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B lymphocytes contribute to indirect pathway T cell sensitization via acquisition of extracellular vesicles

Pablo D. Becker[1] | Kulachelvy Ratnasothy[1] | Monica Sen[1,2] | Qi Peng[1] | Marco Romano[1] | Jordan Bazoer[2] | Erik Suvitra[2] | Anas Stout[2] | Shannon G. Hylton[2] | Anthony Dorling[3] | Robert I. Lechler[1] | Lesley A. Smyth[1,2] | Giovanna Lombardi[1]

[1]MRC Centre for Transplantation, Peter Gorer Department of Immunobiology, School of Immunology & Microbial Sciences, King's College London, London, SE1 9RT, UK

[2]School of Health, Sports and Biosciences, University of East London, London, UK

[3]MRC Centre for Transplantation, Department of Inflammation Biology, Faculty of Life Sciences & Medicine, King's College London, London, UK

Correspondence
Lesley A. Smyth
Email: l.smyth@uel.ac.uk

Giovanna Lombardi
Email: giovanna.lombardi@kcl.ac.uk

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B cells have been implicated in transplant rejection via antibody-mediated mechanisms and more recently by presenting donor antigens to T cells. We have shown in patients with chronic antibody-mediated rejection that B cells control the indirect T cell alloresponses. To understand more about the role of B cells as antigen-presenting cells for $CD4^+$ T cell with indirect allospecificity, B cells were depleted in C57BL/6 mice, using an anti-CD20 antibody, prior to receiving MHC class I–mismatched (K^d) skin. The absence of B cells at the time of transplantation prolonged skin graft survival. To study the mechanisms behind this observation, T cells with indirect allospecificity were transferred in mice receiving a K^d skin transplant. T cell proliferation was markedly inhibited in the absence of recipient B cells, suggesting that B cells contribute to indirect pathway sensitization. Furthermore, we have shown that a possible way in which B cells present alloantigens is via acquisition of MHC-peptide complexes. Finally, we demonstrate that the addition of B cell depletion to the transfer of regulatory T cells (Tregs) with indirect alloresponse further prolonged skin graft survival. This study supports an important role for B cells in indirect T cell priming and further emphasizes the advantage of combination therapies in prolonging transplant survival.

KEYWORDS

animal models: murine, antigen presentation/recognition, B cell biology, immune regulation, immunobiology, immunosuppression/immune modulation, translational research/science

Abbreviations: Ab, antibody; AlloAg, alloantigen; APCs, antigen-presenting cells; BM-DCs, bone marrow–derived dendritic cells; Breg, regulatory B cell; CFSE, carboxyfluorescein succinimidyl ester; DCs, dendritic cells; EVs, extracellular vesicles; GM-CSF, granulocyte monocyte colony-stimulating factor; LNs, lymph nodes; MHC, major histocompatibility complex; MZ, marginal zone; PBS, phosphate-buffered saline; Tregs, regulatory T cells; WT, wild-type.

Lesley A. Smyth and Giovanna Lombardi are co-joint last authors

1 | INTRODUCTION

Solid organ transplantation is the best treatment available for organ failure; however, many transplants are lost within 12 years. The biggest single cause of transplant failure is immune-related injury.

T cell–mediated rejection is initiated when T cells are stimulated in response to graft alloantigens (alloAgs) via three major pathways of allorecognition. (1) The "direct pathway" where recipient T cells recognize intact donor major histocompatibility complex (MHC) molecules from donor antigen-presenting cells (APCs), and (2) the "indirect pathway" where presentation of donor alloAgs occurs after processing and presentation by host recipient APCs.[1] In the third pathway of allorecognition, the "semi-direct," recipient APCs acquire intact donor MHC molecules and present to recipient T cells with direct allospecificity.[2] We have recently demonstrated in a mouse model of skin transplant that the semi-direct pathway lasts for the entire duration of the life of the transplant, implying that the direct recognition of donor alloAgs plays a role not only early posttransplant but also at late time points posttransplant.[3] Recently, Morelli et al and Marino et al have suggested that "cross-dressing" of recipient dendritic cells (DCs) with donor MHC molecules via the acquisition of extracellular vesicles (EVs), is the main contributor of priming of the direct alloresponse in skin graft rejection early posttransplant.[4,5]

The presence of antibodies (Abs) against HLA is in most cases a predictive marker of transplant failure, and the level of circulating Abs against donor antigens correlates with chronic Ab-mediated rejection.[6,7] B cells are best known for their unique role as Ab-producing cells and Abs binding to the graft lead to the deterioration of the organ by Ab-mediated rejection mechanisms. Recent data have shown an additional role for recipient B cells, demonstrating that these cells can contribute to graft survival/rejection by presenting alloAgs to recipient T cells.[8-10] As discussed later, we have shown that B cells specific for alloAgs are particularly efficient in amplifying T cell responses with indirect allospecificity.[9] It is important to note that there is compelling data from murine models suggesting that the antigen-presenting functions of B cells, rather than antibody formation, are the primary reason why B cells are required for chronic rejection.[11-14]

Depletion of B cells has been applied in different murine models to improve transplant survival of MHC mismatched allografts. However, a diverse range of outcomes has been observed. In one study, B cell depletion did not alter the survival of fully mismatched kidney or heart allografts,[8] whereas in other studies accelerated rejection was observed.[15] The latter was hypothesized to be a consequence of depletion of IL-10–producing regulatory B cells (Bregs), which serve to suppress antigen-specific responses.[16-20] Furthermore, although some of the early experiments using depleting Abs suggested that B cells play an important role during T cell priming,[21-23] further research using genetically modified mice deficient in the B cell compartment produced conflicting results.[14,24-29]

In the clinical setting, several desensitization therapies aiming to target donor-specific antibody (DSA) levels in highly sensitized patients prior to transplantation are being tested, many of which involve B cell depletion. However, the differences in protocols, particularly the time of administration of the B cell–depleting drug, such as an anti-CD20 Ab rituximab, make the interpretation of the results difficult. One study has reported that rituximab induction therapy may promote rather than inhibit acute cellular rejection in some patients,[30] with one of the mechanisms suggested being depletion of B cells with regulatory function. In agreement with this idea, we have recently shown that operationally tolerant kidney-transplanted patients have "transitional" regulatory B cells that maintain high levels of IL-10 expression, further supporting the concept that some subpopulations of B cells might enhance graft survival.[20]

Furthermore, we have recently published that B cells make complex and dynamic contributions to the patterns of alloresponses seen in patients with chronic Ab-mediated rejection, including patterns consistent with suppression of alloresponses in some. The data suggest that B cells, in addition to allo-Ab production, are able to induce indirect T cell alloresponses by acting as APCs in some, but suppress indirect alloreactive T cell responses, at least in vitro, in others.[9]

In this study, the role of B cells as APCs for indirect $CD4^+$ T cell alloresponses was examined in vivo. B cells were depleted (using an anti-CD20 Ab) in recipient mice receiving MHC class I–mismatched (K^d) skin grafts. We demonstrate that B cells contribute to the activation of $CD4^+$ T cells with indirect alloresponses via acquisition of donor MHC molecules through the uptake of extracellular vesicles.

2 | MATERIAL AND METHODS

2.1 | Mice

Female BALB/c, C57BL/6 (B6), CBA/Ca mice were purchased from Harlan UK Ltd. (Biscester, Oxford, UK). TCR75$Rag^{-/-}$ (TCR75) and C57BL/6-Tg(K^d)Rpb (B6.K^d) were kindly provided by P. Bucy, IL-10–deficient mice were kindly provided by W. Müller and B6.H2K^{bm1}, OT-1$Rag^{-/-}$, and B6.mOVA were bred at Charles River, UK. All mice were maintained under sterile conditions (Biological Services Unit, New Hunt's House, King's College London).

2.2 | Flow cytometry

Cells were stained and analyzed as described elsewhere.[31] (For more details see Supporting information.)

2.3 | B cell isolation

B cell purification was achieved using untouched CD43 conjugated Dynal beads (Thermofisher) following manufacturer's instructions. (For more details see SI.)

2.4 | Skin transplantation

Donor tail skin grafts were performed and monitored as previously described.[31] In some experiments, anti-CD8 antibody (clone YTS169, 250μg/injection/mouse) was injected intraperitoneally at day 1 before and day 1 after skin graft, and weekly thereafter. When described, B cells were depleted with 200μg of anti-CD20 (clone 5D2, kindly provided by Genentech) by intravenous injection 7 days before skin transplantation.

2.5 | Generation of $CD4^+$ $CD25^+$ Treg line

Tregs with indirect specificity were created as outlined by Tsang et al.[32] (For more details see SI.)

2.6 | CFSE labeling of DCs and isolation of EVs

Bone marrow (BM)–derived dendritic cells (BM-DCs) were generated as previously described.[33] EVs were prepared as described in the SI.[34] To measure the concentration and size of the particles isolated, EVs were assessed on a NanoSight LM10 under a constant flow injection. Five videos of 30-second duration were recorded per sample. EVs isolated had a mean size of 159.2 nm and they expressed CD63 as assessed using an ExoELISA-ULTRA complete kit (CD63 detection), following the manufacturer's instructions (Systems Biosciences, California, USA). EV characterization is shown in Figure S1.

2.7 | DC, EV, and B cell co-culture and functional analysis

Recipient B cells were co-cultured for 18hours with carboxyfluorescein succinimidyl ester (CFSE)–labeled donor DC derived EVs at a ratio of EVs derived from 100 DCs: 1 B cell. B cells were cultured alone as control. Following co-culture, B cells were incubated with anti-CD16/32 Ab followed by anti-B220/CD45R APC conjugated Ab. Where described, B cells were incubated with anti-K^d APC-conjugated Ab. For functional analysis using EVs, recipient B6 B cells were co-cultured for 1hour with donor BALB/c or B6 DC-derived EVs as above before TCR75$Rag^{-/-}$ T cells were added at a 1:1 ratio of B-to-T cells. Cell culture supernatants were removed on day 3 and IL-2 assessed by IL-2–specific ELISA (ThermoFisher).

2.8 | Statistical analysis

Graft survival data were analyzed using the Kaplan–Meier method, with the Wilcoxon rank test and the log-rank test used to verify the significance of difference between the groups (GraphPad Prism). Statistical analysis of other data was performed using the two-tailed Student's *t* test for unpaired samples with unequal variance.

3 | RESULTS

3.1 | B cell subpopulations in the spleen express high levels of CD20

To further understand the contribution of B cells as APCs in transplant rejection, an anti-CD20 Ab was used to deplete B cells. CD20 is first expressed in human pre-B cells in the BM, and its expression continues until B cells differentiate into plasma cells.[35,36] In mouse, several reports have shown a similar CD20 expression pattern.[37] In this study, we used a multi-parametric analysis to investigate the expression of CD20 by B cells during their development.[38,39] From pre-pro-B cell ($B220^+CD43^+CD24^{low}BP\text{-}1^{neg}$) through pro-B cell ($B220^+CD43^+CD24^+BP\text{-}1^{neg}$) until pre-B-I cell ($B220^+CD43^+CD24^+BP\text{-}1^+$), stage CD20 was not expressed (Figure 1, Figure S2). However, CD20 was expressed at low densities at pre-B-II cell stage ($B220^+CD43^+CD24^{high}BP\text{-}1^+$) and increased further during B cell maturation (Figure S3). This is in line with previous reports, where it was shown that CD20 expression is parallel to the expression of immunoglobulin heavy chain.[37]

B cells egress from BM and migrate to secondary lymphoid organs. We analyzed the expression of CD20 on B cell subpopulations from spleen. Although all subpopulations analyzed expressed CD20, transitional 1 (T1) and T2 B cells ($B220^+CD21^{high}sIgM^{high}CD93^+CD23^{neg}$ and $CD23^+$, respectively), as well as marginal zone (MZ) and MZ precursors ($B220^+CD21^{low}sIgM^{high}CD23^{neg}$ and $CD23^+$, respectively) expressed CD20 at very high levels (Figure 1, Figure S3). Of note, MZ precursors are also described by some authors as T2 $CD21^{low}$.[38] Although CD20 expression increases with B cell maturation, it has been reported that once B cells enter the $B220^{high}$ B cell pool, CD20 expression decreases. This suggests that transitional B cells that express high levels of CD20 are recent BM emigrants, whereas mature B cells (eg, follicular B cells) express lower densities of CD20 compared to T1, T2, and MZ B cells (Figure S3).

3.2 | Anti-CD20 antibody efficiently depletes mature B cells

After showing the distribution of CD20 in the different B cell subpopulations in the BM and in the periphery, we injected B6 naïve mice intravenously with the anti-CD20 Abs, and the B cells populations were monitored. In our hands, the 5D2 clone administered in a single dose efficiently depleted B cells rapidly from blood, and by day 14, late pre-B cells to mature stages (Figure 2) could not be detected in BM, whereas early precursors (ie, pre-pro-B cells to early pre-B cells) remained intact (Figure 2). These results are in line with those of previous reports using anti-CD20 Abs and consistent with CD20 expression patterns by BM B cells (Figure S2).

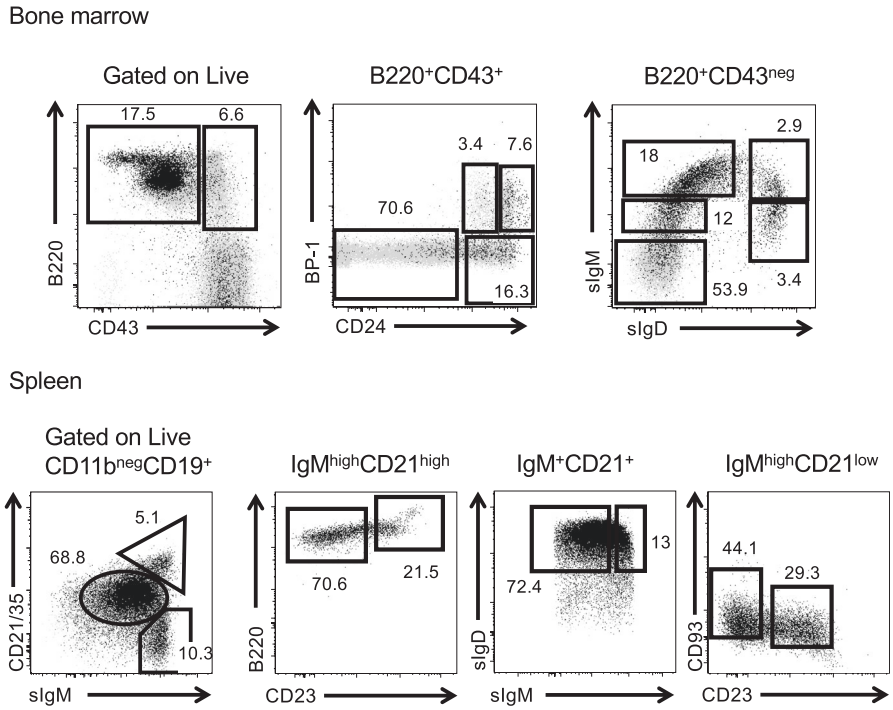


FIGURE 1 CD20 expression on bone marrow and splenic B cell subsets in normal adult C57BL/6 mice. Bone marrow cells were gated on lymphocyte live cells. Then, $B220^+$ cells were subdivided based on their CD43 expression. The earliest B lineage–committed stage ($CD43^+$) was divided into Pre-Pro-B, Pro-B, late Pro-B/early Pre-B, and early Pre-B, whereas $CD43^-$ was divided into late-Pre-B, transitional, immature, early mature, and late mature B cells following Hardy's fractions, as detailed in Figure S1. Splenocytes were gated on lymphocyte live $CD11b^{neg}$ $CD19^+$ cells. Following Allman and Pillai gating strategy, cells were gated based on their CD21 and IgM surface (sIgM) expression. $sIgM^{high}CD21^{high}$ cells were further divided into transitional 1 and transitional 2; $sIgM^+CD21^+$ were divided into follicular I and follicular II; $sIgM^{high}CD21^+$ were divided into marginal zone (MZ) and MZ precursor cells, as detailed in Figure S2. Overimposed black dot plots showed CD20-expressing cells. Data are representative of 3 independent experiments

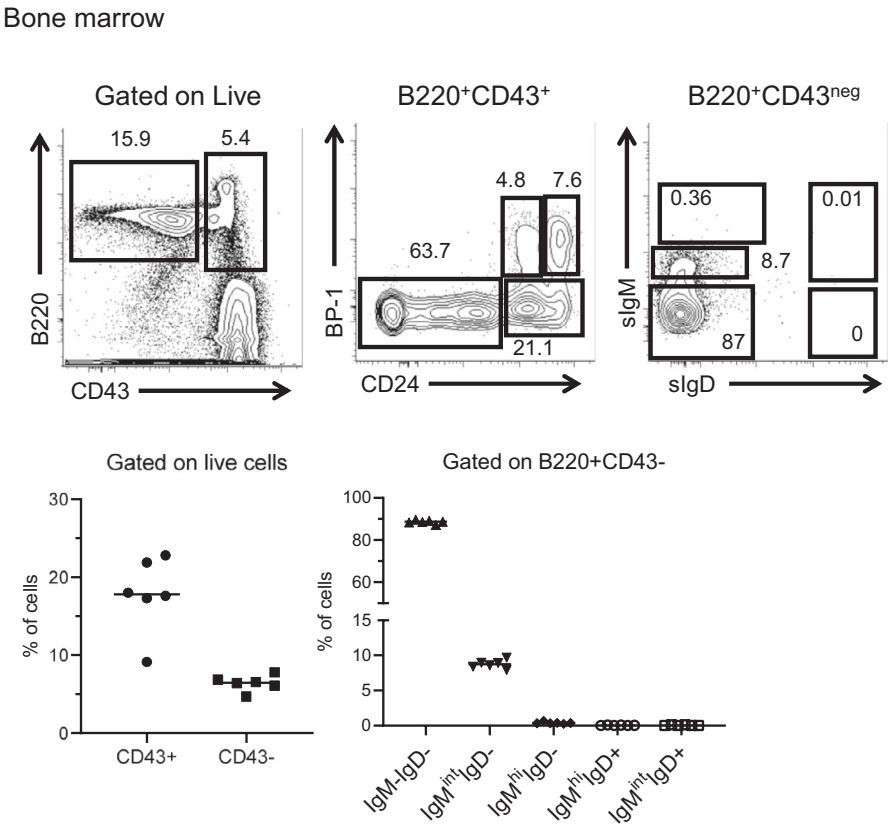
In the spleen, CD20 is highly expressed by recent BM emigrants and transitional B cells and MZ precursor cells (Figure S3). Concurrently, these $IgM^{high}CD24^{high}$ B cell populations with the highest CD20 expression levels were the first cells to disappear from the spleen after treatment with anti-CD20 Abs (Figure S4, day 4). Mature B cells, such as follicular B cells, which express CD20 at a lower density than recent emigrants were not completely depleted until day 7 in spleen (Figure 3) and lymph nodes (LNs) (Figure S5). We confirmed that the depletion of B cells was long-lasting in the absence of inflammation, as after 2months B cells were still depleted (Figure S4, day 60).

3.3 | The absence of B cells at the time of skin transplant is necessary to induce prolongation of skin graft survival

After we confirmed that anti-CD20 Ab treatment depletes B cells, B6 mice transplanted with skin from a B6 mouse strain transgenic for K^d molecules ($B6.K^d$) received anti-CD20 Ab either 7days before (−d7) or 2days after (+d2) transplantation (Figure 4A). Furthermore, to focus on the antigen-presenting function of B cells for T cells with indirect allospecificity, recipient mice were injected with an anti-CD8 Ab at day −1 and +1, and then every week until mice completely rejected the skin graft, as published previously.[31] Of note, anti-CD8 treatment depletes both $CD8^+$ T cells and $CD8^+$ DCs (Figure S6). Skin graft survival was prolonged for 8 days but only when anti-CD20 Ab was injected 7 days before transplantation but not 2days after (phosphate-buffered saline [PBS]; +d2 and −d7 mean graft survival time were 11, 11.5, and 19 days, respectively) (Figure 4B).

Although anti-CD8 Ab alone did not prolong graft survival, anti-CD20 prolonged skin graft survival only when combined with anti-CD8 Abs (Figure S7 and Figure 4B). These results indicate that B cell depletion prolongs graft survival only after depletion of both $CD8^+$ T cells and $CD8^+$ DCs, strongly suggesting that B cells are presenting the K^d alloantigen to $CD4^+$ T cells, most likely because graft rejection in this experimental setup is dependent on indirect allorecognition by $CD4^+$ T cells.

A regulatory IL-10–producing B cell population has been reported[16-18] and if enriched in our experimental setting,[15,40] could be responsible for the extended skin graft survival. Thus, IL-10–deficient mice were treated with anti-CD8 Ab and anti-CD20 Ab using the same protocol applied to wild-type (WT) mice before transplanting $B6.K^d$ skins. Skin graft survival was extended in IL-10–deficient mice to an extent similar to that seen in WT mice (Figure S8), suggesting that prolonged survival seen in association with anti-CD20 was achieved independently of IL-10.



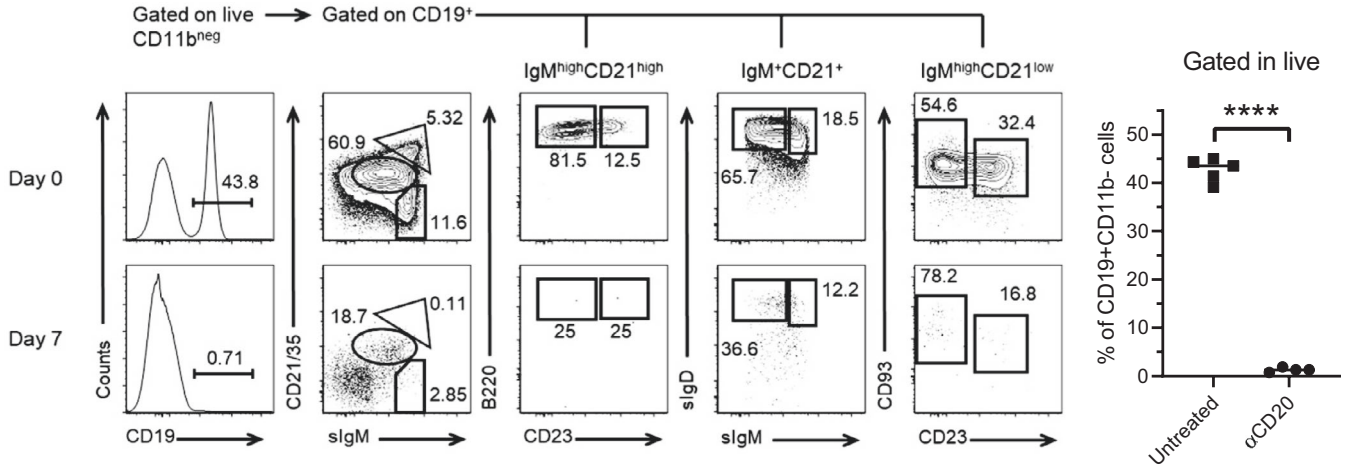


FIGURE 2 Anti-CD20 Ab treatment depletes mature B cells in bone marrow. Stained bone marrow cells were gated on lymphocyte live cells. Cells were gated as shown in Figure 1. Representative plots of B6 adult mice 14days after anti-CD20 Ab (200μg) intravenous injection are shown. The earliest B lineage–committed stage ($CD43^+$) is not affected by anti-CD20 treatment, although there is a shift toward more developed B cell stages (middle panel), whereas mature B cell stages are almost absent (right panel). Percentage of cells in BM from mice treated with anti-CD20 Ab is shown as individual mice and mean (lower panels). Experiments were performed with 4-5 mice per group. Data are representative of 2 independent experiments

FIGURE 3 Anti-CD20 Ab treatment depletes mature B cells in spleen. Stained splenocytes were gated on lymphocyte live $CD11b^{neg}$ cells. Cells were gated as shown in Figure 2. Representative histograms and plots of B6 adult untreated mice (top panels) and day 7 after anti-CD20 Ab (200μg) intravenous injected mice (bottom panels). Percentage of CD19+cells in spleen from mice with and without anti-CD20 Ab treatment is shown as individual mice and mean (right panel). Experiments were performed with 4-5 mice per group. Data are representative of 2 independent experiments. Statistics are calculated using an unpaired *t test*. **** $P < .0001$

3.4 | B cell depletion led to a delay in priming of $CD4^+$ T cells with indirect allospecificity

To understand the mechanisms behind the increased skin survival following B cell depletion, and in the absence of $CD8^+$ T cells and $CD8^+$ DCs, T cells with indirect allospecificity obtained from a B6 mouse strain transgenic for a T cell receptor (TCR)specific for K^d and restricted by A^b (TCR75)[32,41] were adoptively transferred into B6 mice receiving a B6.K^d skin transplant following the injection of anti-CD8 Ab or anti-CD20 Ab alone or the 2 Abs in combination. CFSE-labeled TCR75 was injected at day−1. After 5 days, the mice were culled and CFSE dilution was evaluated (Figure 5A). TCR75 cells proliferated significantly less in mice treated with anti-CD8 Ab or with anti-CD20 Ab alone compared to TCR75 cells from untreated mice (Figure 5B). These results, which support the accepted role of DCs in initiating the clonal expansion of $CD4^+$ T cells with indirect

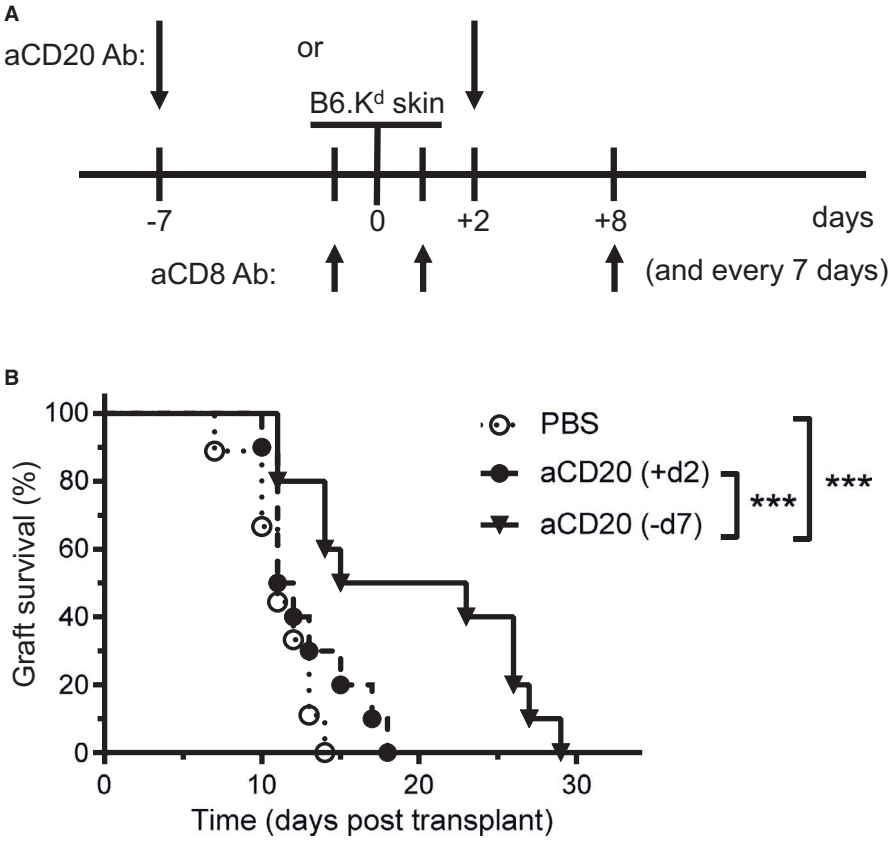


FIGURE 4 Anti-CD20 Ab treatment prolonged skin graft survival only when B cell depletion is achieved before transplantation. B6 mice were injected intravenously with anti-CD20 Ab (200μg) 7days before or 2days after receiving dorsal B6.K^d skin transplant (day 0). Controls received PBS. Anti-CD8 depleting Ab was administered at day −1 and day +1, and every 7days thereafter. Skin survival was monitored daily. (A) Experimental design (B) plots show cumulative data of skin graft survival from 3 independent experiments (n = 5-6/group for each experiment). Statistics are calculated by log-rank (Mantel-Cox) test with Bonferroni correction for multiple comparisons. *** $P < .001$

alloreactivity at this time point, demonstrate that B cells also play a crucial role as APCs for indirect presentation of alloAgs at early time points following skin transplantation.

3.5 | B cells are efficient in donor antigen capture

We have shown previously that graft rejection was attributed to the continuous acquisition of donor MHC class I by recipient DCs.[31] Recently Liu et al have shown that during skin transplantation, T cell priming and graft rejection are mediated via the release of donor DC–derived EVs, which are acquired by recipient conventional DCs.[4] In addition, Marino et al also observed that recipient B cells capture donor antigens via DC EVs.[5] Based on this evidence and on our results so far we hypothesized that recipient B cells activate CD4$^+$ T cells with indirect allospecificity by acquiring donor antigen through the uptake of EVs. One prediction of this is that we should find donor-derived K^d and I-A^d molecules on the surface of recipient B cells immediately after transplantation, and our data using congenic markers indicate that a proportion of recipient B cells from both spleen and draining lymph node, purified 4 days posttransplant, did express surface K^d and I-A^d (Figure S9).

We further confirmed in vitro that purified B cells can acquire MHC class I and II molecules from BM-DCs (Figure S10). To prove that MHC transfer from donor DCs to recipient B cells could be mediated via EVs, purified B6 B cells were co-cultured with CFSE-labeled BM-DC EVs derived from BALB/c mice. As shown in Figure 6A, B cells became CFSE positive after co-culture, demonstrating that B cells were capable of acquiring CFSE-labelled DC-derived EVs and subsequently express acquired K^d molecules on their surface (Figure 6B). The acquisition of CFSE by B cells suggests that the EVs are fusing with the B cell membrane and then the donor MHC class I is processed and presented in the context of MHC class II, as shown previously to be one of the mechanisms of EV acquisition.[42-45] Furthermore, by culturing purified B cells B6 (H-2^b) and CBA (H-2^k) with BALB/c (H-2^d) DC-derived EVs, we were able to detect intact MHC class I (K^d) (Figure S11A) and MHC class II (I-A^d) (Figure S11B) on the surface of recipient B cells, suggesting an alternative way that the donor MHC peptide complex is presented without need of further processing and presentation.[42-45]

Next we addressed whether T cells with indirect allospecificity could be activated by recipient B cells following acquisition, processing, and presentation of donor EVs bearing K^d molecules in the context of recipient I-A^b molecules. To evaluate this, TCR75 T cells were used. Only B6 B cells co-cultured with BALB/c-derived EVs induced the activation of the TCR75 T cells, as measured by the release of IL-2, suggesting that the transferred MHC molecules were being internalized, processed, and loaded onto recipient I-A^b to drive indirect CD4$^+$ T cell responses (Figure 7A).

Asking a similar question, we investigated whether MHC class II–peptide complexes can be transferred from BM-DC EVs to B cells. To this end, EVs were isolated from B6.K^d BM-DCs and added to CBA (H-2k)–derived B cells prior to co-culture with TCR75 T cells. We observed that CBA B cells were able to activate TCR75 T cells

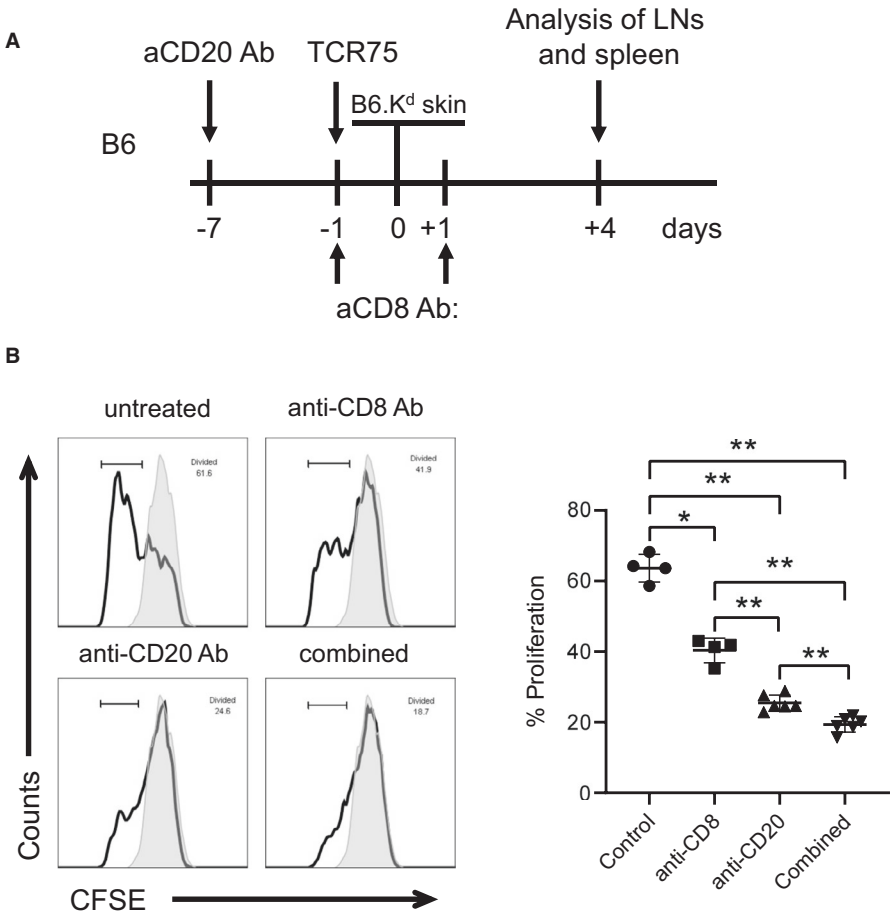


FIGURE 5 B cell depletion delays $CD4^+$ T cells indirect alloresponses. B6 mice (CD90.2) were injected intravenously with anti-CD20 Ab (200μg) 7days before receiving a dorsal B6.K^d skin transplant (day 0). Anti-CD8 depleting Ab was administered at day −1 and day +1 to mice that have received or not anti-CD20 Ab. Congenic (CD90.1) CFSE-labeled TCR75 cells were adoptively transferred at early and late time points as indicated in the experimental design (A). Proliferation by CFSE dilution of $CD90.1^+$ cells of 2 independent experiment from pooled draining lymph node and spleens (n = 4-5/group for each experiment) was analyzed by flow cytometry (B). $^*P < .05$, $^{**}P < .01$

following co-culture with B6.K^d DC–derived EVs but not with B6 DC–derived EVs (Figure 7B). This suggests that intact class II (I-A^b) molecules presenting K^d peptide were transferred onto CBA B cells.

Altogether these results demonstrate that recipient B cells can acquire both intact MHC class I molecules and MHC class II molecules containing class I–derived peptides via EV transfer, and that both can activate T cells with indirect specificity, the first requiring re-cycling of the newly acquired, EV-transferred membrane MHC class I via the endogenous MHC class II antigen processing pathway for presentation onto MHC class II, but the second not. Therefore these data support our hypothesis that B cells can acquire intact MHC via EV transfer to efficiently stimulate $CD4^+$ T cells recognizing alloantigen in a self-MHC class II–restricted manner.

3.6 | Antigen-specific Tregs synergize with B cell depletion for skin graft survival prolongation

Having shown that B cells contribute to priming of T cells with indirect allospecificity and that Tregs have been shown previously to increase skin transplant survival in this skin transplantation model by controlling the indirect $CD4^+$ T cell responses, we hypothesized that B cell depletion might synergistically prolong the graft survival induced by administration of Tregs with indirect allospecificity. We tested this by depleting B cells at day −7 and injecting Tregs with indirect allospecificity one day before transplantation as previously published[32] (Figure 8A). As shown in Figure 8B, although either anti-CD20 Ab treatment or the adoptive transfer of Tregs alone improved skin graft survival to a similar extent, for 9-11days beyond controls to a maximum of 31days, when the two therapies were combined, skin grafts survived up to 41days, demonstrating a synergistic effect. This result further emphasizes the advantage of combined therapies in transplantation to promote graft survival.

4 | DISCUSSION

In this study we have investigated the contribution of B cells in skin graft transplant survival/rejection. Uchida J et al observed that the effectiveness of anti-CD20 Abs to deplete B cells in vivo closely correlated with the subtype. The most efficient depletion was achieved by using immunoglobulin G isotype 2a (IgG2a), through a mechanism involving both Fc-γ receptor and macrophages.[46] In this study we used a murine version of rituximab to deplete B cells, the monoclonal IgG2a anti-CD20 Ab, clone 5D2, which mediates cellular phagocytosis of circulating B cells in the liver.[47] The anti-CD8–depleting Ab was administered to prevent the $CD8^+$ T cell direct alloresponse to donor K^d, but by also depleting the major population of lymphoid resident DC, it allowed us to assess the importance of B cells as APCs to prime $CD4^+$ T cell indirect alloresponse in the absence of $CD8^+$ DCs. Of

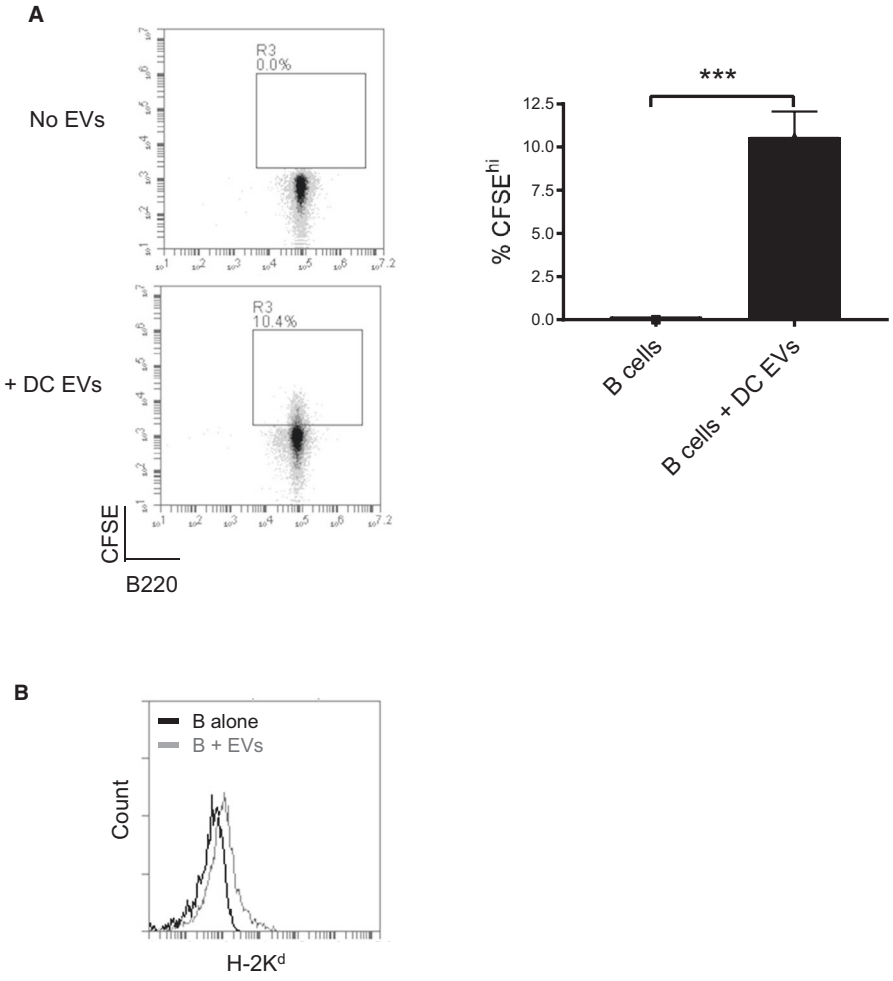


FIGURE 6 B cells acquire MHC molecules from DCs via EV transfer. EVs were isolated from CFSE-labeled BALB/c DCs and co-cultured with B6 B cells. Controls were non–EV-treated B cells. B cell acquisition of EVs was assessed by flow cytometry. Dot plots show representative data of 1 of 3 independent experiments, and the cumulative data are shown in the bar chart (right) (A). Levels of MHC class I K^d on B cells following exposure to BALB/c EVs were assessed by flow cytometry (B). ***$P < .001$

note, the removal of DCs alone did not affect the survival time, suggesting that in their absence, the B cells can substitute effectively to prime the indirect alloresponse. The timing of administration of the anti-CD20 Ab was critical. Administration 2 days after transplantation had no impact on survival, whereas in mice transplanted 7 days after B cell depletion, when B cells are highly depleted in all immunological compartments, skin graft survival was significantly prolonged. These results indicate that B cell priming of T cells with indirect allospecificity occurs in the immediate posttransplant period, and is further supported by experiments using adoptive transfer of $CD4^+$ T cells with indirect specificity, which underwent significant clonal expansion within 4 days posttransplant, but only when B cells were present. We also present data to support the recently proposed idea that following skin transplantation, acquisition of donor MHC molecules onto the B cell membrane via EV transfer ("cross-dressing") is a mechanism by which recipient B cells can acquire donor antigen for uptake, processing, and loading into recipient class II MHC capable of priming T cells with indirect allospecificity.

Recently, the dogma suggesting that allograft responses are driven by trafficking donor passenger leukocytes has been challenged by two groups.[4,5] Both groups have shown that direct T cell alloresponses were primed early following transplantation despite very few donor DCs[4] or no donor leukocytes[5] trafficking from the graft, and that significant numbers of recipient cells expressed donor MHC molecules. In fact Marino et al identified that around 60%-70% $CD11c^+$ recipient DCs and 10%-15% recipient $CD20^+$ B cells were "cross-dressed" by day 7 following skin transplantation. In addition, Lui et al have shown that "cross-dressing" of recipient cDCs with donor MHC molecules is the main contributor of priming direct alloresponses early postheart transplant leading to rejection. Both groups attributed cross-dressing to the release and acquisition of donor EVs, although the origin of the EVs is still not known. Martino et al found expression of CD3, CD20, and CD11c on the EVs, suggesting their origins to be T, B, and DCs, respectively, whereas Lui et al suggest that the donor EVs were derived from donor DCs. Marino et al showed that EV-derived B6 MHC molecules on BALB/c splenocytes could activate alloreactive T cells both in vitro and in vivo, although they did not assess whether this was due to activation of T cells with direct or indirect allospecificity. Our results highlight that B cells efficiently acquired, via EV transfer, donor MHC molecules that are expressed by DC and that these B cells were functional and could activate $CD4^+$ T cells with indirect allospecificity; in vivo, this was unveiled in the absence of $CD8^+$ DC. Although the level of MHC transferred may seem low, the levels were similar to those observed previously between DCs.[48] Based on the evidence that B cells contribute to the priming of T cells with

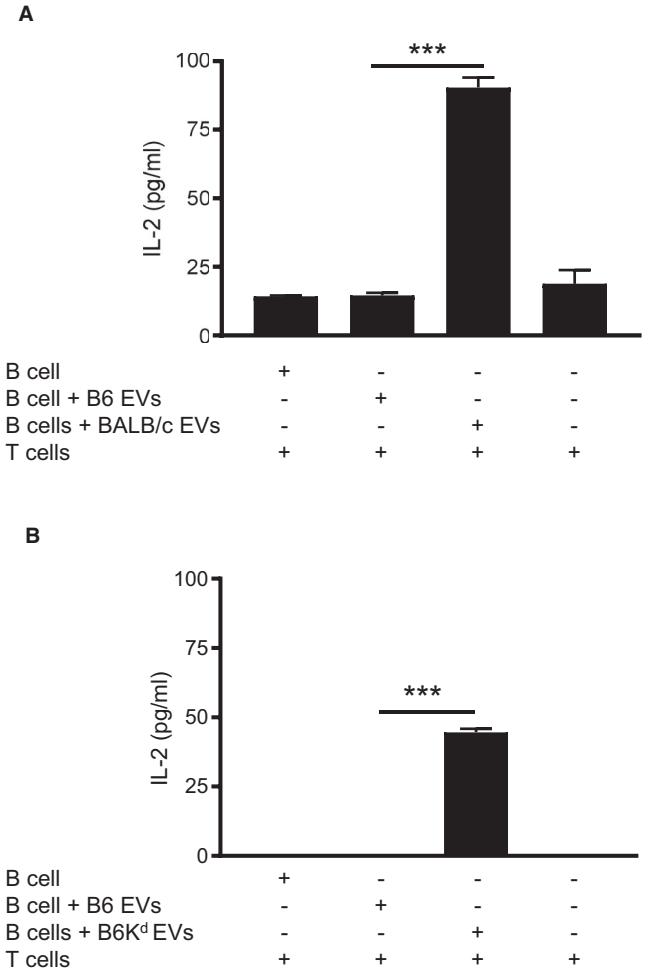


FIGURE 7 Transferred EV-derived MHC drives indirect $CD4^+$ T cell responses. EVs derived from BALB/s (A) or B6.K^d (B)–derived DCs were added to B6 (A) or CBA (B) B cells before co-culturing with TCR75 cells. Control B6 EVs were included. IL-2 was assessed after 3 days using an IL-2 ELISA. Data represent 1 of 3 experiments and are shown as a technical replicate. ***$P < .001$

indirect allospecificity, we provide evidence that B cell depletion generated a permissive environment for the enhanced function of alloAg-specific Tregs, leading to increased transplant survival.

Our in vitro data indicate that the antigen specificity of the B cell is not relevant to the uptake of MHC from EV onto the B cell surface, or the ability of the B cell to process and present these newly acquired membrane MHC via the class II pathway. However, our work does not address the relevance of the antigen specificity of the B cells that acquire MHC from EV in vivo postskin transplantation. Given that antigen-specific B cells are highly likely to bind the donor-derived MHC-expressing EVs with high affinity, and process the allogeneic MHC bound to the B cell receptor for presentation on MHC class II with extremely high efficiency, we speculate that in vivo, the relevant B cells involved in our model are probably K^d specific.

Transplantation across histocompatibility barriers results in acute rejection in the absence of immunosuppression. Nonselective immunosuppressive agents leave patients vulnerable to infections and cancers.[49] Thus, a major effort in the field of transplantation immunology is to generate therapies able to induce donor-specific tolerance leaving intact the rest of the immune system. B cells are key players in inducing adaptive immune responses. Further to their role as the precursors of Ab-producing plasma cells, B cells play an important role as APCs in T cell responses. Several studies have established the requirement of B cells for the generation of $CD4^+$ memory T cells in most antigenic contexts (eg, viral, autoimmunity),[50-52] and although DCs are key in the initiation of $CD4^+$ T cell activation, B cells are required for their subsequent activation.[29,53]

In the clinic, rituximab is currently being added to immunosuppressive regimens, mostly when transplanting across blood-group antibody barriers, however, with no real understanding of whether it gives benefit above other depleting agents. Anti-CD20 Abs have no effect on plasma cells, since they do not express CD20; therefore, rituximab does not immediately decrease the level of circulating anti-donor Abs. However, it is important to highlight that recent trials with multiple sclerosis patients have shown that B cell depletion had a beneficial effect, but the clinical response was not associated with reduction in Ab levels.[54-57] Similarly, in type 1 diabetes where auto-Abs do not play a key role, B cell depletion improves outcome. This suggests the importance of B cells as APCs and their role in sustaining $CD4^+$ effector and memory responses. Furthermore, B cell depletion in mouse models of autoimmune diseases significantly reduced $CD4^+$ T cell responses to foreign and self-antigens, whereas $CD8^+$ T cell reactivity remains unaffected.[21] Recently, we have shown that ex vivo B cell depletion in some patients with chronic Ab-mediated rejection led to reduced indirect T cell alloresponses in a recall assay, suggesting their role as APCs.[9] However, in another group of patients, presented in the same study, ex vivo B cell depletion led to increased responses, suggesting they also are able to suppress indirect T cell responses.[9] Thus, understanding the mechanism governing graft rejection at different stages and the cellular components that play a key role is paramount for the rationale in generating new immunotherapies. Here we demonstrated that injecting anti-CD20 Ab 7 days before skin transplant so that B cells were absent at the time of transplantation (in the absence of DCs), led to increased transplant survival and prevented the proliferation of adoptively transferred T cells with indirect allospecificity, both demonstrating the contribution of B cells at inducing the indirect alloresponses that underpin acute rejection in this model system. Mechanistically, we were able to confirm that our results were not due to the preferential survival of IL-10–producing regulatory B cell populations that control immune responses against the graft, since the same results were obtained in IL-10–deficient mice. Although we cannot completely rule out that Bregs play a role in the early phase of rejection, we did not observe any difference under our experimental settings.

It has also been reported that rituximab treatment could promote the expansion of Treg populations.[58,59] Tregs are key players in maintaining immune homeostasis and tolerance to self. Currently, the clinical use of Tregs in the clinic for transplanted patients has being explored.[60-63] Furthermore, Tregs can control

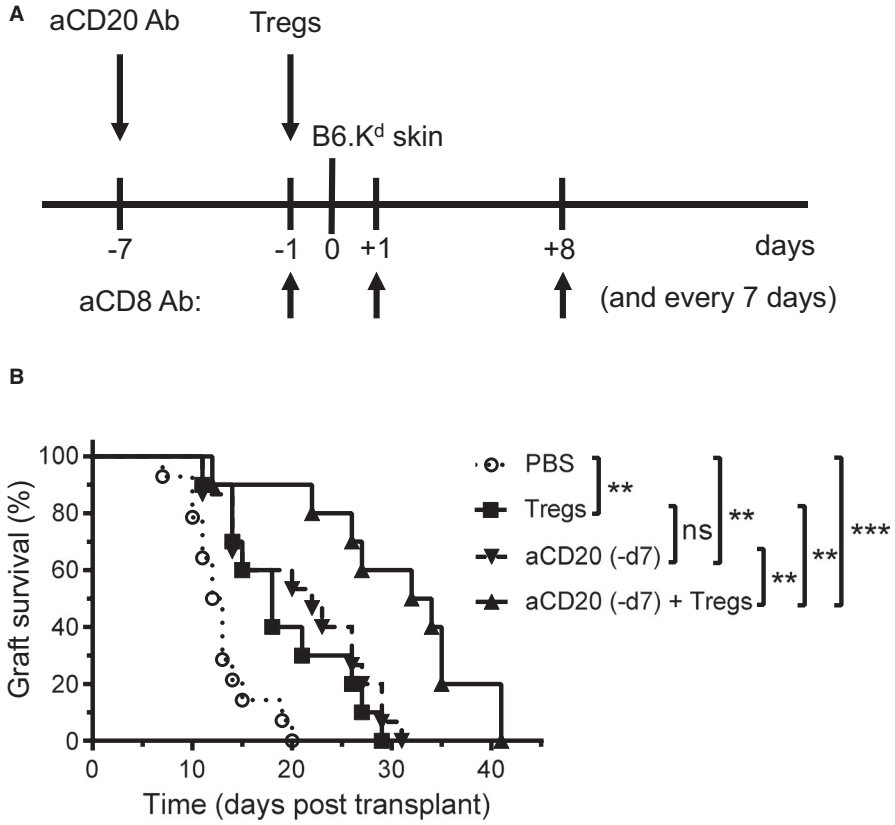


FIGURE 8 Tregs combined with anti-CD20 Ab treatment have a synergistic effect extending skin graft survival. B6 mice were injected intravenously with Tregs 1 day before or with anti-CD20 Ab (200μg) 7days before receiving dorsal B6.K^d skin transplant (day 0) or the combined treatment. Control mice received PBS. Anti-CD8–depleting Ab was administered to all groups at day −1 and day +1 and every 7days thereafter. Skin survival was monitored daily. (A) Experimental design (B) plots show cumulative data of skin graft survival from 4 independent experiments (n = 5-6/group for each experiment). Statistics are calculated by log-rank (Mantel-Cox) test with Bonferroni correction for multiple comparisons. $^{**}P < .01$, $^{***}P < .001$

B cell responses by constraining T cell help.[64-66] Here we demonstrated that by combining B cell depletion with the transfer of Tregs with indirect allospecificity, a synergistic effect is observed. It is important to highlight that B cells are important players during the early phase of graft rejection and that although CD4$^+$ T cells responses are impaired in the absence of B cells and skin graft survival is prolonged, the graft is ultimately rejected. However, we showed here that there is room for improvement of current therapies and understanding the mechanisms behind graft rejection and that survival is important to combined therapies in a rational manner.

Altogether our results further emphasize an important contribution by B cells as APCs in T cell priming, and highlight the potential value of B cell depletion as adjunctive immunosuppression.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, G.L., upon reasonable request.

ORCID

Pablo D. Becker https://orcid.org/0000-0003-1980-1230
Qi Peng https://orcid.org/0000-0002-9223-5856
Marco Romano https://orcid.org/0000-0001-6089-5828
Anthony Dorling https://orcid.org/0000-0003-3102-2600

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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