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The dynamics of T cells during persistent Staphylococcus aureus infection: from antigen-reactivity to in vivo anergy

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 25 February 2011

Thank you for the submission of your manuscript "The Dynamics of αβT Cells during Persistent Staphylococcus aureus Infection: from Effector Functions to in vivo Anergy" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, reviewers #2 and #3 highlight that it is critical to demonstrate the efficiency of the depletion of specific immune cells as well as the purity of cells transferred in the adoptive transfer experiments. Reviewer #3 also highlights that the data suggesting in vivo anergy need to be strengthened and makes specific suggestions for that. Importantly, both reviewers #1 and #2 point out that B and T cell numbers should not only be derived from spleen.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model system):

Good model for persistent S. aureus infection, gooed experimental evidence for role of T cells in this infection with gene expression analysis, RAGKO mice and adoptive transfer.
It is important for the medical community to know that B cells play no role in S.a. infection, explains many earlier observations including 2D gel of sera from patients and should discourage developping new antibodies against S.a. infeciton.,

Referee #1 (Other Remarks):

The authors address the important question of the adaptive immune response in a persistent S. aureus infection.
They follow mice 8 weeks after iv infection and document the persistence by quantifying cfu and describing abscesses in the kidneys. They measure kidney transcript expression 2 days and 4 weeks after infection and show a late upregulation of chemokines for T cells, of chemokine receptors on T cells and of components of B cell and TcR complexes.
They document the early role of PMN and macrophages by myeloid cell ablation assays and complement these observations by infecting RAGKO mice, where they show a higher bacterial load with a lack of abscess formation late in infection. Further evidence for the late role of the adaptive system is the reversion of the phenotype in RagKO mice by adoptive lymphocyte transfer in particular of the T cell fraction. In the kidney of persistently infected C57Bl/6 mice the authors show many plasma cells and CD3 cells. In spleens an increased number of B cells contrasts with an unchanged number of T cells with a increased proportion of T effector and memory cells. The antigen-mediated (heat-killed S. aureus) proliferative response of splenic T cells at 14 days is abolished, it is restored if the TcR + PKC are stimulated.

This comprehensive study of late immune responses in S. aureus infection is novel. The important role of T cells and the negligible role of B cells are well documented and discussed by the animal and human studies. However the recent study by Schmaler et al in J. immunol. Jan 6 2011, should be discussed since these authors also showed no role of B and T cells on bacterial clearing early in systemic S. aureus infection. Further they also documented with RAGKO mice the role of B and T cells for abscess formation and containment of the infection. Further they observed, what they called an Ñimmunosuppression" with increased IL-10 and less IL-17 after repeated vaccination with heat-killed S. aureus before infection. This phenomenon may shed light on, help explain and resemble the exhaustion observed in the present study of a prolonged infection.

Specific comments:
The picogram levels of serum IL-1b and TNF show a large variability. These data should not be shown, first because the levels are just above detection limit, second because it is not clear why TNF would be high later than Il1b, - in human sepsis the TNF peak is the earliest, but transient as the one of IL-1b -. Third, there is no TNF transcript increase in the kidney on day 2 and 28 (perhaps wrong time point!). It is well known that serum cytokines are not informative in sepsis. To test for persisting inflammation, transcript analysis - as done here -, in situ hybridization and immunocytochemistry from tissues are the method of choice. The inflammation should therefore be discussed based on the array data.

SInce the microarray was performed on whole kidney on day 28, kidneys should be studied on the same day by immunohistochemistry for T and B cells. The gene expression changes should be described while taking into account the infection-related change in leukocyte number and distribution, this is particularly important as the authors do describe on day 56 such changes in lymphocyte populations (high numbers of plasma cells) in the kidney.

Is the kidney the only organ with bacteria in the chronic infection, are there no cfu in bone or joints, heart, lung or brain ? Please test.

The B and T cell numbers in the spleen are not meaningful, they should be better discussed or left out. Is it known that total T and B numbers in the spleen are at all related to a functional change? This is different from the kidney, where T and B cells are normally rare and where the infection

takes place, thus these immune cells actively migrated to the kidney.

Minor points: in the paragraph on the array, the organ kidney should be mentioned early

Referee #2:

The manuscript by Ziegler et al. explores the immune response to Staphylococcus aureus using an intriguing persistent infection model of the bacterium and attempts to dissect the mechanism by which the bacterium evades the adaptive immune response against it. Using RAG2 and gamma-c deficient mice, the authors show that T cells, B cells and NK cells play a major role in the protective responses against this pathogen. They further use reconstitution experiments in RAG2-deficient mice to suggest a potent role for T cells - especially in controlling the bacterial load during the persistent infection. Finally, the authors suggest that the T cells in persistently Staphylococcus aureus infected mice are rendered anergic by the mechanism of adaptive tolerance - resulting in their failure to clear the bacteria completely.
The manuscript is quite interesting and the data are clearly presented. The question of whether a chronic bacterial infection evades immunity by triggering adaptive tolerance in T cells is certainly an important one. Previous studies demonstrating the induction of this form of in vivo anergy have relied on model antigen systems or chronic viral infections. However, several concerns (listed below) remain to be addressed, before this manuscript can achieve the task of providing a definitive answer to this question.
Specific comments:
1.Staphylococcus aureus and its products such as superantigens have been previously shown to trigger a massive inflammatory response (as the authors also observe) followed by a state of tolerance. In the T cell compartment, this tolerance has been shown to involve both the differentiation of the T cells to a regulatory/suppressive phenotype as well as the induction of anergy/adaptive tolerance (Sundstedt et al., J.Immunol.158:180, 1997; Miller et al., J.Exp.Med.190:53,1999). In this manuscript, the authors offer as evidence for the latter, the striking data that the proliferative responses of the T cells in persistently infected mice are severely blunted (Figure 8) and that this defect can be rescued by an activator of Protein Kinase C (PKC) such as phorbol-myristate-acetate (PMA) (Figure 9). However, they ignore the alternate explanation for the blunted proliferative responses - which is that the T cells in the infected mice have differentiated to acquire a regulatory phenotype (potentially characterized by the secretion of IL-10 and TGF-beta). This is likely to be a more plausible explanation for their observed proliferative defect, since the defect is observed in bulk stimulation of ALL the T cells in the animal with ConA or anti-CD3 (Figure 8 B & C). If the S. aureus-specific T cells were alone anergic, they would not be expected to dampen the total T cell proliferation in these cultures. However, if they had become regulatory, then the products of their activation could inhibit the proliferation of the remaining T cells. Fortunately, this can be easily resolved by performing a mixing experiment in which they culture T cells from uninfected mice together with titrations of T cells from the infected mice. The mixed cultures can then be stimulated with anti-CD3. If indeed the S. aureus infected mice have developed regulatory T cells, then the total proliferation in the mixed cultures (which includes the non-tolerant T cells from uninfected mice) will be down-regulated. Furthermore, in addition to proliferative responses, the authors could measure IL-2 in these cultures to validate their findings. It should also be noted that there is a recent report (Zanin-Zhorov et al., Science 328:974,2010) of PKC activation abrogating the activity of regulatory T cells - which may be consistent with the observed effect of PMA in the authors' model. We should emphasize that the distinction is critical. The idea that regulatory T cells can arise in some infectious disease models to dampen immunopathology has been demonstrated before. However, the question of adaptive tolerance being the critical regulator in such a context is yet to be reported and would therefore be quite novel.

2.The authors attempt to rule out the role of macrophages and neutrophils by depleting those cells using reagents such as Carrageenan and RB6. Both agents are notorious for lab-to-lab variation in efficacy. In order to be confident that the model system used does not rely on macrophages or neutrophils for protection, the authors should evaluate and display the efficiency of these treatments in depleting the targeted cell types. Along the same lines, it is interesting that even in the RAG2 and gamma-c-deficient mice (Figure 4), there is no continued growth of bacteria beyond the first week (at least at the same initial rate). Does this not suggest that the innate system does control some

amount of bacterial growth? And if macrophages, neutrophils and NK cells are removed from the picture, which cells do the authors suggest contribute to this residual protection? An experiment depleting macrophages or granulocytes in the RAG2-/- gamma-c -/- mice would be illuminating in this regard.

3.The experiments in Figures 5 and 6 do support the conclusion that T cells are critical for the control of persistent S. aureus infection. However, the authors dismiss the role of B cells in this process without evaluating the presence of B cells in the T cell transfer experiments. Since the authors purify T cells by MACS columns to 90% purity, there could still be contaminating B cells co-transferred with the T cells. These few B cells can receive adequate T cell help and differentiate to plasma cells - playing a critical role in the protection. This can be tested by measuring serum Ig in the T cell transfer recipients or looking for B cells in such mice.

4.The authors interpret the decrease in inflammatory responses as the consequence of changes in the immune response over time. However, they also show in Figure 1 that there are variant forms of bacteria that can be recovered from persistently infected mice. Is it possible that the variant forms are adapted to trigger less inflammatory responses? This can be easily tested by culturing colonies of variant S. aureus and infecting naive mice with these. It is also possible that these variants induce the altered T cell functionality reported here.

5.It seems that the proliferation assays to heat killed S. aureus shown in Figures 8 and 9 are performed using bulk splenocytes from persistently infected mice. Therefore, these cell populations are likely to carry significant amounts of bacterial antigens and superantigens along with them. It is well known that effector T cells show a bell-shaped curve in their proliferative responses to antigen - due to activation induced cell death at high doses of antigen. Therefore, the authors need to rule out the trivial explanation that the absence of proliferation is not simply a high dose effect in their cultures (the S.aureus they add, together with bacterial antigens carried over from the infection). There are a few ways of approaching this issue. One would be to purify the T cells away from the antigen-presenting cells (APCs) of the infected mice, before stimulating them with APCs from uninfected mice. Second would be to do the mix experiments detailed earlier. Alternatively, short term assays of cytokine measurements could be used instead of proliferation.

6.The authors evaluate T and B cell dynamics using the spleen of mice for most of the figures. This can be problematic if there is disproportionate expansion of the T cells in subsets of lymph nodes. At the very least the data in Figure 7 should be refined by incorporating cell numbers in lymph nodes.

Referee #3:

The article entitled "The Dynamics of αβT Cells during Persistent Staphylococcus aureus Infection: from Effector Functions to in vivo Anergy" by Ziegler, et al, addresses the nature of the adaptive host immunologic response to this pathogen. The authors utilize an in vivo persistence model of infection in which S. aureus remains present in the kidneys of infected mice for as long as 56 days post-infection. Several distinguishing features of the early and late response to infection are delineated in Figs. 1-3, leading the authors to hypothesize that T and/or B cell immune responses may be required to mitigate late infection. The presented data conclusively reveal a role for the adaptive immune response in curbing the number of S. aureus that can be recovered from the renal tissue of infected animals, most clearly documented through studies in RAG2-/- mice. These studies are of general interest to the fields of immunology and staphylococcal biology, and address an important facet of the host-pathogen interaction that has not been explored to any substantial degree to date. However, a number of essential controls are lacking in the present version of the manuscript, and additional data will need to be supplied to support the authors' conclusions.

Major comments:
1. The studies presented do not specifically address the effector functions of T cells (ie, directed cytolysis or polarized cytokine secretion), rather analyze T cell proliferative responses and infection outcome. As such, the title is inappropriate and should be revised to more accurately reflect the findings of the paper. Phrases in the manuscript citing the role of T cell effector functions should

similarly be modified.
2. The conclusion that neutrophils are required early in the response but are dispensable late in the response cannot be made from the data as presented. The authors should provide controls to document effective depletion of neutrophils, which may be very difficult to achieve on day 28 post-infection owing to the marginalization of these cells into infected tissues. It is similarly essential to document the degree of macrophage depletion achieved with carageenan.
3. The histopathologic findings as presented in Fig. 4C do not lend strong support to the authors' conclusions. The single image presented provides a limited view of the tissue architecture making it difficult to appreciate what is meant by 'intense areas of tissue destruction'. Likewise, additional images covering the broad time course of infection should be presented to document the failure of abscess formation in the RAG2-/- mice.
4. The purity of cell populations delivered to knockout mice in adoptive transfer experiments must be carefully documented by flow cytometric analysis and presented in the manuscript. In addition, data supporting reconstitution (such as Figs. 5D and 6B) should be presented in comparison to wild-type control mice, permitting an assessment of the degree of reconstitution.
5. It is not surprising that naÔve B cells from C57BL/6 mice are unable to contribute significantly to immunoprotection against S. aureus in the persistence model (Fig. 5E), as these cells in the context of the RAG2-/- background are devoid of T cell help that is essential to the establishment of B cell responses (ie, plasma cell differentiation and antibody secretion) against protein antigens. B cells should therefore be harvested from infected mice and transferred to RAG2-/- mice to most clearly appreciate the relevance of this cell population.
6. The authors' suggestion that S. aureus induces a state of adaptive tolerance or in vivo anergy is difficult to conclusively determine from the present data. The failure of T cell production of IL-2 after antigenic stimulation and an impaired response to recombinant IL-2 is a central tenet of in vivo anergy. These data should be presented. Also, in the setting of in vivo anergy, transfer of anergized T cells from a mouse harboring the offending antigen to a naÔve mouse will result in a loss of tolerance - ie, the T cells should resume a normal state of responsiveness. This should be examined by CFSE labeling of anergized, transferred T cells to confirm that normal proliferative responses are regained in the absence of antigen.

Minor comments:

1. There are multiple spelling and grammatical errors throughout the paper, some of which include the following:
-use of a comma following the word 'both' (multiple occurrences)
-"As commensal..." line 2 of Introduction should read "As a commensal"
-"activation of adaptive immune responses pursues..." seems as if it should read 'ensues' (Introduction)
- misspelling of 'Methods' in Supporting Information section
-'spleenocytes' and 'controll' in Results

2. RT-PCR results as presented in Fig. 3B should include appropriate statistical analysis. It is not clear from the current presentation that these results well mirror the microarray data as stated in the manuscript (see observations for cxcl13). It is curious why the authors did not elect to present RT-PCR data confirming the expression profile of chemokines CCL5/7/8 and CXCL9 that seem most directly relevant to the primary conclusions of the manuscript.

3. The display of data derived form the RAG2/IL-2R mice does not contribute substantial information relative to RAG2-/- mice alone; it therefore does not need to be displayed in the main results section.

4. SLP-76 is an essential component of the T cell receptor-proximal signaling cascade, serving as the docking site for Vav1. (1) This should be noted in the text.

5. The authors should delineate which experiments are performed with S. aureus strain 564 as mentioned in the Materials and Methods section.

References
1. Jordan MS, Koretzky GA. Coordination of receptor signaling in multiple hematopoietic cell lineages by the adaptor protein SLP-76. Cold Spring Harb Perspect Biol. 2010;2(4):a002501.

1st Revision – Authors' Response 17 June 2011

We would like to thank the reviewers for their constructive comments and careful critique of our work. Based on the reviewers' comments the revision has incorporated a significant amount of new experimental data that we believe has resulted in a stronger manuscript. Our responses to the reviewer's comments are addressed on a point-by-point basis as detailed below and changes denoted in red text throughout the revised manuscript. Due to the enormous amount of additional information and further experimental data requested by the reviewers, the length of the revised manuscript exceeds 60,000 characters.

The microarray raw data has been uploaded in NCBI´s GEO Database (http://www.ncbi.nlm.nih.gov/geo/) with restricted access during the publication-processing phase.

Reply to the Editor and Reviewer's comments:

Editor

In particular, reviewers #2 and #3 highlight that it is critical to demonstrate the efficiency of the depletion of specific immune cells as well as the purity of cells transferred in the adoptive transfer experiments.

Additional figures showing the efficiency of neutrophil (Supplemental Fig S1) and macrophage (Supplemental Fig S2) depletion as well as the purity of the sorted B (Supplemental Fig S7) and T (Supplemental Fig S8) cell population used in the adoptive transfer experiments have been included in the revised manuscript.

Reviewer #3 also highlights that the data suggesting in vivo anergy need to be strengthened and makes specific suggestions for that.

We have performed the experiments suggested by the reviewers to strength the *in vivo* anergy data (Fig 10D and Fig 10E). The results of those experiments are extremely interesting since they imply that the T cell unresponsiveness during persistent *S. aureus* infection involves a combination of two different mechanisms: (1) intrinsic molecular alteration in antigen-specific T cells, which is irreversible even in the absence of persistent antigenic stimulation; and (2) the presence of extrinsic suppressive mechanisms responsible for the dampening of T cell responses at the population level. The latter mechanism is reversible in the absence of persistent antigenic stimulation.

Importantly, both reviewers #1 and #2 point out that B and T cell numbers should not only be derived from spleen.

Additional experiments have been performed to determine the number of B and T cells in the peripheral lymph nodes. The results are displayed in Fig 6B (B cells), Fig 8B ($CD4^+$ T cells) and Fig 8D ($CD8^+$ T cells) of the revised manuscript.

Reviewer #1

The important role of T cells and the negligible role of B cells are well documented and discussed by the animal and human studies. However the recent study by Schmaler et al in J. immunol. Jan 6 2011, should be discussed since these authors also showed no role of B and T cells on bacterial clearing early in systemic S. aureus infection. Further they also documented with RAGKO mice the role of B and T cells for abscess formation and containment of the infection. Further they observed, what they called an „immunosuppression" with increased IL-10 and less IL-17 after repeated vaccination with heat-killed S. aureus before infection. This phenomenon may shed light on, help explain and resemble the exhaustion observed in the present study of a prolonged infection.

A paragraph has been added to the Discussion section of the revised manuscript addressing the data published by Schmaler *et al.* (page 18).

The picogram levels of serum IL-1b and TNF show a large variability. These data should not be shown, first because the levels are just above detection limit, second because it is not clear why TNF would be high later than Il1b, - in human sepsis the TNF peak is the earliest, but transient as the

one of IL-1b -. Third, there is no TNF transcript increase in the kidney on day 2 and 28 (perhaps wrong time point!).

As suggested by the reviewers, the graphs depicting the serum levels of IL-1beta and TNF-alpha have been removed from the revised manuscript.

Since the microarray was performed on whole kidney on day 28, kidneys should be studied on the same day by immunohistochemistry for T and B cells. The gene expression changes should be described while taking into account the infection-related change in leukocyte number and distribution, this is particularly important as the authors do describe on day 56 such changes in lymphocyte populations (high numbers of plasma cells) in the kidney.

A new figure (Fig 4) showing the immunostaining of B (Fig 4A) and T (Fig 4B) cells in the kidneys of *S. aureus*-infected mice at day 28 post-inoculation has been included in the revised manuscript. In addition, the text has been re-arranged in the revised manuscript in accordance with the reviewer's suggestion (page 8-9).

Is the kidney the only organ with bacteria in the chronic infection, are there no cfu in bone or joints, heart, lung or brain ? Please test.

In addition to the kidneys, we have assessed the bacterial loads in the joints (Fig 1B), heart (Fig 1C), liver (Fig 1D), and lungs (Fig 1E). The brains of infected mice were free of bacteria.

The B and T cell numbers in the spleen are not meaningful, they should be better discussed or left out. Is it known that total T and B numbers in the spleen are at all related to a functional change? This is different from the kidney, where T and B cells are normally rare and where the infection takes place, thus these immune cells actively migrated to the kidney.

In addition to the spleen, we have determined the kinetic of B and T cells in the lymph nodes. The results are displayed in Fig 6B (B cells), Fig 8B ($CD4^+$ T cells) and Fig 8D ($CD8^+$ T cells) of the revised manuscript.

Minor points: in the paragraph on the array, the organ kidney should be mentioned early

This has been made in the revised version.

Reviewer #2

1.Staphylococcus aureus and its products such as superantigens have been previously shown to trigger a massive inflammatory response (as the authors also observe) followed by a state of tolerance. In the T cell compartment, this tolerance has been shown to involve both the differentiation of the T cells to a regulatory/suppressive phenotype as well as the induction of anergy/adaptive tolerance (Sundstedt et al., J.Immunol.158:180, 1997; Miller et al., J.Exp.Med.190:53,1999). In this manuscript, the authors offer as evidence for the latter, the striking data that the proliferative responses of the T cells in persistently infected mice are severely blunted (Figure 8) and that this defect can be rescued by an activator of Protein Kinase C (PKC) such as phorbol-myristate-acetate (PMA) (Figure 9). However, they ignore the alternate explanation for the blunted proliferative responses - which is that the T cells in the infected mice have differentiated to acquire a regulatory phenotype(potentially characterized by the secretion of IL-10 and TGF-beta). This is likely to be a more plausible explanation for their observed proliferative defect, since the defect is observed in bulk stimulation of ALL the T cells in the animal with ConA or anti-CD3 (Figure 8 B & C). If the S. aureus-specific T cells were alone anergic, they would not be expected to dampen the total T cell proliferation in these cultures. However, if they had become regulatory, then the products of their activation could inhibit the proliferation of the remaining T cells. Fortunately, this can be easily resolved by performing a mixing experiment in which they culture T cells from uninfected mice together with titrations of T cells from the infected mice. The mixed cultures can

then be stimulated with anti-CD3. If indeed the S. aureus infected mice have developed regulatory T cells, then the total proliferation in the mixed cultures (which includes the non-tolerant T cells from uninfected mice) will be down-regulated. Furthermore, in addition to proliferative responses, the authors could measure IL-2 in these cultures to validate their findings. It should also be noted that there is a recent report (Zanin-Zhorov et al., Science 328:974,2010) of PKC activation abrogating the activity of regulatory T cells - which may be consistent with the observed effect of PMA in the authors' model. We should emphasize that the distinction is critical. The idea that regulatory T cells can arise in some infectious disease models to dampen immunopathology has been demonstrated before. However, the question of adaptive tolerance being the critical regulator in such a context is yet to be reported and would therefore be quite novel.

We have performed the mixing experiments mentioned by the reviewer and a new graph (Fig 10E) containing the new data has been added to the revised manuscript. The results of these experiments show that proliferation of naive splenic T cells is inhibited by the addition of splenocytes from infected mice in a dose-dependent manner. These results indicate the presence of suppressive mechanisms in the spleen of persistently infected mice. Furthermore, we have performed additional experiments to determine if anergic T cells remain unresponsive when transferred into an antigen-free environment. Our results (Fig 10D) show that antigen-specific T cells remain unresponsive to antigenic re-stimulation whereas the bulk T cell population recovers the capacity to respond to TCR stimulation (anti-CD3+anti-CD28). These results are very interesting since they imply that the T cell unresponsiveness during persistent *S. aureus* infection involves a combination of two different mechanisms: (1) intrinsic molecular alteration in antigen-specific T cells, which is irreversible even in the absence of persistent antigenic stimulation; and (2) the presence of extrinsic suppressive mechanisms responsible for the dampening of T cell responses at the population level. The latter mechanism is reversible in the absence of persistent antigenic stimulation.

2.The authors attempt to rule out the role of macrophages and neutrophils by depleting those cells using reagents such as Carrageenan and RB6. Both agents are notorious for lab-to-lab variation in efficacy. In order to be confident that the model system used does not rely on macrophages or neutrophils for protection, the authors should evaluate and display the efficiency of these treatments in depleting the targeted cell types.

The efficiency of macrophage (>95%) and neutrophil (>90%) depletion has been determined and two additional figures showing the corresponding FACS data have been added to the supplemental material of the revised manuscript (Supplemental Figure S1and Supplemental Figure S2).

Along the same lines, it is interesting that even in the RAG2 and gamma-c-deficient mice (Figure 4), there is no continued growth of bacteria beyond the first week (at least at the same initial rate). Does this not suggest that the innate system does control some amount of bacterial growth? And if macrophages, neutrophils and NK cells are removed from the picture, which cells do the authors suggest contribute to this residual protection? An experiment depleting macrophages or granulocytes in the RAG2-/-gamma-c -/- mice would be illuminating in this regard.

We have performed the experiments suggested by the reviewer regarding the depletion of neutrophils or macrophages in *S. aureus*-infected RAG2/IL-2R$\gamma^{-/-}$ mice. The results show that depletion of macrophages or neutrophils resulted in exacerbation of infection in the kidneys of RAG2/IL-2R$\gamma^{-/-}$ mice indicating that in the absence of specific immunity, these two populations of the innate immune system exert certain levels of control over *S. aureus* during the persistent phase of infection. These results are depicted in the Supplemental Figures (Fig S5).

3. The experiments in Figures 5 and 6 do support the conclusion that T cells are critical for the control of persistent S. aureus infection. However, the authors dismiss the role of B cells in this process without evaluating the presence of B cells in the T cell transfer experiments. Since the authors purify T cells by MACS columns to 90% purity, there could still be contaminating B cells co-transferred with the T cells. These few B cells can receive adequate T cell help and differentiate to plasma cells - playing a critical role in the protection. This can be tested by measuring serum Ig in the T cell transfer recipients or looking for B cells in such mice.

As suggested by the reviewer, we have determined the amount of B cells in RAG2$^{-/-}$ mice after adoptive transfer of purified T cells. A new panel (iv) has been added to Fig 7A demonstrating the absence of B cells in T cell-reconstituted RAG2$^{-/-}$ mice. The purity of the transferred B cells (>95%) is shown in Supplemental Fig S8.

4. The authors interpret the decrease in inflammatory responses as the consequence of changes in the immune response over time. However, they also show in Figure 1 that there are variant forms of bacteria that can be recovered from persistently infected mice. Is it possible that the variant forms are adapted to trigger less inflammatory responses? This can be easily tested by culturing colonies of variant S. aureus and infecting naive mice with these. It is also possible that these variants induce the altered T cell functionality reported here.

We agree with the reviewer that the change in the bacterial phenotype towards small colony variants (SCVs) may be a bacterial adaptation to persist within the host and this can influence the T cell responses. However, we cannot perform the experiments suggested by the reviewer since we have previously reported (Tuchscherr et al., *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med. 2011. 3:129-41) that the SCV is not a stable phenotype and rapidly reverts to the wild-type form during subculture. It is therefore impossible to prepare an inoculum constituted by SCVs since we will need to perform a subcultivation in order to get enough bacteria to challenge animals. After subculture, we only will have the reverted wild-type form.

5.It seems that the proliferation assays to heat killed S. aureus shown in Figures 8 and 9 are performed using bulk splenocytes from persistently infected mice. Therefore, these cell populations are likely to carry significant amounts of bacterial antigens and superantigens along with them. It is well known that effector T cells show a bell-shaped curve in their proliferative responses to antigen - due to activation induced cell death at high doses of antigen. Therefore, the authors need to rule out the trivial explanation that the absence of proliferation is not simply a high dose effect in their cultures (the S.aureus they add, together with bacterial antigens carried over from the infection). There are a few ways of approaching this issue. One would be to purify the T cells away from the antigen-presenting cells (APCs) of the infected mice, before stimulating them with APCs from uninfected mice. Second would be to do the mix experiments detailed earlier. Alternatively, short term assays of cytokine measurements could be used instead of proliferation.

If the loss of T cell function is antigen-driven as proposed by the reviewer, it can be speculated that the removal of the stimulating antigens might reverse the T cell anergy. To address this issue, we have isolated spleen cells from 28 days-infected C57BL/6 mice and transferred them into uninfected RAG2$^{-/-}$ mice. Spleen cells were harvested from the recipient mice after 7 and 28 days of rest and restimulated *in vitro* with different concentrations of heat-killed *S. aureus*. The proliferative response to antigen-specific stimulation was irreversible and could not be recovered after antigenic resting. These results are shown in Fig 10D in the revised manuscript.

6.The authors evaluate T and B cell dynamics using the spleen of mice for most of the figures. This can be problematic if there is disproportionate expansion of the T cells in subsets of lymph nodes. At the very least the data in Figure 7 should be refined by incorporating cell numbers in lymph nodes.

In addition to the spleen, we have determined the kinetic of B and T cells in the lymph nodes. The results are displayed in Fig 6B (B cells), Fig 8B (CD4^{+} T cells) and Fig 8D (CD8^{+} T cells) of the revised manuscript.

Review #3:

Major comments:
1. The studies presented do not specifically address the effector functions of T cells (ie, directed cytolysis or polarized cytokine secretion)., rather analyze T cell proliferative responses and infection outcome. As such, the title is inappropriate and should be revised to more accurately

reflect the findings of the paper. Phrases in the manuscript citing the role of T cell effector functions should similarly be modified.

We have exchanged the term "effector" by "antigen-reactive" T cells. The phrases in the manuscript mentioning "T cell effector functions" have been also modified.

2. The conclusion that neutrophils are required early in the response but are dispensable late in the response cannot be made from the data as presented. The authors should provide controls to document effective depletion of neutrophils, which may be very difficult to achieve on day 28 post-infection owing to the marginalization of these cells into infected tissues. It is similarly essential to document the degree of macrophage depletion achieved with carageenan.

The efficiency of macrophage (>95%) and neutrophil (>90%) depletion has been determined and two additional figures showing the corresponding FACS data have been added to the supplemental material of the revised manuscript (Supplemental Figure S1and Supplemental Figure S2).

3. The histopathologic findings as presented in Fig. 4C do not lend strong support to the authors' conclusions. The single image presented provides a limited view of the tissue architecture making it difficult to appreciate what is meant by 'intense areas of tissue destruction'. Likewise, additional images covering the broad time course of infection should be presented to document the failure of abscess formation in the RAG2-/- mice.

We have included an additional panel (ii) in Fig. 5B showing the kidney tissue architecture at day 28 of infection.

4. The purity of cell populations delivered to knockout mice in adoptive transfer experiments must be carefully documented by flow cytometric analysis and presented in the manuscript.

A new figure showing the purity of the B (Figure S8) and T (Figure S9) cells used in the adaptive transfer experiments have been included in the Supplemental material. The purity of sorted B cells was >95%.

In addition, data supporting reconstitution (such as Figs. 5D and 6B) should be presented in comparison to wild-type control mice, permitting an assessment of the degree of reconstitution.

Two new panels showing the B (Fig. 6Ci) and the T (Fig. 7Ci) cell populations in the spleen of wild type mice has been included in Figure 6 and 7, respectively. The reconstitution efficiency was ~50% for B cells, ~60% for CD4+ T cells and ~50% for CD8+ T cells.

5. It is not surprising that naive B cells from C57BL/6 mice are unable to contribute significantly to immunoprotection against S. aureus in the persistence model (Fig. 5E), as these cells in the context of the RAG2-/- background are devoid of T cell help that is essential to the establishment of B cell responses (ie, plasma cell differentiation and antibody secretion) against protein antigens. B cells should therefore be harvested from infected mice and transferred to RAG2-/- mice to most clearly appreciate the relevance of this cell population.

We have performed the experiment requested by the reviewer consisting in the transfer of B cells isolated from infected animals (day 21 of infection) to $RAG2^{-/-}$ mice to determine the relevance of primed B cells for the infection process. The results of this experiment show that the transfer of primed B cells did not significantly improve the response of $RAG2^{-/-}$ mice to *S. aureus* infection. This data have been included in the revised manuscript as "data not shown" (page 11).

6. The authors' suggestion that S. aureus induces a state of adaptive tolerance or in vivo anergy is difficult to conclusively determine from the present data. The failure of T cell production of IL-2 after antigenic stimulation and an impaired response to recombinant IL-2 is a central tenet of in vivo anergy. These data should be presented.

A new panel showing the impaired response of T cells to stimulation in the presence of rIL-2 data has been included in Fig 9 (Fig 9D).

Also, in the setting of in vivo anergy, transfer of anergized T cells from a mouse harboring the offending antigen to a naive mouse will result in a loss of tolerance - ie, the T cells should resume a normal state of responsiveness. This should be examined by CFSE labeling of anergized, transferred T cells to confirm that normal proliferative responses are regained in the absence of antigen.

We have performed additional experiments to determine if the loss of T cell function is antigen-driven and the removal of the stimulating antigens might reverse the T cell anergy. In these experiments we have isolated spleen cells from 30 days-infected C57BL/6 mice and transferred them into uninfected RAG2$^{-/-}$ mice. Spleen cells were harvested from the recipient mice after 7 and 28 days of rest and restimulated *in vitro* with different concentrations of heat-killed *S. aureus* or anti-CD3 + anti-CD28. The proliferative response to antigen-specific stimulation was irreversible and could not be recovered after antigenic resting. In contrast, the unresponsiveness of T cells at the population level to TCR stimulation is reversible in the absence of persisting antigen and it is most probably induced by the presence of extrinsic suppressive mechanisms. Therefore, T cell unresponsiveness during persistent *S. aureus* infection seems to involve a combination of two different mechanisms: (1) intrinsic molecular alteration in antigen-specific T cells, which is irreversible even in the absence of persistent antigenic stimulation; and (2) the presence of suppressive mechanisms responsible for the dampening of T cell responses at the population level. The latter mechanism is reversible in the absence of persistent antigenic stimulation. These results suggest that are shown in Fig 10D in the revised manuscript.

Minor comments:
1. There are multiple spelling and grammatical errors throughout the paper, some of which include the following:
-use of a comma following the word 'both' (multiple occurrences)
-"As commensal..." line 2 of Introduction should read "As a commensal"
-"activation of adaptive immune responses pursues..." seems as if it should read 'ensues' (Introduction)
- misspelling of 'Methods' in Supporting Information section
-'spleenocytes' and 'controll' in Results

We apologize for the grammatical errors. The revised manuscript has been checked by a native English speaker.

2. RT-PCR results as presented in Fig. 3B should include appropriate statistical analysis. It is not clear from the current presentation that these results well mirror the microarray data as stated in the manuscript (see observations for cxcl13). It is curious why the authors did not elect to present RT-PCR data confirming the expression profile of chemokines CCL5/7/8 and CXCL9 that seem most directly relevant to the primary conclusions of the manuscript.

Statistical analysis has been included in Fig. 3B.

3. The display of data derived form the RAG2/IL-2Rg-/- mice does not contribute substantial information relative to RAG2-/- mice alone; it therefore does not need to be displayed in the main results section.

The Figure showing the course of *S. aureus* infection in the kidneys of RAG2/IL-2R$\gamma^{-/-}$ mice has been moved to the Supplemental Figures (Fig 4S).

4. SLP-76 is an essential component of the T cell receptor-proximal signaling cascade, serving as the docking site for Vav1. (1) This should be noted in the text.

This information has been included in the revised manuscript (Page 14).

5. The authors should delineate which experiments are performed with S. aureus strain 564 as mentioned in the Materials and Methods section.

All experiments have been performed with *S. aureus* strain SH1000. This has been corrected in the Materials and Methods section.

2nd Editorial Decision 20 July 2011

Thank you for the submission of your revised manuscript "The dynamics of T cells during persistent Staphylococcus aureus infection: from antigen reactivity to in vivo anergy" to EMBO Molecular Medicine. We have now received the enclosed reports from the referees whom we asked to re-assess it.

As you will see, both Reviewers acknowledge that the manuscript was significantly improved during revision. However, both Reviewers also raise concerns about the interpretation of the newly added data in figure 10 and Reviewer #3 suggests an additional experiment to support the conclusion that the suppressive mechanism is reversible.

We agree that it would be ideal to perform the suggested experiment, however, we realize that the addition of more data would be time-consuming. Should you be able to provide the data, we would encourage you to include them into the present study. Otherwise, we would strongly encourage you to include a brief discussion of this issue into the manuscript and to tone down the respective conclusions regarding figure 10.

On a more editorial note, in your revised manuscript, please include the accession number for the microarray data in the manuscript. Data of gene expression experiments described in submitted manuscripts should be deposited in a MIAME-compliant format with one of the public databases. We would therefore ask you to submit your microarray data to the ArrayExpress database maintained by the European Bioinformatics Institute for example. ArrayExpress allows authors to submit their data to a confidential section of the database, where they can be put on hold until the time of publication of the corresponding manuscript. Please see http:www.ebi.ac.uk/arrayexpress/Submissions/ or contact the support team at arrayexpress@ebi.ac.uk for further information.

Please submit your revised manuscript within one month.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #2:

The Manuscript by Ziegler et al, in its revised form addresses most of the concerns raised by us and other reviewers last time. The authors must be commended on their efforts and in the process, the manuscript has greatly improved.

The experiments performed during the revision have also added some interesting dimensions to the work. For example, it is now clear that the model system instills tolerance using a combination of anergy and suppression. The authors attempt to reverse the anergy by adoptively transferring T cells to uninfected mice (Figure 10). While this is certainly a good experiment, we are not necessarily in complete agreement with the interpretation of the data. It is clear that the responses to staph antigens are still depressed 21 days after this transfer, while the broader response to anti-CD3 recovers. However, this by itself is not evidence of the loss of suppression and the maintenance of anergy. It is still possible that the data points to a further level of complexity - an antigen specific suppression

component that is not reversed after antigen removal for 21 days, in addition to the anergy. The global suppression (potentially mediated by large amounts of suppressive cytokines such as IL-10 and TGF-b secreted by the regulatory cells chronically activated by the infection) wanes after clearance of infection, but a more narrow subset of suppressive functions could still be retained in the antigen specific T cells, as a future safeguard against immunopathology from robust anti-staph responses.Therefore, it is still likely that the behavior of T cells here continues to reflect a mixture of anergy and immuno-supression.

Referee #3:

The revised manuscript entitled, "The dynamics of T cells during persistent Staphylococcus aureus infection: from antigen reactivity to in vivo anergy' by Ziegler, et al now provides a more carefully refined analysis of the anergic response of T cells following S. aureus infection. A number of modifications, including the presentation of additional controls and new, supportive experimental data enhance the findings as originally presented. The specific addition of new data presented in Fig. 10D demonstrating the inability of antigen-exposed T cells to regain normal proliferative function even after removal from the antigen is interesting, and highlights the potency with which S. aureus alters the adaptive immunologic response.
In this figure (10E), the authors also provide new evidence that a suppressive effect may globally impair T cell responses, in that spleen cells harvested from infected mice are able to inhibit proliferative responses of naive T cells upon coculture in the presence of anti-CD3. Distilling data from Figs. 10D and E, the authors conclude in the last sentences of the manuscript, "...the presence of suppressive mechanisms responsible for the dampening of T cell responses at the population level. This last mechanism is reversible in the absence of persistent antigenic stimulation." This conclusion is not well supported by the data as presented. While Fig. 10D shows the ability of some T cells to regain the ability to respond to the potent stimulation provided with anti-CD3 and anti-CD28 crosslinking after a period of rest, the design of this experiment is distinct from that presented in Fig. 10E from which a conclusion about suppression effects was correctly drawn. To make the conclusion stated above, the authors would need to recapitulate the expt. performed in Fig. 10E with naive cells exposed to infected cells that had been subjected to antigen rest for 7 or 28 days (as in panel D). If naive cells retained the ability to proliferate in response to anti-CD3 alone under these conditions, the above conclusion of reversibility of the suppressive effect would be supported. In the absence of this data, the authors should probably discuss the reversibility of suppression as a possible outcome that can be further investigated in subsequent studies.

2nd Revision - Authors' Response 22 July 2011

We would like to thank the reviewers for their appraisal of the revised manuscript. As suggested, we have included a brief discussion in the re-revised version of the manuscript addressing the issues that has been raised by the reviewers (blue text, page 18-19 of the Discussion section). We have also toned down the conclusions regarding the data presented on Figures 10(page 15 of the Results section). The accession number for the microarray data has been included in the current version of the manuscript (page 23 of the Materials and Methods section).

The microarray raw data has been uploaded in NCB's GEO Database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25244) with restricted access during the publication-processing phase.

We hope that the manuscript would now become acceptable for publication in EMBO Molecular Medicine.

