European Journal of Immunology

Peer review correspondence

Mineralocorticoid receptor signaling reduces numbers of circulating human naïve T cells and increases their CD62L, CCR7 and CXCR4 expression

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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 – 3 December 2013

Dear Dr. Lange,

Manuscript ID eji.201344265 entitled "Mineralocorticoid receptor signaling reduces numbers of circulating human naïve T cells and increases their CD62L, CCR7 and CXCR4 expression" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referee(s) are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referee(s) will be reconsidered for publication.

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 referee(s) before a decision is rendered.

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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,
Laura Soto Vazquez

On behalf of Prof. Andreas Radbruch

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Reviewer: 1
Comments to the Author

Interesting study implicating MR signalling in redistribution of T cells during early sleep. However, a few questions:

Why is CXCR4 affected in all subsets in vitro, however the in vivo study did only show changes in naive and central CD4 cell numbers. It would be nice to present all data (also subsets that have not been changed) for the in vivo study.

Why don't the authors show CCR7 and CD62L in vivo?

The authors refers to an increase in aldosterone during sleep, yet don't show aldosterone data of these patients. Was there an increase?
The authors show that effects on investigated markers take some hours to produce changes. However, looking at cortisol levels in Fig. 1, they start to decrease at +3,75h, whereas changes in T cell # and CXCR4 start between +1,5 and +3,0. Therefore, the changes in cellular compartment are not related to cortisol changes. Therefore, aldosterone changes are responsible? Anything known about 11beta HSD2 activity in T cells? Otherwise MR acts only as cortisol receptor.

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Might it be possible to show all subsets in Fig.2?

Please comment on the specificity of the results for MR, since Fludrocortisone (MR, AR, GR) as well as Spironolacton (MR, AR) are not specific for only the MR receptor.

Fludrocortisone is approved as drug for orthostatic dysregulation and replacement therapy in e.g. Addisons disease. A rise in blood pressure is expected, at least with a maximal dose of the drug like used in this study (0.2mg). Why didn't the authors observe a rise in blood pressure? Did the medication work at all? If blood pressure was elevated couldn't this impact lymphocyte distribution in itself?

Reviewer: 2
Comments to the Author
The authors examined their hypothesis that mineral corticoid receptors (MR) mediate migration of naïve and central memory human T cells involving augmentation of CD62L, CCR7 and CXCR4 expression of these cells in a human double-blinded randomized crossover design trial in which MR specific agonist fludrocortisone or placebo was orally administrated. The study was designed based on their previous study in which they found significant decreases in naïve and central memory T cells during early sleep when aldosterone level increases. They found significant decreases in the circulating naïve CD4, central memory CD4 and naïve CD8 T cells. They demonstrated in a complementary in vitro experiment that fludrocortisone augments CD62L and CXCR4 expression of T cells, and specific MR antagonist spironolactone reduced the expression of CD62L and CCR7 on naïve T cells.

This is a well and carefully designed human study, which may partly explain the diurnal fluctuation in the efficiency of immune responses in human. However, I have several concerns in the interpretation and the presentation of their experimental data.

1)The authors state the reduction of certain T cell subsets as extravasation, but the possibility of T cell margination should not be ruled out, especially when the role of CD62L, which mediates rolling of T cells on endothelial surface, is considered.
2)All the data regarding the expression of CD62L, CXCR4 and CCR7 and T cell subset numbers are shown as the difference from control. Why didn’t the authors show the absolute level? I guess the individual variance was very large, but if this is the case, they could have shown proportional changes and compare them with placebo condition. Aldosterone should be secreted during early sleep as the authors stated, which might have led to fluctuations in the T cell subsets and their expression of CD62L and CXCR4. I also appreciate if the absolute concentration of cortisol is shown.
3)Why could they detect naïve T cells with increased CXCR4 expression after fludrocortisone administration? Since CXCR4 is a potent chemokine receptor, it is very likely that the cells with enhanced CXCR4 expression would quickly migrate to bone marrow, lymph nodes, and other tissues with high

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CXCL12 expression, and will quickly be lost from circulation. It is well know that in the WHIM syndrome, a congenital immunodeficiency disorder characterized by gain of function mutation of CXCR4, persistent activation of CXCR4 results in retention of cells expressing CXCR4 in tissues expressing CXCL12. Was increased CXCR4 inactive? An in vitro transmigration assay of fludrocortisone/spironolactone treated T cells to CXCL12 may answer this question.

First Revision – authors' response – 17 January 2014

Reviewer: 1

1) Why is CXCR4 affected in all subsets in vitro, however the in vivo study did only show changes in naive and central CD4 cell numbers. It would be nice to present all data (also subsets that have not been changed) for the in vivo study.

Authors' response:
We can only speculate about the changes in CXCR4 expression observed for the non-naïve subsets only in vitro but not in vivo. In the non-naïve subsets, in contrast to the findings for the naïve subsets, spironolactone did not block the effect of fludrocortisone on CXCR4 expression and paradoxically even increased it. Therefore, the observed changes in CXCR4 levels on non-naïve subsets might reflect non-physiological conditions of in vitro culture, possibly caused by indirect effects of the drug. We concisely discuss this on page 12. We now also show the data for subsets that remained unchanged following fludrocortisone administration in Figs. 1 and 2.

Discussion, page 12: "Such increasing effects of fludrocortisone and spironolactone on CXCR4 expression of non-naïve subsets were not observed in vivo in the present and a former study [11], respectively, raising the suspicion that they reflect non-physiological conditions of in vitro culture. Likewise, such MR-agonistic actions of spironolactone were revealed in other in vitro systems employing human cell lines [47] and isolated rat hearts [48]."

2) Why don't the authors show CCR7 and CD62L in vivo?

Authors' response:
Unfortunately, CCR7 was only measured in the in vitro experiment. As to CD62L, we had decided not to show the results of the in vivo study because there were no changes following systemic fludrocortisone administration. However, we have now included these results as supplemental data.

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3) The authors refers to an increase in aldosterone during sleep, yet don't show aldosterone data of these patients. Was there an increase?
The authors show that effects on investigated markers take some hours to produce changes. However, looking at cortisol levels in Fig. 1, they start to decrease at +3,75h, whereas changes in T cell # and CXCR4 start between +1,5 and +3,0. Therefore, the changes in cellular compartment are not related to cortisol changes. Therefore, aldosterone changes are responsible? Anything known about 11beta HSD2 activity in T cells? Otherwise MR acts only as cortisol receptor.

Authors' response:
Differing from the findings of Charloux et al. (1999. Am J Physiol Endocrinol Metab, 2001. J. Sleep Res.), in our study aldosterone levels appeared to be higher after awakening than during sleep. However, this discrepancy can be explained by the low blood sampling rate in our study that did not allow to identify single sleep-dependent pulses in aldosterone secretion as observed in the studies by Charloux et al.. In addition, the steep aldosterone increase in the morning in our subjects reflects an orthostatic response as the subjects left the bed after awakening and thereafter remained in an upright position. We now discuss this issue on pages 5-6:

Results, pages 5-6: "ACTH itself and aldosterone levels were not significantly different between conditions (Fig. S2). Differing from the findings by Charloux et al. [7;8], in our study aldosterone levels appeared to be higher after awakening than during sleep. However, this can be explained by our low blood sampling rate that did not allow to identify single pulses in sleep-dependent aldosterone release and by the orthostatic response occurring in the morning as our subjects got up and left the bed after awakening."

The increase in cortisol, indeed, cannot be taken to explain the changes we observed in the T cell parameters as changes in cortisol levels started later and a reduction in cortisol concentration would be expected to increase rather than decrease blood T cell counts. We discuss this on page 9. As aldosterone was not affected by fludrocortisone, we assume that changes in T cell parameters were directly mediated by the drug itself. 11-β HSD2, which protects MR from being activated by cortisol, has not been found in human leukocytes under non-pathological conditions (Chapmann et al., 2009. Mol Cell Endocrinol). However, there seem to be other mechanisms capable of conferring aldosterone selectivity at MR (Gomez-Sanchez, 2011. Trends Endocrinol Metab). The higher affinity of lymphocytic MR for aldosterone than cortisol suggests that an important part of the effects observed during sleep on T cell distribution is indeed mediated via aldosterone rather than cortisol. Moreover, there are studies indicating that cortisol might even be entirely inactive at lymphocytic MR (Wehling et al., 1989. Am J Physiol. Endo Met), thus further supporting the view of a principal role for aldosterone in mediating MR effects on T-cell numbers. We now discuss this issue in more detail on pages 8-9 and 10.

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Discussion, pages 8-9: “Our studies also show that peripheral MR are not saturated during sleep and thus can be further activated by an exogenous ligand like fludrocortisone, as has been similarly demonstrated for central nervous MR [18;25]. This finding is important given that human leukocytes normally do not express the enzyme 11-β hydroxysteroid dehydrogenase type 2, which protects MR in the kidney from being constantly occupied by cortisol [26]. Therefore, T cells seem to have other MR protecting mechanisms, as has already been discussed for brain MR [27]. As a consequence of MR activation at HPA axis level, cortisol concentration was also reduced in the present study, which could have influenced the observed effects on T cells. However, because cortisol diminishes T-cell numbers via GR activation [4;6], decreased cortisol levels are rather expected to counteract the effects of fludrocortisone to some extent.”

Discussion, page 10: “Aldosterone, which is released in a tightly sleep-dependent fashion and seems to be more relevant as endogenous agonist for lymphocytic MR than cortisol [7;8;38;40], conceivably mediates this effect of sleep, as blocking MR during sleep affects T-cell numbers in the same way as depriving subjects from nocturnal sleep [11;12;39].”

4) Might it be possible to show all subsets in Fig.2?

Authors’ response: We now show all CD62L+ and CCR7+ subsets, i.e. naïve and central memory T cells in Fig. 4 (former Fig. 2). Effector memory and effector T cells are not shown because they are CD62L- and CCR7- by definition.

5) Please comment on the specificity of the results for MR, since Fludrocortisone (MR, AR, GR) as well as Spironolacton (MR, AR) are not specific for only the MR receptor.

Authors’ response: Indeed, both drugs can have MR-independent effects. However, at the dose used in our experiments fludrocortisone does not exert strong glucocorticoid activity and, to the best of our knowledge, fludrocortisone can activate the androgen receptor only in patients with a certain mutation (Krishnan et al., 2002. Endocrinology; Matias et al., 2002. J. Med. Chem.). As to the effects of spironolactone, we cannot entirely rule out that they were also mediated at least in part by its antagonistic activity on androgen receptors. However, the complementing results observed for spironolactone and fludrocortisone strongly suggest that the effects were indeed specific to both drugs' action on MR. We now discuss this aspect in the Discussion on page 8.

Discussion, page 8: “We show that a single administration of fludrocortisone to healthy men selectively reduced numbers of circulating naïve CD4+, central memory CD4+ and naïve CD8+ T cells. The results are in line with a recent study of ours showing that the MR antagonist spironolactone increases blood cell counts of the same subsets during early sleep, while other T-cell subpopulations remained unaffected

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[11]. The present findings provide conclusive evidence that these effects of spironolactone were specific to its MR blockade and not secondary to changes in cytokine release that have been found following spironolactone administration and are MR-independent [14]. Moreover, although fludrocortisone displays some glucocorticoid potency, such activity is negligible at the low dose of the drug used in our experiments [24]. Together, the highly complementary findings observed following fludrocortisone and spironolactone administration strongly suggest MR to be the specific receptor involved in the effects of both drugs"

6) Fludrocortisone is approved as drug for orthostatic dysregulation and replacement therapy in e.g. Addisons disease. A rise in blood pressure is expected, at least with a maximal dose of the drug like used in this study (0.2mg). Why didn't the authors observe a rise in blood pressure? Did the medication work at all? If blood pressure was elevated couldn't this impact lymphocyte distribution in itself?

Authors' response: Blood pressure was not affected by fludrocortisone administration, which was likely due to the still rather low dose used and/or the fact that the drug was administered only once. Even higher doses fail to significantly increase blood pressure following single fludrocortisone intake (Morise et al., 1986. Endocrinol. Japon.) and other studies show that also repeated administration of 0.1 – 0.4 mg of fludrocortisone does not change blood pressure (Peterson et al., 1998. Arch. Intern. Med.; Westerdahl et al., 2009. Scand.J.Clin.Lab Invest). Therefore, our result of fludrocortisone failing to affect blood pressure is not surprising. Nevertheless, this does not speak for an ineffectiveness of the drug as the changes observed in cortisol and T cell parameters (as well as on memory function as reported elsewhere (Groch et al., 2013. PNEC)) clearly show that the drug was indeed effective. The Results section was adapted according to the reviewers comment:

Results, page 6: "Fludrocortisone produced no side effects and subjects were not able to correctly indicate whether they had received placebo or fludrocortisone. In line with previous studies, blood pressure was also not changed after fludrocortisone administration [19-21]."

Reviewer: 2

1) The authors state the reduction of certain T cell subsets as extravasation, but the possibility of T cell margination should not be ruled out, especially when the role of CD62L, which mediates rolling of T cells on endothelial surface, is considered.

Authors' response: Indeed, we cannot state with certainty that the reduction in circulating T-cell numbers is due to an increased extravasation. We now discuss this point in the discussion and avoided the use of the word extravasation when appropriate.

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Discussion, pages 9-10: “Increases in the expression of CD62L and CCR7 suggest a redistribution of the cells to lymph nodes as these are the main lymph node homing receptors on T cells [16]. This is in line with our findings that only T-cell subsets with lymph node homing capacity (i.e., naïve and central memory T cells) were affected by fludrocortisone administration in this study, and by spironolactone in our former study [11]. The reduction in T-cell numbers in blood does not provide definite proof for an extravasation of the cells as they might have only attached to the endothelium, thus remaining undetected in the circulating blood. Nevertheless, an enhanced adhesion to the endothelium following increases in CD62L and CCR7 expression is the first step in the migratory cascade und would also favor a subsequent extravasation and homing to lymph nodes.”

2) All the data regarding the expression of CD62L, CXCR4 and CCR7 and T cell subset numbers are shown as the difference from control. Why didn’t the authors show the absolute level? I guess the individual variance was very large, but if this is the case, they could have shown proportional changes and compare them with placebo condition.

Authors’ response:
The purpose of showing the data as difference from control was to remove the strong and well-known circadian variation in the parameters, so that the pure effect of the drug is visible and the kinetics of this effect can be more easily detected at first sight. We now included this rationale in the text. The main focus of the present study was not on these circadian changes but on the effects of fludrocortisone, and we believe that discussing the circadian aspect of T cell migration in detail would be beyond the scope of the study. Nevertheless, if desired, we would add such figures with absolute values as supplemental data.

Figure legends, page 29: “Values for the fludrocortisone condition are indicated as difference from the placebo condition to eliminate the well-known strong circadian variation in T-cell numbers.”

3) Aldosterone should be secreted during early sleep as the authors stated, which might have led to fluctuations in the T cell subsets and their expression of CD62L and CXCR4. I also appreciate if the absolute concentration of cortisol is shown.

Authors’ response:
We now included absolute concentrations for cortisol, aldosterone and ACTH to the supplemental data. A similar comment (# 3) has been raised by Reviewer #1. Differing from the findings by Charloux et al. (1999, 2001), in our study aldosterone levels did not appear to be highest during the sleep period, which can be explained by our low blood sampling rate that did not allow to identify sleep-dependent high aldosterone secretion pulses as observed in the studies by Charloux et al.. In addition, our subjects left the bed and walked around after being woken up, which induces an orthostatic response and accounts for the strong increase in aldosterone levels in the morning. This information is now included in the text:

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Results, page 5-6: ”ACTH itself and aldosterone levels were not significantly different between conditions (Fig. S2). Differing from the findings by Charloux et al. [7;8], in our study aldosterone levels appeared to be higher after awakening than during sleep. However, this can be explained by our low blood sampling rate that did not allow to identify single pulses in sleep-dependent aldosterone release and by the orthostatic response occurring in the morning as our subjects got up and left the bed after awakening.”

4) Why could they detect naïve T cells with increased CXCR4 expression after fludrocortisone administration? Since CXCR4 is a potent chemokine receptor, it is very likely that the cells with enhanced CXCR4 expression would quickly migrate to bone marrow, lymph nodes, and other tissues with high CXCL12 expression, and will quickly be lost from circulation. It is well know that in the WHIM syndrome, a congenital immunodeficiency disorder characterized by gain of function mutation of CXCR4, persistent activation of CXCR4 results in retention of cells expressing CXCR4 in tissues expressing CXCL12. Was increased CXCR4 inactive? An in vitro transmigration assay of fludrocortisone/spironolactone treated T cells to CXCL12 may answer this question.

Authors’ response:
This is an important point. In contrast to a persistent activation of CXCR4 in WHIM syndrome, the acute administration of fludrocortisone is likely too short to induce a complete extravasation of naïve T cells following increases in CXCR4 expression, which should therefore allow to detect increases in CXCR4 levels on those T cells that are still in the circulation. However, we indeed cannot be sure that the observed increase in CXCR4 expression was functional and transmigration assays as well as animal studies would help to clarify this issue. Due to the repeated blood sampling, we were limited as to the amount of blood we could sample from our subjects and thus in the numbers of assay we could run in this study. Therefore, further studies are clearly warranted to elaborate the role of MR in T cell migration in more detail, and we are in fact preparing such studies. We now discuss this limitation of our study on page 11. Nevertheless, there are some studies suggesting that increases in CXCR4 expression on cells that are still in the circulation are functional. For instance, CXCR4 expression on circulating T cells shows a strong glucocorticoid receptor-mediated circadian increase that is paralleled by a concomitant decline in T-cell numbers (Besedovsky et al., 2013. FASEB J). Both, the increase in CXCR4 expression and decrease in T-cell numbers are abolished following blockade of cortisol synthesis. In vitro studies that showed increases in CXCR4 following cortisol administration also demonstrated an increased migration of T cells towards CXCL12 (Okutsu et al., 2005. Am J Physiol Regulatory Integrative Comp Physiol). In another experimental setting, increases in CXCR4 expression on circulating endothelial precursor cells were also paralleled by a concomitant reduction in blood cell numbers (Colombo et al., 2012. J Physiol). Together, these findings underpin the notion that the observed increase in CXCR4 expression in our study was indeed functional.

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Discussion, page 11: “Therefore, the consequences of enhanced CXCR4 expression might crucially depend on the time of day (see also Fig. 6). However, future animal and in vitro studies are warranted to clarify whether the changes we observed in the expression of different migration molecules following MR signaling indeed are functional in that they cause the extravasation and recruitment of T cells to lymph nodes or other body compartments during sleep.”

Second Editorial Decision – 13 February 2014

Dear Dr. Lange,

It is a pleasure to provisionally accept your manuscript entitled "Mineralocorticoid receptor signaling reduces numbers of circulating human naïve T cells and increases their CD62L, CCR7 and CXCR4 expression" 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. Andreas Radbruch

Dr. Karen Chu
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