European Journal of Immunology

Maintenance of murine CD8$^+$ memory T lymphocytes in the spleen but not in the bone marrow is dependent on proliferation

Francesco Siracusa, Özen Sercan-Alp, Patrick Maschmeyer, Mairi McGrath, Mir-Farzin Mashreghi, Shintaro Hojyo, Hyun-Dong Chang, Koji Tokoyoda and Andreas Radbruch

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Review Timeline:
Submission date: 04-Apr-2017
First Editorial decision: 08-May-2017
Revision received: 29-Jun-2017
Accepted: 07-Aug-2017

Handling Executive Committee member: Prof. Francesco Annunziato

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

08-May-2017

Dear Dr. Siracusa, dear Prof. Radbruch,

We are sorry for a slight delay in the peer review of your Manuscript ID eji.201747063 entitled "Maintenance of CD8 memory T lymphocytes in the spleen but not in the bone marrow is dependent on proliferation" which you submitted to the European Journal of Immunology. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. 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. In addition, we encourage you to more thoroughly discuss all the points raised by the Referee 2, since this referee has pointed to several technical points, mainly the use of cyclosphosphamide, which may impact the interpretation of your findings.

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 referees 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,
Nadja Bakocevic

On behalf of
Prof. Francesco Annunziato

Dr. Nadja Bakocevic
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Reviewer: 1

Comments to the Author
This brief report of Radbruch and colleagues shows that CD8 memory T-cells are depleted by cyclophosphamide in the spleen, but not in in the bone marrow following vaccination in the memory phase. This is additional and quite convincing evidence for the provocative claim by these authors that CD8 memory maintenance does not require homeostatic proliferation, but that survival in the resting state in the bone marrow is sufficient. Needless to say that this is a highly relevant issue.
Specific points:
It is stated in the abstract that also memory cells generated by natural infections were analyzed; however

this relevant data is not included in the manuscript. Since this is an intense ongoing debate I strongly recommend to show this data, to avoid the future interpretation that the findings are limited to artificial immunizations and do not apply for memory T-cells generated by infections. In addition, I recommend to expand the Discussion section; in its current form the manuscript is difficult to fully understand without referring to the previously published papers/comments on this issue. In particular it is unclear how the authors explain the different behaviour of splenic and bone marrow memory cells. I guess the idea is that memory cells in the bone marrow become tissue-resident cells. CD69 was however not analyzed here, which is really a pity. Also the interesting concept of Il-7 and IL-15-dependent niches of CCR7+ and CCR7- memory cells proposed by Kaech and colleagues (published in PNAS recently) was not considered, for example the expression of the relevant cytokine receptors would be interesting. Is it possible that CCR7+ memory T-cells require Il-7 to survive in a quiescent state, while the CCR7- memory cells require Il-15 and need to proliferate to compensate for cell death? Finally, experiments with TCR transgenic mice could demonstrate that the different behaviour of splenic and bone marrow memory cells is independent of the TCR clonotype. All these experiments would of course be a lot of work, but would strengthen the point the authors want to make and should at least be discussed and addressed in later studies.

Reviewer: 2

Comments to the Author

This is an interesting and well-written Short Communication, in which the former dogma that memory CD8 T cells in the bone marrow would be maintained by homeostatic proliferation is challenged. The Radbruch group has a strong history in analyzing the physiology and function of T cells in the bone marrow, and they have already shown before in a very convincing and elegant paper (Sercan Alp et al. EJI 2015) that memory CD8 T cells are not proliferating, but rather quiescent. Their finding that BrdU-incorporation not only reflects, but also induces cell cycle progression, was an important finding that provided a proper explanation for their opposing results with previous reports.

The current manuscript supports their former findings, in which the authors use the cytostatic drug Cyclophosphamide (Cyp) to delete dividing T cells. Although the data illustrate a clear difference between memory CD8 T cells isolated from bone marrow and spleen, there are several caveats to both the experimental set-up as well as the conclusions.

1. The major problem I have is that the authors do not take into account, neither experimentally, nor in their discussion, that memory CD8 T cells are highly migratory cells, and it is not clear from this manuscript to what extent this affects the outcome of the experiments. During the Cyp-treatment, which lasts either 1 or 2 weeks, it is very likely that most memory T cells have pass through the spleen and bone marrow multiple times. It is therefore not possible to conclude in which organ the cells are depleted during the treatment.

2. Related to this, the authors argue that the findings from the Ahmed lab that led to the aforementioned dogma were based on experiments, in which the fate of adoptively transferred splenic memory CD8 T cells was studied. This experimental approach is quite reasonable if one assumes that memory CD8 T cells are highly migratory cells (which has been shown in many experiments) and that the source of the donor cells does not matter. Yet, if the authors argue that it does matter, it would be imperative that they repeat these former experiments and compare side by side the fate of memory CD8 T cells purified from either the spleen or the bone marrow.

3. Another inherent problem with the approach taken in this manuscript is that Cyp has a massive impact on many cell types, particularly in the bone marrow. The loss of organ integrity, both in spleen and bone marrow, may well affect the memory T cells in an indirect manner. Cyp depletes many cell types, including Tregs, which can by itself already affect T cell homeostasis. Cyp has been shown to induce expansion of dendritic cells; given that DCs can be a major source of IL-15, it is plausible that Cyp-treatment increases the bio-availability of IL-15 in the bone marrow, thereby enhancing the proliferation and/or survival of memory CD8 T cells. Related to this, the Cyp-mediated lymphodepletion itself will increase the bioavailability of IL-7 and IL-15; if this is happening more efficiently in the bone marrow than in the spleen, this will also benefit the remaining (or re-entering) memory CD8 T cells in the bone marrow. Support for these notions can be seen in Figure 1D, where the fraction of Ki67+ memory CD8 T cells in the pentamer-negative fraction increases from 2.66% to 7.83% in the bone marrow; this is not seen in the spleen, where this fraction rather decreases from 9.83% to 4.07%. This finding and its implications are not discussed by the authors.

4. Even if we would consider only the direct effects of Cyp on the T cells, it is still unclear why the memory CD8 T cells in the bone marrow would be more protected than in the spleen. As bone marrow memory CD8 T cells are largely quiescent (shown here and in their former paper), it is logical that they are less sensitive to Cyp. However, it is not clear whether the DNA of quiescent memory CD8 T cells is equally alkylated as the DNA of non-quiescent CD8 T cells, and whether the quiescent cells would also die if they would receive proliferation cues. This could be tested by restimulating CD44+ CD8 T cells from the bone marrow of Cyp-treated animals in vitro with anti-CD3 and examine whether they subsequently die, in contrast to memory T cells taken from PBS-treated mice.

5. Furthermore, the authors postulate that the memory CD8 T cells in the bone marrow are protected from Cyp, as they are largely quiescent. However, the majority of the splenic B cells as well as the Ki67-negative pentamer+ T cells in the spleen are also depleted by the Cyp treatment, whereas they are also quiescent. This reinforces the notion that the quiescent state of the memory T cells in the bone marrow alone is not sufficient to explain the observed effects and that indirect effects of Cyp-treatment (as

elaborated upon above) may play an important role as well.

6. It is laudable that the authors used antigen-specific, in vivo generated, long-term surviving memory CD8 T cells to address their research question. However, the numbers of Ki67+ SIINFEKL-specific CD8 T cells remaining at day 105 after the immunization is extremely low (I count 7 cells in the spleen of the PBS-group in Fig. 2E; 0.013%), which makes it an unreliable experimental approach to draw firm conclusions from.

First Revision – authors' response

29-Jun-2017

Dear Editor,

Thanks for the overall positive evaluation of our manuscript to you and the reviewers involved. It was stimulating and we think it has improved the manuscript to a state when it should be acceptable, by now.

Point-by-point we would like to reply to the arguments of the reviewers and indicate our measures:

Reviewer: 1

Comments to the Author

This brief report of Radbruch and colleagues shows that CD8 memory T-cells are depleted by cyclophosphamide in the spleen, but not in in the bone marrow following vaccination in the memory phase. This is additional and quite convincing evidence for the provocative claim by these authors that CD8 memory maintenance does not require homeostatic proliferation, but that survival in the resting state in the bone marrow is sufficient. Needless to say that this is a highly relevant issue.

Needless to say that we fully agree to this introductory statement.

Specific points:

It is stated in the abstract that also memory cells generated by natural infections were analyzed; however this relevant data is not included in the manuscript. Since this is an intense ongoing debate I strongly recommend to show this data, to avoid the future interpretation that the findings are limited to artificial immunizations and do not apply for memory T-cells generated by infections.

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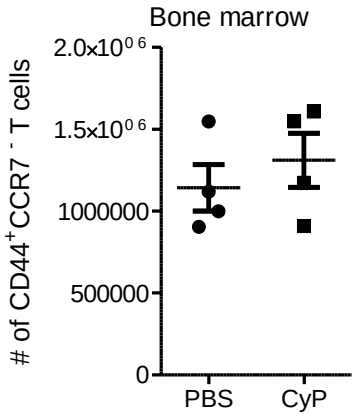
As we point out in the manuscript, we have determined the maintenance of CD8 memory T cells generated in an intentional immunization (to Ova), but also of all memory $CD8^+CD44^+$ T cells in both spleen and bone (Fig. 1C, 2D, 3C). Clearly these memory cells had been generated by natural, unintentional infections in the lifetime of the animals, and not by intentional immunization. We do not think that intentional infections would add any important information beyond that.

In addition, I recommend to expand the Discussion section; in its current form the manuscript is difficult to fully understand without referring to the previously published papers/comments on this issue. In particular it is unclear how the authors explain the different behaviour of splenic and bone marrow memory cells. I guess the idea is that memory cells in the bone marrow become tissue-resident cells. CD69 was however not analyzed here, which is really a pity.

We have done as requested and extended the discussion section a little bit to elaborate on the concept of "(cell) cycling and circulating" versus "resting and resident" memory T cell maintenance. This is to discuss the new Figure 3, which shows that the results obtained are independent of circulation, i.e. cannot be blocked with FTY720. With respect to CD69, we and others had shown previously that only about 30% of CD8 memory T cells in bone and less than 10% in spleen are $CD69^+$. Since CyP eliminates 50% of the splenic memory cells and none of the bone marrow, also in the presence of FTY720 (Fig. 3), it is clear that not only $CD69^+$ memory CD8 cells are resting and not ablated by CyP. We have also stated that in the discussion section, by now.

Also the interesting concept of Il-7 and IL-15-dependent niches of CCR7+ and CCR7- memory cells proposed by Kaech and colleagues (published in PNAS recently) was not considered, for example the expression of the relevant cytokine receptors would be interesting. Is it possible that CCR7+ memory T-cells require Il-7 to survive in a quiescent state, while the CCR7- memory cells require Il-15 and need to proliferate to compensate for cell death?

We have determined the frequencies and absolute numbers of $CCR7^+$ and $CCR7^-$ CD8 memory T cells in the bone marrow and can show that they do not change upon CyP treatment, i.e. $CCR7^-$ memory cells are not maintained by proliferation in the bone marrow. We now also mention this in the discussion section as "data not shown". Data are shown below for the attention of the reviewer:



Finally, experiments with TCR transgenic mice could demonstrate that the different behaviour of splenic and bone marrow memory cells is independent of the TCR clonotype. All these experiments would of course be a lot of work, but would strengthen the point the authors want to make and should at least be discussed and addressed in later studies.

This point comes back to the first point, namely generalization. As elucidated above, we have measured not only the response to one antigen, but also the global population of CD8 memory cells. This means that our argument is “global”. We do not agree that analysis of clonotypes would strengthen our argument, apart from the fact that it would be endless work. It is also not clear to us, what the reviewer is asking for? If it comes to the question which memory cells are maintained in the spleen and which ones in the bone marrow, we have analysed transgenic and wild-type cells before, also different specificities in humans and came up with the published concept that bone marrow hosts the long-term memory to systemic antigens/pathogens. But it is by no means the intention of the present manuscript to address this question. This manuscript just makes the point that the CD8 memory T cells of the bone marrow are maintained as resting (and resident) cells, contrary to established paradigms.

Reviewer: 2

Comments to the Author

This is an interesting and well-written Short Communication, in which the former dogma that memory CD8 T cells in the bone marrow would be maintained by homeostatic proliferation is challenged. The Radbruch group has a strong history in analyzing the physiology and function of T cells in the bone marrow, and they have already shown before in a very convincing and elegant paper (Sercan Alp et al. EJI 2015) that

memory CD8 T cells are not proliferating, but rather quiescent. Their finding that BrdU-incorporation not only reflects, but also induces cell cycle progression, was an important finding that provided a proper explanation for their opposing results with previous reports.

Thank you.

The current manuscript supports their former findings, in which the authors use the cytostatic drug Cyclophosphamide (Cyp) to delete dividing T cells. Although the data illustrate a clear difference between memory CD8 T cells isolated from bone marrow and spleen, there are several caveats to both the experimental set-up as well as the conclusions.

1. The major problem I have is that the authors do not take into account, neither experimentally, nor in their discussion, that memory CD8 T cells are highly migratory cells, and it is not clear from this manuscript to what extent this affects the outcome of the experiments. During the Cyp-treatment, which lasts either 1 or 2 weeks, it is very likely that most memory T cells have pass through the spleen and bone marrow multiple times. It is therefore not possible to conclude in which organ the cells are depleted during the treatment.

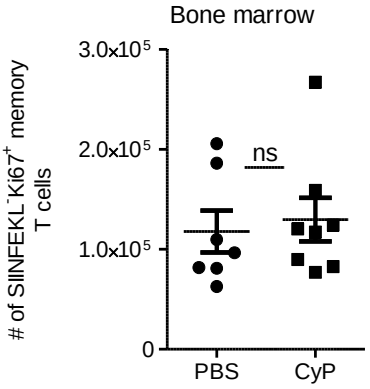
We have addressed this argument now, by inhibition of circulation with the sphingosin-1-phosphate analog FTY720. The results are shown in the new Figure 3. We demonstrate that we can efficiently block circulation, but nevertheless numbers of CD8 memory T cells in the bone marrow are not affected by CyP, while in spleen about 50% of the CD8 memory T cells are depleted. This result shows that the cell counts in the bone marrow are not the result of cells dying and being replaced by immigrating cells. It is also in line with the notion that the cells in the bone marrow are resident cells, at least for the time of observation.

2. Related to this, the authors argue that the findings from the Ahmed lab that led to the aforementioned dogma were based on experiments, in which the fate of adoptively transferred splenic memory CD8 T cells was studied. This experimental approach is quite reasonable if one assumes that memory CD8 T cells are highly migratory cells (which has been shown in many experiments) and that the source of the donor cells does not matter. Yet, if the authors argue that it does matter, it would be imperative that they repeat these former experiments and compare side by side the fate of memory CD8 T cells purified from either the spleen or the bone marrow.

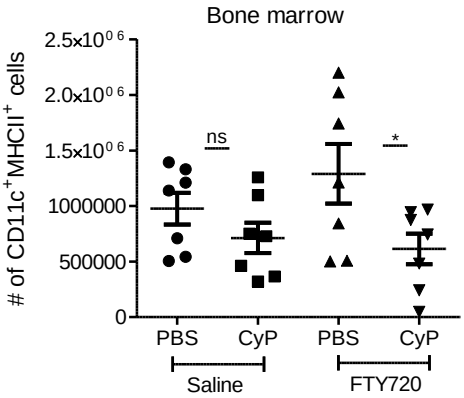
We discuss that the experiment of the Ahmed lab could have two potential pitfalls, namely that it is an adoptive transfer experiment, quasi selecting for circulating cells, and that the cells were not obtained from the bone marrow but from the spleen. On purpose we here choose an approach aiming at the analysis of memory cells *in situ*, rather than after adoptive transfer.

3. Another inherent problem with the approach taken in this manuscript is that Cyp has a massive impact on many cell types, particularly in the bone marrow. The loss of organ integrity, both in spleen and bone marrow, may well affect the memory T cells in an indirect manner. Cyp depletes many cell types, including Tregs, which can by itself already affect T cell homeostasis. Cyp has been shown to induce expansion of dendritic cells; given that DCs can be a major source of IL-15, it is plausible that Cyp-treatment increases the bio-availability of IL-15 in the bone marrow, thereby enhancing the proliferation and/or survival of memory CD8 T cells. Related to this, the Cyp-mediated lymphodepletion itself will increase the bioavailability of IL-7 and IL-15; if this is happening more efficiently in the bone marrow than in the spleen, this will also benefit the remaining (or re-entering) memory CD8 T cells in the bone marrow.

The new Figure 3 shows that the survival of CD8 memory T cells in the bone marrow is independent of immigrating CD8 memory T cells. CyP also does not change the frequencies (Fig. 2) and numbers (see below, additional information for the reviewers) of Ki-67$^+$ CD8 memory T cells in the bone marrow, in the memory phase of an immune response, as analysed in Figure 2:

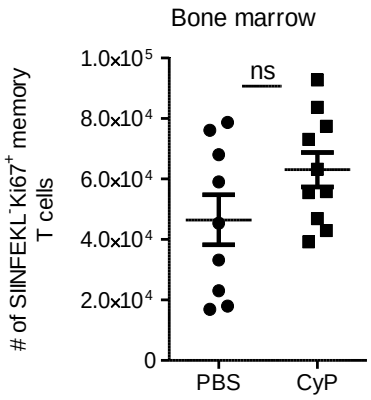


Neither did CyP increase the numbers of dendritic cells, see below, additional information for the reviewer.



Support for these notions can be seen in Figure 1D, where the fraction of Ki67+ memory CD8 T cells in the pentamer-negative fraction increases from 2.66% to 7.83% in the bone marrow; this is not seen in the spleen, where this fraction rather decreases from 9.83% to 4.07%. This finding and its implications are not discussed by the authors.

In Figure 1D we analyse the effect of CyP on CD8 cells of an ongoing immune reaction, depleting the Ova-reactive cells in spleen and bone marrow. Below are shown the absolute numbers of SIINFEKL$^-$Ki67$^+$ memory CD8 T cells, which show a not significant difference, for the information of the reviewer:



In Fig. 2E and F, we show the frequencies of Ki-67$^+$ cells in spleen and bone marrow. They are about the same in the bone marrow, with and without CyP, and drop from about 15% to 5% in the spleen. We find it even more remarkable that although only 15% of the splenic CD8 memory cells express Ki-67 at any time point, 50% are depleted during the window of treatment, implying that within two weeks about 30% of the splenic CD8 memory cells switch from Ki-67$^-$ to Ki-67$^+$, impressive dynamics. On the other hand, in the bone marrow, Ki-67$^+$ memory cells are not ablated by CyP (Figure 2F), suggesting that they are probably not in G_0, but also not cycling. We have discussed this in the manuscript.

4. Even if we would consider only the direct effects of Cyp on the T cells, it is still unclear why the memory CD8 T cells in the bone marrow would be more protected than in the spleen. As bone marrow memory CD8 T cells are largely quiescent (shown here and in their former paper), it is logical that they are less sensitive to Cyp. However, it is not clear whether the DNA of quiescent memory CD8 T cells is equally alkylated as the DNA of non-quiescent CD8 T cells, and whether the quiescent cells would also die if they would receive proliferation cues. This could be tested by restimulating CD44+ CD8 T cells from the bone marrow of Cyp-treated animals in vitro with anti-CD3 and examine whether they subsequently die, in contrast to memory T cells taken from PBS-treated mice.

We have done this experiment and it is now included as Figure 2G in the revised version of the manuscript. The result confirms the efficient alkylation of the DNA of CD8 memory T cells in the bone marrow. When reactivated, they cannot expand efficiently.

5. Furthermore, the authors postulate that the memory CD8 T cells in the bone marrow are protected from Cyp, as they are largely quiescent. However, the majority of the splenic B cells as well as the Ki67-negative pentamer+ T cells in the spleen are also depleted by the Cyp treatment, whereas they are also quiescent. This reinforces the notion that the quiescent state of the memory T cells in the bone marrow alone is not sufficient to explain the observed effects and that indirect effects of Cyp-treatment (as elaborated upon above) may play an important role as well.

As we show in the manuscript, the maintained numbers of CD8 memory T cells in the bone marrow, even in the situation when the circulation is blocked (Figure 3) and controlled for efficient alkylation of their DNA (i.e. impairment in their expansion when they receive proliferation cues (new Fig. 2G), argue that these cells are maintained as not proliferating, "quiescent" or "resting" cells, for the time of observation, i.e. 14 days. In contrast, Ki-67 indicates a momentary state of the cell in G_1 to M phases of the cell cycle, propidium iodide (PI) indicates S to M phases, at the time of observation. We think that it is a major point of our present manuscript that in contrast to Ki-67 and PI, we now determine proliferation over time. As discussed already in response to point 3, this reveals a remarkable dynamics of splenic CD8 memory T cells to switch from quiescence to proliferation. We think we have discussed this in the manuscript.

6. It is laudable that the authors used antigen-specific, in vivo generated, long-term surviving memory CD8 T cells to address their research question. However, the numbers of Ki67+ SIINFEKL-specific CD8 T cells remaining at day 105 after the immunization is extremely low (I count 7 cells in the spleen of the PBS-group in Fig. 2E; 0.013%), which makes it an unreliable experimental approach to draw firm conclusions from.

We agree that those low numbers weaken our argument. We thus replaced the former Figure 2F, with data on SIINFEKL-specific cells, with the new Figure 2F, with data on SIINFEKL$^-$ cells. The conclusion is the same, but now substantiated by large numbers of cells.

European Journal of Immunology

Second Editorial Decision

31-Jul-2017

Dear Prof. Radbruch,

It is a pleasure to provisionally accept your manuscript entitled "Maintenance of CD8 memory T lymphocytes in the spleen but not in the bone marrow is dependent on proliferation" 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.

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,
Nadja Bakocevic

on behalf of
Prof. Francesco Annunziato

Dr. Nadja Bakocevic
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