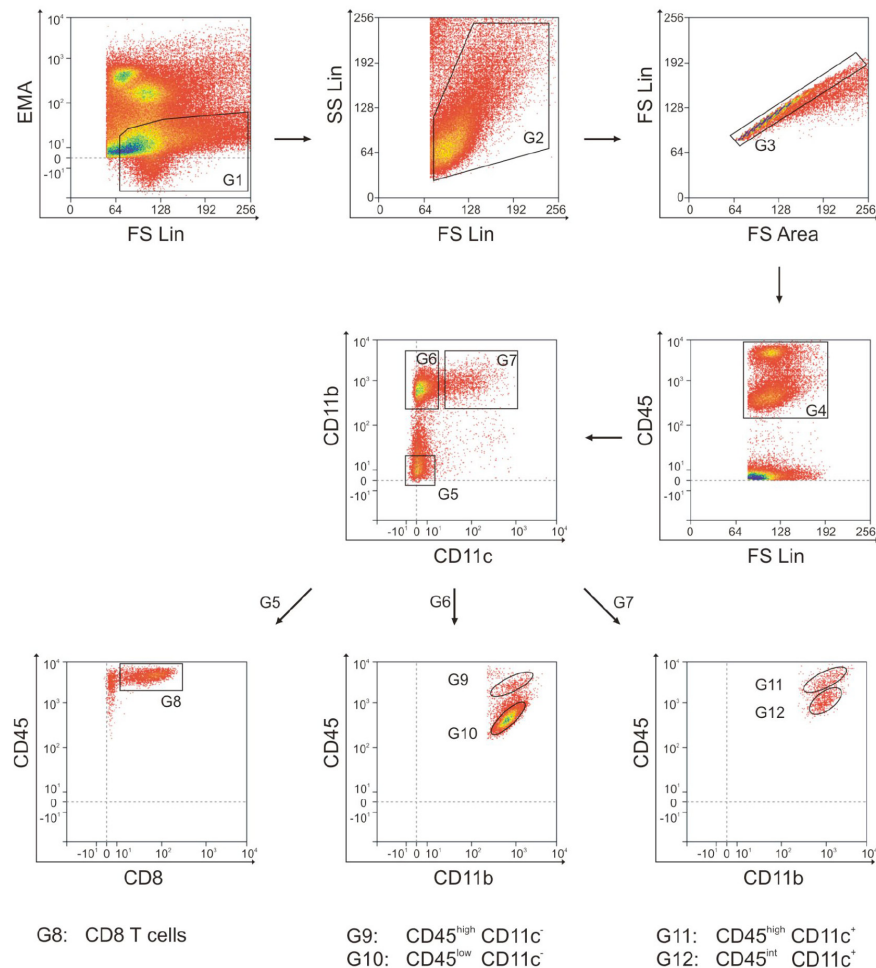
Figure S1. Flow cytometry gating scheme. CNS-derived and Percoll-purified cells were gated on living cells by excluding EMA$^+$ cells (G1). Appropriate Forward and Side scatter were chosen (G2) and doublets excluded (G3). CD45$^+$ cells were chosen to select for hematopoietic origin (G4). The expression of CD11b and CD11c defined three distinct populations. G5 (CD11b$^-$CD11c$^-$) contained by majority CD8$^+$ T cells (G8), G6 (CD11b$^+$CD11c$^-$) contained CD45^{low} conventional microglia (G10) and CD45^{high} MΦ (G9) and G7 (CD11b$^+$CD11c$^+$) CD45^{int} dendritic-like microglia (G12) and CD45^{high} MΦ/DC (G11). This scheme was used for all experiments.
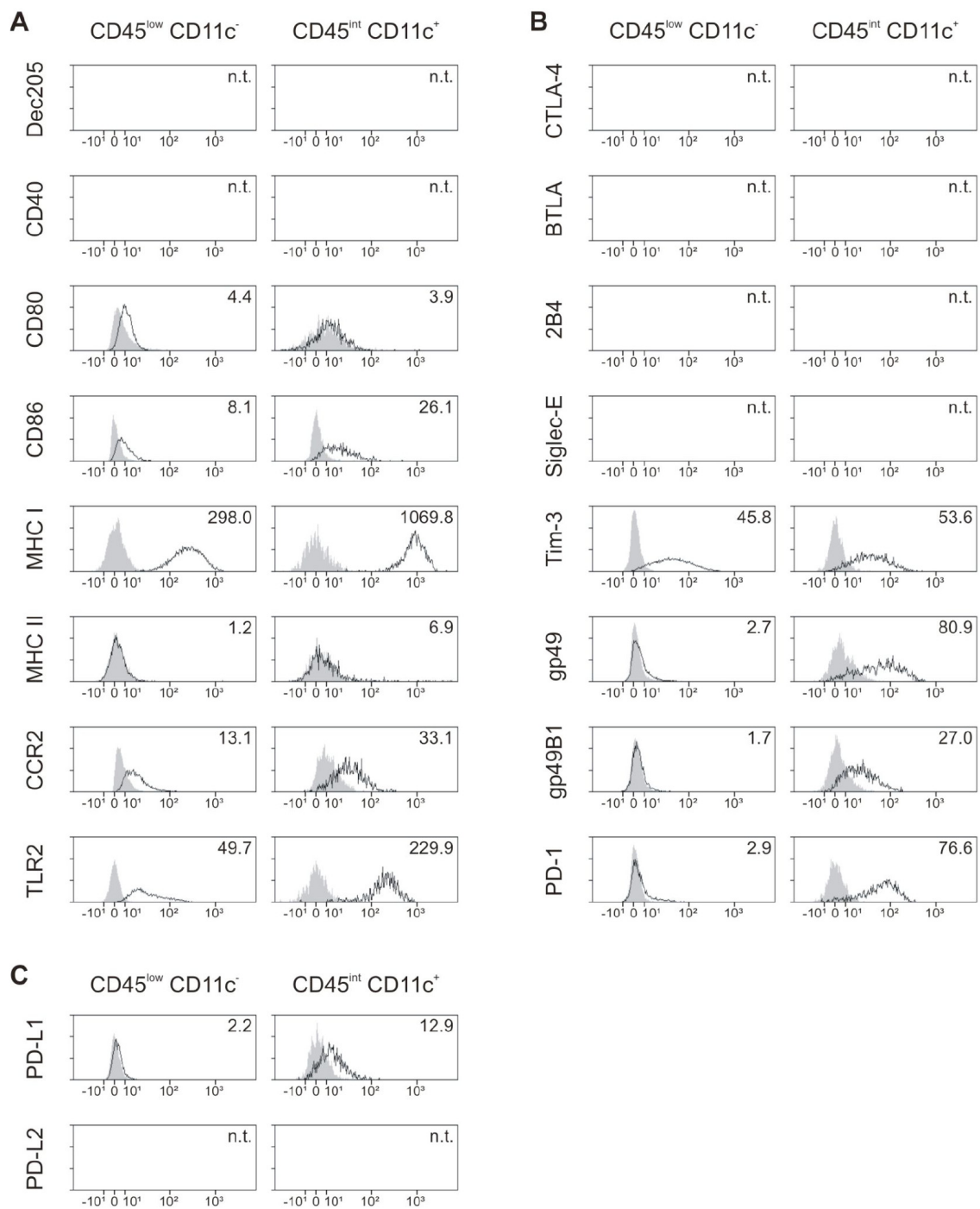
Figure S2. Activated phenotype and inhibitory markers on dendritic-like microglia. CD11b$^+$ CNS-derived cells of (thy1)-h[A30P]SYN mice were analyzed as CD45^{low}CD11c$^-$ conventional microglia and CD45^{int}CD11c$^+$ dendritic-like microglia. (A) The expressions of indicated functional and (B) inhibitory surface marker, as well as (C) ligands for PD-1 were examined. Numbers represent Δ MFI of isotype (gray area) and test signal (open area), n. t. = not tested. Markers were measured at least twice.
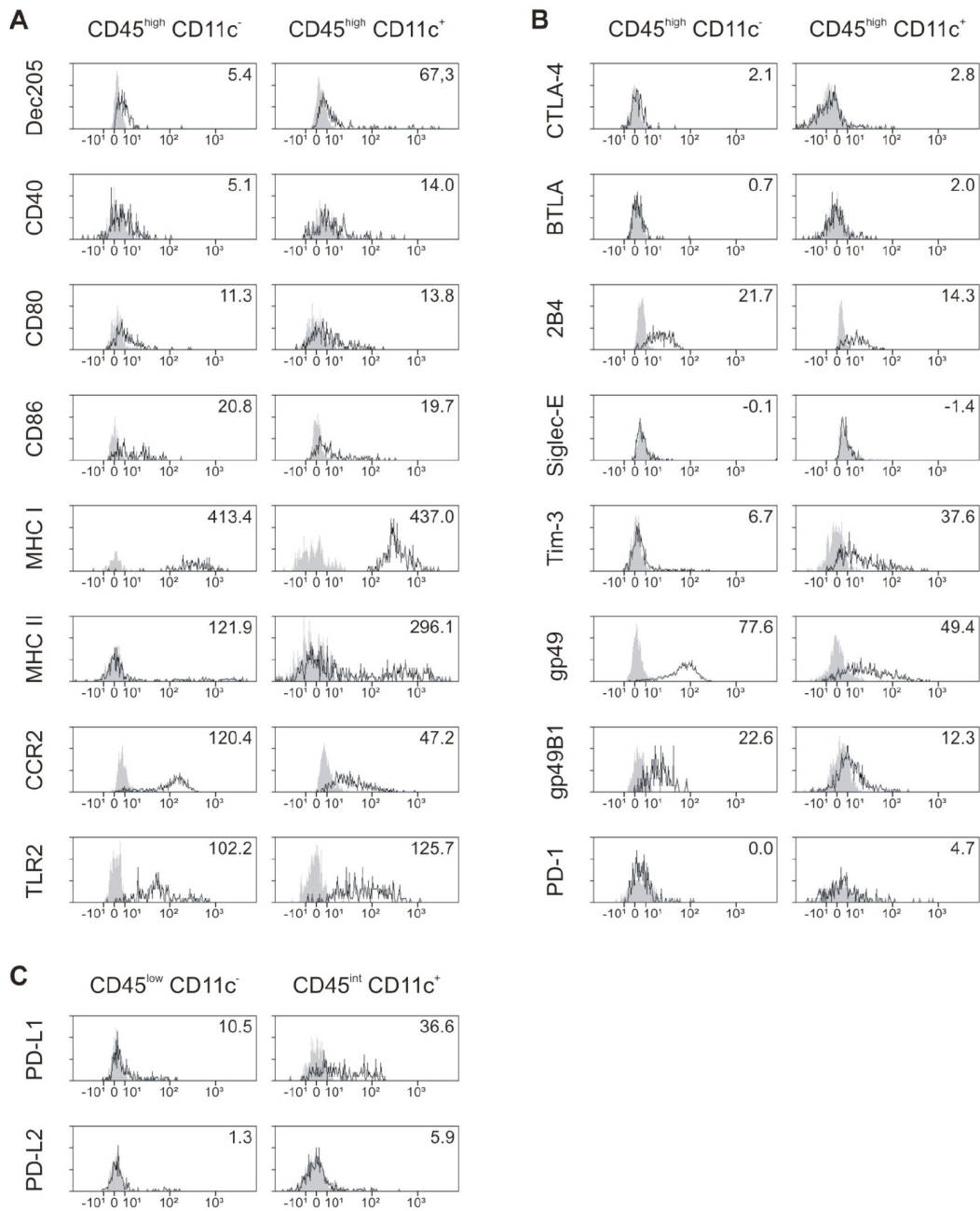
Figure S3. Phenotype of CNS MΦ/DC. CD11b$^+$ CNS-derived cells of tg6799 mice were analyzed as CD45^{high}CD11c$^-$ MΦ and CD45^{high}CD11c$^+$ activated MΦ or DC. (A) The expressions of indicated functional and (B) inhibitory surface marker, as well as (C) ligands for PD-1 were examined. Numbers represent Δ MFI of isotype (gray area) and test signal (open area). Markers were measured at least twice.

10 weeks **17 weeks** **23 weeks**

81.5% 71.4% 91.2%

PD-1
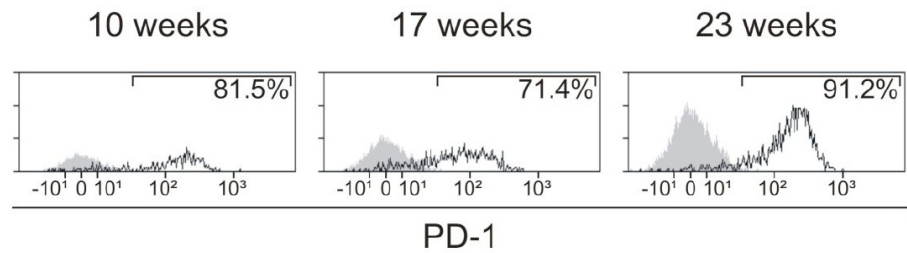
Figure S4. Constant PD-1 expression on dendritic-like microglia. The PD-1 expression kinetics on dendritic-like microglia of tg6799 mice was determined for 10, 17 and 23 week-old mice. The majority of cells stained PD-1$^+$ with constant levels over the indicated time frame. Gray area marks isotype controls. Data are from individual mice and the experiment was repeated three times with similar results.

Table S1. Characteristics of patients involved in the study. The nomenclature of frontotemporal lobar degeneration (FTLD)-patients follows reference [26]. MND = motor neuron degeneration, n = number of cases tested.

Disease	n	Age
Control	6	38 -100
Alzheimer	10	47 - 94
(Protein: Aβ, tau)		
Parkinson	5	67 - 79
(Protein: α-syn)		
Multiple system atrophy (MSA)	6	58 - 80
(Protein: α-syn)		
FTLD-TDP43 (type 1 and 3, type 2 with MND)	3	60 - 77
(Protein: TDP-43)		

Results and discussion

Table S2. Ab used for flow cytometry and immunohistology are listed.

Ag	Species	Label	Supplier	Clone
Anti-mouse				
CD8a	Rat	Pacific Orange	Caltag	5H10
CD11b	Rat	Pacific Blue	Caltag	M1/70.15
CD11c	Hamster	PE-Cy7	Biolegend	N418
	Hamster	FITC	Biolegend	N418
	Hamster	-	eBioscience	N418
CD16/CD32	Rat	-	eBioscience	93
CD40	Rat	PE	eBioscience	1C10
CD45	Rat	APC	Caltag	30-F11
CD80	Hamster	PE	eBioscience	16-10A1
CD85 (gp49)	Hamster	PE	eBioscience	H1.1
CD85k (gp49B1)	Rat	-	Howard Katz	B23.1
CD86	Rat	FITC	eBioscience	GL1
CD152 (CTLA-4)	Hamster	PE	Biolegend	UC10-4B9
CD170 (Siglec-E)	Rat	FITC	Biozol	8D2
CD244.2 (2B4)	Rat	FITC	eBioscience	eBio244F4
CD272 (BTLA)	Mouse	PE	eBioscience	8F4
CD273 (PD-L2)	Rat	PE	eBioscience	122
CD274 PD-L1)	Rat	PE	eBioscience	MIH5
CD279 (PD-1)	Rat	PE	eBioscience	RMP1-30
CD282 (TLR2)	Rat	PE	eBioscience	6C2
CCR2	Rat	FITC	Matthias Mack	MC-21
Tim-3	Rat	PE	Biolegend	RMT3-23
H-2Kb / H-2Db	Mouse	PE	Biolegend	28-8 6
I-Ab	Mouse	PE	Biolegend	AF6-120.1
TNF	Rat	Alexa Flour 488	eBioscience	MP6-XT22
Anti-human				
CD11c	Rabbit	-	Abcam	EP1347Y
Aβ	Rabbit	-	Innovagen AB	DR1-Polyclonal
	Mouse	-	Covance	6E10
tau	Rabbit	-	Dako	Polyclonal
α-syn	Mouse	-	Novocastra	KM51
	Rabbit	-	In house	15G7
TDP-43	Rabbit	-	Proteintech	Polyclonal
Secondary Ab				
Hamster IgG	Goat	Biotin	Vector Laboratories	Polyclonal
Rabbit IgG	Goat	Biotin	Vector Laboratories	Polyclonal
Mouse IgG	Goat	Biotin	Ventana	Polyclonal
Rabbit IgG	Goat	AP	Dako	Polyclonal
Rat IgG	Rabbit	-	Dako	Polyclonal
Rat IgG	Swine	Biotin	Dako	Polyclonal
Rat IgM	Mouse	PE	eBioscience	HIS40

Conclusions

Chronic disorders have major medical, social and economical consequences. It has been estimated that they account for 60 % of all deaths per annum worldwide and in regions with a Western lifestyle, such as Europe, this figure is as high as 86 % [1]. In addition to personal suffering of those affected, the economic impact of chronic diseases is truly staggering. The annual impact on the American economy of the most common chronic diseases is calculated to be more than US$ 1 trillion, and this is expected to escalate to nearly US$ 6 trillion by the middle of the century [2]. SLE and AD are described as chronic disorders associated with abiding inflammation and tissue damage, caused by several different autoAg. Discrimination of self and non-self is based on both structural features of the autoAg as well as on the context in which it is encountered. Unraveling the intricate relationships between the effectors of immunity and autoAg by immunological research continues to be a serious scientific challenge paving the way for the development of new disease-modifying strategies and therapeutic interventions. These novel research avenues require the integration and evaluation of comparative in detail analyses in well defined experimental systems as well as translational approaches to human disorders. These latter points were the common aim of this PhD thesis. It sheds new light on important aspects of inflammation: significance of immune receptors, autoAg-induced activation of immune cells and tissue-specific autoimmunity in the context of SLE and AD.

In SLE, the nucleosome composed of DNA and histone proteins, has been suggested to play a key role as a major autoAg in disease development [3]. Indeed, levels of circulating nucleosomes correlate with the SLEDAI and increased concentrations are associated with the development of central and peripheral nervous manifestations [4]. Nucleosome-restricted autoAb are observed in patients suffering from SLE and strongly correlate with SLEDAI scores (reviewed in [5]). We have previously shown that nucleosomes activate PMN in vivo and in vitro in an HMGB1-independent manner but the triggering pathway involved is unknown [6]. Both normal PMN and PMN from SLE patients were sensitive to nucleosome-induced activation. Whereas most studies analyzed the effects of chromatin-containing IC, we focused on free nucleosomes. Since nucleosomes are partly composed of DNA, we have investigated whether TLR9 was required for nucleosome-induced PMN activation. Whether TLR9 is involved or not is of prime importance since the role of TLR9 in SLE development is still controversial.

Conclusions

We show that circulating nucleosomes from SLE plasmas trigger innate immunity independently of TLR9, leading to the secretion of pro-inflammatory cytokines by PMN and CD11b up-regulation. Indeed, nucleosomes activate human PMN independently of the presence of unmethylated CpG-motifs in nucleosomal DNA and activation does not require endosomal acidification and in not impaired in the presence of a TLR9 inhibitor. Moreover, both PMN isolated from WT and TLR9-KO mice were activated by nucleosomes. Although TLR9 is not required for nucleosome-mediated PMN activation, nucleosomes induce the up-regulation of TLR9 in some donors. Finally, nucleosomes are endocytosed by PMN and accumulate in the cytoplasm but are not translocated to the nucleus. Importantly, nucleosomes act as free complexes without the need for IC formation. The role played by TLR9 during SLE development is still not elucidated. TLR9 has been shown to be activated by unmethylated CpG-motifs present in pathogen-derived DNA. Nevertheless, natural phosphodiester ODN lacking CpG-motifs and even vertebrate or mammalian DNA can trigger TLR9 under certain circumstances, although TLR9-independent pathways are also triggered in addition. Whereas TLR9 is required for anti-nucleosomal autoAb production, TLR9 was also suggested to protect against SLE. However, other receptors than TLR9 have been found to recognize DNA and/or nucleosomes and cytosolic receptors able to recognize DNA have been reported [7]. Therefore it will be of interest determine the role of AIM-2 or DAI (ZBP-1) in PMN towards the innate response to nucleosomes. New functions have been attributed to autoAg, such as activation of chemokine receptors on immature DC. Likewise, increasing efforts are concentrating on the involvement of the innate immune system in SLE development. PMN play an important role in innate immunity and are the first cells recruited at inflammation sites [8]. They have been suggested to link innate and adaptive immunity by influencing DC and PMN activation may favor adaptive immunity. Nucleosome-induced PMN activation may thus partly explain why the peripheral tolerance is broken in SLE patients. Our results support the key role of nucleosomes in SLE and suggest that nucleosomes are not only passive targets for autoAb but may play an active role in SLE pathogenesis. TLR9 may thus be differently required in the triggering of nucleosome-induced innate immunity and anti-nucleosome B cell autoimmunity. So far, most studies investigated autoAb secretion against this autoAg and disease development but did not analyze nucleosomes as a potential trigger of innate immune responses. The induction of a pro-inflammatory context by nucleosomes had not been explored as yet. Preliminary results in our group indicate that the nucleosome belongs to the group of IFN-α-inducing factors in SLE. At present, these factors are described as small IC with anti-DNA specificity and DNA as essential components [9]. Thus, we have identified for the first time that nucleosomes

without the need for IC-formation have the capacity to induce IFN-α secretion in PMN. Therefore it is not surprising that the interest in these immune cells emerges. NET formation by PMN and its immunological activity is currently an interesting issue to be investigated in disease treatment [10] and chronic autoinflammatory conditions [11]. In addition, we report the startling finding that primary PMN express functional TLR9 at the cell surface. This discovery generates new clues to the TLR9 biology since scientists had long thought that this PRR only resides in endolysosomal compartments. This mechanism may predispose PMN critically to activation in the presence of microbial products making them more susceptible to aberrant reactions turning onto self. Cell surface TLR9 may recognize different ligands as its intracellular counterpart responsible for the induction of SLE. Interestingly, the intracellular localization of nucleic-acid sensing TLR does not seem to apply for all cell types equally. For example, TLR3 is predominantly expressed on the cell surface of astrocytes [12], but intracellularly in DC [13]. This suggests that the innate immune response is not homogeneous but rather tailored according to cell type and the environmental signal.

The leading hypothesis in AD is that an imbalance between the production and clearance of Aβ leads to its accumulation in senile plaques, which in turn promotes disease progression. However, this theory has not yet given rise to effective drugs [14]. Recent clinical trials have produced disappointing results to the treatment of AD, and have given rise to the sobering realization that the majority of vaccines currently under development are unlikely to provide a truly effective cure [15, 16]. Hence, there is now a broad consensus that the field needs to return to some fundamentals. Bridging the physiological and pathological role of Aβ, and its interaction with the immune system is of most interest [17]. It is has recently be suggested that Aβ serves the physiological function of fighting off microbes as part of the body's innate immune system [18]. In addition studies concentrating into the role of CNS-resident microglia and peripheral MΦ receive much attention in AD. Certainly the understanding of neurodegeneration would have been limited without the creation of mouse models, but research in APP-tg mice can also be controversial and is largely depending on the mutant used. Worthy of note is that an obscure list of molecules, ranging from cholesterol drugs and painkillers to blueberries and curry spice, has been shown to reduce pathology in APP-tg mice [19-24]. Thus, the rumors become louder and were already summarized by the editors of Nature Medicine that investigations in the causes and treatment of AD yield one culprit time and time again using rodents [25, 26]. Most of the currently used mouse models never exhibit the extent of neurodegeneration seen in people afflicted with AD. Because these mice are based on FAD mutations, it may be that they only model the

Conclusions

rare form of the disease and not the more common SAD [26]. More importantly, these mice have never shed much light on the mechanisms involved in the disease, such as the relationship between Aβ and tau [25, 26]. Evidence now supports the idea that both protein species actually define, but do not fully represent the disease process [27]. Maybe there has been too much focus on early events of the amyloid cascade [14, 26], exaggerating research novelty and boosting apparent significance [27], e.g. prion-like mechanisms in AD nowadays. Modern tools of genetic approaches may indicate that microglia do not play a role in Aβ clearance in mice [28] but at a time of self-criticism one should remember that research dating back to the 1990s already implicating that microglia in vivo cannot phagocytose Aβ in humans [29]. Progressive AD further involves CNS inflammation, neuronal, axonal and synaptic loss as well as dysfunction, which are modeled inadequately in APP-tg mice [30]. The AN1792 trial has best illustrated that many scientists run risk of overlooking aspects of human immunology that do not manifest, or cannot be replicated, in APP-tg mice [14-16]. The differences between mouse and human immunology should always be kept in mind when using mice as preclinical model of human disease as 65 million years of evolution might suggest [31, 32]. One may also criticize if an immunologically humanized AD mouse model would be the correct choice to get off these limits [33]. Encounters with Ag activate T cells in these mice [34], but questions remain as to the consequences of these interactions, in particular, whether these lymphocytes are selected and restricted to mouse or human MHC. This is an important issue, as obvious future applications of humanized animal models might include screening of candidate vaccines [35]. To provoke new lines of discussion novel techniques and experimental strategies have to be implemented in AD research. The existing AD mice begin to allow investigations of human pathology in a small animal model, but the current system is not ideal. The extreme overexpression of mutated transgenes forces aberrant reactions likely to be misinterpreted in comparison to the real life situation. For example there is compelling evidence that one consequence of FAD mutations is dependent upon microglia, since when these cells express mutant forms of PS1 they alter the fate of neural progenitor cells [36]. Senile plaques are only one hallmark of AD, thus further investigations into the cascade of upcoming events, synergistic effects of Aβ and tau, different synaptic protein isoforms and mitochondrial impairment are of major interest. And while we are struggling for new ideas, simply taking an extra pot of coffee [37] after a nap [38] and calling our friends using the mobile phone [39] could already help to reduce the Aβ plaque load and the cognitive impairment seen in AD. But again, since most of these results were obtained in APP-tg mice, it may be difficult to extrapolate these findings to human beings.

For all that better models of AD are needed that are evolutionary closer to humans. It is unnecessary to mention that disorders of higher brain functions are especially challenging to model. Consequently non-human primates might offer an alternative to kick AD research into a new direction [40-44].

Yang et al. have already established a genetically mutated non-human primate model of a neurodegenerative disease [41]. Furthermore cerebral amyloid-β protein develops upon aging in ntg monkeys [42] and their usage is highly discussed [43].

Meanwhile the disadvantages of long-lived non-human primates are disabled because advanced technologies become available to generate small monkeys that pass a transgene on to their offspring [40]. These monkeys are short-lived and show genetic homogeneity which makes them a powerful tool to study neurodegenerative diseases. In this light current research already indicates an important role of vervet monkeys in a series of novel therapeutic strategies [44], showing that naturally occurring autoAb specific for known amyloidogenic species can protect primary neurons from Aβ toxicity.

Thus, helping to gain true knowledge is of major interest rather than pretending that primates are not used in AD research. Surely, it should be possible to do better than that and some scientists do so by providing alternatives which avoid ethical constraints. Induced pluripotent stem (iPS) may offer a more sophisticated strategy for modeling AD. Recent studies have reported the derivation and differentiation of patient-specific iPS cells. Disease-related phenotypes, the ability to model neuropathology and its treatment in iPS cells have been shown [45]. Creating human iPS cells gets easier [46] and patient-specific iPS cells are currently established to study AD, PD and ALS [Harald Neumann, University of Bonn, personal communication]. This may represent an even more powerful and elegant model system than animals, and an approach to produce first relevant functional assays for the identification of candidate drugs. Human disease experimentation suffers necessarily from bioethical restrictions and from the lack of appropriate predictive model systems. To this end, mice will continue to be one model of AD up to a certain degree but with patent defects. Nevertheless, experiments with mice may gain refreshment from time to time when they show a clear convergence between animal models and human data. We were luckily able to tell such a story by showing that a new phenotype of CNS-resident microglia is present in several human proteopathies, suggesting a tight regulation of inflammation in response to aggregation (Fig. 1). Multiple regulatory molecules control the response of this protein misfolding-specific inflammatory cell type and manipulating such regulation would be a promising

Conclusions

target to influence these diseases. Indeed, inflammatory events may help CNS-resident phagocytes to clear aggregates in humans and APP-tg mice [47]. While these innate immune responses receive much interest in AD reserach, the relevance of invading T cells into the CNS is largely unknown. To this end future directions will not only concentrate on further regulatory functions of CNS-phagocytes but also on the involvement of adaptive immunity in AD. Identifying the role of T cells in a beneficial versus detrimental dialogue during the course of AD would be of special interest in humans. If these immune cells are playing any active role in any neurodegenerative disease then enhancing favorable behaviors or suppressing harmful ones may be capable of slowing the progression of the disease. Even if the immune response cannot be manipulated by a therapeutic intervention, its derangements could perhaps be used as markers of diagnosis or disease progression [48].
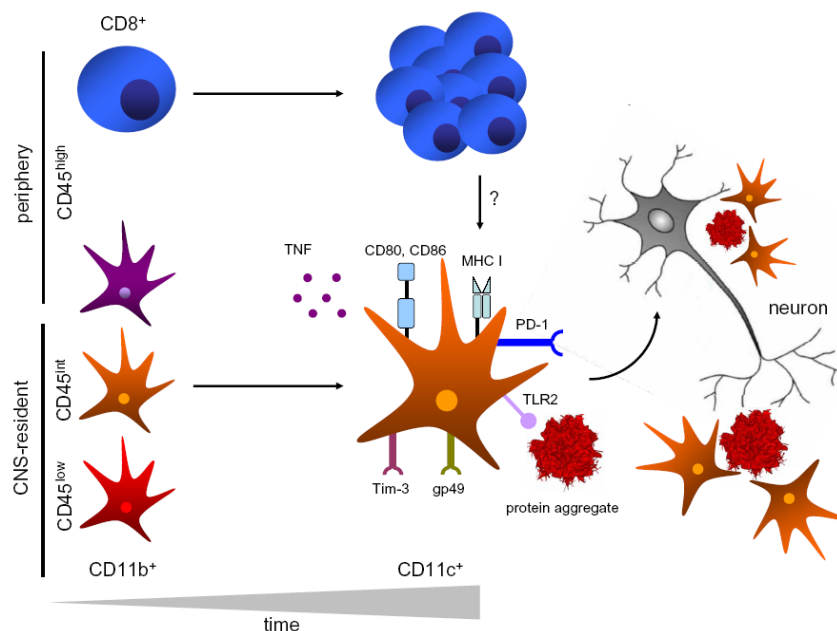
Figure 1. Immune cells present in the proteopathic CNS. Different hematopoietic cells were identified within the CNS of Aβ and α-syn depositing mice. Besides infiltrating CTL, peripheral MΦ and conventional microglia, a new subtype of microglia which we termed dendritic-like microglia was identified. These cells were even more pronounced in human cases of AD and most interestingly also found in other proteopathies. Highly activated dendritic-like microglia cells accumulated together with CTL upon aging and increasing pathology in the CNS. Enhanced expression of MHC class I, CD80 and CD86 on these cells suggests the possibility of communication with CNS-infiltrating CTL, but this assumption needs further proof. Although the dendritic-like microglia showed a very high activity of phagocytosing Aβ ex vivo, this ability could not be restored by deleting the expression of the inhibitory PD-1 gene, which suggests a more complex situation in vivo.

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Future directions

Nucleosome-activated PMN represent a new source of IFN-α

IFN-α has received particular attention for its disease-promoting role in SLE, although the triggering stimulus in its secretion has not been explored [1]. In a first series of experiments we have induced IFN-α secretion by PMN, pDC and monocytes cultured with nucleosomes, which represents a previously unknown pathway of innate immunity (Fig. 1A-C). It is anticipated to confirm these results and to evaluate the mechanism.
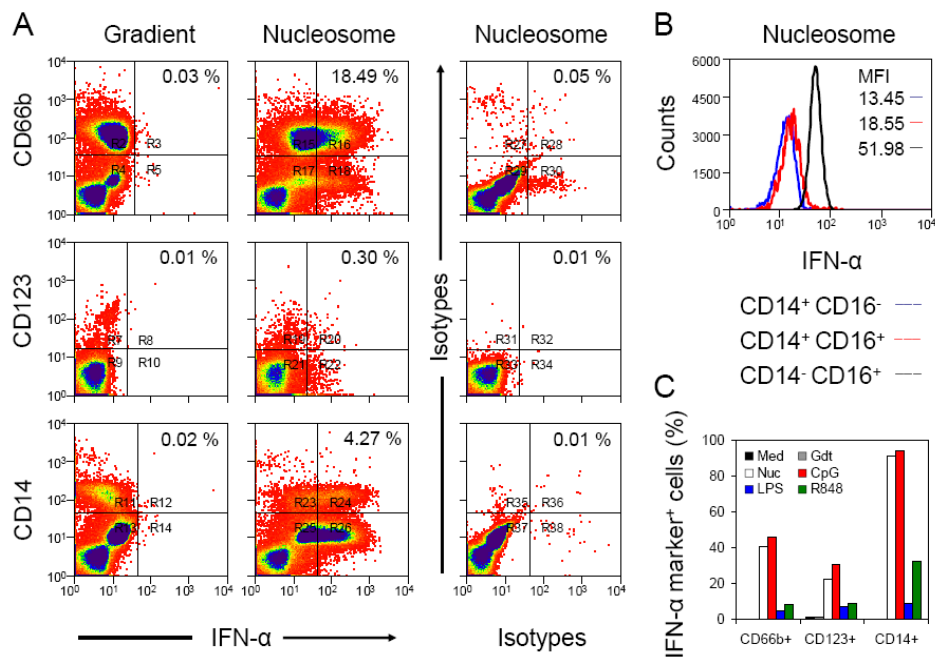
Figure 1. Nucleosome induces IFN-α secretion. (A) Myeloid and lymphoid cells were stimulated simultaneously with nucleosomes or different TLR-ligands. Intracellular cytokine staining revealed IFN-α secretion in neutrophils (CD66b^{+}), pDC (CD123^{+}) and monocytes (CD14^{+}). **(B)** IFN-α secretion was most strongly up-regulated by neutrophils (CD14^{-}CD16^{+}) as compared to conventional (CD14^{+}CD16^{-}) and pro-inflammatory (CD14^{+}CD16^{+}) monocytes. **(C)** In all cells tested the nucleosome was together with CpG-ODN the most potent inducer of IFN-α. Data is representative from four independent experiments. Gradient (Gdt) is the nucleosome purification buffer.

Ag-specificity of CNS-infiltrating CTL

We were clearly able to demonstrate an accumulating effect of cytotoxic T cells in the CNS of aging ntg and tg animal models of neurodegenerative disorders. Massive infiltrates of CTL have been demonstrated in AD patients [2, 3] and as initial approach we have further established the HLA tetramer technology to identify Aβ-specific CTL (Fig. 2, Lindau et al. unpublished). Thus, the logical consequence would be to imply in situ tetramer staining on frozen brain material of HLA-A2+ AD patients to determine the Ag-specificity of CNS-infiltrating CTL in AD [4].
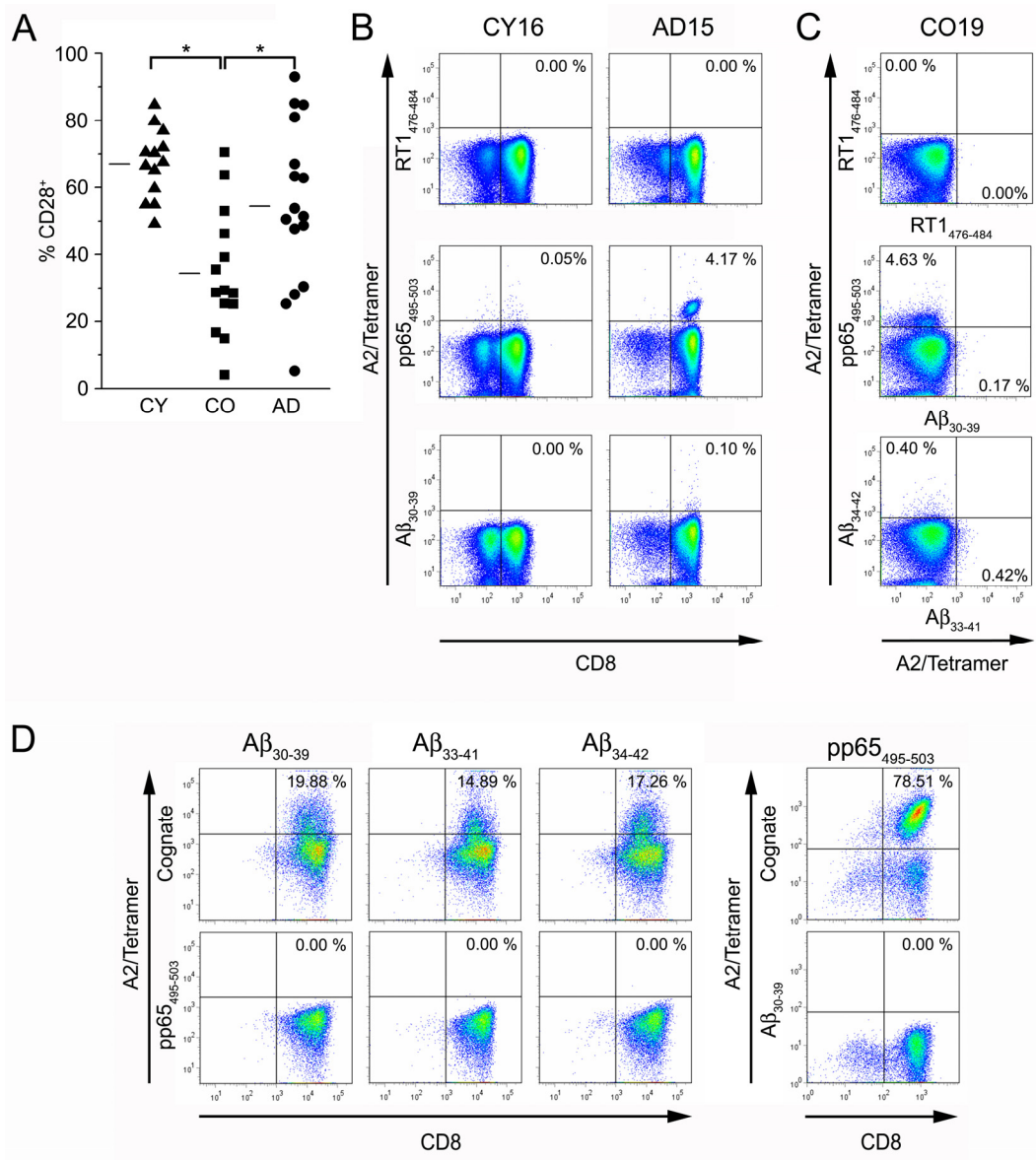
Figure 2. Ex vivo detection and recall of Aβ-specific CTL. (A) PBMC from HLA-A*0201$^+$ healthy young and elderly controls as well as AD patients were analyzed by 10-color flow cytometry. Dead cells were excluded and lymphocytes gated according to forward/side scatter, excluding CD4$^+$CD14$^+$CD32$^+$ cells from the analysis. Depicted is the % of CD28$^+$ cells within the total CD8 population. Comparing either the young group (CY, p < 0.001) or the AD group (p = 0.0058) with the elderly group (CO) revealed a highly significant difference in CD28 expression (Student's t test). CY, n = 13; CO, n = 14; AD, n = 16. **(B)** HLA class I tetramers were produced essentially as described and loaded with predicted Aβ-specific epitopes [5]. Histograms represent CD8 expression and HLA-A2 PE-tetramer staining on gated CD3$^+$ cells. This revealed the existence of directly visible Aβ-specific CTL in some but not all individuals tested in comparison to staining for HIV (RT$_{476-484}$)-specific CTL. Representative data from an Aβ-specific negative-stained young donor (CY16) and an Aβ$_{30-39}$ positive stained individual with AD are shown. These results were independent from the presence of CMV (pp65$_{495-503}$)-specific CTL in the donors. **(C)** Some individuals allowed detection of all three Aβ-specific CTL types as depicted for an elderly control (CO19). Histograms represent HLA-A2 PE-tetramer (Aβ$_{30-39}$, Aβ$_{33-41}$, RT$_{476-484}$) against APC-tetramer staining (Aβ$_{34-42}$, pp65$_{495-503}$, RT$_{476-484}$) on gated CD3$^+$CD8$^+$ cells. Numbers in all histograms represent % of tetramer-positive cells within the CD8$^+$ population. **(D)** Human Aβ-specific CTL were expanded from PBMC of AD patients and young as well as elderly unaffected controls with depicted peptide-HLA monomer-loaded artificial APC as well as IL-2 and IL-12. After 22 days, cultures were stained with the relevant PE-labeled Aβ-specific tetramer and anti-CD8 Ab as published [6]. Cells were analyzed by flow cytometry and depicted histograms show individual cultures for the given peptides gated on living lymphocytes as determined by forward/side scatter. Numbers indicate % tetramer$^+$ cells of the CD8$^+$ population. Aβ histograms are from one single donor and for comparison a histogram of a pp65$_{495-503}$-expanded culture of a CMV serum-positive donor is shown.

Generation of mAb against Aβ-peptide loaded HLA class I molecules

Based on the identification of immunodominant $A\beta_{42}$-derived CTL epitopes this project was started by Katja Grgur during her diploma thesis [7]. My contribution involved technical assistance in the generation of Aβ-loaded HLA-A2 class I complexes which were used by Katja to generate and select specific mAb reactive with these structures. Furthermore I generated HLA-A2$^+$ APP-KO and APP-WT transfectants. When used in immunofluorescent staining reactions, the anti-Aβ-HLA-A2 mAb produced by Katja specifically stained these transfectants in a peptide-dependent manner. It is desirable to continue with this study because anti-Aβ-HLA-A2 mAb could be used in various approaches such as flow cytometry, immunoprecipitation, blotting and intracellular immunofluorescence to detect specific Aβ-peptide-HLA class I molecules formed by either peptide exposure or natural processing of $A\beta_{42}$. Other issues, especially for in vivo tracking of specific ligand-bearing APC using two-photon microscopy, will be useful to determine where CNS-Ag presentation takes place.

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Abbreviations

α	alpha		CD	cluster of differentiation
β	beta		CMV	Cytomegalovirus
γ	gamma		CNS	central nervous system
Δ	delta		CpG	cytosine-phosphate-guanine
ζ	zeta		CRP	C-reactive protein
κ	kappa		CTB	cholera toxin subunit B
μ	my		CTL	cytotoxic T cell
Φ	phi		CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
Ω	omega		CO	old control
α-syn	alpha-synuclein		CQ	chloroquine
aa	amino acids		CSA	cyclosporine
autoAb	autoantibody		CY	young control
autoAg	autoantigen		DAI	DNA-dependent activator of IRF
Ab	antibody		DC	dendritic cell
Aβ	amyloid-beta		ds	double stranded
AC	ammonium chloride		DNA	deoxyribonucleic acid
AD	Alzheimer's disease		DNase	deoxyribonuclease
AD	anno domini		EAE	experimental autoimmune encephalitis
ADAM	a disentegrin and metalloproteinase		eCFP	enhanced Cyan Fluorescent Protein
AFU	arbitrary fluorescence units		EDTA	ethylenediaminetetraacetic acid
Ag	antigen		ELISA	enzyme linked immunosorbent assay
AICD	antigen induced cell death		EM	electron microscopy
AK	Alzheimer-Krankheit		EMA	ethidium monoazide
ALS	Amyotrophic lateral sclerosis		ER	endoplasmatic reticulum
AIM-2	absent in melanoma-2		fAβ	pre-aggregated Aβ
ANA	anti-nuclear AAb		FACS	fluorescent activated cell sorting
APC	antigen presenting cells		FAD	familiar Alzheimer's disease
APC	allophycocyanin		FcR	Fc-receptor
APP	amyloid precursor protein		FCS	fetal calf serum
APPsα	N-terminus of APP after α-secretase cleavage		FITC	fluorescein isothiocyanate
			FTLD	Frontotemporal lobar degeneration
APPsβ	N-terminus of APP after β-secretase cleavage		FTLD-MND	FTLD with motor neuron disease
			GA	glatiramer acetate (Copaxone)
Apoe4	apolipoprotein E4		Gdt.	gradient
AU	arbitrary units		GFAP	glial fibrillary acidic protein
AZA	azathioprine		GN	glomerulonephritis
BBB	blood-brain barrier		h	hour
BCR	B cell receptor		HC	healthy control
BM	bone marrow		HCQ	hydroxychloroquine
bp	base pairs		HIV	Human immunodeficiency virus
BSA	bovine serum albumin		HLA	human leukocyte antigen
BTLA	B and T lymphocyte attenuator		HMGB-1	high motility group box-1
C1q	complement component 1q		Iba-1	ionized calcium binding adaptor molecule-1
CAA	cerebral amyloid angiopathy		IC	immune complex
CCR2	chemokine (C-C motif) receptor 2		IFN	interferon
			Ig	immunoglobulin

Abbreviations

IL	interleukin
IRF	IFN regulatory factor
IPS cells	induced pluripotent stem cells
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
KO	knockout
LCMV	Lymphocytic Choriomeningitis Virus
LPS	lipopolysaccharide
MΦ	macrophage
mAb	monoclonal antibody
MACS	magnetically activated cell sorting
MBL	mannose-binding-lectin
MFGE8	milk fat globule EGF-factor 8
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minutes
MIP	macrophage-inflammatory protein
MMF	mycophenolate mofetil
MRL	Murphy Roths Large
MS	Multiple sclerosis
MSA	multiple system atrophy
MTX	methotrexate
MyD88	Myeloid differentiation primary response gene 88
n	number
N	number of backcross generations
NALP	NACHT-, LRR- and pyrin domain-containing protein
Nct	nicastrin
NET	neutrophil extracellular traps
NFT	neuropfibrillary tangles
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
n. k.	not known
n. t.	not tested
ntg	non-transgenic
Nuc	nucleosome
ODN	oligodeoxynucleotide
P	probability
PAMP	pathogen-associated molecular patterns
PB	polymyxin B
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PD	Parkinson's disease
PD-1	Programmed Death-1
PD-L	Ligand of PD-1
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PercP	Peridinin Chlorophyll Protein Complex
PK	proteinase K

PK-PET	PK-digested paraffin-embedded tissue
PMN	polymorphonuclear leukocytes
Pred	prednisolone
PrP	prion protein
PrP^C	PrP cellular
PrP^{Sc}	PrP Scrapie
PRR	pattern recognition receptors
PS	presenilin
PTX	Pentraxin
R	Spearman correlation coefficient
R848	resiquimod
RAGE	receptor for advanced glycation endproducts
RIP	regulated intramembrane proteolysis
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	room temperature
sAβ	soluble Aβ
SAD	sporadic Alzheimer's disease
SAP	serum amyloid P
SFI	specific fluorescence index
Sigirr	single Ig IL-1R-related molecule
SLE	Systemic lupus erythematosus
SLEDAI	SLE disease activity score
SLPI	secretory leukocyte protease inhibitor
ss	single stranded
TB	trypan blue
TBS-T	Tris-Buffered Saline Tween-20
TDP-43	transactive response DNA binding protein DNA binding protein-43
tg	transgenic
TGF	transforming growth factor
T$_H$	T helper cell
Thy 1	Thymocyte antigen 1 (CD90)
TICAM	Toll-like receptor adaptor molecule
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain containing adaptor protein
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TLR	Toll-like receptor
TM	transmembrane
TNF	tumor necrosis factor
TRAM	TIR-containing adaptor molecule
TRIF	TIR-domain-containing adapter-inducing IFN-β
T$_{REG}$	regulatory T cell
UV	ultraviolet
WT	wildtype
yr	year
ZBP-1	Z-DNA binding protein-1

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