European Journal of Immunology

Peer review correspondence

Post-arrest stalling rather than crawling favors CD8$^+$ over CD4$^+$ T-cell migration across the blood-brain barrier under flow in vitro

Henriette Rudolph, Armelle Klopstein, Isabelle Gruber, Claudia Blatti, Ruth Lyck and Britta Engelhardt

Corresponding author: Britta Engelhardt, Theodor Kocher Institute, University of Bern, Bern, Switzerland

---

Review Timeline:	Submission date:	12 December 2015
	First editorial decision:	11 January 2016
	Revision received:	12 May 2016
	Accepted:	20 June 2016

Handling Executive Committee member: Prof. Shimon Sakaguchi

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 – 11 January 2016

Dear Prof. Engelhardt,

Manuscript ID eji.201546251 entitled "Post arrest stalling rather than crawling favors CD8+ over CD4+ T-cell migration across the blood-brain barrier under flow in vitro" which you submitted to the European Journal of Immunology has been reviewed. 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.

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.*

European Journal of Immunology

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. Shimon Sakaguchi

Dr. Karen Chu
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

****

Reviewer: 1

Comments to the Author
The authors elegantly showed that CD8 T cells show a different kinetics in crossing the brain EC than CD4 T cells. This work is likely to bring the field forward. The data are solid and proper controls are included. Moreover, the conclusions drawn from the data are sound and interesting. However, there are a few minor points that I would like to see addressed prior to acceptation. I listed them below.
1. Arrest and migration were induced only by cytokine (i.e. TNF/IL1)-treated ECs. What about chemoattractant cytokines? Do the authors still see a preference (CD8 over CD4) when the ECs are immobilized with a certain chemokine, or will this favor one cell type over the other due to chemokine preference? I would suggest to at least discussing this point in the discussion section, for example following MHC discussion (line 45).
2. Please explain better why the CD4 and 8 cells are harvested from two different mice models. Why did the authors not try to isolate the CD4 and 8 cells form the same WT mouse line?

European Journal of Immunology

3. The authors elegantly show that CD8 cells prefer the transcellular route, by isolating brain ECs from VE-cad GFP mice. I wonder if these T cells cross the EC transcellularly in other vascular beds (e.g. lung vs skin) as well. Can the authors comment on this?
4. I am somewhat worried about the culture conditions, in particular the extracellular matrix where the ECs are cultured on, since in particular the brain is known for its soft environment and the culture conditions do not reflect this particular environment; on the contrary, the ECs are cultured on plastic/glass (I assume since I could not find this back in the method section). The authors should at least discuss this point, and potentially add an experiment that may show if there are differences for the migratory capacity of CD4 and 8 cells when migrating on a soft (e.g. 2kPa) vs stiff surfaces (25 kPa and plastic).
5. The discussion is rather long. I would appreciate a shorter version.
6. The conclusion (page 14, line 41) that the increased arrest of CD8 cells over Cd4 cells was dependent on LFA1 and the fact that the integrin expression levels are equal between both sub-populations suppl fig 2) suggest to look at the activated epitope of LFA1 (i.e. beta 2 integrin). Is beta2 activation epitope in a more open conformation in the CD8 cells vs Cd4? Or do they believe that the CD8 cells make more use of the downstream effector pathways, as they suggest in their discussion (page 15, line 15). What is the comment of the authors on this?

Reviewer: 2

Comments to the Author
The manuscript by Rudolph et al takes a different approach to addressing the question of whether there are different mechanisms underlying the ability of activated CD8 T cells to cross the blood brain barrier (BBB) compared to CD4+ T cells. Previous studies have indicated that both T cell subsets utilize similar mechanisms that depend heavily on alpha 4 integrin; however, the experimental set-up in these studies did not permit a side-by-side comparison of both CD8 and CD4 T cells extravasating across the BBB. The rational for the studies described here was to perform a side-by-side comparison, and for this purpose an artificial “BBB” was used consisting of a membrane formed from cultured primary mouse brain microvascular endothelial cells that can be studied under physiological flow conditions. The BBB was employed in a non-activated state as well as after activation by exposure to either TNF-a or a combination of TNF-a and IFN-g. OT-1 cells were used to study CD8 T cell behavior and OT-2 cells were used for CD4 T cells. The experiments are technically well performed and the analysis of the data is sound. The major conclusions reached by the authors are:
• CD8 T cells arrest on the BBB more frequently than CD4 T cells regardless of BBB activation, and this ultimately led to increased efficiency of CD8 T cells crossing the BBB.
• CD4 T cells exhibit a greater degree of crawling on the non-activated BBB prior to diapedesis, but this crawling was reduced when the BBB was activated.

European Journal of Immunology

• In contrast, CD8 T cells exhibited little crawling on non-activated BBB and instead remained stalled and then proceeded to diapedesis.
• CD4 T cells crossed the BBB primarily via a paracellular pathway while CD8 T cells predominantly used a transcellular route.
• Increased arrest of CD8 T cells depended in large part on expression of ICAM-1 (and to a lesser extent ICAM-2) on the BBB.
• ICAM-1 and ICAM-2 expression are more important for diapedesis of CD4 T cells than CD8 T cells.

From this reviewer's perspective, the overall finding reported here, i.e., that there are differences in mechanisms used by CD8 T cells versus CD4 T cells to interact with and cross the BBB is interesting and important. It is difficult to extrapolate from this experimental setting to in vivo extravasation across the BBB, but the differences noted here reinforce the need to investigate the issue further and point to some mechanisms that could be tested in vivo. There are some caveats to the experimental approach that should be discussed in the manuscript, and some weaknesses in interpretation that should be addressed, as noted below.

• The authors are attempting to generate comparable conditions to compare CD4 versus CD8 T cell interactions with the BBB. It was useful to see the variation in expression of cell-surface markers on the different preparations of CD4 and CD8 T cells (Sup Fig 2). The extent of variation is fairly high (more than an order of magnitude in some cases) between preparations. However, the author's argument that this variation equally affected preparations of both CD8 and CD4 T cells, as well as the large number of observations recorded for most of the experiments, was sufficiently convincing to believe that the variation did not introduce bias into the results. A bigger concern was the assumption that OT-1 and OT-2 T cells are reasonably equivalent to each other with respect to the TCR interaction with their cognate ligands. The OT-1 TCR is an unusually high affinity TCR, and this is not the case for the OT-2 TCR. This caveat should have been considered when the authors draw conclusions about the differences between CD4 and CD8 T cell interactions with the BBB in general, and especially in the discussion where they comment that CD8 T cells may more efficiently trigger local endothelial signaling cascades compared to CD4 T cells (page 16, lines 33-47). It is always risky to generalize from experiments that utilize T cells expressing only particular TCR, but in the case of the OT-1 TCR, this is a significant concern and should at least be mentioned.
• The authors should clarify what the difference is between "stalling" and "arresting". This is an important distinction that is not well articulated in the paper.
• It appears that only CD8 T cells were analyzed for their use of transcellular versus paracellular routes of diapedesis in these experiments; the authors only reference earlier findings with CD4 T cells (page 9, line 11). As part of the rationale for these studies was to compare the two T cell subsets under the same conditions, it would have been better to include CD4 T cells in the experiments shown in Fig 4.
• The authors implicate ICAM-1 expression on endothelial cells as the main mediator of increased CD8 T cell arrest as expression of this molecule was most affected by cytokine-mediated activation of the BBB.

European Journal of Immunology

They employed ICAM-1 (null)/ICAM-2-/- mice to demonstrate the role of these molecules in the arrest of CD8 T cells, and they conclude that their data support the idea that ICAM-1 is the most influential molecule. It would have been straightforward to conduct the same experiment using ICAM-1(null) ICAM-2 (wild type) cells, such there was only a deficiency in ICAM-1 to really prove that ICAM-1 was more important in the outcome compared to ICAM-2. Such experiments would strengthen the paper. Furthermore, on page 15 line 39, they suggest that the increased arrest of CD8 T cells was “exclusively mediated” by interaction with ICAM-1 and ICAM-2 rather than alpha4 integrin. To prove this point, the same experiments should have been conducted using a blocking antibody to alpha4 integrin to show that there is no effect.

• Figure 6C is confusing, it was not clear to this reviewer what the difference was between “stationary” and “stalling”. There also is no mention of Fig 6D in the paper and no legend for this panel, adding to the confusion.

• The statement on page 15 line 19 that CD8 T cells “make different use than CD4 T cells of adhesion and/or signaling molecules …to breach this barrier” is confusing. The authors concluded that CD8 T cells do depend on ICAM-1 for increased arrest, are they suggesting some novel function for interaction with ICAM-1 by CD8 T cells that does not occur in CD4 T cells?

• The authors state that their data suggest a higher expression level of LFA-1 on CD8 T cells (page 16 line 3), but the data in Sup Fig 2 do not support this. There is also little experimental support for their suggestion that CD8 T cells “more efficiently use LFA-1 mediated downstream signaling cascades” leading to their increased arrest (Page 16 line 15).

• Finally, it would be interesting to know the author’s speculation on how differences in mechanisms of extravasation across the BBB by CD8 versus CD4 T cells could be exploited therapeutically. Do they believe it is more important to inhibit one subset versus the other? Natalizamab seems to prevent trafficking of both T cell subsets – do the authors believe this can be improved upon?

Minor points:

• Fig 2B is discussed before Fig 2A, perhaps the panels should be reversed.

• On page 12, line 5, the authors say they assigned T cells to “six groups as described above”. It was not clear what the six groups are.

In summary, the approach used by the authors to compare CD8 and CD4 T cells interactions with a pseudo BBB in vitro under physiological flow conditions is interesting and they have made novel observations. Many of the concerns above could be addressed by clarifying the writing and more caution in interpretation of results. The caveat of the high affinity of the OT-1 TCR is significant and should be mentioned. Some additional experiments using ICAM (null) endothelial cells and alpha4 blocking antibody in this experimental setting would strengthen some of the conclusions.

European Journal of Immunology

Peer review correspondence

Reviewer: 3

Comments to the Author
Manuscript: Post arrest stalling rather than crawling favors CD8+ over CD4+ T-cell migration across the blood-brain barrier under flow in vitro

Rudolph and colleagues compared the behavior of mouse activated ovalbumin specific CD4 and CD8 T cells upon contact with primary mouse brain microvascular endothelial cells (pMBMEC) using in vitro live cell imaging. They assessed the number of CD4 and CD8 T cells arrested, stalled or crawling when put on non-stimulated or cytokine treated pMBMEC. They observed that a greater number of CD8 compared with CD4 T cells arrested on pMBMEC regardless of treatment. Moreover, most CD8 T cells stalled prior to cross pMBMEC. Finally, in the absence of ICAM-1 and ICAM-2, less CD4 and CD8 T cells arrested on pMBMEC.

Comments:
The current manuscript addresses a highly relevant topic. Indeed, a better understanding of the mechanisms controlling the migration of CD8 vs. CD4 T cells into the central nervous system (CNS) could offer key information in the context of several diseases including multiple sclerosis. Unfortunately, the current manuscript does not provide a significant amount of novel data on the mechanisms controlling the extravasation of T cells into the CNS. The authors did not reveal novel contribution of specific integrins or adhesion molecules; the important role of ICAM has already been demonstrated by numerous groups. It will be important to confirm that the differences they observed between CD4 and CD8 T cells do not apply only to OVA specific T cells and whether the polarization and activation/memory status of T cells influence the interaction with the pMBMEC. Moreover, the authors did not evaluate whether the differences between CD4 and CD8 T cells revealed by in vitro assays reflect in vivo differences.

It will be important to perform additional assays to determine whether T cells that cross pMBMEC are still alive.

Did the authors evaluate the expression of MHC class I vs. class II expression on the pMBMEC ?

p. 13 The authors wrote:
‘that the interaction of activated OVA specific CD8 T cells with inflamed spinal cord microvessels in mice suffering from CD4 T cell mediated EAE pointed to components missing in the set of traffic signals required to induced efficient OVA-specific CD8 T cell migration across the inflamed BBB into the CNS . In contrast, activated OVA-specific CD4 T cells readily cross the BBB during (…) EAE underlining that the activation state rather than the antigen-specificity controls at least CD4 T cell migration across the BBB during EAE. It is therefore tempting to speculate that efficient CD8 T cell migration across the BBB

European Journal of Immunology

requires trafficking cues that are absent in classical EAE. Indeed, in contrast to CD4 T cells, recognition of their cognate antigen on the BBB might contribute to CD8 T cell migration across the BBB. ’

To support such statement, it will be necessary to compare side by side trafficking of OVA-specific CD4 and CD8 T cells in vivo. In fact, the data presented in the current manuscript do not support this statement; the number of CD8 T cells crossing the in vitro BBB model is not reduced compared to CD4 T cells. Did the authors test whether the presence of the cognate antigen (OVA) could change the behavior of T cells?

Did the authors assess the proliferation and cell surface expression of adhesion molecules (Supporting figures 1-2) just before adding these cells onto pMBMEC? What was the purity of CD8 and CD4 T cell samples?

Figure 1: Were the increased numbers of arrested CD4 or CD8 T cells on cytokine treated pMBMEC significantly greater than those observed on NS pMBMEC? It will be important to complete statistical analyses throughout the manuscript; for example, there is no statistics provided in Fig. 6A

Intensity of cell tracker staining is variable between cells (different intensities of green or red). How do the authors explain such variation? In video 8, there is no red cell (CD4) visible. Therefore, quantification in Figure 6 is problematic.

In one or two videos, it will be appropriate to identify at least one representative T cell showing each behavior characterized on p. 7: i) Stalling T cells detaching during the observation period, ii) T cells remaining stalled and iii) stalling T cells that crossed the pMBMEC monolayer in the observation period, iv) T cells which crawled and detached, v) T cells which crawled for the entire observation period and finally vi) T cells which crossed the pMBMEC monolayer after crawling.

For the paracellular vs. transcellular migration experiments, it will be useful to have red labeled T cells to visual their migration on the VECadehrin-GFP cells.

Supporting Fig.2 Could the authors provide the MFI for the integrins to determine whether the staining intensity is similar between CD8 and CD4 T cells?

Some references are not properly presented in the text (e.g. p. 17: Bullard 2007)

First revision – authors’ response – 12 May 2016

Reviewer: 1

European Journal of Immunology

The authors elegantly showed that CD8 T cells show a different kinetics in crossing the brain EC than CD4 T cells. This work is likely to bring the field forward. The data are solid and proper controls are included. Moreover, the conclusions drawn from the data are sound and interesting. However, there are a few minor points that I would like to see addressed prior to acceptation. I listed them below.

1. Arrest and migration were induced only by cytokine (i.e. TNF/IL1)-treated ECs. What about chemoattractant cytokines? Do the authors still see a preference (CD8 over CD4) when the ECs are immobilized with a certain chemokine, or will this favor one cell type over the other due to chemokine preference? I would suggest to at least discussing this point in the discussion section, for example following MHC discussion (line 45).

It has previously been shown by the laboratory of Ronen Alon that in contrast to human naïve T cells, which do require immobilized chemokines on the endothelial surface for integrin mediated arrest and crawling under flow, activated human T cells do not depend on GPCR signaling to arrest and crawl on human umbilical vein endothelial cells under flow in vitro (Shulman et al., Nature Immunology, 2011, 1, 67–76). To investigate if these observations hold true for the activated CD4+ and CD8+ T cells interacting with the BBB we have included a set of novel experiments in the revised version of our manuscript. We now show that inhibition of G□i-dependent GPCR signaling by pretreating the T cells with pertussis toxin did not reduce CD4+ and CD8+ T cell arrest under flow. Rather, also in accordance to the findings of the Alon laboratory, diapedesis of both CD4+ and CD8+ T cells was almost completely abrogated leading to increased T cell crawling on the BBB under flow. These observations demonstrate that chemokines are not required for shear resistant arrest and crawling of activated T cells on the BBB under flow. These data have been included in Supporting Information Figure 3.

2. Please explain better why the CD4 and 8 cells are harvested from two different mice models. Why did the authors not try to isolate the CD4 and 8 cells form the same WT mouse line?

To obtain highly pure, defined and homogenously activated T cell subsets we chose to isolate CD8+ and CD4+ T cells from the well characterized T cell receptor transgenic mouse lines OT1 and OT2, respectively. Isolation of CD4+ and CD8+ T cells from these mice allowed to achieve defined and comparable antigen-specific T cell activation for both T cell subsets. We improved explaining our rational in Material and Methods. Polyclonal CD4+ and CD8+ T cell subsets would have added an additional degree of complexity regarding variability of cell surface expression of adhesion molecules on the T cell subsets and antigen-specific T cell activation profiles. Please be also referred to additional experiments we have now included to address the role of TCR/peptide-MHC affinity on CD8+ T cell activation and subsequent interaction with pMBMECs (Supporting Information Figure 2).

3. The authors elegantly show that CD8 cells prefer the transcellular route, by isolating brain ECs from VE-cad GFP mice. I wonder if these T cells cross the EC transcellularly in other vascular beds (e.g. lung vs skin) as well. Can the authors comment on this?

European Journal of Immunology

We agree with the reviewer that this is indeed an interesting question but this will require further investigations. We cannot answer this at present and hope this reviewer agrees that addressing this issue is beyond the scope of the present study. In a previous study focussing on CD4+ T cells (Abadier et al., European Journal of Immunology, 2015; 45(4), 1043–1058) we did observe that cell surface levels of endothelial ICAM-1 rather than barrier integrity influences the cellular pathway of T cell diapedesis across pMBMECs under flow in vitro.

4. I am somewhat worried about the culture conditions, in particular the extracellular matrix where the ECs are cultured on, since in particular the brain is known for its soft environment and the culture conditions do not reflect this particular environment; on the contrary, the ECs are cultured on plastic/glass (I assume since I could not find this back in the method section). The authors should at least discuss this point, and potentially add an experiment that may show if there are differences for the migratory capacity of CD4 and 8 cells when migrating on a soft (e.g. 2kPa) vs stiff surfaces (25 kPa and plastic).
We do understand the concerns of this Reviewer that our in vitro model does not include all the cellular and matrix components present at the BBB in vivo. We are well aware of this fact and have investigated in the past the influence of individual matrix proteins, e.g. agrin on barrier integrity of in vitro models of the BBB (Steiner et al., Cell Tissue Res. 2014; 358(2):465-79). Having said that, the in vitro BBB model used in the present study has been in depth characterized for its barrier properties and suitability to mimic T cell interactions with the BBB as they occur in vivo and published by us. To just highlight one example, which is also mentioned in our manuscipt, in previous studies we have shown the extended crawling of CD4+ T cells against the direction of flow in this in vitro model (Steiner et al., J Immunol. 2010; 185(8):4846-55) as it is observed by others in vivo (Bartholomäus et al., Nature. 2009; 462(7269):94-8.).
The technical setup of the flow chamber has been developed almost 10 years ago to mimic physiological shear forces. The details of the flow chamber have been summarized by us in Coisne et al., Fluids Barriers CNS. 2013; 10(1):7. This reference is now better highlighted in the Methods section. The matrix chosen for the in vitro model cannot be changed as it has been found to be prerequisite for the differentiation of the barrier properties of the brain endothelial cells. The rigid surface the cells are grown on is prerequisite for the flow chamber in order to maintain the focus level when imaging under flow conditions. A softer bottom would bend when applying shear and would prohibit continuous imaging of T cell/BBB interactions under flow in the plane of focus over time. Thus we consider the experiment as suggested by the Reviewer an entire project on its own, namely finding materials of different stiffness allowing a) pMBMECs to grow and remain attached under flow b) differentiate into a barrier with high TEER and low permeability and c) allowing for continuous live cell imaging in the plane of focus. We hope this explanations convince the Reviewer about the suitability of our in vitro model.

5. The discussion is rather long. I would appreciate a shorter version.
We have significantly shortened the discussion.

European Journal of Immunology

6. The conclusion (page 14, line 41) that the increased arrest of CD8 cells over Cd4 cells was dependent on LFA1 and the fact that the integrin expression levels are equal between both sub-populations suppl fig 2) suggest to look at the activated epitope of LFA1 (i.e. beta 2 integrin). Is beta2 activation epitope in a more open conformation in the CD8 cells vs Cd4? Or do they believe that the CD8 cells make more use of the downstream effector pathways, as they suggest in their discussion (page 15, line 15). What is the comment of the authors on this?

Encouraged by this question of the reviewer we have performed additional experiments aiming to answer this questions. Unlike for human LFA-1 there are no antibodies allowing to reliably distinguish low and high affinity states of mouse LFA-1 on the cell surface by flow cytometry. We therefore investigated the presence of high-affinity LFA-1 on CD8+ versus CD4+ T cells by studying the binding of soluble ICAM-1 by the two T cell subsets. We found that soluble ICAM-1 can slightly bind to CD8+ but not to CD4+ T cells and that pre-incubation of the T cells with manganese readily induced sICAM-1 binding to CD8+ but only slightly to CD4+ T cells. We thus conclude that indeed activated CD8+ T cells display higher proportions of high-affinity LFA-1 on their surface than activated CD4+ T cells and that LFA-1 on activated CD8+ T cells can more rapidly be triggered to change to the high affinity conformation in the presence of manganese. These data have been included into the manuscript and are shown in Supporting Information Figure 5.

Reviewer: 2

…….There are some caveats to the experimental approach that should be discussed in the manuscript, and some weaknesses in interpretation that should be addressed, as noted below.

• The authors are attempting to generate comparable conditions to compare CD4 versus CD8 T cell interactions with the BBB. It was useful to see the variation in expression of cell-surface markers on the different preparations of CD4 and CD8 T cells (Sup Fig 2). The extent of variation is fairly high (more than an order of magnitude in some cases) between preparations. However, the author's argument that this variation equally affected preparations of both CD8 and CD4 T cells, as well as the large number of observations recorded for most of the experiments, was sufficiently convincing to believe that the variation did not introduce bias into the results. A bigger concern was the assumption that OT-1 and OT-2 T cells are reasonably equivalent to each other with respect to the TCR interaction with their cognate ligands. The OT-1 TCR is an unusually high affinity TCR, and this is not the case for the OT-2 TCR. This caveat should have been considered when the authors draw conclusions about the differences between CD4 and CD8 T cell interactions with the BBB in general, and especially in the discussion where they comment that CD8 T cells may more efficiently trigger local endothelial signaling cascades compared to CD4 T cells (page 16, lines 33-47). It is always risky to generalize from experiments that utilize T cells expressing only particular TCR, but in the case of the OT-1 TCR, this is a significant concern and should at least be mentioned.

European Journal of Immunology

This Reviewer has raised an important issue that has prompted us to perform additional experiments with more CD8+ and CD4+ T cell preparations and most importantly we considered the influence of the TCR peptide/MHC affinity on increased CD8+ T cell over CD4+ T cell arrest on the BBB under physiological flow in vitro. To this end, we relied on the well characterized interaction of the OT-1 TCR with ovalbumin peptides harboring single amino acid differences that were shown to exhibit differing stimulatory potencies on the OT-1 cells (Zehn et al., Nature, 2009; 458(7235): 211–214). We confirmed that the altered peptide Q4 (SIIQFEKL) reported to have intermediate affinity interaction with the OT-1 TCR showed lower potency in stimulating OT-1 activation than the N4 (SIINFEKL) peptide (Moreau et al., Immunity, 2012; 37(2), 351–363). These data are now added as Supporting Figure 2C. At the same time, Q4 stimulated OT-1 cells did not show reduced arrest on pMBMECs under physiological when compared to N4-stimulated OT-1 cells excluding a direct role for TCR-peptide/MHC affinity in mediating ICAM-1/ICAM-2 mediated enhanced arrest of CD8+ over CD4+ T cells on the BBB in vitro (Supporting information Figure 2D).

• The authors should clarify what the difference is between “stalling” and “arresting”. This is an important distinction that is not well articulated in the paper.
We have improved our explanation on the definitions of arrest and stalling. Shear resistant arrest is defined as the cells that stay arrested on the pMBMEC monolayer at 30 seconds after onset of increases, e.g. physiological shear (1.5 dynes/cm2). In contrast to CD4+ T cells, which after shear resistant arrest polarize and crawl to sites permissive for diapedesis, CD8+ T cells were observed to remain mostly at the precise spot of their shear resistant arrest, however, not in an inert fashion but rather by probing the local environment for diapedesis. The latter behavior we defined as stalling to avoid implementation of inactivity of the CD8+ T cells.

• It appears that only CD8 T cells were analyzed for their use of transcellular versus paracellular routes of diapedesis in these experiments; the authors only reference earlier findings with CD4 T cells (page 9, line 11). As part of the rationale for these studies was to compare the two T cell subsets under the same conditions, it would have been better to include CD4 T cells in the experiments shown in Fig 4.
As mentioned by the Reviewer we did analyze the cellular pathway of encephalitogenic CD4+ T cell diapedesis across pMBMECs in depth before, which is mentioned in the manuscript and which has been published in Abadier et al., European Journal of Immunology, 2015; 45(4), 1043–1058. We found and published that cell surface levels of endothelial ICAM-1 influence the transcellular or paracellular CD4+ T-cell diapedesis across the BBB under flow in vitro. We have not included repetition of this analysis for OT2-derived CD4+ T cells in the present study as visual and molecular interaction of OT2 derived CD4+ T cells with pMBMECs was very similar to that previously observed for encephalitogenic CD4+ T cells (Abadier et al., European Journal of Immunology, 2015; 45(4), 1043–1058).
We agree with the reviewer that videos showing the different cellular pathways of CD8+ versus CD4+ T cell diapedesis side by side would be very nice, but this is technically almost impossible. Please be aware that for this analysis we have to use a higher magnification (63x) objective at the microscope, which

European Journal of Immunology

results in a smaller FOV. It is already challenging to record sufficient events of diapedesis allowing for a quantitative analysis when studying one T cell subset due to numerous T cells leaving the field of view during the recording. Thus most FOVs only show 1 T cell on its way of diapedesis and the number of recordings allows to quantify the preferred pathway used. Thus to catch a CD4+ and a CD8+ T cells within one FOV during diapedesis using this analysis might be expected in 1 of 20 recordings. Quantification would require hundreds of videos and thus sacrifice of an unbearable number of mice for the pMBMEC isolatinos needed. We hope that it is therefore acceptable to this reviewer that we did not pursue to perform these experiments.

• The authors implicate ICAM-1 expression on endothelial cells as the main mediator of increased CD8 T cell arrest as expression of this molecule was most affected by cytokine-mediated activation of the BBB. They employed ICAM-1 (null)/ICAM-2-/- mice to demonstrate the role of these molecules in the arrest of CD8 T cells, and they conclude that their data support the idea that ICAM-1 is the most influential molecule. It would have been straightforward to conduct the same experiment using ICAM-1(null) ICAM-2 (wild type) cells, such there was only a deficiency in ICAM-1 to really prove that ICAM-1 was more important in the outcome compared to ICAM-2. Such experiments would strengthen the paper.
This reviewer raised a critical issue as we assumed that due to its upregulation ICAM-1 will play most probably a more prominent role than ICAM-2 in mediating increased CD8 T cell arrest on the pMBMECs. Thus we did include study of T cell interaction with ICAM-1null pMBMECs in our revised manuscipt as this will allow to delineate the role of endothelial ICAM-1 versus ICAM-2 in CD8+ T cell interaction with the BBB. These investigations showed that although lack of endothelial ICAM-1 slightly reduced the increased arrest of CD8+ T cells versus CD4+ T cells to pMBMECs, CD8+ T cells still showed increased arrest to pMBMECs when compared to CD4+ T cells on ICAM-1null pMBMECs. These data have been included as Supporting Information Figure 4. Abrogation of increased arrest of CD8+ versus CD4+ T cells on pMBMECs needed absence of both LFA-1 ligands, ICAM-1 and ICAM-2. These observations underscore that both ICAM-1 and ICAM-2 mediate the increased shear resistant arrest of CD8+ T cells compared to CD4+ T cells to pMBMECs in our model.

Furthermore, on page 15 line 39, they suggest that the increased arrest of CD8 T cells was “exclusively mediated” by interaction with ICAM-1 and ICAM-2 rather than alpha4 integrin. To prove this point, the same experiments should have been conducted using a blocking antibody to alpha4 integrin to show that there is no effect.
The increased arrest of CD8+ T cells versus CD4+ T cells observed on wild-type pMBMECs is fully abrogated on endothelial cells lacking both, ICAM-1 and ICAM-2, thus it is ICAM-1 and ICAM-2 that mediate the increase arrest. If □□/VCAM-1 contributed to this difference we would expect to still see increased arrest of CD8+ over CD4+ T cells on ICAM-1null/ICAM-2-/-pMBMECs. This does however not mean that interaction of □□-integrins with endothelial VCAM-1 does not contribute to the shear resistant arrest of CD4+ and CD8+ T cells to pMBMEMCs. To clarify this issue and to avoid misunderstandings we

European Journal of Immunology

have performed the experiment requested by the reviewer. We found that indeed antibody-mediated inhibition of □□□integrins and as expected to a lesser degree inhibition of endothelial VCAM-1 abrogated both, CD4+ and CD8+ T cell arrest on ICAM-1null/ICAM-2-/-pMBMECs. These data have been added as Figure 6C.

• Figure 6C is confusing, it was not clear to this reviewer what the difference was between “stationary” and “stalling”. There also is no mention of Fig 6D in the paper and no legend for this panel, adding to the confusion.
We apologize for this confusion. We have chosen to refer to the cells as stalling rather than stationary in order to avoid impression of inactivity. All cells are now correctly referred to as stalling. Please note that these data have moved to Figure 7.

• The statement on page 15 line 19 that CD8 T cells “make different use than CD4 T cells of adhesion and/or signaling molecules …to breach this barrier” is confusing. The authors concluded that CD8 T cells do depend on ICAM-1 for increased arrest, are they suggesting some novel function for interaction with ICAM-1 by CD8 T cells that does not occur in CD4 T cells?
Due to the additional experiments performed we have corrected our statement and conclude that increased arrest of CD8+ T cells on pMBMECs compared to CD4+ T cells depends on both, ICAM-1 and ICAM-2. Furthermore, we have included additional experiments demonstrating that activated CD8+ bind more soluble ICAM-1 than activated CD4+ T cells suggesting the presence of LFA-1 in its high affinity conformation on the surface of CD8+ but less on CD4+ T cells. In addition in the presence of Mn2+ LFA-1 on CD8+ but not on CD4+ T cells was found to readily change into its high affinity conformation engaging soluble ICAM-1. These data underscore a difference in the ability of activated CD8+ versus CD4+ T cells to trigger the conformational change of cell surface LFA-1 towards its high affinity conformation and therefore allowing CD8+ T cells in a faster fashion than CD4+ T cell to engage sICAM-1 and on the pMBMECs immobilized ICAM-1 and ICAM-2. The precise molecular mechanisms behind this difference in LFA-1 activation on CD8+ versus CD4+ T cells remains to be shown. This has now been explained in the revised version of the manuscript.

• The authors state that their data suggest a higher expression level of LFA-1 on CD8 T cells (page 16 line 3), but the data in Sup Fig 2 do not support this. There is also little experimental support for their suggestion that CD8 T cells “more efficiently use LFA-1 mediated downstream signaling cascades” leading to their increased arrest (Page 16 line 15).
This is a misunderstanding of the Reviewer. We found no difference of cell surface LFA-1 on CD8+ versus CD4+ T cell populations. However, the different T cell populations isolated were not absolutely identical in their cell surface expression of LFA-1 and □□□integrins. Thus cell surface expression of LFA-1 did not allow to understand the increased arrest of CD8+ over CD4+ T cells to endothelial ICAM-1 and ICAM-2. We have now added additional experiments suggesting that CD8+ T cells can more rapidly shift their cell

surface LFA-1 to its high affinity conformation, these data have has been added a Supporting Information Figure 5. We hope this clarifies this misunderstanding.

• Finally, it would be interesting to know the author's speculation on how differences in mechanisms of extravasation across the BBB by CD8 versus CD4 T cells could be exploited therapeutically. Do they believe it is more important to inhibit one subset versus the other? Natalizumab seems to prevent trafficking of both T cell subsets – do the authors believe this can be improved upon?
Yes indeed, our ultimate hope is that this research will serve to significantly improve our understanding of the cellular and molecular mechanisms guiding different T cell subsets into the CNS during immunosurveillance and neuroinflammation. In general, this will set the stage to more accurately foresee CNS specific adverse effects of the increasing numbers of therapies targeting T cell trafficking or even depleting T cells in many chronic inflammatory diseases. Additionally, this will allow to identify novel therapeutic targets at the level of the BBB suited to specifically block CNS recruitment of destructive T cells, while leaving the migration of protective T cell subsets into the CNS unaffected. We have adapted the last paragraph of the discussion to better highlight this issue.

Minor points:
• Fig 2B is discussed before Fig 2A, perhaps the panels should be reversed.
We see the point of the Reviewer. We chose this unlogical sequence of mentioning the Figures in the text to allow a better visual arrangement of the Figure itself, where Figure 2C directly correlates to Figure 2B and thus allows to more easily see the data in the Figure.

• On page 12, line 5, the authors say they assigned T cells to "six groups as described above". It was not clear what the six groups are.
We have repeated description of the 6 categories to allow for easy reading. In addition, we have labeled an exemplary cell for each category in supporting information video 3. We hope this clarifies the definition of the 6 categories of T cell behaviour on the pMBMECs as defined by us.

In summary, the approach used by the authors to compare CD8 and CD4 T cells interactions with a pseudo BBB in vitro under physiological flow conditions is interesting and they have made novel observations. Many of the concerns above could be addressed by clarifying the writing and more caution in interpretation of results. The caveat of the high affinity of the OT-1 TCR is significant and should be mentioned. Some additional experiments using ICAM (null) endothelial cells and alpha4 blocking antibody in this experimental setting would strengthen some of the conclusions.
We thank the Reviewer once more for the productive critique which has been addressed with additional experiments as outlined above. These experiments have helped to significantly improve this study.

European Journal of Immunology

Reviewer: 3

…..

Comments: The current manuscript addresses a highly relevant topic. Indeed, a better understanding of the mechanisms controlling the migration of CD8 vs. CD4 T cells into the central nervous system (CNS) could offer key information in the context of several diseases including multiple sclerosis. Unfortunately, the current manuscript does not provide a significant amount of novel data on the mechanisms controlling the extravasation of T cells into the CNS. The authors did not reveal novel contribution of specific integrins or adhesion molecules; the important role of ICAM has already been demonstrated by numerous groups. It will be important to confirm that the differences they observed between CD4 and CD8 T cells do not apply only to OVA specific T cells and whether the polarization and activation/memory status of T cells influence the interaction with the pMBMEC. Moreover, the authors did not evaluate whether the differences between CD4 and CD8 T cells revealed by in vitro assays reflect in vivo differences.

It will be important to perform additional assays to determine whether T cells that cross pMBMEC are still alive.

The T cells that have crossed the pMBMEC monolayers can be seen as phase dark cells rapidly moving below the endothelial monolayer. Thus in our experience T cell viability is not impaired upon diapedesis. In Supporting Information video 3 we have now tracked one cell in red allowing to see an example of such a cell.

Did the authors evaluate the expression of MHC class I vs. class II expression on the pMBMEC?

We are presently investigating inducibility of MHC class I on pMBMECs in another study that addresses the role of antigen–presentation/cross-presentation by BBB endothelium in CD8+ T cell migration across the BBB. CD4+ T cells have previously been shown by us and others (laboratories of Wekerle, Flügel) to cross the BBB independent of their antigen-specificity and rather due to their activation state. In the present study using ovalbumin specific CD8+ and CD4+ T cells isolated from TCR tg mice antigen-presentation does not play a role as ovalbumin is not present in the assays. Thus we consider expression of MHC molecules on the pMBMECs not relevant in the context of the present study.

p. 13 The authors wrote:

‘that the interaction of activated OVA specific CD8 T cells with inflamed spinal cord microvessels in mice suffering from CD4 T cell mediated EAE pointed to components missing in the set of traffic signals required to induced efficient OVA-specific CD8 T cell migration across the inflamed BBB into the CNS . In contrast, activated OVA-specific CD4 T cells readily cross the BBB during (…) EAE underlining that the activation state rather than the antigen-specificity controls at least CD4 T cell migration across the BBB during EAE. It is therefore tempting to speculate that efficient CD8 T cell migration across the BBB requires trafficking cues that are absent in classical EAE. Indeed, in contrast to CD4 T cells, recognition of their cognate antigen on the BBB might contribute to CD8 T cell migration across the BBB. ’

To support such statement, it will be necessary to compare side by side trafficking of OVA-specific CD4 and CD8 T cells in vivo. In fact, the data presented in the current manuscript do not support this statement; the number of CD8 T cells crossing the in vitro BBB model is not reduced compared to CD4 T cells.
The reviewer criticizes a speculative statement made by us in the discussion of the manuscript which is based on previous in vivo live cell imaging studies by us and others as well as on our previous and present in vitro live cell imaging observations. Based on the data available we consider a speculative statement integrating all these observations as appropriate when placed in the discussion.

At the same time we respect the request of the reviewer to aim for a comparative analysis of CD8+ versus CD4+ T cell trafficking to the CNS. As neither EAE nor CD8+ T cell driven models are appropriate to side-by-side compare CD8+ and CD4+ T cell homing to the inflamed CNS for the reasons outlined in the discussion of our manuscript, we decided to test if cytokine stimulation of the vasculature – as performed in vitro – will allow to side-by-side compare CD8+ and CD4+ T cell homing to the CNS in vivo. To this end C57BL/6 mice were i.v. injected with 25 or 50□g recombinant mouse TNF□□□which has been shown by us and others to allow for induction of adhesion molecules such as E-and P-selectin as well as increased expression of ICAM-1 and VCAM-1 on vascular endothelial cells in many organs of the mouse including the CNS within 4 hours (Engelhardt et al., 1997, Blood 90, 4459-4472). 4 hours after TNF□ injection the mice received a systemic injection of a 1:1 mixture of fluorescently labeled CD8+ and CD4+ T cells exactly as described for the in vitro experiments via a carotid artery catheter as described by us before (Sathiyanadan et al., EJI, 2014; 44(8), 2287–2294). 2 or 4 hours after T cell infusion mice were intracardially perfused with 1% PFA/PBS, and the brains and spinal cords were dissected, snap frozen, and stained for laminin to determine intravasular, perivascular or parenchymal localization of the T cell subsets as described previously (Sathiyanadan et al., EJI, 2014; 44(8), 2287–2294). A total of 4 mice were analyzed. To our surprise 90% of the T cells detected - mostly still within the CNS microvessels - were CD8+ T cells, while CD4+ T cells could hardly be detected. The majority of CD8+ T cells was found in cerebellar microvessels while the few CD4+ T cells detected were found in the spinal cord. One could interpret these in vivo data such that they support our in vitro findings. However, the very few CD4+ T cells detected in the CNS of these mice suggests to us that there are other issues to consider using such a systemic in vivo approach, e.g. preferential accumulation of CD4+ versus CD8+ T cells to other vascular beds not investigated in this study. We thus hope it is acceptable to this Reviewer that we prefer to omit inclusion of these preliminary findings with such highly speculative conclusions into our manuscript.

The reviewer further emphasizes that our in vitro study does not support the in vivo observations made in EAE, namely that in this CD4+ T cell mediated neuroinflammatory model trafficking signals are missing on the BBB promoting CD8+ T cell homing to the CNS. The reviewer is of course absolutely right that our present in vitro studies provide evidence for increased CD8+ T cell diapedesis across the BBB when side-

European Journal of Immunology

by-side compared to CD4+ T cells. However, due to the previous and now present in vivo observations that underscore that a side-by-side comparison of CD8+ and CD4+ T cell interaction with the BBB in vivo is rather difficult if not impossible to investigate (please see our detailed discussion) we have specifically chosen this in vitro approach allowing to directly compare equal numbers of CD8+ and CD4+ T cells interaction in a defined setting, namely on cytokine stimulated pMBMECs. We do not claim that our in vivo model is mimicking the entire complexity of a neuroinflammatory environment as found in EAE or MS.

Did the authors test whether the presence of the cognate antigen (OVA) could change the behavior of T cells?
Previous studies by us and others (Wekerle, Flügel) have shown that CD4+ T cell migration across the BBB in vivo and in vitro is antigen-independent. It still remains to be shown if antigen-presentation/cross-presentation by BBB endothelial cells is influencing CD8+ T cell migration across the BBB. We are indeed investigating this at present in a separate project which due to its complexity is beyond the scope of the present manuscript. Please consider that such a study begins by investigating the regulation of expression of MHC class I on pMBMECs.

Did the authors assess the proliferation and cell surface expression of adhesion molecules (Supporting figures 1-2) just before adding these cells onto pMBMEC? What was the purity of CD8 and CD4 T cell samples?
To answer these questions we have now provided additional information in Material and Methods. T cell proliferation was routinely measured by 3H-thymidine incorporation during the last 16 hours of the 4 day primary culture. Purity of T cell populations and cell surface expression of adhesion molecules was tested on days 3, 4 and 5 of the T cell expansion culture. T cells were used for functional studies on days 3 to 5 in expansion culture.

Figure 1: Were the increased numbers of arrested CD4 or CD8 T cells on cytokine treated pMBMEC significantly greater than those observed on NS pMBMEC? It will be important to complete statistical analyses throughout the manuscript; for example, there is no statistics provided in Fig. 6A
We agree with the Reviewer that statistical analysis needs to be complete. Adding however statistics above the bar-graphs for both, e.g. CD4 versus CD8 and the different cytokine stimuli is very confusing. We have therefore decided to graphically display statistical differences for CD4 versus CD8 T cells and to add mention of the p values comparing e.g. non-stimulated versus cytokine-stimulated conditions in the respective Figure legends.

Intensity of cell tracker staining is variable between cells (different intensities of green or red). How do the authors explain such variation? In video 8, there is no red cell (CD4) visible. Therefore, quantification in Figure 6 is problematic.

European Journal of Immunology

The apparent difference in intensity of cell tracker staining visible in the videos is due to a combination of factors. We did indeed observe slight differences in cell tracker staining intensities between different preparations of T cells as documented by flow cytometry. Influences of the staining reagents on T cell behavior was excluded by swapping the dyes between the different movies and experiments.
In addition optical setting at the microscope are not exactly identical between each assay as for each live cell imaging session settings have to be optimized for optimal imaging quality.
In consequence, after recording the brightness/ contrast differed in between different movies. Additionally, to provide movies for publication that highlight specific cellular behaviour, we readjusted brightness, contrast and color intensities during the processing of the original movie file to the “mov” format with the Image J software.
We agree with the reviewer that in Supporting information video 8 the red fluorescence is quite dim. But as each cell is followed individually from the timepoint of arrest, apparent loss of fluorescence upon e.g. polarization and flattening on the pMBMECs will not influence accuracy of the analysis as the cell is identifiable by its unique interaction track.

In one or two videos, it will be appropriate to identify at least one representative T cell showing each behavior characterized on p. 7: i) Stalling T cells detaching during the observation period, ii) T cells remaining stalled and iii) stalling T cells that crossed the pMBMEC monolayer in the observation period, iv) T cells which crawled and detached, v) T cells which crawled for the entire observation period and finally vi) T cells which crossed the pMBMEC monolayer after crawling.
We thank the Reviewer for this excellent suggestion and apologize that it has not been fully clear how the T cells were categorized. We have labeled an exemplary cell for each category in supporting information video 3. We hope this clarifies the definition of the 6 categories.

For the paracellular vs. transcellular migration experiments, it will be useful to have red labeled T cells to visual their migration on the VECadehrin-GFP cells.
Fluorescent labeling of the T cells was necessary for those studies investigating side by side CD8+ and CD4+ T cells allowing to distinguish the T cell subsets based on their color. The cellular pathway of CD4+ T cell migration was investigated and published by us before therefore in the present study we have added investigation of CD8+ T cells only which can easily be observed by differential interference contrast (DIC) imaging as routinely performed by us. Thus, fluorescent labeling of the CD8+ T cells although it can be considered a “nice to have” is not necessary and was omitted by us as it also avoids further complexity and controls for the impact of fluorescent labeling on T cell behavior.

Supporting Fig.2 Could the authors provide the MFI for the integrins to determine whether the staining intensity is similar between CD8 and CD4 T cells?

European Journal of Immunology

We uploaded an additional supporting information Table 1 with the MFI-values as requested. As described in our manuscript cell surface expression of LFA-1 and □□-integrins varied between the different preparations of CD4+ and CD8+ T cells but were not different between CD4+ and CD8+ T cells.

Some references are not properly presented in the text (e.g. p. 17: Bullard 2007)
We apologize for this oversight and have carefully edited the references in our revised manuscript.

Second Editorial Decision – 10 June 2016

Dear Prof. Engelhardt,

It is a pleasure to provisionally accept your manuscript entitled "Post arrest stalling rather than crawling favors CD8+ over CD4+ T-cell migration across the blood-brain barrier under flow in vitro" 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,
Karen Chu

on behalf of Prof. Shimon Sakaguchi

Dr. Karen Chu
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu