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Distinct T helper cell dependence of memory B cell proliferation versus plasma cell differentiation

Franziska Zabel[1], Antonia Fettelschoss[1], Monique Vogel[3], Pal Johansen[2], Thomas M. Kündig[2], and Martin F. Bachmann[1,3]

[1] Department of Dermatology, Zurich University Hospital, Wagistrasse 14, 8952 Schlieren/Zurich, Switzerland

[2] Department of Dermatology, Zurich University Hospital, Gloriastrasse 31, 8091 Zurich, Switzerland

[3] Department of Immunology, Inselspital, University of Bern, Salihaus 2, 3007 Bern, Switzerland

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Address correspondence:

Franziska Zabel, PhD, Dermatology, University Hospital, Wagistrasse 14, 8952 Zurich, Switzerland

Phone: 0041 44 556 32 63

Fax: 0041 44 556 34 44

Mobile: 0041 79 192 01 70

Email: Franziska.zabel@usz.ch

Abstract

Several memory B cell subclasses with distinct functions have been described of which the most effective is the class-switched (CS) memory B cell population. We have previously shown using virus-like particles (VLPs) that the proliferative potential of these CS memory B cells is limited and they fail to re-enter GCs. However, VLP specific memory B cells quickly differentiated into secondary plasma cells (PCs) with a virtue of elevated Ab production compared to primary PCs. Whereas the induction of VLP+ memory B cells was strongly dependent on Th, we were wondering whether re-stimulation of VLP+ memory B cells and their differentiation into secondary PCs would also require Th. Global absence of Th cells lead to strongly impaired memory B cell proliferation and PC differentiation. On the contrary, lack of IL21R-dependent follicular Th cells or CD40L signaling strongly affected proliferation of memory B cells but differentiation into mature secondary PCs exhibiting increased Ab production was essentially normal. This contrasts with primary B cell responses, where a strong dependence on CD40L but limited importance of IL21R was seen. Hence, Th cell dependence differs between primary and secondary B cell responses as well as between memory B cell proliferation and PC differentiation.

Introduction

B cells are activated when antigens such as viral particles bind to and crosslink their B cell receptors (BCRs). After binding, antigen (Ag) is taken up and processed for Ag presentation on MHCII molecules to enable interaction with Th cells[1, 2] which provide the concomitant 2nd signal for integral B cell activation. Th is provided by cell surface molecules such as CD40L[3, 4] as well as secreted cytokines such as IL4, IFNγ or IL21[5, 6]. With the help of these T cell derived co-stimulatory factors, B cells efficiently expand and initiate GC formation[7]. Within GCs, follicular Th cells (Tfh) further aid the B cell response, eventually leading to affinity maturation and the formation of memory B cells and PCs. These events represent the hallmarks of the humoral immune response and provide the means to avoid re-infections for years or even lifelong. In the absence of Th cells, B cells fail to respond to soluble proteins. However, early B cell responses may occur independently of Th upon exposure to antigens exhibiting a repetitive structure, allowing efficient crosslinking of the BCRs[8-10] and/or binding to TLRs[11-13]. Nevertheless, Ab responses induced in the absence of Th are usually more short-lived and dominated by the IgM isotype. Moreover, the induction of B cell memory and long-lived PCs is inefficient. These T cell independent (TI) B cell responses usually occur in the extra-follicular space and can be prolonged by cytokine secretion of the tumor necrosis factor (TNF) superfamily such as BLyS (also called BAFF) or APRIL[14, 15] produced by Th as well as dendritic cells (DC) and macrophages. Of note, while repetitive viral particles are able to induce transient Th independent B cell responses, they induce strong and long-lived Ab responses in the presence of Th. Hence, viral particles are both TI and Th cell dependent (TD) antigens. In the presence of Th, B cells form GCs, where the interplay between B cells, Tfh and FDCs occurs. The H chains of the BCRs undergo

isotype switching; mutations accumulate within the CDRs followed by subsequent selection for best fit for the antigen. Hypermutation and affinity maturation are largely restricted to B cells, as evidence for a similar process happening for TCRs is very limited[16]. The question of how BCR affinity affects the B cell response remains ill defined. Earlier studies suggested the BCR affinity may affect the differentiation of an activated B cell into plasma blasts, GC B cells or memory B cells[17-19]. On the other hand, it has been shown that the affinity of the BCR does not influence the differentiation but rather the expansion and survival of the differentiated B cells[20-22]. Whereas Abs secreted by early PCs mediate protection against primary infection, there is evidence that CS memory B cells are important for mediating protection during secondary infections as they rapidly differentiate into secondary PCs secreting increased levels of Ab upon antigenic re-exposure[23-25]. In contrast to CS memory B cells, IgM^+ memory B cells or naïve B cells are partly recruited to GCs to generate a new memory B cell pool after Ag re-exposure[23, 24, 26]. Although it has been shown that a memory B cell's fate is dictated by the isotype expressed, it has been proposed that CS memory B cells can re-enter GCs and acquire additional mutations within the variable regions of the BCR providing enhanced protection[25, 27]. Recent findings even suggested that memory B cell function is dependent on expression of main surface markers (CD80, CD73, PD-L2) instead of their BCR isotypes[25]. While different functions of memory B cells have been described in recent years, the role of Th during memory B cell recalls requires further investigation.

We used virus-like particles (VLPs) derived from the bacteriophage Qβ as model antigen which induces strong B cell responses due to the particulate and repetitive structure as well as the packaging of RNA as a natural TLR7/8 ligand. It is known that Qβ-VLPs can drive TI IgM responses followed by long-lived TD IgG responses[28-

[30]. Here, we demonstrate that VLP specific memory B cell responses exhibit a hierarchical dependence on Th. Memory B cells were generated in a primary host and adoptively transferred into different secondary hosts globally deficient in Th cells (MHC class II-deficient) or specifically lacking CD40L or IL-21R (as a model for Tfh-deficiency). We observed that proliferation of VLP specific memory B cells showed a strong Th dependence and furthermore required CD40L and IL21R signaling. In contrast, differentiation of Qβ specific memory B cells to PCs and fully mature secondary PCs were only strongly reduced in global absence of Th cells, while CD40L and IL-21R were dispensable to a large degree.

Material & methods

Mice

Wild-type C57BL/6 mice were purchased from Harlan (Horst, The Netherlands). Mice strains such as Ly5.1 (B6.SJL-Ptprc<a> Pepc<b>/BoyJ (#002014)), IgHa (B6.Cg-Gpi1<a> Thy1<a> Igh<a>/J(#001317)), and $MHCII^{-/-}$ (B6.129S2-H2<dlAb1-Ea>/J(#003584) mice were obtained from Jackson Laboratory (USA). We thank Prof. Manfred Kopf for the kind donation of $IL21R^{-/-}$ (B6N.129-Il21r<tm1Kopf>/J (#019115)) and Prof. Annette Oxenius for $CD40L^{-/-}$ mice (B6-CD40L<tm1Renshaw>). All mice were kept under specific pathogen free condition at the BZL mouse facility at the University Hospital (Zurich, Switzerland) and experiments were performed in accordance to ethical principles and guidelines of the Cantonal Veterinary Office, Switzerland.

Antigen

The Qβ bacteriophage derived virus like particles (VLPs) self-assemble and enclose mRNA (ssRNA) during the production process in *E. coli*. They reveal a particulate and repetitive structure rendering them highly immunogenic. The purification process is described elsewhere[31].

Immunization

Memory B cells were generated by immunization with 50 µg of Qβ-VLP intravenously (i.v.). Splenocytes of immunized mice were isolated and adoptively transferred into recipient mice which were challenged with 20 µg Qβ-VLP (i.v.). The VLPs were diluted with sterile PBS and administered intravenously in a volume of 150 µl.

Adoptive transfer of memory B cells

Congenic mice (Ly5.1 or IgH^a) were immunized with Qβ-VLP. After at least 4 weeks, spleens were collected in DMEM media supplemented with 2% FCS, antibiotics and 10 mM HEPES. A single cell suspension of the splenocytes was prepared. Red blood cells (RBC) were lysed using ACK buffer (0.15 M ammonium chloride, 0.01 M potassium hydrogen carbonate, pH 7.2-7.4). Splenocytes were either directly transferred or negatively purified by $CD4^+$ MACS beads and transferred into recipients which were Ly5.2 positive. Purification by $CD4^+$ MACS beads (Miltenyi) was performed according to the manufacture's protocol. Purities of CD4 depleted splenocytes used for adoptive transfers of 98-100% were achieved.

To be able to directly follow the Ab response either derived from transferred memory B cells or from newly activated host B cells, we used IgH^a mice to generate memory B cells and transferred them into C57BL/6 which expressed the IgH^b-allotype. In

general, recipient mice (Ly5.2, IgH^b, CD40L KO, MHCII KO and IL21R KO) received 1/10 of a donor-mouse (Ly5.1 or IgH^a) derived memory spleen. A number of ~ $1x10^7$ cells of total splenocytes containing ~ 0.03-0.08% ($3\text{-}8x10^3$) VLP specific memory B cells were transferred. Control mice received either no splenocytes or 1/10 of a whole spleen of naïve Ly5.1 or naïve IgH^a mice, respectively.

ELISA

Blood was collected at indicated time points. To determine Qβ-VLP-specific IgG Abs, ELISA plates (Nunc Immuno MaxiSorp, Rochester, NY) were coated with Qβ-VLPs (1 µg/ml) overnight. Binding of specific serum antibodies was detected by horse-radish peroxidase-labelled goat anti-mouse IgG (Jackson ImmunoResearch). To identify allotype specific Abs (IgH^a or IgH^b) pairs of IgH^a-specific (biotin ms anti-ms IgG1[a] (10.9), biotin ms anti-ms IgG2a[a] (8.3) from BD) and IgH^b-specific (biotin ms anti-ms IgG1[b] (B68-2), biotin ms anti-ms IgG2a[b] (5.7) from BD) markers were chosen. Ab binding was determined by horse radish peroxidase labeled streptavidin (BD). Absorbance readings at 450 nm of the 1, 2-Phenylenediamine dihydrochloride color reaction were analyzed as OD50.

ELISPOT

Numbers of Qβ-VLP specific PCs in BM were detected as previously described[32]. Briefly, 24-well plates were coated with 10 µg/ml Qβ-VLPs overnight. A single cell suspension of BM cells was seeded in DMEM containing 2% FCS and incubated for 5 h at 37°C. Subsequently, the cells were washed off and Abs produced by specific PCs were detected by binding to goat anti-mouse IgG (EY Labs), followed by alkaline phosphatase-conjugated donkey anti-goat IgG Ab (Jackson

ImmunoResearch). ELISPOTS were identified by the development of an alkaline phosphatase color reaction.

Flow Cytometry (FCM)

Splenocytes were analyzed by FCM analysis. Prior to staining, a single cell suspension and RBC lysis of samples were performed. Fc receptors were generally blocked with an anti-CD16/32 Ab. Isotype switched memory B cells were stained with and identified as PE-Cy7 - $B220^{+}$, negative for PE – IgD, IgM, CD4, CD8, CD11b, CD11c and Gr-1 and positive for binding to the fluorochrome Alexa Fluor 488 labeled Qβ-VLP. In addition, specific plasma cells in spleens were stained and characterized as PE-Cy 7 - $B220^{low}$ and negative for PE – CD4, CD8, CD11b, CD11c and Gr-1. Subsequently, cells were permeabilized using FACS lysing solution (BD) and intracellular binding of labeled Qβ-VLP (Alexa 488) was detected. Prior staining and fixation, surface binding was blocked by unlabeled Qβ-VLP. All B cells derived from the transfer were analyzed for allotype marker (Ly5.1) expression by binding to Ly5.1-APC antibody. VLPs were labeled by the fluorochrome Alexa flour 488 in accordance to the producer's instructions (Invitrogen). The cell acquisition was performed by the FACS Canto II (BD) and data was analyzed by Flow Jo version 7.6.4 (Tree Star software). All Abs were purchased from Becton Dickenson (BD) Biosciences and eBioscience (eBio).

Statistics

Statistical analysis was obtained by the software Graph Pad Prism 6. Statistically significant difference of two groups was calculated by an unpaired t-test. For the

comparison of three groups, the statistical analysis was performed with an F-test (ANOVA). Statistical significance was defined as $p < 0.05$.

Results

Purification of memory splenocytes comprising memory B cells does not alter the responsiveness of memory B cells

We have previously shown that adoptively transferred unpurified splenocytes containing memory B cells from mice primed with Qβ-VLP into WT recipient mice mount a secondary B cell response to VLP upon re-challenge. Memory B cells poorly proliferated but rapidly differentiated into PCs with an increased capacity to produce Abs[23]. To study the role of Th in a naive environment during Ag re-call responses of memory B cells, we first wanted to address whether memory B cell proliferation and differentiation was affected by the presence of memory Th cells in the splenocyte preparations. To this end, 8 weeks old congenic mice ($Ly5.1^+$) were immunized with Qβ-VLP. After 4 weeks, spleens containing memory B cells and memory Th cells were isolated and an adoptive transfer of splenocytes which were either left untreated ("memory transfer") or $CD4^+$ T cell depleted by MACS purification ("CD4-depleted memory transfer") into $Ly5.2^+$ mice was performed (Fig. 1A). Mice were challenged with Qβ-VLP 24 h after the adoptive transfer on day 0. WT mice ($Ly5.2^+$) which received no adoptive transfer ("No transfer") served as a control and mounted a primary response. Detection of VLP specific CS B cells in spleen was assessed by flow cytometry (FCM) analysis within the $B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11b^-$, $CD11c^-$, $Gr1^-$ population (Fig. 1B, Suppl. Fig. 1A). Transferred memory B cells were identified within the specific CS B cell population using the allotype marker Ly5.1.

As observed before[23] upon transfer of memory splenocytes and subsequent challenge, the capacity of specific CS B cells of host (Fig. 1B, Suppl. Fig. 1A) and transferred B cells (Fig. 2A) to proliferate was limited (Fig. 1B, Fig. 2A). No difference between purified and unpurified memory splenocyte transfer could be observed indicating that the co-transferred memory Th cells had no influence on the proliferation of specific B cells. In comparison, $Q\beta^+$ CS B cells of control mice ("No transfer") immunized with Qβ-VLP proliferated more extensively and mounted a strong primary VLP specific B cell response at day 20 after immunization (Suppl. Fig. 1A).

However, transferred memory B cells quickly differentiated into Qβ-VLP specific PCs characterized as $Q\beta^+$ (intracellular), $B220^-$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11b^-$, $CD11c^-$ and $Gr1^-$ in spleen at day 6 after challenge compared to control mice ("No transfer") (Fig. 1C, Suppl. Fig. 1B). Of note, the majority of the Qβ-specific PCs in mice which had received an adoptive memory splenocyte transfer was positive for Ly5.1 and therefore donor derived (Fig. 2B). Compared to control mice ("No transfer") we not only detected an increased number of VLP specific PCs in spleen but also in BM by ELISPOT. The increased PC populations were only found at a very early time point (d6) upon transfer of memory splenocytes containing memory B cells indicating homing of PCs to the BM (Suppl. Fig. 1C). This observation is in contrast to the primary response, in which a significant specific PC population in the BM is usually only detected at later time points. The increased donor derived Qβ-VLP specific PC populations in spleen and BM caused an elevated Ab level detected in serum at early time points (d4 & d6) compared to immunized control mice ("No transfer") (Suppl. Fig. 1D). In comparison, upon transfer of CD4-depleted memory splenocytes containing memory B cells, the differentiation of memory B cells into PCs (Fig. 1 C,

Fig. 2B), the kinetic and magnitude (Suppl. fig. 1B-D) of specific IgG responses were similar as in the presence of memory $CD4^+$ Th cells.

As described before[23], memory B cell derived secondary PCs exhibited an enhanced capacity to produce Abs. In comparison to non-memory derived primary PCs the mean fluorescence intensity (MFI) of intracellular Qβ-VLP binding in secondary PCs in spleen and ELISPOT sizes of secondary PCs in BM was increased, indicating an increased Ab production. Consistent with this previous finding we observed increased capacity for Ab production in secondary PCs derived from both transferred CD4-depleted or unpurified memory splenocytes (Fig. 2C, D) compared to mice, which did not receive an adoptive transfer ("No transfer").

In order to assess the induction of secondary PCs, we quantified the production of host vs. donor derived Abs by using congenic mice expressing allotype a (IgH^a) instead of b (IgH^b) for adoptive transfer (Fig. 1A). As for Ly5.1 congenic mice PCs of the allotype a which had differentiated from transferred memory B cells produced more Abs especially at early time points than PCs which were derived from newly activated host B cells of the allotype b (Fig. 2C, D). Transfer of either untreated (Fig. 2E) or CD4-depleted (Fig. 2F) memory splenocytes containing memory B cells from immunized congenic IgH^a into IgH^b mice could directly show that the majority of Abs was secreted by donor derived PCs.

Altogether our findings showed that induction of secondary PCs was not affected by the absence of $CD4^+$ memory T cells indicating a limited influence of co-transferred memory Th cells on the VLP specific memory B cell response. Hence the purification of memory splenocytes by $CD4^+$ MACS beads treatment did not affect the responsiveness of VLP specific memory B cells. Thus, the transfer of CD4-depleted memory splenocytes containing memory B cells without T cell correlates into

different knock out strains is a suitable means to investigate the role of Th in VLP specific memory B cell responses.

Qβ-VLP-induced memory B cell responses are strongly reduced in the absence of $CD4^+$ T cell help

Whether $CD4^+$ Th cells are required at all for memory B cell responses against VLPs was addressed next. To this end, we performed adoptive transfer experiments of memory B cells into MHC class II-deficient mice which have virtually no $CD4^+$ T cells[33]; therefore no T cell help is provided. In MHCII knock out (KO) B cells still develop normally from BM progenitors and home to the spleen forming B cell follicles.

Congenic ($Ly5.1^+$) mice were immunized with Qβ-VLPs and after 4-6 weeks spleens were isolated. To deplete specific donor Th, splenocytes of immunized mice were negatively purified by $CD4^+$ MACS beads and transferred into WT as well as MHCII KO mice followed by immunization with Qβ-VLPs after 24 h. In order to observe differences of primary vs. memory B cell responses, control WT and MHCII KO mice did not receive an adoptive transfer and were only immunized.

As described above $Q\beta^+$ CS B cells and $Q\beta^+$ PCs in spleen were analyzed by FCM and identified as $Q\beta^+$ lymphocytes (Fig. 1B, C) at representative time points (combined data are shown in figure 3: first experiment analyzed on day 5 and 15; second experiment analyzed on day 5 and day 18).

During the primary response, the Qβ specific CS B cell and PC populations in spleen were strongly reduced upon Qβ-VLP immunization in MHCII KO compared to WT mice (Fig. 3A, B). A few specific PCs were detected on day 5 in the BM by ELISPOT analysis in WT and MHCII KO mice (Fig. 3D). Along with the reduced cellular

response[34], the IgG Ab production in mice lacking the MHCII molecule was predominantly impaired at a late time point (day 15/18) when compared to WT mice (Fig. 3C) . Transferred CD4-depleted memory splenocytes containing memory B cells proliferated in WT mice, whereas the proliferation of memory B cells in MHCII KO mice was strongly impaired (Fig. 3E). The differentiation of transferred memory B cells into PCs found in spleen (Fig. 3F) and BM (Fig. 3H) was also significantly reduced in MHCII KO mice compared to WT mice. The reduced specific B cell response in MHCII KO mice upon adoptive transfer of memory B cells was accompanied by a strongly reduced IgG Ab response on analyzed time points (d5 and day 15/18) (Fig. 3G).

As observed before[28, 29] Qβ-VLP immunization of WT mice lead to a strong cellular activation reflected by rapid and strong CS B cell and PC populations in spleen as well as humoral response during a primary response. When memory B cells were transferred into WT mice, they poorly proliferated, as the number of transfer derived $Q\beta^+$ CS B cells was overall strongly reduced (Fig. 3E, F) compared to the $Q\beta^+$ CS B cell population in a primary response (Fig. 3A, B). However, the PC population particularly in the BM was increased (Fig. 3H) and lead to an increased $Q\beta^+$ IgG Ab level especially at an early time point. In the absence of Th, Qβ-VLP immunization of MHCII KO mice lead to a poor cellular activation of $Q\beta^+$ CS B cells and PCs in spleen (Fig. 3A, B) and low $Q\beta^+$ IgG Ab titer (Fig. 3C) during the primary response when compared to WT mice. Transferred memory B cells poorly proliferated and failed to differentiate into PCs in spleen and BM in MHCII KO (Fig. 3E, F, H). Nevertheless, IgG humoral responses in MHCII KO mice were elevated in the presence of memory B cells (Fig. 3G) compared to the primary response in MHCII

KO mice (Fig. 3C), indicating that some differentiation of memory B cells into PCs occurred in complete absence of Th.

In summary, the induction of Qβ specific B cell responses was strongly impaired in MHCII KO mice upon primary injection of Qβ-VLP. Specific memory B cell responses however were even more affected as almost no proliferation nor differentiation was observed in the absence of Th during VLP re-challenge.

Memory B cells can efficiently differentiate into PCs and produce vast amounts of Abs independent of CD40L signaling

The most important molecule for cognate Th is CD40L which triggers CD40 on B cells[35]. To address the role of CD40L on memory B cell responses, we performed the same set of adoptive transfer experiments as described above for MHCII deficient mice using CD40L-deficient recipient mice. Briefly, memory B cells were generated in congenic mice (Ly5.1+) in an un-manipulated environment in the presence of Th. Isolated splenocytes were $CD4^+$ T cell depleted prior transfer into WT and CD40L KO mice. These mice were re-challenged with Qβ-VLP 24 h after the transfer. To be able to compare memory B cell responses with primary B cell responses, control WT and CD40L KO mice were only immunized with Qβ-VLP without adoptive transfer of splenocytes.

$Qβ^+$ B lymphocytes of primary and memory B cell responses were analysed by FCM (Fig. 1B, C) at representative time points (combined data is shown in figure 4: first experiment analyzed on day 5 and 15; second experiment analyzed on day 5 and day 18). BM derived specific PCs were assessed in ELISPOT analysis.

The induction of Qβ-VLP specific CS B cells (Fig. 4A) and PCs (Fig. 4B) in spleen as well as IgG Abs (Fig. 4 C) were strongly reduced during primary responses in mice

lacking CD40L compared to WT mice. In CD40L KO mice, transferred CD4-depleted memory B cells proliferated to a low extent detectable on day 5 and 15/18 (Fig. 4E) and differentiated into memory B cell derived PCs (Fig. 4F) analyzed on day 5 in spleen. The number of specific PCs in BM on day 5 was comparable between WT and CD40L KO mice upon transfer of memory B cells (Fig. 4H). Although the Qβ specific B cell populations were reduced in spleen (Fig. 4E, F), the BM derived PC population was similar between KO and WT recipient mice (Fig. 4H) resulting in to only slightly reduced VLP specific IgG responses in sera at an early time point (d5) and almost comparable levels at a later time point (d15/18) when compared to WT mice (Fig. 4G).

Taken together, the primary response to Qβ-VLP is strongly reduced in CD40L KO mice compared to WT mice. In contrast, only the proliferation of VLP specific memory B cells is highly impaired in the absence of CD40L but differentiation into PCs found in spleen and BM and IgG Ab responses were almost normal in the absence of CD40L signaling.

The influence of Il21R signalling on memoy B cell responses

To address whether Tfh play a role in memory B cell responses we performed memory B cell adoptive transfer experiments as described above in IL21R KO mice. IL21R KO mice exhibit strongly reduced levels of functional Tfh cells as IL21 signaling is essential for their differentiation and localisation in B cell follicles[36, 37]. They also show reduced granulocyte numbers and activity, reduced proliferation of lymphocytes as well as decreased antibody levels especially of the IgA and IgG1 isotype[38]. The primary immune response induced by VLP vaccination is however only marginally affected as granulocytes are not involved in the response and TLR7

engagement by the packaged RNA largely overcomes IL21R/IL21 signaling dependence [39, 40]. The IL21/IL21R signaling has a strong influence on the formation of GCs in B cell follicles during the primary B cell response. In particular, IL-21 has an impact on the differentiation and proliferation of B cells and promotes the interaction of GC B cells with Tfh as well as the formation of PCs[41]. In our recent study, we found that VLP specific memory B cells differentiated into PCs and did not form or re-enter GCs[23]. Hence, the influence of IL21R signaling in GC reactions mediated by $Q\beta^+$ memory B cells can be neglicted in our setting, as it does not happen.

Congenic ($Ly5.1^+$) WT mice were immunized to develop memory B cells in an immuno-competent environment. Spleens were collected after 4-6 weeks after immunization. CD4-depleted splenocytes were subsequently adoptively transferred into WT and IL21R KO mice. Animals were challenged with Qβ-VLP 24 h later. Control WT and IL21R KO mice were only immunized ("Primary response"). The analysis of specific B cell responses was performed as already described above (Fig. 1B, C). As shown previously, during the primary response, the generation of specific CS B cells was unaffected or even increased (Fig. 5A), whereas differentiation into PCs found in spleen (Fig. 5 B) as well as $Q\beta^+$ IgG Abs (Fig. 5C) were slightly reduced in IL21R-deficient mice when compared to WT mice[40]. No difference in the early specific PC population in BM was observed (Fig. 5D).

The proliferation of transferred Qβ specific memory B cells was highly reduced in IL21R KO mice compared to WT mice (Fig. 5E). In contrast, memory B cell derived PCs found in spleen (Fig. 5F) and BM (Fig. 5H) on day 5 post VLP re-challenge were comparable in IL21R KO and WT mice. In line with this, Qβ-specific IgG Ab

responses were similar in IL21R KO mice compared to WT mice at the analyzed time points (d5 and d15/16) (Fig. 5G).

Taken together, primary B cell responses to Qβ-VLP upon immunization in IL21R KO compared to WT mice were only partly reduced as shown for the PC population in spleen and Ab levels. These observations go in line with B cell responses described by Zotos et al.[42]. In comparison, absence of Tfh cells caused a similar phenotype as CD40L-deficiency for memory B cell responses, as memory B cell proliferation was reduced while PC differentiation and IgG production was normal. Hence, CD40L and IL21R signalling are more important during primary than secondary Qβ-specific responses.

Generation of fully mature secondary PCs is Th cell dependent

We recently found that adoptively transferred VLP specific memory B cells into WT recipient mice differentiated into PCs upon cognate Ag re-encounter exhibiting the unique feature to produce more Abs than primary PCs generated in a primary response. Whether the generation of fully mature secondary PCs was dependent on Th was addressed next. Secondary PCs derived from CD4-depleted memory splenocyte transfer containing memory B cells were compared to primary PCs derived from non-memory host B cells in recipient mice in the spleen for their intracellular binding of Qβ-VLP. The increased MFI reflects the increased amount of Abs within these PCs.

To this end, memory B cells were generated in congenic ($Ly5.1^+$) mice upon Qβ-VLP immunization. After 4-6 weeks spleens were isolated and CD4 depleted by MACS bead purification prior to transfer into WT, MHCII KO, CD40L KO and IL21R KO mice. The animals were challenged with Qβ-VLP 24 h after the transfer. On day 5

and/or 6, spleens of mice which had received the adoptive CD4-depleted memory splenocyte transfer were analyzed. Specific PCs in spleens were identified as $Q\beta^+$ $B220^-$ lymphocytes (being negative for IgM, IgD, CD4, CD8, Gr1, CD11b, CD11c) (Fig. 1B). Memory B cell derived PCs were distinguished from host derived PCs by the expression of the Ly5.1 congenic cell marker and could be directly compared in one host. In WT mice, memory B cell derived PCs ($Ly5.1^+$) showed an increased binding for intracellular Qβ-VLP compared to host ($Ly5.1^-$) derived PCs (Fig. 6A-C, WT panels) reflecting the generation of secondary PCs from memory B cells with an enhanced capacity to produce Abs. In MHCII KO mice almost none of the memory B cell derived PCs ($Ly5.1^+$, only in 2 out of 7 mice) (Fig. 3F) nor host derived PCs ($Ly5.1^-$) (data not shown) were detected due to complete failure of PC generation in the absence of global Th. Although general Th was critical for generation of secondary PCs, absence of CD40L and IL21R signaling was dispensable for the generation of secondary PCs. Memory B cell derived PCs in CD40L KO and IL21R KO mice showed increased intracellular Qβ-VLP binding compared to their primary counterparts (host $Ly5.1^-$) (Fig. 6B, C).

In summary, proliferation of memory B cells and differentiation into PCs were highly dependent on general Th. However, CD40L and IL21R signaling were not essential for the differentiation of $Q\beta^+$ memory B cells into secondary PCs exhibiting increased capacity for Ab production.

Discussion

Primary B cell responses are generally strongly dependent on Th. While a transient IgM response may be induced by TI antigens, long-lived IgG responses are usually dependent on $CD4^+$ Th cells and, moreover on Tfh cells in GC reactions and the

concomitant interaction of CD40L with CD40. We used three different mouse KO strains to study the role of T cell help: MHCII KO for absence of all Th cells and CD40L KO to investigate the role of one of the key signaling interactions between B and T cells. IL21R KO mice were considered to serve as a model of Tfh deficiency as Tfh are dependent on IL-21 signaling in a number of systems. Th cell-dependence may vary amongst antigens, as very high antigen load upon viral infection may enable to overcome Th dependence of IgG responses[43] and antigens linked to TLR-ligands are able to induce almost normal IgG responses in the absence of Tfh cells[40]. In this study, we used Qβ-VLPs to investigate the Th dependence of memory B cell responses. This antigen induces a Th cell independent IgM response, followed by a Th cell dependent IgG response[44]. During primary responses, global absence of Th cells as well as CD40/CD40L strongly reduces specific IgG responses. In contrast, absence of Tfh cells leaves IgG responses largely unaffected, as the VLPs are loaded with bacterial RNA, a TLR7/8 ligand.

Memory B cell responses differ from primary B cell responses in various aspects. But both of them take place in B cell follicles of secondary lymphoid organs[45] and strictly require the presence of specific antigen[46, 47]. Activated Th cells or TLR-ligands alone are not able to drive a memory B cell response in the absence of an antigen[46]. An important difference between primary and secondary B cell responses is that the latter occurs with accelerated kinetics[16] compared to primary responses. A critical reason for the increased speed of memory B cell responses is the fact that memory B cells remain present in the host at increased frequencies and rapidly differentiate into PCs upon antigenic re-encounter[17, 23, 48]. While it has been shown that memory B cell populations are maintained in the absence of Th and antigen[49], the role of Th

during memory B cell responses is less clear. The majority of model antigens used to study B cell responses are soluble proteins or cells[24, 26] which are Th dependent antigens and do not as efficiently cross-link BCRs as VLPs do and are therefore unable to efficiently activate B cells in the absence of Th cells. In addition, these antigens are not linked to TLR-ligands. VLPs are able to induce strong T cell independent primary IgM responses in complete absence of Th and primary IgG responses occur almost normally in the absence of Tfh cells. Therefore it may be expected that VLPs drive memory B cell responses in a more Th cell independent fashion than other soluble antigens. In line with this, we have previously shown that adoptively transferred memory B cells mounted partially TI IgG responses in irradiated recipient mice upon immunization with viral particles but not with recombinant protein[50]. In another study, adoptive transfer experiments were performed using RAG1 deficient mice to compare memory B cell responses to intact virus and soluble viral protein [51]. The authors reported that the transferred memory B cells mediated a humoral response to the viral particle which was independent of cognate as well as bystander Th. To assess the immune response of memory B cells transferred into RAG1 KO mice may be difficult, as B cells are transferred into a non-physiological environment free of lymphocytes and secondary lymphoid organs with appropriate structures[52]. To avoid this issue, we generated VLP-specific memory B cells in un-manipulated WT mice and transferred them into less cumbered hosts deficient of T cell help, which allows us to study their responses in an environment containing B cell follicles in secondary lymphoid structures. Another study approached by Weisel et al. from the same group revealed that virus-specific memory B cells may differentiate into PCs in the absence of Th as indicated by the detection of Abs after VLP challenge[45]. This study, however, failed to assess

memory B cell and PC responses in parallel. Nevertheless, these observations made by Weisel et al.[45] are partly in line with our findings. We also detected IgG production in the absence of $CD4^+$ Th cells; however, these responses were markedly reduced. In addition, PC formation and expansion of CS memory B cells were strongly dependent on the presence of $CD4^+$ T cells during $Q\beta^+$ memory B cells responses. Ab responses and PC generation however were apparently not dependent on cognate Th, as they occurred rather efficiently in the absence of CD40L/CD40 as well as IL21R signaling. In contrast, expansion of specific CS memory B cells was almost completely abrogated in the absence of CD40L or IL21R. This demonstrates that cognate Th is required for expansion of memory B cells but not for their differentiation into PCs. Hence interaction with Ag alone in an environment containing $CD4^+$ Th cells is sufficient for differentiation of VLP specific memory B cells into PCs. In contrast, a direct interaction of memory B cells with cognate Th cells is required for their proliferation. These findings are consistent with the observation that GC B cells preferentially differentiate into PCs in the absence of CD40L *in-vitro*[17].

Interestingly, CD40L and Tfh were also not required for differentiation of VLP specific memory B cells into secondary PCs producing increased amounts of Abs. In contrast, secondary PCs were not generated in global absence of Th cells. It will be interesting to determine whether the few PCs generated in absence of Th cells mount more short-lived responses compared to fully differentiated secondary PCs. The observation that secondary PC responses depended strongly on Th cells but were rather independent of CD40L and IL-21R signaling indicates that either non-cognate functions of Th cells can induce secondary PCs from memory B cells or that a cognate function of Th cells other than CD40L or IL21R is involved. Therefore it will

be interesting to investigate the role of other co-stimulatory interactions such as CD28/CD80 or ICOS/ICOSL or other cytokines (e.g. IL4, IFNγ) for the generation of secondary PCs. Although IL21R signaling was absent in IL21R KO recipient mice, the transferred memory B cells were still capable to conduct IL21 signaling. It will be interesting to investigate whether IL21 signaling in B cells was key for the differentiation of transferred memory B cells into mature secondary PCs.

Failure to differentiate into fully mature secondary PCs in the absence of Th may be interpreted as a safety measure as this step is not only controlled by the presence of antigen but also by the presence of Th. As Th cell tolerance is usually stricter than B cell tolerance, in particular after B cells have undergone rounds of hypermutation, control by Th cells may avoid generation of self-specific secondary PCs producing maximal amounts of Abs. Nevertheless, differentiation of memory B cells to IgG producing PCs is less strictly controlled than the generation of IgG producing PCs during primary responses. This may reflect the fact that memory B cells are present at increased frequencies compared to “specific” naïve cells. Therefore, less proliferation is required to generate measurable frequencies of specific PCs from memory B cells. In addition, generation of memory B cells has been cross-checked previously by the presence of specific Th cells during the primary response.

As shown before, Qß-VLPs induced largely normal primary B cell responses in the absence of Tfh cells both at the humoral and cellular level. It is therefore rather unexpected that the expansion of CS memory B cells was strongly dependent on Tfh. This may be a way the immune system restricts proliferation of memory B cells to allow naïve B cells to enter the memory B cell pool during secondary B cell responses.

Taken together, this study reveals distinct novel mechanisms regulating primary vs. secondary B cell responses as well as memory B cell proliferation vs. PC differentiation.

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The authors have no financial conflicts of interest.

Figure legends

Figure 1. Adoptive transfer of VLP specific memory B cells and the detection of Qβ-VLP^{+} CS B cells and PCs in spleen by FCM analysis. (A) Congenic mice of the Ly5.1 or IgH^{a} strain were immunized to generate memory B cells in an un-manipulated environment. After 4 weeks of immunization, spleens containing memory B cells were isolated and either left untreated or CD4-depleted by MACS purification. Unpurified or purified splenocytes were transferred into recipient mice (Ly5.2 or IgH^{b}). 24h after the adoptive transfer, recipient mice were challenged with Qβ-VLP. (B) Representative FCM plot to identify CS Qβ positive B cells in the spleen

of mice 9 days after Qβ-VLP immunization. B220-positive lymphocytes negative for IgM, IgD, CD4, CD8, CD11b, CD11c, Gr1 were analyzed for Qβ-VLP binding. Transfer derived specific CS B cells in spleen were identified by simultaneous binding to anti-CD45.1 (Ly5.1) mAb. (C) Representative FCM plot to detect Qβ specific PCs in the spleen of mice 6 days after immunization with Qβ-VLP. B220-negative lymphocytes (also negative for IgM, IgD, CD4, CD8, CD11b, CD11c and Gr1) were analyzed for intracellular Qβ-VLP binding upon permeabilization of the cell membrane. Transfer derived PCs were identified by simultaneous binding to anti-CD45.1 (Ly5.1) mAb. (D) Depletion of CD4+ T cells of the splenocyte population prior adoptive transfer of memory B cells. A representative example of FCM analysis of murine splenocytes from immunized mice is shown that have been purified by CD4 MACS beads. The proportion of CD4+ T cells within the splenocyte population of naïve mice ("Naïve") and immunized mice ("Undepleted") is ~20-25% and of CD4-depleted immunized mice 1-2%. Shown are all splenocytes stained for B220 and CD4 pre-gated on living cells.

Figure 2. Comparison of adoptive transfers of unpurified or CD4 depleted memory splenocytes containing memory B cell in WT mice after Qβ-VLP challenge. Congenic mice (Ly5.1 or IgH^a) were immunized with Qβ-VLP to generate memory B cell in an un-manipulated environment. After 4 weeks of immunization, spleens containing memory B cells were isolated and transferred into WT recipient mice (Ly5.2 or IgH^b). Splenocytes were either directly and non-purified transferred as "Memory transfer" or CD4-negatively purified (MACS beads) and transferred as "CD4 depleted memory transfer". Mice were challenged with Qβ-VLP 24 h after the transfer on day 0. WT control mice (Ly5.2) received "No transfer" and were only

immunized on day 0. (A) Detection of donor ($Ly5.1^+$) Qβ specific B cells within the CS B cell population ($B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen by FCM. Mean with SEM. *P* values represent significances and were obtained by an unpaired t-test. The control group "No transfer" did not receive $Ly5.1^+$ splenocytes from donor mice and was therefore not considered for statistical analysis. (B) Detection of donor ($Ly5.1^+$) Qβ specific PCs in spleen within B220 negative lymphocyte population characterized as $B220^-$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$ by FCM. Mean with SEM. *P* values were obtained by an unpaired t-test. The control group "No transfer" did not receive $Ly5.1^+$ splenocytes from donor mice and was therefore not considered for statistical analysis. (C) Detection of the mean fluorescence intensity (MFI) of intracellular binding of Qβ-VLP of host ($Ly5.1^-$) or transfer-derived ($Ly5.1^+$) PCs in spleen by FCM. *P* value was obtained by an unpaired t-test. (D) Analysis of spot diameter of BM derived PCs specific for Qβ-VLP in ELISPOT. *P* values were obtained by an unpaired t-test. (E) Qβ-VLP specific IgG1 and IgG2a Ab ELISA titer analyzed in serum originated from allotype H^a (derived from transferred memory B cell) or H^b (derived from newly activated host B cells) upon transfer of unpurified memory splenocytes. Mean with SEM. *P* values were calculated by an unpaired t-test. (F) Qβ-VLP specific IgG1 and IgG2a Ab ELISA titer analyzed in serum originated from allotype H^a (derived from transferred memory B cell) or H^b (derived from newly activated host B cells) upon transfer of CD4-depleted memory splenocytes. Mean with SEM. *P* values were calculated by an unpaired t-test. *p<0.05, **p<0.01, ***p<0.001. Mice per group n = 3. Data are representative of at least three independent experiments.

Figure 3. Primary and memory B cell responses to Qβ-VLP in WT and MHCII KO mice. WT and MHCII KO mice received no adoptive transfer and were only immunized with Qβ-VLP on day 0. The primary response of those mice is shown in figure A to D. Splenocytes of Qβ-VLP immunized congenic ($Ly5.1^+$) mice were isolated and negatively purified by $CD4^+$ MACS beads. Purified splenocytes containing memory B cells were transferred into WT and MHCII KO mice and challenged with Qβ-VLP 24 h after the adoptive transfer on day 0. The secondary response of the transferred memory B cell is shown in figure E to H. *Primary response (No splenocyte transfer).* (A) Detection of specific B cells within the B220 positive lymphocyte population (numbers of $Q\beta^+$, $B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen of WT and MHCII KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (B) Detection of specific PCs within the B220 negative lymphocyte population (numbers of $Q\beta^+$, $B220^-$, IgM^-, IgD^- ,$CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen of WT and MHCII KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (C) Measurement of Qβ-VLP total IgG antibody ELISA titer in sera of WT and MHCII KO mice. Mean with SEM. *P* values were calculated by an unpaired t–test. (D) ELISPOT analysis of BM derived PCs specific for Qβ-VLP. Mean with SEM. *P* values were calculated by an unpaired t–test. *Memory response (CD4-depleted memory splenocyte transfer).* (E) Detection of memory B cell transfer derived (Donor, $Ly5.1^+$) specific CS B cells within the B220 positive lymphocyte population (numbers of $Ly5.1^+$, $Q\beta^+$, $B220^+$, IgM^- , IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen of WT and MHCII KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (F) Detection of memory B cell transfer derived (Donor, $Ly5.1^+$) specific PCs within the B220 negative lymphocyte population (numbers of $Ly5.1^+$, $Q\beta^+$, $B220^-$, IgM^-, IgD^-,$CD4^-$,

CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$) in spleen of WT and MHCII KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (G) Measurement of Qβ-VLP total IgG antibody ELISA titer in sera of WT and MHCII KO mice. Mean with SEM. *P* values were calculated by an unpaired t–test. (H) ELISPOT analysis of BM derived PCs specific for Qβ-VLP. Mean with SEM. *P* values were calculated by an unpaired t–test. Combined data of two independent representative experiments. First experiment shows data from day 5 and 15 and second experiment shows data from day 5 and 18. Mice per group first experiment n = 3 and second experiment n = 4 (or 8 on day 5 in C and G).

Figure 4. Primary and memory B cell responses to Qβ-VLP in WT and CD40L KO mice. WT and CD40L KO mice received no adoptive transfer and were only immunized with Qβ-VLP on day 0. The primary response of those mice is shown in figure A to D. Splenocytes of Qβ-VLP immunized congenic (Ly5.1$^+$) mice were isolated and CD4 T cell depleted (by MACS beads). CD4-depleted memory splenocytes containing memory B cell were transferred into WT and CD40L KO mice and challenged with Qβ-VLP 24 h after the adoptive transfer on day 0. The secondary response of the transferred memory B cells is shown in figure E to H. *Primary response (No splenocyte transfer).* (A) Detection of specific B cells within the B220 positive lymphocyte population (numbers of Qβ$^+$, B220$^+$, IgM$^-$, IgD$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$) in spleen of WT and CD40L KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (B) Detection of specific PCs within the B220 negative lymphocyte population (numbers of Qβ$^+$, B220$^-$, IgM$^-$, IgD$^-$,CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$) in spleen of WT and CD40L KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (C)

Measurement of Qβ-VLP total IgG antibody ELISA titer in sera of WT and CD40L KO mice. Mean with SEM. *P* values were calculated by an unpaired t–test. (D) ELISPOT analysis of BM derived PCs specific for Qβ-VLP. Mean with SEM. *P* values were calculated by an unpaired t–test. *Memory response (CD4-depleted memory splenocyte transfer).* (E) Detection of memory B cell transfer derived (Donor, $Ly5.1^+$) specific CS B cells within the B220 positive lymphocyte population (numbers of $Ly5.1^+$, $Q\beta^+$, $B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen of WT and CD40L KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (F) Detection of memory B cell transfer derived (Donor, $Ly5.1^+$) specific PCs within the B220 negative lymphocyte population (numbers of $Ly5.1^+$, $Q\beta^+$, $B220^-$, IgM^-, IgD^-,$CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen of WT and CD40L KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (G) Measurement of Qβ-VLP total IgG antibody ELISA titer in sera of WT and CD40L KO mice. Mean with SEM. *P* values were calculated by an unpaired t–test. (H) ELISPOT analysis of BM derived PCs specific for Qβ-VLP. Mean with SEM. *P* values were calculated by an unpaired t–test. Combined data of two independent representative experiments. First experiment shows data from day 5 and 15 and second experiment shows data from day 5 and 18. Mice per group first experiment n = 3 and second experiment n = 4 (or 8 on day 5 in C and G).

Figure 5. Primary and memory B cell responses to Qβ-VLP in WT and IL21R KO mice. WT and IL21R KO mice received no adoptive transfer and were only immunized with Qβ-VLP on day 0. The primary response of those mice is shown in figure A to D. Splenocytes of Qβ-VLP immunized congenic ($Ly5.1^+$) mice were isolated and CD4-depleted (by MACS beads). CD4-depleted memory splenocytes

containing memory B cells were transferred into WT and IL21R KO mice and challenged with Qβ-VLP 24 h after the adoptive transfer on day 0. The secondary response of the transferred memory B cells is shown in figure E to H. *Primary response (No splenocyte transfer).* (A) Detection of specific B cells within the B220 positive lymphocyte population (numbers of $Q\beta^+$, $B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen of WT and IL21R KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (B) Detection of specific PCs within the B220 negative lymphocyte population (numbers of $Q\beta^+$, $B220^-$, IgM^-, IgD^- ,$CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen of WT and IL21R KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (C) Measurement of Qβ-VLP total IgG antibody ELISA titer in sera of WT and IL21R KO mice. Mean with SEM. *P* values were calculated by an unpaired t–test. (D) ELISPOT analysis of BM derived PCs specific for Qβ-VLP. Mean with SEM. *P* values were calculated by an unpaired t–test. *Memory response (CD4-depleted memory splenocyte transfer).* (E) Detection of memory B cell transfer derived (Donor, $Ly5.1^+$) specific CS B cells within the B220 positive lymphocyte population (numbers of $Ly5.1^+$, $Q\beta^+$, $B220^+$, IgM^- , IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen of WT and IL21R KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (F) Detection of memory B cell transfer derived (Donor, $Ly5.1^+$) specific PCs within the B220 negative lymphocyte population (numbers of $Ly5.1^+$, $Q\beta^+$, $B220^-$, IgM^-, IgD^-,$CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$) in spleen of WT and IL21R KO mice by FCM. Mean with SEM. *P* values were calculated by an unpaired t–test. (G) Measurement of Qβ-VLP total IgG antibody ELISA titer in sera of WT and IL21R KO mice. Mean with SEM. *P* values were calculated by an unpaired t–test. (H) ELISPOT analysis of BM derived PCs specific for Qβ-VLP. Mean with SEM. *P* values were calculated by an

unpaired t–test. Combined data of two independent representative experiments. First experiment shows data from day 5 and 15 (A-C, E-G) and second experiment shows data from day 5 and 16 (A-H). Mice per group first experiment n = 3 and second experiment n=4 (or 8 on day 5 in C and G, in D and H only n = 4).

Figure 6. Comparison of intracellular binding of Qβ-VLP of host derived (Ly5.1$^-$) vs. memory B cell transfer derived (Ly5.1$^+$) plasma cells. Congenic mice (Ly5.1$^+$) were immunized with Qß-VLP and their splenocytes were isolated after 4-6 weeks. Isolated splenocytes containing memory B cells were CD4 depleted by MACS purification and transferred into Ly5.2$^+$ recipient mice (WT, MHCII KO, CD40L KO and IL21R KO). Mice were challenged with Qβ-VLP after 24 h upon adoptive transfer on day 0. Mean fluorescence intensity of intracellular binding of Qβ-VLP of host derived (Ly5.1$^-$) and memory B cell transfer derived (Ly5.1$^+$) PCs were measured by FCM characterized as B220$^-$, IgM$^-$, IgD$^-$, CD4$^-$, CD8$^-$, CD11c$^-$, CD11b$^-$, Gr-1$^-$ in WT and MHCII KO mice (A), in WT and CD40L KO mice (B) and in WT and IL21R KO mice (C) on day 5 and 6 post challenge with Qβ-VLP. Mean with SEM. *P* values were obtained by an unpaired t-test. Combined data of two independent representative experiments for MHCII KO (A) and CD40L KO (B); first experiment n = 4, day 5 & 6; second experiment n = 3, day 5. Results from two experiment for IL21R KO; first experiment n = 4, day 5; second experiment n = 3, day 5.

Supplementary figure 1. Tracking of transferred memory splenocytes containing memory B cell in WT mice after Qβ-VLP challenge. Splenocytes of Qβ-VLP immunized congenic mice (Ly5.1 resp. IgH^a) containing Qβ positive memory B cells were either unpurified transferred as “Memory transfer” or CD4-negatively

purified (MACS beads) and transferred as “CD4-depleted memory transfer” into WT recipient mice (Ly5.2 resp. IgH^b). Mice of “Memory transfer” and “CD4-depleted memory transfer” were challenged with Qβ-VLP on day 0, 24 h after the adoptive transfer. Control WT mice received no splenocyte transfer (“no transfer”) and were immunized on day 0. (A) Detection of $Q\beta^+$ CS B cell in spleen within the specific B220 positive lymphocyte population characterized as $Q\beta^+$, $B220^+$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$ by FCM. Mean with SEM. *P* values were obtained by an unpaired t-test. (B) Detection Qβ-VLP positive PCs in spleen within the specific B220 negative lymphocyte population characterized as $Q\beta^+$, $B220^-$, IgM^-, IgD^-, $CD4^-$, $CD8^-$, $CD11c^-$, $CD11b^-$, $Gr\text{-}1^-$. Mean with SEM. *P* values were obtained by an unpaired t-test. (C) Analysis of BM derived Qβ-VLP specific PCs by ELISPOT. Mean with SEM. *P* values were obtained by an unpaired t-test. (D) Measurement of Qβ-VLP total IgG Ab titer in serum by ELISA. Mean with SEM. *P* values were obtained by an unpaired t-test. *p<0.05, **p<0.01, ***p<0.001; Mice per group n = 3 (A-C) or 6 (D). Data are representative of at least three independent experiments.

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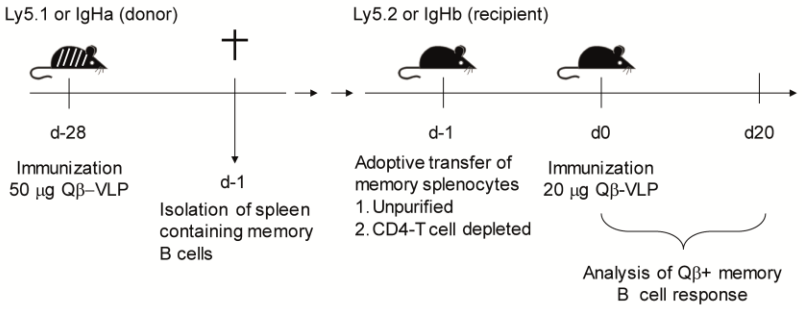
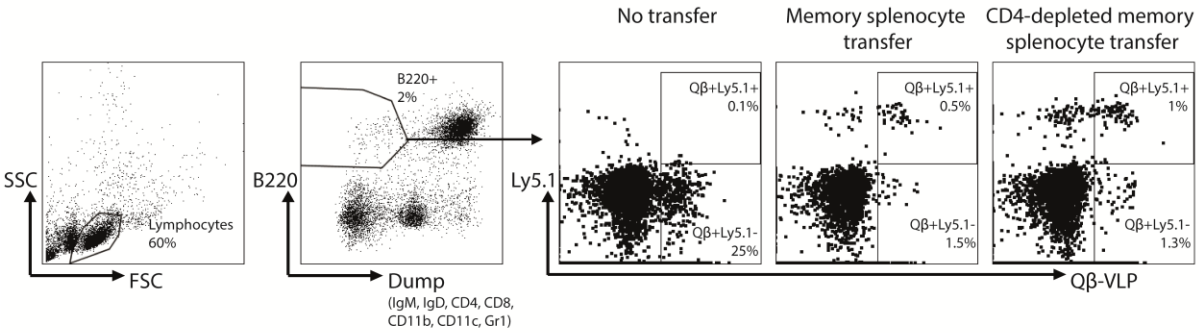
Figure 1

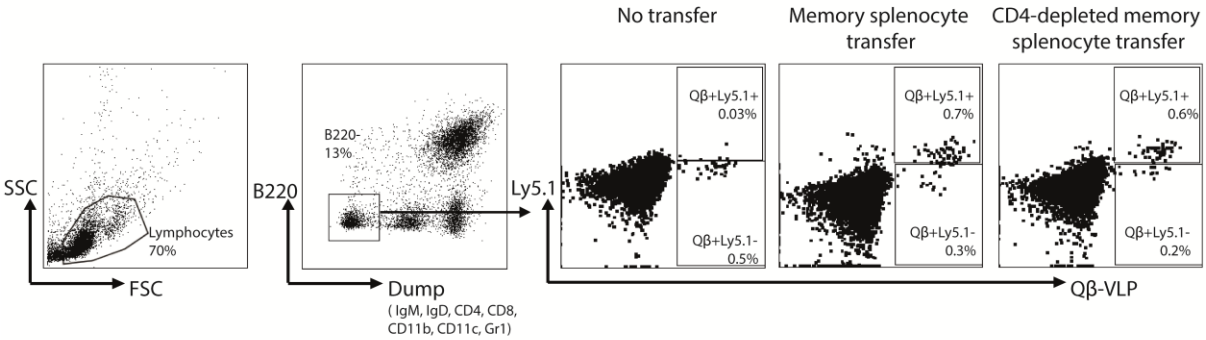
A Experimental set up



B Detection of CS Qβ+ B cells in spleen, day 9 p.i.



C Detection of Qβ+ PCs in spleen, day 6 p.i.



D Depletion of CD4+ T cells prior adoptive transfer

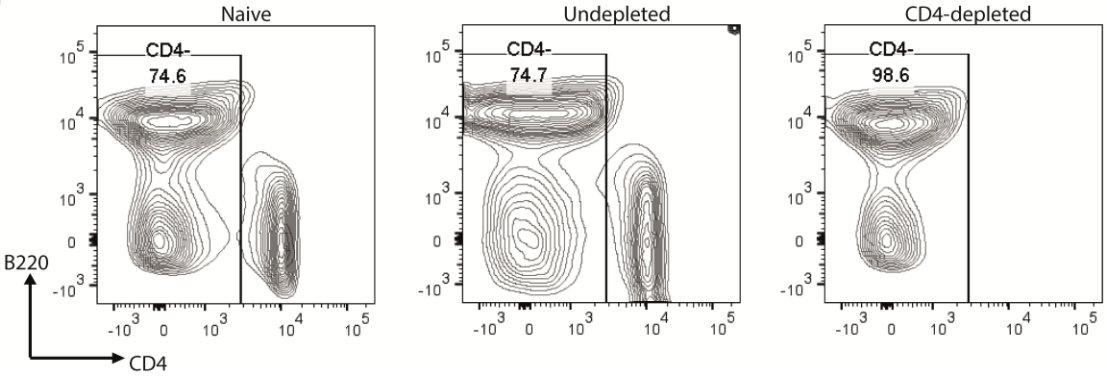


Figure 2

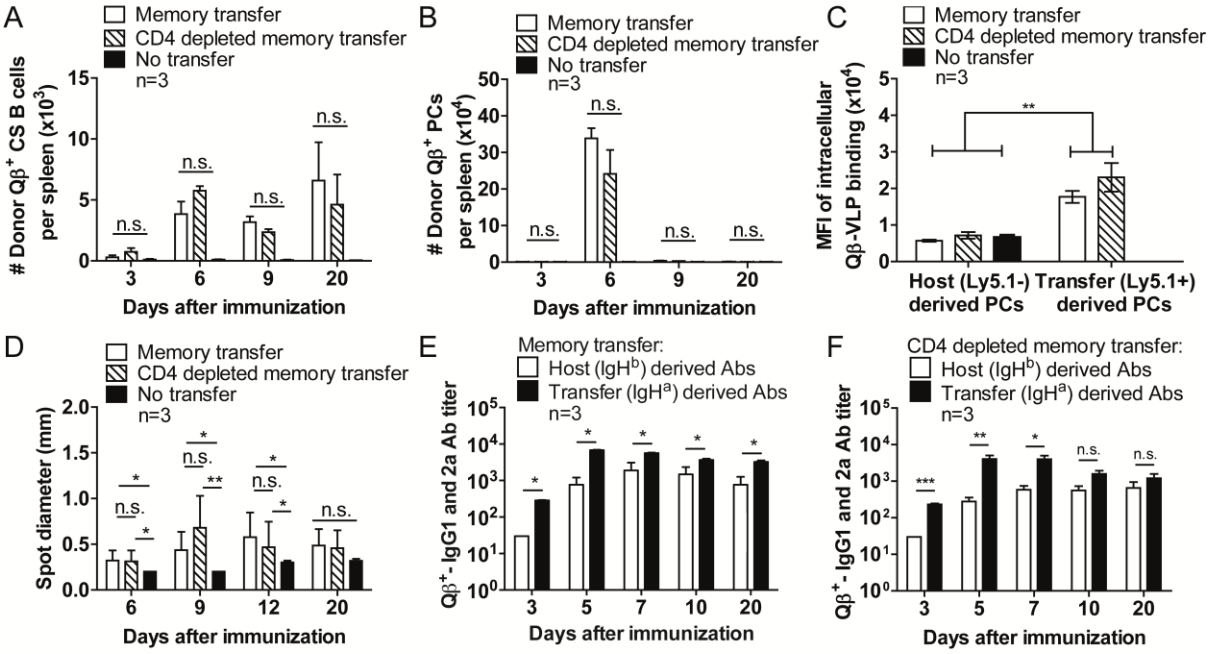


Figure 3

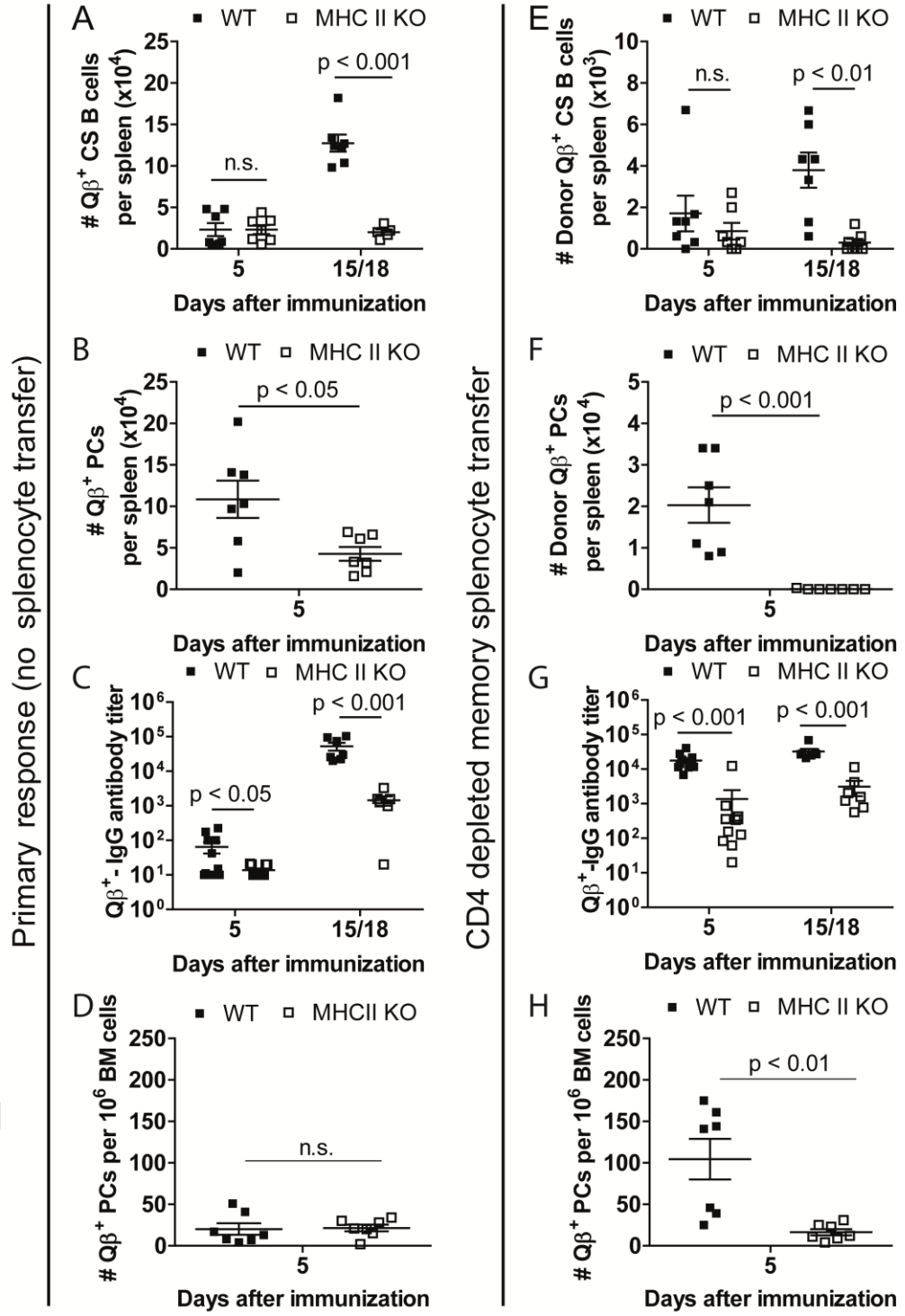


Figure 4

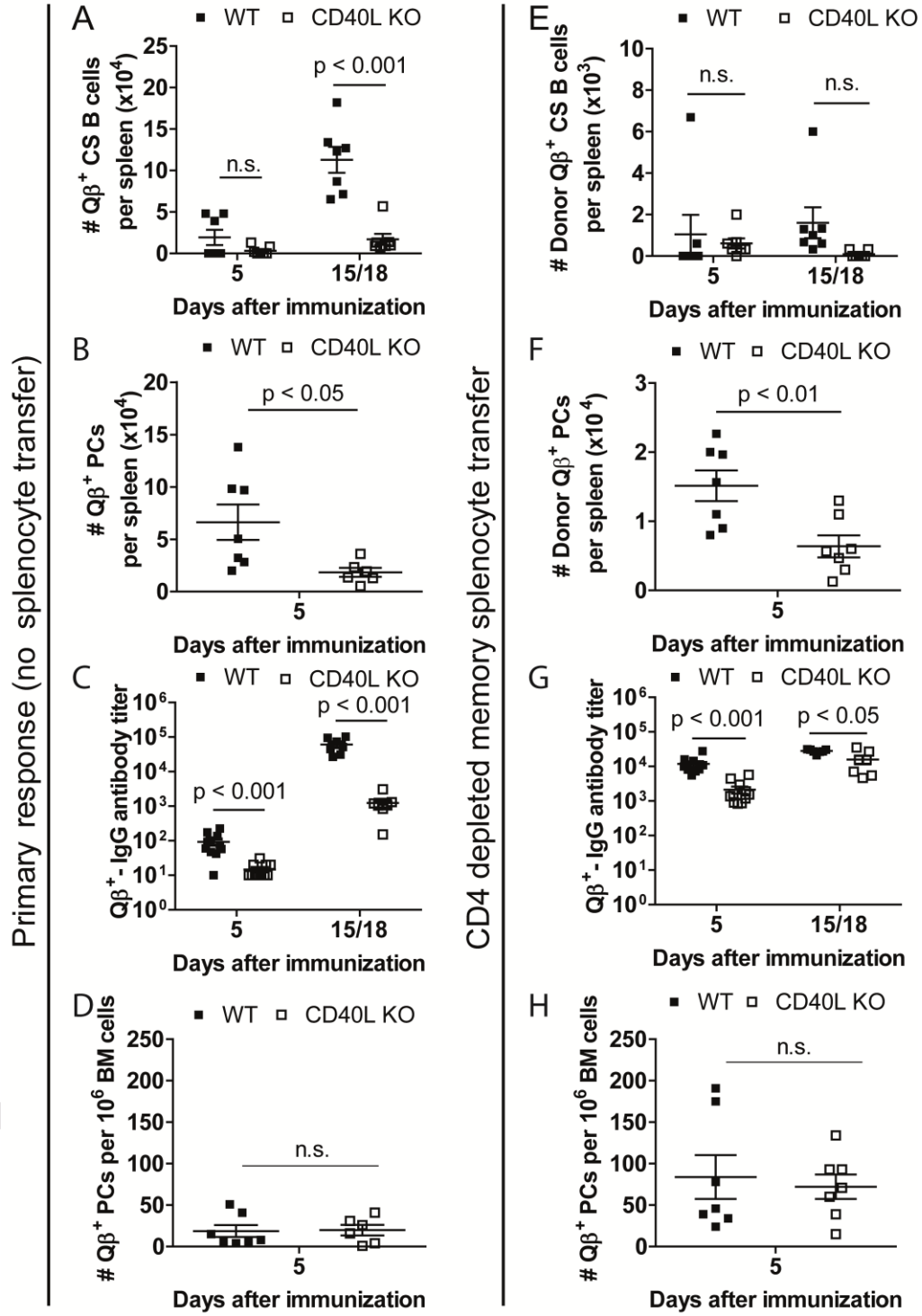


Figure 5