European Journal of Immunology

Aged B cells alter immune regulation of allografts in mice

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Review Timeline:	Submission date:	11-Feb-2016
	First Editorial decision:	16-Mar-2016
	First Revision received:	04-Jul-2016
	Accepted:	17-Aug-2016

Handling Executive Committee member: Radbruch

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision - 16-Mar-2016

Dear Dr. Goldstein,

Thank you for your patience while the Executive Editor assessed your appeal of our decision to reject your manuscript ID eji.201646353 entitled "Aged B cells alter immune regulation of allografts."

The Executive Editor is impressed to see that you have data in hand, showing in vivo data for a possible mechanism by the adoptive transfer of aged B cells into young mice. Given this, and your claim that you could address also the other concerns of all 3 referees, the Editor would like to extend the opportunity to submit a revised version of your manuscript which addresses the concerns of all referees. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should know that this revision will be sent to all 3 original referees for re-assessment as a revision, and as such, there are no guarantees of eventual acceptance.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this

will result in delays in the re-review process.*

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Prof. Andreas Radbruch

Dr. Karen Chu
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Reviewer: 1

Comments to the Author

The authors describe the different response of young compared to aged C57BL/6 mice to anti CD20 depletion in an allogeneic skin graft setting. While in young mice the depletion impaired immune regulation after anti-CD154/CD45RB treatment, skin graft survival did not differ much in aged mice. This was not due to differences in number or function of Bregs, but as the authors suggest to a numerical increase in Age associated B cells (ABCs). These cells expressed higher levels of MHCII, TLR2, CD73 and CD80 in older mice and were good APCs for anti donor T cells. I understand and follow the hypothesis but I am not sure that the title has yet been sufficiently substantiated by the experiments.

Major:

1) In figure 1A it looks like that CD20 depletion in young mice changed the allograft rejection kinetics to what is seen in old mice without treatment. It looks like that at old age there is no significant difference between CD20 depleted and non-depletion in regard to allograft rejection. Given the observation that there is no really numerical difference in Bregs between young and old mice, the question is what is the true effect of the depletion of B cells and why is B cell depletion not accelerating the allograft rejection. The fact that ABCs are expanded and can drive activation of allo specific T cells shows an association but does not prove that they are the cause of this phenomenon. Is there a direct or indirect effect of ABCs on Breg function, or are there two independent contrary B-cell dependent mechanisms, Breg and ABC so that the netto effect is almost none?
Can one try to measure an effect of ABC on Breg if this is postulated?

2) ABCs are thought to be rather part of a Th1 like environment (see also Rubtsova J Immunol. 2015 Sep 1;195(5):1933-7). What other cytokines beside IL-2 are induced by ABCs in the alloreactive setting? I would be especially interested in IFNg.

3) Was there a difference in B cell depletion between young and old?

4) Can the authors state from the histology whether the mode of rejection is the same between young and old mice?

5) Throughout the manuscript including the title it needs to be made clear that this is not only a quantitative, but also a qualitative difference in ABCs in old vs young mice. The described phenomenon isn't just explained by the difference in number but also by the altered function of ABCs with increasing age. So do the authors have additional information whether there is a general difference between young and old ABCs or "only"• the activation status?

6) Is it possible to transfer ABCs from old mice into young mice in order to measure a direct effect on graft survival?

7) Was the experiment shown in Figure 3D also performed in young mice? If so did the anti CD20 depletion affect the IL2 producing T cell?

Minor:

1) Figure 3 C: Is the IL2 production of T cells co-cultured with old vs young ABCs significant differently? If so please indicate.

Reviewer: 2

Comments to the Author

Although the findings are interesting, they are pretty descriptive, and do not include cause-result

relationships.

Reviewer: 3

Comments to the Author

In this manuscript, the authors compare how B cell depletion with anti-CD20 modulates the beneficial effect of anti-CD45RB plus anti-CD154 treatment in a model of skin transplantation in young versus aged mice. They found that B cell depletion reduced the beneficial effect of anti-CD45RB plus anti-CD154 in young mice, while it had an opposite effect in aged mice. The authors then attempt to provide an explanation for this observation. They found that B cells from young and aged mice did not differ in their capacity to produce IL-10 upon stimulation in vitro. In contrast, they found that old mice display a marked accumulation of CD19+CD21-CD23-CD43-CD93- cells. These cells represent a heterogeneous population since they comprise CD73(plus) and CD73(neg) cells. These B cells have a slightly more activated phenotype in aged compared to young mice. The authors then follow the concept that these B cells are responsible for the enhanced pathogenic activity of the B cell compartment in old mice, compared to young mice. This is however not clear since B cell depletion on its own does not modulate the course of transplant rejection in old mice. The different effect of anti-CD20 in young versus old mice is only documented in the context of the treatment with anti-CD45RB plus anti-CD154, yet there is no comparison or characterization of B cell subsets and functions in young versus old mice in such treatment condition. It would therefore be necessary to characterize B cell subsets and functions in young and old mice treated with anti-CD45RB plus anti-CD154 in order to identify why B cells have opposite roles in these conditions. At present the claim of the authors that CD19+CD21-CD23-CD43-CD93- cells are responsible for the beneficial effect of anti-CD20 in old mice is highly speculative.

First Revision – authors' response 04-Jul-2016

The reviewer comments are italics, with our response following in normal text. Please note that changes in the manuscript are denoted by yellow highlighting.

Reviewer #1:

Point #1:" *In figure 1A it looks like that CD20 depletion in young mice changed the allograft rejection kinetics to what is seen in old mice without treatment. It looks like that at old age there is no significant difference between CD20 depleted and non-depletion in regard to allograft rejection. Given the observation that there is no really numerical difference in Bregs between young and old mice, the question is what is the true effect of the depletion of B cells and why is B cell depletion not accelerating the allograft rejection. The fact that ABCs are expanded and can drive activation of allo specific T cells shows an association but does not prove that they are the cause of this phenomenon. Is there a direct or indirect*

effect of ABCs on Breg function, or are there two independent contrary B-cell dependent mechanisms, Breg and ABC so that the netto effect is almost none? Can one try to measure an effect of ABC on Breg if this is postulated?
"

Our response: We thank the reviewer for these thoughtful comments. The revised manuscript provides new data showing that the adoptive transfer of aged ABCs, but not young ABCs, into young skin allograft recipients impairs the ability of anti-CD45RB and anti-CD154 to extend allograft survival (Figure 3F). These data provide direct *in vivo* evidence that ABCs from aged mice affect the immune regulation of allografts.

Our *in vitro* data show that ABCs isolated from aged mice are superior to ABCs isolated from young mice in their alloimmune T cell priming function (Figure 3C-D). As the reviewer correctly points out, we did not discern a difference in the number of regulatory B cells (B regs) or a difference in their function (IL-10 production) with aging (Figure 1C-F). Combined, these data support our conclusion that the enhanced T cell priming function of the ABC subpopulation in the aged transplant recipient overrides the immune regulatory properties of B regs. However, we agree with the reviewer that it is possible that ABCs directly interact with B regs. As our *in vitro* data indicate a clear interaction between ABCs and alloreactive T cells, and given that there is no published *in vitro* assay to examine the immune regulatory function of B regs, we have included comments in the concluding remarks of our manuscript (page 12) that discuss the possibility that ABCs directly interact with B regs.

2) ABCs are thought to be rather part of a Th1 like environment (see also Rubtsova J Immunol. 2015 Sep 1;195(5):1933-7). What other cytokines beside IL-2 are induced by ABCs in the alloreactive setting? I would be especially interested in IFNg

Our response: We thank the reviewer for this helpful remark. In the revised manuscript, we have measured IFN-γ and IL-17 in the culture supernatants of T cells stimulated with alloreactive ABCs from either young or aged mice and found that, compared to ABCs from young mice, ABCs purified from aged mice induce higher levels of IFN-γ, but not IL-17, via alloreactive T cells, suggesting that ABCs from aged mice enhance Th1 but not Th17 alloimmune responses. (Figure 3D and Supplemental Figure 2F, revised manuscript).

3) Was there a difference in B cell depletion between young and old?

We find that B cell depletion is similarly efficacious in young and aged mice. Supplemental Figure 1A of the revised manuscript shows that non-transplanted aged and young mice exhibit similar degrees of B cell depletion in the spleen when treated with anti-CD20 monoclonal antibody (mAb; 100 μg anti-CD20 mAb every 10 days for four serial doses or with one dose of 100 μg anti-CD20 mAb). Supplemental Figure 1D shows similar B cell depletion in the spleen of young and aged mice when we combined anti-CD20 treatment with anti-CD45RB and anti-CD154 following skin transplantation.

4) Can the authors state from the histology whether the mode of rejection is the same between young and old mice?

Our response: We previously demonstrated via histology that skin allograft rejection in an aged transplant recipient treated with anti-CD45RB and anti-CD154 exhibits higher neutrophil infiltration than similarly treated young transplant recipients, which show predominantly lymphocytic infiltration [1]. In our prior study, we demonstrated that alloreactive $CD8^+$ T cells from aged transplant recipients secrete higher

levels of IFN-γ than $CD8^+$ T cells from young transplant recipients. It is possible that the enhanced alloimmune priming response of ABCs from aged transplant recipients documented in the current study synergizes with the increased alloreactive $CD8^+$ T cell responses upon aging as described previously to alter the mode of graft rejection. This issue is outside the scope of the current study and will require future investigation.

5) Throughout the manuscript including the title it needs to be made clear that this is not only a quantitative, but also a qualitative difference in ABCs in old vs. young mice. The described phenomenon isn't just explained by the difference in number but also by the altered function of ABCs with increasing age. So do the authors have additional information whether there is a general difference between young and old ABCs or "only" the activation status?

Our response: We agree with the reviewer that aging exerts both qualitative and quantitative differences in ABCs. In addition to documenting the expression of co-stimulatory molecules (e.g., CD80/86) and markers of B cell memory (e.g., CD73), we now also present data that demonstrate that the expression of the B cell chemokine receptors CCR7, and the adhesion molecule, VCAM-1 (CD106), are upregulated in ABCs isolated from aged mice but not in ABCs isolated from young mice (Figure 2B-C). Thus, in the concluding remarks of the revised manuscript (page #12), we state that both quantitative and qualitative changes in ABCs with aging could impact immune regulation of allografts.

6) Is it possible to transfer ABCs from old mice into young mice in order to measure a direct effect on graft survival?

Our response: The revised manuscript contains new data (Figure 3F) that show that the adoptive transfer of ABCs isolated from aged mice into young mice impairs the ability of anti-CD45RB and anti-CD154 to extend allograft survival. As stated in point #1 above, these new data provide direct *in vivo* evidence that ABCs isolated from aged mice impair the immune regulation of allografts.

7) Was the experiment shown in Figure 3D also performed in young mice? If so did the anti CD20 depletion affect the IL2 producing T cell?

Our response: We have examined the impact of anti-CD20 treatment on anti-donor IL-2 responses in young transplant recipients that were administered anti-CD45RB and anti-CD154. As shown in Supplemental Figure 2G, we did not discern that anti-CD20 significantly affects anti-donor IL-2 T cell production in young transplant recipients treated with anti-CD45RB and anti-CD154 at three weeks post transplantation.

Minor:
1) Figure 3 C: Is the IL2 production of T cells co-cultured with old vs young ABCs significant differently? If so please indicate.

Our response: The difference is statistically significant, and the revised manuscript indicates this (Figure 3D).

Reviewer: 2
Although the findings are interesting, they are pretty descriptive, and do not include cause-result relationships.

Our response: Our manuscript indeed includes cause-effect experiments and documents a disparate effect of recipient age on how B cells impact the immune regulation of allografts, both of which we have

now highlighted more clearly in the revised manuscript. We show a novel role of ABCs isolated from aged mice in impairing the immune regulation of allografts. This finding is significant as prior experimental studies that demonstrated that B cell depletion impairs the immune regulation of allografts only tested the effect in young transplant recipients in experimental models [4, 5]. In addition, a human study that demonstrated that B cell depletion precipitates acute graft rejection did not record recipient age [6]. In contrast, our study includes mechanistic approaches to uncover the novel impact of aging B cells on the immune regulation of allografts.

First, through B cell depletion mediated by monoclonal anti-CD20 antibodies (vs. isotype control), we demonstrate that B cell depletion augments the ability of an immunoregulatory protocol that consists of anti-CD45RB and anti-CD154 to enhance skin allograft survival in aged transplant recipients. In sharp contrast, in young skin allograft recipients, B cell depletion impairs the ability of the protocol to extend allograft survival (Figures 1A-B).

Second, we determined the identity of the subpopulation of B cells that contribute to the disparate effect of immune regulation with aging. Because we found that aging impacted neither the numbers of regulatory B cells (B regs) nor their function (IL-10 production), we examined other B cell subpopulations and found that aging led to an increase in non-follicular, non-marginal B cells, termed age-associated B cells [2, 3] (ABC phenotyped as: $CD19^{+ve}$, $CD21^{-ve}$, $CD23^{-ve}$, $CD43^{-ve}$,$CD93^{-ve}$). In addition to the quantitative effect of aging on ABCs, we noted that ABCs isolated from aged mice expressed higher levels of co-stimulatory molecules (e.g., CD80) and memory markers (e.g., CD73) and exhibited a larger capacity to activate alloreactive T cells *in vitro* than ABCs isolated from young mice (Figures 2-3). Thus, these data indicate that ABCs from aged mice impair the immune regulation of allografts by enhancing the priming of alloreactive T cells.

Third, as there is no *in vivo* approach to specifically deplete ABCs, the only strategy to directly link ABCs to the impaired immune regulation of allografts is to adoptively transfer ABCs from aged mice into young skin allograft recipients treated with anti-CD45RB and anti-CD154. Accordingly, the revised manuscript demonstrates that the adoptive transfer of ABCs from aged non-transplanted mice impairs the ability of anti-CD45RB and anti-CD154 to extend allograft survival in young recipients, providing direct *in vivo* evidence that ABCs impair the immune regulation of allografts (Figure 3F).

Reviewer #3

1)The authors then follow the concept that these B cells are responsible for the enhanced pathogenic activity of the B cell compartment in old mice, compared to young mice. This is however not clear since B cell depletion on its own does not modulate the course of transplant rejection in old mice.

Our response: We thank the reviewer for this thoughtful comment. Given the rapid rejection kinetics of full MHC mismatched skin allografts (BALB/c donor and C57BL/6 recipient, typically < 12 days), it is not surprising that B cell depletion alone has no effect on the tempo of skin allograft rejection in aged mice. We have stated this in the revised manuscript (page 7, paragraph 1). The effect of B cell depletion only becomes evident when acute allograft rejection is delayed under the cover of the immunoregulatory regimen of anti-CD45RB and anti-CD154.

2) The different effect of anti-CD20 in young versus old mice is only documented in the context of the treatment with anti-CD45RB plus anti-CD154, yet there is no comparison or characterization of B cell subsets and functions in young versus old mice in such treatment condition. It would therefore be necessary to characterize B cell subsets and functions in young and old mice treated with anti-CD45RB plus anti-CD154 in order to identify why B cells have opposite roles in these conditions. At present the

claim of the authors that CD19+CD21-CD23-CD43-CD93- cells are responsible for the beneficial effect of anti-CD20 in old mice is highly speculative.

Our response: We thank the reviewer for raising this important point. The revised manuscript provides new data that demonstrate that the adoptive transfer of splenic ABCs isolated from aged mice that are not treated with anti-CD45RB and anti-CD154 into young skin allograft recipients impairs the ability of anti-CD45RB and anti-CD154 to extend allograft survival (Figure 3F). These data provide direct *in vivo* evidence that ABCs from non-treated aged mice impair the immune regulation of allografts. Thus, the data indicate that the changes that occur within the ABC subpopulation upon aging without treatment (i.e., at baseline) are sufficient to impair the ability of anti-CD45RB and anti-CD154 to extend allograft survival. Nevertheless, in comparing the activation phenotype of ABCs from aged and young transplanted mice treated with anti-CD45RB and anti-CD154, we found that the increase upregulation of CD40, CD80, CD86, CD106 and TLR2 persist in aged ABCs after transplantation and treatment (Figure 2C-D). Functionally, the alloimmune priming capability of enriched splenic B cells remain higher in aged transplanted mice treated with anti-CD45RB and anti-CD154 than that of enriched splenic B cells from similar treated young mice (Figure 3B), consistent with the findings noted in B cells enriched from non-transplanted mice (Figure 3A-B).

References

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2. Hao, Y., et al., *A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice.* Blood, 2011. **118**(5): p. 1294-304.
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4. Lal, G., et al., *Interleukin-10 From Marginal Zone Precursor B-Cell Subset Is Required for Costimulatory Blockade-Induced Transplantation Tolerance.* Transplantation, 2015. **99**(9): p. 1817-1828.
5. Deng, S., et al., *Cutting Edge: Transplant Tolerance Induced by Anti-CD45RB Requires B Lymphocytes.* J Immunol, 2007. **178**(10): p. 6028-6032.
6. Clatworthy, M.R., et al., *B-cell-depleting induction therapy and acute cellular rejection.* N Engl J Med, 2009. **360**(25): p. 2683-5.

Second Editorial Decision - 03-Aug-2016

Dear Dr. Goldstein,

It is a pleasure to provisionally accept your manuscript entitled "Aged B cells alter immune regulation of allografts" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

European Journal of Immunology

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Laura Soto Vazquez

on behalf of
Prof. Andreas Radbruch

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