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XXXII CICLO DEL DOTTORATO DI RICERCA IN
NEUROSCIENZE CLINICHE E SPERIMENTALI

HUMAN B CELLS EXPRESS NEURON/GLIAL 2 ANTIGEN. POSSIBLE IMPLICATIONS FOR HEALTH AND AUTOIMMUNE DISEASE OF THE CENTRAL NERVOUS SYSTEM

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Ai miei genitori Federica e Gino.
A Luca.

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List of abbreviations

ab antibody
aBmem atypical B memory cells
APC antigen presenting cell
BBB blood-brain barrier
BCR B cell receptor
Beff effector B cells
BM bone marrow
Bmat B mature cells
Bmem B memory cells
Breg B regulatory cells
BSA bovine serum albumin
CD cluster differentiation
CNS central nervous system
CSF cerebrospinal fluid
CSPG4 chondroitin sulfate proteoglycan 4
DC dendritic cells
EAE experimental autoimmune encephalomyelitis
EBV Epstein–Barr virus
EDSS expanded disability status scale
ELISA enzyme-linked immunosorbent assay
ERK extracellular signal-regulated kinases
FACS fluorescence-activated cell sorting
FOXP3 forkhead box protein P3
FSC forward scatter
Gapdh glyceraldehyde 3-phosphate dehydrogenase
GITR glucocorticoid-induced tumor necrosis factor receptor
GM-CSF granulocyte–macrophage colony- stimulating factor
HD Healthy donor
HLA human leucocyte antigen genes
IFN-γ interferon-gamma
Ig immunoglobulin
IL interleukin
MHC major histocompatibility complex
MLL myeloid/lymphoid leukemia (or mixed lineage leukemia)
MoAb monoclonal antibody
MOG myelin oligodendrocyte glycoprotein
MS multiple sclerosis
NG2 neuron/glial 2 antigen
NG2KO NG2 knock-out
OCB oligoclonal bands
OPCs oligodendrocyte precursor cells
PBMCs peripheral blood mononuclear cells
PCR polymerase chain reaction
PD programmed death
PDGFR-α platelet derived grow factor receptor alpha
PBS phosphate buffered saline

PKCα protein kinase C-alpha
Poly-NG2 polyclona anti-NG2 antibody
RRMS relapsing remitting multiple sclerosis
SD standard deviation
SEM standard error of the mean
SLO secondary lymphoid organs
SSC side scatter
STAT3 signal transducer and activator of transcription 3
t-SNE T-distributed stochastic neighbor embedding
Th T helper cells
TNF tumor necrosis factor
Treg T regulatory cells
WT wild type

1. Introduction

Neuron/Glial 2 antigen (NG2) is a proteoglycan expressed inside and outside central nervous system (CNS). In the CNS, NG2 is expressed on oligodendrocyte precursor cells (OPCs)[1] and on pericytes, which are components of the neurovascular unit of the blood-brain barrier (BBB)-microvessels[2]. Outside the CNS, NG2 is mostly expressed on stem cells and, albeit to a lesser extent, on differentiated cells, like macrophages[3–5]. NG2 is believed to play a role in cell survival, migration and angiogenesis[6], but its function is not completely clear.

1.1 Neuron/glial Antigen 2 chondroitin sulfate proteoglycan structure

NG2 is a single membrane-spanning chondroitin sulphate proteoglycan[6,7] product of the *Chondroitin sulfate proteoglycan 4 (CSPG4)* gene (chromosome 15 in human). The *CSPG4* gene encodes a surface type I transmembrane core protein of ~300 kDa[3,6] NG2 is made of a large extracellular domain with 2225 amino acids (95% of the protein), a transmembrane domain with 25 amino acids and a short intracytoplasmatic part of 76 amino acids[8,9] **(Fig.1).** The extracellular N-terminal domain of this protein is post-translationally modified by chondroitin sulfate glycosaminoglycan chains and disulfide bonds. It also contains two putative proteolytic cleavage sites[10].

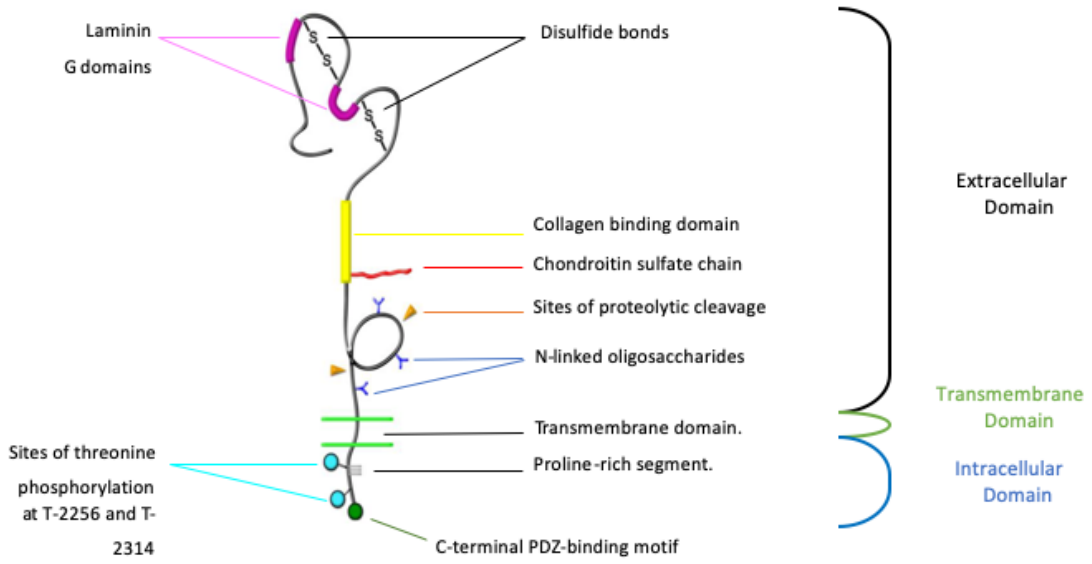

Fig.1: **Structure of NG2**: Domain 1. Bold magenta bars = laminin G domains; S-S = disulfide bonds. Domain 2. Bold yellow bar = collagen binding domain; Irregular red line = chondroitin

sulfate chain attached at S-999. Domain 3. Blue Y shapes = N-linked oligosaccharides; Orange arrowheads = sites of proteolytic cleavage. Transmembrane domain. Double green lines = plasma membrane. Cytoplasmic domain. Blue circles = sites of threonine phosphorylation at T-2256 and T-2314; Green circle = C-terminal PDZ-binding motif; Gray grid lines = proline-rich segment. Taken from Stallcup et al. 2008[11].

1.2 NG2 core protein function

NG2 is crucially involved in cell survival, migration and angiogenesis[6], although its role is not completely clear. In this context, the function of each single part of NG2 molecule needs to be further studied. NG2-mediated molecular events are articulated through the interaction with more than 40 putative ligands and the concurrent involvement of the ectodomain and cytoplasmic tail[12] (Table 1). The function of the extracellular domain fragments is still unknown, but a growing body of evidence suggests that they are involved in the regulation of neuronal networks[13] or endothelial and pericyte functions[14]. The primary and higher order structures of the NG2/ CSPG4 ectodomain shapes multivalent binding sites for structurally and compositionally diversified molecules (Table 1). These may range from those associated with the cell membrane to those involved in paracrine signaling. Primary extracellular ligands of NG2/CSPG4 remain components of the ECM and early studies examining such interactions disclosed binding of the recombinant ectodomain of the NG2 to tenascin-C, laminin 111, perlecan, and collagen types II, V, and VI, but not other collagens[15–17]. The intracellular C-terminal domain of NG2 acts as an acceptor site for the extracellular signal-regulated kinases (ERK) 1/2 and protein kinase C-alpha (PKCα), with the two phosphorylation sites on the intracellular domain, Thr 2256 and 2314, being phosphorylated by PKCα and ERK, respectively[18]. Phosphorylation of each of these sites leads to colocalization of NG2 with β1-integrin, promoting cell proliferation upon phosphorylation of Thr2314 by ERK[18], and increase in cell motility upon PKCα-mediated phosphorylation of NG2 at Thr2256[19]. β1 integrin-mediated signaling of cell motility and proliferation has been shown to be balanced through interaction with differentially phosphorylated NG2, which results in localization of integrin protein to the cell surface[18]. Cell surface localization is followed by β1 integrin binding to the NG2 extracellular domain which also contains binding sites for collagens II, V, and VI, galectin, laminin, and tenascin. NG2 binding to these proteins facilitates cellular adhesion mechanisms[20]. In NG2-dependent signal transduction, NG2 functions as a co-receptor in conjunction with Platelet derived grow factor receptor-alpha (PDGFR-α) for receptor tyrosine kinase PDGF, to activate focal adhesion kinase and Mitogen-activated

protein kinase pathways[15,21,22]. The intracellular domain of NG2 contains also binding sites for multi-PZD domain protein 1, which facilitates the physical interaction of NG2 with the key structural and/or signaling components in the cytoplasm[23]. The cytoplasmic domain also contains binding sites for the synaptic glutamate receptor-interacting protein1 and for syntenin-1, which are important for NG2 mediated cellular migration[24,25]. Altogether, these interactions activate key signaling pathways involved in cell proliferation, migration, invasion, cytoskeletal reorganization, survival, chemoresistance, modulation of neuronal network cell survival and angiogenesis[12,18,19,23–25]

Cytoplasmic domain						Eptodomain		
Cell adhesion/movement/polarity		Cell proliferation and growth		Cell-cell adhesion/polarity				
Direct binding	Indirect linkage	Direct binding	Indirect linkage	Direct binding	Indirect linkage	Direct Binding	Indirect linkage	Effector
PKCα Ezrin MUPP-1 NEDD9 Syntenin-1 BAIA/IRSP53 mDia2	Cofilin-1 Fascin Ena/VASP					Ca^{2+} Plasminogen/tPA MMP-2 MT-MMP3/MMP16 MT1-MMP/MMP14 α2 integrin subunit α4 integrin subunit Fibronectin Tenascin-C Laminin 111 Perlecan Col V, Col VI	pro-MMP2 Col II, Col IV FAK Stonin-1 β1 integrin	cdc42 Ack1 Tnk2 Rac1 PI-3K
		ERK PKCα PI-3K Akt-1 mTOR				Ca^{2+} FGFs PDGFAA FGFR1 FGFR3 PDGFRα PDGFRβ	MT-MMP/MMP14 ADAM-10	ERK1/2 PKCs Phospholipase D
				Ezrin MUPP-1 GRIPP-1 Syntenin-1 HtrA2/OMI	Fascin AMPA receptors Tiam-1 Syx γ-secretase	Ca^{2+} Galectin3 Galectin9 Angiostatin α3 integrin subunit	P-selectin ?	Rac1 Par3 Par6 PKCζ

Ack1, activated cdc42 kinase 1; ADAM-10, disintegrin and metalloproteinase domain-containing protein 10; AMPA-R, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BAIA/IRSP53, brain-specific angiogenesis inhibitor 1-associated protein 2; Col, collagen; Ena/VASP, actin regulator/vasodilator stimulated phosphoprotein; GRIP-1, glutamate receptor interacting protein 1; HtrA2/OMI, HtrA serine peptidase 2; mDia2, diaphanous-related formin-3; mTOR, mechanistic target of rapamycin kinase; MUPP-1, multi-PDZ domain protein 1; Rac1, Rac family small GTPase 1; Syx, synectin-binding RhoA exchange factor; Tiam-1, T-cell lymphoma invasion and metastasis inducing Rho/Rac1-activating exchange factor; Tnk2, tyrosine kinase non receptor 2; tPa, tissue plasminogen activator.

Table 1: Summary of the molecular interactions of NG2/CSPG4

1.3 NG2 expression

1.3.1 NG2 expression in CNS

In CNS, NG2 is expressed on OPCs[1] and on pericytes which are components of the neurovascular unit of BBB-microvessels[2]. The role of OPCs is not completely understood; OPCs differentiate to oligodendrocytes which myelinate axons and release metabolic factors necessary to the integrity and survival of neurons in developing and mature CNS[26], and serve as a primary source of remyelinating cells in demyelinated lesions[27]. They have a complex role in different types of CNS lesions and together with microglia, which in inflammatory condition, removes dysfunctional synapses and cell debris, and modulates neuronal activity[28], constitute the cellular response of resident CNS cells within the first few days after injury[29]. The response of OPCs to lesions involves increased proliferation, migration and differentiation[30], and has largely been interpreted as a response of the oligodendrocyte

lineage to damage of myelin. Together with PDGFRα, NG2 is a marker of OPCs; both of these markers downregulate as OPCs differentiate into mature oligodendrocytes[8,15,31,32]. OPCs have been associated with injury-dependent increase of NG2 cleavage [33,34] leading to release of biological active fragments[35]. Sakry et al. showed that activity dependent cleavage of NG2 by α and γ secretase results in releasing of extracellular and intra cellular domains. The cleaved peptides then become biologically functional molecules regulating neuronal network by bidirectional communication between neurons and oligodendrocyte precursors. In addition, the two conserved N-terminal domains of NG2 extracellular domain (laminin neurexin sex-hormone binding globulin domains) were found to be important for neuromodulation[13].

Pericytes are contractile cells inserted in basement membrane of small blood vessels, including those of the BBB, and cooperate to maintenance of integrity and trophism of BBB endothelial layer. NG2 is a marker of initial activation of pericytes[2], and it has a role in pericyte localization and their interaction with endothelial cells[19,36]. Although NG2 is not expressed in adult CNS pericytes[2], it is an early marker of pericyte activation during CNS development[26] and in pathological conditions[2,37,38]. Thus, upon CNS injury, NG2-reactive pericytes are found along microvessels, where they act as sensors for inflammation and support the immunosurveillance of extravasated neutrophils and macrophages[39]. The role of NG2 in CNS is highly debated, with studies reporting an inhibitory role of NG2 on neuritis growth[40,41], whereas others suggest a positive role of NG2 on axonal regeneration[42,43].

1.3.2 NG2 expression outside CNS

NG2 is highly expressed in many cellular types. In extra-neural tissues the expression of NG2 is highly elevated during development in undifferentiated tissues and it is less expressed during differentiation. NG2 is expressed mainly in stem cells, like mesenchymal stem cells, and progenitor cells (muscle progenitor, melanocytes, osteoblasts etc.)[3,8]. Others studies show that NG2 is expressed also on differentiated cells of various tissues and organs, such as bone marrow, smooth muscle, interfollicular epidermis, muscoskeletal junctions, pancreas, lung, eyes, heart and kidneys[3–5]. NG2 is also expressed by several types of highly malignant neoplasms, including melanomas, glioblastomas, leukemia and lymphomas[44]. Moreover, NG2 is a marker for pericytes which play a crucial role in the stabilization of microvessels, in the modulation of capillary blood flow and in angiogenesis[45]. However, NG2

expression may differs considerably depending on the tissue of origin of analyzed pericyte. For example, arteriolar but not venular pericytes express the proteoglycan in the mesenteric tissue [46,47], while all pericytes (arterioles, capillaries, and venules) are positive for NG2 in the retinal microvascular network [48]. Taken together, this evidence suggests that the expression of NG2 is differently regulated and it depends on specific cell functions in different tissues. Which mechanisms and factors are involved in NG2 expression regulation are still to be explained.

1.3.3 The NG2+ cells in EAE

Considering that NG2 apparently plays a role in OPCs, which are involved in myelination and remyelination, studies in our laboratory[38] analysed the possibility that NG2 could play a role in experimental autoimmune encephalomyelitis (EAE), the purported animal model of multiple sclerosis (MS), associated with increased BBB permeability, inflammatory infiltrates, demyelination, and CNS damage. EAE is a T-cell-mediated experimental disease actively induced in animals, most frequently in mice, by immunization with myelin antigens and/or peptides thereof, or passively transferred by myelin-reactive T cells. These studies showed that NG2 knock-out (NG2KO) mice displayed a milder disease at clinical and neuropathological levels. While such a study showed a role for NG2 at CNS level, it also demonstrated for the first time the expression of NG2 by healthy immune cells, including most T cells and 50% of dendritic cells (DC). Most importantly, the use of chimeric mice indicated that chimera reconstituted with NG2KO bone marrow cells developed a milder EAE, regardless of their inherent WT or NG2KO phenotype, suggesting a role for NG2 in the immune cell response[38].

Recent reports suggest that NG2 is expressed on activated microglia and CNS-infiltrating macrophages that accumulate in CNS lesions[34,49–51]. The signals triggering NG2 expression in macrophages and microglia are unknown, but activated macrophages and microglia play pivotal roles in CNS injury and can cause extensive CNS damage[52–54] . The data obtained by Ferrara et al. [38] in mice suggested that NG2 might play a role in activation of DC. Indeed, in NG2KO mice, milder EAE was associated with a skewed cytokine profile of T cells specific for the inducing myelin antigen, myelin oligodendrocyte glycoprotein (MOG). Thus, MOG-specific T cells expressed lower levels of interferon-gamma (IFN-γ), the typical T helper (Th)1-like pathogenic cytokine, and higher levels of interleukin (IL)-4 and IL-10, characteristic of the

less inflammatory Th2-type T cells, than MOG-specific T cells from wild-type (WT) EAE-affected mice. This was not apparently related to an intrinsic feature of NG2KO T cells as primary line T cells generated from NG2KO and WT mice did not differ in their response to the antigen and/or their cytokine profile. Accordingly, the shift from a Th1- to a Th2-type response suggested a role of DC, antigen-presenting cells involved in the stimulation of T cells. DC from NG2KO mice secreted significantly less IL-12 than DC from WT mice, suggesting that they are less capable than WT DC of inducing the pathogenic Th1 response. To understand if NG2 expression was linked to the ability of DC to produce IL-12 and thereby to their activation and ability to stimulate T cells towards the Th1 type, Ferrara et al. [38] analyzed NG2+ and NG2- populations in WT mice for their expression of IL-12 by intracellular fluorescence-activated cell sorting (FACS) analysis. The proportion of IL-12-expressing cells was found to be lower in DCs that do not also express NG2. These data suggested that NG2 might be involved in the activation of DC and thereby the stimulation of T cells, through control of IL-12 expression.

Considering the expression of NG2 on OPCs and activated pericytes and the results obtained on EAE (animal model of multiple sclerosis), we speculated that NG2 could play a role also in multiple sclerosis, an inflammatory, immune-mediated and neurodegenerative disease of CNS.

1.4 Multiple Sclerosis

MS is the most common chronic an inflammatory, demyelinating and neurodegenerative disease of CNS in young adults[55]. With a prevalence of 50–300 per 100 000 people[56], about 2 million people are estimated to live with MS globally[57]. Environmental, genetic, and epigenetic factors have a causal role in MS and potentially interact with modifiable risk factors[58]. For what concerns genetic risk, genome-wide association studies have identified >200 genetic risk variants for MS[59]; most of these variants encode molecules involved in the immune system (such as the human leucocyte antigen genes - HLA) and are associated with a higher risk of other systemic immune disorders.

1.4.1 Clinical and neuropathological MS features

The pathological hallmark of MS is the accumulation of demyelinating lesions that occur in the white matter and the grey matter of the brain and spinal cord. Demyelinating lesions are

caused by immune cells infiltration, they are typically located around postcapillary venules and are characterized by breakdown of BBB[55,60]. Genetic and pathological studies point towards the adaptive immune system, which consists of T cells and B cells, as a key player in the pathogenesis of MS[61]. Inflammation in MS only affects the CNS, strongly suggesting that T cells and B cells are selectively recruited by specific target antigens (probably autoantigens) that are only expressed in the CNS. Although several candidate antigens have been proposed, none has been confirmed[62,63]. The inflammatory process involves not only peripheral immune cells but also CNS resident cells such as microglia and astrocytes[64]. Together with peripheral immune cells, CNS-resident cells secrete inflammatory mediators that can recruit other inflammatory cells into the CNS, leading to neuronal demyelination and inducing inflammation within the CNS parenchyma. This self-sustaining inflammatory mechanism leads to neuro-axonal damage and neurodegenerative processes typical of MS[60]. Inflammatory and neurodegenerative processes coexist since the earliest phases of MS and they correlate with clinical course of disease. Inflammatory plaques, typical of earliest MS, occur in both white matter and grey matter and are typically found throughout the CNS, including in the brain, optic nerve and spinal cord[65–67]. The anatomical location of white matter lesions is associated with specific clinical manifestations of MS. Although no clinical findings are unique to MS, some are highly characteristic of the disease. Typically, the onset of MS is characterized by an initial clinical attack (defined as “Clinically isolated syndrome”) in ~85% of patients, which is an episode of neurological dysfunction related to an inflammatory plaque in the optic nerve (leading to optic neuritis), spinal cord (leading to myelitis), brainstem or cerebellum (leading to brainstem and/or cerebellar syndromes) or the cerebral hemispheres (cerebral hemispheric syndrome). Relapsing-remitting MS (RRMS) is the most frequent phenotype of disease, it is characterized by clinical episodes that can occur (known as relapses). Most patients with RRMS will develop secondary progressive MS, which is characterized by progressive, irreversible disability that occurs independently of the presence of relapses. Approximately 10–15% of patients present with primary progressive MS, which is characterized by disease progression from the onset, resulting in gradual, progressive and permanent neurological deficits for >1 year without relapses[68,69] (Fig. 2)

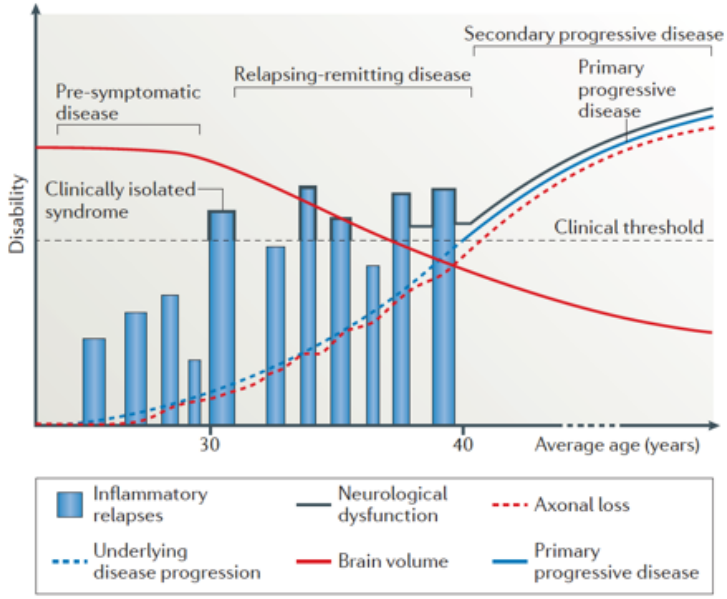

Fig. 2: Clinical Course of MS: The disease course and symptomatology of multiple sclerosis are heterogeneous, although efforts to categorize patients by general patterns of disease presentation have allowed several disease subtypes to be recognized. Taken from Dendrou et al[60]**.**

1.4.2 Immunopathogenesis

Immune cells play a major role in MS pathogenesis; many studies in humans and in the purported animal model of MS, EAE, have shown the crucial role of innate and adaptive immunity. Moreover, the importance of adaptive immune cells in MS is supported by the positive results obtained by treating patients with immunosuppressive and immunomodulatory drugs which target B and T cells[70] (Fig. 3).

T cells: The presence of T cells within CNS lesions is detectable in the early stages of multiple sclerosis[71], Th1 cells (IFNγ-secreting CD4+ T cells) and Th17 cells (IL-17-expressing CD4 T cells) are the main CD4+ T cell subsets implicated in disease. However, CD8+ T cells are found in higher frequency than CD4+ T cells in the white matter and in grey matter cortical demyelinating lesions, and their numbers closely correlate with axonal damage[72]. The aberrant T cell activation in MS requires antigen presentation to T cells by antigen-presenting cells (APCs) such as B cells and myeloid cells (macrophages, dendritic cells and microglia) in the periphery and the CNS, although the responsible antigens have not been routinely identified[73]. After activation in the periphery, immune cells upregulate cell surface molecules such as chemokine receptors and adhesion molecules, which enables efficient tissue infiltration, including to the CNS.

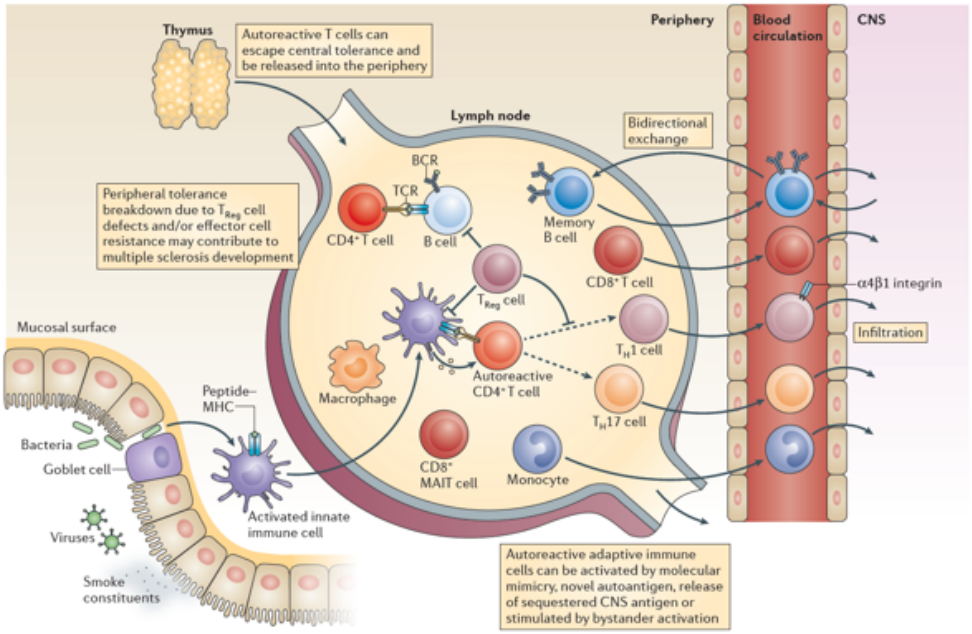

Fig. 3. Immune system dysregulation in MS patients. During maturation of the immune system, mechanisms of central tolerance are established in the thymus. At the end of thymic selection, most of the autoreactive clones are eliminated. However, this selection is not perfect and some autoreactive T cells can be released in peripheral circulation. In healthy subjects, peripheral tolerance mechanisms are activated and can control the activation of autoreactive cells. When tolerance does not work properly, for example in reducing regulatory T-cell activity or increasing resistance of effector cells to suppressive mechanisms, autoreactive immune B and T cells can be activated. Once activated, CD8+ and CD4+ Th1 and Th17 cells, B cells, and innate immunity cells can infiltrate CNS leading to inflammation and tissue damage. Taken from Dendrou et al[60]**.**

One potential cause of aberrant effector T cell activation is an insufficiency in the function of regulatory T (Treg) cells and resistance of CNS-specific effector T cells to Treg cell-mediated regulation[73]. Indeed, several abnormalities in circulating Treg cells have been observed and implicated in MS, including decreased expression of forkhead box protein P3 (FOXP3) by Treg cells and/or deficient regulatory capacity of FOXP3-expressing CD25hiCD127low Treg cells[74–76]. The main mechanisms behind the alterations in regulatory functions are related to the thymic release of Treg with an inadeguate ability to suppress autoreactive effector T cells[77], or, alternatively, to the dysfunction of peripheral suppressor cells, indirectly driven by the dysregulation of tolerogenic APCs, as shown in EAE[78]. An alternative but non-mutually exclusive explanation for the action of autoreactive effector T cells in multiple sclerosis is that, rather than being the outcome of inadequate peripheral suppression, the effector T cells themselves are actively resistant to suppressive mechanisms, with the suggestion that IL-6-induced signal transducer and activator of transcription 3 (STAT3)-mediated signalling contributes to this resistance[79,80]

B cells: B cells, together with T cells, constitute the adaptive branch of immune system, which exhibits antigen- specific memory. Depending on the differentiation status, B cells are able to perform a variety of cellular and humoral functions. Arising from haematopoietic stem cell precursors, naive B cells expressing IgM and IgD emerge from the bone marrow and circulate in the peripheral blood or migrate to lymphoid organs. B cells are activated by the recognition of distinct antigens by the unique rearranged B cell receptor (BCR)[81]. Activated B cells undergo a germinal centre reaction in secondary lymphoid tissues. In germinal centres, B cells expressing a specific BCR undergo clonal expansion and proliferate rapidly, becoming memory B cells (Bmem)[82], ab-secreting CD38+ plasmablasts and long-lived CD138+ plasma cells[83]. The BM is an important reservoir of plasma cells. Both memory B cells and plasma cells produce antibodies, which bind to antigens on cellular targets and cause complement- dependent cytotoxicity, antibody-dependent cellular phagocytosis or antibody-dependent cellular cytotoxicity. Mature, activated B cells can undergo immunoglobulin class switching, a T cell- dependent process that enables them to express other antibody isotypes (IgG, IgA or IgE)[81]. Class- switched autoreactive B cells can be present at high frequencies in autoimmune CNS conditions. B cells can also work as APC presenting antigens to T cells through BCR-mediated internalization of extracellular antigens. B cells secrete a variety of proinflammatory cytokines, leading to type Th1 cell or IL-17-secreting Th17 polarization[84]. B regulatory cells (Breg) also secrete anti-inflammatory cytokines, including IL-10 and IL-35, which can suppress proinflammatory T cells[85] (Fig. 4).

Autoreactive B cells are present in the immunological repertoires of healthy individuals. Their physiological roles is still incompletely understood, although they are normally maintained in a tolerant state. Two major checkpoints contribute to normal elimination and control of autoreactive B cells: central tolerance and peripheral tolerance. Central B cell tolerance is established in the BM and involves the elimination of approximately 75% of self-reactive B cells, whereas peripheral tolerance takes place in the secondary lymphoid organs, where most other self-reactive B cells are controlled. B cell receptor– and Toll-like receptor -signaling pathways play important roles during the selection of B cells in the BM, whereas the CD40–CD40L receptor–ligand pair, the MHC and T regulatory cells are considered important for control of autoreactive B cells in the periphery. Through analysis of self-reactive antibody profiles, deficiencies in both central and peripheral B cell tolerance have been documented in people with systemic autoimmune diseases, such as rheumatoid

arthritis and type 1 diabetes. In contrast, B cells from people with MS appear to display abnormalities only in peripheral tolerance[64].

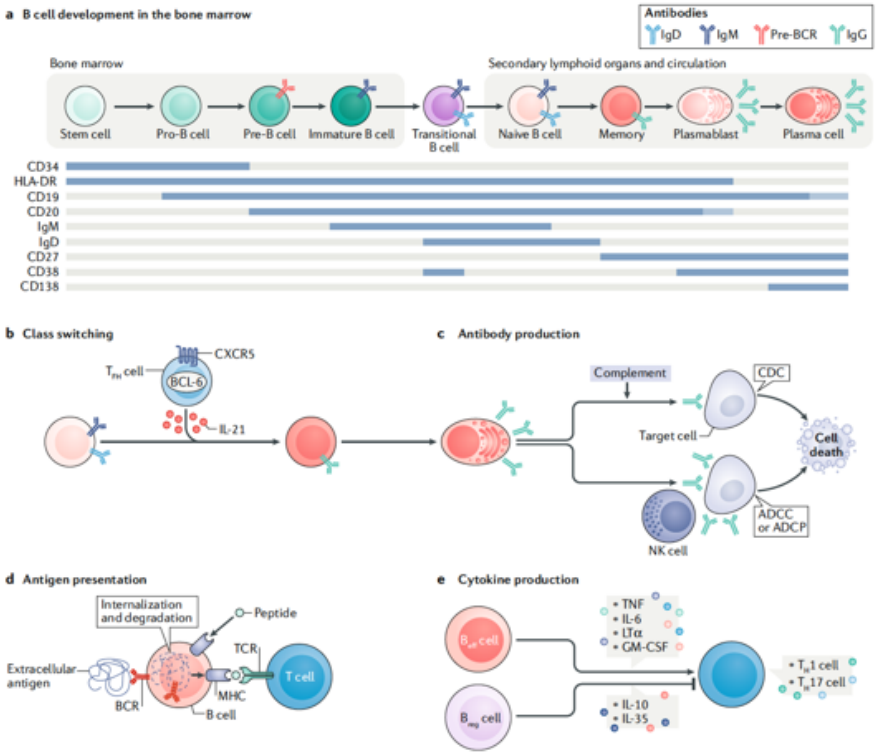

Fig. 4: B-cell maturation and functions. Taken from Sabatino et al.[81]

It has been known for many years that B cells must play a role in MS; however, despite intense research, this role is still unclear. Their importance has been more emphasized by the impressive positive results obtained by anti-CD20 treatment[86,87]. More recent somatic hypermutation studies have demonstrated that, in individual patients, identical B cell clones can be shared between the CNS and the periphery[88–91]. These studies provide evidence of bidirectional trafficking of distinct B cell clones (both into and out of the CNS) and in fact suggest that much of the clonal expansion of these B cells occurs in the deep cervical lymph nodes rather than in the CNS (Fig. 5).

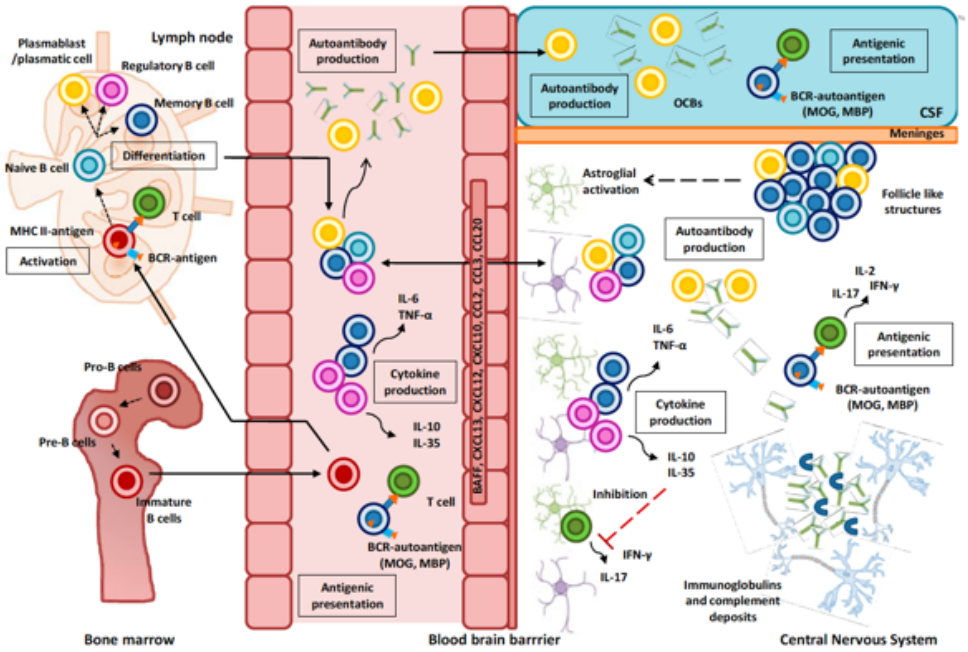

Fig. 5: Role of B cells in MS pathogenesis[81,92]**.** B cells begin their maturation in the bone marrow (BM) and undergo B cell receptor (BCR) rearrangement and upregulation of HLA-DR cell-surface protein. Immature B cells leave the BM and migrate to secondary lymphoid organs (SLO) where they differentiate into naïve mature B cells which are activated by specific antigen binding to its BCR. The antigens are internalized by B cells and their fragments are presented on the cell surface via major histocompatibility complex (MHC) class II to T cells. These T cells activate B cells, leading to B-cell proliferation and proinflammatory cytokines (IL-6, GM–CSF and TNF) secretion. There is also a different way of B activation in which the antigens themselves stimulate antibody production in the absence of T cells. After antigen activation, B cells differentiate into antibody-secreting plasmablasts/plasmatic cells or memory B cells. Activated B and T cells move to the CNS in various routes, including crossing the BBB. In the perivascular space, activated B and T cells interact with macrophages and can then enter the CNS parenchyma ("outside-in" inflammation). Memory B cells (Bmem), T cells and plasma cells enter the cerebrospinal fluid (CSF) through the blood–CSF barrier or the blood–meningeal barrier. Antibody-producing memory B cells and plasma cells in the CSF of patients with MS generate oligoclonal bands. The communication between CSF, periphery and brain parenchyma is demonstrated by the clonal relation of B cells in patients with MS (although B cells in the CSF can undergo further somatic hypermutation). In humans and mice, a specific subset of B cells can exert a regulatory anti-inflammatory function (B regulatory cells) by producing cytokines, such as IL-10 and IL-35 and by expressing inhibitory molecules that suppress proliferation of pathogenic T cells and autoreactive B cells. Moreover, they can enhance the function of regulatory T cells in a cell-to-cell contact-dependent manner. Taken from Torres et al[92].

Role of auto-ab: The presence of oligoclonal bands (OCB) in cerebrospinal fluid (CSF) of MS patients was discovered between 1959 and 1960[93] and their discovery supported the hypothesis that B cells have a crucial role in MS pathogenesis. OCB are constituted by immunoglobulin G (IgG) and M (IgM) produced by plasma cells in the CNS. The existence of OCB within the CSF, but not within the serum, is a strong indicator of intrathecal antibody synthesis [94] and is found in 87,7% of MS patients[95]. Neuropathological studies indicate that

antibody-mediated demyelination is one of the predominant pathogenetic mechanisms involved in white matter lesion formation in a proportion of multiple sclerosis patients[71,96]. Capping of surface IgG on microglia/macrophages engaged in myelin breakdown and co-deposition of IgG and activated complement fragments or complexes at the borders of active MS lesions strongly implicate ab as effectors of demyelination[97,98]. As demyelination is a key feature of multiple sclerosis neuropathology, myelin protein-derived antigens have been hypothesized to be the main autoreactive targets. Myelin basic protein, proteolipid protein and MOG, for example, have been demonstrated to be recognized by circulating CD4+ T cells in MS patients but also in healthy individuals, and there is conflicting evidence regarding potential differences in the frequency an avidity of these cells between the two groups[99,100]. Despite detection of ab recognizing myelin and neuronal antigens in serum, CSF and demyelinating lesions of MS patients, it is still unclear whether such auto-ab have a pathogenic role[101,102]. Thorough examination of the immunoglobulin making up the CSF OCB in MS has identified antibodies that primarily recognize ubiquitous intracellular self-proteins, thus suggesting that the OCB might be generated as a response to dead-cell debris rather than being primary perpetrators of the injury[103,104]. Other studies demonstrate that CSF oligoclonal IgG bind Epstein–Barr virus (EBV) proteins indicating that the compartmentalized B-cell response in MS could be sustained by a viral infection (EBV infection is the most studied exogenous risk factors for MS development) [105,106]

Antibody-independent functions of peripheral B cells in MS: The most compelling implication of the ab-independent roles of B cells in MS has come from the robust and rapid decrease in new relapsing MS disease activity observed after B cell depletion with anti-CD20 therapy[86,87,107–110]. Indeed, the reduction in relapse rate with anti-CD20 therapy is associated with little or no change to the CSF Ig profile in patients suggesting an ab-independent role of B cells in MS relapses[111,112]. These ab-independent functions are the contribution of B cells to cascades of cellular immune interaction in the periphery and in CNS where B cells are able to attract and activate T cells and myeloid cells[64]. Indeed, B cells from MS patients produce higher amounts of pro-inflammatory cytokines (including IL-6, GM-CSF, TNF and lymphotoxin-α) and are deficient in regulatory cytokines such as IL-10[113–118] compared to healthy controls. The abnormal cytokine response profile of B cells from patients with MS can induce abnormal Th1- and Th17-cell responses through TNF and IL-6 and can induce pro-

inflammatory myeloid cell responses which could contribute to the cellular immune cascades involved in relapses[113–118] (Fig. 6).

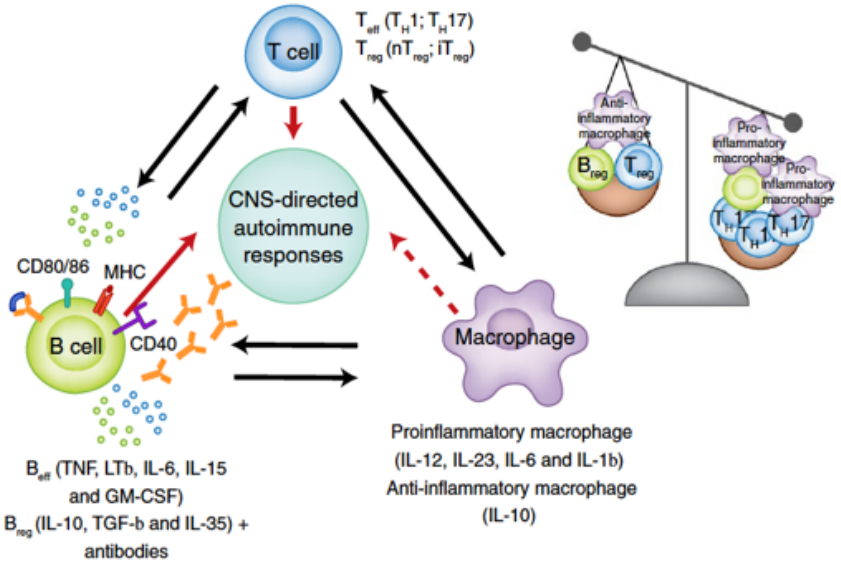

Fig. 6: B cells have a central role in MS pathogenesis independently of ab response. Antibody-independent roles of B cells are mediated through cytokine release resulting in proinflammatory effector B cells (Beff) or anti-inflammatory regulatory B cells (Breg). These cells can respectively activate and downregulate proinflammatory responses of T cells and myeloid cells. Taken from Li et al. [64].

In line with this finding, anti-CD20 B cell-depleting therapy reduces the pro-inflammatory responses of Th1 cells and Th17 cells and reduces myeloid cell pro-inflammatory responses in the periphery of patients with MS[113,114]. In contrast, the (largely naive) B cells that re-emerge after discontinuation of anti-CD20 treatment[113,114] have reduced secretion of GM-CSF, IL-6, and TNF, but produce greater amounts of IL-10; whether these cells have an immune-regulatory effect that potentially contributes to the durability of the therapeutic effect and whether the therapeutic effect lasts until the re-emergence of pro-inflammatory memory B cells is under debate. The regulatory function exerted by anti-CD20 treatment could also impact on the risk to develop a relapse[64], which is also driven by alterations in the balance between pro-inflammatory and anti-inflammatory B cells[113–118].

In the context of CNS inflammatory disease, the potential antigen-presenting cell (APC) function of B cells is supported by findings in EAE experiments in which B cell-specific MHCII-KO mice have been found to be resistant to recombinant MOG-induced disease[119,120]. As part of APC–T cell interactions, the integration of co-stimulatory signals plays a key role in defining the T cell response. Of the more than 20 co-stimulatory molecule–receptor pairs

identified to date, CD80/86 and their T cell–activating binding partner[121] CD28 are among the best characterized. Human B cells also stimulate T cells through both CD80 and CD86[64,122], and in MS, the frequency of circulating CD80+ B cells is abnormally elevated in patients with active disease[123]. In addition to expressing co-stimulatory molecules, B cells express co-inhibitory molecules involved in downregulating the responses of effector T cells. For example, the ligand programmed death(PD)-ligand1, expressed by B cells, protects against EAE by downregulating T cell responses through its receptor, PD-1[124]. B cell expression of the ligand of glucocorticoid-induced tumor necrosis factor receptor (GITR) ligand, another co-inhibitory molecule, directly induces Treg cell differentiation through its receptor, GITR[125].

Progressive MS: In addition to cascades of the cellular immune interactions in the periphery that contribute to MS relapses, ongoing inflammation in the CNS might contribute to the propagation of injury in patients with progressive disease. Chronic inflammatory response leads a self-sustaining chronic neurodegenerative process. This proceeds even in the absence of continued immune cell infiltration from the periphery and it is characterized by the immune cell exhaustion, associated with chronic antigenic exposure[126]. Indeed, in the progressive phase of disease, inflammation may differ from previous more active phases and is characterized by a lower frequency of inflammatory relapses (waves of infiltration of activated immune cells into the CNS in a perivascular distribution)[55]. In 2007, Magliozzi et al. showed the presence of B lymphoid follicle-like structures in the cerebral meninges of some patients with secondary progressive MS (Fig. 7), this finding indicates that B-cell maturation can be sustained locally within the CNS and contributes to the establishment of a compartmentalized humoral immune response[127,128]. Autopsy findings of more substantial meningeal inflammation are associated with more aggressive MS course before death[129] and more severe cortical pathology involving loss of oligodendrocytes and neurons as well as microglial activation[128,129]. This form of cortical injury, which appears to be unique to MS, develops in the most superficial part of the cortex subjacent to the meninges (referred to as 'subpial' cortical injury) and is now considered an important contributor to the pathology of progressive MS[55]. In this context, it is important to reiterate that B-cell-depleting therapy, was shown to be the first therapeutic approach with some effect in progressive MS[130,131].

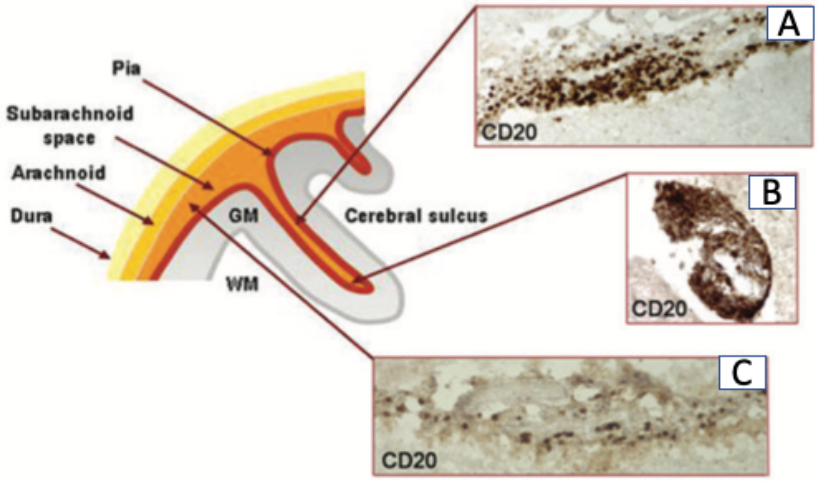

Fig. 7: Localization of ectopic B-cell follicles in the progressive MS patient brains. Ectopic B-cell follicles develop along (A) and in the depth (B) of the cerebral sulci, whereas scattered B lymphocytes (C) are detected in the meninges covering the external brain surface. Taken from Magliozzi et al[128].

2. Material and Methods

2.1 Healthy donors and MS patient cohort

Healthy donors (HD) and MS patients were age and sex matched (Table 2). HD were enrolled according to the following criteria: age 18 years and older, free from infections in the past three months, able to provide written informed consent. For the cross-sectional evaluation of NG2 expression, patients were enrolled according to the following criteria: age 18 years and older, with a diagnosis of RRMS (Poser or McDonald criteria) and an expanded disability status scale (EDSS) score of 0–6.5, free from immunomodulating or immunosuppressant drugs, in acute phase of disease (clinically and/or radiologically) and able to provide written informed consent. The longitudinal study of NG2 expression on B cells upon immunosuppression was performed on 7 alemtuzumab-treated MS patients at different time points: before the first drug administration (T0), after 3 (four patients), 9 (four patients), 27 and after 39 months (T39). Alemtuzumab (Lemtrada®, Genzyme Ltd, UK) was administered in two cycles every twelve months according to the European Medicines Agency approved scheme[132,133] (see Table 2 for demographic characteristics).

The study on NMO patients were performed in two patients age 18 years and older, free from infections in the past three months, free from any type of sustained pharmacological treatment and able to provide written informed consent (see Table 2 for demographic characteristics).

The study of B cells in BM was performed on four anonymous HD and four patients with MS aged 18 years and older, free from infections in the past three months, candidate for BM transplantation and able to provide written informed consent (see Table 2 for demographic characteristics)

Demographic data of HD	
Females	8
Males	8
Mean age	41,7 ± 16,4

Demographic data of MS patients	
Females	12
Males	4
Mean age	36,9 + 9,9
Form of disease	15 RRMM, 1 SPMS
Treatment	IFN, dimetylfumarate or glatiramer acetate
Duration of disease	8,9 ± 7,6
Mean EDSS	1,6 ± 1,1

Alemtuzumab treated patients						
	Gender	Age at treatment iniziation	Year of MS onset	Disease form	EDSS	Previous treatment
PT1	F	39	2010	RRMS	1	Fingolimod
PT2	M	38	2004	RRMS	5.5	Fingolimod
PT3	F	26	2007	RRMS	5	Fingolimod
PT4	F	26	2006	RRMS	1	Fingolimod
PT5	F	45	2008	RRMS	1	Fingolimod
PT6	F	41	2003	RRMS	4,5	Dimetylfumarate
PT7	F	48	2007	SPMS	6	Fingolimod

Table 2: Patient demographics

2.2 Ethics approval

This study was approved by the Ethics Committee of Ospedale Policlinico San Martino—IRCCS, Genoa, Italy, No. 190/12.

2.3 Cell isolation

Fresh blood (18 mls) was collected from HD and patients with MS for cross-sectional study of NG2 expression on immune cells. The same amount of blood was collected from patients treated with alemtuzumab at each single selected time point. For the experiments which involved the sorting of B cells by flow cytometry, 60-80 mls of peripheral blood were collected. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (Lympholyte-H, Cedarlane). 1-3 x 10^6 of cells were isolated from 1ml of blood from HD or untreated MS patients. PBMC isolated from patients treated with alemtuzumab were frozen in a solution of 90% fetal bovine serum (Thermofisher Scietific, Waltham,

California, USA) containing 10% dimethyl sulfoxide (Thermofisher Scietific, Waltham, California, USA), and stored under liquid nitrogen.

2.4 Flow cytometry analysis

Flow cytometry analysis, using the BD FACS CANTO II flow cytometer (Becton Dickinson, New Jersey, USA) and FACSDiva 7 Software (Becton Dickinson, New Jersey, USA), was performed on 1 x 10^6 cells, using the following conjugated antibodies (all from Becton Dickinson, Franklin Lakes, NJ): lineage (Pacific Blue), CD11c (APC) and CD33(PE) anti-CD3 (V500) and anti-CD4 (allophycocyanin [APC]-H7) for T cells, anti-CD56 (BV421) for natural killer (NK) cells, and anti-CD19 (PE.Cy7) for B cells. Further analysis of B-cell subsets was performed with anti-CD38 (APC), and anti-CD24 (PE) antibodies[134].

For analysis of NG2 expression we used one FITC-conjugated polyclonal antibody and four monoclonal antibodies (MoAb) (See Results section). As secondary antibody we used goat anti-mouse IgG1-FITC (Southern Biotech, Birmingham, Alabama, USA). Each antibody was tested with its own isotype control: Purified Mouse IgG1, k isotype antibody (Biolegend, San Diego, California) was used as control for MoAb2, MoAb3 and MoAb4. Purified mouse IgG2a k isotype (Biolegend, San Diego, California) was used for MoAb1. The amount of antibody used was according to the manufacturer's instructions.

2.5 B-cell isolation and B-cell sorting

B cells were negatively selected from PBMC using the B-cell isolation kit II, human (Miltenyi, Germany) according to the protocol given. The purity of the resulting B cell population obtained, which was tested by FACS for each experiment, was $\geq$ 90%. The isolated B cells were sorted into NG2+ and NG2- cells by staining with MoAb2 (1ul for 10^6 cells) followed by its secondary FITC-conjugated anti-IgG antibody, using the BD FACS ARIA sorter (Becton Dickinson, New Jersey, USA).

2.6 B-cell stimulation protocol

B cells isolated from HD were cultured for 72h with with 5 µg/ml CpG (ODN2006, Invivogen, San Diego, California, United States) and 10 ng/ml IL-15 (R&D System, Minneapolis, Minnesota, United States) for 72h at 37°C, 5% CO_2, in flat-bottomed 96-well plates (5 x 10^5 cells/well in 200 ul RPMI 1640 (Thermofisher Scietific, Waltham, California, USA) containing

10% heat-inactivated fetal bovine serum (Thermofisher Scietific, Waltham, California, USA), 1% penicillin/streptomycin (Thermofisher Scietific, Waltham, California, USA), 1% glutamine (Stemcell, Vancouver, Caneda), 1% HEPES (Thermofisher Scietific, Waltham, California, USA), 1% non-essential amino acids (Thermofisher Scietific, Waltham, California, USA), and 1% sodium pyruvate (Thermofisher Scietific, Waltham, California, USA) per volume. Sorted NG2- and NG2+ B cells were stimulated with CpG and IL-15 as above. Upon stimulation the viability of cells was test by cell viability solution (Becton Dickinson, New Jersey, USA).

2.7 Melanoma cell line

The melanoma cell line was as previously described[135]

2.8 Immunofluorescence

Isolated B cells ($1x10^6$) were fixed in suspension with 4% paraformaldehyde (Sigma Aldrich, Missouri, USA) for 30 minutes at room temperature. The cells were washed with phosphate buffered saline (PBS) and blocked for non-specific binding with 1% bovine serum albumin (BSA) (Sigma Aldrich, Missouri, USA) at room temperature for 30 min. B cells were stained with MoAb2 (1ul/100ul of 1% BSA) overnight at 4°C. Cells were washed with PBS and incubated with the secondary ab, Goat anti-mouse IgG1-FITC (Southern Biotech, Birmingham, Alabama, USA) (1:500 in 1% BSA) for 1h at room temperature. B cells were washed with PBS and stained with the nuclear stain, 4',6-diamidino-2-phenylindole (DAPI, 300 nM, Biolegend, California, USA) for 10 minutes at room temperature, washed again in PBS and mounted on polarized glass slides.

2.9 PCR

Total RNA was isolated using QIAzol® Reagent (Qiagen, Hilden, Germany). First-strand cDNAs were generated from 1 µg RNA using QuantiTec Reverse Trascption Kit (Quiagen, Hilden, Germany). Real-time PCR was performed with 20 ng cDNA using a Roche Light Cycler 480 (Roche, Basel, Switserland). Experiments were performed in duplicates with a final reaction volume of 20 µl containing 50 ng cDNA, 1 µl of each primer pair (20 µM), and 10 µl of LightCycler 480 SYBR Green I Master Mix (Roche). Amplification of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used to normalize the expression data. The primer

pairs for the indicated genes were synthetized by Tib Molbiol (Genova, Italy) according to the sequences shown in Table 3.

	Forward	Reverse
Primer1	5'-TTGTCCTGATGGCTAATGCTT-3'	5'-TGGGCTGCTCGATGGTGTA-3'
Primer2	5'-CGCTTCATCGATGGCTC-3'	5'-TGCTGCGGTGGATCTGTATG-3'
Primer3	5'-GAGCCCAGGCACGAAAAATG-3'	5'-GTATGTTTGGCCCCTCCGAA-3'
Primer4	5'-TTGTCCTGATGGCTAATGCCT-3'	5'-TGGGCTGCTCGATGGTGTA-3'
Primer5	5'-CCTCTGGAAGAACAAAGGTCTC-3'	5'-GAACTGTGTGACCTGGAAGAG-3'
Gapdh	5'-GCCCAATACGACATC-3'	5'-AGCCACATCGCTCAGACAC-3'

Table 3: Sequences of the primers used

The PCR was performed according the protocol described in Table 4.

Program name	Activation						
Cycles	1	Analysis mode	none				
Target (°C)	Acquisition mode	Hold	Rampe rate (°C/sec)	Acquisition (per °C)	Sec. Target (°C)	Step size (°C)	step delay (cycles)
95	none	00:08:00	4.40		0	0	0

Program name	Amplification						
Cycles	50	Analysis mode	Quantification				
Target (°C)	Acquisition mode	Hold	Rampe rate (°C/sec)	Acquisition (per °C)	Sec. Target (°C)	Step size (°C)	step delay (cycles)
95	none	00:00:05	4.40		0	0	0
68	single	00:00:05	2.20		60	0.4	1
72	none	00:00:15	4.40		0	0	0

Program name	Melting						
Cycles	1	Analysis mode	Melting curves				
Target (°C)	Acquisition mode	Hold	Rampe rate (°C/sec)	acquisition (per °C)	Sec. Target (°C)	Step size (°C)	step delay (cycles)
72	none	00:00:30	4.40		0	0	0
50	none	00:01:00	2.20		0	0	0
98	continuous		0.11	5	0	0	0
50	none	00:00:30	2.20		0	0	0

Table 4: PCR protocol

2.10 ELISA

Human IgG enzyme-linked immunosorbent assay (ELISA) development Kit and Human IgM ELISA development Kit (Mabtech, Stockholm, Sweden) were used to evaluate levels of IgG and IgM in supernatants (100 ul) from B-cell cultures, according to the manufacturer's protocol, using 96-well high protein-binding ELISA plates. The plates were read at OD 405 nm on a Multilabel Victor3 reader (Perkin Elmer). IL-10 levels were measured in B-cell culture supernatants using the pre-coated human IL-10 ELISA plates (Fine Test, Hubei, China) according to the manufacturer's protocol. Plates were read at OD 450 nm.

2.11 Statistical analysis

Statistical analyses were performed using Graph-Pad Prism v.6.00 (GraphPad Software, San Diego, CA). Statistical significance was evaluated using the paired Student t test (Wilcoxon matched-pairs signed rank test). P values $\leq$ 0.05 were considered statistically significant.
Where appropriate, data were visualized using T-distributed stochastic neighbor embedding (t-SNE)[136], a nonlinear data reduction algorithm that enables the visualization of high-dimensional data represented as two dimensional maps which preserve the original spacing of the data sets.

2.12 Isolation of bone marrow cells

Bone marrow was obtained by aspiration from the iliac bone from healthy donors (HD) for transplantation and from MS patients with severe disease prior to undergoing cell mobilization for auto-transplantation[137]. The BM cells were isolated by ficoll gradient centrifugation.

3. Results

The status of NG2 in human immune cells is mostly unclear. Using the monoclonal antibody (MoAb) 7.1, different groups demonstrated that NG2 is expressed by B cell lymphoblastic leukemia cells with the rearrangement of myeloid/lymphoid leukemia (or mixed lineage leukemia) (MLL) gene [138–144]. This feature is now used as adjunct to diagnosis for this type of leukemia[145,146]. However, NG2 expression was not detected on healthy immune cells with the MoAb 7.1.[138,143].

3.1 Validating the expression of NG2 in human immune cells

3.1.1 Reactivity with the polyclonal anti-NG2 antibody (Poly-NG2) used for the murine study[38].

The expression of NG2 on human immune cells was addressed by FACS analysis, using the polyclonal antibody tested in mice by Ferrara et al[38]. Over 20% of PBMC were stained by the poly-NG2; of these about 70% CD19+ B cells, 50% CD3+ T cells (Th CD4+ and Th CD8+ cells) and 85% CD56+NK cells reacted, while DCs, in contrast to what had been observed in the mouse, did not express NG2 (Fig. 8).

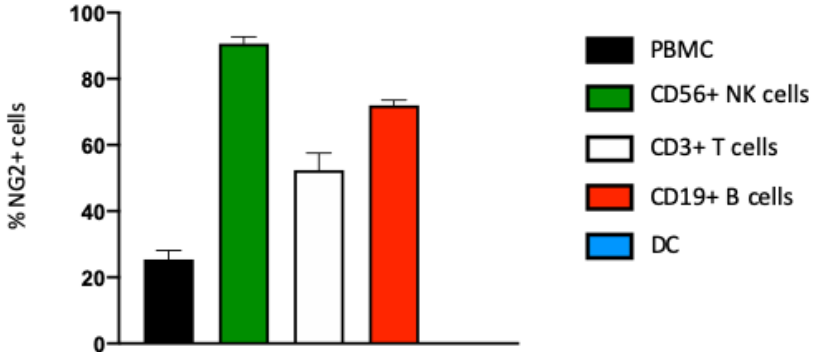

Fig. 8: **Expression of NG2 on main immune cell subsets.** The expression of NG2 by the different cell subsets was assessed by flow cytometry analysis using Poly-NG2.

We performed the analysis of NG2 expression on single immune cell subsets: CD56+dim and bright NK cells, CD3+CD4+ T cells, CD3+CD8+ T cells, CD19+ B cells and DC (Fig. 9).

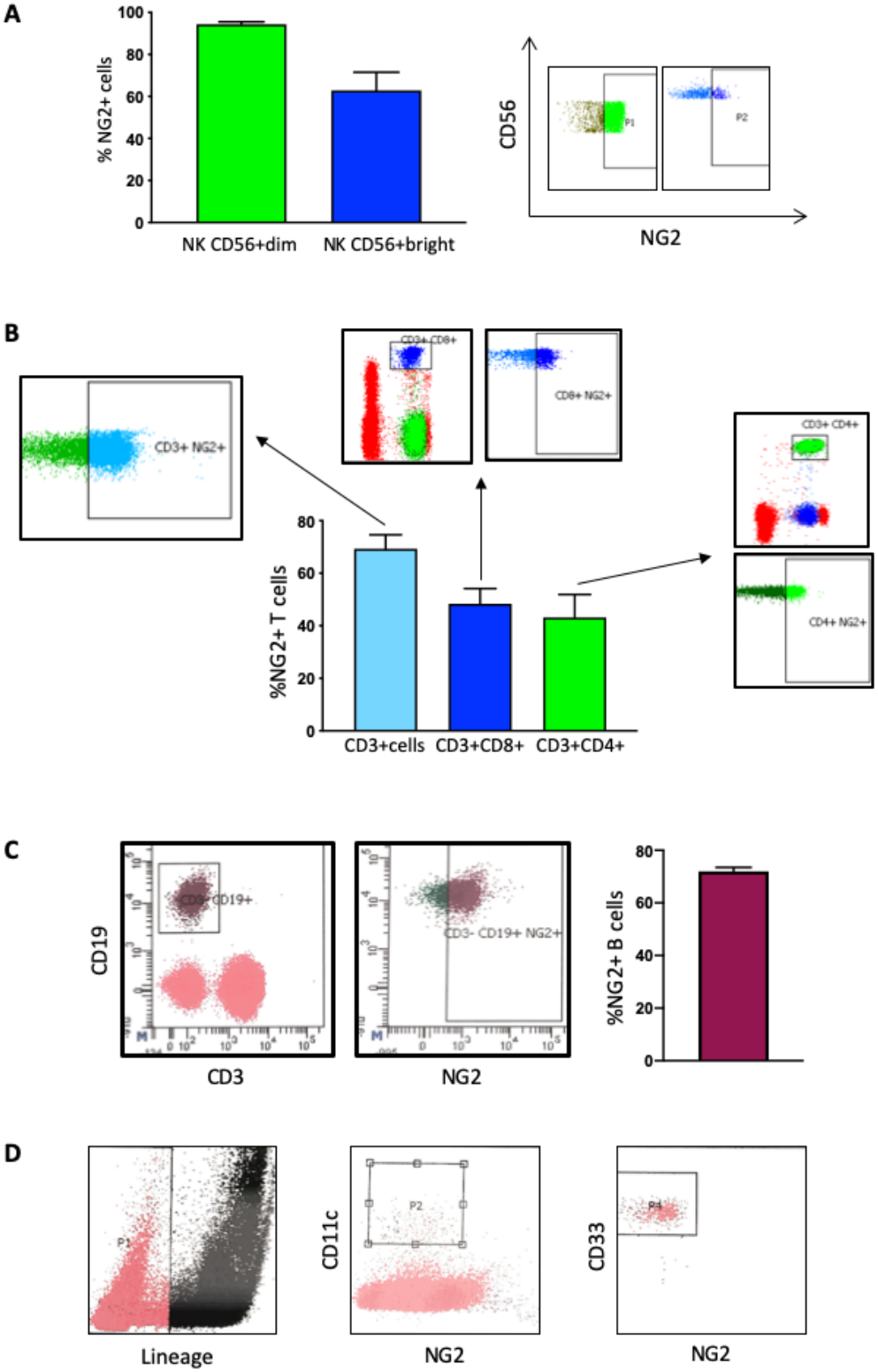

Fig. 9: NG2 expression on immune cell subsets. The expression of NG2 by the different cell subsets was assessed by flow cytometry analysis using Poly-NG2. Panel A: NK cells identified as bright and dim according to CD56 expression. Panel B NG2 expression was evaluated on CD3+ T cells and on CD4+ and CD8+ cells. Panel C: the percentage of NG2+ cells was represented on total CD19+ cells. Panel D: DC are gated as lineage -, CD11c+ and CD33+. All the bar-graphs shown here are represented with mean ± SEM.

Poly-NG2 gave a high percentage of positivity in all studied populations, apart for the DC which did not stain. These data differ from those obtained in other studies [138,143,144] which did not detect NG2 on healthy human immune cells. In the EAE study, we were able to validate the expression of NG2 on immune cells through the absolute negative control of NG2KO cells, which is not possible in humans. Because Poly-NG2 is a polyclonal antibody, there is no appropriate negative control, i.e. isotype control, for flow-cytometry. Accordingly, we needed to demonstrate the expression of NG2 on human immune cells through other means.

3.1.2 Controlled FACS analysis with monoclonal antibodies

Because reactivity with a MoAb, which recognizes a single epitope, in contrast to polyclonal antibodies, is likely to be considerably more specific for the protein under investigation, we have tested four MoAb directed against melanoma cell extract or purified rat NG2 (Table 5).

	Anti-NG2 MoAb 1	**Anti-NG2 MoAb 2**	**Anti-NG2 MoAb 3**	**Anti-NG2 MoAb 4**	**Poly-NG2**
Species Reactivity	Human	Human, Rat	Human	Human, Rat	mouse, rat, human, monkey
Host/ Isotype	Mouse / IgG2a, kappa	Mouse / IgG1, kappa	Mouse / IgG1, kappa	Mouse / IgG1, kappa	Not Applicable
Clone	9.2.27	N143.8	LHM 2	Mix of D120.43/D4 .11/N143.8/ N109.6	
Immunogen	Human M14 melanoma cell extract	Purified rat NG2 protein	Tissue / cell preparation (Human - a 375P cell crude extract)	Purified rat NG2 protein	Purified rat NG2 protein
Comapny		ThermoFisher Invitrogen	ThermoFisher Invitrogen	ThermoFisher Invitrogen	Merk Millipore

Table 5: Antibody specifications

MoAb 1 was selected because it is frequently used in clinical practice to identify NG2+ B cell lymphoblastic leukemia and is also used in our hospital. We used anti CD3, anti-CD56 and anti-CD19 antibodies to identify T cells, NK cells, and B cells, respectively, in PBMC from an HD, together with anti-NG2 MoAb 1 or its isotype. Our data showed that MoAb 1 did not show any reactivity with healthy human immune cells (Fig. 10).

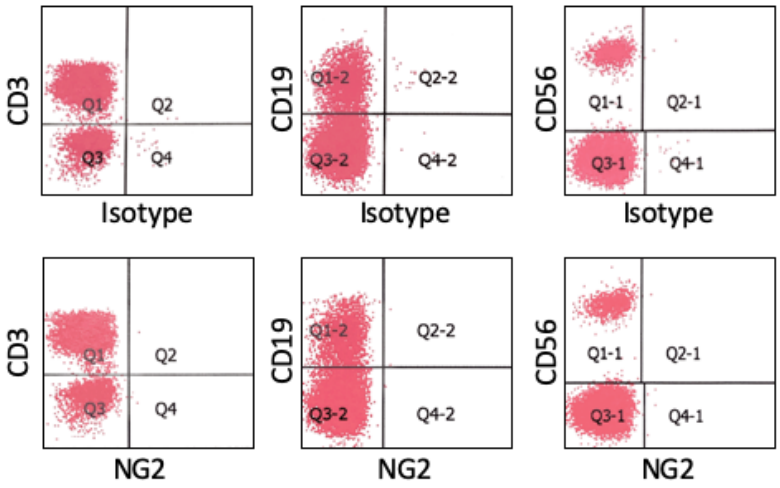

Fig. 10: Flow cytometry staining of PBMC from HD with MoAb 1

Based on our speculation that this MoAb might only detect a neo-epitope on cancerous cells (see also below), we tested three other MoAb preparations, one of which is a mixture of several clones (Table 5). As can be seen on Fig. 10, all 3 MoAb reacted, albeit to different extent, and the study was continued with MoAb 2, which showed the highest reactivity and was also contained in MoAb 2 preparation (Fig. 11).

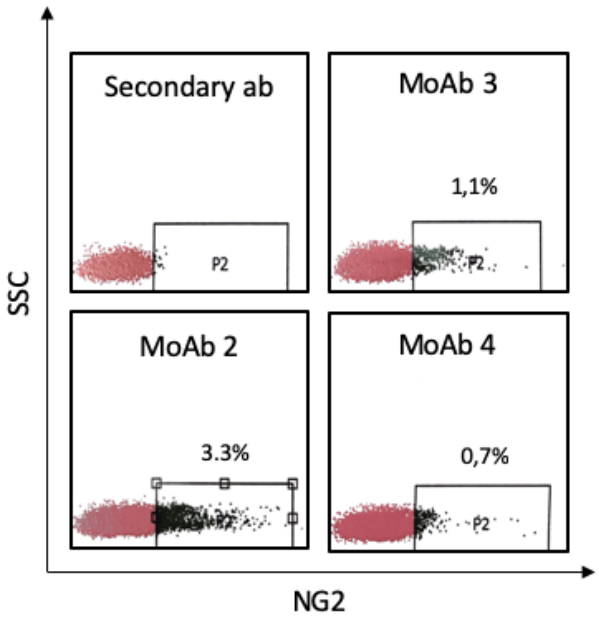

Fig. 11: Flow cytometry staining of PBMC from HD with MoAb2, MoAb3, and MoAb4

3.1.3 Optimization of flow cytometry conditions with positive and negative controls

In order to further validate our flow cytometry data, we selected two positive controls. NG2 is well expressed in many different neoplasms, in particular on melanomas[147] and on B-cell lymphoblastic leukemias[145,148]. Hence, we assessed one melanoma cell line raised from a biopsy sample[135] and lymphocytes from a B-cell lymphoblastic leukemia patient already identified as NG2+ by the clinical laboratory of our hospital. As can be seen on Figs. 12 and 13, more than 90% of the melanoma cells and about 50% of the leukemia cells reacted with MoAb2, providing appropriate positive controls for reactivity to this MoAb**.**

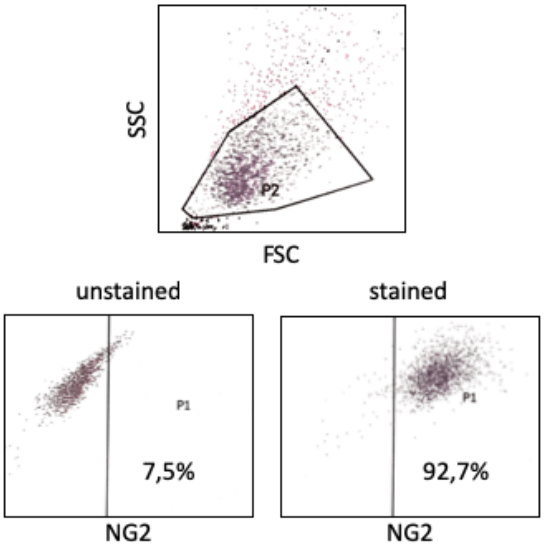

Fig.12: Reactivity of melanoma cells to MoAb2. While some autofluorescence was observed in the absence of MoAb3, more than 90% of melanoma cells reacted to the antibody (MoAb 2).

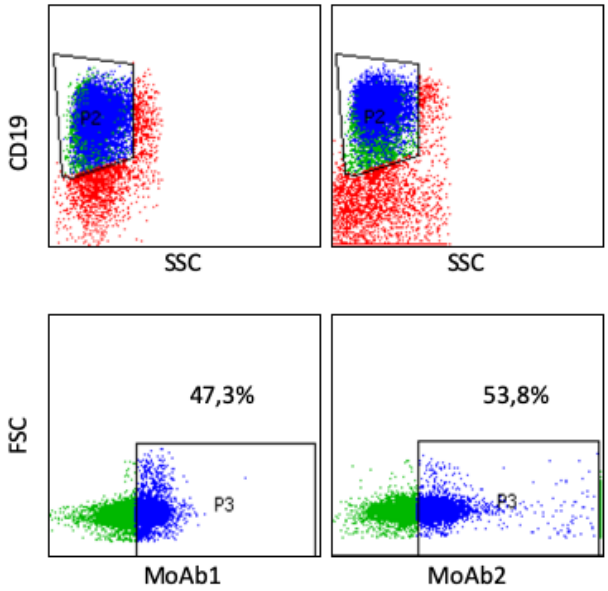

Fig. 13: NG2 expression on B cells from a B-cell lymphoblastic leukemia sample. The percentage of NG2+ cells in a sample of B-cell lymphoblastic leukemia was similar using MoAb2 and MoAb1 (MoAb used in the clinic for B-cell leukemia diagnosis).

In contrast to what happens in the mouse, where we could use immune cells from NG2-KO mice, there are no known NG2-deficient human mutations. Accordingly, we assessed reactivity of B cells from two HD with the IgG1 isotype, which is relevant for MoAbs 2-3-4. As can be seen in Fig. 14, very few cells were stained by the isotype**.**

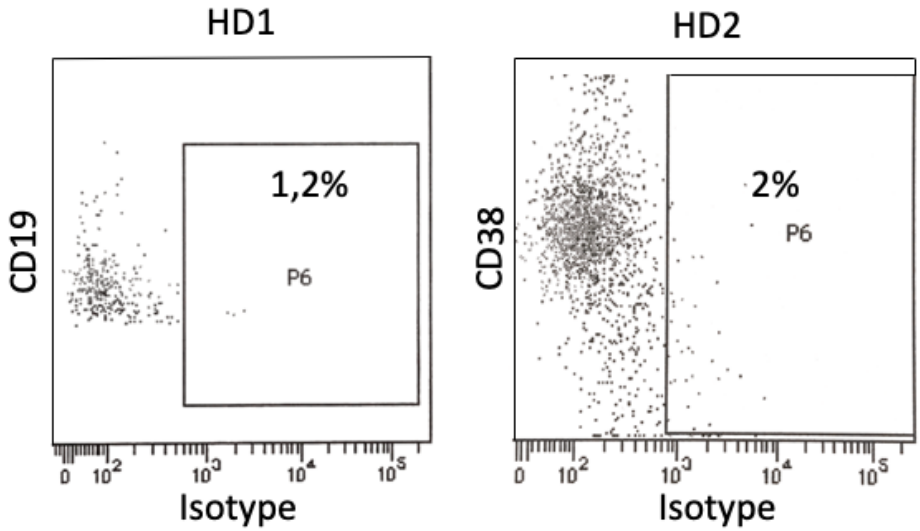

Fig. 14: Assessment of B-cell reactivity to isotype control for NG2 MoAb supports the specificity of MoAb2.

3.1.4 MoAb2 identifies B cells as the main NG2-expressing cells

On the basis of the above experiments, we selected MoAb2 as the NG2 MoAb relevant to monitor NG2 expression in immune cells. As can be seen in Fig. 15A-C, flow cytometry of

PBMC with MoAb2 indicated that CD3+ T cells, CD56+ NK cells, and monocytes express little or no NG2. In contrast, around 10-15% of B cells were stained by MoAb2 (Fig. 15D), as also summarized in Fig. 15E.

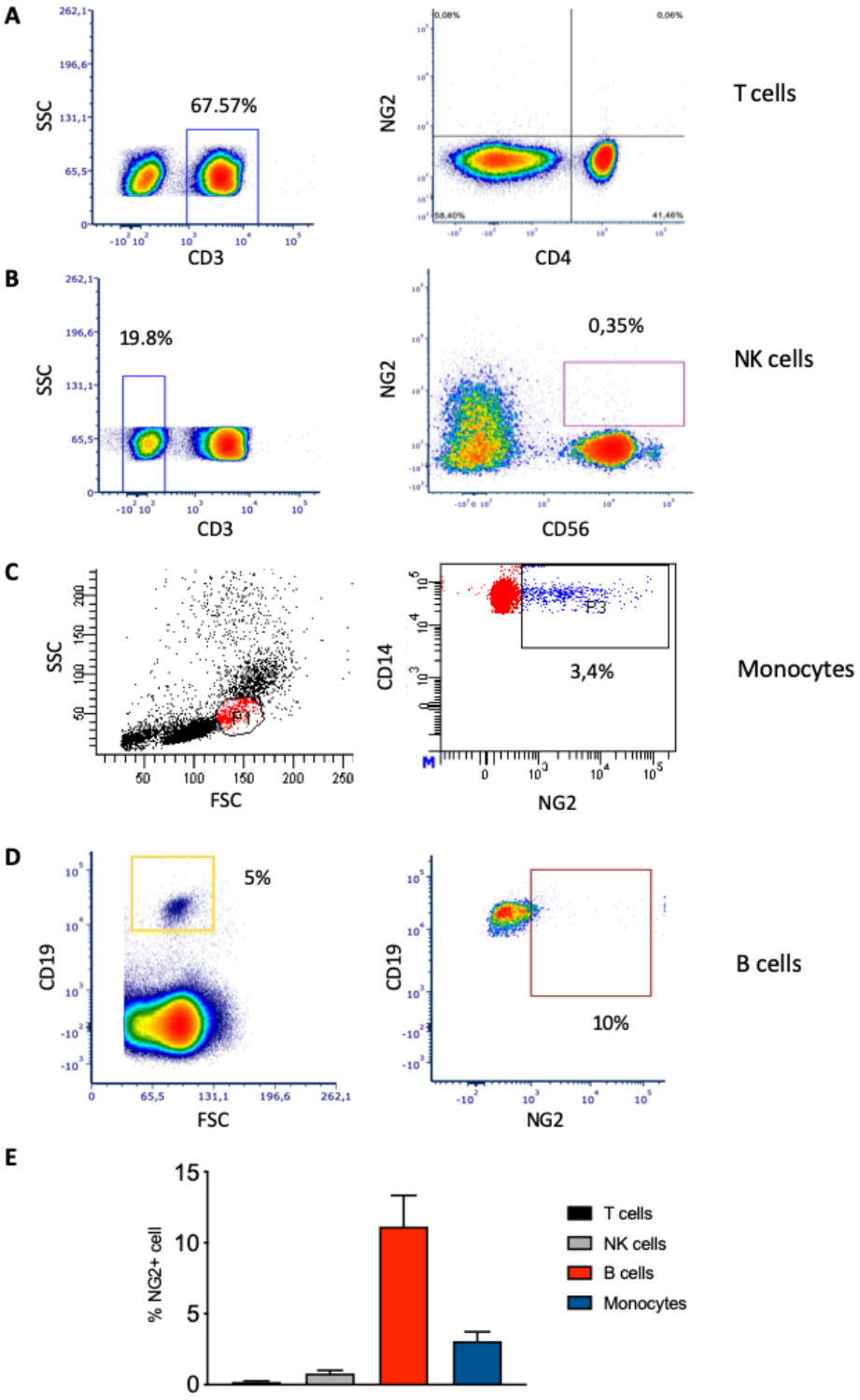

Fig.15: B cells are the main NG2+ lymphocyte population in PBMC. Panels A, B, C and D are representative flow cytometry plots obtained with PBMC from three HD. T cells are identified as CD3+ and CD4 (A), NK cells are identified as CD56+ (B), Monocytes are identified as CD14+

cells (C), B cells are gated as CD19+ (D). Lower panels represent the concomitant reactivity for NG2. Panel E represents the mean ± SEM of analyses from three HD.

3.1.5 Validation of NG2 expression by Immunofluorescence using MoAb2

We further validated NG2 expression by immunofluorescence. Considering the small percentage of NG2+ cells in PBMCs and that most of the positive cells are B cells, we performed immunofluorescence analysis with MoAb2 on B cells isolated by negative selection on magnetic beads (purity >90%, data not shown). Immunofluorescence confirmed that NG2 was expressed in about 1/10 of DAPI-positive cells (Fig. 16), which is commensurate with the 10-15 % positive B cells in PBMC**.**

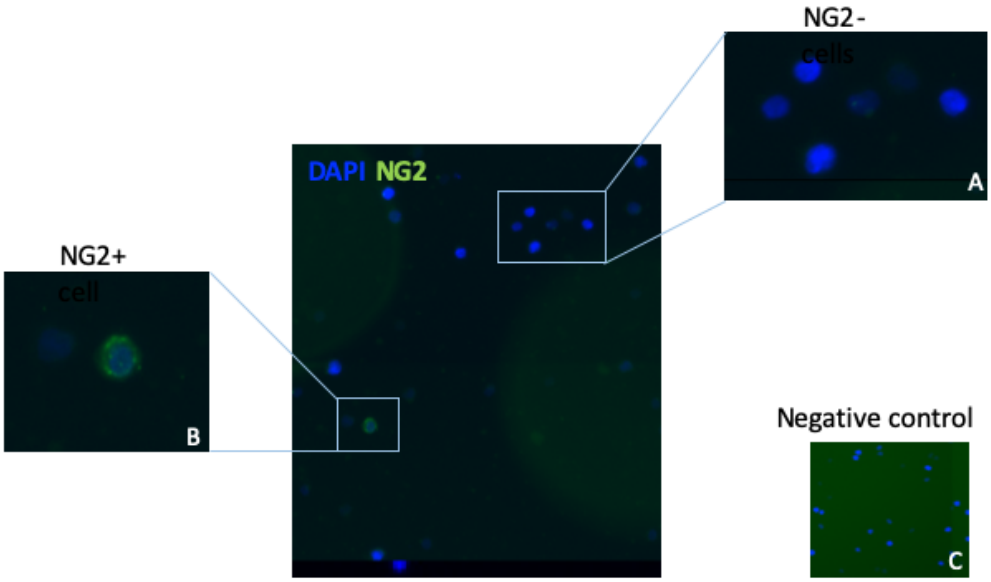

Fig. 16: NG2 expression on a small percentage B cells was confirmed by immunofluorescence assay. Total B cells were processed for immunofluorescence with MoAb2**.** Zoomed images (A) and (B) show non-reactive cells and MoAb2-reactive cells, respectively. (C) shows the image obtained using the secondary anti-IGg1 ab alone.

3.2 Validation of NG2 expression by PCR

To validate the expression of NG2 by peripheral immune cells, we measured the NG2 mRNA content in isolated B cells, shown by FACS to be the main population of NG2-expressing cells in PBMC, as compared to that in T cells which were mostly negative for NG2 by FACS. Melanoma cells which are known to express NG2 were used as positive control. We tested different couples of primers (listed in Table 3, see Materials and Methods, section PCR) and selected the Primer1 pair, which gave the strongest, most reliable results (data not shown), for the successive experiments. As shown in Fig. 17, NG2 mRNA was clearly expressed in B

cells (2 HD tested), at levels comparable to those in melanoma cells; as can be seen, NG2 mRNA could not be detected in T cells, corroborating our flow cytometry and immunofluorescence findings.

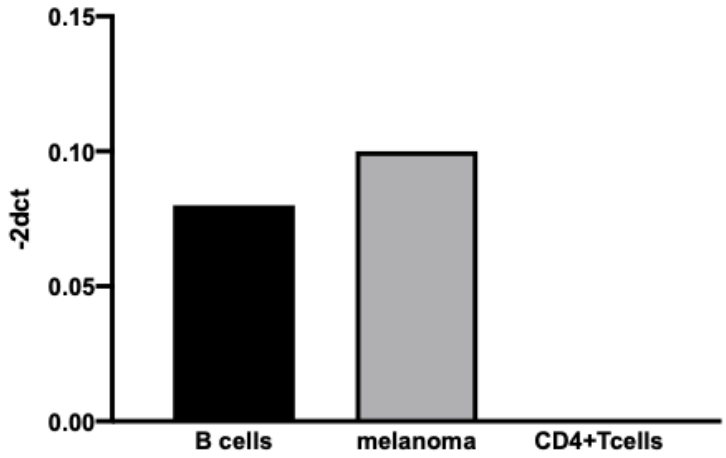

Fig. 17: PCR analysis of sorted NG2+ B cells. PCR was performed with the Primer1 pair on B cells obtained from 2 HD. CD4+ T cells isolated by negative selection, were used as negative control based on their negativity in flow cytometry analysis (see Fig. 15).

3.3 NG2 expression on B-cell subsets in health and disease

To further understand the possible role of NG2 expression on B cells, we assessed if this expression was specific or not to a particular subset.

3.3.1 Flow cytometry gating strategy for B-cell subsets

We identified B-cell subsets by flow cytometry[134,149–151] on the basis of their expression of CD19, CD24 and CD38, distinguishing atypical B memory cells (aBmem), B memory cells (Bmem), B regulatory cells (Breg), B mature cells (Bmat) and plasma cells (Table 6). The gating strategy is presented in Fig. 18.

B CELLS	
aBmem	CD19+CD14-CD24+CD38-
Bmem	CD19+CD14-CD24+CD38low
Bmat	CD19+CD14-CD24lowCD38low
Breg	CD19+CD14-CD24+CD38high
plasma cells	CD19+CD14-CD24-CD38high

Table 6: B cell populations identified as per the indicated markers

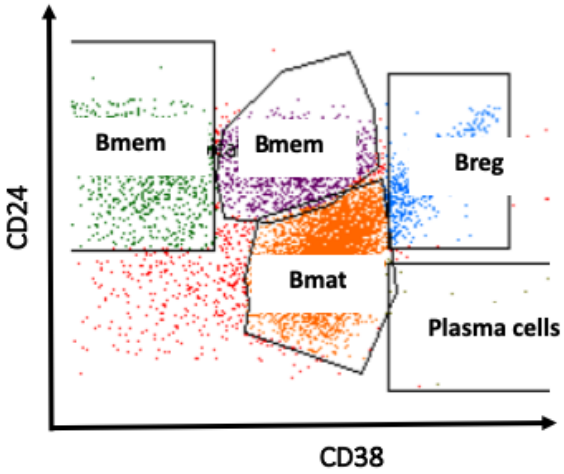

Fig. 18: Flow cytometry gating strategy for B cells.

3.3.2 NG2 expression in different B-cell subsets in HD compared to MS patients

We used this gating strategy to analyze NG2 expression on B-cell subsets in 16 HD and compared it to that in 16 patients with MS. As can be seen in Fig. 19A, the percentage of cells in the various B-cell subsets did not change between HD and MS patients, nor did the frequencies of NG2+ cells in the total B-cell population or in B-cell subsets, except for the NG2+ plasma cell populations, which showed some statistical difference (P =0.014). However, the mean % of plasma cells in the B-cell population is very small (around 3%), and of these 6 and 12 % were NG2+ in HD and MS patients, respectively, hence it is difficult to make conclusions on changes in such small populations, i.e. about 0.2 and 0.4% NG2+ in total B cells in HD and MS patients, respectively. Both in HD and MS patients the B memory cell population (indicated as aBmem+Bmem) displayed an increased frequency of NG2+ cells, but there was no difference in frequency of these NG2+ cells between HD and MS patients (Fig. 19B).

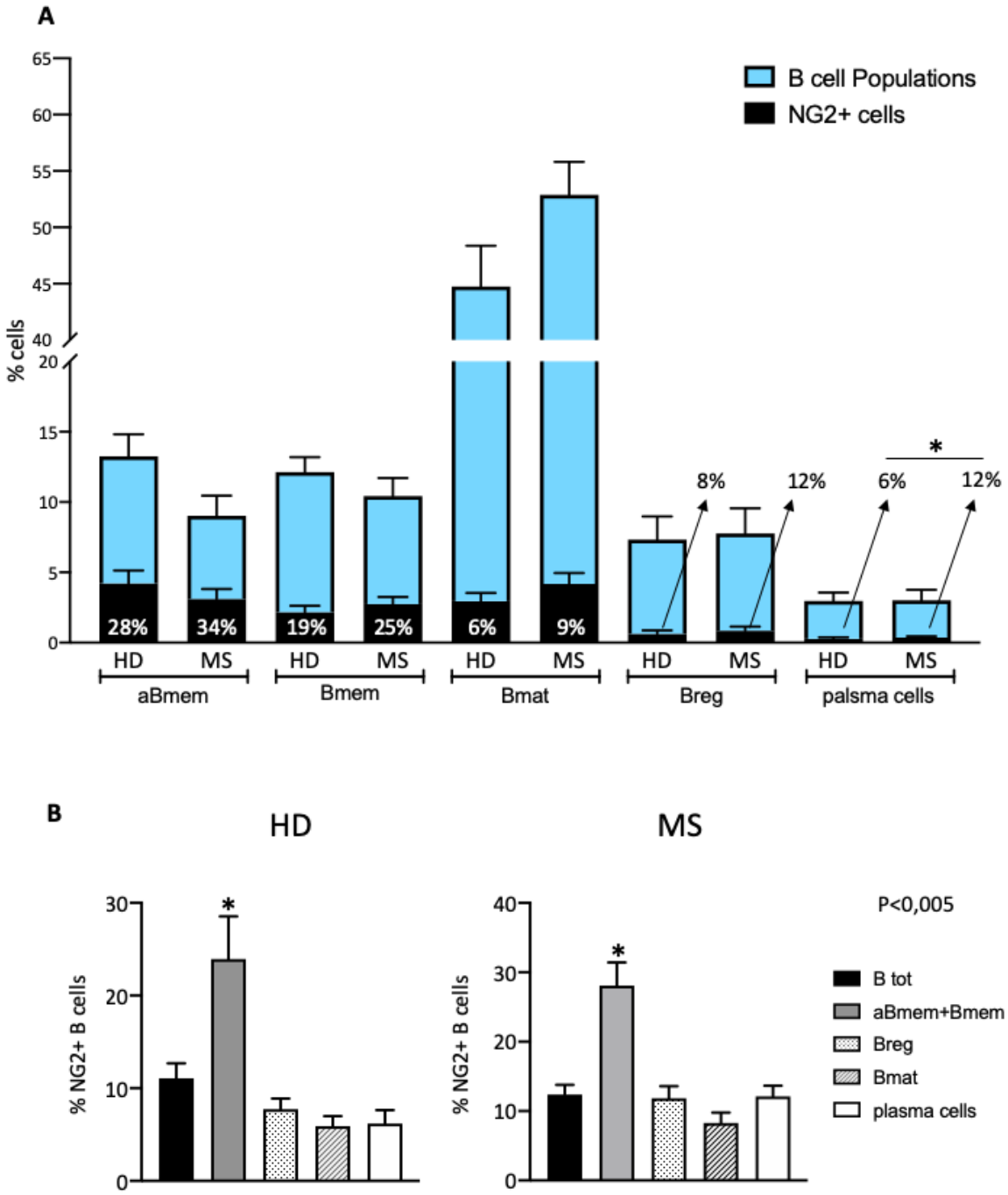

Fig. 19: Human B cell subpopulations express different levels of NG2. NG2 expression was measured by flow cytometry using MoAb2 and antibodies against relevant B-cell markers as indicated in Table 6. Results are reported as mean ± SEM of 16 MS patients or HD. (A) shows the frequency of cells in each subpopulation, together with the % of NG2+ cells within that population. (B) shows the comparison of the percentage of NG2+ cells in each subpopulation in HD (left panel) and MS patients (right panel). *P < 0.05 (NG2+ cells in MS vs HD plasma cells); **P < 0.005 (NG2+ cells in memory B cells vs all other subsets in HD and MS patients).

3.3.3 NG2 expression changes dramatically in B cells from 2 patients with B-cell-mediated demyelinating disease, neuromyelitis optica (NMO)

MS is considered a T-cell-mediated disease. We speculated that the expression of NG2, which is found predominantly, if not exclusively, on B cells in PBMC, might differ in diseases that are predominantly B-cell-mediated, such as NMO. We chose NMO as a neurological disease that is associated, as is MS, with demyelination and axonal loss[152]. As NMO is a rare disease (prevalence 1-2/100,000 individuals[153]), we could only test 2 untreated newly diagnosed patients. Despite the very low number of patients tested, there was a drastic increase in percentage of NG2+ B cells in the total B-cell population in both patients as compared to HD or MS patients, from 10-15 % in HD or MS patients to about 70% in the 2 NMO patients (Fig. 20A). In HD and MS patients, the total NG2+ memory B-cell population (Bmem+aBmem) amounts to 25-30%. Comparison of the frequencies of NG2+ B cells in the different populations between HD or MS patients and both NMO patients, indicated that the frequency of NG2+ cells appeared considerably increased in each subpopulation analyzed (Fig. 20B and C); obviously we cannot analyze the data statistically, but in each of the 2 NMO patient, the frequencies in Breg, Bmat and plasma cells, which were $\leq$ 10-15% in HD or MS patients, were considerably increased to at least 30%, albeit with variations between the two patients (Fig. 20B and C). The memory B-cell population frequency was also increased to 64 and 87% in the NMO patients (Fig. 20B-C).

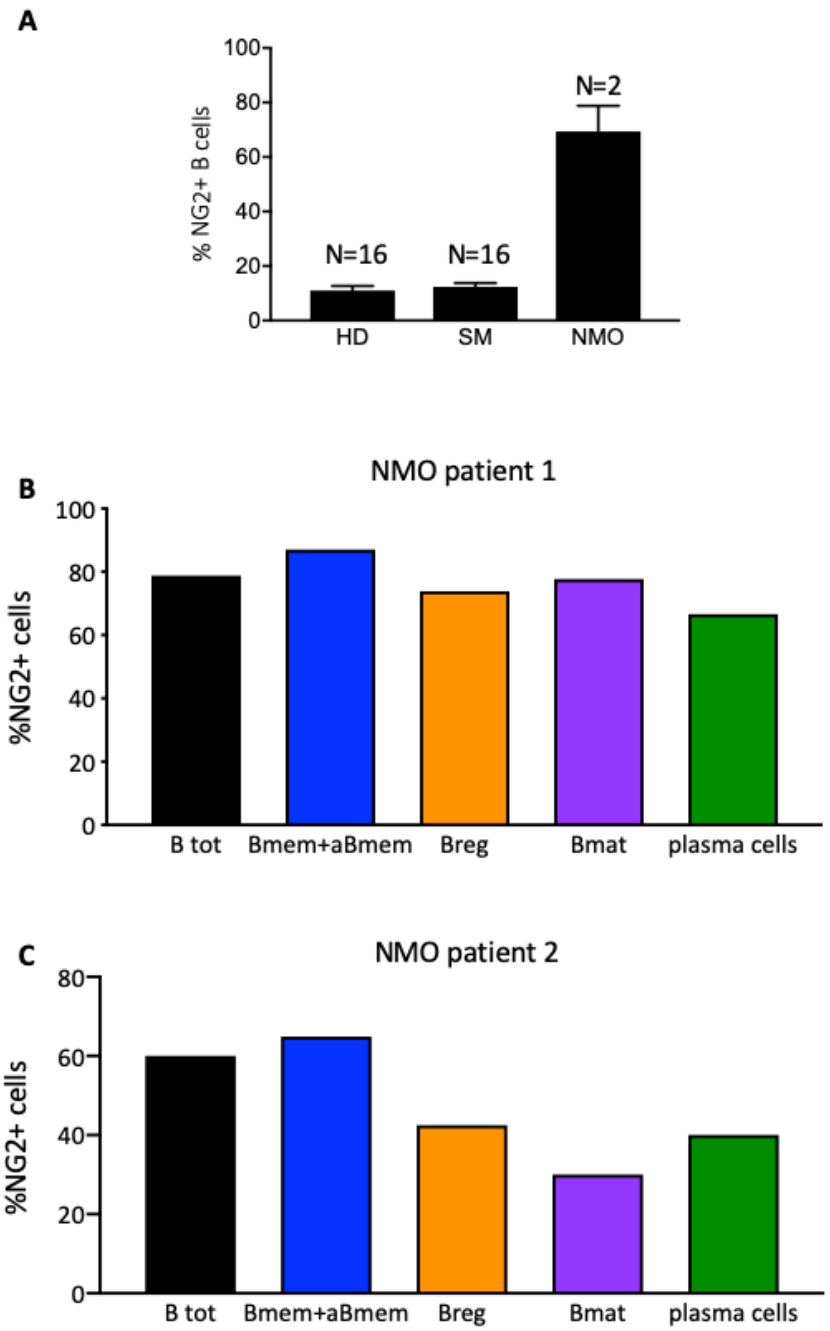

Fig. 20: NG2 expression on total B cells and B cell subsets in two patients with NMO. (A) shows the comparison of mean ± SEM NG2+ B-cell frequency in the total B-cell population between HD, MS and NMO patients. (B) and (C) shows the NG2+ B cell percentages in each B-cell subpopulation in each of the two NMO patients.

3.4 Effect of B-cell stimulation on NG2 expression

In mice, the expression of NG2 on immune cells is associated with activation of dendritic cells and, thereby, potentially with T-cell stimulation[38]; it is also associated with activation of progenitors, such as OPCs and pericytes[32,36]. Therefore, we postulated that NG2 could be

also playing a role in the functionality of human B cells. Accordingly, we have investigated how the expression of NG2 changes upon B-cell stimulation.

3.4.1 Effect of stimulation on B-cell subsets

We first assessed activation of isolated HD B cells upon culture with CpG and IL-15 by evaluating physical parameters through flow cytometry. We tested the activation of B cells by verifying the increase in cell size by FACS after 72 hours of stimulation (data not shown). The percentage of dead cells was 30% in stimulated cells and 40-45% in not stimulated (data not shown). The evaluation of B cell populations and of NG2 expression was performed on live cells. Flow cytometry analysis of the resulting B-cell subsets showed a significant reduction in Bmem and Plasma cells (Fig. 21).

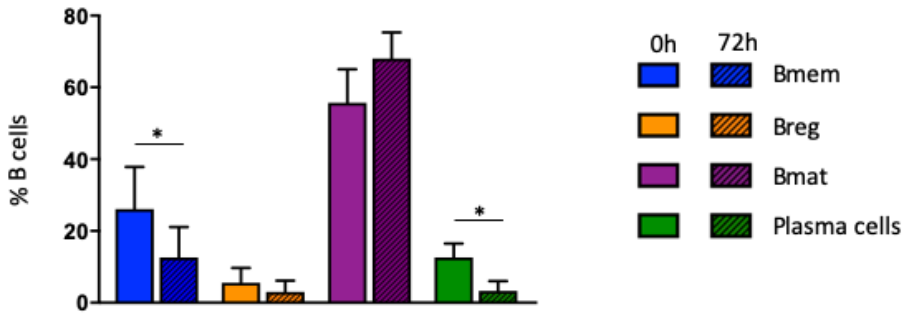

Fig. 21: Stimulation of total B cells with CpG and IL-15 affects cell frequencies differentially in B-cell subsets. The analysis was performed on live cells gated by FACS (live/dead staining). The graph is representative of 6 experiments (mean ± SEM). *$P < 0.05$ (NG2+ Bmem or plasma cells at 0 vs 72 hours).

The percentage of total NG2+ B cells decreased after 72h stimulation of isolated HD B cells with CpG and IL-15 (Fig. 22A). However, although there was a decreasing trend in NG2+ cells in all subsets, except plasma cells, upon stimulation, particularly in Bmem and Breg cells, the decreases were not significant (Fig. 22B). Nevertheless, tSNE analysis indicated that the intensity of NG2 expression was reduced in stimulated cells [see different scales in left (89 to 1129 AU) and right (29 to 210 AU) panels of Fig. 22C].

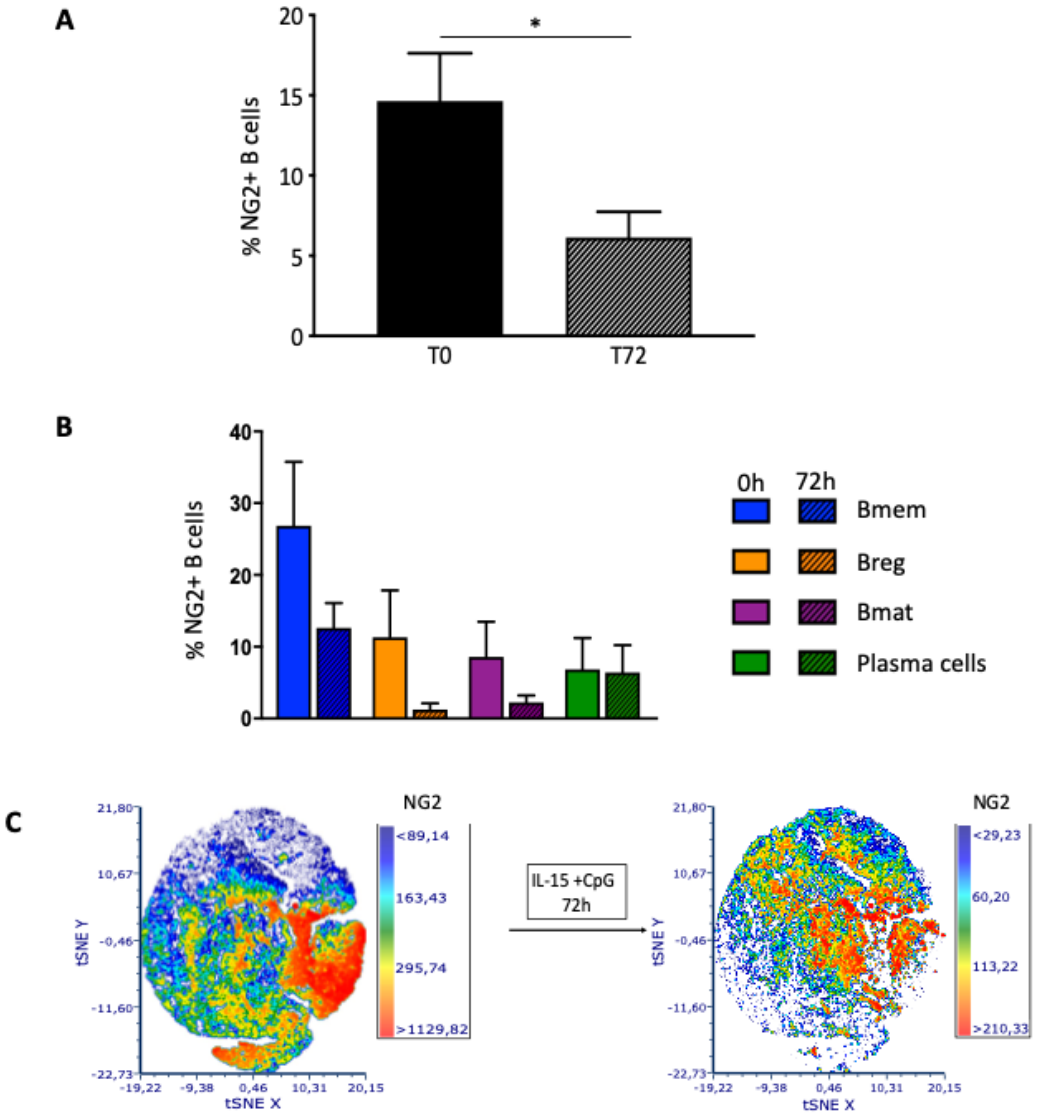

Fig. 22: Effect of B-cell stimulation on NG2 expression. Isolated B cells were stimulated with CpG and IL-15 at 0 and 72 hours and analyzed by FACS for their expression of NG2. (A) and (B) present the percentage of NG2+ cells in the total B-cell and B-cell subsets, respectively. Data are presented as mean ± SEM of 6 experiments. (C) shows the tSNE analysis of the results.*$P < 0.05$ (NG2+ cells in total B cells at T0 vs T72).

We confirmed the activation of B cells by evaluating the release of IL-10, IgM, and IgG (Fig. 23). B activated cells from all 3 HD produced a higher amount of IL-10 as compared with unstimulated cells (Fig. 23A), which corroborates the data obtained by Blair et al.[150]. There was a clear trend to increased IgG (Fig. 23B) and IgM (Fig. 23C) levels in supernatants obtained from activated cells.

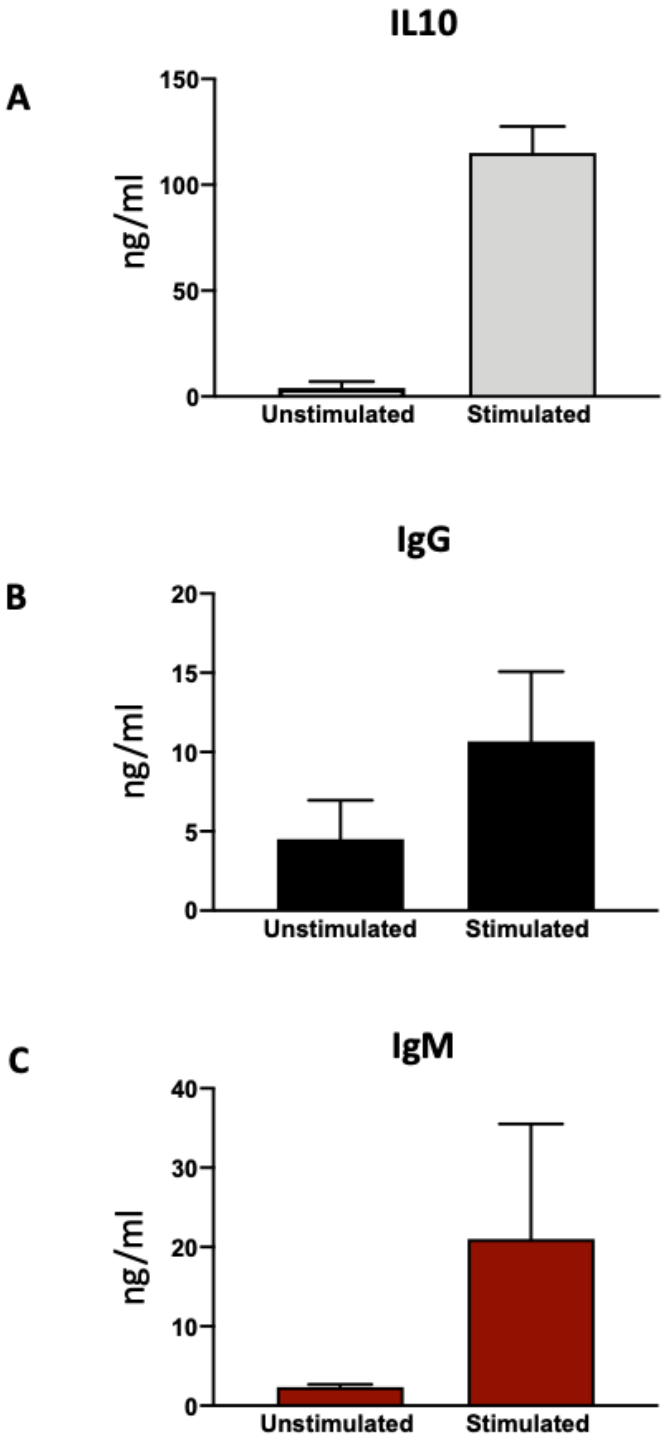

Fig. 23: IgM, IgG and IL-10 levels in supernatants from stimulated and unstimulated B cells. IgM, IgG and IL-10 levels in supernatants of B cells isolated from 3 HD and activated with CpG and IL-15 were measured in duplicates by ELISA. Graphs are represented as mean ± SEM. *$P < 0.05$ (IL-10 expression of unstimulated vs stimulated B cells).

3.4.2 Effect of activation on NG2- and NG2 + sorted cells

To assess if activation of B cells was associated with changes in NG2 expression, we isolated B cells from PBMC by magnetic bead-sorting and used MoAb2 to flow-sort the resulting total B cells as NG2+ and NG2- populations. We confirmed that the B-cell populations were indeed

NG2+ and NG2- by FACS immediately upon flow-sorting, and stimulated, or not, each sorted population with CpG and IL-15 for 72 hours. In order to confirm the activation of both populations, we evaluated the production of IgG (Fig. 24A), IgM (Fig. 24B) and IL-10 (Fig. 24C) by ELISA of supernatants from stimulated and unstimulated cells. In both NG2+ and NG2- stimulated B cells, an increase in IgM and IgG production was observed, but there was no difference in IgM and IgG release between NG2+ and NG2- B cells (Fig. 24A and B). In contrast to what we observed with total unsorted B cells, IL-10 release by NG2+ and NG2- B cells did not change upon activation (Fig. 24C).

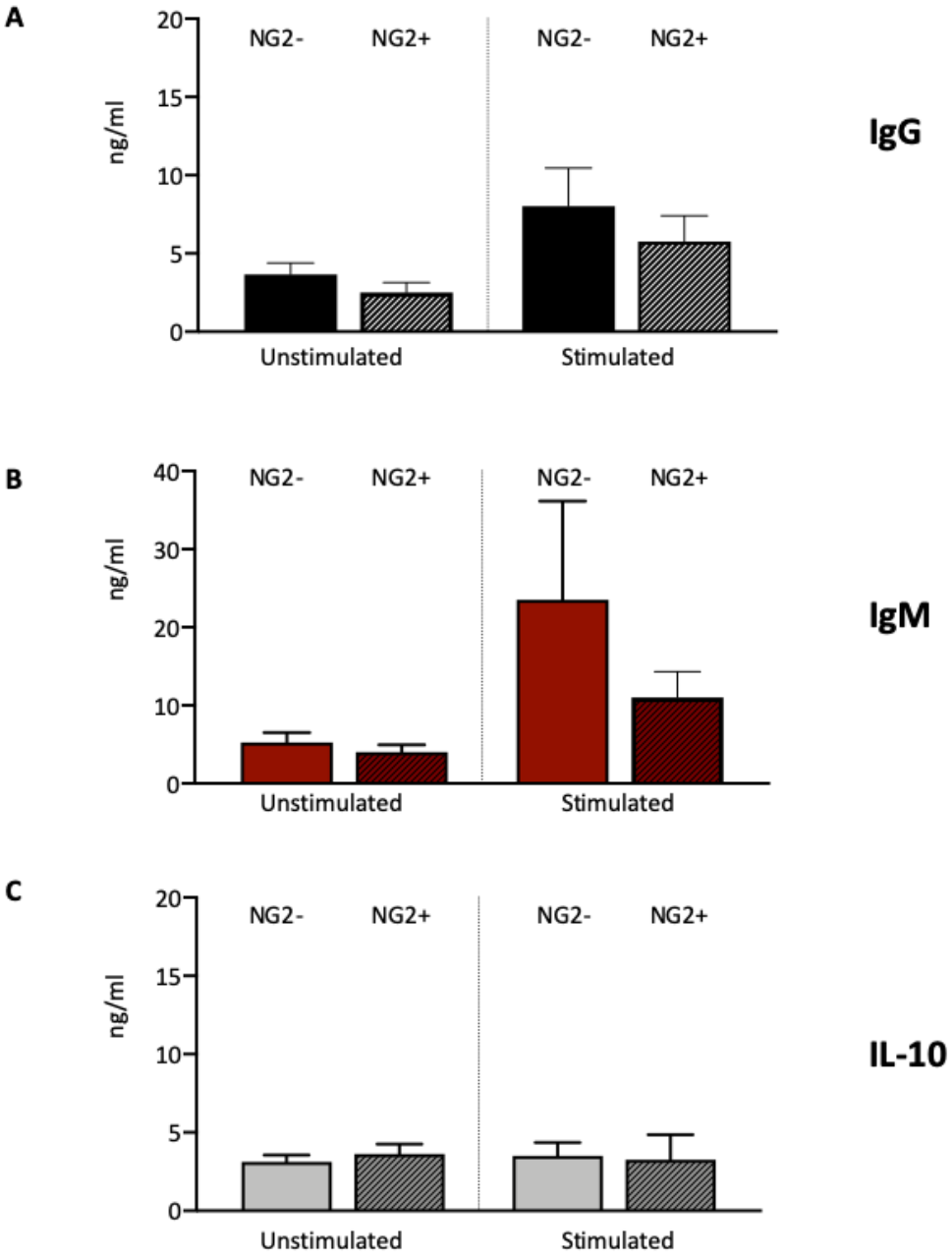

Fig. 24: IgG, IgM and IL-10 release from NG2+ and NG2- sorted cells. B cells isolated from 3 HD were flow-sorted into NG2+ and NG2- populations and stimulated with CpG and IL-15 for

72 hours. Supernatants were tested for IgG, IgM and IL-10 by ELISA. Data are presented as mean + SEM of 3 experiments.

Upon B-cell activation, we observed a mild decrease in NG2 expression on NG2+ sorted cells, whereas some NG2+ cells were observed in the NG2- sorted cells (Fig. 25). Thus, about 30% of sorted NG2- cells expressed NG2 upon 72hrs of culture independently of the presence of stimulating factors (Fig. 25A). It is well known that flow-sorting can lead to cell activation and we speculated that this increase might be due to such an effect. However, as there was a similar increase in NG2- B cells after stimulation, we cannot propose that the de-novo NG2 expression at the surface of the activated B cells is due to stimulation. On the other hand, after stimulation, the percentage of sorted NG2+ cells had decreased (Fig. 25B).

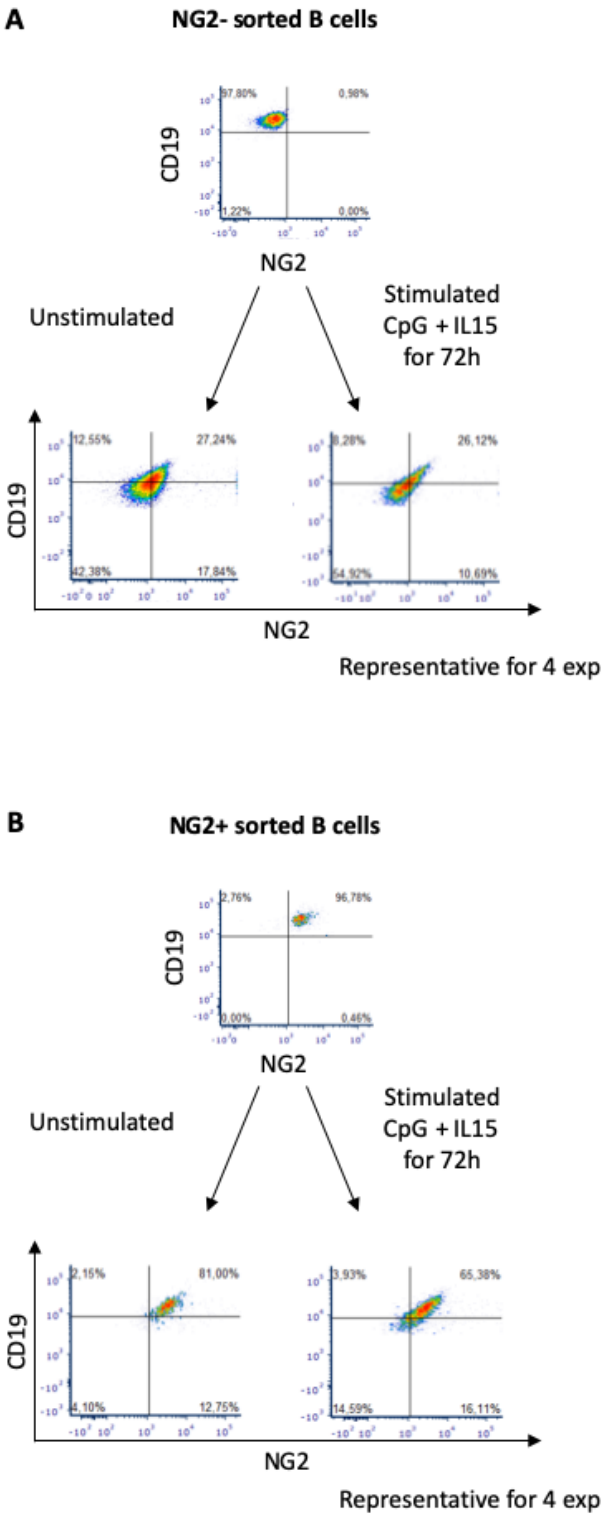

Fig. 25: Changes in NG2 expression on stimulated and unstimulated NG2+ and NG2- sorted cells. B cells isolated from 4 HD were flow-sorted into NG2+ and NG2- populations, stimulated with CpG and IL-15 for 72 hours, and assessed for NG2 expression by flow cytometry. The shown plots are representative of 4 experiments.

3.5 Effect of alemtuzumab treatment on NG2-expressing B cells

Alemtuzumab is a humanized monoclonal anti-CD52 antibody that selectively depletes circulating CD52-expressing B and T lymphocytes[154,155]. After lymphocyte depletion, the pattern of immune cell repopulation shows a reprogramming of the immune system towards a less inflammatory phenotype[156,157]. Most interestingly in the context of our study is the observation that at least 30% of alemtuzumab-treated MS patients develop a secondary B-cell-mediated autoimmune disease, including thyroiditis, immune thrombocytopenia, and nephropathies[133,158]. Wiendl et al. [159] have recently showed that neither the total count of B cells nor the dynamics of T- or B-cell repopulation differed between patients who developed secondary autoimmunity or were non responders, and patients who did not develop such secondary effect or were responders. We therefore postulated the possibility that the autoimmunity risk might be associated with changes in NG2 expression on B cells and/or changes in frequency of NG2-expressing B cells. Accordingly, we investigated the impact of anti-CD52 treatment on NG2 expression on different B-cell subsets, in particular Bmem cells, at times when secondary autoimmunity generally develops, in 7 treated patients.

We observed that total B-cell frequencies were not affected by alemtuzumab treatment (Fig. 26A). Indeed, B cells fluctuated between 17% and 22% throughout the period of observation up to 39 months after the first treatment. When we analyzed fluctuations in specific B-cell subsets, we found that the frequencies of Bmat and Breg cells changed dramatically after the first cycle, albeit with the proviso that only 4 patients were tested at these time points (T3 and T9), but reverted to the original levels two years from the second cycle (Fig.24B, green and red lines). Plasma cells did not show any variations during the period of observation (Fig. 26B, blue line). Interestingly, Bmem showed a steady decrease from the first treatment and their level remained low throughout the remaining of the observation period (39 months; Fig. 26C). This suggests that the beneficial effect of treatment on MS course is probably related to the significant decrease in B memory cells, which lasts throughout the period of observation. The percentage of NG2+ cells did not change significantly between the two cycles or after the second, suggesting that alemtuzumab treatment does not impact on NG2+ cell frequency and NG2+ cells are probably not involved in autoimmune adverse reactions related to the treatment.

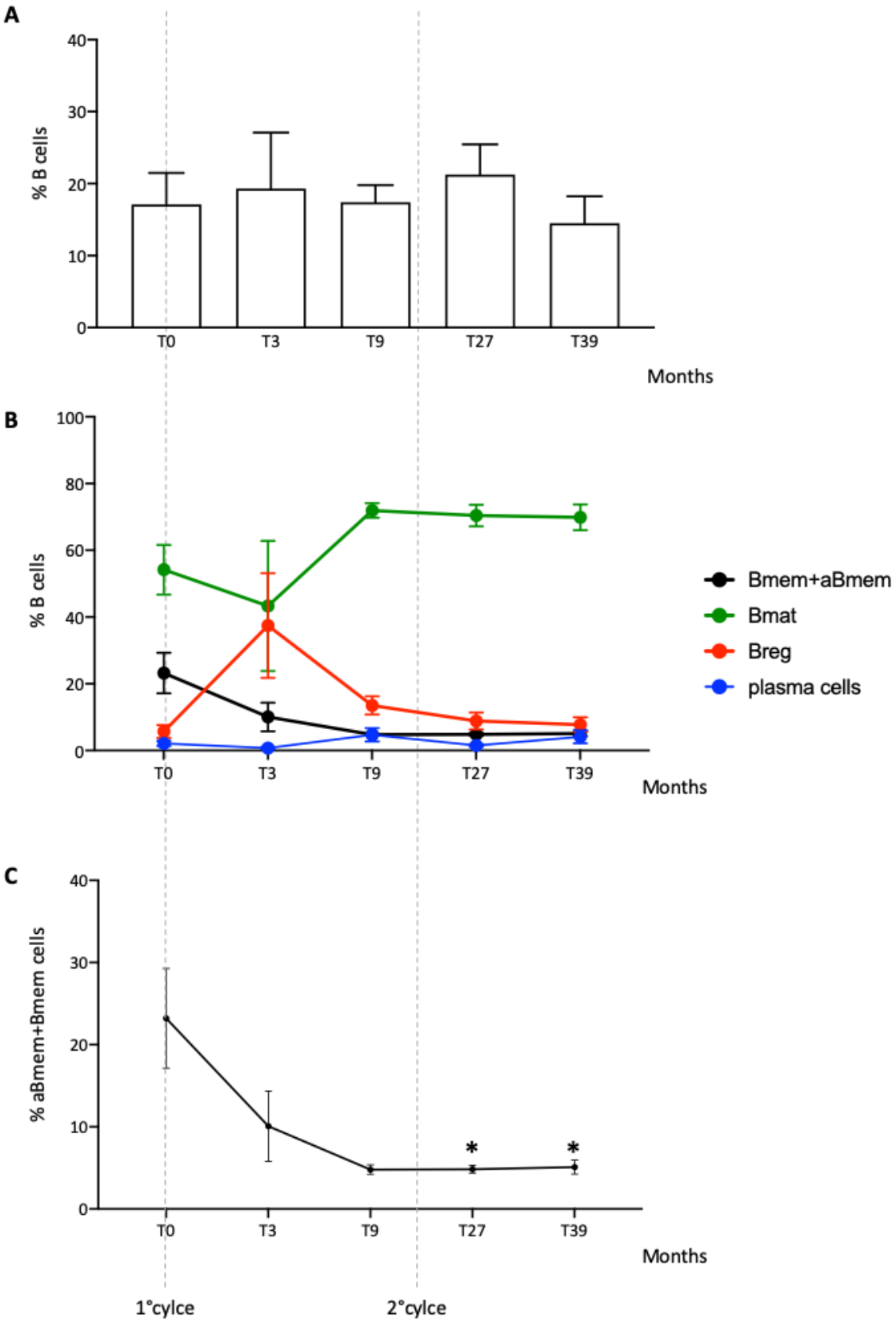

Fig. 26: Dynamic of total B cells and B-cell subsets during alemtuzumab treatment. Flow cytometry analysis of B-cell subsets. (A) Percentages of total B cells in isolated PBMC. (B) Time course of B-cell frequencies throughout the observation period. (C) Time course of B memory cells throughout the observation period. Data are presented as mean ± SEM. *P < 0.05 (B memory cells at T0 vs T27 and T39). N = 7 at T0, T27, and T39; N = 4 at T3 and T9.

The percentage of total NG2+ B cells remained stable throughout the time of observation (Fig. 27A). Analysis of NG2 expression on the single B-cell subsets revealed a clear trend in decreased frequency of NG2+ B memory cells (aBmem plus Bmem) at T27 and T39, independently of the decrease in Bmem cells. The percentage of NG2+ cells on the other B-cell subsets was not impacted by alemtuzumab treatment (Fig. 27B).

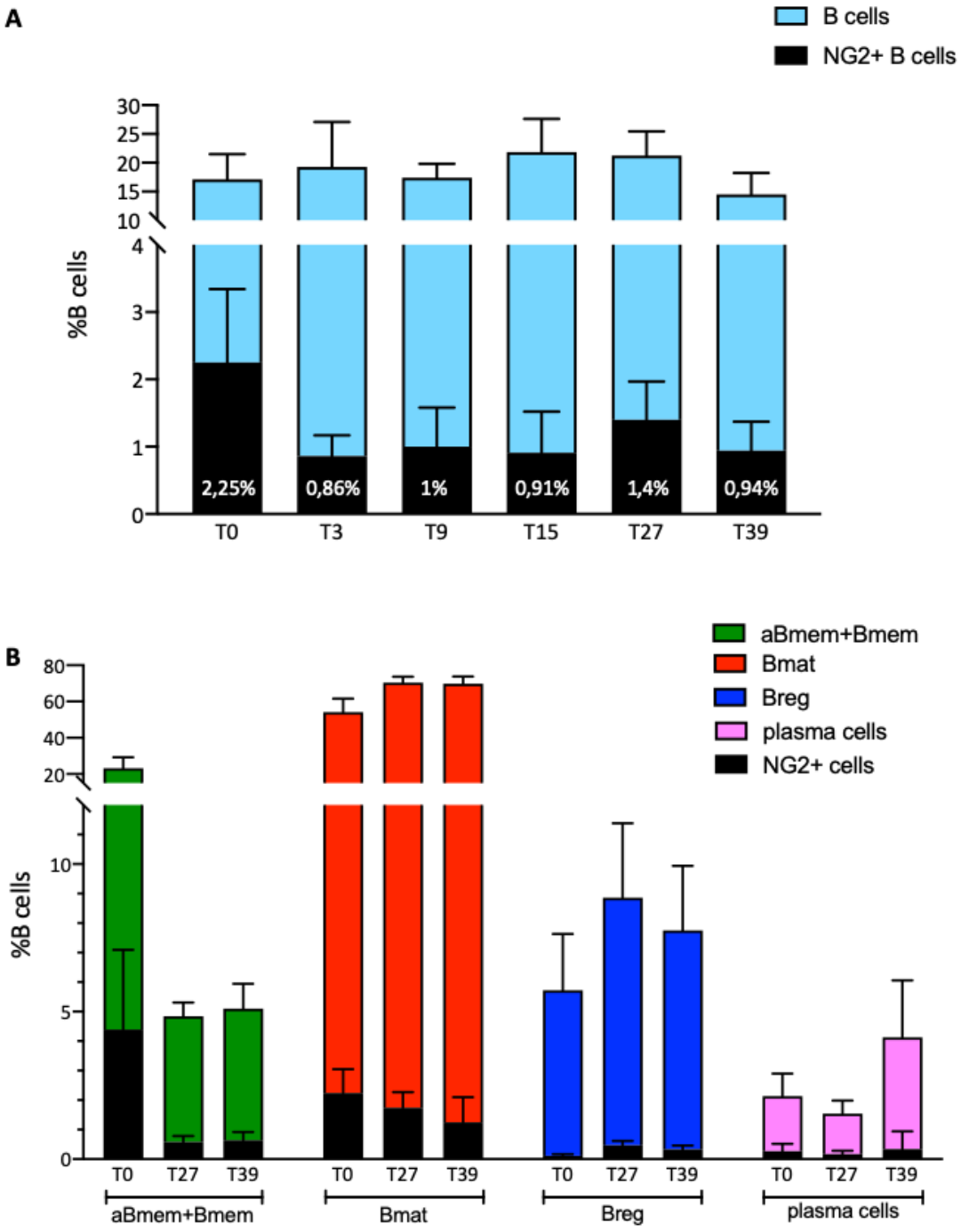

Fig. 27: NG2 expression on total B cells and on B-cell subsets before and after alemtuzumab treatment. Flow cytometry data of NG2+ cells in (A) the total B-cell population and (B) within each B-cell subset. Data are represented as mean ± SEM**.**

3.6 NG2 expression on B cells in BM

Throughout the body, the expression of NG2 has often been associated with progenitor-type cells, such as OPC and pericytes, and we postulated that NG2 expression on transitional B cells in the BM, defined for the most part by their expression of CD38 and CD24[160,161], might be relevant in the context of B-cell development in health and disease. We therefore compared NG2 expression on total B cells and CD24high CD38high B cells in BM sampled from 4 HD (BM donors for transplantation) and 4 patients with highly active MS. As can be seen in Fig. 28, there was a decrease in total B-cell frequency in MS as compared to HD, albeit not significant. Similarly, there was no significant difference in the frequency of NG2-expressing total B cells or transitional B cells in BM from HD or MS patients.

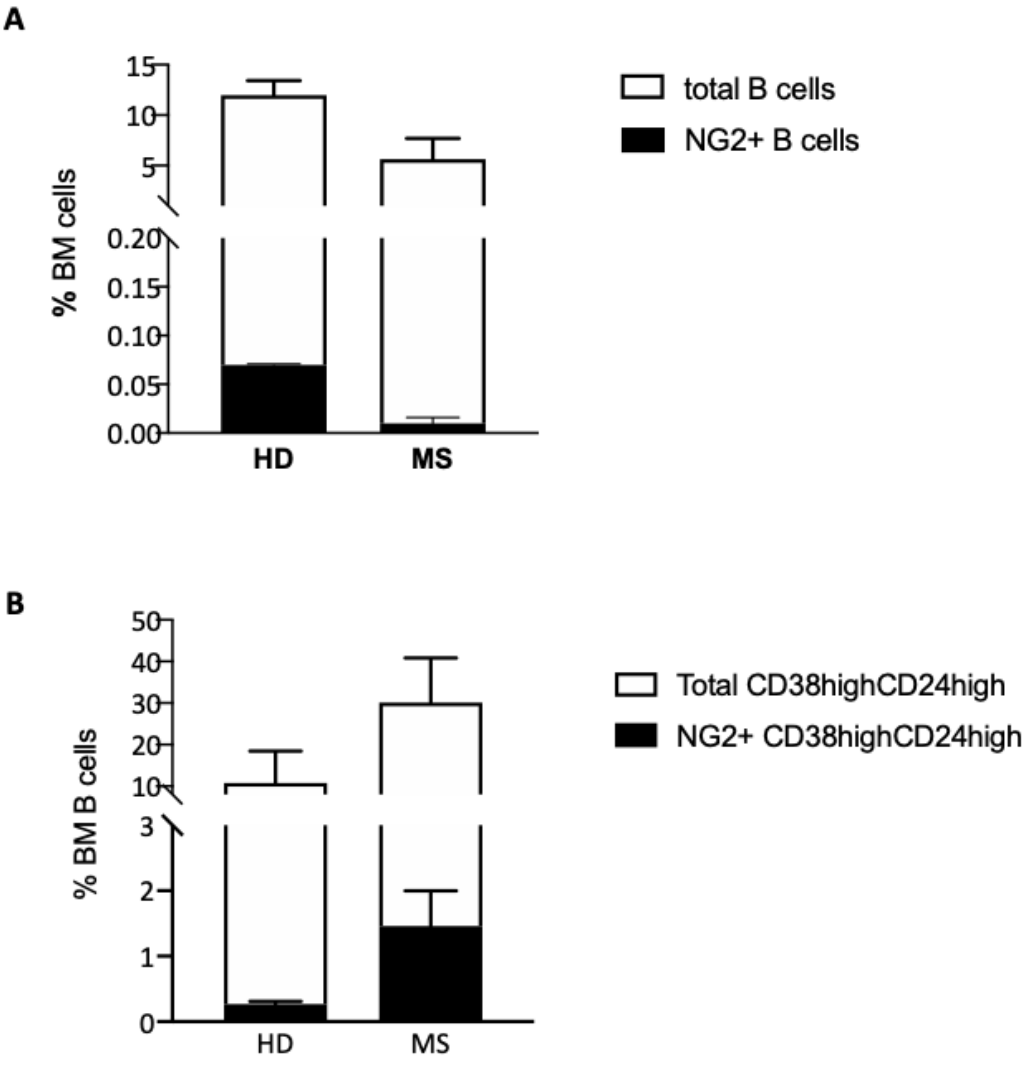

Fig. 28: **NG2-expressing B cells do not differ in frequency in BM from HD and MS patients**. Isolated BM cells were analyzed by FACS. (A) shows the NG2-expressing total B cell frequency and (B) shows the NG2-expressing frequency of transitional B cells in BM from HD vs MS patients. Data are represented as mean ± SEM of 4 HD and 4 MS patients**.**

4. Discussion

1. Validation of expression of NG2 on immune cells

In this study, for the first time, we have demonstrated that human immune cells express NG2. Previous studies had shown that circulating healthy immune cells do not express NG2. In these studies the expression of NG2 was assessed with a single MoAb (clone 7.1), which was developed against SV-40-transformed human marrow stromal cell lines, and recognizes a 220–240 kDa protein [138–144] .

Our speculation that healthy immune cells could express NG2 was based on the results obtained by Ferrara et al.[38], who demonstrated the expression of NG2 on immune cells, particularly on DC, T-lymphocytes and monocytes, using NG2KO mice as negative control of the reactivity in flow cytometry and immunofluorescence experiments. This data encouraged us to investigate further NG2 expression on human immune cells. As we had observed reactivity of healthy immune cells with Poly-NG2 in a pilot study, we proposed that the NG2 reactivity observed in cancerous cells (i.e. melanoma, glioblastoma, and B-cell leukemia), but not on healthy human immune cells, by other groups[138–144], could be related to an unmasking of a neo-epitope on these pathogenic cells. Indeed, there are a number of post-translational variants of NG2, which are differentially glycanated[12], including non-glycanated forms especially in cancer cells[12]. It could also be related to the high expression of NG2 on the cancerous cells, as compared to healthy cells. Indeed, it is possible that MoAb7.1 is an antibody of low affinity, resulting in low sensitivity of reactivity with healthy cells that express little NG2, but high enough in cancerous cells that express it in much greater amounts.

Our pilot FACS study with Poly-NG2 showed reactivity with a very high percentage (~ 70%) of healthy human immune cells (monocytes, T, B and NK cells), in clear contrast with the negative results obtained with MoAb7.1[138–144]. Accordingly, we needed to ascertain the specificity of Poly-NG2 binding. Ferrara et al.[38] had tested Poly-NG2 on NG2KO mice, ruling out any possible cross-reactivity of the antibody with another protein. In contrast, such a negative internal control is unknown in humans, and the use of a polyclonal ab excludes the use of other negative control such as an isotype control. Hence, we sought to select a MoAb that would react with NG2 on healthy immune cells. Out of four different commercially available MoAbs that we tested, we selected one, MoAb2, that showed good reactivity to healthy immune PBMCs and could demonstrate that the FACS reactivity was apparently

specific using an isotype control. In addition, other observations of the FACS data argue in favor of MoAb2 being indeed specific for NG2: firstly, the high reproducibility of our data showing that the NG2-positive population in PBMC is always that of Bmem cells in more than 40 subjects, including HD and MS patients. Secondly, the coexisting presence in a single FACS acquisition of clearly negative (negative internal control) and clearly positive populations (T cells vs B cells and within B-cell subsets). Thirdly, when we tested MoAb2 on leukemic cells, which we know express NG2, we observed the same frequency of reactivity than with MoAb1; as MoAb2 was raised to purified NG2, in contrast to MoAb1 that was raised to melanoma cell extract, it likely recognizes a different epitope than MoAb1, but still reacts with the same molecule and to the same extent than MoAb1, confirming leukemic cells as positive controls for MoAb2. As support for the specificity of MoAb2, and therefore for confirmation of NG2 protein expression by immune cells, we could show the presence of NG2 on sorted B cells by immunofluorescence. Finally, we were able to confirm NG2 expression also at the transcriptional level, albeit at a low level, which would be commensurate with the known low turnover of proteoglycans. In this context, it is interesting to speculate that the changes observed at surface protein level upon activation of the B cells could be related to translocation of the protein, rather than to direct induction of its transcription. Experiments are ongoing to assess this possibility.

2. NG2 is mainly expressed on B memory cells

Among circulating human immune cells, CD19+ B cells were the most frequent NG2-positive population. Interestingly, the subset showing the highest NG2 positivity was represented by Bmem cells. The importance of this result has to be considered in the context of the crucial role of Bmem in the pathogenesis of many autoimmune diseases, including MS[64,81]. Additional confirmation of the pivotal role of Bmem cells in MS is further provided indirectly by the evidence that has emerged from the use of B cell-depleting treatments. Indeed, many different studies have shown that the inhibition of Bmem cell function improves MS course, reducing relapse frequency and severity of disability progression[64]. The mechanism of action of alemtuzumab, one of the most effective approved treatment for MS, has been demonstrated to be related to its capability to persistently reduce the level of circulating Bmem cells [162]. Moreover, other evidence comes from the expanding use of anti-CD20 MoAbs (i.e. ocrelizumab and rituximab) in MS and NMO. Although, the established protocol

for these drugs is a six-monthly administration regardless of the different dynamics of immuno- reconstitution in individual patients[163], studies have shown that a personalized, repeat-treatment based on levels of circulating Bmem cells may be sufficient to control relapsing disease[164], indicating a strong correlation between Bmem cell level and disease activity. Consistent with this observation, atacicept, a B-cell activating factor inhibitor able to enhance Bmem cell function, causes disease worsening[165]. Natalizumab is the only approved drug in MS that induces an increase in blood levels of Bmem cells[166] without being associated with a clinical worsening. These mechanisms are probably related to the capability of natalizumab to prevent lymphocytes from entering CNS[167]. Our data show that the percentage of circulating NG2+ Bmem cells from HD and MS patients does not significantly differ. This is not surprising because the peripheral immune cell populations rarely reflect the immune mechanisms beyond the BBB, during brain inflammation. Moreover, the possibility exists that the presence of possible differences in NG2 expression among HD and MS patients could be underestimated if, for example, the expression of NG2, and/or its different post-translational isoforms, on Bmem cells differs between HD and MS, and/or that the MoAb we used does not react to the same extent with HD and MS cells, similarly to what we observed with HD and cancerous cells. In this context, albeit with few samples, we observed a drastic increase in NG2-expressing total B cells (up to 60-80% of total B cells, as compared to 10-15% in HD or MS) in each of the 2 patients with NMO, as compared to HD or MS patients. This increase was spread out to all the B-cell subsets analyzed, and not just to Bmem cells. In contrast to MS, which is a primarily T cell-mediated disease, NMO, also a demyelinating CNS disease, is characterized by the presence of circulating abs against the astrocyte water channel aquaporin-4. The key role of B cells in this disease has been largely proven in different studies[168,169] and it was confirmed by the results obtained with anti-CD20 depleting treatments[1164]. Unfortunately, because NMO is a very rare disease, we could only analyze 2 patients. Nevertheless, the dramatic increase in NG2+ B cells in these patients suggest the possibility that these cells might play a role in the disease. In this context, it has been shown that B-cell activation is a feature of NMO; we could therefore speculate that the increased frequency of NG2+ cells might be related to B-cell activation. Further studies with additional NMO patients are necessary to investigate this possibility.

3. Possible function of NG2

a) Possible role of NG2 in B cell activation

In this project, we investigated the role of NG2 in the activation of immune cells. The data on stimulated B cells have shown that B-cell subset composition is impacted by the activation of total B cells. Indeed, we observed a decreasing trend in frequency of B memory cells and plasma cells within the total B-cell population after 72 hours of stimulation. These data contrast with the study of Capolunghi et al. who showed that, upon CpG stimulation, B memory cells differentiate toward plasma cells and that CD24highCD38high B cells could shift toward a B-memory phenotype[134]. The non-concordance with our results might be related to the differences in stimulation protocol: Capolunghi et al. used a double concentration of CpG without the addiction of any other stimulus, and, most importantly, rather than stimulating the whole B-cell population, they stimulated cells from each flow-sorted B-cell subset[134].

In the activated B cells, we observed a reduction in the frequency of NG2+ cells, together with a significant decrease in intensity of expression of NG2 at the cell surface. This data encouraged us to investigate further if NG2 expression was related to B-cell activation. The analysis of stimulated NG2-sorted B cells showed that about 30% of these cells acquired NG2 expression. However, a similar percent increase was observed in the sorted NG2- B cells that had been incubated for 72 hours in the absence of stimulus. We can speculate that the change in NG2 expression on non-stimulated cells could be related to the possible mechanical activation induced by flow sorting, which we and others have observed, notably with dendritic cells. This observation, however, does not permit us to conclude on the possible effect of specific activation on NG2 expression. In contrast, we observed a mild decrease in the number of NG2+ cells when sorted NG2+ B cells were stimulated. The levels of IgG and IgM in both NG2+ and NG2- activated B cells did not differ significantly suggesting that the level of NG2 expression apparently does not influence the ability of B cells to be activated upon stimulation. It should however be noted that IL-10 levels were not increased in the stimulated cells that had been sorted, in contrast to what we observed with total B cells and what would be expected, at least for the Breg cells[150]. On the other hand, we cannot exclude that NG2 expression might be impacted by cell activation and that its intensity of expression can change during the different phases of B-cell activation upon CpG stimulation, a TLR9 ligand. Notably, this hypothesis is further supported by the evidence that NG2

expression on macrophages can be induced by others TLR ligands, such as lipopolysaccharide, as shown by Stallcup et al.[170]. In addition, other studies [49,171] have demonstrated that NG2 expression can be transiently upregulated on activated macrophages/microglia. Interestingly, Cejudo-Martin et al.[171] suggested that NG2 expression may be up-regulated on circulating activated monocytes in vivo in response to inflammation signals, and that this event may be part of the transition from monocytes to macrophages. According to this hypothesis, NG2 expression could be considered as a transient phenomenon that, *in vivo*, might facilitate the migration of cells rather than their activation[171]. In our *in vitro* samples, the changes in NG2 expression in NG2- and NG+ sorted cells could reflect a transient cellular condition triggered by activation. The mechanism through which the frequency of NG2 cells is reduced upon activation has to be further investigated; we excluded that NG2 expression is related to a reduction in cell viability as we did not observe any increase in cell death upon activation. On the other hand, we cannot exclude that the lower frequency of NG2+ B cells upon activation might be related to internalization of NG2 in endosomal vesicles, as was shown in Stonin-/- mouse embryonic fibroblasts[172]. Interestingly in that study, the fibroblasts were shown to display markedly elevated NG2 levels that were associated with altered focal adhesion and increased cell migration[172].

b) Possible role of NG2 in cell migration

Increasing evidence suggests that NG2 plays a crucial role in cell migration mechanisms[6]. In the last years, many studies have shown that NG2 expression on cancer cells can be related to a worse prognosis linked to a high capability of tumor growth and diffusion in NG2+ cancers[173]. Indeed, different trials are ongoing to test if NG2 can be used as a target of MoAbs in order to limit tumor metastasis[173]. Recently, Lopez-Millan et al. demonstrated that pre-treatment of NG2+ B-lymphoblastic cells with anti-NG2 MoAb (clone 9.2.27 and clone 7.1) reduced to one third the possibility of cell engraftment in an animal model of B-cell lymphoblastic leukemia[148]. Interestingly, they also showed that the administration of anti-NG2 antibodies, together with traditional chemotherapy, reduces the risk of disease relapses through their ability to mobilize cancerous cells from BM, thereby rendering them accessible to cytotoxic treatments. Moreover, at the same time, Prieto at al. showed that NG2 expression on MLLr B-lymphoblastic leukemia cells is associated with a higher capability of the cancerous cells to infiltrate the CNS[145]. Most interestingly, they also demonstrated that

the gene expression profile of the NG2+ B lymphoblasts differs significantly from that of the NG2- ones. In particular, genes associated with cell migration and movement were significantly upregulated in NG2+ clones, as compared to NG2- ones, and this feature was particularly observed in CNS-infiltrating cells[145].

These data suggest that NG2 can play an important role in migration of cells toward the CNS. The mechanism through which NG2 can contribute to the homing of cells toward CNS has not been studied. One hypothesis is that this capability is mediated by the interaction of NG2 and integrins[12]. Indeed, many studies have shown a crucial interplay between NG2 and integrins, in particular β1-class integrin, which plays a role in highly dynamic integrin-dependent mechanisms, such as proliferation, motility and survival[12]. Interestingly, it has been shown that, in melanoma cells, NG2 can modulate the adhesive function of α4β1 integrin[174]. These observations are particularly relevant considering the role of α4β1 integrin in leukocyte trafficking to inflamed tissues, especially brain, skin and gut[175–178]. These data all together support the hypothesis that the interaction between NG2 and α4β1 can play a role in migration of cells towards CNS. We can speculate further that the higher levels of NG2 expression on B memory cells might be a factor that influences the susceptibility of these cells to migrate towards the CNS. This hypothesis is supported by data derived from inflammatory CNS diseases, in particular MS. Indeed, many studies support the hypothesis that B cells, and in particularly B memory cells, play a crucial role in MS pathogenesis by migrating to the CNS[167]. Among the most relevant observations are the presence of B-lymphoid follicle-like structures in the cerebral meninges of some MS patients[128] and the accumulation of B memory cells in the CSF[179]. That the detrimental role of immune cells in MS is highly correlated to their possibility of entering the CNS is demonstrated by the data obtained with natalizumab-treated MS patients. Indeed, natalizumab, a MoAb directed against α4β1 integrin that acts by preventing infiltration of the CNS by immune cells, is one of the most effective treatments for MS[180].

In conclusion, we have shown for the first time that NG2 is also present on human healthy immune cells, more specifically on B cells, in particular B memory cells. The results suggest that NG2 expression fluctuates with B-cell activation, but further studies are necessary to better understand the possible role of NG2 on human immune cells, in particular at the level of migration.

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