Identification of Clec4b as a novel regulator of bystander activation of auto-reactive T cells and autoimmune disease

Liselotte Bäckdahl1*, Mike Aoun1*, Ulrika Norin1, Rikard Holmdahl1,2*

1 Medical Inflammation Research, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden, 2 The Second Affiliated Hospital of Xi’an Jiaotong University (Xibei Hospital), Xi’an, China

* These authors contributed equally to this work.
* rikard.holmdahl@ki.se

Abstract

The control of chronic inflammation is dependent on the possibility of limiting bystander activation of autoreactive and potentially pathogenic T cells. We have identified a non-sense loss of function single nucleotide polymorphism in the C-type lectin receptor, Clec4b, and have shown that it controls chronic autoimmune arthritis in rat models of rheumatoid arthritis. Clec4b is specifically expressed in CD4+ myeloid cells, mainly classical dendritic cells (DCs), and is defined by the markers CD4+/MHCIIhi/CD11b/c+. We found that Clec4b limited the activation of arthritogenic CD4+αβ T cells and the absence of Clec4b allowed development of arthritis already 5 days after adjuvant injection. Clec4b sufficient CD4+ myeloid dendritic cells successfully limited the arthritic T cell expansion immediately after activation both in vitro and in vivo. We conclude that Clec4b expressed on CD4+ myeloid dendritic cells regulate the expansion of auto-reactive and potentially pathogenic T cells during an immune response, demonstrating an early checkpoint control mechanism to avoid autoimmunity leading to chronic inflammation.

Author summary

To identify early disease regulatory mechanisms in autoimmune diseases such as rheumatoid arthritis (RA) is challenging not only because of the genetic and environmental complexity but also because of the critical autoimmune time-period that precedes the clinical diagnosis. Therefore, we set out to study the complex disease pathways in a more restricted setting. Through genetic segregation of rat crosses, followed by the selection of recombinants to produce minimal congenic strains, we have identified a single nucleotide polymorphism regulating the expression of Clec4b2 that in turn controls the development of arthritis. The Clec4b gene is normally expressed in a population of antigen-presenting cells that can limit enhanced activation of bystander autoreactive T cells during an immune-priming response. This previously unknown type of immune regulation reveals the existence of a mechanism protecting against autoimmune diseases by the avoidance of...
bystander activation of autoreactive T cells during a normal immune response to foreign antigen.

Introduction

A tissue-specific autoimmune disease process starts decades before the clinical onset of autoimmune diseases, such as rheumatoid arthritis (RA) [1]. Most likely the first trigger involves the activation of autoreactive T cells, which are normally regulatory or anergic, into a more aggressive state. The activation requires strong costimulation, which during an immune response is mediated by adjuvants carried by infectious organisms or possibly damaged endogenous cells, or environmental hazards such as tobacco smoke [2]. These challenges trigger the innate immune system, leading to the activation of autoreactive T cells. Innate immune cells interpret infectious intruders or danger signals via a range of pattern-recognizing receptors (PRRs) on their cell surfaces. When the innate cells sense enhanced risk in the environment, these cells are able to activate other cells, such as T cells. If the activation of adaptive responses displays joint specificity, the situation could initiate clinical arthritis. Animal models of arthritis mimic these disease stages [3]. They are initiated by adjuvant immunization followed by an autoimmune response to a tissue-specific protein. In the case of collagen-induced arthritis (CIA), it is the type II collagen (CII) that is involved and in the case of arthritis induced by various type of adjuvants, such as pristane-induced arthritis (PIA), or mineral-oil induced arthritis (OIA), a bystander response is raised to a pattern of unknown endogenous auto-antigens [4]. Clinical arthritis starts to develop 2 weeks after the injection, as a result of an inflammatory attack on peripheral cartilaginous joints, involving the autoimmune response, that later can develops into a chronic inflammatory disease.

To determine the basic mechanisms leading to an autoimmune disease we searched for the genetic polymorphisms that allow the development of disease in certain inbred strains. For our investigation, we selected a cross between the DA rat, which is highly susceptible to autoimmune diseases, and the disease resistant E3 rat strain. The rats were injected intra-dermally with pristane, a simple alkene adjuvant oil which triggers a disease that fulfils the classification criteria for RA [5]. Through genetic linkage mapping, we identified 20 arthritis-associated loci in the DA rat [6]. One of the major loci was localized to chromosome 4 and was denoted Pia7. The same locus was identified using several different arthritis models, including CIA, PIA and OIA [7–9]. With the use of recombinant congenic strains, the disease association could be mapped to a gene family encoding C-type lectin receptors, denoted hereafter as APLEC (antigen presenting cell expressing lectin like receptor gene complex) [10,11]. APLEC contains a cluster of 8 genes, 4 of which have a carbohydrate recognition domain highly similar to the full-length Clec4a/DCir and are thus denoted Clec4a1-3. The complex also covers 4 non-DCir like C-type lectins: Clec4b2b/Dcar, Clec4d/MCL, Clec4e/Mincle and pClecn/Dectin2 (a non-functional pseudo-gene in both the DA and E3 strains). It has been proposed that both Clec4a and Clec4b are important in antigen presentation and uptake [12,13] as well as in T cell responses [14] and can be assumed to be of importance early in an immune response. Furthermore, there is a genetic association to human DCIR in RA [15]. In contrast, Clec4d and Clec4e are involved in later inflammatory responses [16]. We have now located a single nucleotide polymorphism (SNP) in the Pia7 locus, regulating the Clec4b gene. Only E3 derived cells express the Clec4b/Dcar receptor and it is expressed on CD4+ Dendritic cells. The loss of Clec4b/Dcar1 expression leads to dysregulation of CD4+ myeloid antigen presenting cells, which lose the ability to control bystander activation of autoreactive T cells. This previously

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unknown physiological regulator of autoreactive T cells, are of critical importance to the development of autoimmune disease.

Results

A SNP within the Clec4b gene explain the arthritis-association

The DA.E3-Pia7 congenic strain is less susceptible to arthritis compared to the DA strain [11]. We chose to fine map the underlying polymorphisms within the Pia7 locus for association with arthritis using OIA, which gives a milder arthritis compared with PIA. In fact, the same locus as Pia7 has also been mapped in OIA and designated Oia2 [7]. We aimed to produce recombinant congenic strains to identify the experimental arthritis regulating polymorphism in Pia7. Whole-genome sequencing of both the DA and the E3 genomes was advantageous as it enabled us to identify at least 1 microsatellite marker/50 Kb within the APLEC region [6]. More than 5000 pups were screened for recombination events using a panel of 37 microsatellite markers in the original 1.5 Mb DA.E3-Pia7 interval. Two recombinant congenic strains were produced. The first identified sub-congenic strains, DA.E3-Clec4a contained arthritis protective E3 alleles only in the 4 Clec4a isoforms of the APLEC region where the recombination event occurred immediately after the Clec4a gene but before Clec4b2 between MSAplec710 and MSAplec740. The DA.E3-Clec4bde sub-congenic strain was the product of two separate recombination events, containing E3 alleles in Clec4a, Clec4b2, Clec4d, Clec4e, and the Clec4n/Dectin2, which is a pseudogene in both DA and E3 (Fig 1A). Limited on the centromeric side by a recombination between MSAplec573 and MSAplec633 and distally by a recombination event occurring right after Clec4e between the markers D4rat90 and MSAplec981.

OIA assessment in the DA.E3-Clec4a strain showed no protection within this region, contrary to the full DA.E3-Pia7 congenic strain (Fig 1C). The sub-congenic strain DA.E3-Clec4bde on the other hand showed almost complete protection against OIA, as well as to PIA and CIA (Fig 1C–1E). Sequencing the DA.E3-Clec4bde fragment identified two coding polymorphisms: a nonsense mutation in the DA strain that causes a truncated protein at exon two of Clec4b2; and a non-synonymous SNP in the membrane domain of Clec4e (Fig 1B). The mouse has two isoforms of Clec4b; Clec4b1 and Clec4b2. Since the rat only has the Clec4b2 homolog we refer to this gene as Clec4b. The Clec4d gene was excluded as a candidate since it does not contain any coding or regulatory polymorphisms and does not show any differences in gene expression between strains (Fig 1F). A comparison of the genome sequence with other inbred rat strains showed that the arthritis protective PVG and E3 share the same Clec4bde haplotype [6], making them distinct from the DA Clec4bde haplotype. The arthritis protective F344 strain, on the other hand, have a Clec4bde haplotype that is identical to DA. When trying to identify strains that deviate in their Clec4bde haplotype, only 1 strain was different. The BN strain shares alleles with DA only in the Clec4e locus (see Fig 1B), whereas the remaining sequence of the BN Clec4bde region is shared with E3, the arthritis protective haplotype. In a literature search to compare F2 crosses between DA and PVG, and between DA and BN strains, the arthritis quantitative trait locus at APLEC was reproduced in both crosses despite the arthritis susceptible version of Clec4e in BN. An APLEC arthritis QTL could not be reproduced in crosses between DA and the arthritis protective F344 strain, principally because F344 has the DA version of the full APLEC [9]. The inherent ability to reproduce linkage in an F2 cross between DA and BN provided sufficient evidence to us to conclude that arthritis regulation most probably stems from variations in Clec4b rather than Clec4e. We identified a causative nonsense SNP in the Clec4b DA allele that causes a truncation of the DA allelic protein that is nonfunctional but with expressed transcripts. Thus, the DA strain is a natural functional knock-out for Clec4b, which further substantiates the gene as the arthritis regulating gene in
Fig 1. Identification of the arthritis-regulating gene in the Oia2/Pia7/Cia13 locus on rat chromosome 4. a) An illustration of the distribution of the 8 C-type lectin genes across the APLEC interval on rat chromosome 4 and the congenic fragments with genomic regions harboring arthritis-susceptible DA alleles (grey) and arthritis-protective E3 alleles (black) for the three congenic strains DA.E3-Pia7 to the far right, DA.E3-Clec4a to the left of the chromosome and DA.E3-Clec4bde to the far left. b) An illustration of the distribution of SNPs across the DA.E3-Clec4bde congenic fragment and the Clec4bde encoded genes, together with an illustration of the two non-synonymous SNPs and nearby SNPs in five different inbred strains including the reference rat strain BN. c) OIA development in DA strain and the 3 congenic strains. d) PIA development over time in the original DA strain and DA.E3-Clec4bde congenic strain. e) CIA development over time for the DA strain and the DA.E3-Clec4bde strain. Significance stars illustrate Mann Whitney Test * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 and SEM. f) Gene expression of all APLEC encoded genes for DA and Clec4bE3 strains in naïve splenocytes. House-keeping gene is b2m and all genes are compared to one DA sample.

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the APLEC region. The two DA strains with different Clec4b alleles are hereafter denoted DA and DA.Clec4bE3, abbreviated as Clec4bE3 and occasionally "E3. To investigate if other non-coding variations from the fragment could be regulatory, expression of all APLEC encoded genes were measured in DA and Clec4bE3 in naive splenocytes. Only Clec4b showed differential expression (Fig 1F). All raw data to figures are available in the S1 Data file.

**Clec4b is expressed in steady-state/immature CD4⁺ myeloid-derived spleen cells**

In order to identify possible mechanisms by which Clec4b could regulate experimental arthritis we first aimed to identify which cell types express the Clec4b gene. We analysed gene expression in blood, lymph nodes, thymus, bone marrow and spleen (Fig 2A). The highest Clec4b expression was found in the spleen, but lower expression levels could also be observed in blood and lymph nodes. Bone marrow showed lower expression compared to spleen whereas
no expression could be detected in thymus. Clec4b gene-expression was significantly down regulated in DA spleen compared to the Clec4b<sup>E3</sup> strain.

Next, we aimed to identify which splenic cell population expresses Clec4b. First, we pooled three naïve Clec4b<sup>E3</sup> and three DA spleens and selected T cells and B cells using anti-pan-T and B cell labelled magnetic beads. The T and B cell negative flow-through was elected in two new magnetic labelled fractions, one for CD4<sup>+</sup> and another for CD8<sup>+</sup>. The CD4<sup>+</sup> flow-through was lastly also selected for CD11b/c<sup>+</sup> cells. Clec4b expression was low in all cell types tested except for CD4<sup>+</sup> T and B cell negative cells (Fig 2B). Then unpooled individual magnetic bead selected splenocyte samples were assessed, Clec4b expression was significantly enriched in cells from Clec4b<sup>E3</sup> compared to DA, and more than a tenfold increase in CD4<sup>+</sup> TCR<sup>+</sup> compared to the CD4<sup>+</sup> TCR<sup>−</sup> cells was observed (Fig 2C). More than a sixtyfold difference in Clec4b expression was confirmed between CD4<sup>+</sup> and CD4<sup>+</sup> non-T/B cells when sorted with a flow sorter. This showed Clec4b expression exclusively in CD4<sup>+</sup>αβTCR CD45RA<sup>−</sup> (a B cell-marker) cells, whereas CD4<sup>+</sup> TCR CD45RA<sup>+</sup> had close to undetectable levels of Clec4b (Fig 2D). Interestingly, plasmacytoid dendritic cells (pDCs) are known to be CD4<sup>+</sup> in the rat, and to determine if pDCs also express Clec4b we used a previously established rat pDC sorting protocol [17] followed by gene-expression assay. Lower levels of Clec4b were observed in pDCs, albeit without any strain difference (Fig 2D). A recent study by Daws et al [18] supports both of these findings, suggesting that rat Clec4b is highly expressed in CD4<sup>+</sup> myeloid cells but not in CD4<sup>+</sup> pDCs.

The fact that OIA is a rapid arthritis disease model, with an onset of clinical arthritis within 10–12 days of injection, suggests that disease regulating genes are already operating within the first few days of injection. In order to examine changes in the kinetic expression of Clec4b during early arthritis-induction, CD4<sup>+</sup> TCR<sup>+</sup> bead selected splenocytes were isolated from both naïve animals and at different time points after mineral oil injection. The highest levels of Clec4b expression were observed in naïve splenocytes. A daily decrease in gene-expression was shown until day 5 after injection, when Clec4b levels were close to undetectable (Fig 2E). This supports the argument that regulation of Clec4b expression is sensitive to rapid environmental changes, either transmitted by instant general immune responses to the oil injection. Alternatively, it indicates that the receptor is sensitive to the oil itself. The rapid downregulation of Clec4b after oil injection suggests that the C-type lectin receptor is expressed in naïve or steady-state DCs as a possible steady-state off-switch and that this signal could be important for accurate immune-activation.

**The CD4<sup>+</sup>Clec4b<sup>+</sup> cell population consists of two distinct subtypes, classical and granular/plasmacytoid dendritic cells**

The Clec4b gene is expressed in CD4<sup>+</sup> cells that are TCR<sup>+</sup>. CD4 is frequently expressed on pDCs in both rats and humans, [17,19]. It has been suggested that CD4 is expressed on a sub-population of conventional dendritic cells, cDC2 [20] but this population is poorly characterized in the rat. Rat CD4<sup>+</sup> DCs have been described as being better regulators of the MHCI<sup>+</sup> Th1 adaptive responses than CD4<sup>+</sup> DCs [21], therefore, we wanted to investigate the phenotype and function of the CD4<sup>+</sup> TCR<sup>+</sup> DC population. The CD4<sup>+</sup> Clec4b<sup>+</sup> population was selected from naïve splenocytes with magnetic labelled pan T cell microbeads and, in the second step, with beads isolated TCR CD4<sup>+</sup> cells. The selected cells were then labelled with fluorescent antibodies and studied in a flow cytometer.

The yield from 100 x10<sup>6</sup> splenocytes was 3.0 x 10<sup>6</sup> ± 0.3 CD4<sup>+</sup> TCR<sup>+</sup> cells and 26.1x 10<sup>6</sup> ± 3.6 CD4<sup>+</sup> TCR<sup>+</sup>. More than 80% of the CD4<sup>+</sup> selected fraction expressed CD4 and had less than 2% T cells (Fig 3A and 3B). Within the CD4<sup>+</sup> selected cell fraction, CD11b/c and the granulocyte
Fig 3. Clec4b is expressed in CD4+ MHCII+ DCs in two distinct sub-populations that are either MHCIIhi classical dendritic cells or MHCIIdim pDCs. a) Frequencies of fluorescence labeled cells (n = 8 for all groups), first selected to be TCR-, and after a second sorting separated into either CD4+ or CD4- fractions using a CD4 antibody coated beads. NK cells are CD161hi, Neutrophils are His48+ and CD11b/c+, pDC are CD4+ and (CD45R) His24+, B cells are CD45RA+ (OX33), CD11b/c cells are OX42+ and His48 cells are granulocytes. b-d) Antibody labeled cell frequencies for CD4+ and CD4- fractions in the total mononuclear cell population. b) Shows CD4 and TCR (clone R73) expression. c) Expression of CD11b/c and His48. d) Illustrate MHCII+ expression (clone OX17) separated by cellular size by the forward scatter, and the three populations identified are also

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**Identification of Clec4b as a novel regulator of bystander activation of auto-reactive T cells**

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marker His48 were expressed in noticeably differently patterns in different sub-populations (Fig 3C). When the expression of major histocompatibility complex type II molecules (MHCII) was analysed in combination with forward scatter (FSC), three distinct populations were detectable, each representing a third of the total CD4+ pan T population (Fig 3D). One population expressed high levels of MHCII, a second population expressed intermediate levels of MHCII, and a third population was found negative for MHCII. The MHCIIbright population showed classical dendritic cell characteristics, such as high CD11b/c and CD172 (Sirpα) expression and negative for the granulocyte marker His48 (Fig 3E). The MHCIIdim population, on the other hand, was granulocyte-like with very high expression of His48. The population also expressed His24, one of the markers for pDCs, here defined by CD45RA /TCR /His24+/ CD4+ expression [17]. The MHCII− population was more monocyte-like, expressing CD68 and CD11b/c at levels comparable to the MHCII− population in CD4+ TCR−, S1 Fig. iNOS expression was elevated in both MHC expressing populations (Fig 3E), from which one may infer that reactive nitrogen species are involved in shared regulatory functions. In conclusion, the Clec4b expressing CD4+ cells were shown to include two unique populations. The first is a dendritic cell-like population corresponding to the CD4+ classical DC subset that has been previously described in the rat spleen [22], and which are similar to human cDC2. A second granulocyte-like population, which was undetected in the CD4+/Clec4b− fraction and showed lower MHCII expression, corresponded to the pDC population. Since the pDC population was shown to express very low levels of Clec4b (see Fig 2D), we conclude that the CD11b/c+ DC population should be the main Clec4b expressing CD4+ population in the spleen. The production of a monoclonal antibody for rat Clec4b is described in recently published data from Daws et al. [18]. They also detailed how Clec4b is expressed mainly on CD4+ DC/monocytes [18]. We found that Clec4b was only expressed in Clec4bE3 CD4+DCs but not in B or T cells (Fig 3F). Comparing cellular expression of the Clec4b receptor showed significant differential expression between DA and DA congeneric rats, expressing Clec4bE3 (Fig 3G).

**Clec4b deficient DA rats showed enhanced expansion of CD4+DCs at day 3 after oil injection.**

With the intention of identifying how the non-sense mutations in the Clec4b gene affect the cellular immune response during the early phase of experimental arthritis, splenocytes were harvested from the first days after oil-injection and then studied by flow cytometry. Assessing the cellular distribution in naïve spleens did not show any differences in the composition of cell sub-set populations between DA and Clec4bE3 rats, S2 Fig. However, three days after intradermal injection of arthritogenic oil we observed an increase of CD4+ myeloid cells in DA spleens, as measured ex vivo (Fig 4A). Furthermore, the MHCII expressing subset of CD4+ DCs had expanded after activation and the difference between the two genotypes was more marked, discernible in the FACS plots (Fig 4B). After day three, MHCII expression was two-fold in DA compared to Clec4bE3, and DA populations also showed enriched expansion of the adhesion molecule CD11b (Fig 4C). On day three no differences were seen for either T or B
cell subsets in the spleen, S3 Fig. Populations of antigen-presenting cells were generally more activated in DA rats, so that there were more CD4+$\text{MHCII}^+$ and CD80$^+$ cells (Fig 4D). However, neither pDC nor monocyte populations expanded differentially between DA or Clec4bE3 during the first days of activation. Kinetic dissection of the early cellular responses to injection of an arthritogenic oil further supports dendritic cells as the cells initially involved in the Clec4b mediated responses and hence the arthritis development control.
Aiming to delineate the expression differences in the CD4\(^+\) DCs, after oil injection, a set of DC-activation associated genes was compared by quantitative PCR between naïve cells and cells harvested three days after oil injection. CD4\(^+\) DCs at day three had increased gene expression of *IL12p40*, *Ifna*, CD4\(^+\)DC activation marker *Ifr4*, and the pro-inflammatory regulator *Inos* suggesting an enhanced activated phenotype at day three. A comparison of DA to *Clec4b\(^{E3}\)* expression showed significantly higher expression of both *Inos*, and *Ifr4* in DA. Furthermore, there was already a significant difference in *Stat1* expression between DA and *Clec4b\(^{E3}\)* in the naïve CD4\(^+\) myeloid cell population (Fig 4E).

In summary, the DA CD4\(^+\) DC population expanded at day 3 compared to rats with *Clec4b\(^{E3}\)*, displaying a distinct pro-inflammatory expression profile that promoted up-regulation and expansion of MHCII and adhesion markers such as CD11b. Thus, *Clec4b* deficiency lowers the threshold for classical CD4\(^+\) DC activation and MHCII expression.

**Clec4b regulates activation of pro-inflammatory T cells before day five of OIA pathogenesis**

Adjuvant induced arthritis models in the rat, such as PIA and OIA, are mediated by arthritogenic auto-reactive T cells activated a few days after adjuvant injection (see Fig 1C and 1D). Arthritogenic T cells can be isolated in draining lymph nodes as early as day 7 whereas the disease onset occurs around day 12 after adjuvant injection [23]. To investigate the *Clec4b*-mediated regulation of auto-reactive T cells during OIA development and to identify the earliest time point by which T cells can transfer disease, we transferred bead selected T cells (98% purity) from rats primed with mineral oil IFA into naïve recipient rats. Sufficient T cell activation for mild joint swelling was seen already at day 5 after oil injection (Fig 5A), whereas T cells from day 6 induced severe arthritis. Importantly, spleen T cells from DA but not DA. *Clec4b\(^{E3}\)* induced arthritis in naïve DA recipient rats. However, the presence of *Clec4b* in the recipient was irrelevant. As *Clec4b* is expressed in DC and not on T cells, these data demonstrate that *Clec4b* suppress T cell activation before day 5 during the immune response. Moreover, the absence of T cell regulation in *Clec4b* sufficient recipient rats shows that this early regulatory effect was absent after the T cell activation has advanced to autoreactive. T cells from draining lymph nodes transfer arthritis similarly to T cells from spleen (Fig 5B).

Since the T cells isolated at day 5 after oil injection were already arthritogenic, we sought to assess the *in vivo* primed T cells from day 0 by flow cytometry. As T cells from both sources transfer arthritis T cells [23], subsets and activation markers were analyzed from both joint draining lymph nodes and splenocytes. Surprisingly, no expansion of CD4\(^+\)CD25\(^+\) T cells was observed within the first 6 days after arthritis induction (Fig 5C). However, a dramatic decrease of CD62L\(^-\)CD4\(^+\) T cells among all live cells was seen in the spleen, from 15% to almost undetectable numbers at day two. This dramatic reduction of naïve T cells did not differ between the genotypes (Fig 5C) but provides a clue that this population is downregulated immediately only hours after oil injection.

**Co-cultures of CD4\(^+\) DCs and T cells identifies differential limiting effects on naïve T cell already 24hrs after stimulation**

The CD4\(^+\)DC subset is able to efficiently present antigen to T cells, and induce T cell activation [21,24]. Since no clear population differences between DA and *Clec4b\(^{E3}\)* rats could be identified for T cells *in vivo* within the first six days after disease induction, T cell activation was evaluated during *in vitro* stimulation where proliferative responses can also be studied. Co-cultures were made to assess the CD4\(^+\)DC regulatory function on T cell proliferation by labeling bead- selected T cells with CFSE. Moreover CD4\(^+\)DC have been shown to be superior in
Identification of Clec4b as a novel regulator of bystander activation of auto-reactive T cells

A

B

C

Spleen

DLN

Spleen

DLN

CD25+ CD4 T cells

CD62L+ CD4 T cells

D5 d.

D6 DA to DA r.

D6 DA to *E3 r.

D6 *E3 to DA r.

D5 DA to DA r.

D5 DA to *E3 r.

D5 *E3 to DA r.

Arthritis score (0-60)

days after transfer

Freq. in total

Days

Freq. in total

Days

Freq. in total

Days

Freq. in total

Days

Freq. in total

Days

Freq. in total

Days

Freq. in total

Days

Freq. in total

Days
stimulation of allogeneic T cell responses in the rat compared to CD4 DCs. We harvested allogeneic T cells from rats with the MHC haplotype RT1<sup>a</sup>, which differ from RT1<sup>a</sup> in the DA rat, from both naïve rats and day three after IFA injection. Syngeneic DA T cells were also harvested from day three after IFA, from both Cle4<sup>b</sup><sup>E3</sup> and DA rats. The different T cells were co-cultured with bead-selected CD4<sup>+</sup>DCs from either DA or Cle4<sup>b</sup><sup>E3</sup> in all combinations. Naïve allogeneic T cells were co-cultured with naïve CD4<sup>+</sup>DCs, and the day three derived T cells, either allogeneic or syngeneic, were all co-cultured with day three in vivo derived CD4<sup>+</sup>DCs. Since arthritogenic T cells in the rat have been characterized as activated CD4<sup>+</sup> T cells, proliferation was assessed in CD25<sup>+</sup> CD4<sup>+</sup> T cells [23]. Proliferation was near 100% for both naïve and OIA day three harvested allogeneic T cells however no difference in DC activation could be seen from Cle4<sup>b</sup>. This implies that Cle4<sup>b</sup> regulation on arthritogenic T cell development operates independent of MHCII. The CD4<sup>+</sup> DC mediated T regulation occurs, most likely, by limiting bystander activation via cytokine secretion. Interestingly, syngeneic Cle4<sup>b</sup><sup>E3</sup> derived T cells showed dramatically lower proliferation compared to DA. Since no effect could be seen for the two CD4<sup>+</sup>DCs populations in the co-cultures, the restricted proliferation seen for Cle4<sup>b</sup><sup>E3</sup> derived T cells must be due to regulatory differences during the first days in vivo rather than in the in vitro culture (Fig 6A and 6B).

In order to find an appropriate stimulatory agent triggering down-regulation of Cle4<sup>b</sup> expression, known pro-inflammatory compounds (ConA, LPS, mannan, thioldimycholate and CpGs) were incubated with freshly isolated naïve spleen cells. Remarkably, there was down-regulation of Cle4<sup>b</sup> expression already after 24h of ConA stimulation (Fig 6C). However, there was no difference in viability of DCs after Con A between the two strains. Overnight ConA cultures activated naïve CD4<sup>+</sup>DCs cells to become strong inducers of T cell proliferation compared with CD4 DCs cells (Fig 6D). This ability to induce proliferation of CD4<sup>+</sup>CD25<sup>+</sup> T cells was unique to DA derived CD4<sup>+</sup> DCs (Fig 6D).

Endeavoring to assess the restraining ability on T cell activation/proliferation from Cle4<sup>b</sup><sup>E3</sup> derived DCs, two sets of DC:T cell co-cultures were prepared. Naïve Con A overnight activated CD4<sup>+</sup>DCs from DA and Cle4<sup>b</sup><sup>E3</sup> were combined with naïve T cells from either DA or Cle4<sup>b</sup><sup>E3</sup>. Overnight ConA stimulation of CD4<sup>+</sup>DCs enabled the DCs from Cle4<sup>b</sup><sup>E3</sup> derived rats to limit proliferation of CD25<sup>+</sup>CD4<sup>+</sup> T cells from either DA and Cle4<sup>b</sup><sup>E3</sup> equally efficiently after 72 hours of culture (Fig 6E).

In summary, Cle4<sup>b</sup><sup>E3</sup> DCs from day 3 after oil injection failed to limit T cell activation in co-cultures with day 3 activated T cells, and T cell activation status correlated with the genotype of its origin (Fig 6B). Naïve Cle4<sup>b</sup><sup>E3</sup> DCs, on the other hand, that had been Con A stimulated before co-culture with naïve T cells were able to inhibit activation and proliferation of T cells independent of the T cells Cle4<sup>b</sup> genotypes (Fig 6E). These data reveal that the arthritis regulating gene Cle4<sup>b</sup> expressed in CD4<sup>+</sup> DCs can efficiently limit T cell activation in response to adjuvant/PRR stimulation, thus preventing the expansion of recently activated auto-reactive and potentially pathogenic T cells.
Fig 6. *Clec4b* regulates CD4+ myeloid cell mediated suppression of naive T cells in mixed lymphocyte reactions. 

a) Illustration of the gating for activated T cells, by first gating for live cells, then T cells, and CD4+CD25+ cells followed by proliferation as carboxyfluorescein succinimidyl ester (CFSE) low. And an illustration of CFSE histogram for two samples. b) Graph describes proliferation as CFSE low labeled CD4+CD25+ T cells from a) for Naive or OIA Day 3 activated allogenic T cells and OIA Day 3 activated syngenic DA or *Clec4b* E3 T cells. The T cells have been co-cultured with OIA day 3 *in vivo* activated DCs from both genotypes, or cultured with naive DCs from both genotypes (only for naive allogenic T cells). c) *In vitro* stimulated *Clec4b* gene expression in CD4+
TCR- spleen cells, after 24 h culture with either Con A, TDM, mann, CpG, LPS, complete media only (n = 5 per group) Mann. Whitney Test ** = p<0.01 SEM. d) Comparisons of T cell subset in co-cultures mixing naïve DA T cells, cultured with Con A stimulated selected DCs, either CD4+DCs or CD4-DCs from DA or Clec4bE3. Frequencies of total cells are displayed for the co-culture at different DC:T cell ratios explained in 3 graphs; total CD4+ cells; total CD25+ CD4+ T cells; and proliferating (CFSE low) CD25+ CD4+ T cells. (n = 10 for all groups) Mann Whitney Test ** = p<0.05, *** = p<0.01, **** = p<0.001 and SEM. e) Describes two four-way grouped co-culture experiments comparing the proliferative regulation from either DA or E3 (Clec4bE3) CD4+ DCs on bead selected CFSE labeled T cells from both rat strains and mixed in combinations in co-cultures for 72 h. n = 10 for all groups. 3 Graphs illustrate proliferative T cell subsets: total proliferating T cells, total CD4+CD25+ T cells and the frequencies of proliferating CD4+CD25+ T cells in all cells. Graphs describes regulation of proliferation on recently harvested naïve T cells by in vitro Con A activated CD4+DCs in different T cell to DC ratios/concentrations. Mann. Whitney Test * = p<0.05, ** = p<0.01, *** = p<0.001 and SEM.

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Clec4b in recipient naïve DCs regulates T cell proliferation on day four activated T cells in vivo after challenge by innate stimulus and down-regulation of IL17 production

Given the fact that only DA.Clec4bE3 and not DA derived T cells are prevented from the development into arthritogenic T cells after an intradermal injection of mineral oil, we propose that Clec4b should be able to allow DA donor T cell limiting responses in vivo after a second adjuvant injection in naïve recipients from the arthritis transfer model, as described in Fig 5A and 5B. Since T cells have already become arthritogenic 6 days after arthritogenic oil injection, we investigated the activation and proliferation status of T cells transferred earlier than this time point. CFSE' labeled T cells harvested from day 3 after oil primed DA rats were transferred to naïve DA.Clec4bE3 or DA rats. After transferring the primed T cells, the recipient was also challenged by an intradermal oil-injection to trigger the CD4+DCs. After 3 days in the recipients, the spleens were harvested and more than 30 million splenocytes were analyzed per sample by flow cytometer. 3 distinct population of T cells could be visualized in recipient spleens: an unlabeled population (CFSE'); a proliferative population (CFSElow); and a non-proliferating population (CFSEhigh) (Fig 7A). Whilst the non-proliferating CFSEhigh population corresponds to the main part of the donor T cell population, a considerable part of the donor CD4+ T cells (i.e. the CFSElow population) were proliferating. Intriguingly, all cells in the CFSElow subset were CD4+T cells, which corresponds well with previous findings from the Transfer arthritis model that has described the arthritogenic T cells as mainly CD4+[23]. Since these donor T cells most likely have been exposed to a form of CD4+DC bystander activation of abundantly polyclonal antigen specificity, there will have been no definite starting point for the T cell proliferation. Thus, the CFSE density could not be visualized into distinct cell divisions. Therefore, we used a second proliferation marker (Ki67) to assess proliferation within the CFSE categories. Further analysis of donor T cells in the Clec4bE3 recipients revealed a significant regulation of the proliferating CD4+ T cells in terms of both cell expansion and activation (both lower Ki67 and MHCII expression) (Fig 7B and 7C). Conversely, no difference in activation and proliferation markers was detected in non-dividing (CFSEhigh) and endogenous T cells (CFSE') between both congenic rats. Furthermore, the effects of polyclonal stimulation of transferred cells indicate a role of Clec4b in modulating IL-17 producing T cells on the basis that a reduction in IL-17+ dividing T cells frequency was found in Clec4bE3 compared to DA rats (Fig 7D and 7E). However, no changes in the frequency of IFNγ+ T cells was detected. Notably, CFSEhigh population failed to produce both IL-17 and IFNγ while endogenous T cells (CFSE-) produced IFNγ only. These findings corroborate the role of Clec4b in limiting T cell activation and proliferation in vivo. They also infer that Clec4b plays a part in controlling Th17 cells that together account for a substantial proportion of the DA susceptibility to adjuvant triggers that lead to arthritis development in the rat.
Fig 7. Clec4b regulate T cell proliferation also in vivo and the activation correlate with down-regulation of IL17 production. DA rats were injected intradermally with oil and 4 days later the spleens were used as a source of oil primed T cells. These were CFSE labeled and injected intravenously into naïve DA and Clec4b$^{E3}$ recipients that had been injected intradermally with mineral oil the same day. a) Proliferation FACS plot describing CFSE intensity in CD4 versus CD8 T cells harvested from spleens of the recipients day 3 after oil injection. CFSE negative T cells are the recipient endogenous T cells. The CFSE$^{high}$ are the non-proliferating T cells from the donors and the CFSE$^{low}$ are the donor T cells that have been proliferating in the recipient. b) Histogram over the distribution of the three groups from a and how they differ in the expression of both Ki67 and MHCII on their surface. c) Graphs describing how the two donor groups, non-dividing and dividing T cells, differ in the expression of MHCII and Ki67 between DA and Clec4b$^{E3}$ recipients. d,e) Illustration on how the expression of the two cytokines IFNg and IL17 is distributed between the three groups of differentially proliferating T cells between the Clec4b sufficient and deficient recipient rats. d) Illustrative FACS plot. e) Statistics analysis and graph. n = 4 for all groups, Mann Whitney Test $^*$ = p<0.05, $^{**}$ = p<0.01, $^{***}$ = p<0.001 and SEM.

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Discussion

The identification of a single nucleotide polymorphism controlling the expression of Clec4b in the rat highlights a new role of Clec4b in regulation of autoreactive T cell activation. The suggested identification of the Clec4b SNP to be causative was based different type of evidence. Firstly, it was found within a 200kb chromosomal interval defining the QTL Pia7. This genomic region harbors three protein coding genes, whereof two had non-synonymous SNPs. Of these, only the SNP in Clec4b showed functional evidence as it led to differential gene expression in naïve rats. Secondly, data from several genetic mapping studies aiming to identify arthritis susceptibility regulating loci in the rat, shows that only the DA version of Clec4b segregates with disease susceptibility, and not strains lacking the Clec4b SNP but maintaining the polymorphism outside the Clec4b locus. Thirdly, only the DA Clec4b.E3 congenic, and not the DA rat, expresses the Clec4b protein. More specifically, Clec4b is highly expressed on CD4 + DCs, a population known to be regulating MHCII interaction and T cell activation. Interestingly after an intradermal oil injection, the DA strain displays a rapid onset of autoreactive T cells whereas Clec4b.E3 rats have normal control of T cell activation after oil injection. Clec4b is a C-type lectin receptor, expressed in classical CD4+DCs, and we now find that it is essential for the control of bystander activation of autoreactive T cells during immune priming. Deficient expression of Clec4b allows the escape and expansion of autoreactive T cells leading to arthritis.

T cells from a rat strain deficient in Clec4b expression are able to transfer arthritis to naïve rats already 5 days after being activated by adjuvants. Two-photon imaging of DC T cell interactions in vivo has revealed that, after adjuvant stimulation, T cells quickly attach to the activated DCs via interaction with peptide-loaded MHCII that then leads to T cell viability decisions within the first 12 hours [25]. Our data suggest that Clec4b is a regulator of these viability decisions and thereby helps to prevent T cells from responding to self-peptides that are already present on classical DCs and vulnerable to activation. It is suggested that immature/ steady state DCs have a tolerogenic function during steady-state [26]. During pattern recognition, receptor-mediated activation of DCs will occur at a critical time point when these self-presenting DCs need to differentiate or die in order to prevent development of autoimmunity [27]. We suggest that Clec4b function is a control switch for the tolerogenic DCs to either transform or die during the short interval when the DCs have received secondary inflammatory signals, yet are still capable of activating T cells directed to an immunogen that are, in most cases, derived form a pathogenic intruder. We have identified two triggers of Clec4b, mineral oil and Con A, which will activate DCs lacking Clec4b expression, leading to uncontrolled T cell proliferation. The presence of the lectin receptor Clec4b/Dcar on the DC surface cause a more controlled activation of DC with Clec4bE3, compared with DC with Clec4bDA, inducing less expression of proinflammatory genes such as Stat1, Irf4 and iNos, that could limit bystander T cell activation.

Importantly, mature T cells that possess a variable degree of self-reactivity and have escaped negative selection—through low avidity or by the absence of the relevant tissue antigens during their selection—can potentially cause autoimmune disease [28,29]. During the first priming immune response there are multiple mechanisms leading to activation of T cells with high affinity to peptides derived from the invading pathogen or immunogen. In the same time period, however, other cells with variable degree of self-reactivity need to be suppressed. We have now identified a way in which such autoreactive T cells can be suppressed by dendritic cells expressing Clec4b during a limited period during the immune priming. If Clec4b is not functioning properly, autoreactive T cells could be activated that could be pathogenic and cause an autoimmune arthritis that mimics rheumatoid arthritis.
The CD4+ DCs population in the rat spleen have previously been described to be highly efficient inducers of MHCII restricted T cell activation and to express enhanced levels of co-stimulatory receptors [22]. These also express PRRs on their surface [21]. A possible mouse counterpart to the rat CD4+ DC is CD11b+CD8a- DCs, which have been described as a mediator of peripheral tolerance via MHCII [30]. Targeting antigen bound antibodies to a steady-state associated C-type lectin receptor DEC-205 without antigen can induce tolerance in mice [31, 32]. In the mouse, there are two isoforms of Clec4b and of these Clec4b2 could have a different function as it is expressed on CD8+ DC and involved with cross-presentation of MHCII of peptides circulating in the early endosome [13]. Instead, the less homologous isotype Clec4b1 (DCAR2) could be functionally more similar to rat Clec4b as it is co-expressed with Clec4a4 (DCIR2) [33] on mouse CD8+CD11b+ DCs, the mouse splenic DC subset analogous to the rat CD4+ DCs. Clec4a4 was recently identified as a mediator of inflammation and MHCII:T cell immunity because both its function and the DC specificity show similarities with the identified function of rat Clec4b [14,34]. However the proportion of CD4+ and CD8+ dendritic cell populations differ between rats and mice where rat DCs better resembles human DC subsets [35]. However, rat Clec4b most likely does not have a completely overlapping set of functions with human CLEC4C, since the latter is a human pDC bio-marker [36,37] and Clec4b fails to show significant expression in rat pDCs.

Clec4b expressing CD4+ DCs plays an important role in limiting the expansion of autoreactive T cells during an immune response and, in particular, when the immune response is triggered in the absence of strong antigens. This new role of antigen-presenting DCs is of fundamental importance in understanding autoimmune diseases but may also have important implications for current explanations of the lack of autoreactive T cell responses in growing tumors.

Methods
Selective breeding of congenic animals
DA/ZtmRhd (DA.RT1Av1) and E3/ZtmRhd (E3) were originally obtained from Zentralinstitut fur Versuchstierkunde (Hannover, Germany) but maintained as inbred strains in our laboratory for more than 20 generations. The original 2.3Mb DA.E3-Pia4 congenic strain was produced and back-crossed to DA for at least 16 generations[11]. Two recombinant congenic strains were produced by marker-assisted selective breeding from back-crossed DA.E3-Pia4 heterozygotic parents in an F2 inter-cross. During the process of producing the recombinant strains the congenic strains were back-crossed to DA three more times. The DA.E3-Clec4a recombinant was obtained after 567 meiosis and occurred between MSaplec710 and MSaplec740 (which is downstream of Clec4a before Clec4b). DA.E3-Clec4bde was produced from intercrossed DA.E3-Pia4 in two steps. The first recombination occurred between MSaplec573 and MSaplec633 upstream of Clec4a and happened after 1723 meiosis. The second recombination in the DA.E3-Clec4bde fragment occurred after 4831 meiosis between D4Rat90 and MSaplec1003 right after the Clec4e gene. The congenic fragment DA.E3-Clec4bde is a 340 kb congeneric fragment between 164.61 Mb to 164.95 Mb on chromosome 4 conformed to the Baylor Rn4 2004 assembly and is illustrated in the schematic in Fig 1A. Markers for the production of the two subcongenic strains were: From the distal top D4MIR55F GTGAAAGTTTGG GGTGTATT, D4MIR55R GGTCAGCCGCTTTTCT, D4WOX49F TGTACTCAGACCC ATGTA, D4WOX49R GCAAGCACGCGACACTAC, MSaplec573F ACCAGTAAACACC ATCATTG, MSaplec573R CACTATTGGAAAAAGGCACAA, MSaplec637F CACCTCCCA GACCAAAGTT, MSaplec637F AGGTACAATGGCACACCC, MSaplec710F CTTCT ATTACTAATATCCTTGCCATA, MSaplec710R GGATCAAACTGGCCAGATAC,
MSaplec740F AGTAATGCCCATGTGAAAA MSaplec740R GACCTGCATGCACATGAA
TA D4RA190F CTCAGCATGCTCGCCACT, D4RA190R TGTATTAGACACTGTCTTA
GTGAA, MSaplec981F TGCACCTGAGCATGTAGTGAT, MSaplec981R CCTGAACAG
TCCTGACATAAACC.

Allogenic rats were congenic DA.RT1u. Animals were maintained at the Scheele Laboratory, in the Karolinska Institutet, in a pathogen-free environment according to the Federation of European Animal Laboratory Science Association guidelines (FELASA). All procedures and animal experiments have been approved by the local ethics committee (Stockholms djurför- söksetiska näm). The animals were maintained in a 14h light and 10h dark cycles and held individually in cages containing wood shavings and aspen. Moreover, they had free access to water and were fed with the standard rodent chow.

**Arthritis experiment**

Arthritis was elicited by a single intradermal injection at the dorsal side of the tail base. For PIA induction, 150 μl pristane (2,6,10,14-tetramethylpentadecane, 95%; Acros Organics, Morris Plains, NJ, USA) was injected. For induction of OIA, 200 μl mineral oil (incomplete Freund’s adjuvant /IFA/, BD, Franklin Lakes, NJ, USA) was injected. For induction of CIA, rat collagen type II (CII), dissolved in 0.1M acetic acid and emulsified in IFA, was injected. All used rats were >8 weeks old. All experiments were performed with littermate rats that were age-matched, distributed within the cages and blind evaluated by the investigator. The rats were regularly inspected, including monitoring the limbs for arthritis development by a previously described macroscopic scoring systems. Briefly, 1 point was given for each individual swollen and erythematic toe and up to 5 points for an inflamed ankle (15 points in total per paw) [38,39]. The scoring was carried out every second or third day for 20–30 days after disease induction. No points were given to deformed paws that did not exhibit signs of inflammation.

For transfer OIA, we harvested spleens or inguinal lymph nodes. Concisely, at day 6 or day 5 post injection organs were made in to single cell suspension. T cells were positively selected using magnetic CD6 Macs beads. Cells were then culture for 56 hours in complete media and stimulated with 10 μg/ml αTCR (R73) and 5 μg/ml αCD28 (JJ319) and later injected into recipient naïve rats. Cells from DA or Clec4bE3 were injected into DA or Clec4bE3 recipients and arthritis development was monitored using a 0–60 macroscopic scoring system. All experiments follow established guidelines involving littermate controls, mixing of groups in cages and blinded scoring evaluation.

**Cell preparation and immuno-sorting**

Splenocytes were harvested from naïve spleens 1–5 days after oil injection. Single cell suspensions were prepared as follows. Inguinal joint draining LNs and spleens were harvested from euthanized rats and digested for 30 min at 37˚C with 1 ml digestion buffer (1 mg/ml collagenase IV, Sigma-Aldrich, St. Louis, MO, USA) in DMEM media (Sigma-Aldrich). Spleens were passed through 40-μm cell strainers and washed twice in sterile PBS. Red blood cells were lysed by adding 2 ml of ACK buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA) to the pellet. After incubation for 3 mins at 4–8˚C, lysis reaction was stopped by adding cold PBS, centrifuged (8 min, 300g) and washed in ice-cold PBS.

Immuno-precipitation of samples in single cell suspensions was performed according to the manufacturer’s protocol (Miltenyi biotec. Gladbach, Germany). T cells were selected using OX52 (CD6) Macs beads. T cell negative fractions were harvested from the flow-through. The T cell-purity was never less than 97%, the non-T cell flow-through had less than 3%
contaminant T cells. The non-T cells flow-through was used for sorting the CD4+ non-T cell fraction using Macs CD4+ beads (coated with OX38). CD8+ or CD11b/c+ non-T cells were selected by using streptavidin-coated Macs beads coupled to biotin conjugated anti-CD8 antibodies (clone OX8, BD Franklin Lakes, NJ, USA) or CD11b/c+ (clone OX42, Biolegend San Diego, CA, USA). B cells were selected from single cell suspensions using CD45RA coated Macs beads (Clone OX33). Neutrophils were selected from EDTA-treated whole blood. First from this selection were PBMCs isolated by 6% dextran and centrifugation, followed by neutrophils isolated using Ficoll Hypaque, according to Current Protocols in Immunology 7.23.1.

Gene expression
RNA was extracted from -80C snap frozen tissue samples using RNAeasy (Qiagen, Hilden Germany). cDNA was prepared by reverse transcriptase (iScript Bio-Rad Hercules, CA, USA) and qPCR amplification was performed using q-SYBR green supermix (Bio-Rad Hercules, CA, USA). To eliminate potential splicing effects two different primer sets that cover different regions of the Clec4b gene were used in the tissue and cell type-characterizing experiment. The amplified sequence was cloned and sequenced for both primer sets and both sets were verified to have amplified Clec4b specific sequences and neither of the highly similar other APLEC encoded receptors. All other genes show gene expression from 1 primer set amplicon.

Clec4b2:1F TGCTCCTCGTGTGTTGATCCA, Clec4b2:1R TGTTAAGATAAACCCCAACG AGTGTTCTA, Clec4b2:2F AAAACTGCCCCAAAGTGAAG, Clec4b2:2R GAAGCAGGTGCT TAGAGGATGAA, Clec4e.F TTTTCAAGAGTCTCGAGCTCT, Clec4e.R TTCCCTCATGTC GTGGCACAGT, Clec4d.F CAAAAGGCTAACATGCATCCTA, Clec4d.R GCAAAGTACGTTAGCTAACTGGAATGCT, Clec4a1F CATTCTGGTCAGGAGACAA, Clec4a1R TGCAG AGTCCCTGGAAGT, Clec4a2F CCATAGCAAGGAAGAACAG GACTT, Clec4a2R TGAATCC CAGAGCCAGAGAAGAGAGATT, Clec4a3F AGCCACAAGGAAGAGAGATT, Clec4a3R Clec4a4F ACCAGGAGAGAGAGAGAT, Clec4a4R Clec4a5F AGCCAGGAGAGAGAGAGATT, Clec4a5R Clec4a6F AGCCAGGAGAGAGAGAGATT, Clec4a6R Clec4a7F AGCCAGGAGAGAGAGAGATT, Clec4a7R Clec4a8F AGCCAGGAGAGAGAGAGATT, Clec4a8R Clec4a9F AGCCAGGAGAGAGAGAGATT, Clec4a9R Clec4a10F AGCCAGGAGAGAGAGAGATT, Clec4a10R Clec4a11F AGCCAGGAGAGAGAGAGATT, Clec4a11R Clec4a12F AGCCAGGAGAGAGAGAGATT, Clec4a12R Clec4a13F AGCCAGGAGAGAGAGAGATT, Clec4a13R Clec4a14F AGCCAGGAGAGAGAGAGATT, Clec4a14R Clec4a15F AGCCAGGAGAGAGAGAGATT, Clec4a15R Clec4a16F AGCCAGGAGAGAGAGAGATT, Clec4a16R Clec4a17F AGCCAGGAGAGAGAGAGATT, Clec4a17R Clec4a18F AGCCAGGAGAGAGAGAGATT, Clec4a18R Clec4a19F AGCCAGGAGAGAGAGAGATT, Clec4a19R Clec4a20F AGCCAGGAGAGAGAGAGATT, Clec4a20R Clec4a21F AGCCAGGAGAGAGAGAGATT, Clec4a21R Clec4a22F AGCCAGGAGAGAGAGAGATT, Clec4a22R Clec4a23F AGCCAGGAGAGAGAGAGATT, Clec4a23R Clec4a24F AGCCAGGAGAGAGAGAGATT, Clec4a24R Clec4a25F AGCCAGGAGAGAGAGAGATT, Clec4a25R Clec4a26F AGCCAGGAGAGAGAGAGATT, Clec4a26R

All quantifications were based on the Livak method 2-ddCT and minimum one of 3 house-keeping genes PPIA, Gapdh, TBP, or beta-2-macro.

Flow-cytometry
Cells in single-cell suspension were added to 96-well V-bottom polypropylene plates (BD Falcon) at 10^6 cells/well and incubated with saturating concentrations of mAbs (see below). Cells...
were washed with FACS buffer and incubated in BD Cytofix/Cytoperm for 20 mins at room temperature and washed twice in BD Perm/Wash before staining with Abs to CD68. Fluorescence minus one control were used in all experiments. Dead cells were stained with Live/Dead Violet (Invitrogen) after washing the cells twice in Dulbecco’s PBS without sodium azide and FCS. A SORP BD LSR II analytic flow cytometer (BD Biosciences) was used for acquisition and the data were analyzed with FlowJo (Tree Star). The following Alexa Fluor 488–, PE–, PerCP-Cy5.5–, allophycocyanin–, allophycocyanin-Cy7–, Qdot-655–, and biotin-conjugated Abs were used for flow cytometry: 2.4G2/BD Fc Block (CD32), OX-8 (CD8a), OX-1 (CD-45), HRL-1 (CD62l) and WT.5 (CD11b) were purchased from BD Pharmingen (San Diego, CA); R73 (αβTCR), OX-62 (CD103), W3/25 (CD4), 24F (CD86), 3H5 (CD80) purchased from BioLegend (San Diego, CA USA); OX-39 (CD25), OX-17 (RT1-D), His24 (CD45R/B220), His36 (ED-2) were purchased from eBioscience (San Diego, CA); and ED1 (CD68), purchased from AbD Serotec.

Co-culture assay

Splenocytes from either naïve- or oil-injected rats were prepared in single cell suspension and selected with antibody coated beads, either in a negative or positive selection for the following surface receptors: CD4^+^ CD6^-^ (CD4^+^ DCs), CD4^-^ CD6^-^ (CD4^-^ DCs) or CD6^+^ (total T cells) as previously described. Cells were cultured in complete DMEM media (10% FCS, 1M Hapes (Sigma, St Louis, MO, USA), 50 uM 100U/ml penicillin, 100 ug/ml streptomycin, 50 uM β-mercaptoethanol (Sigma St Louis, MO, USA)). In-vitro stimulation of naïve CD4^-^ DCs was prepared in 24-hour cultures of complete media in addition to one of the following: 3ug/ml Concanavalin A (Sigma, St Louis, MO, USA); 5 ug/ml Trehalose Dimycolate (TDM)(Sigma, St Louis, MO, USA); 10 ug/ml Mannan (Sigma, St Louis, MO, USA); 50 ug/ml CpG-A 1668 (Invivogen, San Diego, CA, USA) or 3 ug/ml LPS (Sigma, St Louis, MO, USA). For proliferation assays magnetic-bead selected T cells were labeled with CFSE 2 uM before 72 hrs culture in complete media with either in vivo activated CD4^-^ DCs taken from day one, day two or day three of after oil injection or CD4^-^ DCs activated in vitro for 24 hours with Con A.

In vivo proliferation assay and cytokine ELISA

T cells were harvested from four DA rats at day four after oil injection. T cells were harvested from whole spleen and lymph nodes and selected using magnetic CD6 Macs beads and subsequently labeled with CFSE. The labeled T cells where then immediately injected to naïve rats from either the DA or Clec4b^{E3} strain. T cells from one rat from day four oil injected donors, were split between one DA and one Clec4b^{E3} recipient. The naïve recipients where then challenged with an intradermal injection of oil and, three days later, spleens and draining lymph nodes were collected. The cells were labeled with antibodies to CD3, MHCII and CD25 in order to measure expression together with CFSE from the donor T cells. More than 100 million cells where assessed per recipient animal using a flow cytometer. Other cells were cultured with PMA, ionomycin and brefeldin A for 6 hours and further stained intracellularly with anti-IL17, IFNg and IL10 antibodies and analyzed with the flow cytometer.

Ethics statement

All animal experiments were approved by the local ethics committee (Stockholms djurförsöksöver nämnd) and all experiments were carried out according to method approved (Ethical Approval Number N35/16). Animal anesthesia was performed using an inhalant gas mixture of isoflurane and air. All euthanasia was carried out using CO2 gas.
Supporting information

S1 Fig. Illustration of cell population subsets within both the CD4+ as well as the CD4- fraction of antigen presenting cells depending on their expression of MHCII. Three spleens harvested from day 3 after oil injection were selected first for T cells were the negative fraction was collected and selected a second time for CD4 expression. The twice selected cells were either CD4+ or CD4-, each represented as 3 samples. The samples were then labeled with fluorescence conjugated antibodies to identify subsets of cells. Since there appeared to be 3 clear subsets of CD4+ DCs depending on the MHCII expression the cells were first gated as either MHCII negative, MHCII high/bright and MHC dim. The value on the x axis is the present of total number of cells.

(TIF)

S2 Fig. Illustration of cell subsets in the naïve spleen of DA versus Clec4bE3.

(TIF)

S3 Fig. Illustration of distribution of B and T cell subset in the spleen of DA versus Clec4bE3 at 3 days after oil injection.

(TIF)

S1 Data. Excel-file that includes data to all graphs for the figures in this manuscript. The file have 1 sheet per figure where the different subset graphs in each figure are named by their index.

(XLSX)

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Author Contributions

Conceptualization: Liselotte Bäckdahl, Ulrika Norin, Rikard Holmdahl.

Data curation: Liselotte Bäckdahl, Ulrika Norin.

Formal analysis: Liselotte Bäckdahl, Mike Aoun.

Funding acquisition: Liselotte Bäckdahl, Rikard Holmdahl.

Investigation: Liselotte Bäckdahl, Mike Aoun, Ulrika Norin.

Methodology: Liselotte Bäckdahl, Mike Aoun, Ulrika Norin, Rikard Holmdahl.

Project administration: Liselotte Bäckdahl, Rikard Holmdahl.

Resources: Rikard Holmdahl.

Supervision: Liselotte Bäckdahl, Rikard Holmdahl.

Validation: Liselotte Bäckdahl, Mike Aoun.

Visualization: Liselotte Bäckdahl.

Writing – original draft: Liselotte Bäckdahl.

Writing – review & editing: Liselotte Bäckdahl, Mike Aoun, Rikard Holmdahl.
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