



Research paper

An optimized assay for the enumeration of antigen-specific memory B cells in different compartments of the human body

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ARTICLE INFO

Article history:

Received 21 December 2009

Received in revised form 9 March 2010

Accepted 11 March 2010

Available online 17 March 2010

Keywords:

Memory B cells
Tetanus toxoid
Cytokines
Immunoglobulin
Lymphoid tissue

ABSTRACT

Object: In the framework of our current study we set out to develop an optimized assay for the quantification of antigen-specific B cells in different compartments of the human body.

Methods: Mononuclear cells (MNC) derived from the peripheral blood, bone marrow (BM), or human tonsils were incubated with different combinations of stimuli. The stimulated cells and culture supernatants were then applied to IgG-ELISPOT and ELISA read-out assays and tetanus toxoid (TT)-specific B cell responses were quantified.

Results: We found that a combination of CD40L, CpG, and IL21 was optimal for the induction of TT-specific IgG-producing cells from memory B cell (mBc) precursors. This cocktail of stimuli led to a proliferation-dependent induction of CD19^{intermediate}CD38^{high}CD138^{high}IgD^{negative} terminally differentiated plasma cells. Applying our optimized methodology we were also able to quantify mBc specific for cytomegalovirus and influenza virus A. Most importantly, the same method proved useful for the comparison of mBc frequencies between different compartments of the body and, accordingly, we were able to demonstrate that TT-specific mBc preferably reside within tonsillar tissue.

Conclusion: Here, we optimized an assay for the quantification of antigen-specific B cells in different human tissues demonstrating, for example, that TT-specific mBc preferably reside in human tonsils but not in the BM or the peripheral blood. We suggest that our approach can be used for the enumeration of mBc specific for a wide variety of Ag (microbial, tumor-related, auto-antigens), which will lead to significant improvements regarding our knowledge about the biology of humoral immunity.

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1. Introduction

Humoral memory to an antigen (Ag) is critical for controlling infections and maintaining long-term antibody

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(Ab)-mediated immunity (Ahmed and Gray, 1996; McHeyzer-Williams and Ahmed, 1999). Memory B cells (mBc) have been suggested to persist for a lifetime and to maintain the plasma cell pool producing serum antibody levels over prolonged periods of time (Bernasconi et al., 2002). Persisting antigen, certain cytokines, or toll-like receptor signals may drive mBc to differentiate into long-lived plasma cells which are the source of serum antibodies. On the other hand, a reduced function of mBc may lead to impaired antigen-specific-immunity (Wasserstrom et al., 2008). Accordingly,

human mBc play a decisive role in mediating long-term protection against a wide variety of infectious diseases.

Persistence of mBc is well documented and mBc have been suggested to reside at the sites of Ag drainage such as the marginal zones of human lymph nodes or the bone marrow (BM). Unfortunately, there is little information regarding differences in the location of certain mBc subsets as characterized by their respective target antigen. Studies have indicated that the number of mBc specific for certain antigens found in the peripheral blood often do not correlate with long-term serum antibody titers against the respective target (Hofer et al., 2006; Lanzavecchia et al., 2006; Sasaki et al., 2007). This suggests that the peripheral blood (PB) does not represent the body compartment where most of the immune globulin (Ig)-secreting cells (ISC) are located. However, it is still not clear for most Ag where else mBc specific for the respective target preferably reside and where they produce clinically relevant antibodies. This seems particularly surprising since such results would have an immediate impact on the understanding of fundamental immunological mechanisms as well as on the development and optimization of vaccination strategies, i.e. against microbial pathogens. Most likely, methodological problems in the enumeration of mBc reactive with specific Ag are the reason for our lack of knowledge regarding the differential accumulation of these cells within certain body compartments.

In our current study, we aimed at developing an improved method for the enumeration of antigen-specific mBc in various compartments of the human body. To this end, we used tetanus toxoid (TT), a potent immunogen that induces persisting and protective humoral immunity, as a model antigen. In a second step, we evaluated different human tissues for the presence of antigen-specific mBc. We further correlated the antigen-specific IgG subsets produced *in vitro* by mBc from respective tissues with those in the PB to find out which body compartments these peripheral antibodies stem from.

2. Materials and methods

2.1. Patients and healthy donors

Tonsils and PB were obtained from adult patients undergoing tonsillectomy. BM and PB samples were obtained from healthy stem cell donors. Healthy subjects and patients gave informed consent in accordance with the revised version of the Declaration of Helsinki. The study protocol was approved by the local ethics committee (decision number OB-038/06).

2.2. Purification of B cells from different tissues

Tonsillar tissue was chopped into small pieces and the resulting cell suspension was washed in culture medium. Peripheral blood mononuclear cells (PBMC), BM mononuclear cells (BMMC) and mononuclear cells (MNC) from tonsils were prepared by density centrifugation on Ficoll-Hypaque. Untouched B cells and non-B cells were separated using the B Cell Isolation kit II (Miltenyi Biotech, Cologne, Germany) containing a cocktail of avidin-conjugated antibodies against

a variety of non-B cells and biotin-conjugated microscopic magnetic beads.

2.3. Memory B cell assay

Briefly, 6×10^6 MNC or 3×10^5 PB-, BM- or tonsil-derived B cells mixed with 5.7×10^6 non-B cells separated from autologous PBMC were re-suspended in 2 ml RPMI 1640 medium (Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA), penicillin, streptomycin, and glutamine. Cells were seeded into 6-well plates and were incubated with combinations of 5 ng/ml polyclonal mitogens pokeweed mitogen extract (PWM; Sigma-Aldrich, St Louis, MO), 1:5000 *Staphylococcus aureus* Cowan (SAC; Sigma-Aldrich), 6 µg/ml 24-mer DNA oligonucleotide containing a phosphorothioated optimized human CpG motif (CpG 2006: 5'-TCGTCGTTTGTGCGTTTGTGCGTT-3'; DNA Technology, Risskov, Denmark), 500 ng/ml human CD40 Ligand (CD40L; GenWay Biotech, San Diego, CA), 50 ng/ml Interleukin (IL)-21 (PeproTech, Hamburg, Germany), 100 U/ml IL-2 (Roche, Mannheim, Germany), and 20 ng/ml IL-10 (ImmunoTools, Friesoythe, Germany). Cells were cultured for 3–6 days at 37 °C with 5% CO₂.

2.4. Enumeration of antibody-secreting cells by ELISPOT

To enumerate antigen-specific immune globulin-secreting mBc, an Enzyme-Linked Immunospot Technique (ELISPOT) was used. Briefly, flat-bottomed 96-well nitrocellulose plates (Multi Screen-HA; Millipore, Bedford, USA) were coated overnight at 4 °C with 30 µl of the following Ags or monoclonal antibodies: 30 LF/ml tetanus toxoid (TT; Chiron Behring, Marburg, Germany), 2 µg/ml Glutathione-S-Transferase (GST; Abnova, Taipei, Taiwan), 4 µg/ml influenza (Flu) A virus nucleoprotein (NP; Biomol, Hamburg, Germany), 4 µg/ml Cytomegalovirus (CMV) pp28 protein (GenScript, Piscataway, USA), 2 µg/ml goat anti-human IgG-Fab antibody (Bethyl Laboratories, Montgomery, USA) in coating buffer made of carbonate-bicarbonate buffer (Sigma-Aldrich). Free protein-binding sites were blocked by incubation with 3% Albumin bovine (BSA; Biomol) at 37 °C for 2 h. PBMC (2.5×10^5 /well) were incubated in RPMI 1640 (Gibco, Paisley, United Kingdom) at 37 °C for 20 h. Plates were then washed thoroughly with water containing 0.05% Tween 20 (Sigma-Aldrich), followed by incubation with 1 µg/ml Alkaline phosphatase (ALP)-conjugated goat anti-human IgG antibody (Mabtech, Stockholm, Sweden) for 2 h at 37 °C. After washing, substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich) was added and plates were developed at room temperature for 15 min. Spots were counted using an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany).

2.5. Enzyme-linked Immunosorbent Assay (ELISA)

Antigen-specific IgA, IgG and IgM antibody responses were determined in plasma and culture supernatants by ELISA as described previously (Atanackovic et al., 2007). Briefly, half-area, 96-well microtiter plates (Corning Costar, Lowell, MA) were coated overnight with 25 µl/well of TT (5.7 LF/ml) or GST (1 µg/ml) or goat anti-human IgG-Fab (1 µg/ml; Bethyl Laboratories Inc., Montgomery, TX). Plates

were washed twice with PBS containing 0.05% Tween 20 and once with PBS. ELISA plates were then blocked with PBS containing 5% non-fat dry milk for 2 h at room temperature. Plasma or supernatants from cell cultures were added to the plates at 30 μ l/well and were incubated at room temperature for 2 h. For the detection of antigen-specific antibody responses, alkaline phosphatase (AP)-conjugated goat anti-human IgG, IgM, IgA (Southernbiotech, Birmingham, AL), goat anti-human pan IgG and IgG1, 2, 3, 4 antibodies, (Southernbiotech) diluted 1/3000 in block buffer were added at 30 μ l/well; and were incubated for 1 h at room temperature. After washing, plates were developed with substrate p-nitrophenyl phosphate (PNPP; Southernbiotech) at room temperature for 30 min. Plates were evaluated using an ELISA reader (Tecan, Männedorf, Switzerland) at a wavelength of 405 nm with a reference wavelength of 620 nm.

2.6. CFSE labelling and flow cytometry

Purified B cells or MNCs were labelled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) and were cultured with CD40L, CpG and IL21 for 3–6 days. *In vitro*-activated cells were harvested and non-specific binding sites were blocked by pre-incubating with normal mouse IgG. Cells were then incubated at 4 °C for 20 min with fluorochrome-conjugated anti-CD19, -CD27, -CD38, -CD138, -IgD monoclonal antibody (mAb). After washing, cells were analyzed on FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using CellQuest software (BD Biosciences).

2.7. Statistical analysis

Since most of the data were not distributed normally, a Mann–Whitney rank sum test was performed to compare values between groups, and Spearman's rank correlation coefficient was calculated to analyze correlations between groups. For all statistical analyses, *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. CD40L, CpG, and IL-21 represent the optimal combination of stimuli to induce TT-specific IgG-secreting B cells

Due to their relatively low frequencies, it is necessary to use polyclonal stimuli to amplify Ag-specific mBc before enumerating them in a read-out assay. In a first step, we investigated which combination of stimuli would be optimal for an efficient *in vitro* induction of IgG-secreting B cells (ISC) from PBMC. To this end, we stimulated whole PBMC from healthy donors separately with PWM, CpG 2006, IL-2, IL-10, IL-21, CD40L, and SAC. After 6 days of *in vitro* incubation with different stimuli, numbers of TT-specific ISC were determined in an ELISPOT read-out assay. In addition, TT-specific antibody produced during the *in vitro* culture was evaluated in an ELISA. We found that PWM represented the most potent inducer of TT-specific ISC from precursor B cells compared with other stimuli when used as single agents (Fig. 1a). CpG 2006 and IL-21 as single agents were also able to amplify significant numbers of ISC from TT-specific mBc, however,

with lower efficiency. In contrast, no TT-specific ISC were induced by IL-2, IL-10, CD40L, and SAC when used as single agents (Fig. 1a). Importantly, these results were corroborated by the ELISA, showing that IL-21, CpG, and PWM led to elevated levels of TT-specific IgG produced by ISC during the *in vitro* stimulation (Fig. 1b).

Next, we aimed at determining the most effective combination of stimuli for the induction of TT-specific ISC from mBc precursors. Incubating PBMC with combinations of 2–5 stimuli for 6 days, we observed that the combination of CD40L, CpG 2006 and IL-21 could most efficiently induce ISC from TT-specific mBc (Fig. 1c), but not IgA or IgM secreting B cells (data not shown). Although PWM, when used as a single stimulus, had shown the strongest capacity for the induction of TT-specific ISC, the addition of PWM to a combination of stimuli did not further increase the yield of TT-specific ISC (Fig. 1c). These findings were largely corroborated by observations made in the ELISA analysis of culture supernatants after *in vitro* stimulation of whole PBMC (Fig. 1d).

Previously, of all cytokines IL-2 and IL-10 have most commonly been used in memory B cell assays because IL-10 can induce the differentiation of memory B cells into ISC and IL-2 enhances the response of CD40L-stimulated B cells (Fluckiger et al., 1993; Zinkernagel et al., 1996; Tangye et al., 2003a,b). However, stimulation of a combination of CD40L/CpG 2006, with cytokine IL-21, which was identified as a very potent stimulant in our current study, led to higher numbers of TT-specific ISC compared to a combination with IL-2 or IL-10 (Fig. 1e, f). Furthermore, the addition of IL-10 and/or IL-2 to our cocktail of stimulants consisting of CD40L/CpG 2006/IL-21 did not further increase Ig secretion (data not shown).

3.2. Antibody-secreting B cells with specificities other than TT can be induced using a CD40L/CpG/IL-21 cocktail of stimuli

Having established an optimized assay for the enumeration of antigen-specific mBc, we next aimed at evaluating whether the same assay was also suitable for the analysis of B cell responses against Ag other than TT. Therefore, we applied our method to viral Ag such as CMV pp28 and influenza virus A nucleoprotein. Stimulating PBMC from healthy donors with the aforementioned combination of CD40L, CpG 2006 and IL-21 for 6 days, we clearly detected ISC specific for both antigens by ELISPOT (Fig. 2a). Corresponding IgG antibodies secreted during *in vitro* stimulation were observed in the ELISA (data not shown).

3.3. Memory B cells proliferate and differentiate into ISC upon stimulation with CD40L/CpG 2006/IL-21

It has long been known that polyclonal stimulation is necessary to induce antigen-specific ISC from memory B cells present among PBMC. However, it is unclear by which mechanisms exactly such stimuli amplify B cell signals. We used CFSE staining in order to monitor the proliferation of different B cell upon unspecific stimulation. We found that after three days, unspecific stimulation had induced division of CD19⁺ B cells. In addition, the number of CD138⁺ plasma cells presented in the culture had significantly increased (Fig. 3a). After 6 days, CD138⁺ plasma cells had all divided multiple times while the proportion of cells that had undergone cell division was

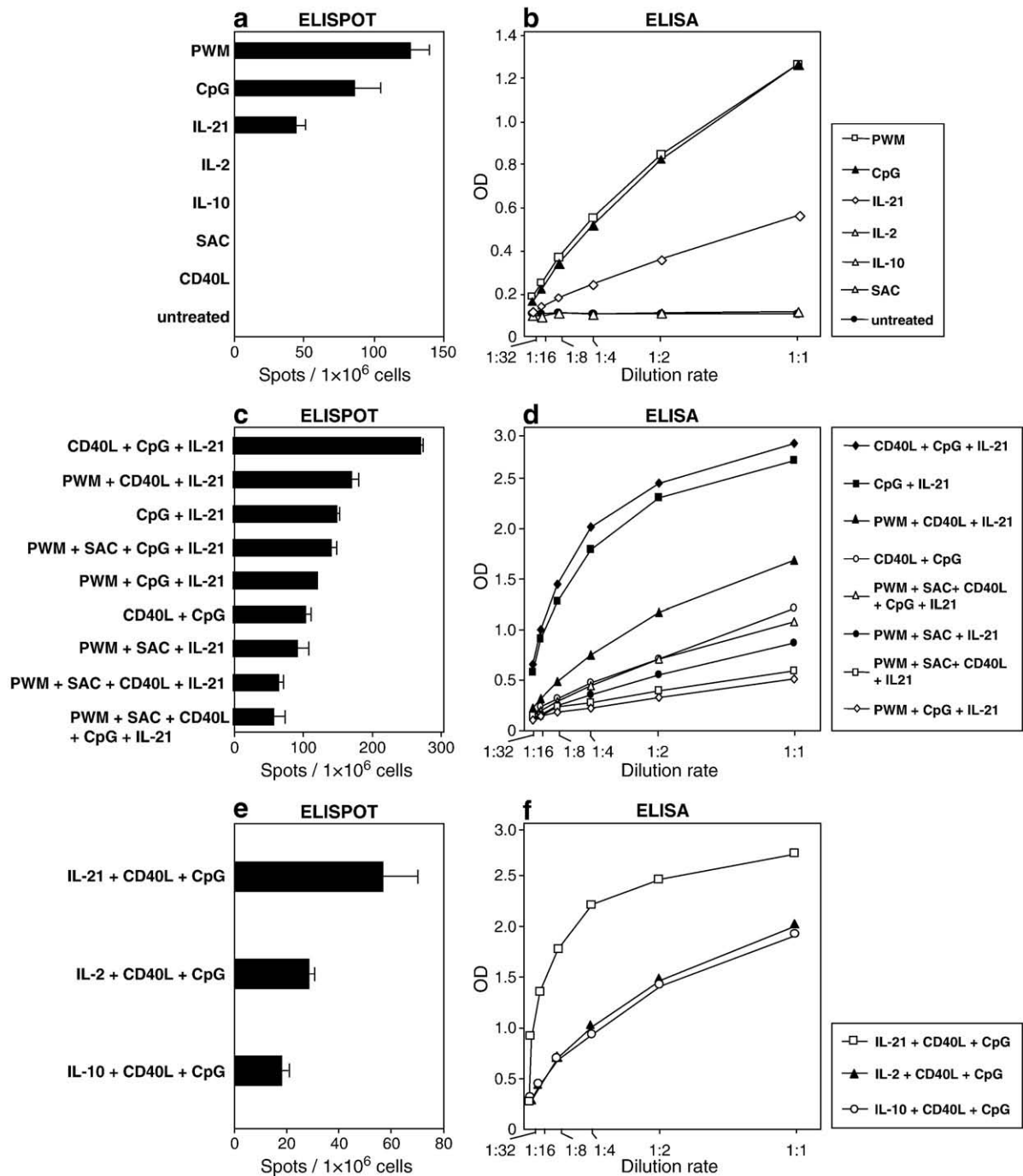


Fig. 1. A cocktail of CD40L, CpG and IL-21 represents the ideal stimulus for the amplification of antibody-secreting B cells from TT-specific mBc. (a, b) PBMC were stimulated with single agents PWM, CpG, IL-21, CD40L, and SAC for 6 days. (c, d) Alternatively, PBMC were stimulated with combinations of 2–5 stimulants for 6 days. (e, f) Finally, PBMC were stimulated with CD40L, CpG, combined with either IL-2, IL-10 or IL-21 for 6 days. TT-specific mBc precursors were detected by ELISPOT (left column) and ELISA (right column). Bars represent the mean ($\pm$ SEM) number of TT-specific ISC per 1×10^6 cells for the ELISPOT and ODs of TT-specific antibody responses as measured in an ELISA.

much lower among CD19⁺ conventional B cells. Importantly, other cell types such as T cells and monocytes had not undergone any cellular proliferation (Fig. 3a).

Further dissecting the B cell-restricted differentiation induced by stimuli CD40L/CpG 2006/IL-21, we found that

after 6 days of stimulation, B cells could be divided into two distinct groups, CD19^{high} and CD19^{intermediate} cells. Most of the CD19^{high} B cells were negative for CD27 which is a marker for memory B cells. These CD19^{high} B cells had proliferated less frequently and showed almost no differentiation into

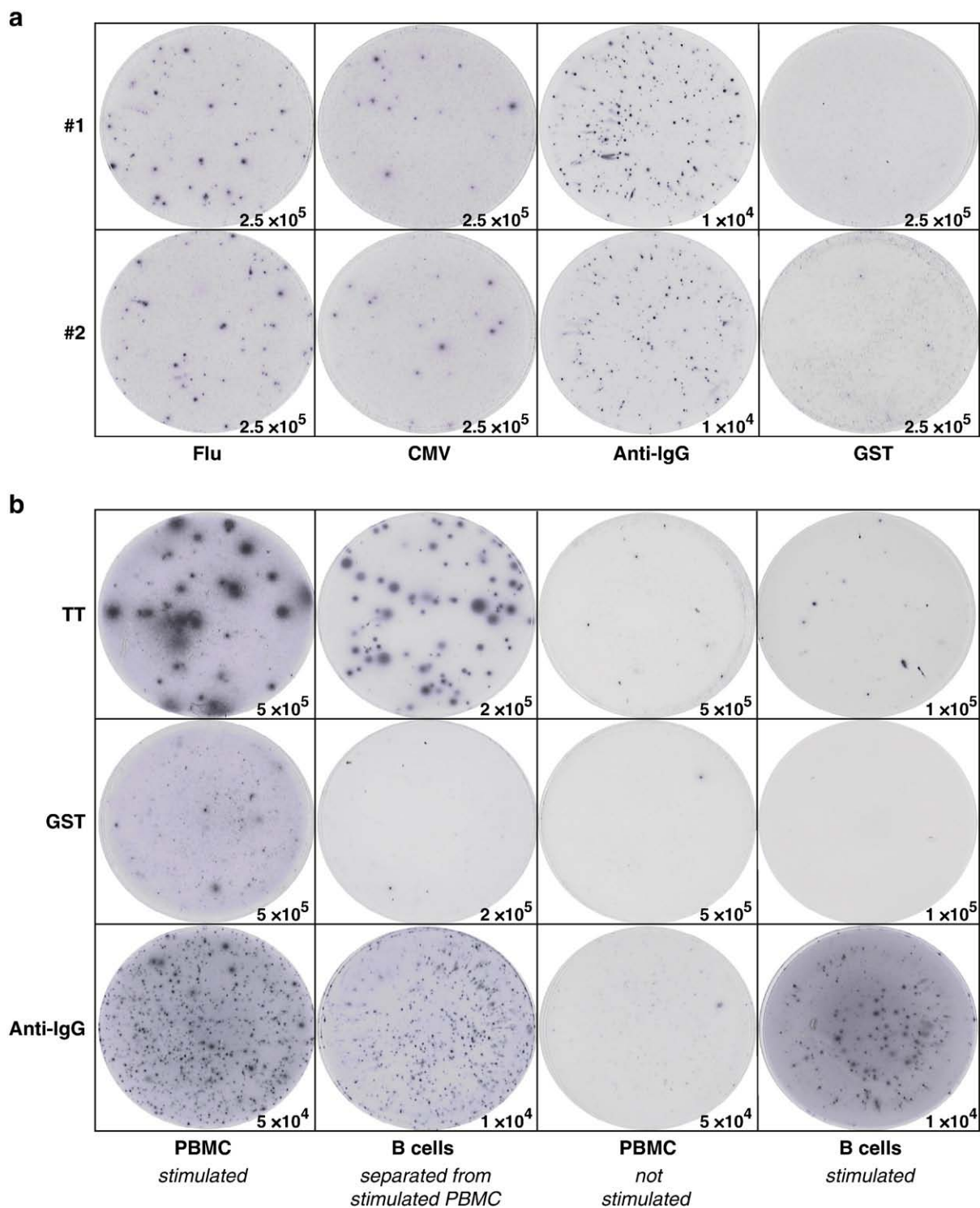


Fig. 2. Antigen-specific responses determined by ELISPOT 6 days after polyclonal stimulation. (a) Based on the analysis of TT-specific B cells, we next determined the number of influenza NP- and CMV-specific B cells among human PBMC. Experiments were performed in duplicate wells (#1 and #2). (b) For the analysis of TT-specific responses, PBMCs were cultured without stimulation or were stimulated with CD40L/CpG 2006/IL-21. After 6 days, cells were collected and applied to an ELISPOT assay. ELISPOT plates were coated with TT and anti-human IgG antibody to enumerate antigen-specific ISC and total IgG-secreting B cells, respectively, GST protein was used as negative control. In some experiments, B cells were isolated before or after polyclonal stimulation. Total B cells were also isolated before stimulation and cultured with CD40L, CpG, and IL-21 for 6 days following with ELISPOT assay.

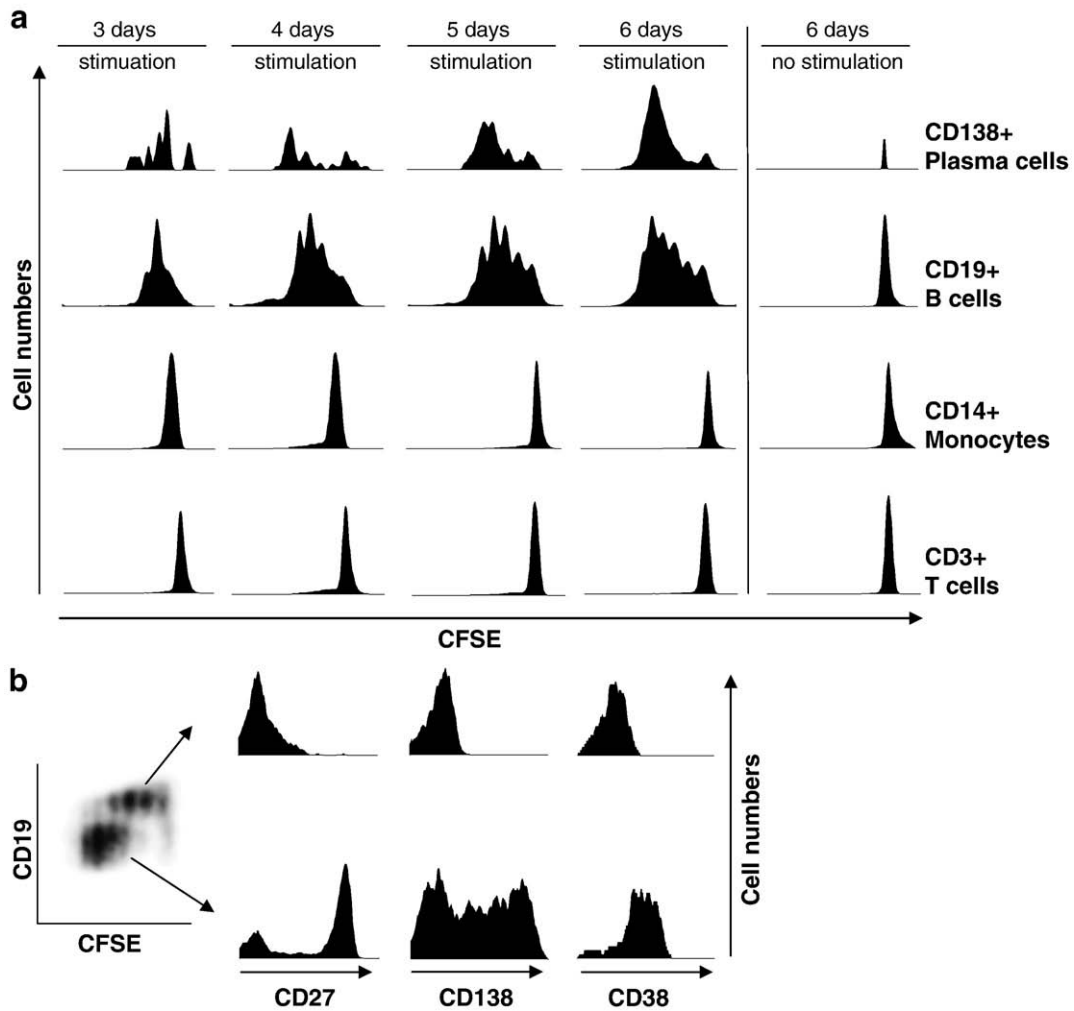


Fig. 3. Selective proliferations of human memory B lymphocyte subsets in response to CD40L, CpG 2006, and IL-21. (a) CFSE-labelled PBMCs were cultured with or without CD40L, CpG 2006, and IL-21 in human B cell medium. After 3, 4, 5 and 6 days, cells were collected. The proliferation of total B cells, plasma cells, monocytes and T cells was determined. (b) Differentiation of memory B cells to plasma cells. CFSE-labelled memory B cells were stimulated for 6 days with CD40L, CpG 2006, and IL-21, the expression of CD19, CD27, CD38 and CD138 were measured as a function of cell activation and differentiation. The cell division was evaluated in variant B cell subgroups.

CD38⁺ or CD138⁺ plasma cells (Fig. 3b). In contrast, the population of CD19^{intermediate} B cells evidenced strong expression of the memory B cell marker CD27, had undergone several cycles of division and had developed into CD38⁺ CD138⁺ terminally differentiated plasma cells. Overall, these combined data suggested that the amplification of the ISC signal caused by our mixture of stimuli was most likely based on the induction of a B cell-restricted proliferation and differentiation.

3.4. IgG antibody responses observed in an ELISPOT assay are generated by TT-specific memory B cells but depend on help from non-B cells

To further confirm that TT-specific IgG responses observed after stimulation of whole PBMC were indeed generated by mBc but not by other cell types present in the culture, we

isolated B cells from the culture after 6 days stimulation. When we applied these cells to our standard ELISPOT assay, we observed further amplified TT-specific IgG responses without any background signal against control protein GST (Fig. 2b). These results demonstrated that stimulated memory B cells were indeed the source of the TT-specific responses observed in our *in vitro* assays.

We also isolated B cells before stimulating them with CD40L, CpG 2006 and IL-21 for 6 days. However, applying these stimulated cells to our ELISPOT read-out assay, we observed that both numbers of detectable TT-specific B cells as well as total numbers of IgG-secreting cells were much lower than those produced by stimulated whole PBMC (Fig. 2b). These observations suggested that B cells present among total PBMC needed the help of some form of non-B cells in order to proliferate and/or fully differentiate into competent antibody-secreting cells upon unspecific

stimulation. As expected, non-B cells themselves could not be transformed into antibody-secreting cells by stimulation (data not shown).

3.5. TT-specific B cells preferably reside in human tonsils compared to the bone marrow and the peripheral blood

We had originally developed our optimized methods for the enumeration of antigen-specific B cells in order to compare numbers of ISC specific for certain Ag between different body compartments. Therefore, in a final step we aimed at evaluating numbers of TT-specific B cells within the PB, the BM, and within tonsillar tissue. To exclude a possible influence of different microenvironments containing different subsets of non-B cells, we standardized the composition of the indispensable “helper” cells by mixing a total of 3×10^5 B cells separated from PBMC, BMMC or tonsil MNCs with 5.7×10^6 B cell-depleted PBMC from the same donor. Consequently, B cells made up approximately 5% of all cells used in the culture, a proportion comparable to that usually seen among PBMC of healthy subjects. This mixture of cells was treated for 6 days with our optimized cocktail of stimuli consisting of CD40L, CpG 2006 and IL-21. Applying the stimulated cells to our ELISPOT read-out assay, we observed slightly higher numbers of TT-specific B cells in the peripheral blood than in the BM of the same individual ($p = 0.23$; Fig. 4a).

However, the frequency of TT-specific B cells was even further increased in tonsillar tissue than among PBMC of the same patients ($p < 0.05$; Fig. 4c). These findings were again corroborated by an ELISA analysis of culture supernatants after *in vitro* stimulation of the B cell/PBMC mixture (Fig. 4b, d). In conclusion, we show here that using our optimized system, TT-specific memory B cells preferably reside in human tonsillar tissue when compared to the BM or the peripheral blood.

In order to further define which body compartments the antibody titers detected in the peripheral blood most likely stem from, we correlated the antigen-specific IgG subsets induced from mBc within respective tissues with the same IgG subsets detected in the PB plasma of the same individual. We found that the pattern of IgG subtypes produced *in vitro* by tonsillar B cells correlated significantly with the one observed in the peripheral blood in 5 out of 6 patients tested (Fig. 5a). In 4 out of 6 donors IgG subtypes produced *in vitro* by peripheral B cells correlated significantly with the IgG subtypes present in the autologous plasma (Fig. 5b). Finally, only half of the 6 donors tested showed a correlation between IgG subtypes produced *in vitro* by BM-derived B cells and the IgG subtypes present in the autologous plasma (Fig. 5c). This finding further supports our view that TT-specific antibodies are primarily produced by mBc present in tissues such as human tonsils.

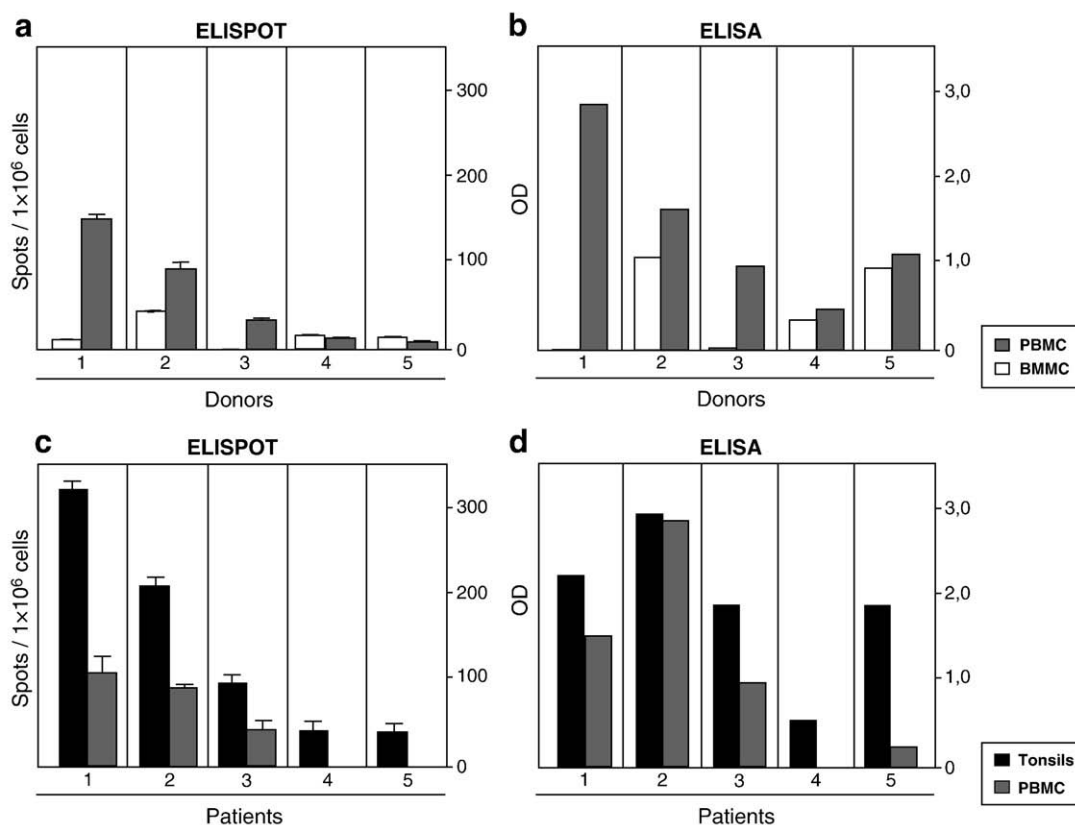


Fig. 4. Higher numbers of TT-specific mBcs in human tonsil than in the PB. 3×10^5 B cells from human PB, BM or tonsils mixed with 5.7×10^6 non-B cells from autologous PBMC were incubated with CD40L, CpG 2006 and IL-21 for 6 days. Cells and supernatants were collected and applied to ELISPOT and ELISA assays, respectively. (a, c) Bar graphs indicate mean numbers of TT-specific B cells derived from tonsils (black), peripheral blood (grey), and bone marrow (white) per total number of 1×10^6 cells. (b, d). Bar graphs indicate TT-specific IgG antibody production by B cells derived from tonsils (black), peripheral blood (grey), and bone marrow (white) in an ELISA assay.

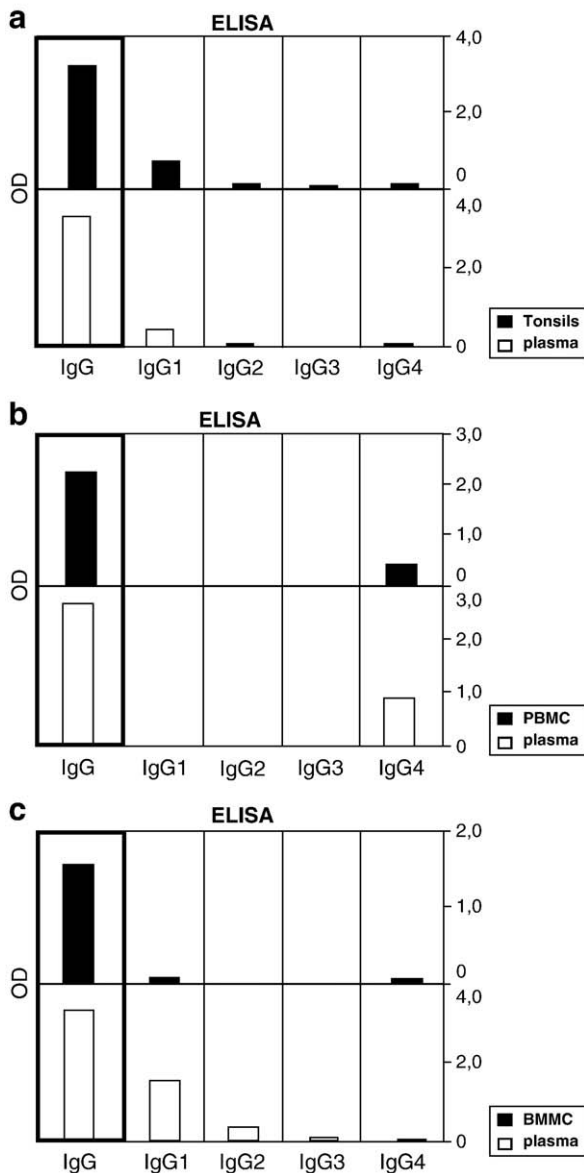


Fig. 5. TT-specific IgG subclass production in culture supernatants of stimulated B cells and plasma samples. Autologous plasma and supernatant from tonsil-, PB- and BM-derived mBc after polyclonal stimulation were analyzed for IgG subclass production using an ELISA assay. Bar graphs present TT-specific IgG1, 2, 3, 4 responses and the TT-specific total IgG responses in the supernatants of polyclonally stimulated B cells derived from tonsillar tissue MNC (a), PBMC (b) and BM (c) from representative individuals. In addition, results for PB plasma of the same subject are shown.

4. Discussion

The enumeration of antigen-specific mBc in human subjects is critical for the analysis of humoral immune responses following vaccination or infection, but has remained technically challenging due to the relatively low frequencies of these cells in the peripheral blood. In the framework of this study, we developed an improved method for the quantification of antigen-specific mBc in samples derived from different body compartments.

An effective amplification of the B cell signal before application to the read-out assay is critical for increasing the sensitivity of any given method for the quantification of Ag-specific mBc. Mitogens such as PWM as well as microbial products have previously been used to amplify mBc signals with varying success (Crotty et al., 2004). Out of all microbial products tested in our current study, we found CpG 2006 to be most effective for the induction of ISC from memory B cells when added to a cocktail of stimuli. CpG 2006 contributed significantly to the proliferation and maturation of mBc, probably by acting through Toll-like receptor 9 (TLR9), which is expressed on human memory B lymphocytes (Hornung et al., 2002; Bernasconi et al., 2003).

The binding of CD40 to CD40L (CD154) expressed on activated T lymphocytes plays a central role in B lymphocyte activation (Banchereau et al., 1994; van Kooten and Banchereau, 2000). Accordingly, T cells activated by a third-party antigen have been used for stimulating B cells in a noncognate fashion via CD40L and cytokine production (Lanzavecchia et al., 1983; Banchereau et al., 1994). Alternatively, CD40L-expressing cell lines (Tangye et al., 2003a,b; Avery et al., 2005; Muehlinghaus et al., 2005; Neron et al., 2005; Amanna and Slifka, 2006; Tuaille et al., 2006), soluble recombinant CD40L or anti-CD40 antibody have been used for B cell stimulation (Ettinger et al., 2005; Larousse et al., 2006; Racanelli et al., 2006; Bryant et al., 2007; Kuchen et al., 2007). We show here that in an assay for the enumeration of Ag-specific mBc, recombinant CD40L represents a critical component for the amplification of mBc signals.

Finally, cytokines such as IL-2, IL-4, and IL-10, that influence B cell development and homeostasis by regulating their proliferation and survival, have also been indicated to be useful for the induction of ISC from mBc (Konforte et al., 2009). We show here for the first time that IL-21 is an important component of any mixture of stimulants used for the induction of antigen-specific ISC from mBc. Importantly, the capacity of IL-21 to induce ISC from mBc greatly exceeded that of other cytokines such as IL-2 and IL-10. The high efficiency of IL-21 in amplifying B cell signals might be related to the fact that it seems to be a surrogate for TLR signalling inducing proliferation and differentiation of human mBc into Ig-secreting plasma cells in the presence of B cell receptor and CD40 signals (Pene et al., 2004; Ettinger et al., 2005; Pene et al., 2006; Ettinger et al., 2008; Jacobi et al., 2008). Most recently, CD40L and IL-21 have been applied to converting peripheral blood memory B cells into highly proliferating, immunoglobulin-secreting B cells (Kwakkenbos et al., 2010). We believe that the introduction of this cytokine into B cell monitoring approaches will significantly facilitate the investigation of antigen-specific humoral immunity.

Previous studies have indicated that an efficient proliferation of human B cells and their subsequent differentiation into antibody-secreting cells require the combination of B cell receptor triggering, cognate T cell help, and TLR stimulation (Ruprecht and Lanzavecchia, 2006). We show here that cellular stimuli in the form of “help” from blood leukocytes are indispensable for the generation of ISC from mBc precursors. In addition to this cellular signal, the most efficient combination of soluble stimuli to induce TT-specific ISC consists of CD40L, CpG 2006 and IL-21. Importantly, we were also able to show that a CD40L/CpG 2006/IL-21-induced proliferation of memory

B cells and their differentiation into antibody-secreting plasma cells are most likely the bases of the amplified ISC signal.

Using our optimized method for the enumeration of Ag-specific B cells we were also able to quantify mBc with specificities other than TT. Thus we could demonstrate significant numbers of mBc specific for viral antigens such as CMV and influenza virus in the peripheral blood of healthy donors. Therefore, we suggest that our methodology is applicable to a wide variety of Ag, probably even Ag inducing specific mBc at a lower frequency, such as tumor antigens.

The most important goal of our current study was to develop a methodology with sufficient sensitivity and specificity to allow for the comparison of antigen-specific B cell numbers between different compartments in the body. Such comparative studies have rarely been performed, probably based on the lack of appropriate methods, and results were contradictory. Accordingly, previous findings have indicated a strong correlation between the frequency of measles- and TT-specific mBc in the peripheral blood and the respective serum antibody levels (Lanzavecchia et al., 2006). In contrast, other groups reported no significant correlation between the frequencies of circulating antigen-specific mBc and the serum titers of antigen-specific IgG (Leyendeckers et al., 1999; Rojas et al., 2008). We show here that TT-specific memory B cells preferably reside in secondary lymphoid organs like tonsillar tissue, and not in the bone marrow or the peripheral blood. We believe that the respective antibody titers, which are detectable in the periphery, primarily stem from this body compartment. It would be of major interest to investigate if the same is also the case for other types of antigens, such as tumor or auto-antigens.

In conclusion, we have developed here an optimized methodology for the enumeration of antigen-specific mBc in different compartments of the human body. We have demonstrated that the combination of CD40L, CpG2006 and IL-21 is optimal for the amplification of the ISC signal causing an expansion and differentiation of mBc. Applying our methodology to samples from healthy donors, we have shown that TT-specific mBc preferably reside in human tonsils but not in the bone marrow or the peripheral blood, and that these B cells are the primary source of TT-specific antibodies. We suggest that our approach can be used for the enumeration of mBc specific for a wide variety of Ag (microbial, tumor-related, auto-antigens) in different human tissues, which will lead to significant improvements regarding our knowledge about the biology of humoral immunity.

Acknowledgements

This work was supported by grants from the Erich and Gertrud Roggenbuck-Stiftung, Eppendorfer Krebs- und Leukämiehilfe, Deutsche José Carreras Leukämie-Stiftung foundation, and from the Cancer Research Institute (to D.A.)

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