

B cells and endogenous retroviruses in multiple sclerosis

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Dekan

To my sister,
my mother and Christoph

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Part I

Investigation of B cell involvement in multiple sclerosis

Abbreviations

ADEM	acute disseminated encephalomyelitis
APCs	antigen-presenting cells
AQP4	aquaporin-4
BCR	B cell receptor
CIS	clinically isolated syndrome
CNS	central nervous system
CSF	cerebrospinal fluid
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunospot
FACS	fluorescence-activated cell sorting
HA	influenza hemagglutinin
IgG	immunoglobulin G
KIR4.1	inward-rectifying potassium channel 4.1
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NMO	neuromyelitis optica
OCBs	oligoclonal bands
RIS	radiological isolated syndrome

Summary

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Although neither the cause nor the pathomechanism of MS is fully understood, it is believed to be caused by autoimmune destruction of the myelin. Various findings in patients with MS suggest the involvement of B cells in the pathogenesis. The most prominent finding is the presence of oligoclonal bands in patients with MS and the success of B cell depleting therapies. Despite tremendous efforts, the target antigens of B cells in MS have not yet been identified.

The goal of our first study (Results I of Part I of the presented work), was to develop a sensitive method for identifying antigen-specific B cells in humans, with the future goal of being able to identify autoantigen-specific B cells in MS patients. However, in many patients we do not know the specific antigens involved, and we expect the frequency of autoreactive B cells to be low. Thus, in order to develop a method for isolating such B cells we needed a model in which the target antigen is known and B cells specific for that antigen are present in normal human donors. Influenza hemagglutinin (HA) was chosen as an appropriate model antigen since healthy individuals are commonly immunized against influenza and the frequency of influenza-specific B cells is relatively high following immunization. Our method is based on culturing human B cells with fluorescently labeled hemagglutinin-expressing cells, followed by a FACS-sort to isolate putatively hemagglutinin-specific B cells from other B cells. With this method we were able to enrich hemagglutinin-specific B cells approximately 150-fold, as shown by ELISpot. We EBV-transformed FACS-sorted B cells to enable the production of monoclonal antibodies needed for the analysis of the B cell specificity. The transformations with EBV were performed with B cells from four different donors by adding 30-60 sorted B cells per well, yielding transformed B cell colonies in 48-75% of wells. Antibodies in the supernatants from those cultures were examined for their binding capacity to HA. Between 15% and 26% of GFP-capturing B cell cultures, and between zero and 3% of non-capturing B cell cultures produced antibodies that showed binding to HA by ELISA. By a cell-based flow cytometric assay 2.7-26% of GFP-capturing B cell cultures and none of the non-capturing B cell cultures produced antibodies that showed binding to HA. In addition, FACS-sorted B cells were subjected to an *in vitro* expansion protocol that also allowed the recombinant expression of immunoglobulins. Seventy-two combinations of heavy and light chains were expressed recombinantly and the cloned antibodies were tested by IgG ELISA for binding to HA, tetanus toxoid, BSA and anti-IgG. Of those 72 combinations, 42 showed binding to HA and 5 showed polyspecific binding to BSA, tetanus or both, in addition to HA.

Because this method isolates intact cells, rather than soluble antibodies, it can be used for the identification of B cells whose B cell receptor (BCR) is specific for unknown antigens, e.g. antigens expressed on the myelin sheath but of unknown identity.

In a connected project that also makes use of the phenomenon of membrane capture by B cells (Results II of Part I of the presented work), we tested a novel hypothesis of B cell autoimmunity in an *in vitro* system. We examined whether B cells induce autoimmunity indirectly by activating autoreactive T cells via the following mechanism: a B cell binds and captures its cognate antigen from an antigen-expressing cell, and simultaneously captures other membrane components including self-antigens. The possibility arises that this B cell, in parallel with processing its cognate antigen, processes and presents this "bystander antigen" to T cells of the appropriate specificity, which in an inflammatory context would induce a self-destructive T cell response. We tested this hypothesis using adherent cells that express the CNS-restricted membrane protein myelin oligodendrocyte glycoprotein (MOG) as a model self-antigen, influenza hemagglutinin (HA) as a model viral antigen, and transgenic mouse B and T cells specific for each antigen. This antigen pair was chosen because both are integral membrane proteins, with a plausible role in autoimmunity. MOG is implicated in autoimmune neurological disorders and animal models thereof, notably in acute demyelinating encephalomyelitis, and influenza is among the most important known viral triggers of this autoimmune condition. In our study we observed the following two mechanisms: (i) cognate antigen is rapidly captured from membranes and induces strong activation of the capturing B cell and (ii) smaller quantities of other co-expressed, "bystander" antigens are co-captured at the same time and can be presented to T cells as well. This non-cognate antigen co-capture was observed in two different paradigms. HA-specific B cells co-capture MOG and present it to autoreactive MOG-specific T cells; and MOG-specific B cells co-capture a fusion protein of HA and ovalbumin and present it to ovalbumin-specific T cells. The fusion protein was generated because HA-specific T cells were not available. This phenomenon thus has two kinds of implications for autoimmunity: viral antigen-specific B cells can activate self-reactive T cells, and conversely, self-antigen-specific B cells can receive T cell help from virus-specific T cells, leading to the production of autoantibodies. These findings offer a possible explanation for the link between autoimmunity and viral infections.

Introduction

Multiple sclerosis – a demyelinating disease

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). It is the most common disabling neurological disorder in young adults. The onset typically occurs between the ages of 20-40 years, and women are about 2 times more likely to develop MS than men [1]. Although the origin of MS is unknown, it is believed to be caused by autoimmune destruction of the myelin [2]. Myelin coats, protects and insulates nerves of both the central and the peripheral nervous system, enabling them to quickly conduct impulses between the brain and different parts of the body. The destruction of myelin, axons and neurons in the central nervous system is what causes many of the signs and symptoms of MS [3,4].

Symptoms and their severity may differ significantly from patient to patient, and over the course of the disease, depending on the location of affected nerve fibers and severity of the damage. MS is typically diagnosed based on presenting symptoms like vision and balance problems, dizziness, fatigue, bladder problems and stiffness and/or spasms in combination with supporting magnetic resonance imaging and laboratory testing [5]. The most commonly used criteria for diagnosing MS are the McDonald criteria, which focus on clinical, laboratory, and radiologic evidence of demyelinating lesions at different time points and affecting different areas of the CNS [6,7]. Treatment of MS typically focuses on speeding recovery from relapses, slowing down the inflammatory activity and managing MS signs and symptoms. Medications used in MS can reduce the frequency and severity of relapses as well as the development of new lesions in the brain, but they do not cure MS [8].

Four different major clinical subtypes of MS have been defined: relapsing-remitting, secondary progressive, primary progressive, and progressive-relapsing MS [9]. **Relapsing-remitting MS** is characterized by clearly defined, often unpredictable relapses followed by periods of weeks to years of remission, in which patients are clinically stable. This subtype is the most common form of the disease. The majority of patients with MS begin with a relapsing-remitting course and this stage can last for years or even decades. **Secondary progressive MS** occurs in patients with an initial relapsing-remitting disease course followed by progression with or without occasional relapses, minor remissions, and plateaus. **In primary progressive MS**, patients show a progression of disability from onset with occasional periods of exacerbation and remission. Although modest fluctuations in neurological disability may be seen, those patients do not experience relapses. **In progressive-relapsing MS**, which is the least common disease course,

patients show a progression of disability from onset but with occasional relapses. The periods between relapses are characterized by a continuous progression [9]. In 2013, an international panel reviewed these definitions by adding two new disease subtypes; clinically isolated syndrome (CIS) and radiological isolated syndrome (RIS) [10]. CIS is defined as first clinical presentation of a disease that could be MS, showing characteristics of inflammatory demyelination, but which yet has to fulfill criteria of dissemination in time [11]. In RIS the situation is more complicated. Incidental imaging findings suggest an inflammatory demyelination but clinical signs or symptoms are not present in the patient [12]. Although these definitions of the disease courses exist, and they are relevant for prognosis and treatment decisions, it should be noted that the course of MS in an individual patient is widely unpredictable.

The role of B cells in multiple sclerosis

It has long been speculated that MS is a disease mediated by myelin-reactive T cells. Evidence supporting this hypothesis includes the observations that MS is characterized by recruitment of activated T cells into the CNS [13] and that T cells outnumber B cells in MS lesions [14,15]. Furthermore, T cells play an important role in the most used animal model of MS, i.e., experimental autoimmune encephalomyelitis (EAE). Inflammatory demyelinating disease can be induced in mice by adoptive transfer of activated, encephalitogenic, myelin-specific T cells [16]. Interest in the role of B cells in EAE was initially limited to their ability to produce antibodies that could mediate demyelination [17].

In the past few years, the concept of MS being a T cell mediated autoimmune disease has been challenged, and the role of B cells reassessed [18,19]. It has been reported that under physiological conditions in healthy people, the frequency of B cells, plasmablasts, and plasma cells in cerebrospinal fluid (CSF), is rather low, contrary to the CSF of patients with MS, which shows an increase in B cells reaching up to 5% of all mononuclear cells therein. The ratio of B cells to monocytes correlates with disease progression and a higher B cell count indicates a more aggressive course of the disease [20].

The presence of oligoclonal bands (OCBs) in the CSF is the most consistent immunological finding in patients with MS and similarly one of the few biomarkers used in clinical practice for the establishment of the diagnosis of MS [21,22]. OCBs arise from the intrathecal synthesis of clonal IgG. These clonally expanded antibodies seem to be stable over a long period of time and are found specifically in the CSF but not in serum of more than 95% of patients with MS [23,24]. Many attempts have been made to find the target antigens of these OCBs but yet none could successfully be identified. In addition to that, recombinant monoclonal antibodies cloned from human B cells in CSF, failed to show binding to well known myelin antigens or to brain sections [25].

More evidence for an important role of B cells in MS is supported by the remarkable success of B cell depleting therapies. One of the most meaningful changes in MS therapy was reached through the development of monoclonal antibodies. Several different, tested molecules promise to show a higher therapeutic efficacy than currently available baseline treatments like interferons [26]. Although the target antigens of these therapeutic antibodies are known, there are still uncertainties about which exact mode of action is responsible for their therapeutic effect, since these antibodies target a wide range of immune cells. The humanized monoclonal antibody

natalizumab for example, blocks the $\alpha 4$ subunit of the VLA-4 receptor that is expressed on most leukocytes and which is needed for migration across the blood-brain barrier [27]. Alemtuzumab, another monoclonal antibody effective in treating MS, is known to deplete all cells expressing the CD52 molecule, including not only B cells, but also T cells, natural killer cells, dendritic cells, most monocytes and macrophages [28]. Rituximab, which is interestingly the only cell-type-specific antibody used in MS, depletes B cells positive for the CD20 antigen. It has shown a high efficacy in treating relapsing-remitting MS by reducing the number of gadolinium-enhancing lesions and the proportion of patients experiencing relapses [29,30]. CD20 is expressed in different stages of B cell differentiation, from pre-B cells to naïve and memory B cells, but is absent in earlier stages and plasma cells [31]. The second generation of anti-CD20 molecules, like ocrelizumab and ofatumumab, showed similar results in clinical trials [32,33]. The success in relapsing-remitting MS of treatments specifically targeting B cells provides not only evidence for the pathogenic role of B cells in MS, but enables a possible elucidation of how B cells may be acting. Interestingly, anti-CD20 molecules neither target plasma cells nor do they influence IgG levels in the CSF or OCBs of patients with MS in the short term, suggesting that the autoantibody production of B cells is not what mediates the pathogenesis in MS [34].

During the last few years, our understanding of the role of B cells in the pathogenesis of MS has progressed tremendously. Although B cells have been known best for their antibody-producing function, they are now recognized as main players in conjunction with T cells by acting as antigen-presenting or cytokine-producing cells, exhibiting stimulatory, regulatory, as well as pro- and anti-inflammatory capacities [35]. The characterization of diverse B cell phenotypes will allow further elucidation of the role of B cells in MS and lead to the development of novel disease treatment and prevention strategies.

The search for autoantigens in demyelinating CNS diseases

The antigen specificity of B cells is assumed to play a leading role in their antigen-presenting and antibody-producing function. What antigen these antibodies may recognize has been controversial for years. Compared to T cells, B cells recognize antigens independently of the major histocompatibility complex (MHC), which has several consequences. B cells can recognize antigens either in solution or located on the surface of cells and quite often they recognize epitopes dependent on their native conformation. These characteristics of B cell antigen recognition play an important role when antibodies are used to identify target antigens.

Myelin oligodendrocyte glycoprotein (MOG) has been the focus of extensive research for more than thirty years and is probably the best studied B cell antigen in MS [36]. MOG is mainly located at the outermost surface of myelin in the CNS, which makes it accessible for antibodies. The role of MOG as an autoantigen for T cells and as a target of demyelinating antibodies has been established in various forms of EAE [37]. However, the role of MOG antibodies in human MS is more controversial. Although MOG antibodies can neither be used for the diagnosis of MS nor for the prediction of the course of the disease [38], they have recently been shown to be associated with pediatric demyelinating disease [39,40]. Antibodies against MOG could be detected in around one quarter of pediatric patients with MS or acute disseminated encephalomyelitis (ADEM) and were shown to correlate with the course of the disease. The presence of MOG antibodies in these children was strongly negatively correlated with age, which interestingly could indicate that the myelination process, which is still ongoing at that age, influences the autoantigenic response [41].

Aquaporin-4 (AQP4) was the first specific molecule and also the first example of a water channel which has been identified as a target for the autoimmune response in CNS demyelinating diseases. AQP4 is the most prominent B cell target in neuromyelitis optica (NMO). Its discovery contributed tremendously to the understanding of this disease. Anti-AQP4 antibodies constitute a highly specific marker of NMO. An additional characteristic which enables differentiation between NMO and MS is the specific loss of AQP4 expression in lesions [42]. The first relevance of anti-AQP4 antibodies was demonstrated by staining brain slices with sera from patients with NMO [43,44]. Further significance was shown in an animal model of NMO, in which recombinant anti-AQP4 antibody or anti-AQP4 antibody-containing sera were transferred into animals, resulting in pathological changes resembling this disease [45,46].

Recently, the detection of autoantibodies against the inward-rectifying **potassium channel 4.1 (KIR4.1)** was described as a possible breakthrough in understanding the pathophysiology of MS. In 2012, Srivastava and colleagues identified anti-KIR4.1 antibodies in sera of MS patients by immunoprecipitation of human brain proteins and reported that over 40% of patients with MS harbor these antibodies in serum, compared to less than 1% of the controls [47]. Several subsequent independent studies have failed to reproduce this finding [48,49], presumably because they relied on a peptide ELISA whereas the original study used a protein ELISA to detect anti-KIR4.1 antibodies. However, the most recent study by Pröbstel and colleagues was performed with an identical method as originally described and neither the KIR4.1 protein nor the peptide ELISA distinguished patients with MS from controls [50].

The search for autoantigens in MS is still an open field of research. The discovery of disease-specific autoantibodies against AQP4 and MOG, as B cell targeted autoantigens, has enabled a big step towards autoantigen-targeted therapies. Hopefully more autoantibodies will be discovered that are clinically useful for diagnosis and stratification of patients with MS.

Antigen capture by B cells

While the uptake of soluble antigen by B cells has been known for decades, only a few years ago it was reported that B cells can capture antigens also when expressed on membranes. In 2006, Fleire and colleagues observed using live cell imaging that B cells capture antigens by spreading over a solid substrate, followed by an internalization of the BCR-antigen complex and a rounding and retraction of the B cell. Furthermore, they demonstrated that subsequently these B cells can present these captured antigens to T cells. The T cell activation was monitored by measuring interleukin-2 production after 24 hours [51].

In 2013, Natkanski and colleagues reported on the mechanical energy B cells use to discriminate antigen affinities. They developed an experimental model for studying immune synapses using immobilized plasma membrane sheets and planar lipid bilayers. B cells interacting with plasma membrane sheets including cognate antigen captured not just the antigen, but large pieces of membrane with it. By contrast, B cells that formed synapses with planar lipid bilayers did not take up any antigen or membrane pieces, suggesting that B cells require flexibility of the presenting membranes to pinch off the antigen together with pieces of the membrane. The authors suggested that a similar phenomenon occurs when B cells interact with cells expressing their cognate antigen, i.e., the B cell captures both the antigen and significant amounts of membrane [52].

Another approach, by Beadling and Slifka, describes a method to quantify T cells without knowing the fine epitope specificity. The method is based on biotinylation and streptavidin-fluorochrome labeling of antigen-presenting cells (APCs), followed by subsequent acquisition of this label by antigen-specific T cells. They used splenocytes from mice that were immunized with lymphocytic choriomeningitis virus (LCMV) or splenocytes from naïve mice. After mixing splenocytes with peptide-pulsed APCs, they labeled the LCMV-specific T cells with MHC tetramers and by intracellular cytokine labeling before subjecting to flow cytometry to examine CD8⁺ T cells. The small population of antigen-specific T cells identified by tetramer labeling and antigen-dependent cytokine production was almost identical to the population identified by acquisition of fluorochrome from the APCs [53]. The goal of the first part of the presented work (Results I of Part I) was to adapt this method for B cells.

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Aim of the Thesis

In the first part of the presented work (Results I of Part I) we aimed to generate a method to identify human antigen-specific B cells recognizing membrane-bound, complex antigens in their real conformation. The identification of B cells specific for particular antigens has shown to be difficult because of their unknown, presumably very low frequency. In the last thirty years, different techniques have been established to isolate antigen-specific B cells that recognize conformational epitopes, most of them using antigens in their soluble form. However, in some cases it is impossible to mimic a real conformation using soluble antigens. Autoimmune antibodies in myasthenia gravis for example bind to acetylcholine-receptors consisting of membrane-protein clusters. At present, no technique exists to isolate B cells that recognize such membrane-bound, complex antigens. Therefore, we aimed in the first part of this work at filling this gap by generating a technique enabling the identification and isolation of exactly these B cells. In a next step we aimed to characterize these isolated B cells and confirm their antigen-specificity by different read-outs, i.e., ELISpot, ELISA and a cell-based flow cytometric assay.

In the second part of the presented work (Results II of Part I) we tested a novel hypothesis linking B cells, viral infection and autoimmunity. It has been known for decades that B cells can specifically capture, process and present antigens that are recognized by their B cell receptors. The antigen recognized by B cells has been generally considered to be soluble. Recently, it was reported that B cells are able to capture their cognate antigens also when expressed on the surface of antigen-expressing target cells. It appears that this antigen capture involves not only the antigenic molecule itself, but also substantial fragments of the target cell membrane, presumably along with numerous other proteins. This led to the question whether B cells can induce autoimmunity indirectly by presenting co-captured self-antigens to self-reactive T cells. To investigate this hypothesis, we addressed the following three main questions: (i) Can B cells capture membrane-bound cognate antigen? (ii) Do they co-capture non-cognate antigens? (iii) Do they present both cognate and non-cognate antigens to T cells? We answered these questions in an *in vitro* model using transgenic B and T cells.

Results

I. Membrane capture as a novel sensitive technique to isolate live antigen-specific B cells

(Manuscript in preparation)

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ABSTRACT

The identification and isolation of B cells specific for particular antigens is difficult because of their very low frequency in peripheral blood. Existing methods to identify antigen-specific cells, like rosetting or cell sorting techniques with fluorescent antigens are well developed. However, these techniques use recombinant antigens in soluble form, which poses the problem that especially complex protein like multichannel proteins may not retain their native conformation. There are several examples showing that the native conformation of the antigen is essential for recognition of antibodies since these antibodies recognize conformational rather than linear epitopes. The aim of this newly developed assay was to retain the native conformation of the target antigen used for isolating specific B cells.

Using influenza hemagglutinin as a model we have established a sensitive technique for isolating intact, living B cells recognizing their cognate antigens in cell membranes in their native conformation. The technique is based on culturing human B cells from peripheral blood for several hours with adherent cell lines expressing a fusion protein of hemagglutinin and GFP, to allow GFP-antigen uptake. GFP-capturing B cells get FACS-sorted and analyzed for their antigen-specificity by ELISpot, ELISA and cell-based flow cytometric assays. A subsequent immortalization of GFP-capturing B cells by Epstein-Barr virus or alternatively in vitro expansion of GFP-sorted B cells enabled us to get single B cell clones to reconstruct recombinant antibodies.

With this novel and sensitive technique we were able to significantly enrich hemagglutinin-specific B cells and confirm their specificity by different read-outs. We believe that this technique will also be applicable to identify B cells directed against membrane associated tumor antigens or autoantigens.

INTRODUCTION

Antibodies have made a dramatic transformation from valuable scientific tools in research to powerful therapeutics introduced in the clinic [1]. One of the current largest growing fields are antibody biopharmaceuticals used as treatment against cancer or autoimmune diseases [2]. A key for understanding how antibodies recognize their target antigen is the identification of the binding site, or epitope. Epitopes are commonly divided into two categories, linear epitopes and conformational epitopes. Antibodies often recognize conformational epitopes in which key amino acid residues are brought together by protein folding, rather than linear epitopes where a stretch of continuous amino acids is sufficient for binding [3].

Production of useful antibodies generally depends on the isolation of B cells recognizing a particular antigen, but the identification of B cells that are specific for any given antigen is difficult because of their very low frequency. It has been reported that the frequency of B cells specific for any particular antigen is usually less than 1% [4,5]. Another difficulty is the lack of appropriate markers to evaluate the purity of the isolated cells.

Common techniques to identify antigen-specific B cells like cell sorting techniques involving staining with fluorescent antigens quite often use antigens in their soluble forms, which makes those techniques unsuitable for antigens that form their real conformation only in a membrane-bound state. B cells, which only recognize such conformation-dependent protein antigens, will not be identified by existing methods. Examples of conformation-dependent B cell recognition include viral envelope proteins (it is known that at least some broadly neutralizing antibodies do not bind to linear epitopes [6]), and autoreactive B cells in myasthenia gravis that recognize acetylcholine-receptors consisting of membrane-proteins densely packed in relatively immobile clusters [7].

While the uptake of soluble antigen by B cells has been known for decades, it has only recently been reported that B cells can capture antigens also from membranes of other cells. Fleire and colleagues observed using live cell imaging that B cells capture antigens by spreading over a solid substrate, followed by an internalization of the BCR-antigen complex and a rounding and retraction of the B cell. They demonstrated that subsequently these B cells can present the captured antigens to T cells [8]. Natkanski and colleagues reported on the mechanical energy B cells use to discriminate antigen affinities. By comparing immobilized plasma membrane sheets and planar lipid bilayers as an experimental model for studying immune synapses, they concluded that B cells require flexibility of the presenting membranes in order to pinch off the

antigen together with pieces of the membrane [9]. To isolate B cells, which recognize membrane-proteins, we postulated that it ought to be possible to develop a method based on this phenomenon of membrane capture.

Beadling and Slifka described a method to quantify T cells without knowing the fine epitope specificity, based on biotinylation and streptavidin-fluorochrome labeling of antigen-presenting cells, followed by subsequent acquisition of this label by antigen-specific T cells. The small population of antigen-specific T cells identified by tetramer labeling and antigen-dependent cytokine production was almost identical to the population identified by acquisition of fluorochrome from the APCs [10]. In our study we tried to adapt this method for B cells.

Using influenza hemagglutinin as a model antigen we aimed to develop a novel and sensitive technique for isolating intact, living B cells recognizing their cognate antigens in membrane-expressed form in their native conformation. The technique is based on culturing human B cells from healthy donors for several hours with an adherent cell line expressing a fusion protein of hemagglutinin and GFP, to allow GFP-antigen uptake. By FACS-sorting of GFP-capturing B cells we tried to enrich them and analyse their specificity by ELISpot, ELISA and cell-based flow cytometric assays.

MATERIALS AND METHODS

Plasmids and cell lines

Cloning:

A plasmid encoding influenza hemagglutinin A/California/04/09 amino acids 1-529 (GenBank: ACP41105.1) was purchased from Sino Biological (Beijing, China, cat no. VG11055-C), the open reading frame was amplified and fused to an oligonucleotide (purchased from Microsynth, Balgach, Switzerland) encoding amino acids 530-566. To incorporate the tyrosine-to-phenylalanine mutation (Y98F) in the sialic acid binding site of hemagglutinin (HA) [11], the 1-529 amplicon was generated in two segments, with overlaps including the target residue, enabling us to include the mutation in the primers, and then all three sections (corresponding to amino acids 1-98, 99-529, and 530-566) were joined by template switching PCR and cloned into the PigLIC vector which confers puromycin (Gibco) resistance. To make the plasmid encoding the fusion protein HA-Y98F-GFP, we amplified GFP from pcDNA6.2C-EmGFP-DEST

(Invitrogen) and fused it to the mutated HA construct described above between amino acid 566 and the STOP codon.

To generate a plasmid coding for the fusion protein MOG-GFP, the open reading frame encoding amino acids 1-204 of rat MOG (UniProt: Q63345.1) was amplified from pRSV-MOG (a kind gift from Nicole Schaeren-Wiemers, University of Basel, Switzerland), and GFP amplified from pcDNA6.2C-EmGFP-DEST (Invitrogen) was added at the carboxyl terminal, before cloning into PigLIC. Accuracy of plasmid sequences after cloning was verified by sequencing.

Cell lines:

TE671 rhabdomyosarcoma cells and HEK293 embryonic kidney cells (referred to as “TE cells” and “HEK cells” throughout the text) were obtained from ATCC (LGC, Wesel, Germany). Cells were cultured in complete RPMI medium or complete DMEM medium (10% heat-inactivated fetal calf serum, 100 units/ml of penicillin and 100 ug/ml of streptomycin; all reagents obtained from Gibco), at 37°C in 5% carbon dioxide. Cells were tested for mycoplasma infection on arrival and subsequently (LookOut Mycoplasma PCR Detection Kit, Sigma Aldrich). Mouse fibroblasts stably transfected with human CD40 Ligand (a kind gift from Edgar Meinel, Ludwig-Maximilians-Universität, Munich, Germany), used as feeder cells for EBV transformation, were cultured in complete DMEM medium supplemented with 0.5mg/ml of the selective antibiotic Geneticin (G418 Sulfate, cat no. 10131-035, Gibco) at 37°C in 5% carbon dioxide. For irradiation, cells were washed once in PBS 1X, trypsinized, resuspended in ice-cold FCS (all reagents obtained from Gibco) and kept on ice during the irradiation (75 Gy).

Transfection:

TE and HEK cells were transfected by exposure to 0.5 µg/ml of plasmid DNA, 0.75 µg/ml of polyethylenimine and 0.125 mg/ml 20% Glucose in complete RPMI medium for six hours. Transfection reagent was removed and replaced with 6 ml of complete RPMI medium. The appropriate selective antibiotic was added from the following day on. Cells resistant to the appropriate selective antibiotic were labeled as described below and sorted by the Department of Biomedicine Flow Cytometry Core.

Verification of transgene expression:

TE cells expressing influenza A H1N1 hemagglutinin (A/California/04/2009) with or without incorporated tyrosine-to-phenylalanine mutation (Y98F) or non-transfected TE cells (referred to as “TE wildtype HA”, “TE mutated HA” (TE mHA) and “TE 0” throughout the text) were incubated with RM10 antibody (rabbit monoclonal antibody anti-influenza A H1N1 hemagglutinin, cat no.

11055-RM10, Sino Biological) 1:1000 diluted in flow buffer or human sera from before and after influenza vaccination 1:300 diluted in flow buffer for 45 minutes on ice, washed three times with cold flow buffer (composed of PBS 1X supplemented with 2% FCS and 0.1% azide), incubated with PE-conjugated anti-human IgG or PE-conjugated goat anti-rabbit IgG, each 1:100 diluted in flow buffer, on ice for 30 minutes, washed twice in cold flow buffer, and subjected to flow cytometry (Attune Flow Cytometer, Applied Biosystems). All centrifugation steps were at 300 g, for 5 minutes at 5°C. DAPI (Sigma Aldrich) was added at 200 ng/ml to enable exclusion of dead cells.

Mice and primary immune cells

C57Bl/6 mice were bred in the University of Basel Mouse Core Facility. IgH^{MOG} mice were a kind gift from Guru Krishnamoorthy and Hartmut Wekerle (Max-Planck-Institut für Neurobiologie, Martinsried, Germany). Primary immune cells were obtained from spleens by mechanical disruption followed by brief settlement under gravity to remove tissue fragments. B cells were obtained by positive selection using mouse CD19 microbeads from Miltenyi (cat no. 130-052-201). All procedures involving animals were authorized by the local veterinary office.

Human samples

Healthy donors, male and female, between 25 and 65 years old, were vaccinated against the seasonal influenza with Agrippal®, containing inactivated influenza virus surface antigens (hemagglutinin and neuraminidase) from type A/H1N1 (A/California/2009 (H1N1)pdm09), type A/H3N2 and type B. All healthy donors gave written informed consent. Use of human blood samples was approved by the local ethics committee (49/06).

Blood was drawn into S-Monovette tubes (Sarstedt, 7.5ml K3E, REF 01.1605.100, 1.6 mg EDTA/ml blood) before the vaccination and 7, 10 or 14 days after vaccination. 15 ml of EDTA-blood was diluted with an equal volume of PBS 1X (Gibco), carefully layered over 15 ml of Ficoll-Paque (Axon Lab, Switzerland) in 50 ml conical tubes and centrifuged in a swinging-bucket rotor at 600 g for 20 minutes without brake at room temperature. Mononuclear cell layers were carefully collected and transferred to new 50 ml conical tubes, washed twice with PBS 1X by centrifuging at 270 g for 10 minutes at room temperature and frozen in cryovials in aliquots of 20-30 million PBMC in 1 ml FCS-10% DMSO (FCS from Gibco, DMSO from Sigma Aldrich). Cryovials were immediately transferred into freezing containers containing 100% isopropanol, put into -80°C freezer and transferred to liquid nitrogen the next day.

Blood for serum was drawn into S-Monovette tubes containing granulate beads with silicate clotting activator (Sarstedt, 7.5 ml Z, REF 01.1601.100) and left at room temperature for 30-60 minutes to allow coagulation, before centrifuging at 2000 g for 10 minutes at room temperature. Serum was aliquoted and frozen at -80°C.

B cell enrichment

PBMC were quickly thawed in 10 ml pre-warmed complete RPMI medium and put into incubator for one hour at 37°C, centrifuged and resuspended in ice-cold separation buffer, counted and enriched for B cells by negative selection using biotinylated antibodies and magnetic beads from Miltenyi (human B cell Isolation Kit II, cat no. 130-091-151). All spinning steps were at 300 g for 5 minutes at 5°C. Separation buffer was made by sterile filtering 500 ml PBS 1X (Gibco) containing 0.5% BSA (Sigma Aldrich) and 2 mM EDTA.

FACS isolation of putatively hemagglutinin-specific B cells

B cells were isolated from PBMC after influenza vaccination, co-cultured for three hours with CTV-labeled TE mHA-GFP cells, retrieved and incubated with PerCP-Cy5.5-conjugated anti-human CD19 (for IgG ELISpot experiments) diluted 1:20 in cold separation buffer or with APC-conjugated anti-human CD45 (for EBV transformation experiments and high-throughput B cell activation) diluted 1:50 in cold separation buffer. Cells were gated on scatter to select live, single cells; on CD19^{high} or CD45^{high} to exclude contaminating TE cells; and two subgates were used for sorting (FACS Aria III Cell Sorter, BD Biosciences): “GFP-capturing”, i.e., GFP-positive, CTV-low-to-intermediate; and “GFP-non-capturing”, i.e., GFP-negative, CTV-low.

ELISpot for detection of HA-specific, IgG-secreting human B cells

Human IgG B cell ELISpot kit was obtained from Mabtech, Sweden (Code: 3850-2A). Plates were coated according to the provided protocol with hemagglutinin at 5 ug/ml, or anti-IgG capture-antibody (provided with the kit) at 15 ug/ml, or bovine serum albumin at 5 ug/ml. Plates were washed with 200 ul/well sterile PBS 1X and blocked with 100 ul/well complete RPMI medium, according to the provided protocol. B cells were isolated from PBMC from after influenza vaccination, co-cultured for three hours with TE mHA-GFP and labeled with anti-human CD19. GFP-capturing and non-capturing CD19-positive B cells were sorted into ELISpot plate containing 200 ul/well complete RPMI medium supplemented with 1 ug/ml R848 and 10 ng/ml recombinant human IL-2 (both provided with the kit). After incubating for three days at 37°C in 5% carbon dioxide, the plates were washed five times with 200 ul/well PBS 1X and developed by incubating with biotinylated anti-human IgG, followed by streptavidin-AP and BCIP/NBT substrate solution to visualize IgG-specific spots. All antibodies and solutions were

provided with the kit and all steps were performed according to the protocol. Plates were analyzed by AID ELISpot reader (AID, EliSpot Reader, software version 7.0, build 14790, AID GmbH, Ebinger Strasse 4, 72479 Strassberg, Germany). Results were shown as number of counted spots and the corresponding overall spot area in mm².

EBV transformation of FACS-isolated hemagglutinin-specific B cells

GFP-capturing and non-capturing B cells were sorted into 1.5 ml Eppendorf tubes containing 200 ul complete RPMI medium, mixed gently with 500 ul of pre-warmed EBV supernatant (ATCC-VR-1492 Epstein-Barr virus, strain B95-8) and incubated for one hour at 37°C in 5% carbon dioxide. Flat-bottom 96WP were prepared containing 30,000 irradiated CD40L mouse fibroblasts in RPMI medium containing 20% non-heat-inactivated fetal calf serum, 100 units/ml of penicillin, 100 ug/ml of streptomycin and 1ug/ml of TLR agonist R848 (Mabtech, Sweden, REF 3611-5X), referred to as “RPMI-20” throughout the text. B cells were added to plates at 30 cells per well and incubated for at least two weeks at 37°C in 5% carbon dioxide.

Proteins, antibodies and vital dyes

Bovine serum albumin (cat no. A4503) was obtained from Sigma Aldrich. Influenza A H1N1 hemagglutinin (A/California/04/2009) protein (cat no. 11055-V08B) and rabbit monoclonal anti-HA antibody RM10 (cat no. 11055-RM10) was both obtained from Sino Biological (Beijing, China).

PerCP-Cy5.5 anti-human CD19 (clone HIB19, BD Biosciences, cat no. 561295), BV510 anti-human CD20 (clone 2H7, BD Biosciences, cat no. 563067), APC anti-human CD45 (clone HI30, BD Pharmingen, cat no. 555485), PerCP-Cy5.5 anti-mouse CD69 (clone H1.2F3, Biolegend, cat no. 104521), APC-Cy7 anti-mouse B220 (clone RA3-6B2, BD Biosciences, cat no. 552094), PE anti-human IgG (Jackson ImmunoResearch, cat no. 109-116-098), Alexa Fluor 488 anti-human IgM (Jackson ImmunoResearch, cat no. 109-545-129), PE anti-rabbit IgG (Jackson ImmunoResearch, cat no. 111-116-144). Anti-human IgG/HRP (cat no. P0214) and anti-human IgM/HRP (cat no. P0215) both obtained from Dako.

Cell Trace Violet was obtained from Thermo Fischer Scientific (cat no. C34557) and DAPI from Sigma Aldrich.

Flow cytometric antibody assay

For each supernatant, 100 ul of flow buffer containing 50,000 each of unlabeled TE mHA and CTV-labeled TE 0 cells were mixed and incubated with 25 ul of supernatant from EBV

transformed B cell clones for 30 minutes on ice, washed three times with cold flow buffer, labeled with PE-conjugated anti-human IgG and Alexa Fluor 488-conjugated anti-human IgM for 20 minutes on ice, washed twice with cold flow buffer and subjected to flow cytometry.

ELISA

96WP from Corning (Costar 3590 96well EIA/RIA plate flat bottom without lid) were used. Bovine serum albumin and Tween were both obtained from Sigma Aldrich, PBS 1X from Gibco, TMB for chromogenic development from KPL (SureBlue RESERVE, TMB Microwell Peroxidase, 53-00-00) and 1N hydrochloric acid from Acros Organics.

Plates were coated with hemagglutinin and BSA, each at 5 µg/ml, overnight at 4°C on the shaker. Plates were washed three times with 250 µl/well PBS-0.05% Tween and blocked with 100 µl/well PBS-2%BSA at room temperature for two hours on the shaker. Supernatants from FACS-isolated, EBV-transformed, putatively hemagglutinin-specific B cell clones, and from GFP-non-capturing, putatively non-hemagglutinin-specific, negative control B cell clones, were diluted 1:3 in PBS-0.5%BSA. Plates were incubated with diluted supernatants for two hours at room temperature on the shaker, washed three times with 250 µl/well PBS-0.05% Tween and incubated with rabbit anti-Human IgG HRP (1:6000 diluted) or rabbit anti-human IgM HRP (1:1000 diluted) in PBS-0.5% BSA for one hour at room temperature on the shaker. Plates were washed three times with 250 µl/well PBS-0.05% Tween and developed with 100 µl/well (pre-equilibrated to room temperature) TMB until a blue color was visible. The reaction was stopped with 100 µl/well 1N HCl and the plates read at 450 nm immediately after stopping.

High-throughput B cell in vitro expansion and ELISA

High-throughput B cell activation and supernatant screening by ELISA was performed according to the method published by Huang and colleagues in 2013 [12].

FACS-isolated, GFP-capturing and non-capturing B cells were plated at approximately 1.6 cells per well into 384WP wells containing IL-2, IL-21 and irradiated mouse CD40L cells to induce activation and expansion of the B cells. After 12 days, supernatants from these B cell clones were assayed as described above (see ELISA section) with the addition of tetanus toxoid coated wells as negative controls and anti-IgM and anti-IgG capture antibody-coated wells as positive controls.

Generation of recombinant monoclonal antibodies

The cDNA encoding heavy and light chains were cloned from 35 GFP-capturing B cell cultures producing anti-HA antibodies, and expressed recombinantly using standard methods.

Assessment of cell membrane protein transfer to human B cells

TE mHA and TE 0 cells were biotinylated (EZ-Link Sulfo-NHS-Biotin, Thermo Scientific, cat no. 21326), plated into 6WP at 300,000 cells per well in complete RPMI medium and incubated at 37°C in 5% carbon dioxide. Adherent biotinylated TE cells in 6WP were labeled with 200 ul Streptavidin-PE (Invitrogen, cat no. SA1004-1) 1:100 diluted in complete RPMI medium for 10 minutes on ice, then washed twice in complete RPMI medium, and allowed to re-equilibrate in 500 ul warm complete RPMI medium. B cells were isolated from PBMC from a healthy donor and were added in 200 ul ice-cold FCS to biotinylated TE cells at 1 million per well and incubated for 1 hour at 37°C in 5% carbon dioxide. B cells were retrieved by gentle resuspension from the adherent layer with a pipette so as to leave the majority of the adherent cells, and labeled with PerCP-Cy5.5-conjugated anti-human CD19, BV510-conjugated anti-human CD20 and PE-conjugated anti-human IgM, for 20 minutes on ice, washed twice in cold flow buffer and subjected to flow cytometry. IgM downregulation of the B cells, and biotin label transfer from TE cells to B cells were measured.

Assessment of specificity and sensitivity of GFP-antigen capture by transgenic B cells

The sensitivity and specificity of antigen-GFP uptake as a marker of cognate antigen capture were assessed using B cells from transgenic IgH^{MOG} mice, whose BCR recognizes the extracellular domain of myelin oligodendrocyte glycoprotein (MOG). Wild type and IgH^{MOG} mouse splenocytes were quickly thawed in 10 ml pre-warmed complete RPMI medium and put into the incubator for one hour at 37°C, centrifuged and resuspended in ice-cold separation buffer, and B cells were isolated using mouse CD19 microbeads from Miltenyi (cat no. 130-052-201). IgH^{MOG} B cells were labeled with cell trace violet, diluted with wildtype B cells at 1:100, 1:1000 and 1:10,000 and co-cultured for 2.5 hours with HEK cells expressing the fusion protein MOG-GFP. B cells were retrieved, labeled with anti-B220 and anti-CD69 antibodies and subjected to flow cytometry. MOG-GFP uptake by the B cells was analyzed by gating on scatter to select live, single cells. The population of putatively antigen-specific, i.e., CD69^{high} and GFP^{high} cells was then examined for CTV labeling to determine the numbers of true and false positives and negatives, and thus the sensitivity and specificity of the technique.

The specificity was calculated as: true negatives divided by the sum of true negatives plus false positives. The sensitivity was calculated as: true positives divided by the sum of true positives plus false negatives.

Statistical analysis

We used GraphPad PRISM 6 to graph and analyze the data for ELISA and flow cytometric antibody assays. To test the significance of differences in numbers of antigen-specific cells between GFP-capturing and non-capturing populations, a threshold was set at mean plus five standard deviations of the negative distribution, and the frequencies of cells above this threshold were compared by fisher's exact test or chi-square test as specified in the figure legends.

RESULTS

Proof of principle using BCR transgenic mouse B cells

To investigate the detectability of different B cell frequencies by flow cytometry, we used transgenic mouse B cells of known specificity. We labeled B cells from transgenic mice, whose BCR is specific for myelin oligodendrocyte glycoprotein (MOG), with cell trace violet, mixed them with unlabeled polyclonal wildtype B cells at three different dilutions (1:100, 1:1000 and 1:10,000) and cultured them for two hours with HEK MOG-GFP cells. Retrieved B cells were labeled with anti-mouse CD69 and anti-mouse B220 antibody and subjected to flow cytometry. After the co-culture a distinct population of B cells with upregulated CD69 and significant uptake of GFP-tagged MOG could be detected (Figure 1A, 1B). To confirm the MOG-specificity of the CD69^{high}-GFP^{pos} B cell population, we plotted GFP against CTV and could detect MOG-specific B cells when diluted at 1:10,000 with a specificity of 99.9% and a sensitivity of 75.8% (Figure 1B). These promising results encouraged us to use the same approach to detect human antigen-specific B cells of physiologically realistic frequencies.

Establishment of HA antigen donor cell line

We generated a TE cell line that stably expresses wildtype HA on the surface (referred to as "TE HA" throughout the text). To verify the HA expression we tested sera from healthy donors from before and after influenza vaccination for binding to HA, compared to binding to non-expressing control cells (TE 0). Figure 2A shows staining of both cell lines using serum from before and after influenza vaccination from one healthy donor. Sera after vaccination showed a

stronger binding to the TE HA cells. This indicates that the HA protein is expressed in an intact protein conformation.

We next examined whether human B cells can capture HA expressed on TE cells. To investigate this question we pre-labeled the surface of HA-expressing cells and non-expressing control cells with biotin, cultured human B cells with these biotinylated cells, retrieved and labeled them with two B cell markers (anti-human CD19 and anti-human CD20) as well as with anti-human IgM and looked at the IgM downregulation and the biotin label transfer from the TE cells to the B cells. It turned out that the majority of the B cells took up HA from the HA expressing cell line (Figure 2B). Due to the expected low frequency of HA-specific B cells, we hypothesized that the binding was rather due to unspecific binding via sialic acid than due to a specific recognition of HA by the B cells. It is known that influenza HA contains a sialic acid binding site that interacts with sialic acids present on the surface of most cell types including to B cells.

To reduce this sialic acid mediated, BCR-independent binding (reported in 2014, by James R. R. Whittle and colleagues) we generated a cell line expressing a mutated form of HA; we incorporated a tyrosine-to-phenylalanine point mutation at HA1 position 98 (Y98F) to the sialic acid binding domain. Repeating the experiments described above, using the TE cell line expressing the mutated HA (referred to as “TE mHA” throughout the text) showed that the antigen-specific binding of antibodies from human sera as well as the binding of the rabbit monoclonal anti-HA antibody RM10 was not compromised by this mutation (Figure 2C), but the antigen-independent interaction between B cells and the HA-expressing cells was eliminated (Figure 2D).

Antigen-GFP capture enables significant enrichment of human antigen-specific B cells

The approach of labeling the membrane of TE cells with biotin shows a lower specificity than using adherent cells expressing an antigen-GFP fusion protein, as described in the first section. Furthermore, the biotin approach contains in addition to the chemical biotinylation also a streptavidin incubation step, which influences the adherence of the TE cells.

The technique described above developed for identifying MOG-specific mouse B cells requires a stably transfected cell line expressing the antigen of interest fused to GFP. Accordingly, we focussed on generating a cell line expressing the mutated HA fused to GFP (referred to as “TE mHA-GFP” throughout the text) and investigated the capture of HA-GFP by human B cells. B cells from a healthy donor after influenza vaccination were cultured with TE mHA-GFP cells for

two hours to allow antigen uptake, retrieved and labeled with anti-human CD19. ELISpot plates were coated with HA, anti-IgG or BSA and either polyclonal non-cultured B cells were added (Figure 3A) or GFP-capturing and non-capturing B cells were FACS-sorted directly into ELISpot plates (Figure 3B), incubated for three days and detected with anti-IgG. Spots on anti-IgG coated wells indicate the total number of IgG secreting B cells, whereas spots on HA-coated wells indicate B cells secreting HA-specific IgG. Comparing non-cultured polyclonal B cells (Figure 3A) and HA-GFP-capturing, HA-specific B cells from the same donor (Figure 3B), we see that the technique enables a significant enrichment (approximately 150-fold; 0.024% to 3.6%) of antigen-specific cells.

B cells isolated by antigen-GFP capture FACS include both IgG- and IgM-secreting antigen-specific clones

To verify the antigen-specificity of the putatively HA-specific B cells and to enable a confident estimate of the quantitative enrichment we took advantage of the high-throughput activation and expansion of single sorted B cells using a mixture of recombinant cytokines described by Huang and colleagues [12].

FACS-sorted, GFP-capturing and non-capturing B cells were plated at approximately 1.6 cells per well into 384WP wells containing a cytokine mixture to induce activation and expansion of the B cells. Antibodies in the supernatants of these B cell cultures were screened by microELISA using anti-IgG or anti-IgM capture antibodies to measure total immunoglobulin, HA, tetanus toxoid, and BSA. Binding to each antigen was detected separately with anti-human IgG and anti-human IgM secondary antibodies, while total immunoglobulins bound to isotype-specific capture antibodies were detected only with detection antibodies with the same isotype specificity as the capture antibody. Figure 4 shows the ODs corresponding to the three antigens and the two immunoglobulin isotypes of 1920 supernatants from GFP-capturing B cell cultures and 1920 supernatants from non-capturing B cell cultures. 39.6% of the GFP-capturing B cells (761 supernatants) and 38.9% of the non-capturing B cells (747 supernatants) produced IgG (Supplementary Figure 1A). Within the GFP-capturing B cells 4.5% (35 supernatants) of the IgG-producing supernatants showed specific HA-binding activity whereas within the non-capturing B cells, none of the IgG-producing supernatants showed specific HA-binding activity. One supernatant from the non-capturing population exhibited HA-binding above our threshold of 2.5 (Supplementary Figure 1B), but this supernatant also bound strongly to BSA and tetanus toxoid (Figure 4).

IgM reactivities to all three antigens were more broadly distributed than the IgG reactivities of the same supernatants, both those from GFP-capturing and non-capturing B cells. Within the supernatants from GFP-capturing B cell clones, no clearly separated population with a strong reactivity to HA is visible. Because of the high level of IgM binding to the negative control antigens, we hypothesized that the HA-specific signal was being masked by non-specific binding. To evaluate this possibility, we plotted the anti-HA reactivities against either anti-BSA (Figure 5A) or against anti-tetanus toxoid (Figure 5B). In these plots, HA-specific reactivity should appear as a high value on the HA axis compared to the control antigen. The clearly separated group of supernatants within the coloured triangles shows that this is the case for the GFP-capturing IgG-reactive B cell clone supernatants.

IgM binding to HA in supernatants from both GFP-capturing and non-capturing B cell clones was frequently associated with high anti-BSA, or anti-tetanus activity, suggesting that IgM is not as specific as IgG. Examining only the region of the plots with high HA and low control antigen binding, there are a small number of supernatants that appear to contain HA-specific IgM, but only in the comparison with tetanus toxoid is the frequency of these supernatants statistically significantly greater in the GFP-capturing cell cultures than in the non-capturing cell cultures.

Antigen-GFP capture enables immortalization and cloning of antigen-specific B cells

The high throughput B cell activation technique we used enabled confirmation of the rate of enrichment and antigen specificity of HA-specific B cells, but produces only limited quantities of antibody, and does not yield viable B cells for further study. To enable characterization of the antibodies by flow cytometry, and to generate immortal B cell clones, we transformed HA-specific B cells with EBV. We repeated the experiment by culturing human B cells with TE mHA-GFP cells, retrieved and labeled them with anti-human CD45 and FACS-sorted them into Eppendorf tubes for a subsequent immortalization by EBV transformation. Antibodies in the supernatants of the B cell clone cultures were re-screened for antigen-specificity by ELISA (Figure 6A) and a cell-based flow cytometric assay (Figure 6B).

Figure 6A shows binding of the IgG and IgM from GFP-capturing or non-capturing B cell clones to hemagglutinin and to BSA. If detected with anti-IgG secondary antibodies, around a quarter of the supernatants (12 out of 46) from GFP-capturing B cell clones showed binding to HA with an OD 450 of > 0.25 , compared to antibodies from GFP-non-capturing B cell clones, which all bound to HA with an OD 450 of ≤ 0.125 . The anti-IgG detected supernatants in both GFP-capturing and non-capturing B cell clones bound to BSA with OD 450 of < 0.125 . If detected with anti-IgM secondary antibodies, supernatants from both GFP-capturing and non-capturing B cell

clones showed binding to HA which correlated with the binding to BSA, suggesting that IgM is not as specific as IgG. Only one supernatant in the GFP-capturing group showed high anti-HA IgM activity in the absence of BSA binding.

The same supernatants were also tested for binding to mHA expressed on TE cells in a flow cytometric assay (Figure 6B), detecting with anti-human IgG and anti-human IgM secondary antibodies. If detected with anti-IgG, around a quarter of the supernatants (13 out of 46) from GFP-capturing B cell clones showed binding to mHA expressed on TE cells. Supernatants from GFP-non-capturing B cell clones did not show binding to mHA. Compared to the results from ELISA, IgM binding was more clearly different between supernatants from GFP-capturing and non-capturing B cell clones. 5 out of 46 supernatants from GFP-capturing B cell clones bound to mHA whereas all supernatants from GFP-non-capturing B cell clones did not show any binding to mHA expressed on TE cells.

Generation of recombinant monoclonal antibodies

Thirty-five GFP-capturing B cell cultures producing anti-HA antibodies (data points marked red in Figure 4) were used for cloning and the generation of recombinant monoclonal antibodies. From those 35 B cell cultures, which initially contained 1.6 cells per well on average, 45 different heavy chains and 59 different light chains were cloned. Seventy-two combinations of heavy and light chains were expressed recombinantly, reproducing the combinations found in the wells, and the secreted antibodies tested by IgG ELISA for binding to HA, tetanus toxoid, anti-IgG and BSA. Of those 72 combinations, 42 showed binding to HA with OD 450 > 1.7 and 5 showed polyspecific binding with OD 450 > 1.7 to either BSA or tetanus or both, in addition to HA. The remaining 25 combinations showed weak binding to HA with OD 450 < 1.7, presumably representing HA-nonspecific antibodies originating from B cells that were co-cultured with specific B cells, or non-binding combinations of heavy and light chains from specific and non-specific donor B cells. The cloning results showed that the binding of the recombinant antibodies to the antigen are determined by both the heavy and light chains, although some particular heavy chains can mediate specific binding regardless of the light chain partner (Supplementary Figure 2). Supplementary Table 1 shows 27 pairs (one derived from each of 27 original GFP-capturing B cell cultures) of immunoglobulin heavy and light chain sequences that when expressed as recombinant antibodies showed binding to HA by ELISA.

DISCUSSION

In this study, we developed a novel method to isolate intact, living B cells that recognize membrane-bound, complex antigens in their native conformation. To develop this method we needed a model in which the target antigen is known and B cells specific for that antigen are present in normal human donors. Influenza hemagglutinin was chosen as an appropriate model since healthy individuals are commonly immunized against influenza which is thought to increase the number of HA specific B cells [13]. In addition, influenza hemagglutinin protein antigens are well studied.

To our knowledge, only little research has been done on isolating human influenza-specific B cells. One study, which reports on the establishment of a flow cytometric based method to enumerate and characterize influenza virus hemagglutinin-specific B cells was performed in a mouse model and focused on comparing the use of purified HA with the use of whole influenza virus to stain virus-specific B cells [14].

Our method is based on culturing human B cells with fluorescently labeled hemagglutinin-expressing cells, followed by a FACS-sort to isolate hemagglutinin-specific B cells from other B cells. With this method we were able to enrich hemagglutinin-specific B cells approximately 150-fold. We could show that B cells could be EBV-transformed or in vitro expanded after FACS-sorting. This enables testing of the specificity of the sorted B cells on a single cell level. Two different assays investigating the binding of antibodies to hemagglutinin were compared: ELISA and a cell-based flow cytometric assay. In the cell-based flow cytometric assay we investigated whether antibodies bind to membrane-bound hemagglutinin expressed in the native conformation on adherent cells, while ELISA uses recombinant protein antigen bound to plastic whose conformation is unknown. Previous studies have suggested that antibody binding differs depending on the antigen conformation, and therefore that results from the two kinds of assay are not interchangeable [15-17]. In 1999, a study by Brehm and colleagues described ten different demyelinating monoclonal antibodies which recognize the extracellular immunoglobulin-like domain of human myelin oligodendrocyte glycoprotein (hMOG) expressed on transfected fibroblasts but failed to recognize MOG peptides when assessed by ELISA [18]. A study by Pröbstel and colleagues reported on the importance of using a cell-based assay instead of ELISA to distinguish anti-MOG immunoglobulin positive patients from controls [19]. In our study we also observed different binding results for individual antibodies depending on the assay paradigm. Not all supernatants from FACS-sorted, EBV-transformed B cell cultures that bound

to hemagglutinin expressed on adherent cells, showed binding to hemagglutinin in ELISA. In contrast, only one single supernatant was positive by ELISA and failed to show a significant binding to hemagglutinin expressed on adherent cells.

In the last thirty years, different techniques have been established to isolate antigen-specific B cells that recognize conformational epitopes, most of them using antigens in their soluble form. However, in some cases it is impossible to mimic a real native conformation using soluble antigens as shown by autoimmune antibodies in myasthenia gravis that bind to acetylcholine-receptors consisting of membrane-protein clusters [7]. At present, no technique exists to isolate B cells that recognize such membrane-bound, complex antigens. We believe that our study fills exactly this gap; the technique enables the isolation of antigen-specific B cells which recognize and capture membrane-bound protein antigens. A comparison of the results from ELISA, which uses soluble protein antigen of unknown conformation, with the results from the cell-based flow cytometric assay, shows that our cell-based technique catches more of the relevant B cells compared to methods using soluble antigens to isolate antigen-specific B cells.

A limitation of our study is that the antigen has to be known or at least assumed in order to label it fluorescently. To overcome this issue, it would be necessary to label the whole surface of the antigen-expressing cells chemically with e.g. biotin and looking at the antigen-uptake of the B cells by analyzing the biotin-transfer from the adherent cells to the B cells, comparing B cells cultured in antigen-expressing and non-expressing cells. Another limiting factor of our method is the EBV transformation rate which is relatively low, as reported in previous studies [20,21]. If only a small percentage of the FACS-sorted B cells get transformed, the chance is high to lose relevant B cells. In our study we were interested in the whole B cell population, and therefore labeled the B cells with one marker to be able to differentiate between B cells and adherent TE cells. For a more specific sorting of the B cell population of interest, activation markers like CD69 could be included. CD69 upregulation is one of the earliest and most sensitive measures of antigen recognition [22], and was associated with antigen capture in our experiments with mouse cells. In addition, markers for various B cell subpopulations could be added, in order to specifically detect memory cells, plasma cells, particular immunoglobulin isotypes, or other B cell subsets.

The precise identification of epitopes, especially in the field of autoimmune diseases, can be a step towards new treatment approaches, like antigen-specific depletions of relevant B cells. A proof of concept for such a therapy has been shown in 2012 by Henry and colleagues in an animal model of diabetes [23]. Other benefits gained from isolated antigen-specific B cells are

the possibility of tracking a vaccine success or to generate possible therapeutic antibodies by cloning the B cell receptor and generating recombinant antibodies. Current studies on the human immunodeficiency virus for example, are investigating in animal models the influence of injections of broadly neutralizing antibodies on the prevention and treatment of the disease [24].

We believe that our technique, using the approach of culturing human B cells with cells expressing the antigen of interest fused to GFP, will enable us to identify B cells whose antigens are known. For unknown antigens, which are found on a particular cell type, the approach of culturing human B cells with biotinylated antigen-expressing cells could be applied. A possible application of this technique would be to identify and clone human anti-tumor antibodies from patients.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Assay to investigate the detectability of different B cell frequencies by flow cytometry using transgenic mouse B cells. CTV-prelabeled MOG-specific B cells were diluted with wildtype B cells, co-cultured with HEK MOG-GFP cells, labeled with anti-B220 and CD69 antibody and subjected to flow cytometry. **(A)** Shows the gating strategy. The right-hand side dot plot contains a purple square indicating the putatively MOG-specific B cells. **(B)** In all three frequencies (1:100, 1:1000 and 1:10,000) GFP-positive and CD69-high cells could be detected (three top plots), as indicated as purple squares showing the percentage of double-positive cells. Those cells were confirmed as MOG-specific B cells by looking at their CTV-signal (three bottom plots). The specificities and sensitivities of this assay are shown underneath.

Figure 2. (A) Binding of human serum antibodies to wildtype hemagglutinin (HA) expressed on adherent TE cells (TE HA). Histogram showing IgG anti-HA binding activity from serum from before and after influenza vaccination to HA-expressing (orange and green lines) and non-expressing cells (TE 0, red and blue lines). **(B)** Binding of human B cells to wildtype HA expressed on adherent TE cells by looking at the biotin label transfer from TE cells to B cells. Both TE HA and TE 0 cells were prelabeled with biotin before adding human B cells for co-culture. Red line shows B cells co-cultured with TE 0, blue line shows B cells co-cultured with wildtype HA-expressing cells. **(C)** HA-expression of mutated cell line (green line) measured by flow cytometry using rabbit monoclonal anti-HA antibody RM10 (left histogram) and human serum after influenza vaccination (right histogram) compared to non-expressing cells (TE 0, blue line). **(D)** Binding of human B cells to Y98F-mutant HA expressed on adherent TE cells (TE mHA) by looking at the biotin label transfer from TE cells to B cells. Both TE mHA and TE 0 cells were prelabeled with biotin before adding human B cells for co-culture. Red line shows B cells co-cultured with TE 0, green line shows B cells co-cultured with mutated HA-expressing cells.

Figure 3. Confirmation of antigen specificity by human IgG B cell ELISpot. Wells were coated with HA, anti-IgG or BSA and detected with anti-IgG. Numbers in blue italics represent the number of counted spots. The black numbers below represent the overall spot area in mm². **(A)** B cells from a healthy donor two weeks after influenza vaccination were added to the plate at 100,000 or 10,000 cells per well and incubated for three days at 37°C. **(B)** B cells were co-cultured with HA-GFP expressing cells for two hours, retrieved, labeled with anti-human CD19 and sorted by FACS. Increasing numbers (100-400, shown on the left of the figure) of

GFP-capturing and GFP-non-capturing B cells were sorted directly into ELISpot plate and incubated for three days at 37°C.

Figure 4. High-throughput B cell activation and supernatant screening by microELISA. B cells from a healthy donor two months after influenza vaccination were co-cultured with HA-GFP-expressing cells for three hours, retrieved and labeled with anti-human CD45. GFP-capturing and non-capturing B cells were sorted and plated at approximately 1.6 cells per well into 384WP containing a cytokine mixture to induce activation and expansion. Supernatants from those B cell clones were measured by ELISA for their binding capacity to three different antigens (HA, tetanus toxoid and BSA) and two controls (anti-IgG or anti-IgM), looking at IgG and IgM specificity. The horizontal axes show the three different coating antigens including the two controls and the vertical axes show the OD 450. Anti-HA IgG-specific supernatants above the OD 450 of 2 are marked in red. The upper graph shows supernatants from both GFP-capturing and non-capturing B cell clones detected with anti-IgG and the lower graph shows the same supernatants detected with anti-IgM.

Figure 5. High-throughput B cell activation and supernatant screening by microELISA. Biaxial plots comparing **(A)** HA binding against BSA or **(B)** HA binding against tetanus toxoid, for the same supernatants as shown in figure 4. The blue shaded triangles represent the regions where the difference (OD 450 HA minus OD 450 BSA) is greater than or equal to the mean plus five SD of this difference for the whole population. The pink shaded triangles represent the regions where the difference (OD 450 HA minus OD 450 tetanus toxoid) is greater than or equal to the mean plus five SD of this difference for the whole population. P value is calculated by chi-square test comparing proportions of HA binding supernatants between GFP-capturers and non-capturers.

Figure 6. Assessment of antigen-specificity of putatively HA-specific sorted B cells. **(A)** Sorted GFP-capturing and non-capturing B cells were immortalized with Epstein-Barr virus (EBV) and cultured for two weeks at 37°C. The immunoglobulin concentrations in the supernatants were measured by ELISA for IgG (top plots) and IgM (bottom plots) specificity. Wells were coated with HA or BSA. Anti-HA and anti-BSA OD 450 were plotted against each other. Vertical broken line shows the cutoff of mean plus five SD (of the HA OD 450 from the GFP-negative population). P value is calculated by fisher's exact test comparing proportions of HA binding supernatants between GFP-capturers and non-capturers. Points colored red are explained below. **(B)** The same supernatants were measured by a cell-based flow cytometry assay, comparing immunoglobulin binding to mutated HA-expressing (TE mHA) and non-expressing cells (TE 0).

Vertical axis shows the ratio of geometric mean immunofluorescence of TE mHA divided by TE 0. Red squares represent supernatants with a geometric mean immunofluorescence-ratio above the cutoff of mean plus five SD (calculated from GFP-negative population) and points representing these supernatants are also colored red in the dot plots above.

SUPPLEMENTARY FIGURE AND TABLE LEGENDS

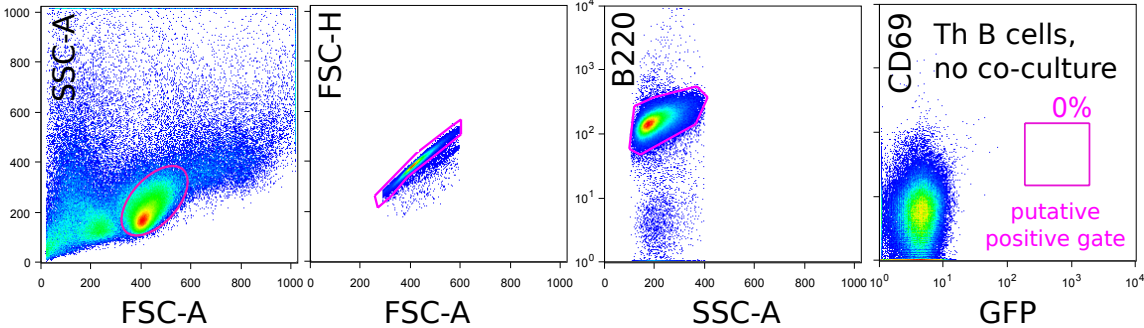
Supplementary Figure 1. Frequency distribution histograms of ELISA ODs to visualize the distribution of binding and non-binding supernatants. The horizontal axis shows the OD 450 and the vertical axis the number of B cell culture wells. **(A)** Frequency distribution of IgG capture ELISA ODs among supernatants from GFP-capturing and non-capturing B cells to enable determination of the cut-offs above which supernatants were considered IgG-positive. Cut-offs of 1.7 for the GFP-capturing B cells and 1.2 for the non-capturing B cells were chosen (dashed lines). **(B)** Frequency distribution of HA IgG ELISA ODs among supernatants from GFP-capturing and non-capturing B cells to enable determination of the cut-offs above which supernatants were considered anti-HA IgG-positive. A cutoff of 2.5 for the GFP-capturing was chosen, and the same cutoff was used for the non-capturing B cells (dashed lines).

Supplementary Figure 2. HA binding propensity of recombinant monoclonal antibodies cloned from GFP-capturing B cell cultures (data points marked red in Figure 4). Different combinations of heavy and light chains were recombinantly expressed and tested by ELISA for their binding capacity to HA. The horizontal axes show the different heavy chains and the vertical axes show the HA OD 450. Binding OD for each combination is plotted on the vertical axis with a circle or square. **(A)** Five examples of heavy/light chain combinations originating from B cell culture wells containing only one single heavy chain (horizontal axis) and 2, 3 or 4 light chains. **(B)** Example of heavy/light chain combinations originating from one B cell culture well containing two heavy chains and three light chains (same light chains are indicated by color). **(C)** Example of heavy/light chain combinations originating from one B cell culture well containing two heavy chains and eight light chains (same light chains are indicated by color).

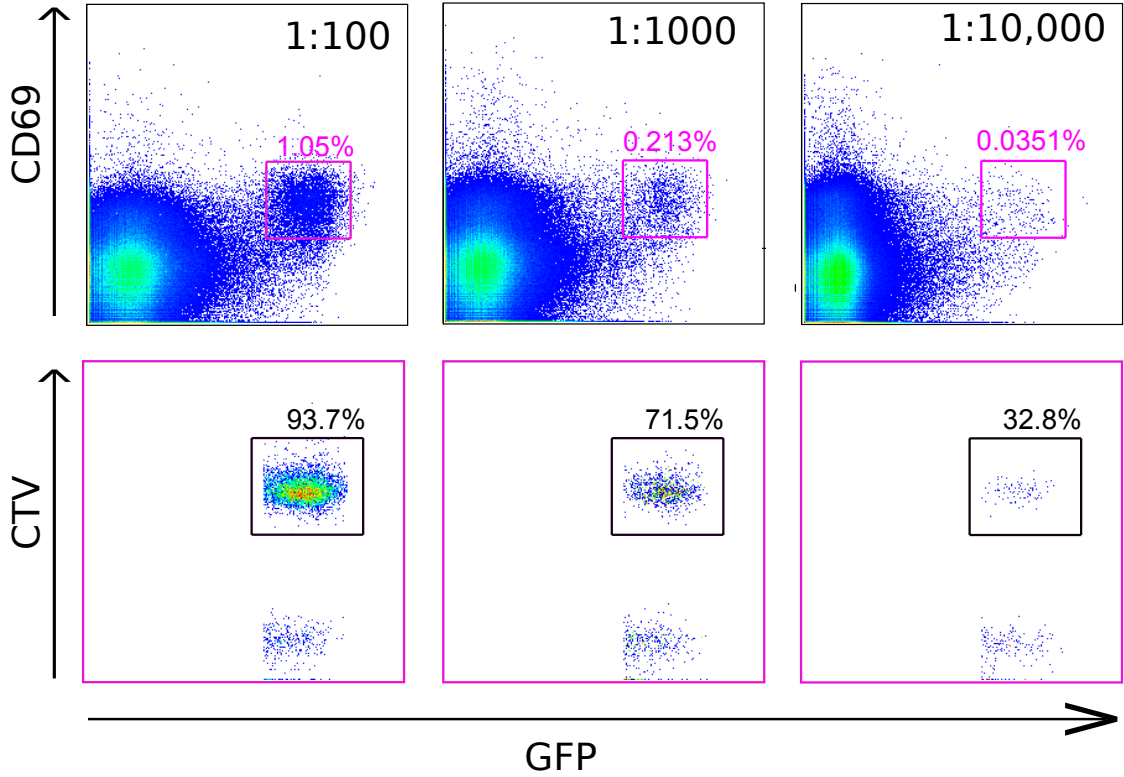
Supplementary Table 1. Sequences of 27 pairs (one derived from each of 27 original GFP-capturing B cell cultures) of immunoglobulin heavy and light chain sequences that when expressed as recombinant antibodies showed binding to HA by ELISA.

Figure 1

A



B



specificity:	99.8%	99.8%	99.9%
sensitivity:	78.6%	79.5%	75.8%

Figure 2

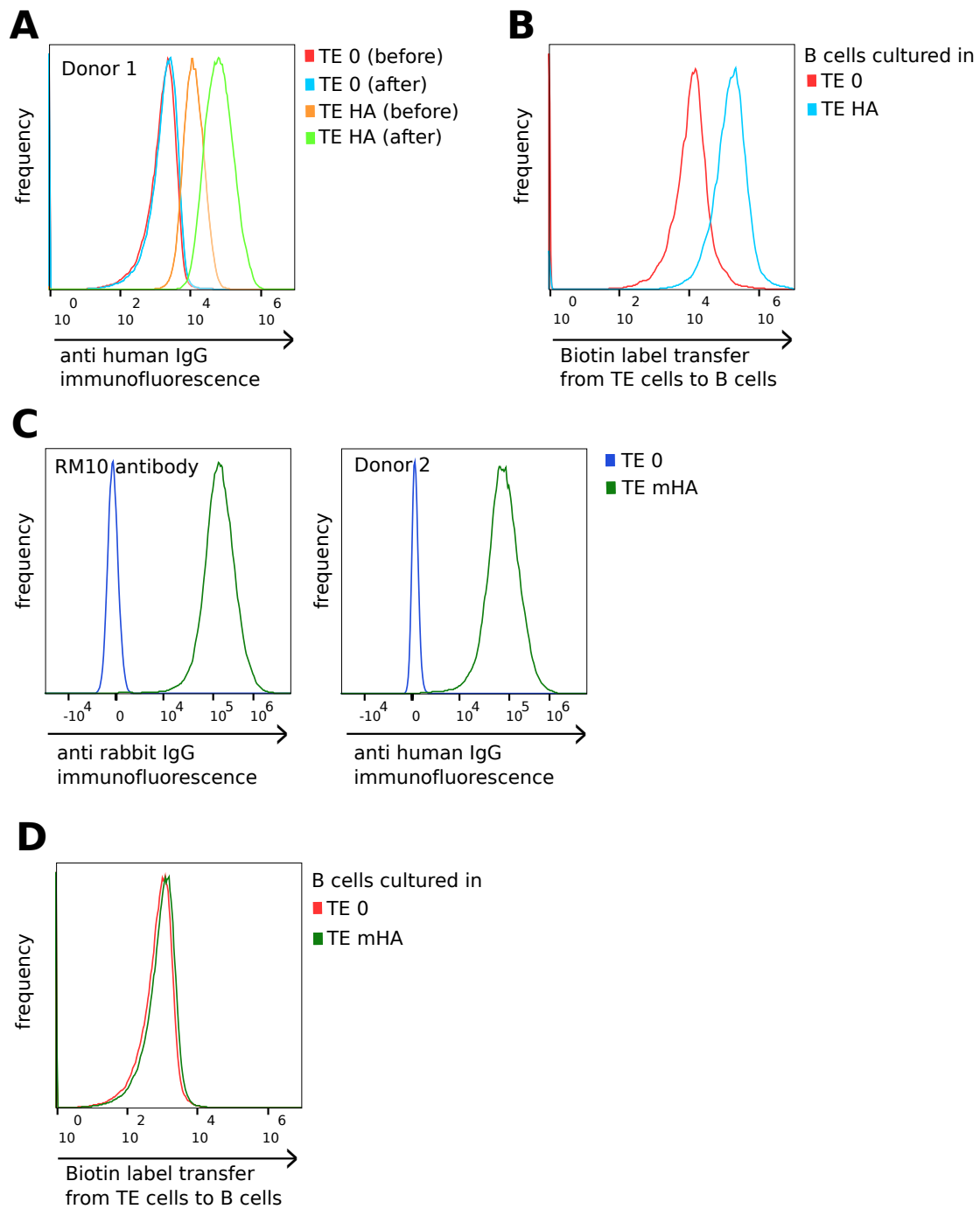


Figure 3

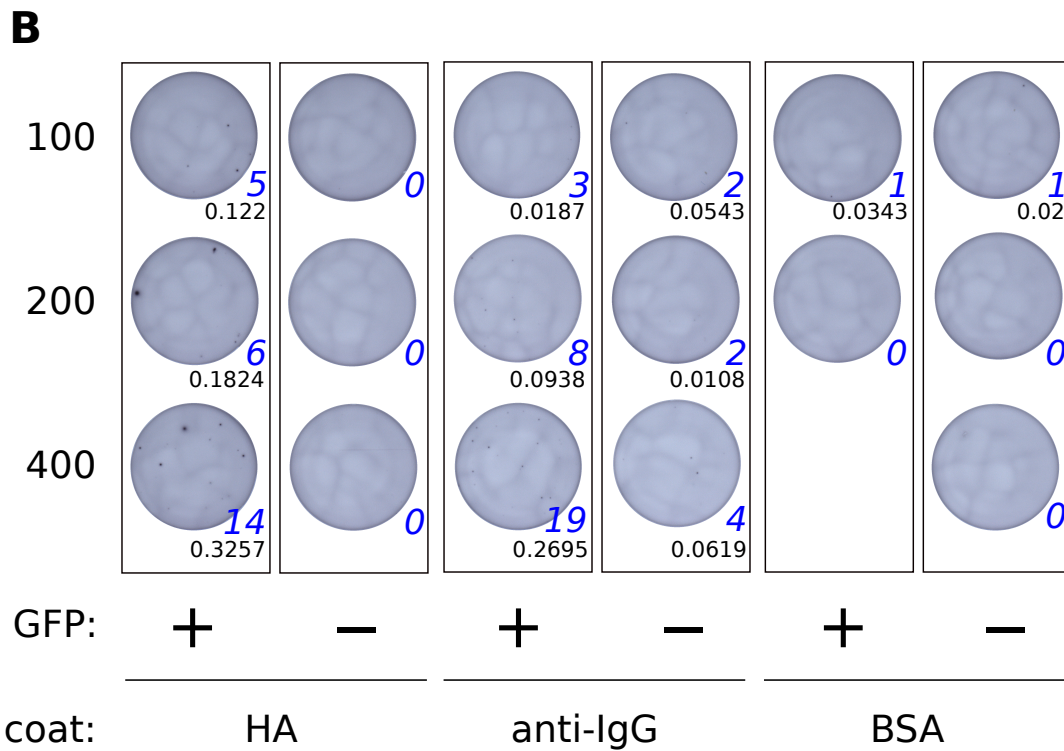
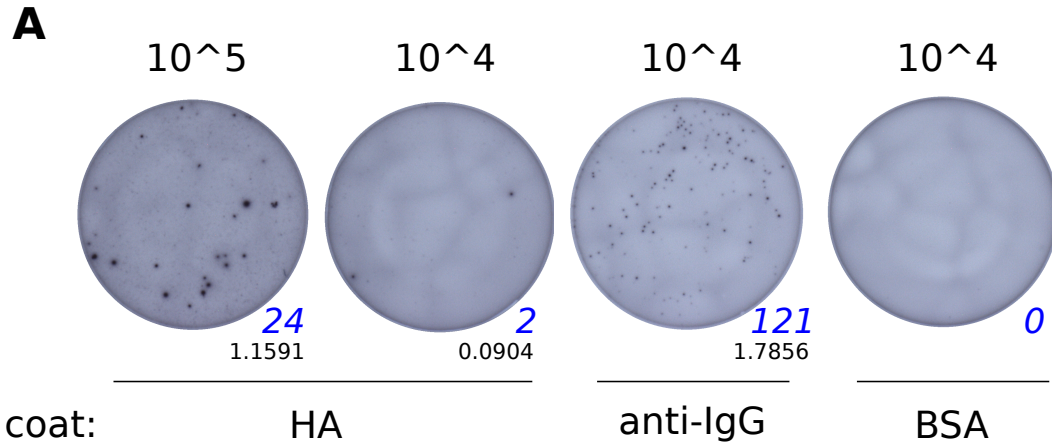


Figure 4

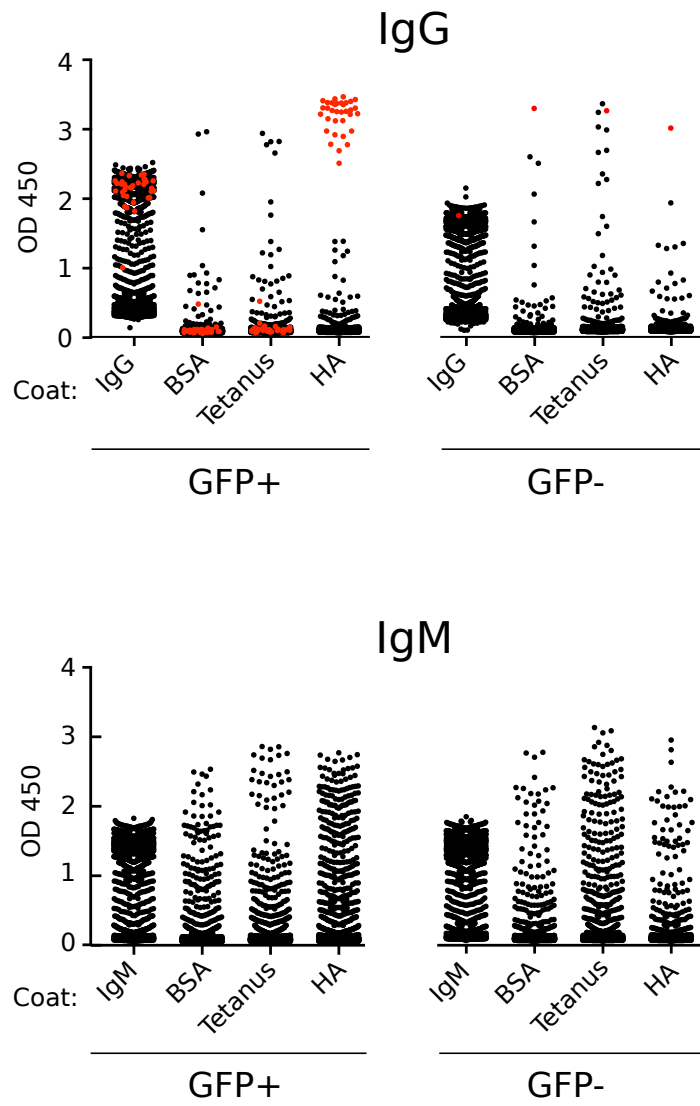


Figure 5

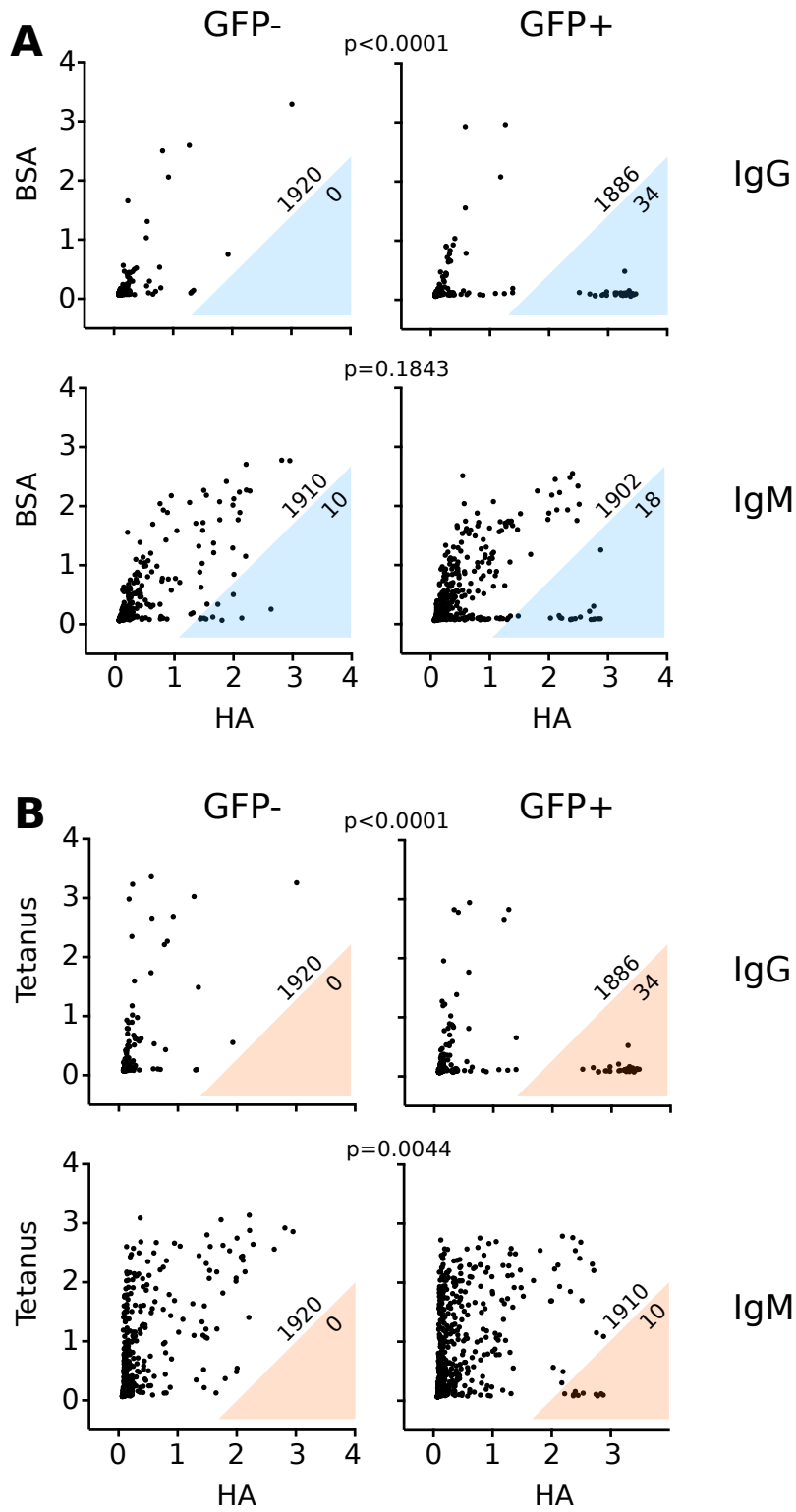
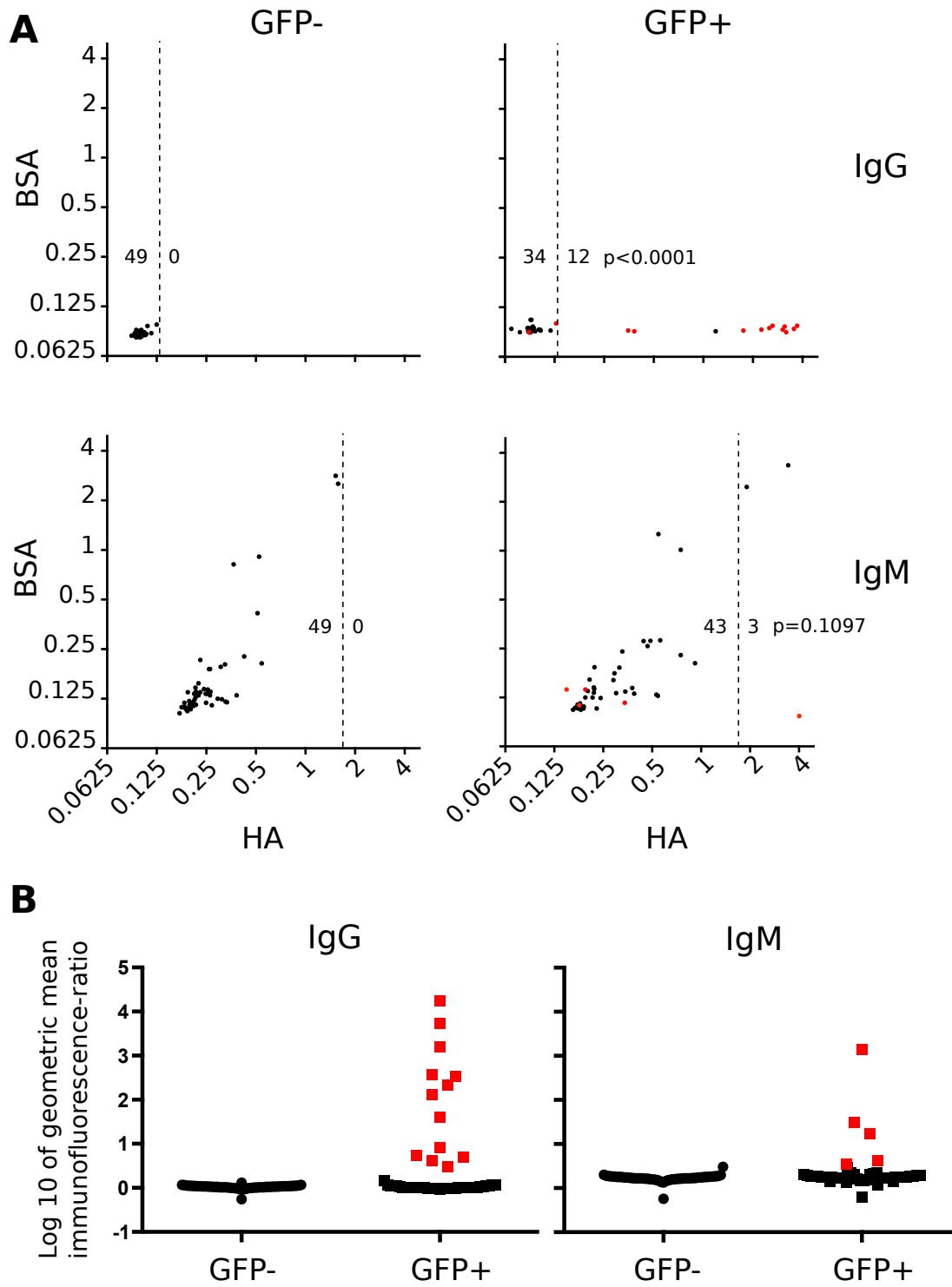
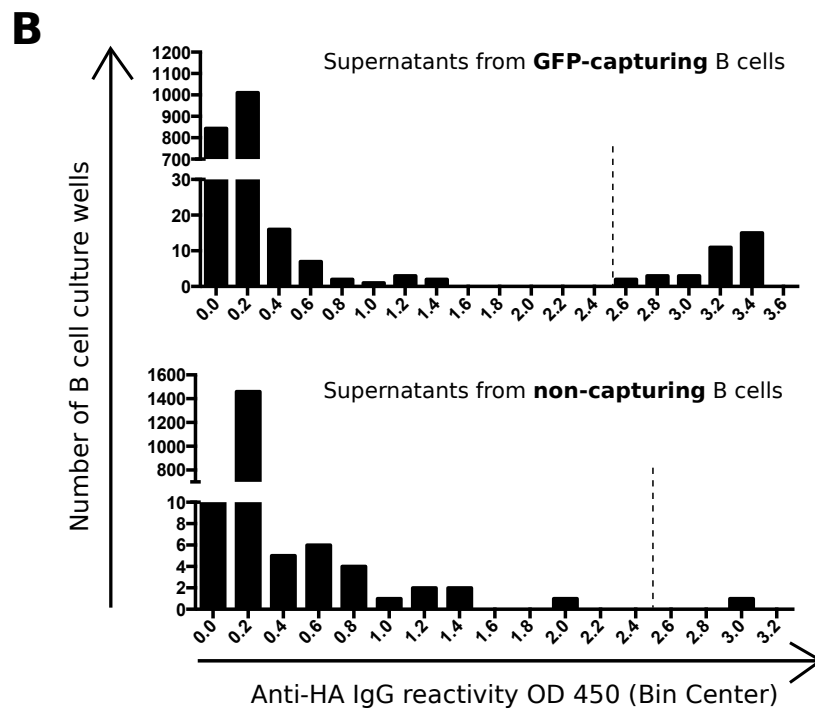
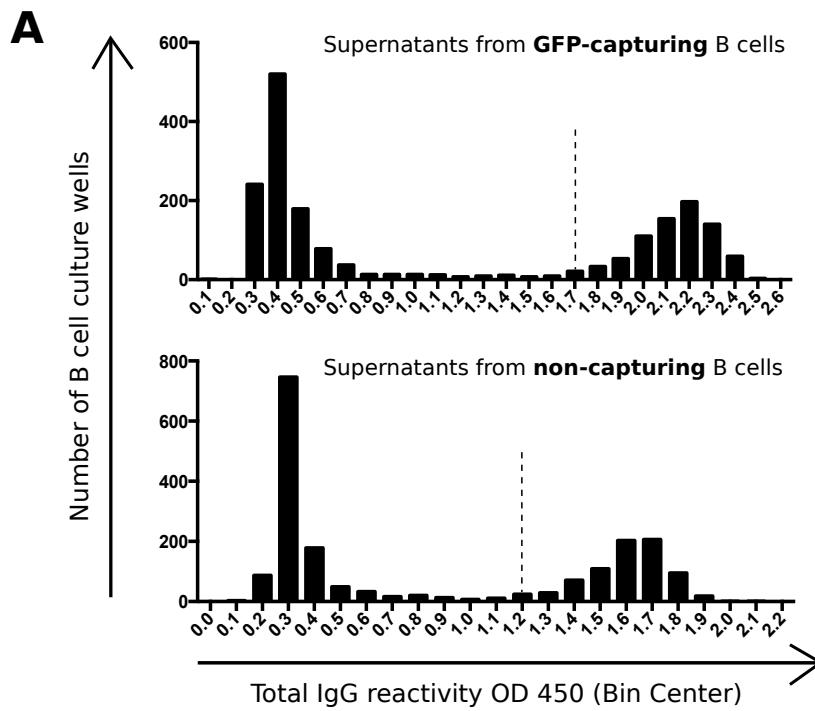


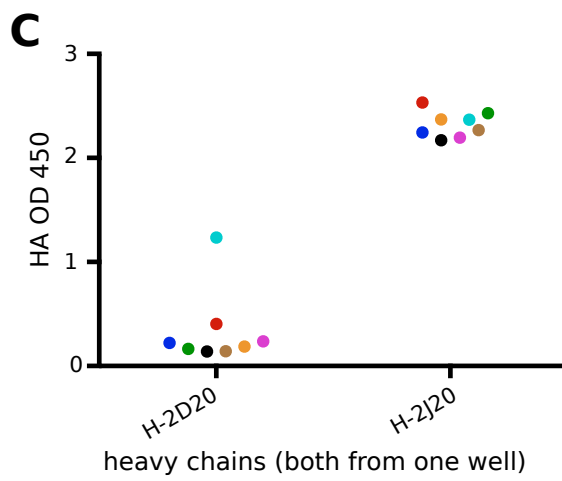
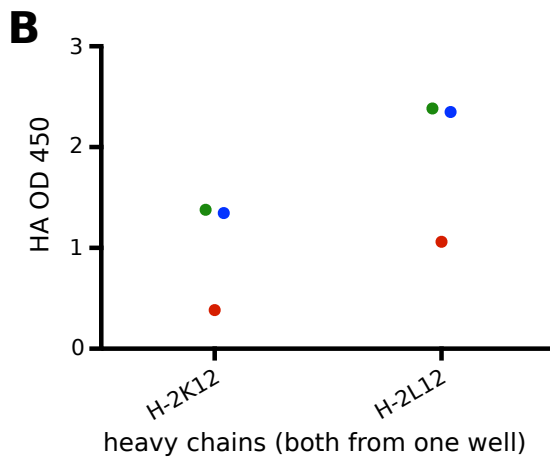
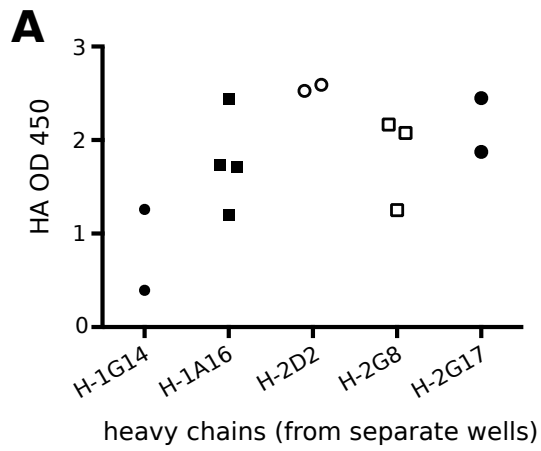
Figure 6



Supplementary Figure 1



Supplementary Figure 2



Supplementary Table 1

VH	Heavy Chain				Light Chain			
	DH	JH	CDR3	VK/VL	JK/JL	CDR3		
1	VH1-18	DH3-10	JH6	ARDRMSPFIWFGATNDYNMDV	VK2-30	JK1	MQGTQWPWT	
2	VH1-18	DH3-9	JH4	ARDRRDLITGPTFDS	VK2-30	JK2	MQGTHRPYT	
3	VH3-23	DH3-3	JH4	AKDRVTVVWESGSVDY	VK3-15	JK4	QQYNNWPPLT	
4	VH3-30	DH4-11	JH5	ARVHSNYFVGWAGSWFDP	VK1-12	JK4	QQAHSFPLT	
5	VH1-18	DH3-9	JH6	ARDRQHTLTGYGMDV	VK2-30	JK2	MQGTHWPVA	
6	VH3-15	DH3-10	JH4	TTMGEERLLNVPVVFHN	VK1-39	JK2	QLGYT	
7	VH1-69	DH2-8	JH4	ARNSGSYGDDY	VK3-11	JK2	QQRSNWLYT	
8	VH1-69	DH2-21	JH4	AIDRDSSDY	VK3-11	JK1	QQRGNWLWT	
9	VH1-18	DH1-26	JH4	ARDKGNLLSGTFLDY	VK2-30	JK2	MQGTHWPYT	
10	VH1-3	DH3-22	JH6	ARARDFLGEAGLIVAFYGMVDV	VK3-11	JK4	QQRSNWPPLT	
11	VH4-31	DH3-10	JH4	ARDRGLDHLVLGY	VK1D-33	JK4	QQSLT	
12	VH3-21	DH6-19	JH4	ARDLPSISVAGPLDY	VK1-17	JK1	LQYNTYPRT	
13	VH1-2	DH1-26	JH6	ARGWEQAGLWEAPFRYYYYGMDV	VK2D-29	JK4	MQSRQLPLT	
14	VH1-3	DH2-2	JH6	ARAVPAVILGVPWNYGMDV	VK1D-12	JK5	QQTKTFPIT	
15	VH1-18	DH1-26	JH5	ARDKGTLSSGLLDF	VK2-30	JK2	MQGSRWPYT	
16	VH3-7	DH6-6	JH4	ARAVIFPNLPIAARPPYFDF	VK1D-33	JK4	QQYADPLT	
17	VH4-4	DH4-17	JH2	AKMDYRDVDLDYWFYFDL	VK3-15	JK1	QQYNHWPPPL	
18	VH4-30-2	DH1-26	JH4	ARADGMTDKLFDY	VL1-40	JL2	QSYDTSLSALV	
19	VH4-59	DH6-13	JH5	ARTIATAGTFRFDP	VL3-1	JL2	QTWDSSTV	
20	VH4-4	DH4-17	JH2	ARTDYGVDLDYWFYFDL	VL3-21	JL2	QVWYTSADHPAV	
21	VH3-64D	DH6-19	JH4	VKALSKYSSGWQIDY	VL3-21	JL2	QVWDSSTDRPAV	
22	VH3-21	DH3-16	JH6	SRDFYDSRGGQYYGMDV	VL4-69	JL1	QVWDSSTDRPAV	
23	VH4-59	DH6-13	JH5	ARGIAAAGAFRFPD	VL3-1	JL2	QAWDTTTTV	
24	VH1-3	DH6-19	JH6	VGPPRQWLAAFYTYGLDV	VL7-43	JL3	LLYIGGTGV	
25	VH2-70	DH1-7	JH4	ARTRTTWAFFS	VL1-47	JL2	AAWDDSLNGVV	
26	VH4-31	DH6-19	JH4	ARKKAVAGTLWSDY	VL3-1	JL2	QAWDIAGVV	
27	VH3-23D	DH3-10	JH5	AKYAYFHYNWFDP	VL1-40	JL3	QSYDSSLSGSV	

Results

II. **B cell co-capture of cognate and bystander antigens activates autoreactive B and T cells**

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ABSTRACT

Antibody secretion by B cells is dependent on antigen capture, followed by presentation of antigenic peptides on major histocompatibility class II molecules to CD4 T cells. Although studied mostly with soluble antigens, B cells also capture antigen from the membranes of other cells. Using influenza hemagglutinin as a model viral antigen, myelin oligodendrocyte glycoprotein as a model self antigen, and transgenic B cells and T cells specific for each, we find that cognate antigen is rapidly captured from membranes and induces strong activation of the capturing B cell. Smaller quantities of other co-expressed, "bystander" antigens are co-captured at the same time and can be presented to T cells as well. This has implications for autoimmunity: viral antigen-specific B cells can activate self-reactive T cells, and conversely, self-antigen-specific B cells can receive T cell help from virus-specific T cells, leading to the production of autoantibodies. These findings offer a possible explanation for the link between autoimmunity and viral infections.

INTRODUCTION

A successful vertebrate immune system must discriminate between self and non-self antigens. Important checkpoints for elimination of self-reactive lymphocytes include the destruction of self-reactive T cells in the thymus (Klein et al., 2014), and suppression of development of self-reactive B cells in the bone marrow (Wardemann et al., 2003). Although AIRE ensures the expression of otherwise tissue-specific antigens in the thymus (Klein et al., 2014), the set of antigens expressed in the bone marrow is narrower, meaning that B cells whose immunoglobulin antigen receptors (B cell receptor, BCR) recognize self antigens restricted to other tissues can escape this selection. Normally this does not lead to autoimmunity, because active production of antibodies requires T cell help - peripheral B cells that encounter their cognate antigen cannot develop into antibody-secreting cells without positive signals from helper T cells. This takes place in secondary lymphoid organs, and involves extensive physical contact with a helper T cell whose antigen receptor (T cell receptor, TCR) recognizes a peptide displayed on the B cell's major histocompatibility (MHC) class II molecules. Efficient negative T cell selection in the thymus therefore safeguards against the production of autoantibodies.

However, in some individuals, autoantibodies are made and can cause debilitating disease. For some diseases, such as myasthenia gravis, the causal relationship between the antibody and the pathology is clear (Ha and Richman, 2015). For others, such as multiple sclerosis, there is a prevalence of certain autoantibodies, but their pathological significance is not understood (Hohlfeld et al., 2016). For none of these diseases is there an obvious reason for the breakdown of B cell tolerance.

Unlike antigen presenting cells of the myeloid lineage, B cells capture very little antigen by non-specific routes such as phagocytosis (Lanzavecchia, 1987). They avidly capture cognate antigen from solution, but this mechanism is very selective, and therefore B cells that recognize soluble antigens require help from T cells with a shared antigen specificity (linked recognition). Alternatively, B cells can capture antigen from membranes of other cells. This process differs significantly from capture of soluble antigen: membrane-bound antigens are multivalent, increasing the binding avidity in comparison to a monovalent antigen in solution (Batista and Neuberger, 2001); moreover they associate with other protein and lipid components of the membrane. We hypothesized that if these bystander molecules were co-captured with the cognate antigen, processed and presented to T cells, it would circumvent the antigen-specificity of T cell help.

We tested this hypothesis using adherent cells that express the CNS-restricted membrane protein myelin oligodendrocyte glycoprotein (MOG) as a model self-antigen, influenza hemagglutinin (HA) as a model viral antigen, and transgenic mouse B and T cells specific for each antigen. This antigen pair was chosen because both are integral membrane proteins, with a plausible role in autoimmunity. MOG is implicated in autoimmune neurological disorders (Pröbstel et al., 2011) and animal models thereof (Molnarfi et al., 2013), notably in acute demyelinating encephalomyelitis (Pröbstel et al., 2011), and influenza is among the most important known viral triggers of this autoimmune condition (Wang et al., 2010).

RESULTS AND DISCUSSION

Capture of cognate antigen from membrane is rapid and robust

Capture of cognate antigen from membrane has been examined in molecular detail (Carrasco and Batista, 2006; Natkanski et al., 2013), mostly using artificial membrane preparations. We examined the capture of cognate antigen from membranes of live cells by IgH^{MOG} transgenic B cells, whose B cell receptor (BCR) recognizes the extracellular domain of MOG (Litzenburger et al., 1998). When IgH^{MOG} B cells were exposed to adherent HEK cells that express a MOG-green fluorescent protein (GFP) fusion, GFP capture was detected in the B cells by flow cytometry as soon as one minute after contact, and continued to increase for more than one hour (Fig. 1 A, B, C, and Supplementary Movie 1). Capture was paralleled by loss of surface IgM, indicating internalization of the BCR-antigen complex (Fig. 1 A, B). Immunolabeling of co-cultures showed that captured antigen and IgM associate with LAMP1-immunoreactive structures in the B cell, presumably lysosomes (Fig 1 D, E). Initially these structures are seen at the interface of B cell and antigen-expressing HEK cell (Supplementary Movie 2), but upon prolonged interaction they distribute throughout the B cell (Fig. 1 D, Supplementary Movie 3), by which point the majority of the immunoglobulin colocalizes with antigen (Fig. 1 B, E).

Membrane antigen capture renders B cells highly antigenic for T cells

We exposed IgH^{MOG} B cells for 8, 12 or 20 hours to adherent TE cells that express MOG (TE MOG) and examined expression of activation markers by flow cytometry. We compared membrane-antigen-capturing B cells with identical B cells exposed to non-antigen-expressing TE cells, or to soluble MOG protein, or to an IgM-crosslinking antibody. Loss of surface IgM was similar for B cells exposed to soluble or membrane-expressed antigen, but upregulation of the activation markers CD69 and CD25 was far greater on the B cells exposed to membrane

antigen (Fig. 2 A). Upregulation of Class II MHC and CD86 was similar for the three kinds of BCR stimulation (Fig. 2 A).

We next examined the presentation of captured membrane antigens to T cells by co-culturing IgH^{MOG} B cells (MHC class II allotype I-A^b) with TE MOG cells, then retrieving and co-culturing them with 2D2 T cells, which recognise MOG 35-55 in the context of I-A^b (Bettelli et al., 2003). 2D2 T cells exposed to IgH^{MOG} B cells after membrane capture proliferated more strongly than those co-cultured with naive B cells in the presence of a high concentration of cognate peptide (Fig. 2 B, C). Soluble MOG protein was no more effective than peptide (Fig. 2 C). Neither B cells exposed to culture supernatants from antigen-expressing cells, nor to TE cells that did not express antigen stimulated any proliferation. This rules out involvement of secreted soluble antigen, or antigen-independent properties of the TE cells (Fig. 2 C). Rather, it appears that the act of antigen capture renders the capturing B cell highly stimulatory for T cells.

To test this interpretation we assessed the stimulatory properties of B cells after membrane capture of antigens that were irrelevant for the responding T cell, with or without additional peptide that matched the T cell specificity. We exposed HA-specific FluBI B cells (I-A^b) to TE cells stably transfected with HA (TE HA) and then retrieved and co-cultured these B cells with 2D2 T cells, in the presence or absence of exogenous MOG 35-55 peptide (Fig. 3 D). Without additional peptide, FluBI B cells stimulated no proliferation of 2D2 T cells, whether or not the B cells had previously encountered HA. With added peptide, even antigen-naive FluBI B cells stimulated T cell proliferation, but much less than B cells that had been previously activated by membrane HA capture (Fig. 2 D). The enhanced ability of B cells after membrane capture to stimulate T cell proliferation may thus be due to the high level of activation of B cells and the concomitant increase in costimulatory ligands (Fig. 2 A).

We also measured 2D2 T cell proliferation in response to MOG-specific B cells exposed to the same TE cells with or without peptide. Again, without peptide, pre-exposure to membrane-expressed MOG rendered the B cells more stimulatory for the T cells than pulsing with peptide, whereas the presence of HA had no effect. Most strikingly, FluBI cells exposed to TE cells that express both HA and MOG stimulated the proliferation of MOG-specific T cells, without the addition of exogenous MOG peptide. This implies that MOG had been co-captured with HA, and processed and presented. The co-capture of irrelevant, "bystander" antigens from cell membranes by B cells has implications for possible causes of autoimmunity. For example, the capture and presentation of T cell antigens by B cells with an unrelated specificity offers a possible explanation for the widely observed phenomenon that viral infections sometimes

appear to promote autoimmune responses (Cappalletti et al., 2015; Selin et al., 2011), either because viral-specific B cells activate autoantigen-specific T cells, or because autoantigen-specific B cells obtain T cell help from viral-antigen-specific T cells.

Co-captured, non-cognate antigen is presented and can qualify B cells for T cell help

To visualize the capture of non-cognate, "bystander" antigen, we generated an adherent HEK cell line stably co-transfected with MOG-GFP and HA (HEK HA MOG-GFP). We co-cultured these cells with IgH^{MOG} B cells, or with FluBI B cells. Capture of MOG-GFP by IgH^{MOG} B cells followed similar kinetics with or without co-expression of HA, but MOG-GFP capture by FluBI B cells was dependent on HA (Fig. 3 A). This bystander co-capture was as rapid as cognate capture, being observable after 3 minutes of contact, but quantitatively less. Not all antigens we examined were co-captured. For example, when FluBI B cells were exposed to TE cells that express HA, MOG-GFP and mOVA-Cherry, MOG-GFP was co-captured but mOVA-cherry was not (Fig. 3 B).

Immunoglobulin-bound antigens may be more efficiently presented to T cells than other endocytosed cargo (Lanzavecchia, 1985, 1987; Drake et al., 2006; Zhang et al, 2007). To address whether co-acquired membrane antigens were processed and presented on MHC class II, we used TE cell lines stably transfected with MOG and HA, or with HA alone. Since WSN/33 HA-specific, class II restricted transgenic T cells were not available, we added a carboxy terminal fusion to the hemagglutinin including amino acids 323-339 of ovalbumin (OVA), a peptide recognized by OT-II T cells in the context of I-A^b. IgH^{MOG} B cells were exposed to TE HA-OVA, or TE MOG HA-OVA for three hours and then retrieved and co-cultured with OT-II T cells for four days. T cell proliferation and activation were assessed by flow cytometry of CTV dilution and CD25 immunofluorescence, respectively. The results in this model were consistent with the degree of co-capture observed previously. After exposure to TE MOG HA-OVA, IgH^{MOG} B cells stimulated robust proliferation in OT-II T cells, and this was dependent on MOG (Fig. 4 A, B). The increase in CD25 expression was not as great as in the cognate capture condition (Fig. 4 A), and the percentage of proliferated T cells was also lower (Fig. 4 B). We also saw secretion of interferon- γ , IL-2 and IL-4 during interaction between B cells presenting a co-captured antigen and T cells responding to that antigen, but less than was seen in the cognate capture condition (Fig. 4 C). Presentation of the non-cognate antigen is dependent on co-capture, because mOVA, which contains the OT-II epitope but is not co-captured with HA or with MOG (Fig. 3 B) is also not presented to OT-II T cells by IgH^{MOG} B cells after exposure to TE MOG mOVA (Fig. 4 D). To be certain that this result was not due to insufficient expression of mOVA, we confirmed

the accessibility of extracellular, membrane-bound OVA in live TE MOG mOVA cells (Fig. 4 E). The preferential co-capture of certain antigen pairs suggests that interactions between the antigens, or localization of proteins in membrane microdomains such as lipid rafts may influence the likelihood of co-capture.

To test whether co-captured antigen would qualify B cells for T cell help, we used the same combination of model antigens, i.e., IgH^{MOG} B cells capturing antigen from TE cells expressing both MOG and HA-OVA, but extended the subsequent co-culture of IgH^{MOG} B cells with OT-II T cells to seven days. To exclude the involvement of BCR-independent antigen uptake, and the possibility that the mere fact of capturing antigen stimulates antibody production, we included negative controls of IgH^{MOG} B cells interacting with TE HA-OVA or with TE MOG HA. As a positive control, another TE cell line was established that stably expresses MOG fused to the OT-II OVA epitope (TE MOG-OVA). We exposed the IgH^{MOG} B cells to the TE cells, retrieved and co-cultured them with OT-II T cells, and measured anti-MOG antibodies in the culture supernatants. Omitting either the T cells, the OVA epitope, or MOG, no antibodies were detected. When OT-II T cells were present and the OVA epitope was included in the B cell cognate antigen, antibody production was robust. We clearly detected antibodies also when the OVA epitope was present on the HA antigen (Fig. 4 F). Autoreactive B cells that present co-captured viral antigen can thus be triggered to produce autoantibodies by unrelated anti-viral T cells.

B cell capture from membranes has been studied mostly in the context of antigen display to B cells by follicular dendritic cells in secondary lymphoid organs (Batista and Harwood, 2009; Cyster, 2010). We here propose that B cells could also capture cognate antigen from the membranes of cells that express it. Exposure to viral proteins expressed as cell membrane antigens is highly likely, because numerous common viruses infect leukocytes. We note that for the FluB1 B cells, the presence of the HA-specific BCR enables influenza to infect them (Dougan et al., 2013). However, exposure to self antigens that are not expressed in bone marrow (and therefore are likely targets of non-tolerized B cells) is less likely inside the blood/lymph compartments where the majority of B cells are located. Extralymphatic B cells are found in the peritoneal and pleural cavities (Höpken et al., 2010) and in the mucosa, notably the lamina propria, of the respiratory, digestive and urogenital tracts (Brandtzaeg and Johansen, 2005). B cells are found in synovial fluid and tissues in rheumatoid arthritis patients (Scheel et al., 2010), as well as in lesions and cerebrospinal fluid in multiple sclerosis (Krumbholz et al., 2006). *In vitro*, bystander antigen co-capture can circumvent the normal B cell / T cell common antigen

checkpoint, and enable autoreactive T cell activation and/or autoantibody secretion (Figure 4 G). Because the amount of co-captured bystander antigen is small, a physiologically relevant, co-capture-dependent autoimmune response is likely to depend also on other predisposing circumstances, such as chronic or repeated infection, or an MHC haplotype that favors the competitive binding of bystander-derived peptides.

MATERIALS AND METHODS

Mice and primary immune cells

C57Bl/6 mice were bred in the University of Basel Mouse Core Facility. Tg(TcraTcrb)425Cbn (OT-II) mice on a Rag2^{-/-} background were a kind gift from Ed Palmer (University of Basel). FluBI mice (Dougan et al., 2013), were bred from founder members from the Whitehead Institute, Cambridge, Massachusetts. IgH^{MOG} (Litzenburger et al., 1998, also known as Th B cells) and C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J ("2D2") mice (Bettelli et al., 2003) were bred from founder members provided by Guru Krishnamoorthy and Hartmut Wekerle (Max-Planck-Institut für Neurobiologie, Martinsried, Germany). Primary immune cells were obtained from spleens by mechanical disruption followed by brief settlement under gravity to remove tissue fragments. B cells and CD4-positive T cells were obtained by negative selection using biotinylated antibodies and magnetic beads from Miltenyi (Pan B Cell Isolation Kit II, mouse 130-104-443, and CD4-positive T Cell Isolation Kit, mouse, 130-104-454). When higher purity of B cells was required, for example for the T cell proliferation assays, the splenocytes were first pre-depleted with anti-CD43 beads (Miltenyi 130-049-801) before following the manufacturer's instructions for the negative selection kit. All procedures involving animals were authorized by the Kantonale Tierversuchskommission.

Peptides, proteins, antibodies and vital dyes

MOG 35-55 peptide and MOG 1-125 recombinant protein were obtained from AnaSpec (Fremont, California). Goat anti-mouse μ -chain-specific F(ab') fragment (cat#115-006-020) was from Jackson ImmunoResearch (West Grove PA), and FITC-conjugated anti-mouse IgM[a] from BD Pharmingen. Cell Trace Violet and Cell Tracker Deep Red were from ThermoFisher.

Plasmids and cell lines

The open reading frame encoding rat MOG was amplified with its STOP codon from pRSV-MOG and cloned into the blasticidin (Gibco) resistance-conferring pcDNA6.2C-EmGFP-DEST (Invitrogen) by gateway cloning, following the manufacturer's instructions. An expression

plasmid encoding A/WSN/33 influenza hemagglutinin under hygromycin (Sigma Aldrich) selection was purchased from Sino Biological (Beijing, China). Fusion constructs MOG-GFP, MOG-OVA, HA-OVA were made by template-switching PCR and cloned by Ligation Independent Cloning into the PigLIC vector, which confers puromycin (Gibco) resistance. The mOVA construct was amplified from pODpCAGGS (Ehst et al., 2003; a kind gift from Marc Jenkins, University of Minnesota) and cloned into PigLIC. Cells transfected with this construct express ovalbumin at the cell surface, which can be detected by flow cytometry on living cells using a polyclonal rabbit anti-OVA antibody (Ab1225, Millipore). HEK 293 embryonic kidney cells and TE671 rhabdomyosarcoma cells (referred to as "TE cells" throughout the text) were obtained from ATCC (LGC, Wesel, Germany). Cells were transfected by exposure to 2 µg/ml of plasmid DNA and 5 µg/ml of polyethylenimine in RPMI medium without serum for four hours. Cells were cultured in complete medium (RPMI medium containing 10% fetal calf serum, 100 units/ml of penicillin and 100 µg/ml of streptomycin, all from Gibco) at 37°C in 5% carbon dioxide. Cells resistant to the appropriate selective antibiotic were sorted by the Department of Biomedicine Flow Cytometry Core after labeling as described below. Cells were tested for mycoplasma infection on arrival and subsequently (LookOut Mycoplasma PCR Detection Kit, Sigma).

Cell Surface Antigen Confirmation and General Flow Cytometry

For each cell line designed to express a cell membrane antigen, we verified expression at the establishment of the cell line, and subsequently periodically checked the expression of the antigen by immunofluorescence flow cytometry (see Supplementary Table 1). Adherent cells were washed in Phosphate-Buffered Saline (PBS), trypsinized, resuspended in complete medium, washed in labeling buffer (PBS, 2% FCS, 0.1% sodium azide), incubated for thirty minutes on ice in primary antibody, washed again, incubated with fluorescent secondary antibodies, washed and measured on a BD LSRFortessa flow cytometer (BD Biosciences). Cells known to be positive and negative for the target antigen were labeled and measured in parallel each time. A typical result from such an experiment is shown in Figure 4 E. When flow cytometry results are shown as contour plots, the contour lines are spaced at 10%. Gating strategies for each figure are shown in Supplementary Figure 1.

B cell Flow Cytometry

IgH^{MOG} B cells isolated by negative selection were co-cultured with TE MOG or TE 0 cells for 8 - 20 hours in complete medium at 37°C in 5% carbon dioxide, then retrieved and labeled with the following antibodies before analysis as above. APC-Cy7 anti-B220 (clone RA3-6B2),

PerCP-Cy5.5 anti-CD69 (clone H1.2F3), PE-Cy7 anti-CD86 (clone GL1), APC anti-I-A/I-E (clone M5/114.15.2, Biolegend), PE anti-CD25 (clone PC61.5), AlexaFluor488 donkey anti-mouse IgM (Jackson ImmunoResearch), FITC R35-95 rat IgG2a kappa isotype control. All antibodies from BD Biosciences unless otherwise specified.

T cell proliferation assays

B cells were exposed to antigen-expressing adherent cells in 25 cm² flasks for three hours, and then retrieved, counted and put into 96-well plates at 50,000 per well in complete medium supplemented with 50 µM 2-mercaptoethanol. T cells were labeled with 5 µM Cell Trace Violet in Hanks' Balanced Salt Solution (Gibco) at 37°C for 15 minutes, then washed thrice in complete medium, counted and added to the B cell wells at 25,000 per well. After four or five days, the co-cultures were retrieved, labeled with anti-CD19, anti-B220, anti-CD4 and anti-CD25 (all from BD Biosciences) and analyzed by flow cytometry on a BD LSRFortessa.

Fixed and live cell microscopy

For high resolution immunolabeling, B cells were added to HEK MOG-GFP cells growing in chambered coverslips (Ibidi, Martinsried, Germany) at 37°C and at various time points were fixed in ice cold 4% phosphate-buffered paraformaldehyde for 20 minutes, then washed in Phosphate-Buffered Saline (PBS), blocked in PBS containing 5% normal goat serum and 0.02% TritonX and immunolabeled with rat anti-mouse LAMP1 (Southern Biotech), followed by Alexa647 conjugated goat anti-mouse IgM and Rhodamine-RedX-conjugated goat anti-rat antibodies, both from Jackson ImmunoResearch. Images were acquired with a Nikon a1r maintained by the microscopy core facility of the Department of Biomedicine, in line-scanning mode with a 60X 1.40 N.A. oil immersion objective, using voxel dimensions set at approximately Nyquist sampling. Acquisition was controlled by Nikon Elements software, and the same software was used to create 3-dimensional reconstructions and maximum intensity projections from the stacks of X-Y planes. Regions of interest were subsequently cropped, and when necessary to enhance visibility brightness and contrast were increased using the Fiji distribution of ImageJ (Schindelin et al., 2012). For Live cell imaging, a similar setup was used, but the B cells were pre-labeled with Cell Tracker Deep Red (Invitrogen) following the manufacturer's instructions, and without fixing the cells, the chambered coverslips with the cells growing in complete medium were placed into a humidified chamber on the microscope stage, held at 37°C and 5% carbon dioxide by an INU-TIZ-F1 controller (Tokai Hit, Fujinomiya, Japan). The pinhole was opened to 5.0 Airy units, and other parameters (PMT voltages, voxel dimensions, etc.)

optimized to minimize laser light exposure. Approximately one frame per minute was captured, and frames were assembled into movies with Nikon Elements software.

Anti-MOG antibody assay

Anti-MOG antibodies were measured in culture supernatants by flow cytometry as previously described (Pröbstel et al., 2011). Supernatants were mixed with two volumes of reporter cell suspension in PBS / 2%FCS / 0.1% sodium azide on ice. The reporter cells include equal numbers of unlabeled TE MOG cells and CTV-labeled TE 0 cells. After 30 minutes on ice, the cells are washed, and antibodies adhering to the TE MOG cells are detected with Alexa 647 anti-mouse IgG secondary antibodies (Jackson ImmunoResearch) and measured by flow cytometry. TE 0 and TE MOG populations are then separated by CTV label and the ratio of fluorescence intensities between TE MOG and TE 0 indicates the abundance of antibody in the supernatant.

Cytokine assays

IFN- γ , IL-2, and IL-4 were quantified in supernatants from B/T cell cocultures by the MSD V-PLEX Proinflammatory Panel 1 (mouse) electrochemiluminescent (ECL) assay (MSD: Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's guidelines. Samples were analyzed in duplicate and the plates were read in a SECTOR Imager instrument (Meso Scale Discovery).

Statistics

Geometric mean fluorescence intensities of activation markers on B cells were analyzed by two-way analysis of variance (time versus condition) and since an effect of condition but not of time was found, the effect of condition was examined by one-way analysis of variance followed by Dunnett's Multiple Comparison Test to compare each of the conditions against "no stimulation". T cell proliferation data (proportions of cells in the CTV-low gate) were analysed by one-way analysis of variance followed by Dunnett's test. Cytokine concentrations were log-transformed to improve normality and subjected to two-way analysis of variance followed by Bonferroni-corrected post-hoc multiple comparisons. Anti-MOG antibody levels, expressed as ratios of geometric mean immunofluorescence TE MOG: TE 0 were analyzed by two-way analysis of variance (presence of T cells versus antigen condition), revealing a strong interaction, with an effect of condition limited to the T cells present condition. The Kruskal-Wallis test was therefore used to assess the effect of antigen condition, and Dunn's Multiple Comparison Test used to compare each of the conditions with the T cells absent condition.

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FIGURE LEGENDS

Figure 1. Capture of cognate antigen from membrane. (A) Flow cytometry of MOG-GFP acquisition and cell-surface IgM loss by MOG-specific IgHMOG or wild-type B cells. B cells were co-cultured with MOG-GFP-expressing HEK cells for the times shown, then retrieved, labeled with fluorescent anti-CD45R (B220), anti-IgM antibodies, washed and measured. Data shown are gated on scatter and B220 immunofluorescence from one of two similar experiments.

Tick marks on axes of bottom left plot show log 10 decades and are the same for all plots. Contour line intervals are 10% of the population, for which the gating strategy is shown in Supplementary Figure 1. (B) Graphical representation of the GFP fluorescence (solid green line) and IgM immunofluorescence (broken purple line) intensities shown in A. Geometric mean fluorescence is shown on a linear scale against time. (C) Single frames from a live cell imaging experiment showing the capture of MOG-GFP (green) from stably transfected HEK cells by IgHMOG B cells labeled with Cell Tracker Deep Red (magenta). When the top left frame was captured, one B cell (broken cyan arrow) was in contact with the HEK cell. Three minutes later (top right frame) a second B cell had made contact (cyan arrow), but has not yet obviously captured antigen. In the subsequent frames at 15 and 45 minutes, the accumulations of antigen (cyan arrows in lower frames) are visibly increasing. The entire time sequence is provided as Supplementary Movie 1. (D) 3D reconstructions of confocal z stacks of B cell - HEK MOG-GFP interactions. B cells were fixed after 3 or 60 minutes of co-culture with HEK MOG-GFP cells and immunolabeled for LAMP1 (red) and IgM (magenta) before confocal microscopy. Stacks of XY planes stacks were processed digitally to generate 3D reconstructions, and rotated to show the view "from the side". The broken white arrows on each image show the level at which the planes shown in E were captured. Bar = 2 μ m. 3D reconstructions provided as Supplementary Movies 2 and 3. (E) "Horizontal" (i.e., parallel with the coverglass) sections through the same two cells shown in D. These images are maximum intensity projections of three XY-planes each, centered vertically at approximately the level shown by the broken white arrows in the left image of panel D. Bar = 2 μ m.

Figure 2. Immunological sequelae of membrane antigen capture. (A) Changes in surface expression of various molecules following antigen capture. IgHMOG B cells were exposed to TE MOG cells, or to various comparison treatments for 8 to 20 hours, then retrieved, immunolabeled and measured by flow cytometry. Responses of IgHMOG B cells exposed to different conditions are plotted in different colors, as shown in the legend. The vertical axis is the geometric mean fluorescence value for the population of cells in the B cell gate. Median values from three independent replicate experiments are shown. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (B) T cell proliferation in response to B cells presenting antigen from different sources. IgHMOG B cells were either exposed to TE MOG cells for 3 hours and then retrieved and co-cultured with MOG-specific 2D2 T cells (orange line), or else were added naive to the T cells in 96-well plates with 30 μ g/ml of MOG 35-55 (cyan line) or without any antigen (red line). The T cells were pre-labeled with Cell Trace Violet (CTV) to enable tracking of proliferation, and after 4 days at 37°C, the co-cultured cells were retrieved, labeled for B220 and TCR alpha 3.2 (expressed on 2D2

T cells) and subjected to flow cytometry. The range labeled “CTV low” was used to calculate the values used for Panel C. (C) T cell proliferation induced by B cells capturing membrane antigen, soluble protein, or soluble peptide. IgHMOG B cells were exposed to adherent cells and then retrieved for co-culture with MOG-specific 2D2 T cells, or else were co-cultured with T cells in the presence of the indicated antigen concentrations. 2D2 cells were pre-labeled with CTV to track proliferation, and after 4 days at 37°C, the co-cultured cells were retrieved, labeled and measured as in Panel B. The proportion of cells in the CTV-low gate is shown as a percentage of the total number of CD4-positive, B220-negative cells. Red bars show results from conditions in which B cells were pre-cultured with adherent cells, green bars from cells cultured with MOG 35-55 peptide, and blue from cells cultured with MOG 1-125 recombinant protein. In the negative control no antigen was added. Other controls include exposure of B cells to antigen non-expressing TE 0 cells, or to supernatants from TE MOG cells. (D) Effect of membrane antigen capture on T cell-stimulating capacity of B cells. MOG-specific IgHMOG B cells, or HA-specific FluBI B cells were exposed to MOG or HA, expressed in TE cell membranes. After exposure, B cells were retrieved and pulsed (solid bars), or not (outline bars) with 10 µg/ml of MOG 35-55 peptide and then co-cultured with CTV-labeled MOG-specific 2D2 T cells for four days. Bars show percentages of T cells in the CTV-low gate. Results from one of three independent experiments.

Figure 3. Flow cytometry of bystander antigen capture. (A) IgHMOG B cells, FluBI B cells, or wild-type B cells were exposed to HEK cells expressing MOG-GFP fusion protein and HA (HEK HA MOG-GFP), or to cells expressing MOG-GFP alone (HEK MOG-GFP), or not exposed to adherent cells for the indicated times, then retrieved, and labeled for B220 and surface immunoglobulin for flow cytometry. Data are representative of two experiments. (B) Co-capture of MOG-GFP and mOVA-Cherry with HA was compared by flow cytometry of FluBI B cells exposed to TE cells expressing all three antigens (third contour plot). Intensity of captured MOG-GFP (excitation 488 nm, emission 530/30 nm) is shown on horizontal axis and of captured mOVA-Cherry (excitation 561 nm, emission 610/20 nm) is shown on vertical axis. TE 0 and TE MOG-GFP without HA were used as negative controls, and TE HA-GFP as a positive control.

Figure 4. Immunological sequelae of bystander antigen co-capture and presentation. (A) IgHMOG B cells or FluBI B cells were exposed to TE cells expressing either HA fused to the OT-II epitope (TE HA-OVA), or co-expressing both HA-OVA and MOG (TE MOG HA-OVA) for 3 h, then retrieved and cultured with CTV-labeled OT-II T cells for 5 d. Dot plots show CTV intensity against CD25 immunofluorescence for CD4-positive, CD19-negative cells. MOG (blue) is

recognized by the IgHMOG BCR; HA (orange) is recognized by the FluBI BCR; and the OVA epitope (red) presented in the context of the I-Ab MHCII expressed by both B cell types is recognized by the OT-II TCR. The range in the upper left plot marked "CTV-low" was used to calculate the percentages displayed in (B). (B) Column scatter graph showing T cell proliferation in three independent experiments like the one shown in A. Vertical axis shows percentages of CD4-positive, CD19-negative cells in the CTV-low gate. Cyan and blue circles show results from co-cultures with IgHMOG B cells previously exposed to TE HA-OVA or TE MOG HA-OVA respectively; orange and red circles show results from co-cultures with FluBI B cells previously exposed to TE HA-OVA or TE MOG HA-OVA respectively. Results from the four conditions pooled from three independent experiments were analysed by one-way ANOVA followed by Dunnett's test. Stars indicate significant difference from the IgHMOG + TE HA-OVA condition (** $p < 0.01$, *** $p < 0.001$). (C) Cytokine concentrations in supernatants from OT-II T cell proliferation assays like that shown in (A) were measured by ELISA. Color scheme is the same as (B). Bars show mean and error bars 95% confidence intervals of data pooled from two similar experiments. Results for interferon- γ (IFN- γ), interleukin-2 (IL-2), and interleukin-4 (IL-4) are shown as indicated on the horizontal axis. Asterisks indicate significant difference from IgHMOG + TE HA-OVA condition ($p < 0.0001$) (D) IgHMOG B cells were exposed to TE cells expressing OVA at the cell surface (TE mOVA, empty red bars) or co-expressing both MOG and mOVA (TE MOG mOVA, violet bars); or a single fusion protein (TE MOG-OVA, blue bars). After three hours, the B cells were retrieved and co-cultured with CTV-labeled 2D2 or OT-II T cells for 4 d, and then the proliferation of the T cells was assessed by flow cytometry. Vertical axis shows percentage of T cells in the CTV low gate. One of two experiments shown. (E) Flow cytometric confirmation of membrane location of MOG and OVA antigens. The TE mOVA and TE MOG mOVA cell lines used in the experiment shown in C were immunolabeled with mouse anti-MOG and rabbit anti-OVA primaries, Dylight 405 anti-mouse and Alexa 488 anti-rabbit secondaries. The quadrants are set to 99% of the TE 0 negative control in the bottom left quadrant. Grey contours show TE 0 cells, red contours show TE mOVA and blue contours show TE MOG mOVA. (F) MOG-specific IgHMOG B cells were exposed to adherent TE cells expressing MOG and HA separately; HA-OVA alone; MOG and the HA-OVA fusion; or MOG-OVA fusion (TE MOG-OVA), then retrieved and co-cultured with (inverted red triangles) or without (open blue circles) OT-II T cells. After 7 d, supernatants were assayed for anti-MOG antibodies by labeling MOG-expressing compared to non-expressing reporter cells by flow cytometry. Vertical axis shows ratio of geometric mean fluorescence between MOG expressing and non-expressing reporter cells. Medians of replicates pooled from three independent experiments were analyzed

by Kruskal-Wallis Test and each condition compared with the no T cells condition (** $p < 0.001$). (G) Hypothetical model of the bystander antigen co-capture and presentation phenomenon. The left side represents the situation in which a viral-antigen-specific B cell co-captures self antigen and thereafter activates an autoreactive T cell. The right side represents the converse situation in which an autoreactive B cell co-captures viral antigen and fraudulently gains T cell help from a viral-antigen-specific T cell, leading to the secretion of autoantibodies.

SUPPLEMENTARY FIGURE AND MOVIE LEGENDS

Supplementary Table 1. Properties and origins of stably transfected cell lines and their parental stocks.

Supplementary Figure 1. Gating strategies for flow cytometry figures. For each labeled Figure panel, gating strategies are shown with the largest population at the top and successively reduced populations going down the page.

Supplementary Movie 1. Live cell imaging of MOG-specific B cells capturing MOG-GFP (green) from stably transfected HEK cells by IgHMOG B cells labeled with Cell Tracker Deep Red (magenta). Frames are separated by approximately one minute.

Supplementary Movie 2. 3D reconstruction of confocal z stack of B cell - HEK MOG-GFP interaction fixed at 3 minutes after contact, and immunolabeled for LAMP1 (red) and IgM (magenta) before laser scanning confocal microscopy. Stacks of XY planes were processed digitally to generate 3D reconstructions, and then rotated to create successive frames of the movie in order to show the spatial location of the B and HEK cells and the antigen-IgM complexes being internalized by the B cell.

Supplementary Movie 3. 3D reconstruction of confocal z stack of B cell - HEK MOG-GFP interaction fixed at 60 minutes after contact. Other than the time before fixation, exactly analogous to Supplementary Movie 2.

Figure 1

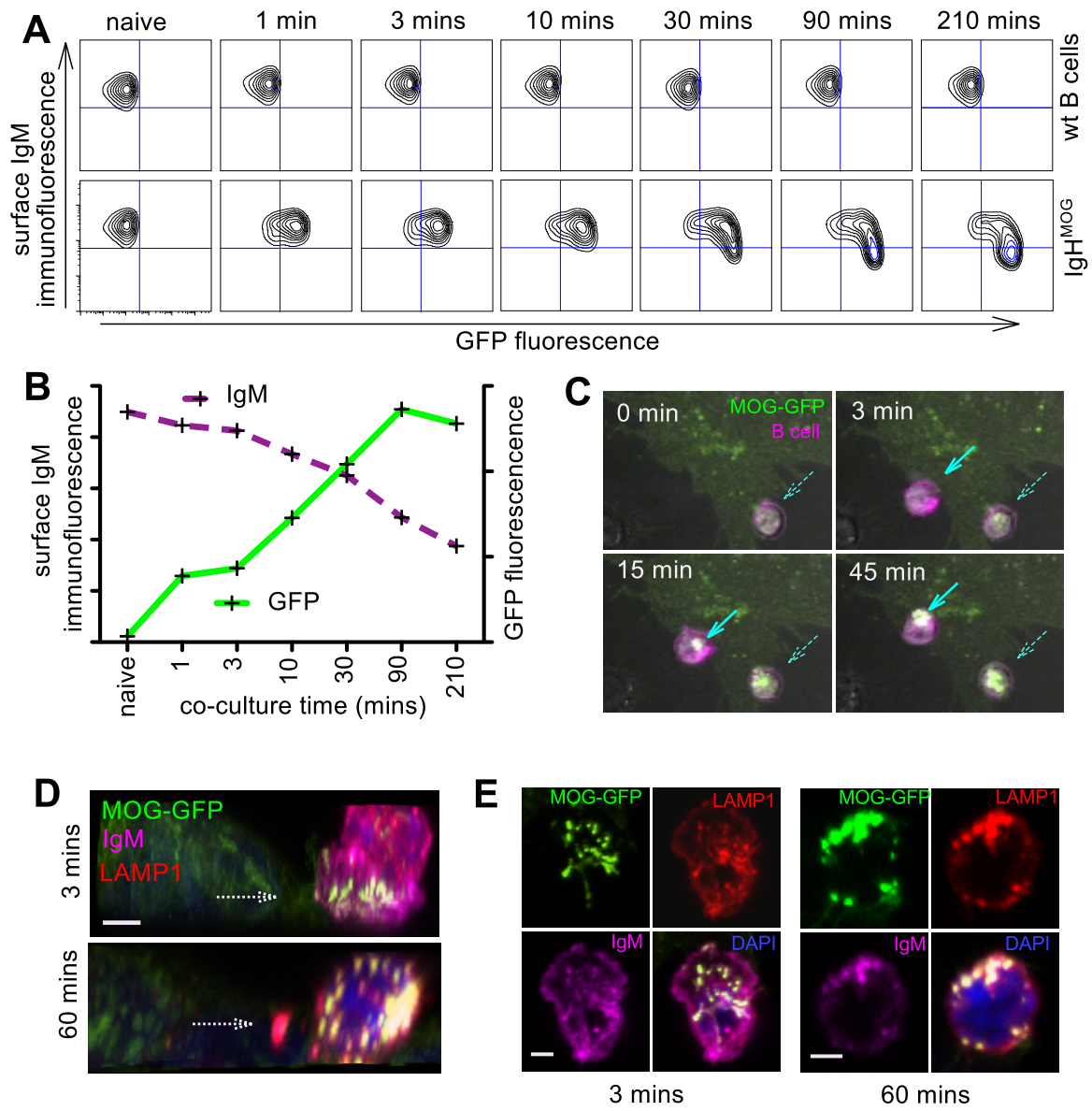


Figure 2

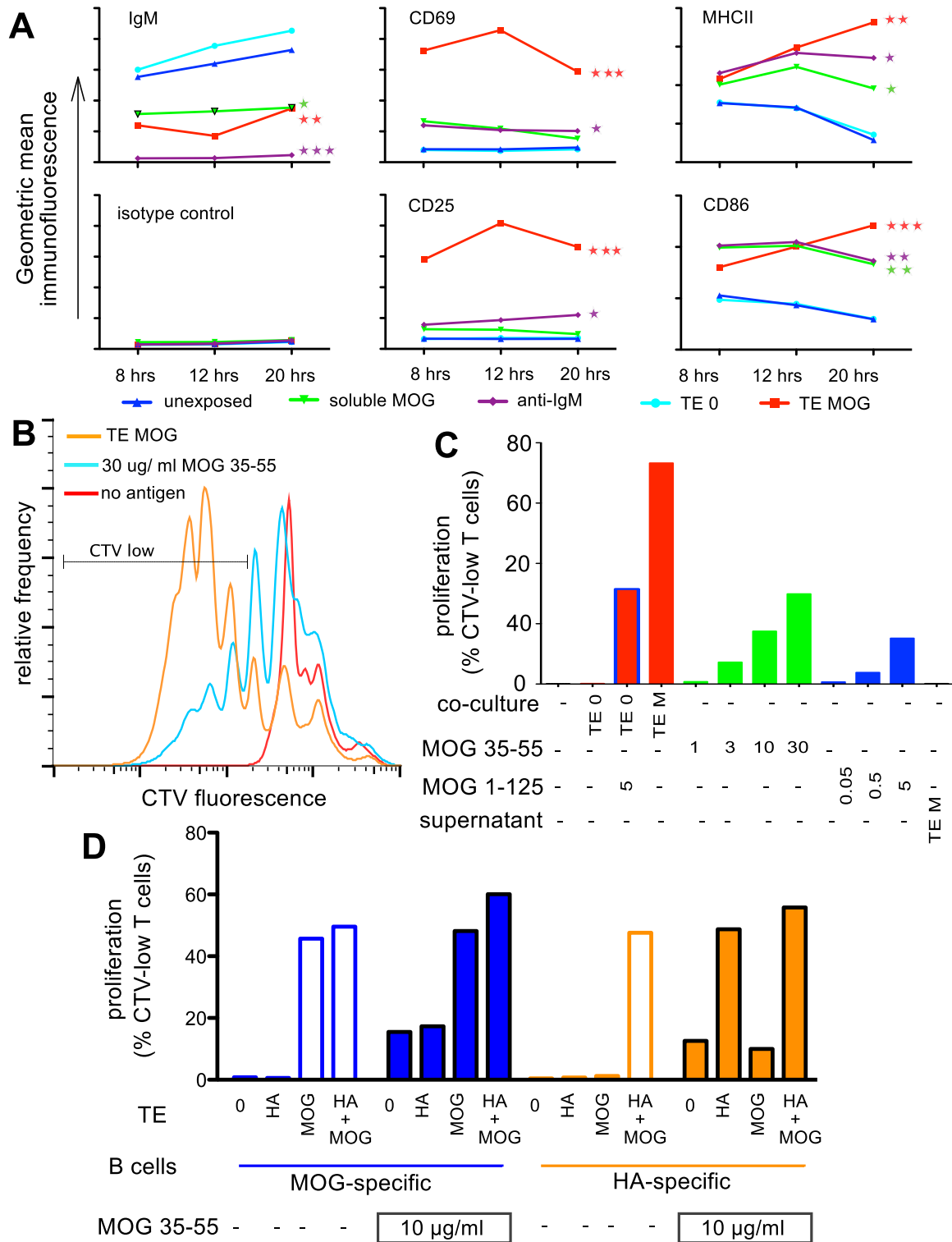


Figure 3

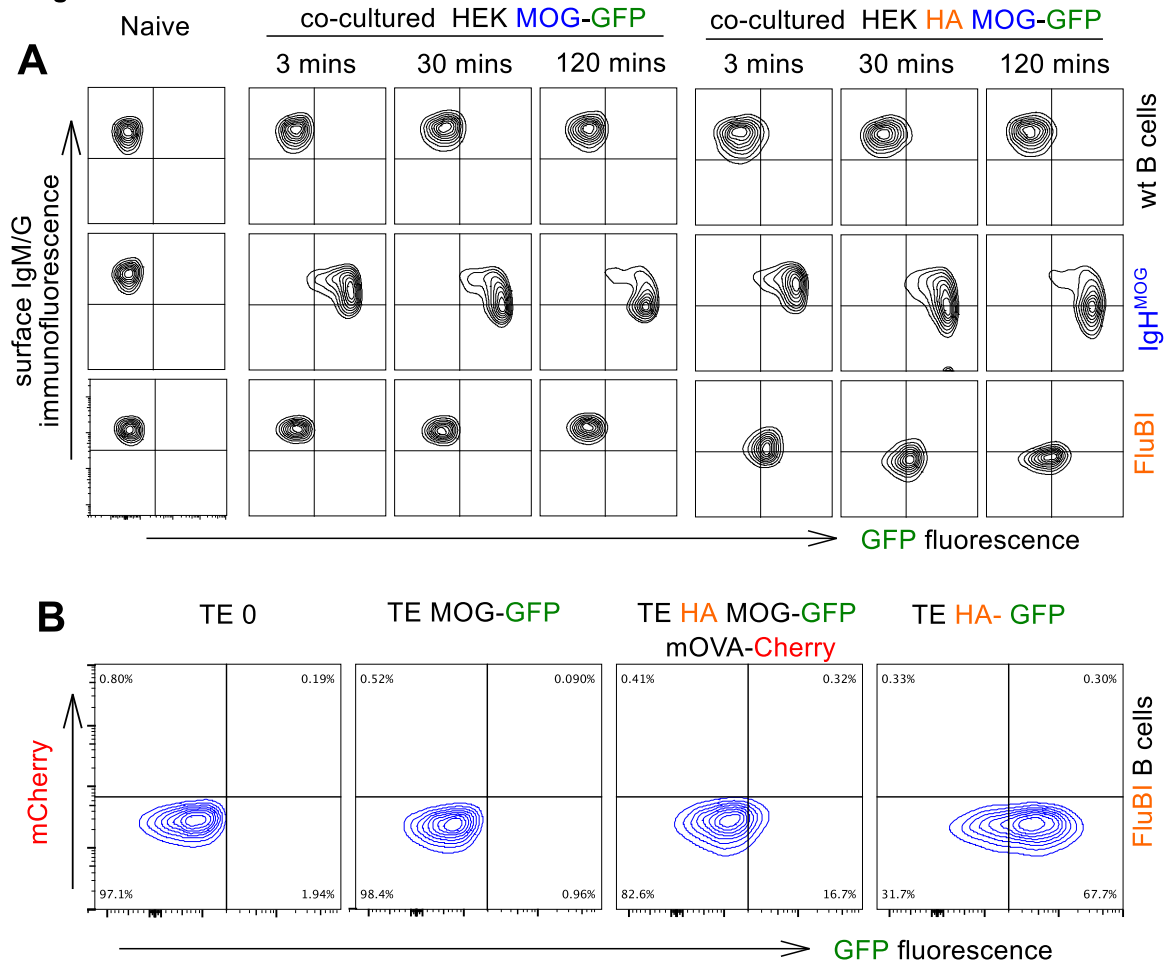
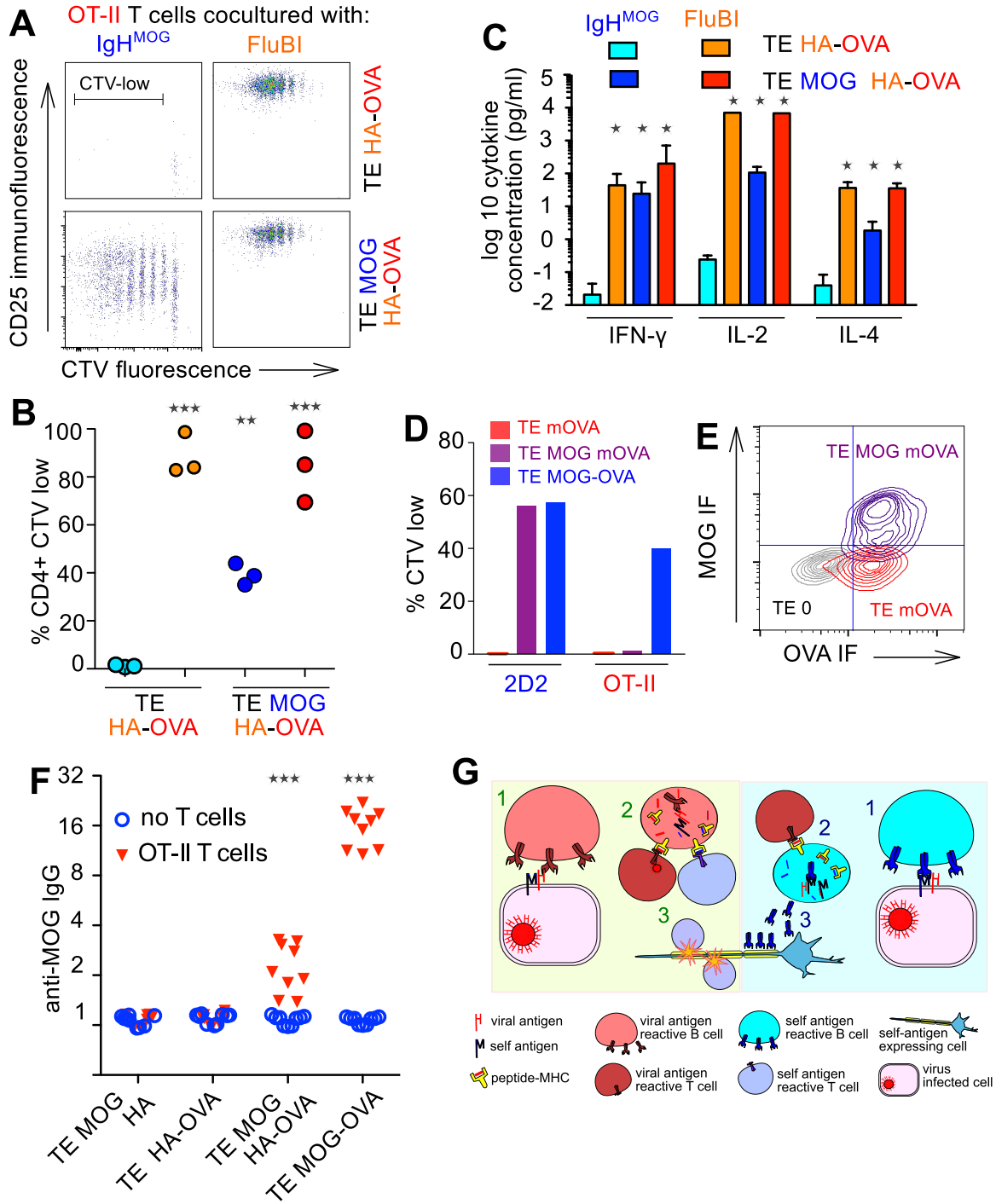


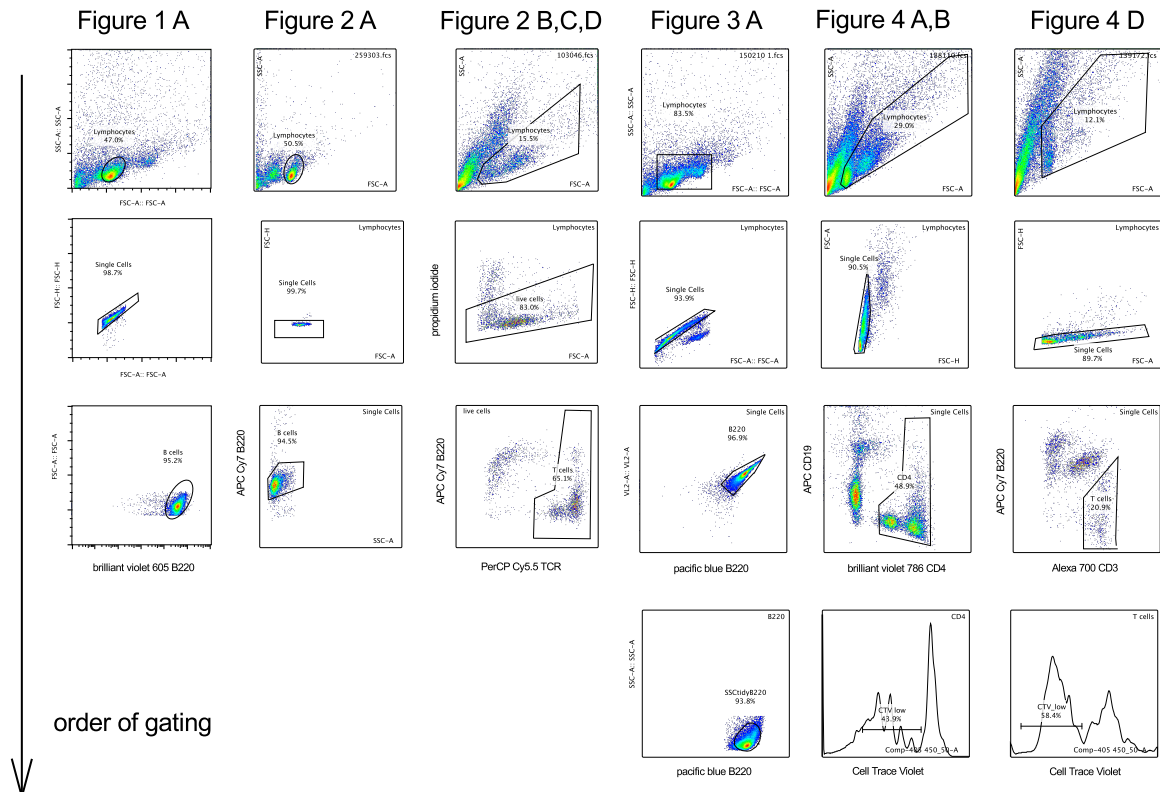
Figure 4



Supplementary Table 1.

cell line	transfected antigen	source	antibody used to verify surface expression
TE 0	no added antigen	ATCC CRL-8805	
TE MOG	MOG, i.e., native Myelin Oligodendrocyte glycoprotein from <i>Rattus rattus</i>	NM_022668.2 PMID: 10428054	8.18C5
TE HA	HA, i.e., native hemagglutinin from influenza A/WSN/33	ACF54598.1	FluBI mouse serum
HEK	no added antigen	ATCC CRL-1573	
HEK MOG-GFP	MOG aa 1-204 fused to GFP		8.18C5
HEK HA MOG-GFP	HA		FluBI mouse serum
	MOG aa 1-204 fused to GFP		8.18C5
TE MOG-GFP	MOG aa 1-204 fused to GFP		8.18C5
TE MOG HA	MOG		8.18C5
	HA		FluBI mouse serum
TE mOVA	mOVA - a fusion protein comprising the signal peptide from H-2 Kb, the full sequence of chicken ovalbumin (OVA), and the transmembrane domain of H-2 Db, which results in extracellular membrane expression.	pODpCAGGS PMID: 14525595	AB1225
TE MOG mOVA	MOG		8.18C5
	mOVA		AB1225
TE MOG-OVA	MOG aa 1-204 fused to full length chicken ovalbumin		8.18C5
TE HA-OVA	HA-OVA i.e., A/WSN/33 HA fused to amino acids 312 to 345 of chicken ovalbumin (this includes the epitope recognized by OT-II T cells)		FluBI mouse serum
TE MOG HA-OVA	MOG		8.18C5
	HA-OVA		FluBI mouse serum

Supplementary Figure 1.



Outlook

In the first part of this study we developed a sensitive method for identifying antigen-specific B cells in humans. The long-term goal of this study is to identify autoantigen-specific B cells in patients and elucidate their role in the pathology of autoimmune diseases. One potential problem could be that the population of antigen-specific B cells may be too small to be detected over background. However, the technique does not need to be perfectly specific. Even an enrichment of 100 or 1000 fold would provide a useful starting point for the application of other techniques like the transformation with EBV for the generation of single B cell clones. For this purpose we have generated a cell line expressing GFP-tagged MOG and we will attempt to isolate and immortalize B cells from patients with serum antibodies against MOG. Ideally this technique will enable us to recombinantly express these autoreactive antibodies to test their pathogenicity in animal models.

In the second part of the presented work we tested a novel hypothesis linking B cells, viral infection and autoimmunity. We showed that B cells capture not only cognate antigens but simultaneously co-capture "bystander" antigens and present both to T cells. The next step is to examine this question with human B cells and also in parallel in an animal system *in vivo*. We want to characterize the ability of B cells to acquire membrane-expressed antigen in different tissues *in vivo*. The future goal is to determine whether the phenomenon of bystander antigen co-capture can lead to T cell activation and antibody secretion *in vivo* as it does *in vitro*. A possible, physiologically realistic paradigm would be that B cells capture antigen *in vivo*, after induction of viral antigen expression using adeno-associated viral vector encoding both cognate and non-cognate antigen.

Part II

**GNbAC1, a humanized monoclonal antibody
against the multiple sclerosis associated retrovirus envelope protein**

Abbreviations

CNS	central nervous system
CSF	cerebrospinal fluid
EDSS	expanded disability status scale
ELISpot	enzyme-linked immunospot
Env	envelope protein
HERV	human endogenous retrovirus
HERV-W	human endogenous retrovirus type W family
IFN- γ	interferon-gamma
IgG4	immunoglobulin G4
IL-1 β	interleukin-1 beta
IL-6	interleukin-6
LPS	lipopolysaccharide
mAb	monoclonal antibody
MBP	myelin basic protein
MS	multiple sclerosis
MSRV	multiple sclerosis associated retrovirus
MSRV-Env	multiple sclerosis associated retrovirus envelope protein
OPCs	oligodendroglial precursor cells
PBMC	peripheral blood mononuclear cells
TLR4	Toll-like receptor 4
TNF- α	tumor necrosis factor alpha

Summary

We reported the results of immunological monitoring of patients enrolled in a randomized, placebo-controlled, single-blind, phase 2a study testing two doses of GNBAC1 in patients with multiple sclerosis (MS). GNBAC1 is a recombinant, humanised, IgG4 monoclonal antibody directed against the envelope protein (Env) of the multiple sclerosis associated retrovirus (MSRV), a human endogenous retrovirus of the HERV-W family. The hypothesis that binding of MSRV-Env protein to Toll-like receptor 4 (TLR4) activates monocytes predicts that blocking this binding with GNBAC1 should decrease monocyte activation in patients with MS.

Ten MS Patients with a mean age of 53 years and a mean expanded disability status scale (EDSS) score of 4.8 were randomized into two groups (2 or 6 milligrams of GNBAC1 per kilogram of body weight given at 4 week intervals for 12 months). In addition to safety and clinical monitoring, three immunological parameters were followed: (i) T cell response to myelin and viral antigens by ELISpot using peripheral blood mononuclear cells (PBMC); (ii) Monocyte TLR4 signaling measured by flow cytometry of p38 phosphorylation; and (iii) general immune status as reflected in proportions of lymphocytes with given phenotypes.

In the immunological substudy (Results I of Part II of the presented work) ten patients and yoked healthy volunteers were followed. T cell response, as measured by interferon-gamma (IFN- γ) ELISpot, to viral antigens was robust throughout the study for both healthy subjects (range 22 to 322 spots per 200,000 PBMC) and patients (range 30 to 534 spots per 200,000 PBMC). Response to the myelin antigen Myelin Basic Protein (MBP) was not different from negative control in any subject at any time point. TLR4 signaling was analyzed before treatment start and at several timepoints after treatment by measuring phosphorylated p38 using intracellular immunofluorescence flow cytometry of CD33-positive cells, with and without lipopolysaccharide (LPS) stimulation. Without stimulation, fewer than 10% of monocytes showed p38 phosphorylation in patients and in controls at all time points, other than one time-point for one patient, in whom elevated C-reactive protein was also detected, suggesting an inflammatory infection. LPS stimulation (1 nanogram per milliliter in whole blood) resulted in almost 100% monocyte p38 phosphorylation in both patients and controls. There was a non-significant trend towards a reduced monocyte activation during treatment compared to before treatment. Immune cell subsets (CD4+ and CD8+ T cells, monocytes, and B cells) were stable over the treatment.

Part II Summary

These results suggest that GNBAC1 administration is not associated with any deficit in T cell response to viral antigens. TLR4 activation might be reduced by the treatment. However, this result did not reach statistical significance probably due to the small number of investigated patients.

Introduction

Human endogenous retroviruses (HERV) represent approximately 8% of the human genome. Researchers assume that during primate evolution, a viral infection led to the integration of retroviral genetic material into the germline of superior primates that was subsequently passed on to their offspring [1,2]. The first reported observation of retrovirus-like particles with reverse transcriptase activity was made in leptomeningeal and macrophage cell cultures from patients with multiple sclerosis (MS), suggesting a viral origin of this disease [3]. Further molecular characterization of this multiple sclerosis associated retrovirus (MSRV) revealed that it was not a classical infectious exogenous retrovirus but expressed by genes from the human endogenous retrovirus type W family (HERV-W) [4]. Most of the contemporary HERV copies are inactivated by mutations, deletions, or silenced by epigenetic modifications, and therefore unable to encode functional proteins [5,6]. However, several chromosomal HERV copies have retained potential open reading frames possibly derived from viral genes [7-9]. Exogenous viral agents, such as Herpesviridae, appear to be able to trigger the reactivation of MSRV transcription and therefore might be associated with the epidemiology of MS without showing a causative role in the disease [10,11]. These observations suggest that the reactivation of silent MSRV might be a possible link between environment and the onset of MS [12].

Following the initial report of isolation of MSRV envelope protein (MSRV-Env) from cerebral tissues of MS patients, various studies have proposed links between the expression of MSRV-Env and MS. It has been reported that the presence of MSRV in cerebrospinal fluid (CSF) correlates with clinical progression and prognosis of MS [13]. In addition, immunohistochemical analyses of post-mortem brain tissue from MS patients have shown that the MSRV-Env is expressed in MS plaques and its level of expression correlates with the plaque activity [14,15].

The MSRV-Env consists of 3 domains: the signal peptide, the transmembrane domain and the surface domain, which has been reported to be an agonist of the Toll-like receptor 4 (TLR4) [16]. Through this activation of the TLR4 pathway, it is proposed that MSRV-Env induces the release of pro-inflammatory cytokines like IL-1 β , IL-6 or TNF- α from mononuclear cells of the peripheral blood and microglial cells from the central nervous system (CNS) [16,17]. Another effect of MSRV-Env mediated by TLR4 interaction is the blockade of oligodendrocyte differentiation, which is necessary for the remyelination process. Oligodendroglial precursor cells (OPCs) are present in the adult brain and can replace lost oligodendrocytes and remyelinate demyelinated axons and are therefore essential for the regeneration of the CNS [18]. This blockade of OPC

maturation by env could be influenced *in vitro* by an antibody targeted against env [19]. Both the pro-inflammatory effect and the direct toxicity on OPCs point to MSR-Env as a relevant therapeutic target for MS.

Based on these considerations, a recombinant humanised monoclonal antibody (mAb) of the IgG4/kappa class was designed to target the MSR-Env [20]. This mAb, GNBAC1, selectively binds with high affinity to the surface domain of the MSR-Env and neutralizes its TLR4 binding potential without interacting with this receptor [17,20]. As a consequence, GNBAC1 is expected to neutralize the effects of MSR-Env in MS plaques and on circulating monocytes, and suppress its inflammatory and neurodegenerative potential [21]. In contrast to most of the monoclonal antibodies developed for the treatment of MS, which have been designed to interact with molecules and cells involved in the immune response [22], GNBAC1 targets a potential key factor in the disease without the need to modulate or suppress the immune system [23]. GNBAC1 is currently being developed as a potential first line therapy for MS, with a particular application for progressive forms of the disease [24]. The first-in-man clinical study with single doses up to 6 milligrams per kilogram of body weight was administered intravenously in 33 young healthy volunteers [20]. In a subsequent study, GNBAC1 was tested in 10 MS patients in a randomized placebo controlled trial (Results I of part II of the presented work) with a one year open label extension [25,26].

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Aim of the Thesis

We aimed to monitor in a phase 2a study the systemic immune response of patients with MS under treatment with GNBAC1, a monoclonal antibody targeting the envelope protein of the multiple sclerosis associated retrovirus. We analyzed the composition of immune cell subsets and the activation level of monocytes by flow cytometry and the response against viral and vaccine antigens by ELISpot.

Results

I. Immunologic monitoring during a phase 2a trial of the GNBAC1 antibody in patients with MS.

Immunologic monitoring during a phase 2a trial of the GNBAC1 antibody in patients with MS

OPEN

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ABSTRACT

Objective: To monitor the systemic immune responses of patients with multiple sclerosis (MS) under treatment with GNBAC1, a monoclonal antibody against the envelope protein of the MS-associated retrovirus, during a phase 2a trial.

Methods: We analyzed the composition of immune cell subsets and the activation level of monocytes by flow cytometry and the response against viral and vaccine antigens by ELISpot.

Results: None of the endpoints measured revealed any immunosuppressive effect of the therapeutic antibody. Activation of monocytes slightly decreased during treatment as predicted by the hypothesized mechanism of action of GNBAC1.

Conclusion: These results support the conclusion that the antibody is safe for use in patients with MS.

Classification of evidence: This study provides Class III evidence that in patients with MS GNBAC1 does not significantly affect several biomarkers of systemic immune response. *Neural Immunol Neuroinflamm* 2015;2:e144; doi: 10.1212/NXI.000000000000144

GLOSSARY

CPT = cell preparation tube; **env** = envelope protein of MSRV; **IFN** = interferon; **LPS** = lipopolysaccharide; **MAPK** = mitogen-activated protein kinase; **MBP** = myelin basic protein; **MS** = multiple sclerosis; **MSRV** = MS-associated retrovirus; **TLR4** = toll-like receptor 4.

Both genetic and environmental factors have been discussed in the pathogenesis of multiple sclerosis (MS). Endogenous retroviruses fall between genetics and environment, having been integrated into the human genome long ago and now being inherited like other genes. Several studies indicate that an endogenous retrovirus, the MS-associated retrovirus (MSRV), might be connected to MS pathogenesis. MSRV has been isolated from brain tissue of patients with MS.¹ Subsequently, different studies found increased viral copy numbers and/or transcripts in blood, CSF, or brains of patients with MS compared with controls.²⁻⁴ Detection of MSRV was also correlated with disease severity and disability progression.^{5,6} MSRV might influence the pathogenesis of MS in 2 different ways: by targeting immune cells and by interacting with oligodendrocytes. It has been shown that the envelope protein of MSRV (env) can act as a ligand of toll-like receptor 4 (TLR4) and can activate cells expressing this receptor, such as monocytes, leading to an aberrant upregulation of immune reactivity, thus contributing to autoimmune pathology.⁷ It has also been suggested that env might directly compromise oligodendrocytes, possibly also via interaction with TLR4.^{8,9} It was therefore speculated that blocking the interaction between env and immune cells and/or oligodendrocytes might be a novel therapeutic option in MS. Recently, results were reported from a phase 2a trial of GNBAC1, a humanized monoclonal IgG4/κ antibody against the

Supplemental data
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extracellular domain of env.¹⁰ This trial showed a favorable safety and pharmacokinetic profile of the therapeutic antibody and showed that MSRV transcripts declined during GNBAC1 treatment, indicating a pharmacodynamic effect. The immunologic substudy reported here examined blood samples from patients enrolled in the previously published phase 2a trial to assess the impact of the therapeutic antibody on the immune system. Additional healthy volunteers were recruited for comparison.

METHODS Patients and design. Blood samples were taken from the 10 patients involved in the phase 2a trial (5 from Basel, 5 from Geneva) and from 5 healthy volunteers recruited additionally for this immunologic substudy. Demographic details are provided in the table. Patients received monthly infusions of 2 or 6 mg/kg body weight over a period of 6 months. Blood was drawn on the day before the first administration of GNBAC1, on the first day after administration, 2 weeks after administration, and at the end of the 6-month treatment period. Two patients received placebo for the first time point. Blood from healthy controls was drawn at each time point on the same day as the patients and processed in parallel. A schematic of the study design is shown in figure e-1 at Neurology.org/nn.

Standard protocol approvals, registrations, and patient consents. The study protocol was approved by the ethics committees of both centers and the Swiss Medicine Agency, Swissmedic. All patients enrolled in this study gave written informed consent.

Assays. Blood taken from patients in Basel was processed immediately. In Geneva, blood was drawn into cell preparation tubes (CPTs), transported to Basel by courier, and processed the same day. The primary research question was: How does the state of the immune system change following treatment with GNBAC1? This was addressed in 3 assays (Class III level of evidence): (1) proportions of various immune cell populations by flow cytometry to monitor the general state of the immune system; (2) interferon (IFN)- γ production in response

to various antigens, using the Ready-SET-go human IFN- γ ELISpot kit (eBioscience, San Diego, CA) to measure physiologic immune reactivity; and (3) p38 mitogen-activated protein kinase (MAPK) phosphorylation by phospho-specific flow cytometry (clone 36/p38, BD Biosciences, San Jose, CA) as a measure of TLR4 stimulation in monocytes. Immune cell subpopulations and ELISpot results are not reported from the Geneva patients, whose peripheral blood mononuclear cells after retrieval from CPTs contained variable levels of erythrocytes, granulocytes, and other cell types, making comparisons unreliable. For the p38 MAPK phosphorylation, this problem was obviated by restricting flow cytometry to CD33-positive monocytes, and results are reported for all 10 patients and 4 controls.

RESULTS Using flow cytometry, we determined the frequencies of the main subfractions of lymphocytes during the trial. We could not detect a difference in the proportions of CD4⁺ and CD8⁺ T cells, B cells, and monocytes between patients and controls. There was also no change in the composition of lymphocytes during 6 months of treatment with GNBAC1 (figure 1A).

We monitored antigen-specific responses by IFN- γ ELISpot. We detected robust immune responses against varicella-zoster virus and tetanus vaccine (Tetanol) throughout the study. The responses were highly variable between individuals and across time but not obviously different between patients and controls (figure 1B). The presence of robust antivaccine and antiviral responses and the absence of antigen-independent IFN- γ production before and after treatment suggest that there was no adverse activation or suppression of the protective immune response. We also tested the T cell response against an established autoantigen in MS, myelin basic protein (MBP). The response against MBP was low in patients and healthy controls and did not significantly change over time (data not shown).

To monitor TLR4 activation in monocytes, we determined the phosphorylation of p38, a key component of the signaling pathway downstream of TLR4, using a phospho-specific anti-p38 MAPK antibody in flow cytometry. We were able to detect the effect of lipopolysaccharide (LPS) down to 100 pg/mL (figure 1D), a level of sensitivity comparable to the gold standard Limulus amoebocyte lysate endotoxin test. Primarily, we examined phosphorylation of p38 in naive monocytes (without exogenous LPS) before and during treatment. The pattern we observed was consistent with the hypothesis underlying the proposed mechanism of action of GNBAC1, i.e., before the trial, p38 phosphorylation was higher in patients with MS than in healthy controls, and treatment reduced the p38 phosphorylation in patients (figure 1C). However, this did not reach statistical significance.

Table Patient and control demographic characteristics

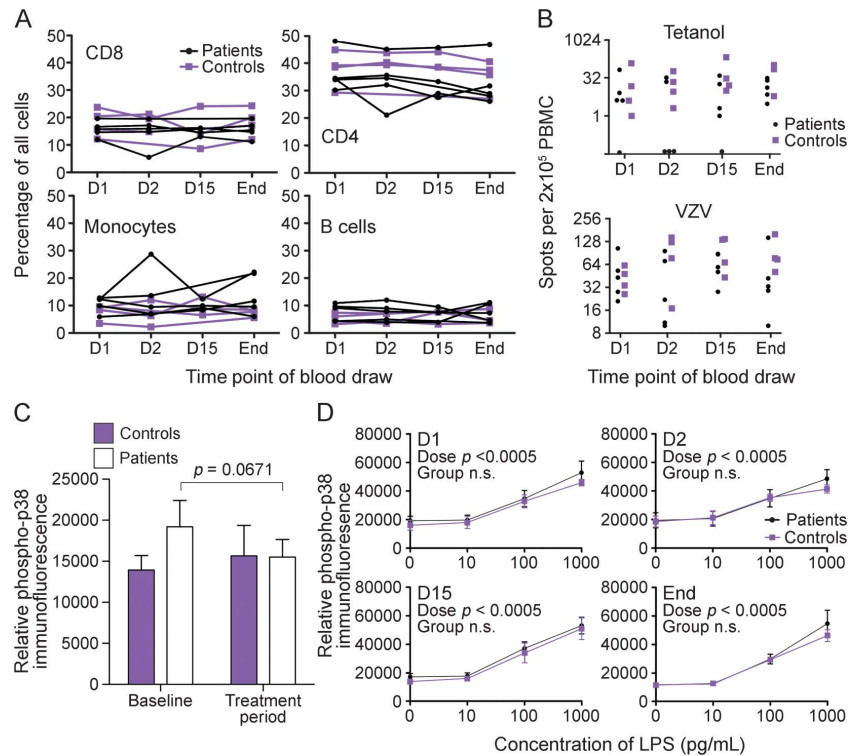
Characteristic	Patients (N = 10)	Controls (N = 5)
Age, y, mean \pm SD	53.4 \pm 6.6	37.6 \pm 8.2
Female/male	3/7	2/3
Diagnosis, n (%)		
RRMS	1 (10.0)	NA
PPMS	3 (30.0)	NA
SPMS	6 (60.0)	NA
EDSS, mean \pm SD	4.8 \pm 1.5	NA

Abbreviations: EDSS = Expanded Disability Status Scale; NA = not applicable; PPMS = primary progressive multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis.

T1

F1

Figure 1 Comparison of immune phenotype, antigen reactivity, and monocyte activation in blood of patients and controls



(A) Cellular composition of peripheral blood mononuclear cells (PBMC) from 5 patients and 4 controls over time (immediately before first infusion: D1, the following day: D2, 2 weeks later: D15, and the conclusion of the study: End). Percentages of CD8⁺ T cells, CD4⁺ T cells, CD33⁺ monocytes, and CD19⁺ B cells in patients (black circles and lines) and controls (purple squares and purple lines) at the 4 time points. Results are only reported from Basel patients. Missing data points are indicated by omitted symbols. (B) Interferon (IFN)- γ response to a vaccine (Tetanol) and a viral antigen (varicella-zoster virus [VZV], Varilrix) throughout the trial in 5 patients and 4 controls. Each condition was assayed in triplicate wells with PBMC exposed to the stimulus using the human IFN- γ ELISpot Ready-SET-Go! (eBioscience, San Diego, CA), and this figure displays the medians from these triplicates at each time point for each of 5 patients and 4 controls. Results are only reported from Basel patients. Missing data points are indicated by omitted symbols. (C) Phosphorylation of p38 mitogen-activated protein kinase (MAPK) in monocytes from 10 patients before and during treatment and from 5 healthy controls at the same time points. Cryopreserved PBMC were permeabilized in methanol and then immunolabeled with anti-CD33 antibodies (eBioscience) to identify monocytes and anti-phospho-p38 MAPK antibodies (BD Biosciences, San Jose, CA) to measure phosphorylation. Vertical axis shows relative phospho-p38 immunofluorescence (arbitrary units). When values from more than 1 time point were available before or during the treatment period, these were averaged, producing 1 before-treatment and 1 during-treatment value for each patient. Time points at which patients were given placebo were included in the pretreatment condition. Error bars show SEM. Averaging for controls followed the patients to whom they were yoked, and the data were analyzed by repeated-measures 2-way analysis of variance comparing effects of group (i.e., patients vs controls) and time point (i.e., before vs during treatment). Neither the main effects nor the interaction were significant. Since the parameter of most interest was the comparison between before and during treatment within the patients, these data were also compared using a paired 2-tailed t test ($p = 0.0671$). (D) Phosphorylation of p38 MAPK in monocytes of 10 patients and 5 controls across the trial in response to lipopolysaccharide (LPS). Vertical axis is the median immunofluorescence in arbitrary units. Purple lines and squares represent healthy untreated controls. Black lines and circles represent patients. Each of the 4 panels shows results from 1 time point from blood exposed to a range of concentrations of LPS, as indicated. The effect of dose was analyzed by linear regression and found to be significant ($p < 0.0005$ for both patients and controls at each time point). This effect of dose (i.e., the slope of the dose-response regression lines) was compared between patients and controls and between time points, but neither the main effects nor the interaction were significant.

We also tracked p38 phosphorylation across the trial in naive monocytes and in response to various doses of LPS and observed that the response to this exogenous TLR4 ligand was similar between patients and controls and stable across the trial (figure 1D).

DISCUSSION These results are consistent with the previously published report of the phase 2a trial in that no evidence was obtained that GNBAC1 administration interferes with the normal functioning of the immune system. This apparent evidence of safety should be interpreted in light of the small size and limited duration of the trial. Flow cytometry of phospho-p38 in monocytes has potential as a biomarker for monitoring response to treatment with this antibody or any other agent targeting the TLR signaling system. Larger studies will be required to corroborate these findings and elucidate the relationship between monocyte activation and clinical response.

AUTHOR CONTRIBUTIONS

Study concept and design: M.Z., N.S.R.S., M.R., P.H.L., R.L.P.L., T.D. Acquisition of data: M.Z., N.S.R.S., M.R., P.H.L. Analysis and interpretation: M.Z., N.S.R.S., M.R., A.B.L., F.C., T.D. Critical revision of the manuscript for important intellectual content: M.Z., N.S.R.S., M.R., P.H.L., A.B.L., F.C., R.L.P.L., L.K., T.D. Study supervision: P.H.L., R.L.P.L., L.K., T.D. Statistical analysis: N.S.R.S.

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DISCLOSURE

M. Zimmerman reports no disclosures. N.S.R. Sanderson received research support from Swiss National Science Foundation and Swiss Multiple Sclerosis Society. M. Rasenack reports no disclosures. P.H. Lalive is on the scientific advisory board for Biogen-Idec and Novartis and received travel funding and/or speaker honoraria from Biogen-Idec, Teva, and Merck Serono. A.B. Lang is employed by GeNeuro. F. Curtin is employed by GeNeuro. R.L.P. Lindberg received research support from Swiss Multiple Sclerosis Society, Swiss National Science Foundation, and Roche Postdoctoral Fellowship Program. L. Kappos is on the editorial board for *Multiple Sclerosis Journal*, *Multiple Sclerosis and Related Disorders*, and *Journal of Neurology* and received research support from Actelion, Addex, Almirall, Bayer, Biogen-Idec, Biotica, CSL Behring, Genetech, GeNeuro, Genzyme, Merck Serono, Mitsubishi, Novartis, Octapharma, Ono Pharma, Pfizer, Receptos, Roche, Sanofi-Aventis, Santhera, Siemens, Teva, UCB, Xenoport, Swiss National Research Foundation, European Union, Swiss MS Society, and Novartis and Roche Research Foundations. T. Derfuss is on the scientific advisory board for Biogen-Idec, Novartis Pharma, Genzyme, Merck Serono, Bayer Schering, Octapharma, GeNeuro, and Roche; received travel funding and speaker honoraria from Bayer Schering, Biogen-Idec, Merck Serono, Novartis Pharma, and Genzyme; is on the editorial board of PLoS One;

is a member of steering committees by Mitsubishi Pharma and GeNeuro; is on the executive board of ECTRIMS; received research support from Novartis Pharma, Merck Serono, Biogen-Idec, Swiss National Foundation, and Swiss MS Society; and his spouse is employed by Novartis Pharma. Go to Neurology.org/nn for full disclosure forms.

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Outlook

Preclinical and early clinical data support the conclusion that GNbAC1, a recombinant, humanized, IgG4 monoclonal antibody directed against the MSRV-Env, presents a favorable safety profile, which is critical for long-term treatments. Recently, GeNeuro SA, the company that developed GNbAC1, announced that it has initiated its planned phase 2b study with GNbAC1 in relapsing-remitting MS (clinicaltrials.gov identifier NCT02782858). The study plans to recruit 260 patients from 68 clinical centers in the European Union and Eastern Europe and expects preliminary results by the end of 2017. Considering our results monitoring of the phosphorylation of p38 in monocytes might be a possible biomarker that reflects the mode of action of GNbAC1.

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