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Reversing Endogenous Alloreactive B cell GC Responses with Anti-CD154 or CTLA-4Ig

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Abstract

Alloantibodies mediate acute antibody-mediated rejection as well as chronic allograft rejection in clinical transplantation. To better understand the cellular dynamics driving antibody production, we focused on the activation and differentiation of alloreactive B cells in the draining lymph nodes and spleen following sensitization to allogeneic cells or hearts. We used a modified staining approach with a single MHC Class I tetramer (K^d) bound to two different fluorochromes to discriminate between the Class I-binding and fluorochrome-streptavidin-binding B cells with a high degree of specificity and binding efficiency. By day 7-8 post-sensitization, there was a 1.5-3.2-fold increase in the total numbers of K^d-binding B cells. Within this K^d-binding B cell population, approximately half were IgD^{low}, MHC Class II^{high} and $CD86^+$, 30-45 % expressed a germinal center (Fas^+GL7^+) phenotype, and 3-12 % were $IRF4^{hi}$ plasma cells. Remarkably, blockade with anti-CD40 or CTLA-4Ig, starting on day 7 post-immunization for 1 or 4 weeks, completely dissolved established GCs and halted further development of the alloantibody response. Thus MHC Class I tetramers can specifically track the *in vivo* fate of endogenous, Class I-specific B cells, and was used to demonstrate the ability of delayed treatment with anti-CD154 and CTLA-4Ig to halt established allo-B cell responses.

Keywords

Alloreactive B cells; MHC Class I Tetramers; Allograft Rejection; GC; PCs; anti-CD154; CTLA-4Ig

Introduction

It has become increasingly clear that alloantibodies can mediate acute antibody-mediated rejection (AMR) as well as chronic allograft rejection, and as a result, play an important role in determining the outcome of allografts in the clinic (1-5). In non-sensitized transplant recipients, alloantibodies are secreted by plasma cells (PCs) that have differentiate from naïve alloreactive B cells, whereas in sensitized individuals, PCs from a pre-existing long-lived pool or arising from memory B cells are responsible for the *de novo* alloantibody produced following the re-exposure to alloantigens (6-8).

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The characterization of endogenous B cells that participate in a *de novo* alloantibody response has been technically challenging because of their low frequencies even during active immunization (9). In experimental models, attempts to circumvent this limitation have involved the use BCR-transgenic models where the frequencies of alloreactive B cells are significantly increased (10, 11). However the caveats of this approach are becoming increasingly apparent, since the frequencies of alloreactive B cells in these mice greatly exceed physiological frequencies and observations with a monoclonal population of B cells with a single affinity may not fully reflect alloreactive B cells with a spectrum of affinities (12, 13). Thus, there has been a renewed interest in tracking endogenous alloreactive B cells in experimental models as well as in humans.

The dominant specificity of alloantibodies is for donor MHC Class I and Class II antigens and donor reactive antibodies quantified in the clinic focus primarily on these specificities (5, 14, 15). MHC Class I tetramers have been extensively used to characterize the change in frequencies of antigen-specific CD8 cells following infection and immunization in both humans and mice (16, 17). MHC Class I tetramers have also been used to identify alloreactive B cells in humans, and more recently in mice (9, 18-22). In humans, the frequencies of B cells in the peripheral blood capable of binding specific HLA-tetramers was reported to be significantly higher in individuals who had detectable circulating alloantibodies of the same specificity compared to those that did not (19). While those reports demonstrated feasibility of approach, the access only to B cells in the blood of transplant recipients significantly limited mechanistic investigations that delve into how alloantibody production by these B cells are orchestrated because much of the B cell response occurs in the secondary lymphoid organs (23, 24). Thus complementary studies in mice are important if we are to develop a better understanding of the regulation of alloreactive B cell responses. Indeed, the recent report by Kwun et al. (22) demonstrated that MHC Class I tetramers could be used to identify an emergent IgD^{-} subset of alloreactive B cells, in parallel with increased titers of DSA, in a murine model of chronic cardiac allograft rejection.

Using MHC Class I tetramers, we here demonstrate in qualitative and quantitative detail the presence of alloreactive B cells in naïve mice, and their entry into germinal centers (GC) and commitment into PC fates during allo-sensitization and following allogeneic cardiac transplantation. We further demonstrate the therapeutic efficacy of delayed treatment with anti-CD154 and CTLA-4Ig in dissolving established GCs and halting established alloreactive B cell responses.

Materials and Methods

Mice

C57BL/6 mice and BALB/c mice were purchased from Harlan Sprague Dawley, while BALB.B (C.B10-H2b/LilMcdJ) mice were from Jackson Labs. μMTmice (B6.129S2-Igh-6tm1Cgn/J) were purchased from Jackson Labs and maintained in our animal facility. All mouse procedures described in this study had been approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Chicago.

Tetramers and Monomers

$H2K^{d}$- biotin monomers, $H2K^{b}$-biotin monomers, $H2K^{b}$ or $H2K^{d}$ tetramers conjugated with PE, APC or FITC were from NIH tetramer Core Facility (Atlanta, GA). The peptides bound to $H2K^{d}$ and $H2K^{b}$ were SYIPSAEKI (Plasmodium berghei circumsporozoite peptide 252-260) (25) and SSIEFARL (herpes simplex virus (HSV) glycoprotein B peptide 498 to

505) (26), respectively. For the staining 5×10^6 cells, saturating (0.5 μg) concentrations of each tetramer were used.

Antibodies and Flow cytometry

Flow cytometry was performed with an LSRII- blue or LSR-Fortessa (BD) and analyzed with Flowjo (Tree Star, Ashland, OR). All antibodies were purchased from Ebioscience, unless specified. For $H2K^d$-specific B cells staining, single cells from lymph nodes or spleens were pre-blocked at 4°C with 2.4G2, followed by lineage-specific antibodies (for dump channel: (Anti-CD3 (17A2), anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-F4/80 (BM8, Biolegend), anti-Gr1 (RB6-8C5, Biolegend), anti-CD49b (DX5, Biolegend)) and anti-B220 (RA3-6B.2), anti-IgD (11-26C.2a). Next, $H2K^d$ tetramers together with other antibodies were added and incubated for 30 minutes: anti-MHC Class II (I-A/I-E) (M5/114/15.2) and CD86 (GL1) or anti-Fas (JO2, BD Pharmingen) and anti-GL7 (GL7). For Interferon response factor 4 (IRF4) intracellular staining, cells were pre-incubated with Live/Dead fixable violet dead cell stain kit for 405 nm excitation (Invitrogen, Cat. L34955) prior to incubation with $H2K^b$- or $H2K^d$-tetramers, anti-B220 and anti-IgD. The cells were fixed, permeabilized (FoxP3 TF staining buffer kit, EBioscience), and then incubated with the anti-IRF4 antibody (clone 3E4) for 1 hour.

Adoptive Transfer

Cell suspensions of spleen and lymph nodes from C57BL/6 mice were incubated with $H2K^d$-PE and $H2K^d$-APC at 4°C for 1 hour, then anti-APC and anti-PE microbeads (Miltenyi) were added. After 30 mins, the cell-bead suspension was passed through magnetized LS column (Miltenyi), the flow through was collected and adoptively transferred into μMT mice. The other half of the non-depleted spleen and lymph node cells suspension was transferred into another cohort of μMT mice. One day after transfer, mice were immunized with BALB/c splenocytes subcutaneously and intraperitoneally, and at 21 days after cell transfer, sera were collected.

Immunization

For comparison of draining and non-draining lymph nodes, B6 mice were injected with 10 million BALB/c splenocytes in two sites on the flank. On day 7 after immunization, draining LN and non-draining LNs were collected (Inguinal LNs, axillary LNs, brachial LNs) respectively. For comparison of BALB/c or BALB.B immunized mice, separate groups of B6 mice were immunized on their flanks. On day 7 after immunization, the draining LNs were collected and analyzed by flow cytometry. Total cell numbers were counted with a hemotocytometry.

ELISA and Donor Specific Antibody (DSA) Assay

For $H2K^d$ antibody detection, plate were coated with streptavidin overnight at 37°C, after several washes, $H2K^d$-biotin monomers were added and incubated for 1 hour at room temperature, cells were then blocked with BSA% 1 hour at room temperature, serum were diluted (1:100) and incubated with $H2K^d$ coated plate at room temperature for 2 hours, plates were washed and anti-IgG conjugated to alkaline phosphatase (AP) (Jackson Immunoresearch, Cat. 115-055-071) or anti-IgM-AP (Jackson Immuoresearch, Cat. 115-055-075) were added. The substrate was used according to product information (Sigma, SIGMAFAST™ r-Nitrophenyl phosphate Tablets), and O.D values ere measured at 405 nm by a multiwall plate reader (BioRad). For donor specific antibody detection, serum were diluted (1:25) and incubated with 10^6 BALB.B splenocytes for 1 hour incubation at 4°C, then the cells were wash and incubated with anti-IgG (Southern Biotech, Cat. 1030-02) or

anti-IgM (Southern Biotech, Cat. 1021-09) for 30 minutes. Mean fluorescence intensity were measured by flow cytometry.

Heart Transplantation

Hearts from BALB/c mice were transplanted into C57BL/6 recipients using techniques described previously (27).

Immunosuppression

C57BL/6 mice were immunized subcutaneously on Day 0 using BALB/c splenocytes and divided into 5 groups: (i) MR1 d0: Anti-CD154 (MR1; 500 μg/mouse) starting on Day −1, 0 and 1; (ii) MR1 d7: Anti-CD154 (500 μg/mouse) starting on Day 7, 8, 9; (iii) CTLA-4Ig D0: CTLA-4Ig (500 μg/mouse) starting on Day −1, 0 and 1; (iv) CTLA-4Ig d7 group: CTLA4-Ig (500 μg/mouse) starting on Day 7, 8 and 9. Mice then received MR1 or CTLA-4Ig (i.p.) every three days till day 35 post-immunization. (v) Immunized but no treatment. Mice were bled every week and on the day of sacrifice, and the spleen, inguinal LNs, brachial LNs and axillary LNs were collected for analysis of GC B cells.

Statistical analysis

The statistical significance of differences in mean values, cell numbers, percentages were analyzed by student's T test (unpaired) or ANOVA using Prism 5 (Graphpad, San Diego, CA). P values ≤ 0.05 were considered statistically significant.

Results

Visualizing allospecific B cells with MHC Class I tetramers

To enhance the detection of K^d-reactive B cell clonotypes, the K^d molecule is tetramerized to increase the avidity and thus half-life of the interaction. This is accomplished by combining purified MHC class I K^d possessing a single biotin molecule at the carboxy terminus with streptavidin (SAV) at a 4 to 1 molar ratio, respectively. Visualization is then possible if the SAV is conjugated to a fluorochrome (16). Early studies by Mulder A et al. (2003) observed high background staining of human B cells to SAV-phycoerythrin (PE), and recent studies by Pape *et al.* (13) reported on a detectable population of B cells in naïve mice that bound specifically to PE. Thus, B cells binding to fluorescently labeled MHC Class I tetramers should comprise of at least two distinct populations of B cells - those recognizing the MHC Class I molecule directly or those recognizing the fluorochome-coupled SAV. Indeed, we observed that approximately 0.2% of B cells from naive C57BL/6 mice bound to SAV-PE or to SAV-allophycocyanin (SAV-APC) in the absence of the MHC Class I (Fig 1a).

To reduce false positive detection of fluorochrome-binding B cells, we used MHC Class I (K^d) tetramers conjugated to PE or APC simultaneously during the staining process. This dual fluorochrome approach has been shown to enable exquisite sensitivity in detecting low frequencies of antigen specific B cells (28). B cells from naïve C57BL/6 mice binding to K^d-PE and K^d-APC could be segregated into three populations: those that only bound to K^d-PE or K^d-APC and those that bound to both the K^d-PE and K^d-APC tetramers; we refer to the latter population as Tet-DP cells for the remainder of this manuscript (Fig 1a). The total numbers of Tet-DP cells, PE-binding and APC-binding B cells in a naïve C57BL/6 mouse was approximately 19,241, 17,604 and 3,270 respectively. To further probe the specificity of the Tet-DP B cells, single-cell suspensions of spleen and lymph node cells of naïve C57BL/6 mice were stained with either the combination of K^d-PE and K^d-APC or SAV-PE and SAV-APC, and then enriched by anti-PE and anti-FITC microbead positive selection. A significantly higher percentage of K^d Tet-DP B cells (1.93%) was observed compared to the

SAV-DP B cells (0.07%), even after K^d Tet enrichment (Fig 1b). In addition, non-enriched single-cell suspensions of spleen and lymph node cells of naïve C57BL/6 mice were co-stained with K^d-PE, K^d-APC and an irrelevant ligand, H-2K^b-APC or SAV-APC. Only 5% of the K^d Tet-DP cells bound to H-2K^b-APC while <1% bound to the SAV-APC, confirming that the K^d Tet-DP cells in naïve mice were highly specific for K^d-reactive B cells (Fig 1c). Similar results were obtained with spleen cells isolated from a C57BL/6 mouse 14 days after receiving a BALB/c heart transplant (Supplemental Fig 1). Finally we confirmed that the K^d Tet-binding was BCR specific, by demonstrating significantly fewer K^d Tet-DP cells in MD4 mice that express a transgenic BCR specific for hen egg lysozyme, compared to non-transgenic littermate controls (Fig 1d).

To determine the efficiency to which the K^d tetramers bound to the B cells that produce anti-K^d antibodies, we used a sensitive functional assay, as described by Pape et al. (13), that enabled the presence of these cells to be analyzed following immunization. K^d tetramer-binding cells were depleted from splenocytes isolated from naïve C57BL/6 mice, transferred into B cell deficient mice and then immunized with BALB/c (H2d) splenocytes; the control group consisted of transferring non-depleted cells (Fig. 2a). Three weeks after immunization, the serum was collected and assessed for circulating anti-K^d antibodies as well as for anti-BALB/c DSA. Mice receiving K^d tetramer-depleted spleen cells did not produce detectable anti-K^d antibodies, confirming in this sensitive assay that the K^d tetramers efficiently bound to virtually all K^d-specific B cells (Fig 2b). Although K^d-specific antibody responses were voided, the remaining allo-specificities were intact as they were able to produce DSA specific for other BALB/c antigens that were expressed and detected on BALB.B targets, comparable to mice receiving non-depleted spleen cells (Fig 2c) and significantly above negative control with no serum added or naïve serum (Supplemental Fig 2).

Quantifying the expansion of allospecific B cells following immunization

We quantified the expansion in the numbers of K^d Tet-DP B cells in the draining (DLN) lymph nodes following a sub-cutaneous injection in the right flank with BALB/c spleen cells (Fig 3a). The negative control consisted of cell in the contralateral non-draining (NDLN) lymph node of the same immunized mice. At 7 day post-immunization, we observed an approximately 6-fold increase in the total number of cells in the DLN versus the NDLN, and a 16-fold increase in total number of K^d Tet-DP B cells.

Because activated B cells can become sticky and prone to non-specific binding of the K^d-tetramers, we additionally compared the K^d Tet-DP B cell response in C57BL/6 mice immunized to BALB/c versus BALB.B spleen cells that are MHC-matched to C57BL/6 but minor-mismatched. Following both immunizations, the total numbers of cells in the DLNs were comparable (Fig 3b) and significantly increased over the NDLN (Fig 3a). However, the percentage and total numbers of K^d Tet-DP B cells were significantly increased by three-fold only in mice immunized with BALB/c splenocytes but not in mice immunized with BALB.B (Fig 3b). Thus collectively, these results confirm the specificity of the dual fluorochrome-single tetramer approach in tracking the fate of alloreactive B cells, and also provide insights into the magnitude of the B cell response to a single MHC Class I antigen in the context of an alloimmune response elicited to complete MHC and minor antigen mismatch BALB/c splenocytes.

Quantifying the activation of allospecific B cells following immunization

Within hours of antigen encounter by the B cell receptor, B cells rapidly down regulate surface IgD, up regulate MHC Class II and CD86, and migrate toward the T-B cell interface, where these B cells present antigen and provide co-stimulatory signals to $CD4^+$ T cells

(reviewed in (23)). These activated T cells then provide pro-survival and instructive signals, comprising CD154-CD40, Icos-IcosL and cytokines, to B cells. After 2-4 days at the T-B zonal interface, the proliferating B cells commit to three different fates – extrafollicular PCs, memory B cells or GC B cells. In the GC, B cells undergo repeated interactions with follicular dendritic cells (FDC) and T follicular helper cells (Tfh), and then further B cell proliferation, somatic hypermutation and class switch recombination. High affinity, class-switched cells emerge from the GC as a second wave of PCs and quiescent memory B cells. We used the K^d-tetramer system to track the activation of alloreactive B cells, and observed that at 7 days after exposure to BALB/c splenocytes cells, 38.4-46.0% of K^d Tet-DP B cells had down-regulated their surface IgD compared to only 1.3-5.4% of the mice immunized with BALB.B splenocytes or in the NDLD (Fig 4). The total numbers of K^d Tet-DP B cells in the DLN was 13,933 and 688 in the BALB/c versus BALB.B immunized mice, reflecting an 18.9-fold increase upon exposure to specific alloantigen (Table 1).

When K^d Tet-DP IgD^- B cells from BALB/c-immunized mice were further analyzed, the majority had up regulated MHC Class II and CD86, indicative of their ability to enter into cognate interactions with T cells. Furthermore the majority (70-80%) of K^d Tet-DP IgD^- B cells expressed the GC markers, Fas and GL7 (29, 30). Approximately 13% of the IgD^- K^d Tet-DP were committed into the PC lineage; expressing high levels of the transcription factor, IRF4 (Fig 5) (31) (32) (33-35). We further confirmed that majority of the $IRF4^{hi}$ B cells co-expressed CD138, another marker of PCs (Supplemental Fig 3). When the total cell numbers were quantified (Table 1), we observed a 4-10-fold increase in IgD^- K^d Tet-DP B cells that had increased expression of MHC Class II/CD86, and a 30-40-fold increase in cells that had acquired the GC phenotype. Finally, committed PCs were only observed in mice immunized with BALB/c and not in the negative control mice immunized with BALB.B splenocytes.

Kwun J et al. (22) focused on the IgD^- K^d Tet-DP B cells that emerge following encounter with alloantigen, but did not comment on the IgD^+ K^d Tet-DP B cells that are present in both naïve and immunized mice because of concerns of a lack of specificity of the MHC Class I tetramers for the IgD^+ B cells. Because our approach of using the dual fluorochrome-single K^d tetramer approach results in the specific identification of K^d-binding B cells, we were able to extend our analysis to the IgD^+ K^d Tet-DP B cells cells in BALB/c and BALB.B immunized mice (Figs 4 & 6). The total number of these IgD^+ B cells increased 2-fold (Fig 4), and exhibited modest signs of activation (increased MHC Class II, CD86, and GL7 expression) that were nevertheless statistically significant. These data suggest that at least some of the IgD^+ K^d Tet-DP B cells had encountered antigen, but it remains to be determined whether these cells go on to develop into GC B cells or early memory B cells as has been recently described (36).

Tracking alloreactive B cells in the spleen following BALB/c heart transplantation

The above experiments involving immunization with BALB/c splenocytes provide important proof-of-principle that we can specifically detect K^d Tet-DP B cells in the DLN. We extended those findings to demonstrate that we can also track alloreactive B cells in the spleen following heterotopic BALB/c cardiac transplantation into C5BL/6 mice (Table 2). Overall the fate of the K^d-binding B cells resembled that observed with subcutaneous immunization with BALB/c spleen cells, with the emergence of IgD^- cells that expressed increased MHC Class II, CD86. At the time of rejection, day 7 post-transplantation, approximately 65% of these IgD^- K^d Tet-DP B cells were GC B cells (Fas^+GL7^+) and 10% were $IRF4^{hi}$ PCs. By day 14 post-transplantation, a significantly increased number and percentage of K^d Tet-DP B cells demonstrated signs of antigen encounter, namely downregulated IgD and upregulated MHC Class II. While the total number of K^d Tet-DP B cells that acquired the GC phenotype remained the same as on day 7, the number of $IRF4^{high}$

PCs on day 14 post-transplantation doubled compared to day 7, consistent with a further maturation of the alloreactive B cell response (Table 2). Finally, while there were significant numbers of B cells in the heart at the time of acute rejection, the percentage of K^d Tet-DP B cells were not further enriched compared to the spleen (data not shown).

Delayed treatment with anti-CD154 or CTLA4-Ig attenuated ongoing GC responses

Recent observations by Taylor et al (36) and Kaji et al. (37) indicated that CD40-mediated interactions are necessary for sustaining GC reactions, and that delayed initiation of anti-CD154 treatment at day 5-6 post-immunization resulted in a dramatic reduction of GC B cells and post-GC B cells. Because these observations could have therapeutic implications in regards to the inhibition of an ongoing antibody response, we defined the effects of delayed administration of anti-CD154 antibodies at 7 days post-sensitization, when the GC response was clearly developed (Table 1). By analyzing serum titers of anti-K^d over time, we observed that anti-CD154 treatment significantly inhibited the ongoing allo-IgG response (Days 14 and 21) but not the initial wave of antigen specific antibodies (Day 7) demonstrating that delayed anti-CD154 treatment prevented the expansion but not the priming of plasma cell precursors. Importantly, this inhibition was comparable to anti-CD154 administered on day 0 (Fig 7a) suggesting that this treatment effectively blocks T cell dependent maintenance of K^d-specific B cells that would have progressed to plasma cell differentiation. This possibility was investigated by enumerating the numbers of K^d Tet-DP B cells 4 weeks after treatment. Indeed, the percentage and total numbers of GC K^d Tet-DP B cells were significantly reduced compared to untreated sensitized mice and comparable to naïve mice and (Fig 7b). Remarkably, delayed intervention with CTLA-4Ig had comparable effects as anti-CD154 (Fig 7a&b).

To gain better insight into the kinetics of the inhibition, we analyzed a cohort of sensitized mice that underwent only 7 days of anti-CD154 or CTLA-4Ig treatment. We did not detect K^d Tet-DP B cells with the GC phenotype in both treatment groups, confirming that delayed anti-CD154 or CTLA-4Ig therapy was able to rapidly prevent further increase in circulating antibody titers by dissolving established GCs and inhibiting the generation of new GCs. These flow-cytometry data were confirmed by immunofluorescence-based staining of tissue sections performed on the draining LN following treatment with CTLA-4Ig for 7 days (from day 7-14 post-immunization) and demonstrating that the GCs (IgD^-PNA^+) after treatment with CTLA4-Ig were significantly smaller even when compared to day 7 post-immunization controls (Table S5).

Discussion

The ability to track endogenous alloreactive B cells at resting state and also following antigen exposure provided us with an unprecedented opportunity to investigate the cellular basis of the alloantibody response. Recent reports of MHC or HLA Class I tetramers being used to detect allospecific B cells by flow cytometry in mice and in humans have used a single tetramer conjugated to a single fluorochrome, which simultaneously identifies B cells that recognize Class I and the SAV-fluorochrome. Molecules comprising the SAV-conjugated fluorochrome are large proteins (475 D - 240 kD) derived from bacteria and algae, thus it is not surprising that significant numbers of B cells in naive C57BL/6 mice display specificities to these structures. With the single tetramer-single fluorochrome approach, approximately half of the B cells identified may actually not be allospecific but binding to the SAV-fluorochrome. To address the problem of SAV-fluorochrome binding B cells we used the approach of a single K^d tetramer bound to two different fluorochromes, which allowed the identification of alloreactive, MHC Class I-specific B cells in a highly specific manner. We have been cognizant of the issue of false positive detection and have validated the use of the K^d-tetramer in identifying the vast majority of allo-specific B cells

by showing (i) their accumulation in response to sensitization (Fig. 1&3) (ii) their acquisition of expected phenotypes; GC B cells, plasma cells, and activation markers in response to sensitization (Fig. 4-6) (iii) negligible binding to antigen-irrelevant (anti-HEL) transgenic B cells (Fig. 1d) and (iv) ≤ 10% binding by irrelevant fluorochrome-conjugated reagents (Fig. 1c). With respect to the last observation, it is unclear whether the "contaminant" population reflects true biological cross-reactivity or idiosyncratic interactions of isolated proteins and cells in a tube and the ultra-sensitive fluorescence detection methods employed with this approach. We were also able to demonstrate that the K^d tetramers bound to essentially all the B cells capable of producing anti-K^d antibodies, as the elimination of B cells binding to K^d tetramers resulted in a remaining B cell population that could no longer produce anti-K^d antibodies but were able to produce DSA of other specificities. These data confirm both specificity and efficiency of the MHC Class I tetramer approach to identify endogenous alloreactive B cells.

This approach has enabled us to quantify the total number of K^d-specific B cells in a naive C57BL/6 mouse to be approximately 20,000, comparable to the total number of APC-binding B cells. Single MHC Class I molecules can associate with a wide array of peptides, and T cell receptors have exquisite ability to discriminate between these peptide-MHC Class I complexes (16). In contrast the allo-determinants recognized by anti-Class I monoclonal antibodies have mapped predominantly to the polymorphic first and/or second external domains of the MHC Class I molecule (14, 38, 39). While alloantibodies and the corresponding BCR can discriminate between peptide-MHC Class I complexes (40, 41) the contribution of the peptide to the ability of the antibody to bind to the peptide Class I complex is relatively subtle. In these studies we used K^d tetramers bound to the *Plasmodium berghei* circumsporozoite peptide 252-260 (SYIPSAEKI) as a proof of principle of the technique; however, it remains to be confirmed experimentally whether and to what degree K^d tetramers bound to self-peptides would alter the absolute numbers of K^d–specific B cells identified.

We traced the activation of K^d–specific B cells following alloantigen exposure, by examining the down-regulation of surface IgD, and the up regulation of MHC Class II and CD86 (Table 1). The expression of the latter two sets of molecules on approximately 45% of the K^d Tet^+ B cells imply involvement of these cells in alloantigen presentation and co-stimulation via the indirect pathway. In addition, approximately 43% of K^d Tet^+ B cells were Fas^+GL7^+ GC cells, while about 12% were committed to the PC lineage ($IRF4^{hi}$). Thus at day 7 post-immunization, we observed that most of the alloreactive B cells were in a GC reaction, consistent with our understanding of alloantibody production being T cell-dependent and class-switched to IgG (42, 43). Importantly, it is the products of the GC that will go on to constitute the pathogenic effects of B cells in sensitized patients – high affinity long lived PCs and memory B cells. While we are able to trace the B cells committed to the PC lineage, a potential limitation of this cell-surface staining approach is inefficient detection of fully-differentiated PCs that are known to down-regulate their BCRs.

While the majority of B cells that expressed the activation markers were in the IgD^- population, we observed a modest 2-fold increase in the numbers of K^d Tet^+ IgD^- B cells in the DLN following immunization with allogeneic spleen cells, with a small but detectable subset of these cells exhibiting increased MHC Class II, CD86 and GL7. While more detailed time course and cell tracking experiments are necessary, we speculate that these cells are most likely poised cells at the earliest stages of antigen encounter or differentiated memory B cells (13).

A current challenge in clinical transplantation is finding immunosuppressive agents that effectively halt an ongoing alloreactive antibody responses. Here we showed that a delayed

administration of anti-CD154 to day 7 post-immunization, when GC had already been established, was able to rapidly halt further increase in circulating allo-IgG titers and dissolve established GCs. Also because anti-CD154 is not approved for human use whereas a high affinity mutant of CTLA-4Ig, belatacept, has recently received FDA approval for use in clinical renal transplantation (44), we also demonstrated that CTLA-4Ig was able to comparably halt an ongoing alloantibody response. This latter observation is surprising because CD28 signaling has been reported to be necessary for the initiation of GC development but is not required for T cell-dependent B cell proliferation within established GCs (45). Thus our demonstration of the ability of CTLA-4Ig to rapidly halt a developing antibody response bodes well for its use in the clinic, and provides insights into the requirement for sustained CD28-B7 interactions in GC maintenance. One caveat is that these studies involved allogeneic spleen cell transfusion, and a theoretical possibility exists that GC activity following solid organ transplantation may be more resistant to these therapies.

The studies with subcutaneous injection of BALB/c splenocytes allowed us to define the precision of this approach in tracing the fate of alloreactive B cells in the draining lymph nodes, but a significant percentage of alloreactive immune responses in the clinic are elicited in the context of solid organ transplantation. Thus we also provide data confirming that this approach can be used to visualize the K^d-specific B cell response in the spleen of mice receiving heterotopically transplanted hearts (BALB/c into C57BL/6 recipients). We report that the magnitude of the K^d-specific B cell response at day 7 post-transplantation in the spleen, namely their entry into the GC and commitment into the PC fate was comparable, albeit reduced in magnitude, to that observed in the DLN following allosensitization with subcutaneous injection with BALB/c spleen cells. At day 14, there is a further increase in the percentage and total number of K^d Tet-DP B cells, and of those committed to the PC fate. Interestingly, K^d-specific B cells were not enriched in the acutely rejecting hearts.

The role of antibodies in mediating acute or chronic transplant rejection in the clinic is now widely accepted (4, 42, 46-48). Furthermore, the consensus is that current immunosuppression is most effective for controlling T cell responses, and is less effective at controlling alloantibody production in pre-sensitized individuals awaiting transplantation (7, 8, 49, 50). Both naïve and memory B cells can differentiate into short-lived or as long-lived PCs that produce circulating alloantibodies. Here we describe in detail the use of MHC Class I tetramers to trace the fate of all MHC Class I-reactive B cells responsible for the systemic production of alloantibodies, thereby providing a versatile and a powerful way to analyze the cellular and molecular processes that lead to the production of alloantibody. This ability to specifically monitor the activation, proliferation and differentiation of naive alloreactive B cells in the secondary lymphoid organs will allow us to understand the mechanistic basis for these events and to identify the efficacy and limitations of current immunosuppressive strategies. The challenge will be to apply this approach to monitor changes in alloreactive B cell subsets in the peripheral blood of transplant patients where lymphoid organs are not readily available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AP	alkaline phosphatase
APC	allophycocyanin
BCR	B cell receptor
DLN	draining lymph node
FITC	fluorescein isothiocyanate
GC	germinal center
IRF4	Interferon response factor 4
MFI	mean channel fluorescence
NDLN	non-draining lymph node
O.D.	optical densities
PC	plasma cell
SAV	streptavidin
Tet-DP	tetramer-double positive

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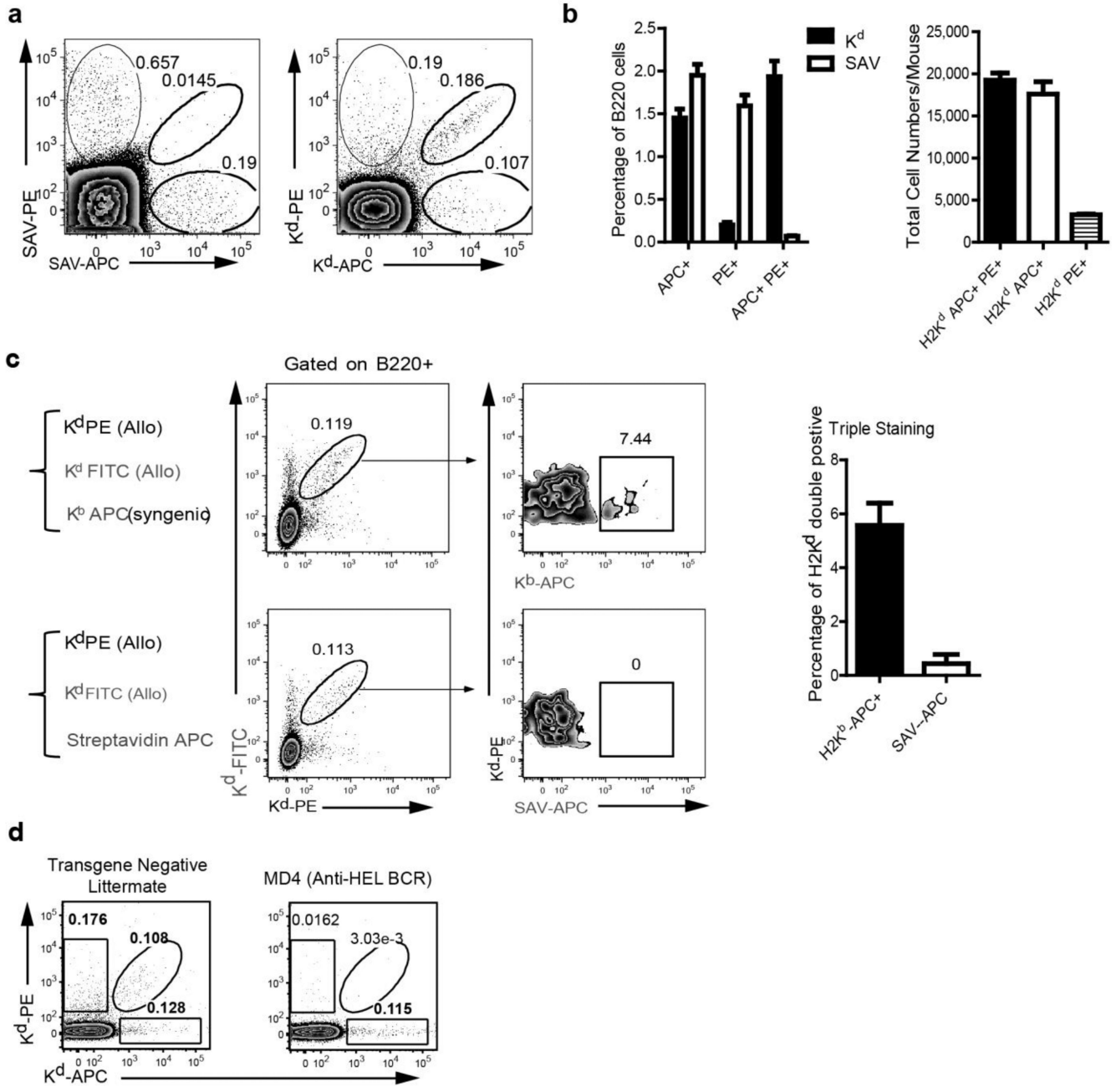

Fig 1.

Visualizing K^d-specific B cells in naïve C57BL/6 mice with fluorescent K^d-tetramers. (a) The percentage of $B220^+$ B cells binding to Streptavidin-phycoerythrin (SAV-PE), Streptavidin-allophycocyanin (SAV-AP), or both (Left), compared to binding of K^d tetramer-PE or K^d tetramer-APC or both (Right). (b) To enhance detection of alloreactive B cells, single-cell suspensions of spleen and lymph node cells of naïve C57BL/6 mice were stained with either the combination of K^d-PE and K^d-APC or SAV-PE and SAV-APC, and then enriched by anti-PE and anti-APC microbead positive selection. The percentage (following enrichment) and total numbers of B220+ B cells in a naïve C57BL/6 mouse binding only to K^d tetramer-PE or K^d tetramer-APC or to both (K^d Tet-DP) were determined. (N=4). (c) K^d Tet-DP (K^d-PE and K^d-FITC tetramers) B cells display minimal

binding to irrelevant molecules, K^b Tet^+ APC or SAV-APC. Data are the mean and SEM of 4 different individuals. (d) K^d Tet DP B cells are reduced in MD4 HEL BCR transgenic mice. Inguinal LNs from MD4 BCR transgenic mice or transgene negative littermates were collected and stained for K^d Tet DP B cells.

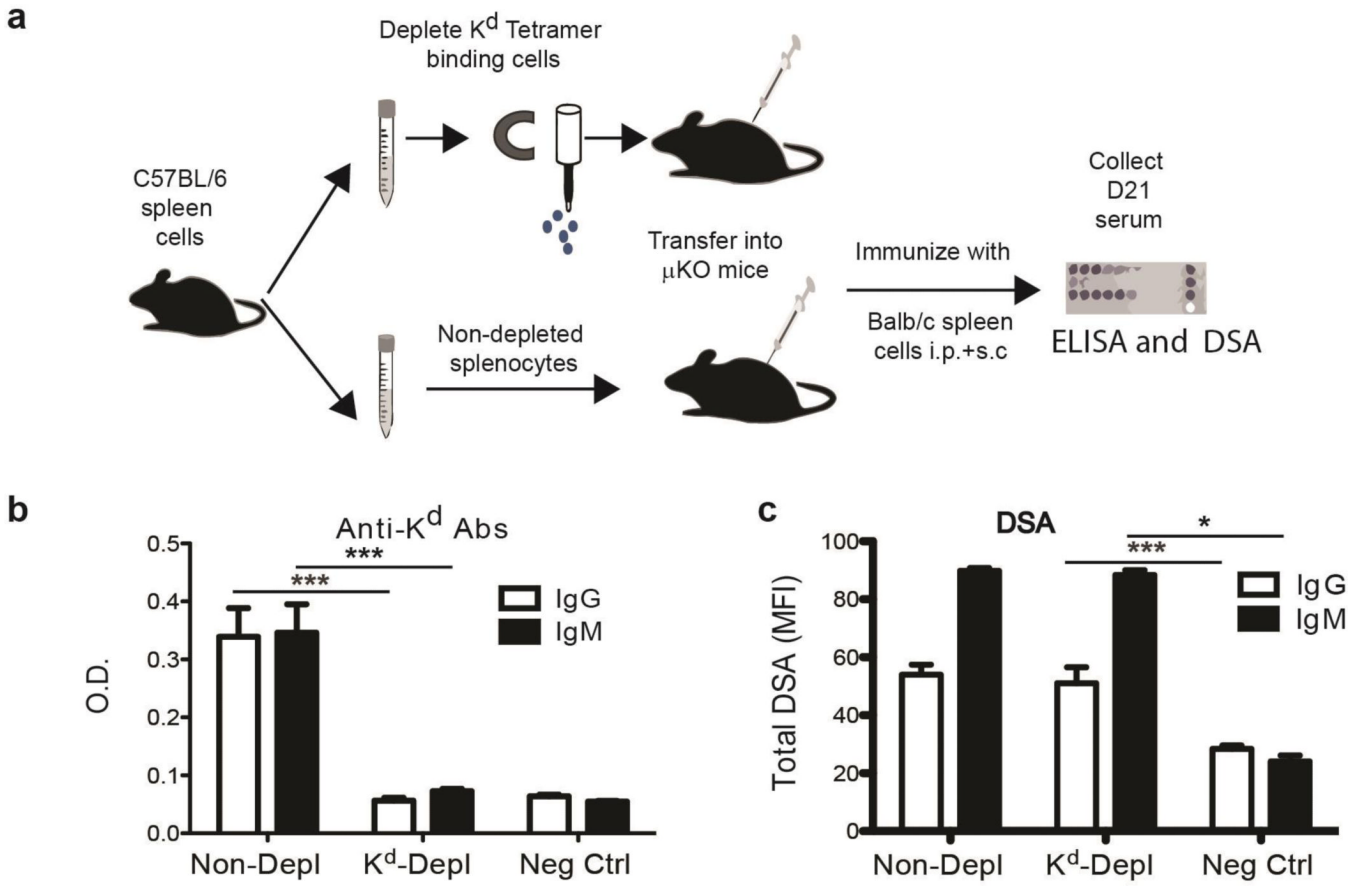

Fig 2.

Efficient binding and depletion of alloreactive K^d-specific B cells with K^d-tetramers. (a) Experimental design where K^d tetramer-depleted or non-depleted spleen cells from naïve C57BL/6 mice were adoptively transferred into B cell-deficient (μMT) mice. Depletion resulted in a 4% reduction of total spleen cells and a 1.25% reduction in total B cells. One day later these mice were immunized with BALB/c spleen cells, and 21 days later the serum was harvested and assessed for the presence of anti-K^d antibodies by (b) ELISA and for (c) DSA using flow cytometry and BALB.B targets. Negative controls were the optical densities (O.D.) or mean channel fluorescence (MFI) of wells or cells not receiving serum. All data are the mean and SEM of 3-4 mice, and statistically significant differences are indicated (*$p<0.05$; ***$p<0.005$).

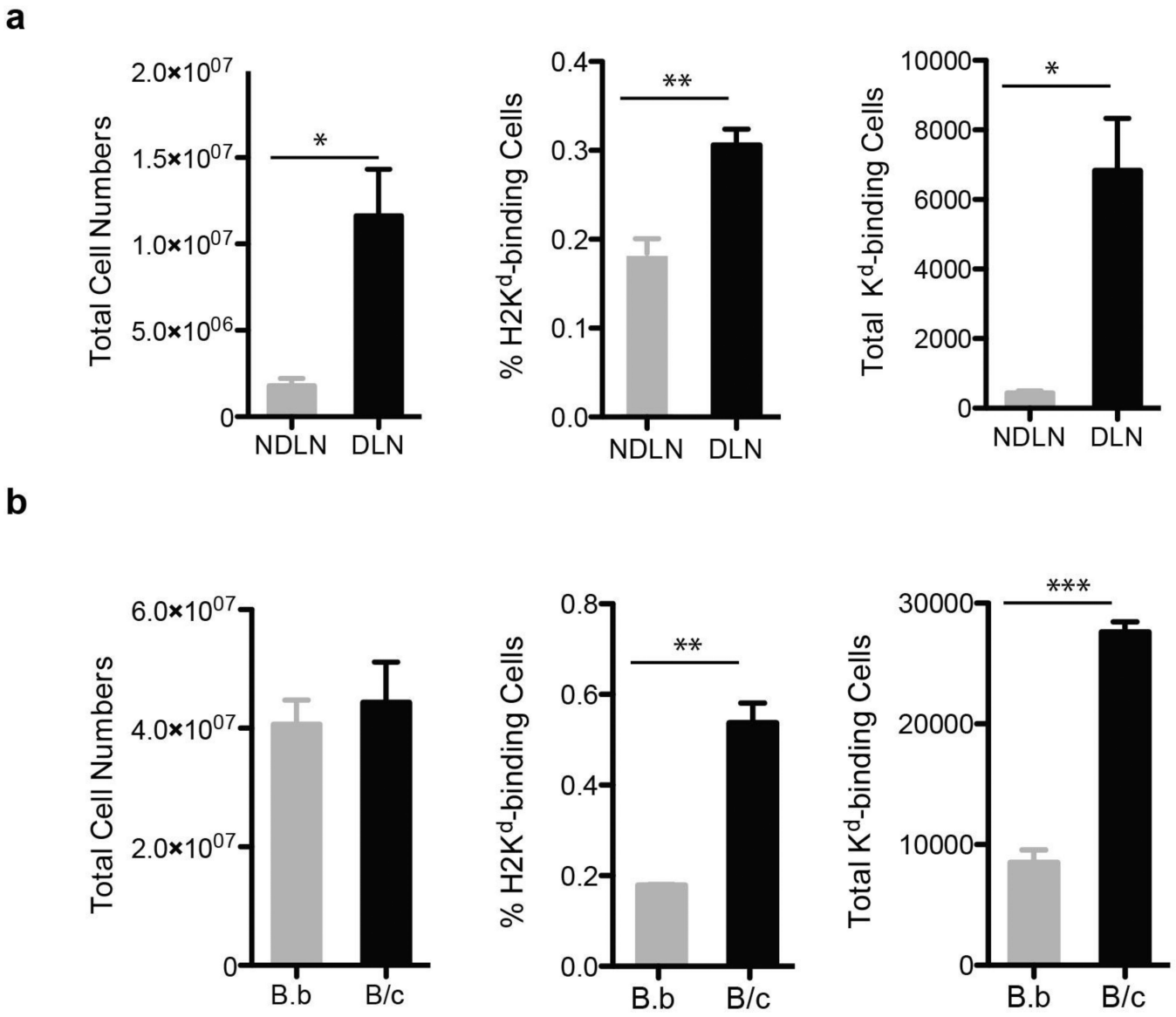

Fig 3.

Quantifying the expansion of K^d-binding B cells at day 7 after immunization with BALB/c (B/c) or BALB.B (B.b) splenocytes. The total number of cells recovered, percentage of K^d Tet-DP B cells of $B220^+$ cells, and total number of K^d Tet-DP B cells from (a) the draining lymph node (DLN) compared to the contralateral non-DLN (NDLN); or (b) from the DLN of mice immunized with BALB/c or BALB.B splenocytes. Data are the mean and SEM of three mice per group. In (a) only one side of the mouse was immunized with BALB/c splenocytes to allow the retrieval of draining and non-draining lymph nodes, whereas in (b) both sides of the mouse were immunized with BALB/c or BALB.B splenocytes.

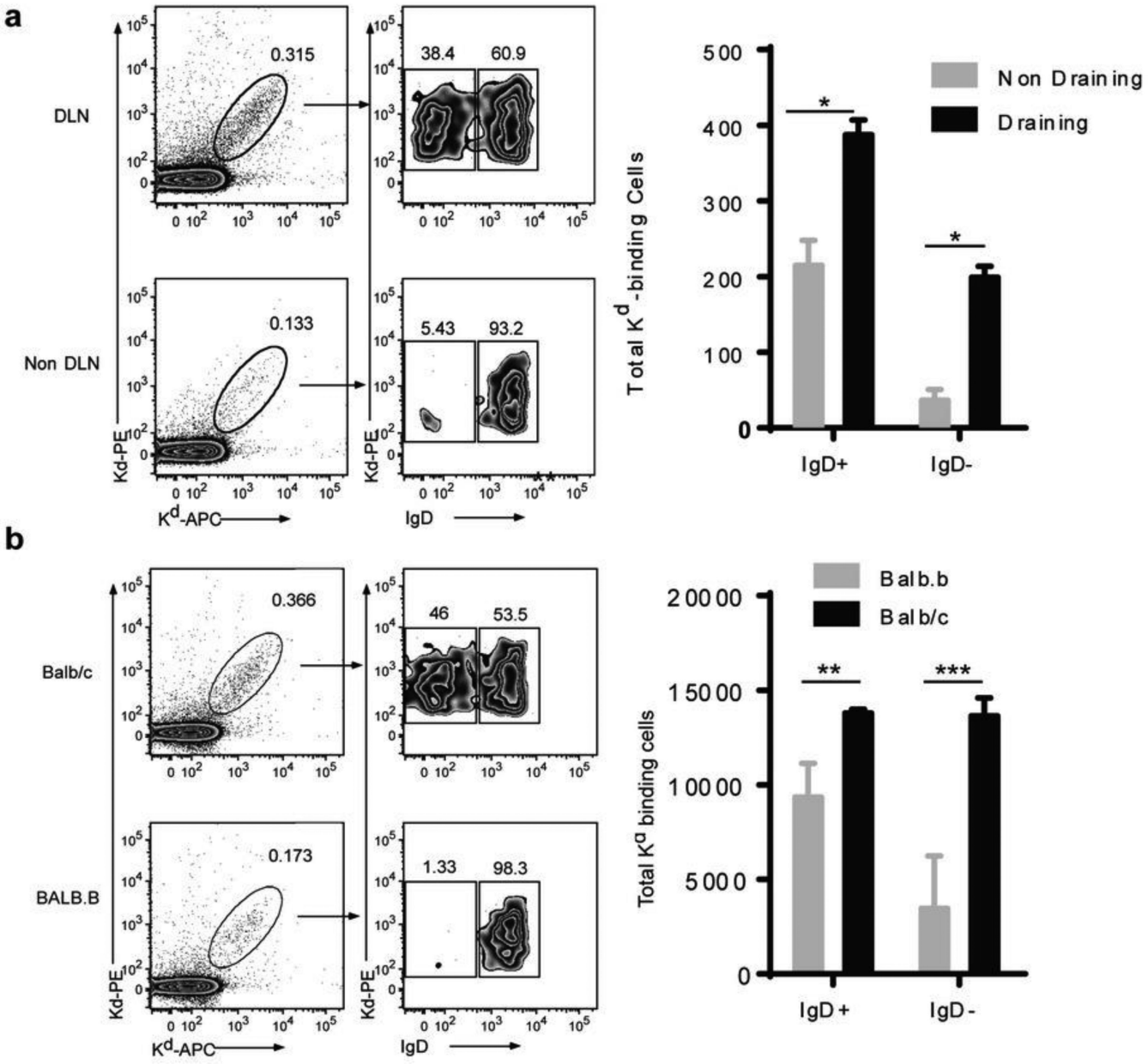

Figure 4.

Emergence of IgD^{-} K^{d} Tet-DP B cells at day 7 after immunization with BALB/c or BALB.B splenocytes: (a) comparing draining versus non-draining lymph nodes, and (b) comparing BALB/c versus BALB.B immunization. Detailed gating strategy is provided in Supplemental Fig 4. All data are the mean and SEM of 3-4 mice, and statistically significant differences are indicated (*p<0.05; **p<0.01; ***p<0.005).

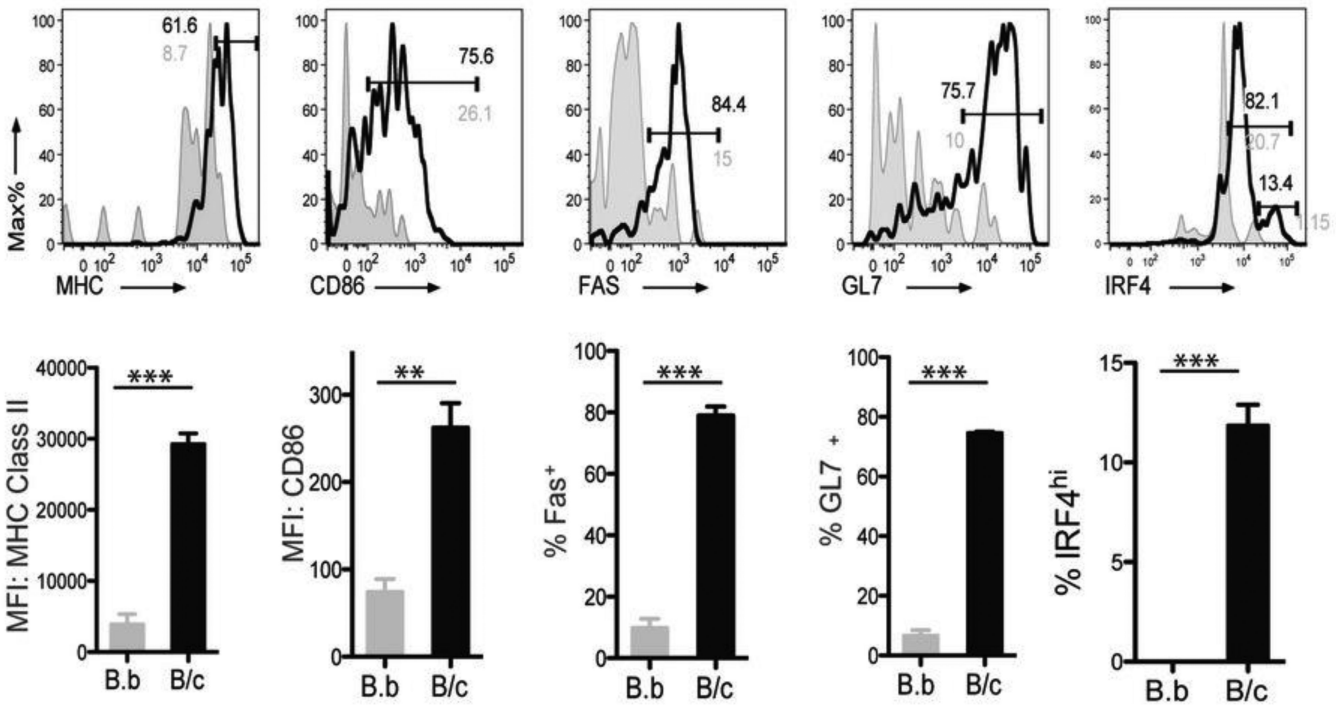

Fig 5.

Tracking the changes in phenotype of K^d Tet-DP B cells at day 7 after immunization with BALB/c or BALB.B splenocytes. Increased percentages (top) of IgD^- K^d Tet-DP B cells expressing MHC Class II and CD86 and their mean fluorescence intensities (MFI) (bottom), as well as increased percentages of IgD^- K^d tetramer-binding B cells expressing Fas, GL7 and IRF4 in the DLN of mice immunized with BALB/c (black) compared to BALB.B (grey) splenocytes. All data are the mean and SEM of 3-4 mice, and statistically significant differences are indicated (**$p<0.01$; ***$p<0.005$).

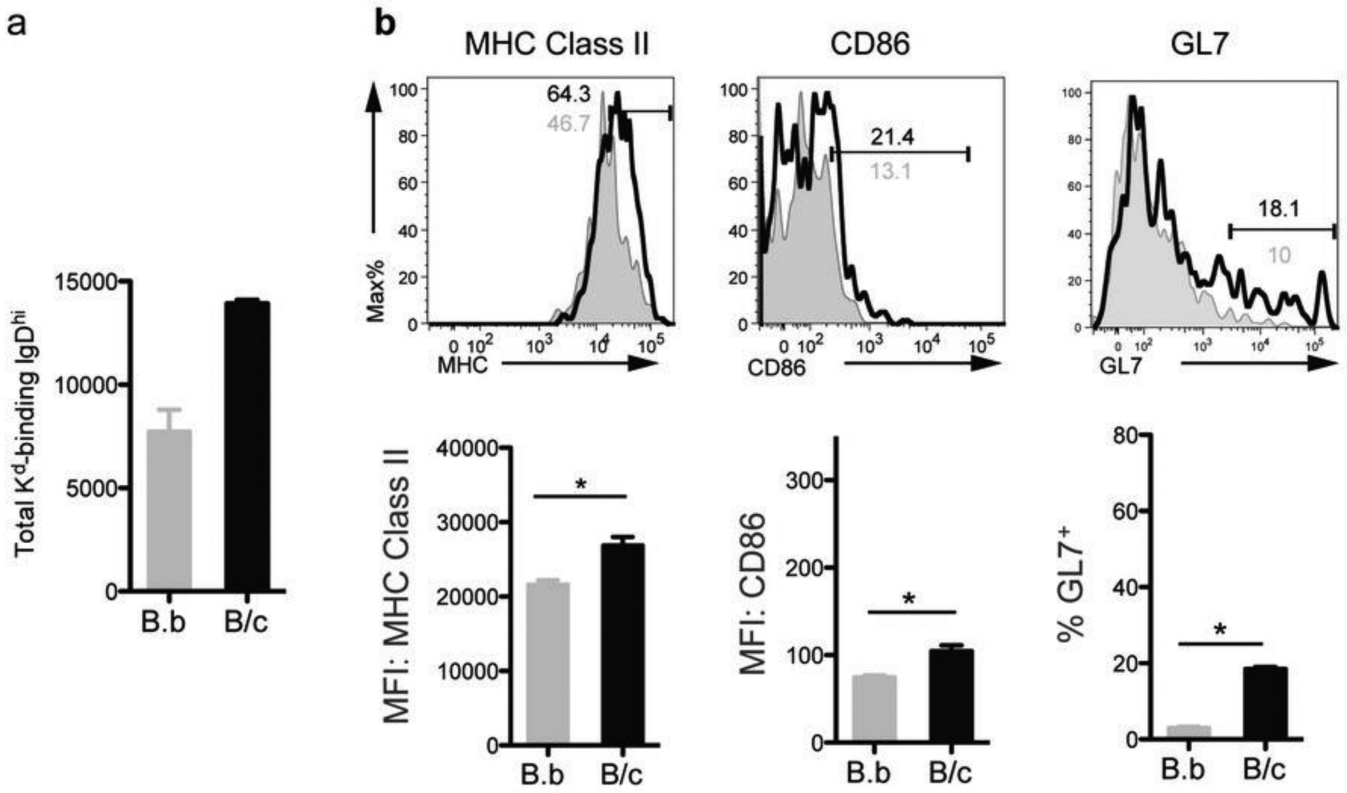

Fig 6.

Tracking the changes in the total number and phenotype of IgD^+ K^d Tet-DP B cells at day 7 after immunization with BALB/c or BALB.B splenocytes. Modest but statistically significant increased percentages of IgD^+ K^d Tet-DP B cells expressing MHC Class II and CD86 (top) and their mean fluorescence intensities (MFI) (bottom), as well as the percentage of IgD^+ K^d Tet-DP B cells expressing $GL7^+$ cells in the DLN of mice immunized with BALB/c (black) compared to BALB.B (grey) splenocytes. Detailed gating strategy is provided in Supplemental Fig 4. All data are the mean and SEM of 3-4 mice, and statistically significant differences are indicated (*$p<0.05$).

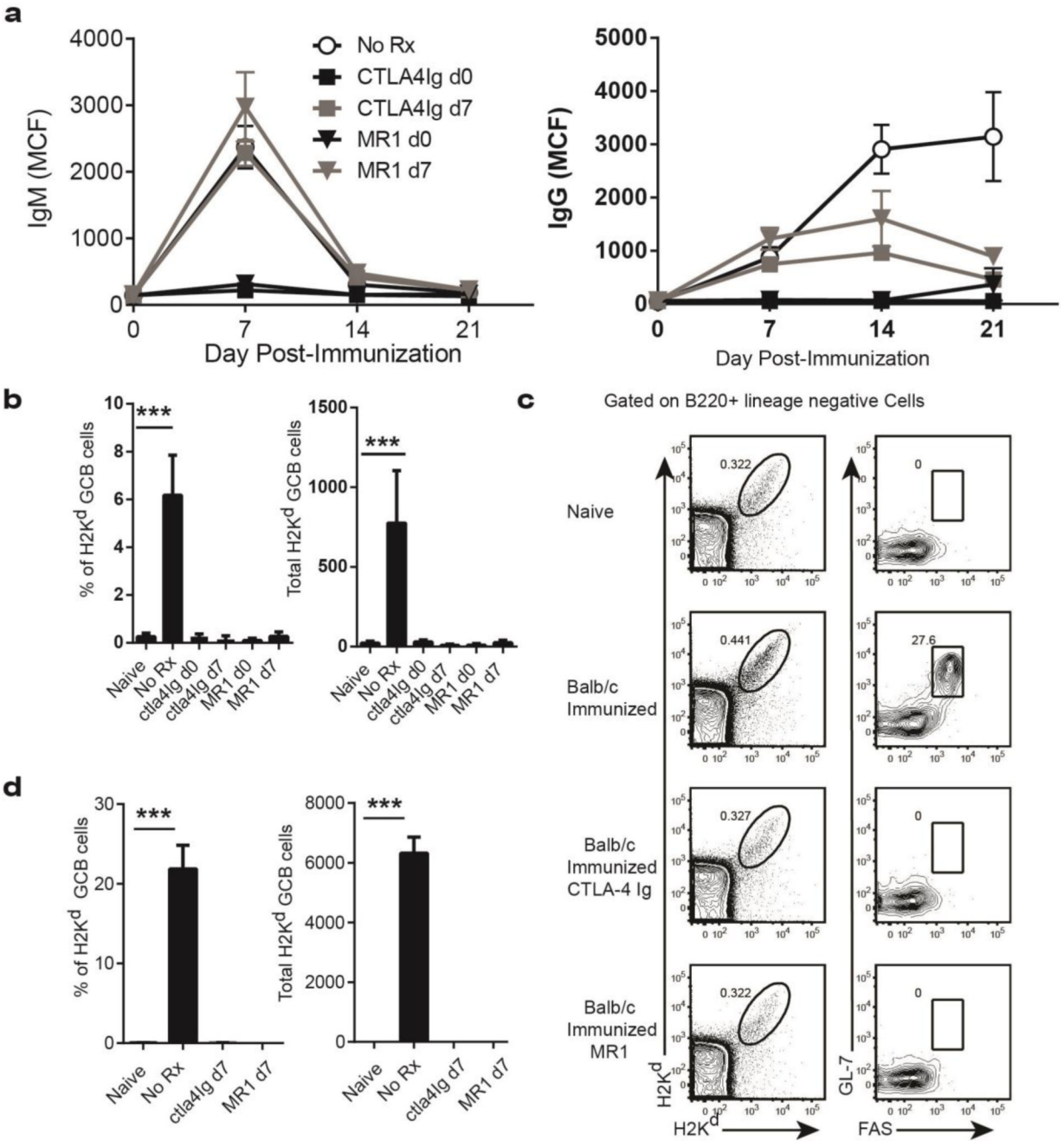

Fig 7.

Effect of delayed anti-CD154 or CTLA-4Ig administration on established GCs and developing alloantibody response. Anti-CD154 (500 μg/mouse) or CTLA-4Ig (500 μg/mouse) was administered starting on the day before (d0) or 7 days (d7) after immunization with BALB/c splenocytes. Positive controls were immunized, untreated (No Rx) mice. All groups receiving anti-CD154 or CTLA-4Ig demonstrated reduced (a) donor-specific antibodies and (b) reduced percentages and total numbers of GC K^d Tet-DP B cells at the end of the 28 day treatment period, compared to the immunized, untreated group. (c & d) Effect of delayed treatment with anti-CD154 or CTLA-4Ig from day 7-14 post-immunization on the percentage and total numbers of GC K^d-Tet DP cells. Untreated (No

Rx) group were sacrificed on day 7 post-immunization. All data are the mean and SEM of 4-5 mice, and statistically significant differences are indicated (***$p<0.001$).

Table 1

Magnitude of the anti-K^d B cell Response in the draining lymph node of mice immunized with BALB/c or BALB.b Spleen Cells

	Total LN	**Tet^+**	**IgD^{hi}**	**IgD^{lo}**	**$MHCII^{hi}$**	**$CD86^{hi}$**	**Fas^+GL7^+**	**$IRF4^{hi}$**
BALB.B	40.7×10^6	8537	7,727	688	4,122	1,310	361	0.0
BALB/c	44.4×10^6	27,633	13,933	13,033	16,231	12,621	12267	3,275
Fold Increase	**1.1**	**3.2**	**1.8**	**18.9**	**3.9**	**9.6**	**33.9**	**>3275**

Table 2

Magnitude of the anti-K^d B cell Response in the Spleen of C57BL/6 recipients at day 7 and day 14 post-BALB/c heart transplantation

	Total Spl	**Tet^+**	**IgD^{hi}**	**IgD^{lo}**	**$MHCII^{hi}$**	**$CD86^{hi}$**	**Fas^+GL7^+**	**$IRF4^{hi}$**
None	81×10^6	23,121	17,607	5,021	460	137	168	0
BALB/c HT× D7	157×10^6	35,419	18,522	16,133	9,611	592	10,460	962
Fold Increase	**1.9**	**1.5**	**1.1**	**3.2**	**20.9**	**4.3**	**62.3**	**>962**
BALB/c HT× D14	135×10^6	44,027	18,865	24,435	12,305	305	10,548	1,905
Fold Increase	**1.7**	**1.9**	**1.1**	**4.9**	**26.8**	**2.2**	**62.8**	**>1905**