Impaired responsiveness to TCR stimulation and defective negative selection of thymocytes in CCR7-deficient mice

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Abstract

The chemokine receptor CCR7 has been implicated in maintenance of thymus morphology and establishment of tolerance to self-antigens. Here, we provide direct evidence that negative selection of maturing thymocytes is defective in CCR7-deficient mice. Impaired negative selection was observed following TCR/CD3-complex stimulation in vivo as well as in vitro and was prominent in both double positive and semi-mature single positive cells (CD4⁺CD8⁻ CD24^high). Importantly, thymocytes of CCR7⁻/⁻ mice display defective negative selection in response to endogenous superantigens, demonstrating that the defect also occurs under physiological conditions. Disturbed negative selection correlated with delayed activation kinetics and decreased calcium flux response of CCR7⁻/⁻ thymocytes following in vitro TCR/CD3 stimulation, suggesting that an impaired response of CCR7⁻/⁻ thymocytes via TCR-mediated signalling is responsible for defective negative selection in these mice.
Introduction

CCR7 is a major regulator of the immune system orchestrating a broad spectrum of fundamental processes ranging from lymphoid organ development to induction of defensive immune responses as well as oral tolerance 1-3. An important role for CCR7 in thymus compartmentalization and coordination of migratory events during thymopoiesis has been recently demonstrated4,5. Mice deficient in CCR7 or in its ligands CCL19 and CCL21-ser (plt/plt mice6,7) display altered thymus architecture, impaired T-cell development, and decreased numbers of thymocytes4,5. Alterations of the thymus architecture are frequently observed in mice suffering from autoimmunity such as mice deficient in the transcriptional regulator autoimmune regulator (Aire) and non-obese diabetic (NOD) mice8,9. In these strains, autoimmunity correlates with breakdown of central tolerance, although in NOD mice peripheral tolerance seems to be affected as well10-12. It has been previously noticed that lack of CCR7-signalling is associated with the manifestation of autoimmunity to lachrymal and salivary glands13. Expanding this view, we recently demonstrated that CCR7-deficient mice in fact display a more generalized autoimmune phenotype affecting several organs14. These observations raised the possibility that CCR7-deficiency affects induction of central tolerance.

The hallmark of central tolerance is the negative selection of potentially self-reactive thymocytes. This process guarantees the generation of a self-tolerant peripheral T-cell repertoire. The major mechanism of negative selection is clonal deletion. During this process thymocytes reacting strongly to self-peptide:self-MHC complexes are eliminated15,16. Several studies using well characterized in vivo and in vitro models of negative selection suggest that clonal deletion can occur in all developmental stages of thymocytes, including double negative (DN), double positive (DP) and single positive (SP)15,17-20. However, it is widely accepted that at least for thymocytes expressing an MHC class II-restricted T-cell receptor, deletion of auto-reactive T-cells occurs predominantly at a semi-mature SP stage of development characterized by high expression levels of CD24. CD4+CD8+CD24high cells are considered to be semi-mature because they become apoptotic in response to TCR stimulation, whereas mature CD4+CD8+CD24low cells proliferate following such stimulus15,21,22.

In the present study we demonstrate that negative selection of CD4+CD8+CD24high and DP thymocytes is strongly impaired in CCR7-deficient mice following in vivo TCR/CD3-complex stimulation. Thymocytes from CCR7+/− mice also resist negative selection occurring physiologically in response to endogenous superantigens. Impaired negative selection was also observed in vitro and was accompanied by delayed activation kinetics and decreased calcium flux response of DP and CD4+ SP CCR7-deficient thymocytes to TCR/CD3-complex ligation. Therefore, our data indicate that CCR7 deficiency lowers the sensitivity and, thereby, the reactivity of thymocytes to TCR triggered signalling at distinct developmental stages.

Material and Methods

Mice

The generation of CCR7−/− mice has been described elsewhere1. CCR7-deficient mice were backcrossed for at least 7 generations either to the C57BL/6 or to the BALB/c genetic background. In vitro stimulation with the OVA323–339 peptide was performed using thymocytes from DO11.10/CCR7+/− and DO11.10/CCR7−/−DO11.10 mice. The animals were bred and maintained under SPF conditions in Hannover Medical School’s animal facility. Wild type C57BL/6 CD45.2, congenic CD45.1 and BALB/c mice were either bred in the same facility.
or obtained from Charles River (Sulzfeld, Germany). Mice were used at the age of 6 to 9 weeks. All animal experiments were performed in accordance with institutional guidelines following approval by the local committees.

**Immunohistology**

Mice were sacrificed by CO₂ inhalation. Thymi were excised, embedded in OCT compound and frozen on dry ice. Eight µm thin cryosections were prepared, air dried and fixed in ice-cold acetone. Sections were blocked with 10% mouse or rat serum and stained with antibodies against the indicated markers. The following mAb and reagents were used: home made anti-CD8β (RmCD8-2) followed by mouse anti-rat IgG-Cy3 (Jackson ImmunoResearch); biotinylated anti-CD4 (RM4-4) followed by streptavidin-Alexa-Fluor-488 (Molecular Probes); unlabelled anti-Ep-CAM1 (G8.8) (BD Bioscience) followed by mouse anti-rat IgG-Cy3 and biotinylated Ulex europaeus agglutinin-1 (UEA-1, Vector laboratories) followed by streptavidin-Cy5 (Caltag laboratories, Invitrogen). Images were acquired using either an Olympus BX61 microscope (Olympus, Hamburg, Germany) with F-View II Camera and cellP software (Soft Imaging System GmbH, Münster, Germany) or a Zeiss Axiovert M200 microscope with Hamamatsu/Orca ER Camera and Axiovision 3.1 software (Carl Zeiss MicroImaging, Oberkochen, Germany). Medullary areas were marked and measured using the cellP software. Cells within the marked areas were counted using the ImageJ software (National Institutes of Health, USA).

**Flow Cytometry**

Single cell suspensions of thymi were obtained by mincing the organs through a nylon mesh. The following antibodies were used: home made anti-CD3 (17A2), anti-CD4 (RmCD4-2) and anti-CD8β (RmCD8-2); anti-CD5 (53-7.3), anti-CD8α (53-6.7), anti-CD24 (M1/69), anti-CD45.2 (104), anti-CD62-L (AME-14), anti-CD69 (H1.2F3), anti-Vβ3 (KJ25), anti-Vβ5 (MR9-4), anti-Vβ11 (RR3-15) and anti-Vβ12 (MR11-1) (BD Biosciences). CCR7 staining was performed using a PE-labeled monoclonal antibody (4B12, eBioscience). The stain was performed at 37°C as recommended by the manufacturer guidelines. For the experiments using DO11.10 mice, thymocytes were additionally stained with the TCR-specific antibody KJ1.26 (Caltag). Flow cytometry was performed either on a FACSCalibur or on a FACS LSRII (BD Biosciences).

**In vitro stimulation of thymocytes**

Freshly isolated thymocytes (3.0x10⁶) from DO11.10/CCR7+/+ or DO11.10/CCR7-/- mice were cultured in RPMI medium (Invitrogen) supplemented with 2-mercaptoethanol, L-glutamine and 10% FCS in the presence of either non-loaded or OVA-loaded bone marrow-derived DC (5x10⁵). After 18 hours, cells were collected and analyzed by flow cytometry. DC were prepared as described². Briefly, 2x10⁶ bone marrow cells from tibia and femur of BALB/c mice were cultured in RPMI, 10% FCS, 2-mercaptoethanol, glutamine, and penicillin/streptomycin supplemented with 100–200ng/ml GM-CSF for 7 or 8 days. Cells were then stimulated for 18 hours with 1µg/ml LPS to induce maturation and then loaded with OVA323-339 peptide for 2 hours before co-incubation with thymocytes. Alternatively, non-TCR-transgenic thymocytes were cultured for 2h, 6h, 8h, 12h or 24h in plates pre-coated with 10µg/ml anti-CD3 antibody (17A2) in medium containing 10µg/ml anti-CD28 antibody (37N).
Calcium flux

Thymocytes (2.5x10^7) were loaded with 4µg/ml Fluo-4 and 10µg/ml Fura Red in the presence of 0.02% Pluronic (all from Molecular Probes) in 2.5ml phenol red-free RPMI containing 25mM HEPES and 10% FCS for 30min at 37°C. Cells were washed and labeled with rat-anti-mouse CD4-Cy5 and rat-anti-mouse CD8-APC-Cy7 for 15 min on ice. After washing, thymocytes were resuspended in 1ml medium containing 2mM CaCl_2 and incubated in the dark for 90min at room temperature. Calcium influx was measured by flow cytometry at 37°C using a custom-built heating device. After measurement of baseline levels for 30s, 5µg rat-anti-mouse CD3 (17A2) plus 24µg mouse-anti-rat IgG (Jackson ImmunoResearch) as crosslinking antibody were added. Events were collected for a total time of 5min. As a positive control, calcium ionophor A23187 (Sigma) was added at a final concentration of 20µg/ml. FlowJo software (Treestar) was used to analyze the calcium flux kinetics of CD4^+ SP and CD4^+CD8^+ DP thymocytes.

Stable Bone Marrow Chimeras

Bone marrow from C57BL/6 CCR7-deficient (CD45.2) and congenic wild-type (CD45.1) females were prepared as described^4, mixed at a ratio of 1:1 and a total of 1.0x10^7 cells were transferred into CD45.1 recipients. Prior to transplantation, recipients were irradiated (5.5 Gy) twice at an interval of 5h. Three months later, chimeric mice were killed, thymi were harvested and analyzed by flow cytometry.

Statistical Analysis

Statistical analysis was accomplished using GraphPad Prism 3 (GraphPad Software, San Diego, CA). Data were analyzed as indicated in the figure legends. P values ≤0.05 were considered significant.

Results

CCR7-deficient thymocytes display impaired negative selection following in vivo TCR/CD3-complex ligation

The presence of autoimmunity in CCR7^-/- and plt/plt mice^13,14 suggests that central tolerance is altered in these mice. To address this possibility, we induced negative selection of thymocytes by injecting wild type and mutant mice with anti-CD3 mAb. In this model of negative selection, depletion of DP as well as semi-mature CD4^+CD8^-CD24^high thymocytes is triggered experimentally in vivo as a consequence of TCR/CD3-complex stimulation^12,21,22. Elimination of DP cells is thought to be largely influenced by corticosteroids and/or inflammatory cytokines produced in response to the activation of peripheral T-cells^20,24. In contrast, depletion of CD4^+CD8^-CD24^high cells is believed to represent clonal deletion of cells undergoing negative selection in the medulla^18,20,22.

Negative selection was induced in wild type and mutant mice by injecting 200 µg of anti-CD3 mAb intravenously. Treated animals were sacrificed 42h later and thymocytes were analyzed for expression of CD4, CD8 and CD24 by flow cytometry. No differences between CCR7^-/- and wild type mice regarding the proportions of DP and SP thymocytes were observed following PBS injection, while injection of 200 µg of anti-CD3 mAb induced depletion of the majority of wild type but not of mutant DP thymocytes (Fig. 1A). The percentage of cells belonging to the semi-mature CD4^+CD8^-CD24^high subpopulation was also similar in wild type
and CCR7-deficient animals receiving PBS. However, whereas the administration of 200 µg of anti-CD3 mAb induced the elimination of the majority of CD4⁺CD8⁻CD24⁺thymocytes in wild type mice, only a slight reduction in this population was observed in CCR7⁻/⁻ mice (Fig. 1B).

The model of negative selection applied in our study assumes that bystander antigen-presenting cells provide co-stimulation. Therefore, to exclude the possibility that impaired deletion of mutant thymocytes is caused by lack of proper co-stimulation by CCR7-deficient APC, we generated chimeric animals displaying a chimerism of 30% to 50% CCR7⁻/⁻ and 50% to 70% wild type cells in the thymus. Chimeric animals were injected either with PBS or 200 µg of anti-CD3 mAb and analyzed 42h later. As shown in Fig. 1C, depletion of both DP and CD4⁺CD8⁻CD24⁺thymocytes of CCR7-deficient origin remained impaired even in the presence of wild type APC. These results indicate that a cell intrinsic defect is responsible for the resistance of mutant thymocytes to undergo deletion following TCR/CD3-complex stimulation.

Negative selection of CD4⁺CD8⁻CD24⁺ cells is Fas-independent following weak to moderate TCR ligation and Fas-dependent following strong TCR ligation. These conditions can be mimicked in vivo by injecting different concentrations of anti-CD3 mAb. Antibody at concentrations between 20 µg and 200 µg induced the elimination of thymocytes in wild type mice in a dose dependent manner. In marked contrast, no apparent reduction in the numbers of total thymocytes was observed in CCR7⁻/⁻ mice (Fig. 2A). The analysis of DP and semi-mature CD4⁺CD8⁻CD24⁺ cells revealed that depletion of both populations was strongly impaired in CCR7-deficient animals at all doses of antibody administered (Fig. 2B and 2C), indicating either that both Fas-dependent and Fas-independent pathways are affected in these mice or that the defect leading to the resistance to negative selection occurs upstream of both pathways. Lack of deletion of DP thymocytes in CCR7-deficient mice seems not to be due to the inability of peripheral T-cells to respond to TCR stimulation, since lymphocytes derived from peripheral lymph nodes proliferate strongly in response to anti-CD3 injection (Fig. 2D). The exaggerated proliferation of lymphocytes observed in peripheral lymph nodes of CCR7-deficient mice following anti-CD3 injection is a likely consequence of the T-cell lymphopenia found in the mutant organs under homeostatic conditions.

**Defective negative selection of CCR7-deficient thymocytes does not correlate with impaired migration of these cells into the medulla**

It has been recently postulated that negative selection of thymocytes reactive to tissue specific antigens should be impaired in the absence of CCR7-signalling due to the inability of positively selected cells to migrate from the cortex into the medulla, where negative selection would normally occur. However, this hypothesis cannot explain why the DP cell population of CCR7-deficient mice resists depletion following anti-CD3 injection, since these cells are virtually restricted to the cortex. Furthermore, only a minute fraction of DP thymocytes express CCR7 (1.4 % of all DP cells, Fig. 3A), indicating that the migratory behavior of DP cells towards CCR7-ligands is similar in wild type and mutant mice. More importantly, less than one fourth of positively selected DP cells (CD4⁺CD8⁺CD69⁺) express CCR7, suggesting that in addition to CCR7, other chemokine receptors might be involved in the transition of these cells from the cortex to the medulla (Fig. 3A). CCR7 is largely expressed on SP cells (Fig. 3A). Notably, CCR7 expression levels are variable within these populations, suggesting that the expression of the receptor is differentially regulated at different maturational stages. To test this hypothesis, we analyzed the expression of CCR7, CD24 and CD69 on CD4 SP cells concomitantly. Surprisingly, CCR7 was either not expressed or expressed at low to intermediate levels on CD4⁺CD8⁻CD24⁺ cells (Fig. 3B, subpopulations I and II). In contrast,
the receptor was highly expressed on CD4+CD8CD24int cells (Fig. 3B, subpopulation III). This result indicates that CCR7 expression might be upregulated following TCR stimulation on cells that escaped clonal deletion. Mature CD4+CD8CD24low cells also express CCR7, although at intermediate levels (Fig. 3B, subpopulation IV). Interestingly, CCR7 expression levels seem to correlate with the activation state of the cells, as CD4+CD8CD24int thymocytes express the highest levels of CD69 (Fig. 3C, subpopulation III). Note that in a small fraction of CD4+CD8CD24int cells CD69 expression is downregulated. It is likely that the decreased CD69 expression in these cells reflects their maturation into CD4+CD8CD24low cells, which are CD69- (Fig. 3C, subpopulation IV).

Intriguingly, our data also demonstrate that CD4+CD8CD69+CCR7+ cells express higher levels of CCR7 than CD4+CD8CD24high cells. Therefore, either CCR7 is downregulated on CD4+CD8CD69+ thymocytes directly following positive selection or the CD4+CD8CD24high cell population develops from positively selected DP thymocytes that do not express CCR7. In the latter case, no impairment in cortex-to-medulla migration of CD4 SP thymocytes would be expected in CCR7-deficient mice. One possibility to address this question is to analyze the distribution of SP cells in the thymus of CCR7-deficient mice. It has been previously reported that the medulla of sublethally irradiated CCR7-deficient mice contain strongly reduced numbers of SP thymocytes5. Similarly, a reduction in the numbers of SP cells found in the medulla of non-irradiated CCR7+/+AND-TCR transgenic mice was observed6. As nothing is known about the distribution of SP cells in the thymus of untreated CCR7+ mice displaying a polyclonal TCR repertoire, we analyzed the presence of SP cells in the medulla of non-irradiated CCR7-deficient and wild type mice. As shown in Fig. 4A and B, many CD4+ and CD8+ SP cells were observed in the medulla of CCR7-deficient mice. Interestingly, even those medullary areas that are misplaced to the outer rim of the organ in the mutant mice are filled with SP cells (Fig. 4A, arrowheads). A quantitative evaluation of the medulla revealed that there is no significant difference in the number of CD4 SP cells found per area between wild type and CCR7-deficient mice (121.9 ± 13.11 cells/0.01 mm² vs. 120.00 ± 13.47 cells/0.01 mm², respectively; Fig. 4C). In contrast, a 2.2 fold decrease in the numbers of medullary CD8 SP cells was observed (20.73 ± 8.62 cells/0.01 mm² in wild type vs. 9.05 ± 2.19 cells/0.01 mm² in mutants; Fig. 4C). The reasons for the reduced numbers of medullary CD8 SP cells observed in CCR7-deficient animals are not known yet but it would be possible that these cells display a partial defect in cortex-to-medulla migration. Nevertheless, our data clearly indicates that the majority of the positively selected thymocytes of CCR7+ animals migrate efficiently from the cortex into the medulla. Therefore, impaired negative selection of CCR7-deficient thymocytes does not causally correlate with impaired cortex-to-medulla migration.

**CCR7+ thymocytes display impaired negative selection in response to endogenous superantigens**

To investigate the capacity of CCR7-deficient thymocytes to undergo negative selection under physiological conditions, we compared the deletion efficiency of thymocytes reactive to endogenous retroviral superantigens (SAg) in wild type and CCR7+/− mice. In this scenario, negative selection evokes clonal deletion of thymocytes bearing distinct TCR Vβ domains that react to SAg derived from mouse mammary tumor virus (Mtv). BALB/c mice are positive for Mtv-6, Mtv-8 and Mtv-926. Accordingly, thymocytes expressing Vβ3 (reactive to Mtv-6), Vβ5, Vβ11 and Vβ12 (reactive to Mtv-8 and Mtv-9) are deleted to a large extent in this mouse strain. As shown in Fig. 5A, significantly higher percentages of DP and SP thymocytes expressing Vβ5, Vβ11 and Vβ12, but not Vβ3, were observed in CCR7-deficient mice. Similar results were also observed for splenocytes (Fig. 5B). Together, the data indicate that negative selection of thymocytes reactive to Mtv-8 and Mtv-9 but not to Mtv-6 SAg is
impaired in the absence of CCR7 signalling. At least for Vβ5+ thymocytes, clonal deletion is thought to be induced by a medullary thymic epithelial cell (mTEC) subpopulation that binds the lectin *Ulex europaeus* agglutinin-1 (UEA1)\(^2^7\). It has been previously described that clusters of mTEC expressing G8.8 and binding UEA-1 are missing in the thymus of CCR7-deficient mice\(^5\). Therefore, it would be possible that the absence of such cell clusters leads to impaired negative selection of thymocytes bearing the Vβ5 domain in CCR7-deficient animals. However, in contrast to the earlier published data, G8.8+/UEA-1+ mTEC clusters were present in CCR7-deficient animals (Fig. 5C). These results demonstrate that impaired negative selection of Vβ5+ thymocytes is not caused by lack of G8.8+/UEA-1+ mTEC clusters in the medulla of CCR7-/- animals analyzed here.

**Impaired responsiveness of CCR7-deficient thymocytes to TCR stimulation *in vitro***

The observations described above would imply that thymocytes of CCR7-/- mice display a cell internal defect affecting their responsiveness to TCR stimulation. To test this hypothesis, we co-incubated thymocytes of DO11.10/CCR7+/+ and DO11.10/CCR7-/- mice for 18 hours with mature bone marrow-derived dendritic cells (DC) loaded with ovalbumin. As shown in Fig. 6A, the majority of the DP thymocytes of DO11.10/CCR7+/+ mice become activated upon TCR ligation. This is demonstrated by down-regulated expression of both CD4 and CD8 surface molecules\(^2^1\). In contrast, a minor fraction of the DP thymocytes of DO11.10/CCR7-/- showed this behavior (Fig. 6A). Similar results were also observed after *in vitro* stimulation of CCR7-deficient thymocytes with anti-CD3/anti-CD28 antibodies (data not shown). Together, these results support the idea that mutant thymocytes are more refractory to TCR stimulation than wild-type thymocytes. To substantiate this idea, we stimulated thymocytes of both strains *in vitro* with anti-CD3/anti-CD28 antibodies and investigated the expression of surface markers which are normally regulated on thymocytes subsequent to TCR activation. After a 12h stimulation period, we observed up-regulated expression of CD69 in two-fold more wild type thymocytes versus CCR7-mutant thymocytes (Fig. 6B). Note that cells with increased CD69 expression had concurrent decreased CD4 surface levels (arrows in Fig. 6B). In addition, while no differences in the expression levels of TCRα/β or CD5 were observed between non-stimulated wild type and mutant thymocytes, the kinetic of CD5 up-regulation as well as of TCRα/β down-regulation was obviously delayed in thymocytes of CCR7-/- mice (Fig. 6C). These results strongly support the hypothesis that the capacity of thymocytes to receive or transduce TCR-mediated signals is impaired in CCR7-deficient mice. Therefore, we subsequently examined the calcium flux responses in SP and DP thymocytes using a FACS-based single cell assay. For this purpose, thymocytes were labeled with the calcium indicators Fluo-4 and FuraRed and were stained with antibodies to CD4 and CD8. Following CD3 cross-linking, calcium flux was measured over time and analyzed separately for the CD4+ SP and DP populations as indicated in Fig. 7A. The calcium signal elicited by CD3 cross-linking was reduced in both CD4+ SP and DP CCR7-deficient thymocytes (Fig. 7B). However, whereas the CCR7-deficient CD4+ SP thymocytes exhibited a reduced peak height of the calcium flux signal in comparison to wild type cells (Fig. 7B, red arrows), the calcium flux response of mutant DP thymocytes was reduced primarily due to a larger fraction of CCR7-deficient DP thymocytes remaining unresponsive to TCR stimulation (Fig. 7B, red arrow heads). In contrast, the response to the calcium ionophore A23187 was comparable for mutant and wild type thymocytes (data not shown), indicating that the cellular mechanisms of calcium mobilization operate normally in CCR7-deficient mice.
Discussion

The presence of autoimmunity in CCR7-deficient mice indicates a failure in the induction of central tolerance in these animals. In the present study, we demonstrate that negative selection is in fact impaired in CCR7 mice. Hampered negative selection of CCR7-deficient thymocytes was observed in all in vivo and in vitro models we applied. We found elimination of DP as well as of semi-mature CD4+CD8+CD24+ SP thymocytes induced by TCR/CD3-complex stimulation to be strongly impaired in CCR7-deficient mice. Although this model for induction of negative selection would be considered non-physiological, our data demonstrate clearly that thymocytes of CCR7 mice do not respond to TCR/CD3-complex stimulation in the same way that wild type thymocytes do. Importantly, negative selection of thymocytes reactive to endogenous SAg also seems to be impaired in CCR7 mice since the extent of deletion of CCR7-deficient Vß5+, Vß11+ and Vß12+ thymocytes was significantly decreased in comparison to control thymocytes. It is of note that the proportions of Vß5+, Vß11+ and Vß12+ thymocytes found in CCR7-deficient mice were within the range described in other mouse strains displaying defective negative selection. For example, similar proportions were reported in mice deficient in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or mice deficient in the co-stimulatory molecules B7-1 and B7-2. Interestingly, the degree of depletion of CCR7-deficient Vß3+ thymocytes was normal, indicating that negative selection of thymocytes reactive to Mtv-8 and Mtv-9 but not reactive to Mtv-6 SAg is defective in CCR7 mice. Normal deletion frequencies of thymocytes expressing Vß3 concomitantly with impaired depletion of thymocytes expressing Vß5, Vß11 and Vß12 was also observed in Ikaros null mice and in CD28-deficient mice. Interestingly, it has been previously reported that BALB/c thymic DC express Mtv-6 but not Mtv-8 and Mtv-9 SAg. This indicates that negative selection of Vß3+ thymocytes but not of Vß5+, Vß11+ and Vß12+ thymocytes may be accomplished by DC. Therefore, the observed differences in deletion efficiency regarding thymocytes bearing distinct subsets of Vß domains may reflect the cell type expressing the respective Mtv-Sag and thereby achieving negative selection. Indeed, because of their co-stimulatory capacities, DC might induce clonal deletion more effectively, and thus compensate for less vigorous TCR-signals. This would explain the equivalent efficiency of DC-mediated depletion of Vß3+ thymocytes in CCR7 deficient and wild type mice. In contrast to the data presented here, it has been previously reported that negative selection of Vß5+ thymocytes is not impaired in plt/plt mice which are deficient in the CCR7 ligands CCL19 and CCL21-ser. The reasons for the discrepancy between our data and the data generated by others using plt/plt mice are currently unclear but may be attributed to the different mouse strains used in the studies. Thymocytes of plt/plt mice express CCR7 and CCL21-leu is present in some non-lymphoid organs of these mice. Therefore, it would be possible that this variant of CCL21 reaches the thymus via the blood stream and binds to stromal cells, thus compensating for the lack of CCL21-ser.

It has been recently proposed that negative selection of thymocytes reactive to tissue antigens is defective in the absence of CCR7-mediated signals due to impaired migration of positively selected cells from the cortex to the medulla. This hypothesis is based on the fact that reduced numbers of SP cells were observed in the medulla of irradiated CCR7 mice. However, although we observed a two-fold reduction in the numbers of CD8 SP cells, we never observed reduced numbers of CD4 SP cells in the medulla of non-manipulated CCR7-deficient mice. Therefore, differences in the applied experimental set-up are likely to generate the discrepancies in the results obtained in our study versus those previously reported. It has been recently demonstrated that sublethal irradiation leads to up-regulation of CCL25, CCL21 and CXCL12 mRNA expression by thymic dendritic and stromal cells. Therefore, the unbalanced chemokine-profile provoked by irradiation may influence the positioning of SP radio-resistant cells in the thymus depending on the presence or absence of CCR7.
the discrepancy observed between non-irradiated CCR7−/+ bearing a polyclonal T cell repertoire and CCR7−/+/AND-TCR transgenic mice is likely to reflect differences between the analyzed mice. Ueno and co-workers reported that the medullary regions were poorly formed in the thymus of CCR7−/+/AND-TCR mice and many CD4 SP cells were found in the cortex of these animals. However, AND-TCR transgenic mice display strongly skewed positive selection towards CD4 SP thymocytes in comparison to non-TCR transgenic mice. Moreover, the skewed positive selection of CD4 SP cells observed in these mice result in disruption of the thymus morphology and disorganization of the medullary epithelium. Therefore, the combination of the transgenic AND-TCR with CCR7 deficiency may create the unique phenotype observed by Ueno and co-workers. Since our analysis of non-irradiated thymi of mice bearing a polyclonal TCR repertoire represents a physiological situation, we conclude that impaired cortex-to-medulla migration is not a major factor leading to defective negative selection of CCR7-deficient thymocytes. The finding that the elimination of DP cells, which reside in the cortex, was also defective in CCR7-deficient animals lends further support to this conclusion. In addition, only a minute fraction of CD4+CD8+ cells express CCR7. Therefore, it seems unlikely that this receptor is directly involved in the failure of these cells to undergo negative selection. The expression pattern of CCR7 on DP thymocytes observed in our present study is in agreement with data reported by others demonstrating that CCR7 mRNA expression by DP cells is extremely low and that these cells do not migrate in vitro in response to the CCR7-ligands CCL19 and CCL21. In addition, CCR7 was expressed on less than one fourth of activated DP thymocytes (CD4+CD8+CD69+), which are thought to represent a positively selected population in transit between cortex and medulla. This result correlates very well with previously published data demonstrating that this cell population expresses low levels of CCR7 mRNA. In fact, it has been demonstrated by others that a similar fraction of these cells is able to migrate in vitro towards CCR7-ligands. Notably, CD4+CD8+CD69+ thymocytes migrate more efficiently to CCL22, CXCL12 and CCL25 than to CCL19 and CCL21. With the exception of CCL25, which is present in the whole thymus at high levels, the expression of the chemokines CCL17, CCL19, CCL21, CCL22 and CXCL12 is either more prominent or restricted to the medulla and cortico-medullary junction. Therefore, chemokine receptors other than CCR7 might guide cortex-to-medulla migration of positively selected thymocytes.

As expected, CCR7 expression was detected in the majority of the CD4 SP cells of wild type mice. Interestingly, the analysis of CCR7 and CD24 co-expression on these cells revealed that only part of the most immature CD4+CD8+CD24high subpopulation express the receptor. Furthermore, CCR7 is expressed on these cells at low to intermediate levels, while the more mature CD4+CD8+CD24int subpopulation express high levels of CCR7. This observation indicates that CCR7 expression positively correlates with the maturation of CD4 SP thymocytes, as the receptor seems to be up-regulated on thymocytes upon TCR stimulation. Expression of CCR7 on SP thymocytes that escaped negative selection could be necessary for thymocyte/medullary stroma cell interactions, thus assuring their proper functional maturation. Actually, our data demonstrate that CCR7 is not crucial for the migration of the majority of positively selected thymocytes into the medulla. A definitive confirmation of this hypothesis will require further experiments analyzing the migratory behavior of positively selected cells by two-photon microscopy. Nevertheless, it seems unlikely that the function of CCR7 in negative selection and maturation of SP cells is restricted to cortex-to-medulla migration. The results of the in vitro experiments clearly demonstrate that thymocytes of CCR7-deficient mice are at least in part hyporesponsive to TCR ligation: i) only a small proportion of DP thymocytes of CCR7-deficient mice express decreased levels of CD4 and CD8 after stimulation with either OVA-peptide or anti-CD3 plus anti-CD28 antibodies, ii) only a fraction of CCR7−/+ thymocytes exhibit increased surface expression of CD69 following anti-CD3/anti-CD28 stimulation, indicating that most of those cells were not activated.
following TCR cross-linking, and iii) thymocytes of CCR7−/− mice show delayed up-regulation of CD5 as well as down-regulation of TCR α/β expression following *in vitro* stimulation. Finally, DP as well as CD4 SP mutant thymocytes display reduced calcium flux responses after CD3 cross-linking.

Remarkably, CCR7 is virtually not expressed on DP cells. This observation suggests that the defect leading to impaired negative selection in CCR7-deficient animals either occurs at a developmental stage preceding the DP stage or is not directly related to CCR7-signalling on selecting thymocytes. The thymus of CCR7/CCR7-ligand-deficient mice displays a strongly disrupted architecture indicating that stromal cells/thymocytes cross-talk is impaired in these animals. Therefore, it seems possible that impaired thymic cross-talk during early developmental stages eventually results in the differentiation of thymocytes displaying an impaired capability to undergo activation. Notably, CCR7-deficiency could influence thymic cross-talk in different ways. One possibility would be that stromal cells developing in the absence of CCR7-signalling are defective and unable to transduce the signals necessary for proper T-cell development during stromal cell/thymocyte interactions. In this case, thymic cross-talk would be impaired independently of the expression of CCR7 on thymocyte precursors. A further possibility would be that lack of CCR7 expression on thymocyte precursors directly hampers their migratory behavior and their capacity to meet stromal cells expressing CCL19/CCL21, thereby impeding stromal cell/thymocyte interactions. Alternatively, absence of CCR7 might influence the stability of stromal cell/thymocyte interactions, as CCR7-mediated signals increase the avidity of integrins favoring the stabilization of cell-cell interactions.

In addition, other mechanisms may contribute to the resistance of CCR7-deficient SP thymocytes to undergo negative selection. It has been recently demonstrated that surface-bound CCL19 and CCL21 capture and polarize CCR7-expressing T-cells. This enhances T-cell capacity to stably interact with antigen-presenting cells, thus favoring immunological synapse formation. Lack of CCR7 on thymocytes undergoing negative selection may result in less stable interactions with medullary thymic epithelial and dendritic cells. These impaired interactions could further contribute to the decreased TCR activation observed *in vivo* in CCR7-deficient thymocytes. An additional factor influencing negative selection of CCR7-deficient thymocytes would be an impaired induction of activation-induced cell death following TCR stimulation. Indeed, recently published data indicate that this process should be impaired in the absence of CCR7-signaling.

In summary, our present study indicates that decreased responsiveness of CCR7−/− thymocytes to TCR stimulation is a major factor leading to impaired negative selection in CCR7-deficient animals. In addition, our findings identify impaired negative selection as a factor contributing to the autoimmune phenotype observed in CCR7/CCR7-ligand-deficient animals.
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Author contribution
Ana Clara Marques Davalos-Misslitz: designed the study, performed research, analyzed data and wrote the manuscript;
Tim Worbs: performed research and analyzed data
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References


Legend to figures

**Figure 1**

**Impaired negative selection of CCR7-deficient thymocytes in vivo.** Nine-week-old C57BL/6 (+/+) and CCR7-/- (-/-) mice were intravenously injected with PBS or 200 µg of anti-CD3 mAb. 42h after injection, thymocytes were quantified, stained with antibodies to CD3, CD4, CD24 and CD8 and analyzed by flow cytometry. CD24 expression was analyzed to identify the semi-mature CD4+CD8+CD24high population undergoing negative selection after TCR ligation. (A) Proportions of CD4 SP and DP cells in the thymus of PBS-injected mice and mice injected with anti-CD3 mAb, demonstrating the depletion of the DP population in wild type but not in mutant animals. (B) Proportions of CD4+CD8+CD24high cells in the thymus of mice injected with anti-CD3 mAb. Note that in contrast to wild type mice, only a minority of CD4+CD8+CD24high cells have been depleted in CCR7-deficient animals after antibody administration. (C) Bone marrow of CD45.2 CCR7-/- mice was mixed with bone marrow of CD45.1 wild type mice at ratio of 1:1 and transferred into irradiated CD45.1 wild type recipients. Lack of depletion of CCR7-deficient DP and CD4+CD8+CD24high cells in a wild type thymus environment indicates that an intrinsic defect in these thymocytes is responsible for the resistance of these cells to undergo depletion after TCR stimulation.

**Figure 2**

**Comparative analysis of thymocyte deletion in wild type and CCR7-deficient mice following in vivo injection of anti-CD3 mAb.** Nine-week-old C57BL/6 (+/+) and CCR7-/- (-/-) mice were intravenously injected with increasing doses of anti-CD3 mAb. 42h after injection, thymocytes were recovered and stained as described for Fig. 1. (A) Total number of thymocytes. (B) Total number of DP thymocytes. (C) Total number of CD4+CD8+CD24high thymocytes. (D) Total number of lymphocytes isolated from peripheral lymph nodes in mice of both genotypes injected with PBS or different doses of anti-CD3 mAb, demonstrating a strong expansion of CCR7-deficient cells. Data shown was obtained from 12 mice of each genotype on C57BL/6 genetic background in three independent experiments. Similar results were also obtained for mutant mice on BALB/c background. Data has been subjected to One-way ANOVA with Dunnett’s multiple comparison post test. P values ≤0.05 were considered significant.

**Figure 3**

**Expression of CCR7 expression on different thymocyte subpopulations.** Thymocytes of adult C57BL/6 mice were stained with anti-CD4, anti-CD8, anti-CD24, and anti-CD69 antibodies. CCR7 was detected using a monoclonal antibody. (A) CCR7 expression on DP and SP thymocytes. Populations were gated based on the expression of CD4, CD8 and CD69 as indicated. Bright gray: CCR7, dark gray: isotype control. Numbers indicate the percentage of cells expressing CCR7 above the background level. (B) CCR7 expression on CD4 SP cells at different maturational stages. The analysis of CCR7 and CD24 co-expression on CD4+CD8+ cells allows the identification of 4 different subpopulations (I to IV). (C) Expression of CD69 on subpopulations I to IV depicted in (B).

**Figure 4**

**Unimpaired migration of CD4+ cells in the medulla of CCR7-deficient mice.** Cryosections of the thymus from adult (6 to 8 week old) wild type (+/++) and CCR7-/- (-/-) mice were stained with antibodies to CD4 (green) and CD8 (red) to identify DP and SP cells. DP cells appear in yellow. Nuclear staining was performed with DAPI (white). Cortex and medulla topography is reflected by the DAPI stain (left panels). CD4 SP cells are normally found in the medulla of CCR7-deficient animals. (A) A representative region (objective magnification 4x/0.16 NA.) of
the thymus of CCR7-deficient animals containing several medullary areas, all of them filled with CD4 SP cells is shown (green). Note that even the medullary areas that are misplaced at the outer rim of the organ contain CD4 SP cells (arrows). (B) High magnification picture (objective magnification 20x/0.75 NA.) showing a representative medullary area of the thymus of wild type (+/+ ) and CCR7-deficient mice (-/- ). Whereas CD4 SP cells are normally found in the medulla of both mouse strains, CD8 SP cells are less abundant in CCR7-/- mice. Images were acquired using an Olympus BX61 microscope. (C) Mean ± SD of the numbers of CD4 and CD8 SP cells per medullary area. Quantitative data was obtained from analysis of 3 mice from each genotype.

Figure 5

Impaired negative selection of CCR7-deficient thymocytes in response to endogenous SAgs. (A) Negative selection of Vß5*, Vß11* and Vß12* but not of Vß3* thymocytes is impaired in CCR7-deficient mice. (B) Increased proportions of Vß5, Vß11 and Vß12 lymphocytes in the spleen of CCR7-deficient mice, indicating that auto-reactive cells escaping negative selection in the thymus are not deleted in the periphery and, therefore, could induce the development of autoimmunity in these mice. Data has been subjected to unpaired T-test. P values ≤0.05 were considered significant. (C) Clusters of G8.87/UEA-1+ epithelial cells are present in the medulla of CCR7-deficient animals, demonstrating that impaired negative selection of thymocytes reactive to endogenous SAgs is not due to the absence of cells able to induce their deletion. Cryosections of the thymus from wild type and mutant mice were stained with antibodies to EpCAM1 (G8.8, red) as well as with UEA-1 (blue). Images were acquired using a Zeiss Axiovert M200 microscope. Objective magnification, 10x/0.45 NA.

Figure 6

Impaired responsiveness of CCR7-deficient thymocytes to TCR stimulation in vitro. (A) Thymocytes of DO11.10/CCR7+/+ (+/+ ) and DO11.10/CCR7-/- (-/-) mice were co-incubated with either unloaded or ova-loaded DC. Cells were harvested after 18 hours of incubation and analyzed by flow cytometry. CCR7-deficient thymocytes were less responsive to TCR stimulation as indicated by the lower number of DP cells with decreased expression of CD4 and CD8 after stimulation. (B) Thymocytes of wild type (+/+ ) and CCR7-deficient (-/-) mice were incubated for either 12 or 24 hours in the absence (unstimulated) or in the presence (stimulated) of antibodies to CD3 and CD28. Similar expression levels of CD69 were observed in unstimulated thymocytes of both strains. Although TCR stimulation induces up-regulation of CD69 on thymocytes of both strains, mutant thymocytes respond more slowly and less efficiently (arrows). For A and B, pseudocolor density plots are gated on living thymocytes according to FSC/SSC profile, numbers indicate the percentage of cells in the specified regions. (C) Impaired responsiveness of CCR7-deficient thymocytes to CD3/CD28 stimulation is also reflected by delayed kinetic of CD5 up-regulation and TCR down-regulation. Dotted lines (unstimulated), continuous line (stimulated). Data shown are representative of three independent experiments.

Figure 7

Impaired calcium flux of CCR7-deficient thymocytes in response to CD3-ligation by cross-linking antibodies. (A) Wild type (+/+ ) and CCR7-deficient (-/-) mice harbor comparable numbers of CD4+ SP and CD4+CD8+ DP thymocytes. Pseudocolor density plots are gated on living thymocytes according to FSC/SSC profile. Numbers indicate the percentage of cells in the specified regions. (B) Impaired calcium flux of CCR7-deficient thymocytes in response to CD3-ligation by cross-linking antibodies. Pseudocolor density plots are gated on CD4+ SP or CD4+CD8+ DP thymocytes according to the gates depicted in (A). Black arrows indicate addition of CD3 and cross-linking antibodies. CCR7-deficient (-/-)
CD4 SP thymocytes show reduced calcium flux in comparison to wild type (+/+) as indicated by the red arrows. Note that a large fraction of the CCR7-deficient DP population remains mostly unresponsive (red arrow heads). Line graphs show median values of Fluo-4/Fura Red-ratio for wild type (red) and CCR7-deficient thymocytes (blue). Data shown is representative of three independent experiments.
Figure 1

A  gate on all cells

+/-

CD8

/-

PBS  anti-CD3

CD4

B  gate on CD4^+CD8^- cells

+/-

CD8

/-

PBS  anti-CD3

CD4

CD24

C  gate on all CD45.2^+ cells (-/-)

PBS  anti-CD3

CD8

CD4

CD24

gate on CD4^+CD8^-CD45.2^+ cells (-/-)

PBS  anti-CD3

CD4

CD24
Figure 5

A

% of vβ3+ cells

CD4 | CD8 | DP

% of vβ11+ cells

CD4 | CD8 | DP

% of vβ12+ cells

CD4 | CD8 | DP

B

% of vβ3+ cells

CD4 | CD8

% of vβ11+ cells

CD4 | CD8

C

+/+

-/-

Images showing cellular distributions and statistical comparisons.
Impaired responsiveness to TCR stimulation and defective negative selection of thymocytes in CCR7-deficient mice

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