

We Stand by You

Tools, services, products, and people to support your research



This information is current as of March 18, 2013.

Dual TCR Expression Biases Lung Inflammation in DO11.10 Transgenic Mice and Promotes Neutrophilia via Microbiota-Induced Th17 Differentiation

Muriel M. Lemaire, Laure Dumoutier, Guy Warnier, Catherine Uyttenhove, Jacques Van Snick, Magali de Heusch, Monique Stevens and Jean-Christophe Renauld

J Immunol 2011; 187:3530-3537; Prepublished online 22

August 2011;

doi: 10.4049/jimmunol.1101720

<http://www.jimmunol.org/content/187/7/3530>

References This article **cites 43 articles**, 31 of which you can access for free at:
<http://www.jimmunol.org/content/187/7/3530.full#ref-list-1>

Subscriptions Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscriptions>

Permissions Submit copyright permission requests at:
<http://www.aai.org/ji/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/cgi/alerts/etoc>



Dual TCR Expression Biases Lung Inflammation in DO11.10 Transgenic Mice and Promotes Neutrophilia via Microbiota-Induced Th17 Differentiation

Muriel M. Lemaire,^{*,†} Laure Dumoutier,^{*,†} Guy Warnier,^{*} Catherine Uyttenhove,^{*} Jacques Van Snick,^{*,†} Magali de Heusch,^{*,†} Monique Stevens,^{*} and Jean-Christophe Renauld^{*,†}

A commonly used mouse model of asthma is based on i.p. sensitization to OVA together with aluminum hydroxide (alum). In wild-type BALB/c mice, subsequent aerosol challenge using this protein generates an eosinophilic inflammation associated with Th2 cytokine expression. By contrast, in DO11.10 mice, which are transgenic for an OVA-specific TCR, the same treatment fails to induce eosinophilia, but instead promotes lung neutrophilia. In this study, we show that this neutrophilic infiltration results from increased IL-17A and IL-17F production, whereas the eosinophilic response could be restored upon blockade of IFN- γ , independently of the Th17 response. In addition, we identified a CD4⁺ cell population specifically present in DO11.10 mice that mediates the same inflammatory response upon transfer into RAG2^{-/-} mice. This population contained a significant proportion of cells expressing an additional endogenous TCR α -chain and was not present in RAG2^{-/-} DO11.10 mice, suggesting dual antigenic specificities. This particular cell population expressed markers of memory cells, secreted high levels of IL-17A, and other cytokines after short-term restimulation in vitro, and triggered a neutrophilic response in vivo upon OVA aerosol challenge. The relative numbers of these dual TCR lymphocytes increased with the age of the animals, and IL-17 production was abolished if mice were treated with large-spectrum antibiotics, suggesting that their differentiation depends on foreign Ags provided by gut microflora. Taken together, our data indicate that dual TCR expression biases the OVA-specific response in DO11.10 mice by inhibiting eosinophilic responses via IFN- γ and promoting a neutrophilic inflammation via microbiota-induced Th17 differentiation. *The Journal of Immunology*, 2011, 187: 3530–3537.

Allergic asthma is a frequent pulmonary disorder caused by dysregulated immune responses that are triggered by inhaled environmental Ags. This chronic disease is characterized by lung inflammation, mucus accumulation, and airway hyperresponsiveness, and leads to tissue remodeling (1, 2). The pathogenic role of CD4⁺ Th2 cells has been well documented throughout mouse preclinical models (3). Following the Th1/Th2 general paradigm, Th1-derived IFN- γ is supposed to antagonize Th2 differentiation, and, conversely, Th2-derived IL-4 inhibits the Th1 axis. Hence, it has been suggested that asthma could develop as a result of an impaired balance of Th1 in favor of Th2. Yet, this dichotomic model has been challenged by the discovery of the Th17 subset, which plays a key role in antibacterial defenses and autoimmunity, but also soon became implicated in asthma (4, 5). An increase of IL-17 production has been observed in airways and plasma from asthmatic patients (6, 7), and the level of this cytokine in the sputum has been correlated with the severity of

bronchial hyperreactivity (8). In addition, the expression level of ROR γ t, the key transcriptional factor for Th17 differentiation, was augmented in asthmatic patients (7). In mouse models, accumulating evidence supports a key role for IL-17 induction in lung inflammation triggered by sensitization and challenge with allergens (9–11). Whereas IL-5-producing Th2 cells are required for lung eosinophilia, IL-17 was shown to be responsible for neutrophil recruitment to the lungs following local Ag stimulation, as this influx was blocked either by anti-IL-17 Ab administration (9, 12) or in IL-17R^{-/-} mice (11). In line with these observations, in vitro differentiated Th17 cells specific for OVA were shown to induce airway neutrophilic inflammation in adoptively transferred wild-type mice after local OVA challenge (13, 14).

Whether Th17 cells influence Th2 lung inflammation remains a controversial issue. In a classical model elicited by i.p. sensitization and aerosol challenge with OVA, it has been reported that IL-17 inhibition could diminish lung neutrophilia and increase eosinophilia (12). Conversely, in a similar model, but with epicutaneous sensitization, the lung eosinophilic response was abrogated in favor of an elevated neutrophil infiltration when mice were deficient in both IL-4 and IL-13 (15). These results suggest that Th17 and Th2 cells are able to antagonize each other during allergen-induced airway inflammation. However, other results also support a synergy between the Th2 and Th17 axes. Using the same OVA sensitization and challenge protocol, one group has shown that anti-IL-17 Abs inhibited not only lung neutrophilia, but also eosinophilia, and decreased Th2 cytokine levels in bronchoalveolar lavage (BAL) fluid (16). Following OVA inhalation, it has also been reported that recipient mice transferred with in vitro differentiated OVA-specific Th cells presented an increase of BAL neutrophil, but also of eosinophil numbers when Th17 cells were injected concomitantly to Th2 cells. Moreover, airway

*Ludwig Institute for Cancer Research, Brussels Branch, B-1200 Brussels, Belgium; and [†]de Duve Institute, Université Catholique de Louvain, B-1200 Brussels, Belgium

Received for publication June 9, 2011. Accepted for publication July 24, 2011.

This work was supported in part by Fonds pour la Formation et la Recherche dans l'Industrie et dans l'Agriculture (Brussels, Belgium), the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming, and the Actions de Recherche Concertées of the Communauté Française de Belgique. L.D. is a research associate of the Fonds National de la Recherche Scientifique.

Address correspondence and reprint requests to Dr. Jean-Christophe Renauld, Ludwig Institute for Cancer Research and Experimental Medicine Unit, Université Catholique de Louvain, Avenue Hippocrate 74, B-1200 Brussels, Belgium. E-mail address: renauld@bru.lir.org

Abbreviation used in this article: BAL, bronchoalveolar lavage.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/\$16.00

hyperresponsiveness was aggravated when both Th cell types were transferred (17). Taken together, all these observations point to an important role of Th17 cells in asthma, although their interactions with Th2 cells remain poorly understood.

To obtain a better insight into this issue, we took advantage of the DO11.10 mice, which are transgenic for a TCR specific for a MHC-II-restricted OVA peptide. These mice are known to develop a neutrophilic lung inflammation upon OVA aerosols without any sensitization (18, 19), which was dependent on a particular population of Th17 cells previously designated as “natural occurring IL-17 producing T cells” (20). In this study, we show that these precommitted Th17 cells actually correspond to memory T cells expressing two different TCR α -chains. In addition, we found that OVA/alum sensitization failed to overcome the prevalence of the Th17-neutrophilic inflammation, but that the repression of the Th2-eosinophilic inflammation was due to IFN- γ rather than to IL-17.

Materials and Methods

Mice

DO11.10 mice (21) were provided by A. Radbruch (German Rheumatism Research Centre, Berlin, Germany) and backcrossed with RAG2^{-/-} mice originally purchased from Taconic (Ejby, Denmark). All mouse strains were bred under specific pathogen-free conditions in the central animal facility of the Ludwig Institute for Cancer Research, and female mice between 2 and 12 wk of age were used for this study. Handling of mice and experimental procedures were conducted in accordance with national and institutional guidelines for animal care.

OVA lung inflammation protocols

To induce the classical lung inflammation model, BALB/c mice (and other strains) received two sensitizing i.p. injections of 20 μ g OVA (chicken-egg OVA grade V; Sigma-Aldrich) with 70 μ l alum (Alum Imject; Thermo-Scientific) in PBS to reach 200 μ l on days 0 and 5. One week after the second sensitization, mice were challenged with three or four daily OVA aerosols (1% w/v in saline buffer, 20 min), generated using an ultrasonic nebulizer (LS290; System). Twenty-four hours after the last aerosol, a BAL was performed for flow cytometry analysis, and lung samples were collected for RT-PCR. In some experiments, aerosols were applied without any sensitization.

Adoptive transfer experiments

Spleen cells were extracted and filtered on 40- μ m nylon filters. RBCs were lysed by osmotic shock. For R2 (CD5^{high} KJ1-26^{low}) and R3 (CD5^{low} KJ1-26^{high}) sorting or V α 2⁺ sorting, CD4⁺ spleen cells were isolated using magnetic beads (MACS; Miltenyi Biotec), stained with anti-CD5 Ab (BD Pharmingen; final concentration, 2 μ g/ml) and the KJ1-26 anti-DO11.10 TCR Ab (Caltag Laboratories; final concentration, 2 μ g/ml) or with anti-V α 2 Ab (BD Pharmingen; final concentration, 2 μ g/ml), and sorted with a FACS Vantage instrument. For spleen cell transfer, 80 $\times$ 10⁶ cells (BALB/c or DO11.10 donors) and 10 $\times$ 10⁶ cells (RAG2^{-/-} DO11.10 donors) were injected i.v. 24 h before initiation of the lung inflammation protocol. For FACS-sorted cell transfer, 2 $\times$ 10⁶ cells were injected i.p. into RAG2^{-/-} mice 24 h before sensitization with OVA and alum. In other experiments, similar cell numbers were injected i.v. into RAG2^{-/-} DO11.10 mice 1 wk before starting aerosol protocol, without any sensitization. For V α 2⁺ cell transfer, 1 $\times$ 10⁶ cells were injected i.v. into RAG2^{-/-} mice 1 wk before beginning aerosol protocol.

Antibiotic treatment

To drastically reduce gut flora in DO11.10 mice, mothers and newborns received antibiotic treatment. Starting 1 wk before birth and during breastfeeding, DO11.10 mothers received s.c. injection of penicillin and streptomycin every other day (4 and 5 mg/mouse, respectively; Duphaphen; Strept; Norbrook Laboratories). In addition, paromomycin (Gabbrovet 70; CEVA) was diluted in drinking water (25 g powder/l) of mothers and pups during the same period and until the end of experiments.

Flow cytometry analysis

Single-cell suspension of splenocytes was prepared in Hanks' medium. One million cells per condition were preincubated with 10 μ g/ml purified rat

anti-mouse CD16-CD32 mAb (Fc block; BD Pharmingen) for 15 min at room temperature. FITC-, PE-, or allophycocyanin-conjugated Abs were then added at a final concentration of 2 μ g/ml and incubated for 1 h at 4°C. For BAL analysis, 5 $\times$ 10⁴ cells were used per condition and stained with anti-Gr1 and anti-CCR3 to differentiate neutrophils from eosinophils. Stained cells were analyzed with a FACScan or FACScalibur instrument using the CellQuest software (BD Biosciences), and postacquisition analysis was achieved with FlowJo software (Tree Star).

Antibodies

For flow cytometry analysis, fluorochrome-conjugated anti-CD5, -V α 2, -CD62L, -Gr1 (BD Pharmingen), anti-CCR3 (R&D Systems), and anti-DO11.10 TCR (Caltag Laboratories) were used, as described above. Cytokine inhibition experiments were performed by in vivo injections of the FXIVF3 rat anti-mouse IFN- γ (22), the MM17F3 mouse anti-mouse IL-17A (23), and the MM17F-8F5 mouse anti-mouse IL-17F (24). Three hundred to 500 μ g each Ab was injected 24 h before starting the lung inflammation protocol.

T cell stimulation assays

For in vitro stimulation of sorted R2 and R3 populations, 1.5 $\times$ 10⁶ cells per condition were seeded in 24-well plates coated with anti-CD3 (clone 145.2C11, 5 μ g/ml). Cells were stimulated for 24 h in Iscove Dulbecco's medium supplemented with 5% heat-inactivated FBS, 0.24 mM asparagine, 1.5 mM glutamine, 0.55 mM arginine, 50 μ M 2-ME, and 2.5 μ g/ml anti-CD28 (clone 37.51). A total of 4 $\times$ 10⁵ total spleen cells from 6-wk-old DO11.10 mice treated or not with antibiotics was cultured for 3 d in 96-well plates with the same medium supplemented with 100 μ g/ml OVA or 1 μ g/ml OVA 323–339 peptide. In each experiment, cell culture supernatants were collected to measure cytokine production. Proliferation was evaluated by adding tritiated thymidine to the cells for 4 h. Cells were then collected on microfiltered plates, and thymidine incorporation was measured with a Top Count microplate scintillation counter (Canberra-Packard, Meriden, CT).

Cytokine measurement

Cytokine production was measured in cell culture supernatants. ELISA specific for murine IL-5, IFN- γ (eBiosciences), and IL-13 (R&D Systems) was performed, according to manufacturer's instructions. IL-17A was measured using mouse Abs generated in our laboratory. Cytokine concentrations were calculated by means of a standard curve generated via the use of calibrated standards.

Reverse transcription-quantitative PCR

Lung samples were collected and frozen in liquid nitrogen. TriPure isolation reagent (Roche) was used to extract total RNA, according to manufacturer's instructions. One microgram RNA was included in reverse transcription with oligo(dT) primers (Roche), and Moloney murine leukemia virus reverse-transcriptase enzyme (Invitrogen) and cDNA were diluted five times. Quantitative PCR reactions were performed using primer pairs and probes specific for murine IL-4, IL-17A, IL-17F, IFN- γ , ROR γ t, and β -actin with qPCR Mastermix for TaqMan (Eurogentec), and using primer pairs specific for murine IL-5, T-bet, and GATA3 with qPCR Mastermix for SYBR Green I (Eurogentec). The sequences of the primers (final concentrations, 300 nM) were as follows: mIL-4, 5'-GAACGAGTCA-CAGGAGAAGG-3' (forward) and 5'-GGACTCATTCATGGTGCAGCT-TA-3' (reverse); mIL-5, 5'-GAAGGATGCTTCTGCACTTGAGTG-3' (forward) and 5'-CAGGAAGCCTCATCGTCTCATTG-3' (reverse); mIL-17A, 5'-GCTCCAGAAGGCCCTCAG-3' (forward) and 5'-CTTTCCC-TCCGATTGACA-3' (reverse); mIL-17F, 5'-GAGGATAACACTGTGA-GAGTTGAC-3' (forward) and 5'-TTCCTGACCTGGGCATTGATG-3' (reverse); mROR γ t, 5'-CCGCTGAGAGGGCTTCAC-3' (forward) and 5'-TGCAGGATAGGCCACATTACA-3' (reverse); mT-bet, 5'-CACTAAG-CAAGGACGGCGAATG-3' (forward) and 5'-GTCCACCAAGACCAC-ATCCACA-3' (reverse); mGATA3, 5'-GACATCCTGCGCGAACTGTC-3' (forward) and 5'-GATGCCTTCTTCTTCATAGTCAGG-3' (reverse); and m β -actin, 5'-CTCTGGCTCCTAGCACCATGAAG-3' (forward) and 5'-GCTGGAAGGTGGACAGTGAG-3' (reverse). The sequences of specific probes were as follows (final concentration, 100 nM): mIL-4, 5'-CCTCACAGCAACGAAGAACCACAG-3'; mIL-17A, 5'-ACCTCA-ACCGTTCCACGTCACCTG-3'; mIL-17F, 5'-CAACAAAACACAGG-GCATTCTGTGTC-3'; mROR γ t, 5'-AAGGGCTTCTTCCGCCGACG-AGCAG-3'; and m β -actin, 5'-ATCGGTGGCTCCATCCTGGC-3'. Samples were first heated 10 min at 95°C. cDNA was amplified as follows: 40 cycles of a two-step PCR program at 95°C for 15 s and 60°C (or 61°C for IL-5) for 1 min. For SYBR Green, melting point analysis was carried out

by heating the amplicon from 60°C to 95°C. Results were analyzed by MyIQ software (Bio-Rad).

Statistical analysis

Statistical significance was analyzed using the Instat3 software. The *p* value was determined with two-tailed Student *t* test when comparing two independent groups, and one-way ANOVA or Kruskal–Wallis test for more than two independent groups. Results were expressed as means $\pm$ SD or SEM.

Results

OVA sensitization and challenge in DO11.10 mice induce a Th17 neutrophilic inflammatory response

After i.p. sensitization with OVA in alum and aerosol challenges, BALB/c mice showed a classical Th2 inflammation, with BAL eosinophilia and lung expression of Th2-related cytokines, such as IL-4 and IL-5. Surprisingly, when applied to DO11.10 transgenic mice, this treatment failed to induce the same response. Instead, we observed BAL neutrophilia and induction of IFN- γ , IL-17A, and IL-17F in inflamed lungs (Fig. 1). As shown in Fig. 2, the combination of both anti-IL-17A and anti-IL-17F almost completely abolished the neutrophilic response, but failed to restore the eosinophilic infiltration in DO11.10 transgenic mice. By contrast, anti-IFN- γ Ab did not affect the neutrophilic response, but restored the eosinophilic infiltration (Fig. 2), suggesting that a potentially concurrent Th2 response in the lungs is blocked by Th1, but not by Th17 cytokines.

A particular subset of T cells is responsible for DO11.10 response

To further study the Th cell populations responsible for the unique phenotype of the DO11.10 mice, we sought to reproduce this model after adoptive transfer of spleen cells into RAG2^{-/-} recipient mice. Upon OVA/alum sensitization and aerosol challenge, recipient mice reconstituted with DO11.10 cells showed the expected neutrophilic lung inflammation, whereas mice reconstituted with BALB/c spleen cells developed an eosinophilic profile (Fig. 3A). Interestingly, transfer of RAG2^{-/-} DO11.10 cells led to an eosinophilic response similar to that observed with wild-type BALB/c spleen cells, indicating that the type of inflammation did not simply result from a high number of Ag-responsive T cells nor from a particular affinity of the DO11.10 TCR. Thus, these data suggest that the spleen from DO11.10 mice, but not from RAG2^{-/-} DO11.10 mice, contains a subset that preferentially drives a Th17 and Th1 response.

To further explore this hypothesis, we compared spleen cells from DO11.10 and RAG2^{-/-} DO11.10 mice by flow cytometry using an Ab directed against the CD5 T cell marker and the clonotypic KJ1-26 Ab (Fig. 3B). T cells from RAG2^{-/-} DO11.10 constituted a single homogenous population expressing high levels of the OVA-specific transgenic TCR, whereas DO11.10 mice with functional RAG2 alleles showed three distinct populations, each representing ~30% of CD5⁺ cells. The first subset contained KJ1-26-negative T cells (R1), the second subset expressed an intermediate level of the OVA-specific TCR (R2), and the third one was composed of cells displaying the same phenotype as in RAG2^{-/-} DO11.10 (R3). Cells from the latter subset expressed lower levels of CD5 than cells from R1 and R2, allowing for a better discrimination (Fig. 3B). Such a lower expression of CD5, as observed for the R3 subset, was expected from DO11.10 T cells and is considered to reflect a relatively low affinity of the TCR–MHC interaction for this particular receptor, as described by Azzam et al. (25).

To determine which of the KJ1-26-positive T cells could be responsible for the induction of the Th17 neutrophilic response of

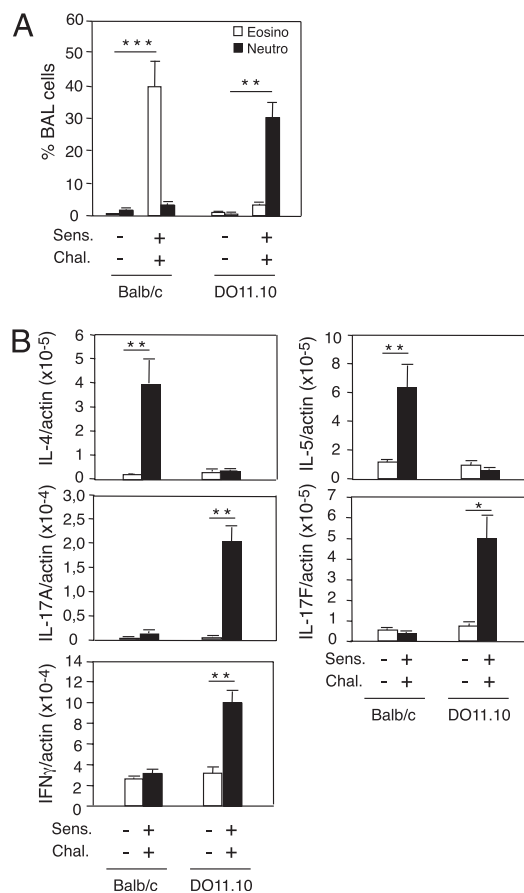


FIGURE 1. DO11.10 mice develop lung neutrophilia and Th17 inflammation after alum sensitization and aerosol challenge with OVA. **A**, Flow cytometry analysis of BAL eosinophils and neutrophils. BALB/c and DO11.10 mice were sensitized twice with OVA in alum on days 0 and 5. Since day 12, they were daily challenged using OVA aerosols during consecutive 4 d. On day 16, BAL cells were collected and analyzed by flow cytometry for neutrophil and eosinophil quantification. Mean values and SEM were calculated from four to six mice for each group. Statistical significance was determined using Kruskal–Wallis test with Dunn's multiple comparisons test (***p* < 0.01, ****p* < 0.001). **B**, Cytokine expression in lungs. Total RNA was extracted from lungs of each group, and quantitative RT-PCR was performed using primers specific for murine IL-4, IL-5, IL-17A, IL-17F, and IFN- γ genes. Transcripts were normalized to the level of the murine β -actin and expressed as mean values and SEM. For IL-4 and IL-5, statistical significance was calculated using unpaired *t* test, and unpaired *t* test with Welch correction for IL-17A, IL-17F, and IFN- γ (***p* < 0.01, **p* < 0.05). Data are from a representative experiment of four independent experiments.

DO11.10 mice, we sorted cells corresponding to the R2 and R3 subsets from DO11.10 spleen cells. Cells were adoptively transferred into RAG2^{-/-} DO11.10 before OVA/alum sensitization and challenge. As shown in Fig. 3C, transferred R2 cells were able to induce a predominant neutrophilic response, whereas R3 promoted a mostly eosinophilic response, in line with the activity of the RAG2^{-/-} DO11.10 T cells. Taken together, these results indicate that the neutrophilic response of DO11.10 mice is mediated by a CD5^{high} KJ1-26^{dull} (R2) population of T cells that requires a functional RAG2 gene for its development.

Neutrophilia-promoting T cells show dual TCR expression and memory surface markers

It has been reported previously that DO11.10 mice (26–28), as well as normal wild-type mice (29–31) and humans (32), produce a nonnegligible proportion of T cells expressing two different

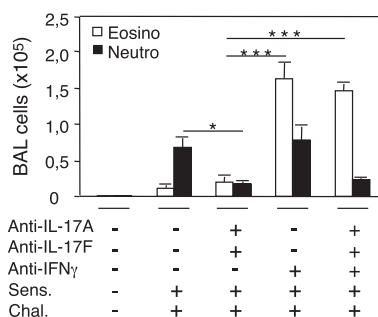


FIGURE 2. IFN- γ , but not IL-17, inhibits Th2 eosinophilia in DO11.10 mice after OVA sensitization and challenge. BAL cell analysis of DO11.10 mice treated with anti-IL-17A, anti-IL-17F, and/or anti-IFN- γ and subsequently sensitized and challenged with OVA. Mice received Abs 24 h before the first sensitization and the first aerosol challenge. The common protocol was applied using two sensitizations and four daily OVA aerosol challenges. BAL cells were collected and analyzed by flow cytometry for neutrophil and eosinophil quantification. Statistical significance was calculated using one-way ANOVA and Bonferroni multiple comparisons test (* $p < 0.05$, *** $p < 0.001$). Data are representative of at least two experiments.

TCRs at the same time. In DO11.10 mice, Zhou et al. (28) showed that dual TCR cells tend to express a lower level of the transgenic TCR. We therefore hypothesized that cells from the R2 subset express the DO11.10 TCR concomitantly with a second TCR α -chain. To test this hypothesis, we stained DO11.10 spleen cells using Abs specific for the V α 2 domain or for the DO11.10 receptor. As the transgenic receptor contains the V α 13.1 domain, double-positive cells express two different TCRs. As shown in Fig. 4, no dual TCR cell population was detectable when gating on R3, whereas up to 15% of R2 cells were recognized by both Abs. Similar results were found with Abs specific for the V α 8.3 domain (data not shown).

Moreover, further analysis of both subsets using common T cell markers revealed that ~50% of R2 cells expressed a low level of CD62L, suggesting that R2 contains a large proportion of memory T cells, in contrast to R3 cell population, which consists of naive CD62L^{high} T cells (Fig. 5A). The memory phenotype of the cells from the R2 subset might explain why DO11.10 mice do not need any sensitization to develop a neutrophilic response following OVA aerosol challenges. To test this hypothesis, we sorted the R2 or R3 cell subsets from DO11.10 mice and adoptively transferred them into RAG2^{-/-} DO11.10 mice before performing OVA aerosol challenges without any sensitization step. As shown in Fig. 5B, these mice, which contain exclusively naive OVA-specific T cells, did not develop any neutrophilia after aerosol challenges, unless they received either total CD4⁺ cells or cells from the R2 subset. To further confirm that dual TCR cells are responsible for this airway neutrophilia, we sorted CD4⁺ V α 2⁺ cells from DO11.10 mice. In this population, all OVA-responsive cells should therefore express a dual TCR. BALB/c V α 2⁺ CD4⁺ T cells were used as a negative control. After transfer, recipient RAG2^{-/-} mice were challenged using OVA aerosols, and BAL cells were analyzed by flow cytometry. As shown in Fig. 5C, neutrophilia was induced only in mice that received V α 2⁺ cells from DO11.10 mice, demonstrating that dual TCR cells from DO11.10 mice are sufficient to trigger airway neutrophilia in vivo.

OVA sensitization of DO11.10 mice fails to activate naive T cells

Although our data show that memory dual TCR cells are responsible for the major part of the in vivo response of DO11.10 mice to OVA administration, the potential contribution of naive T cells to this response is less clear. To address this question, we

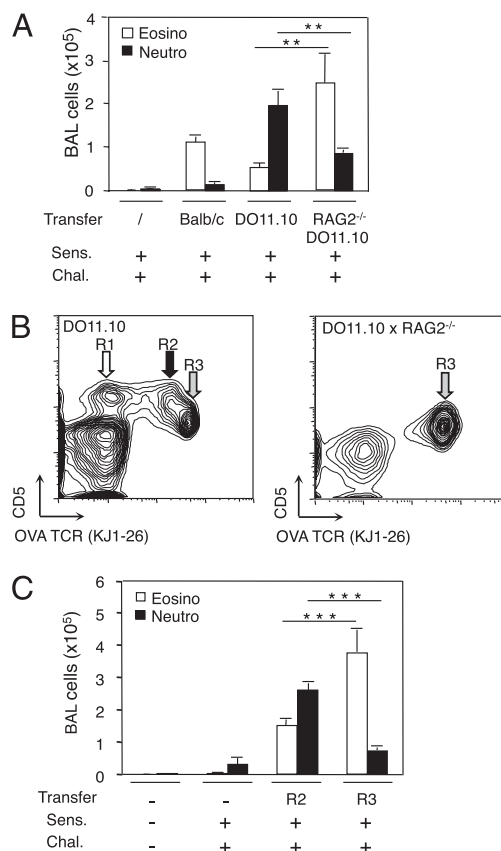


FIGURE 3. CD5⁺ DO11.10 TCR^{int} spleen cells (R2 subset) are responsible for OVA-induced neutrophilia. **A**, Flow cytometry analysis of BAL eosinophils and neutrophils. RAG2^{-/-} mice received total spleen cells from donor BALB/c, DO11.10 (80 × 10⁶ cells), or RAG2^{-/-} DO11.10 mice (10 × 10⁶ cells). Twenty-four hours after the transfer, recipient mice were sensitized twice with OVA in alum on days 0 and 5. Since day 12, they were daily challenged using OVA aerosols during consecutive 4 d. On day 16, BAL cells were collected and analyzed by flow cytometry for neutrophil and eosinophil quantification. Mean values and SEM were calculated from four to five mice of each group. Statistical significance was determined using one-way ANOVA and Bonferroni multiple comparisons test (** $p < 0.01$). Data are representative of more than three independent experiments. **B**, Flow cytometry of splenocytes collected from DO11.10 or RAG2^{-/-} DO11.10 mice and stained with FITC/PE-conjugated anti-OVA TCR and anti-CD5 Abs. The results are representative of more than three independent experiments. **C**, Flow cytometry analysis of BAL eosinophils and neutrophils from RAG2^{-/-} mice transferred with R2 or R3 subsets and sensitized and challenged with OVA. CD4⁺ cells from freshly isolated DO11.10 spleen cells were stained with anti-OVA TCR and anti-CD5 Abs to sort both R2 and R3 subsets. RAG2^{-/-} recipient mice were reconstituted with sorted cells. Twenty-four hours after the transfer, recipient mice were sensitized twice with OVA in alum on days 0 and 5. Since day 12, they were daily challenged using OVA aerosols during consecutive 4 d. On day 16, BAL cells were collected and analyzed by flow cytometry for neutrophil and eosinophil quantification. Results are expressed as the means for three independent experiments. Mean values and SEM were calculated from 9–12 mice of each group. Statistical significance was determined using one-way ANOVA and Bonferroni multiple comparisons test (*** $p < 0.001$).

checked the expression of CD44, as a memory T cell marker, upon OVA-alum sensitization. In RAG2^{-/-} DO11.10 spleen cells, all KJ1-26⁺ T cells express high levels of this TCR (corresponding to the R3 population of DO11.10 mice) and low levels of CD44. Within 10 days after a single injection of OVA in alum, ~10% of these naive T cells became CD44^{high}, reflecting their antigenic stimulation (Fig. 6A). By contrast, we could not observe any increase in the percentage of CD44^{high} when gating on KJ1-26^{high} T cells (corresponding to R3 cells) from OVA-sensitized DO11.10

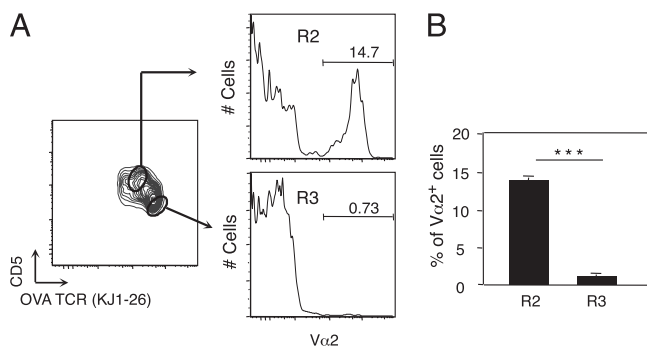


FIGURE 4. DO11.10 R2 subset is composed of dual TCR cells. *A*, Flow cytometry analysis of Vα2 expression among R2 and R3 cell subsets. Total DO11.10 spleen cells were stained for three-color analysis with allophycocyanin anti-OVA TCR, FITC anti-CD5, and PE anti-Vα2 Abs. R2- and R3-restrictive gates were defined on OVA TCR⁺ cells to calculate the proportion of Vα2⁺ cells among each subset. Representative results are shown for one mouse. *B*, Percentage of Vα2⁺ cells among R2 and R3 subsets from DO11.10 spleen cells. Results are presented as mean values and SEM calculated from a group of four mice, and are representative of three experiments. Statistical significance was calculated using unpaired *t* test (***) *p* < 0.001.

mice (Fig. 6B). This suggests that, in DO11.10 mice, naive OVA-specific T cells do not (or only marginally) contribute to any in vivo response to the Ag, whereas memory dual TCR cells predominantly mediate the OVA response.

Dual TCR cells from DO11.10 mice are biased toward the Th17 and Th1 lineages

To further characterize this dual TCR cell population, we monitored the expression of the prototypical transcription factors for the different Th subsets using quantitative RT-PCR. We found that RORγt and T-bet, representative of the Th17 and Th1 subsets, respectively, were significantly overexpressed in R2 compared with R3 cells, whereas the Th2 transcription factor GATA3 was induced in a lesser, nonsignificant extent (Fig. 7A). To refine their differentiation profile, we stimulated cells from the R2 or R3 populations in vitro with anti-CD3 and anti-CD28 and measured cytokine production in culture supernatants after 24 h. Such a short-term stimulation induced cytokine production in cells from the R2 subset, but not in their R3 counterparts. As illustrated in Fig. 7B, these cells produced high concentrations of IFN-γ (60 ng/ml) and IL-17A (6.25 ng/ml) and lower, but significant amounts of Th2-derived cytokines such as IL-5 (1.1 ng/ml) and IL-13 (1.3 ng/ml). Further intracellular staining experiments showed that T cells produced either IFN-γ or IL-17, but did not coexpress these cytokines (data not shown). Taken together, these observations suggest that the R2 population contains memory T cells, whereof the majority is preferentially polarized toward the Th1 and Th17 pathways.

Dual TCR cells in DO11.10 mice expand with age, and their Th17 differentiation depends on gut microflora

To obtain some insight into the ontogenesis of this dual TCR cell subset, we monitored the presence of Vα2⁺-KJ1-26⁺ cells in early age of life using flow cytometry. Between 2 and 8 wk of age, the percentage of such double TCR⁺ cells in the spleen of DO11.10 mice increased 30-fold, whereas the percentage of total KJ1-26⁺ cells increased only 2.8-fold (Fig. 8A, 8B). This relative expansion of dual TCR cells in the spleen might result from continuous stimulation of such cells by intestinal bacterial Ags. Saparov et al. (27) indeed showed that a significant fraction of KJ1-26⁺ cells in the lamina propria and Peyer's patches was in the cell cycle and

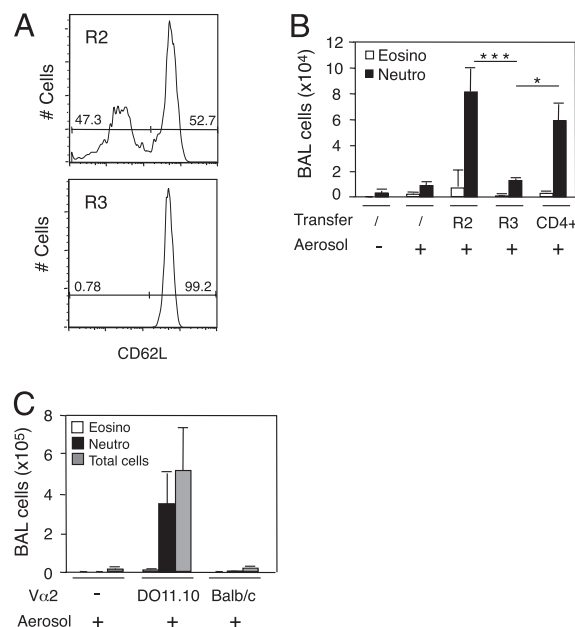


FIGURE 5. R2 cells include memory cells and are able to generate neutrophilia in recipient mice without OVA sensitization. *A*, Flow cytometry analysis of CD62L expression among R2 and R3 cell subsets. Total DO11.10 spleen cells were stained for three-color analysis with FITC anti-OVA TCR, PE anti-CD5, and allophycocyanin anti-CD62L Abs. R2- and R3-restrictive gates were defined on OVA TCR⁺ cells to calculate the cell proportion that expresses a low level of CD62L among each subset. Representative results are shown for one mouse. Data are representative of three experiments. *B*, Flow cytometry analysis of BAL eosinophils and neutrophils from RAG2^{-/-} DO11.10 mice transferred with R2 or R3 cell subsets and challenged with OVA. CD4⁺ cells from DO11.10 spleen cells were purified and stained with anti-OVA TCR and anti-CD5 Abs to sort both R2 and R3 subsets. RAG2^{-/-} DO11.10 recipient mice were reconstituted with sorted cells. One week after the transfer, recipient mice were daily challenged using OVA aerosols during consecutive 3 d. Twenty-four hours after the last challenge, BAL cells were collected and analyzed by flow cytometry. Results are expressed as the means for three independent experiments. Mean values and SEM were calculated from 9–11 mice of each group. Statistical significance was determined using one-way ANOVA and Bonferroni multiple comparisons test (***) *p* < 0.001, **p* < 0.05). *C*, Flow cytometry analysis of BAL eosinophils and neutrophils from RAG2^{-/-} mice transferred with Vα2⁺ cells from DO11.10 or BALB/c mice and challenged with OVA aerosols. CD4⁺ spleen cells from both donor mouse strains were stained with anti-Vα2 Ab to sort positive cells before transfer into RAG2^{-/-} recipient mice. One week after the transfer, recipient mice were daily challenged using OVA aerosols during consecutive 3 d. Twenty-four hours after the last challenge, BAL cells were collected and analyzed by flow cytometry. Mean values and SEM were calculated from four mice of each group.

expressed memory/activation markers. Incidentally, a population of Vα2⁺-KJ1-26⁺ cells also appeared in adult mice, corresponding to T cells that failed to express the transgene encoding the TCR α-chain, as previously described by others (33, 34).

To address the hypothesis that Ags from the gut microflora are responsible for the differentiation of OVA-specific T cells toward the Th17 lineage, we treated DO11.10 mice since birth with broad-spectrum antibiotics to reduce bacterial colonization of gastrointestinal tract. After 6 wk, we restimulated spleen cells in vitro with OVA protein or the 323–339 OVA peptide for 72 h. As illustrated in Fig. 8C, IL-17A production was almost completely abolished following antibiotic treatment, whereas the cell proliferation was not affected at all. The percentage of dual TCR cells was significantly, but only partially decreased by the treatment (Fig. 8D),

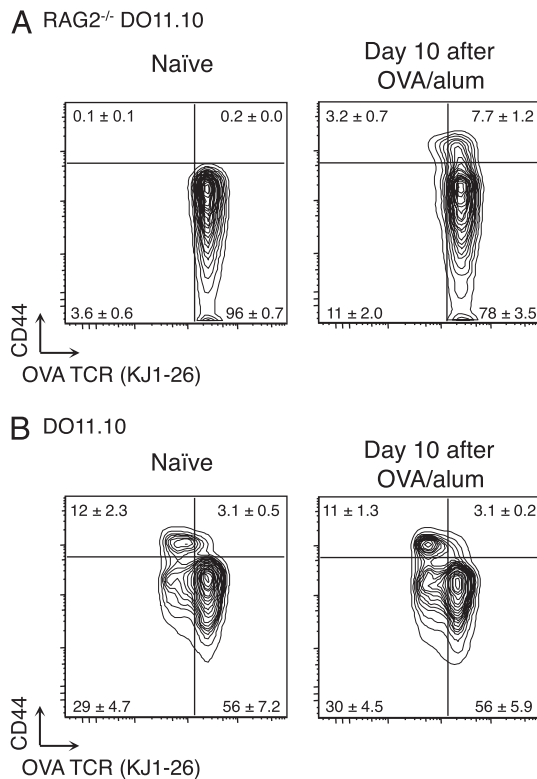


FIGURE 6. OVA sensitization activates naïve T cells from DO11.10 mice only if they are Rag2 deficient. RAG2^{-/-} DO11.10 (A) or DO11.10 (B) mice were sensitized or not with one injection of OVA in alum. Ten days later, spleen cells were stained for FACS analysis with allophycocyanin anti-OVA, TCR KJ1-26, and PE anti-CD44 Abs. A gate was defined on KJ1-26⁺ cells, and the plots show expression levels of CD44 versus OVA TCR. R3 cells correspond to the upper right and lower right quadrants, whereas R2 cells, which express lower levels of the OVA TCR, are located in the upper left and lower left quadrants. Representative results are shown for one mouse per group with the indicated mean percentages and SEM calculated from three to five mice of each group.

suggesting that the expansion of dual TCR DO11.10 spleen cells is less dependent on the microflora than their Th17 differentiation.

To assess the effect of antibiotic treatment in vivo, 6-wk-old treated mice received two daily OVA aerosols, and BAL cells were analyzed 24 h after the last challenge. The antibiotic treatment significantly reduced lung neutrophilia in DO11.10 mice (Fig. 8E). Taken together, these observations demonstrate that the OVA-specific response of DO11.10 mice is dominated by memory cells expressing a dual TCR specificity and that are biased toward the Th17 subset by previous contact with enteric Ags, thereby leading to neutrophilic inflammation.

Discussion

OVA aerosol challenges of DO11.10 mice induce a robust Th17 response associated with a neutrophilic lung infiltration. This response is due to a population of OVA-specific T cells that includes memory T cells, even in OVA-naïve mice, and was previously proposed to correspond to natural Th17 cells generated in the thymus (20). Our results show that this OVA-responsive T cell population contains dual TCR cells, expressing other Vα specificities, and is characterized by a higher level of CD5 expression reflecting a MHC avidity different from cells expressing exclusively the OVA-specific receptor. The absence of this population in RAG2^{-/-} DO11.10 mice, in which all T cells express exclusively the DO11.10 receptor, explains why the latter failed to develop

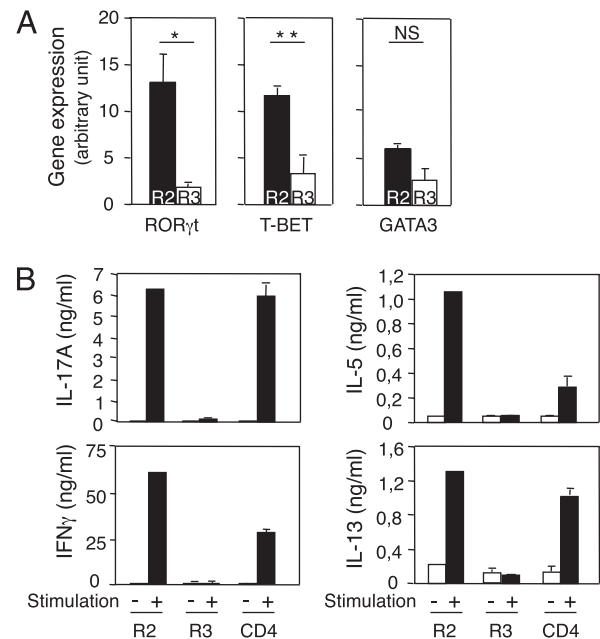


FIGURE 7. R2 cells naturally express higher levels of RORγt and T-bet and produce more IL-17 and IFN-γ following in vitro stimulation. A, Expression levels of key transcription factors for Th differentiation. Total RNA was extracted from cells of the R2 or R3 subset, and quantitative RT-PCR was performed using specific primers for murine RORγt, T-bet, and GATA3 genes. Results are mean values of four independent sorting experiments, and were obtained by normalizing transcripts to the level of the murine β-actin and by applying the 2^{-ΔΔCT} algorithm of the ddCT method. Arbitrary units were calculated by comparison with a reference sample. ***p* < 0.01, **p* < 0.05, unpaired *t* test with Welch correction. B, Cytokine production by R2 and R3 DO11.10 subsets following in vitro stimulation. Purified CD4⁺ spleen cells or further sorted R2 and R3 cells from DO11.10 mice were stimulated for 24 h with anti-CD3 and anti-CD28, and culture supernatants were collected to measure cytokine production by ELISA. Data are representative of two different experiments.

a Th17 response without previous sensitization (20). Besides the expression of an additional TCR α-chain, about half of these lymphocytes present a memory T cell phenotype based on CD62L, CD44, and CD45RB (Fig. 5, data not shown). In line with this memory phenotype, these dual TCR lymphocytes proliferate in response to lower concentrations of OVA peptide than RAG2^{-/-} DO11.10 (data not shown). Interestingly, their cytokine secretion is not limited to IL-17, as they also produced more IFN-γ, and to a lesser extent IL-5 and IL-13, and this cytokine expression pattern correlated nicely with a strong upregulation of RORγt and T-bet, whereas GATA3 was only marginally increased. Thus, these memory T cells are mainly primed toward the Th17 and Th1 subsets. Moreover, our observation that antibiotic treatment abolishes IL-17 production by this population upon in vitro challenge suggests that these T lymphocytes are specific for Ags from the enteric microflora. Thus, an OVA aerosol challenge of naïve DO11.10 mice triggers a secondary response of memory T cells with dual antigenic specificity, leading to an IL-17A/IL-17F-driven neutrophilic lung inflammation.

A second striking characteristic of the DO11.10 mice was that sensitization using OVA in alum failed to induce a Th2-associated eosinophilic response, in contrast with wild-type BALB/c mice. The fact that such a sensitization induced a Th2 response in RAG2^{-/-} DO11.10 mice points to these dual TCR lymphocytes as the cause of the defective Th2 response. Using anti-cytokine Ab injections before OVA sensitization and challenge, we could

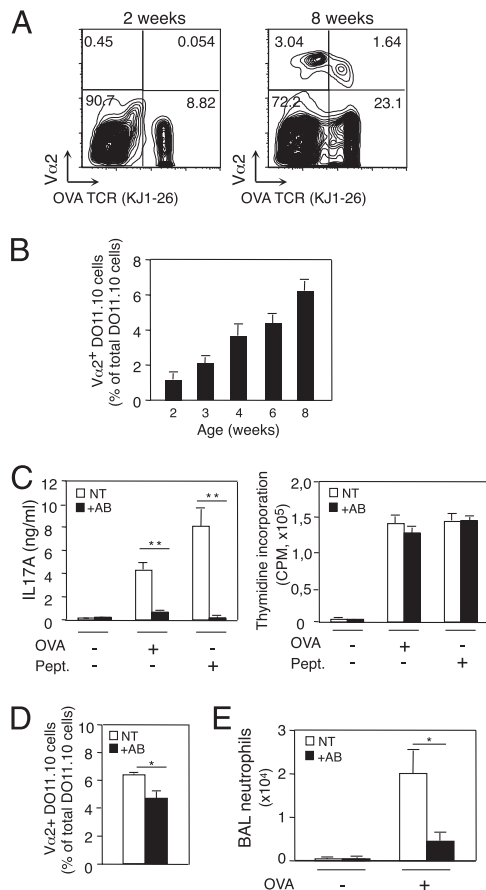


FIGURE 8. Antibiotic treatment reduces OVA-induced IL-17 production and decreases IL-17-dependent airway neutrophilia. **A**, Flow cytometry analysis of dual TCR cell onset with age. Total spleen cells from DO11.10 mice of increasing ages were stained with anti-DO11.10 TCR and anti-Vα2 Abs. Contour plots show the results for 2- and 8-wk-old representative mice. **B**, Age-dependent increase of dual TCR cell proportion among DO11.10 cells. Total spleen cells from 2-, 3-, 4-, 6-, and 8-wk-old DO11.10 mice were stained for DO11.10 OVA TCR and Vα2 domain. Data were compiled from two different experiments and show the proportion of dual TCR cells among total DO11.10 spleen cells. Mean values and SEM were calculated for each group of age (4 mice/group). **C**, OVA stimulation of DO11.10 spleen cells from antibiotic-treated mice. Cells were cultured for 72 h with or without OVA or OVA peptide. Supernatants were collected for cytokine production analysis by ELISA, and cell proliferation was evaluated by tritiated thymidine incorporation. Significance was determined by unpaired *t* test. **D**, Flow cytometry of DO11.10 spleen cells from antibiotic-treated mice. DO11.10 mice were treated since birth with antibiotics to reduce gut flora. Total spleen cells from 6-wk-old mice were stained as in **B**. **E**, BAL cell analysis of antibiotic-treated DO11.10 mice. Antibiotic-treated 6-wk-old mice were challenged twice with daily OVA aerosols. Twenty-four hours after the last challenge, BAL cells were collected and analyzed by flow cytometry. Mean values and SEM were calculated from four mice of each group. **F**, BAL cell analysis of antibiotic-treated DO11.10 mice. Antibiotic-treated 6-wk-old mice were challenged twice with daily OVA aerosols. Twenty-four hours after the last challenge, BAL cells were collected and analyzed by flow cytometry. Mean values and SEM were calculated from four mice of each group. **p* < 0.05, unpaired *t* test. Data are representative of three experiments.

demonstrate that IFN- γ , but not IL-17, is responsible for the inhibition of eosinophil recruitment. This observation that the Th17 axis did not antagonize the Th2 development contrasts with a previous report showing that the lung eosinophilic response of C57BL/6 mice sensitized with OVA and alum was inhibited by intranasal IL-17A administration, but increased by anti-IL-17A Ab treatment during the challenge phase (35). However, several other publications showed that blocking IL-17 can also, depending

on the experimental conditions, inhibit lung eosinophilia (16, 17, 36). Altogether, these observations suggest that the effect of IL-17 on the eosinophilic response is mainly indirect and might involve partially antagonistic pathways.

By contrast, our observation that IFN- γ is the major cytokine responsible for repression of the eosinophilic response is clearly in line with previous observations showing that IFN- γ administration during the challenge phase blocks this process in mice sensitized with OVA and alum (37, 38). Two different hypotheses might explain this activity of IFN- γ in our model. First, early IFN- γ secretion by memory dual TCR cells upon OVA sensitization might prevent naive OVA-specific T cells from undergoing Th2 differentiation. Alternatively, the repressor activity of IFN- γ could be mainly directed against memory Th2 cells that are present in the dual TCR population. The presence of such OVA-responsive Th2 memory T cells in the dual TCR cell population is supported by the fact that these R2 cells express slightly higher levels of GATA3 and release higher levels of Th2 cytokines (IL-5 and IL-13) upon OVA stimulation *in vitro* than naive T cells from the R3 region. Nevertheless, their higher relative expression of T-bet and ROR γ t and the amounts of IFN- γ and IL-17 produced by these cells indicate that Th2 cells remain a minority of dual TCR T cells in this model. This hypothesis that IFN- γ represses memory Th2 cells is supported by the observation that anti-IFN- γ Ab was similarly able to restore the eosinophilic response in DO11.10 mice following OVA aerosol without any sensitization (data not shown and Ref. 18), whereas RAG2^{-/-} DO11.10 mice, which only have naive T cells, do not show any eosinophilic response without sensitization (data not shown).

In addition, our data show that naive T cells are poorly activated by OVA sensitization of DO11.10 mice, and that the major part of the *in vivo* response is due to the dual TCR memory cells. This observation is in line with the theory of original antigenic sin, which assumes that the presence of memory cells prevents activation of additional naive cells upon *in vivo* administration of the Ag (39). Applied to T cells, this implies that the cytokine secretion profile should mainly depend on the conditions of their original activation. Thus, in DO11.10 mice, the cytokine profile of the OVA response is not determined by the context of OVA sensitization, but by the previous context of activation of dual TCR T cells. A large panel of antigenic determinants can be provided by the gut microflora, including bacterial strains that promote Th17 differentiation. Enteric bacterial Ags have previously been shown to activate DO11.10 T cells *in vitro* (26, 27). The fact that oral antibiotic treatment could almost completely abolish the Th17 differentiation of the dual TCR cell population from DO11.10 mice supports the role of such microflora Ags in this process.

Dual TCR T cells have been shown to play a significant role in the immune responses in several mouse models based on transgenic expression of a particular TCR. For instance, in mice transgenic for a MBP-specific TCR, tolerance can be disrupted by a viral infection that activates MBP-specific cytotoxic T cells that coexpress a TCR for viral Ags, pointing to a potential mechanism for autoimmune responses triggered by ubiquitous viral infections (40). In another MBP-specific model, autoimmune encephalitis is prevented by regulatory T cells expressing both transgenic and endogenous TCR chains (41). By contrast, in another model, dual TCR expression allowed T cells expressing low level of autospecific receptors to escape central and peripheral tolerance and to induce autoimmune responses (42).

Dual TCR T cells are not limited to transgenic mouse models, and such cells also have been described in PBMCs from normal human donors, with a frequency that has been estimated to up to one-third of T lymphocytes (32). Interestingly, most human T

regulatory cells were shown to express two different TCRs (43), pointing to a relationship between this property and differentiation toward a regulatory phenotype, and supporting some observations in mouse transgenic models (41). However, the true frequency of T lymphocytes with functional dual specificity and their significance in immune responses remain a matter of controversy. In autoimmune diseases, it has been proposed that, based on the relatively low and variable proportions of dual TCR expression, this mechanism represents one way among others by which infections can trigger autoimmunity. Our data suggest that dual TCR expression might also contribute to the effect of the environment on the response to allergens in a subset of asthma patients.

Acknowledgments

We thank Dr. Andreas Radbruch for providing mice and Dr. André Tonon for help with flow cytometry experiments.

Disclosures

The authors have no financial conflicts of interest.

References

- Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyper-responsiveness. *Annu. Rev. Immunol.* 17: 255–281.
- Renauld, J. C. 2001. New insights into the role of cytokines in asthma. *J. Clin. Pathol.* 54: 577–589.
- Kips, J. C., G. P. Anderson, J. J. Fredberg, U. Herz, M. D. Inman, M. Jordana, D. M. Kemeny, J. Lötvall, R. A. Pauwels, C. G. Plopper, et al. 2003. Murine models of asthma. *Eur. Respir. J.* 22: 374–382.
- Wang, Y. H., and Y. J. Liu. 2008. The IL-17 cytokine family and their role in allergic inflammation. *Curr. Opin. Immunol.* 20: 697–702.
- Nembrini, C., B. J. Marsland, and M. Kopf. 2009. IL-17-producing T cells in lung immunity and inflammation. *J. Allergy Clin. Immunol.* 123: 986–994; quiz 995–996.
- Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, N. Pagé, R. Olivenstein, J. Elias, and J. Chakir. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 108: 430–438.
- Zhao, Y., J. Yang, Y. D. Gao, and W. Guo. 2010. Th17 immunity in patients with allergic asthma. *Int. Arch. Allergy Immunol.* 151: 297–307.
- Barczyk, A., W. Pierzchala, and E. Sozańska. 2003. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir. Med.* 97: 726–733.
- He, R., M. K. Oyoshi, H. Jin, and R. S. Geha. 2007. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc. Natl. Acad. Sci. USA* 104: 15817–15822.
- Kim, S. R., K. S. Lee, S. J. Park, K. H. Min, K. Y. Lee, Y. H. Choe, Y. R. Lee, J. S. Kim, S. J. Hong, and Y. C. Lee. 2007. PTEN down-regulates IL-17 expression in a murine model of toluene diisocyanate-induced airway disease. *J. Immunol.* 179: 6820–6829.
- Wilson, R. H., G. S. Whitehead, H. Nakano, M. E. Free, J. K. Kolls, and D. N. Cook. 2009. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 180: 720–730.
- Hellings, P. W., A. Kasran, Z. Liu, P. Vandekerckhove, A. Wuyts, L. Overbergh, C. Mathieu, and J. L. Ceuppens. 2003. Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 28: 42–50.
- Liang, S. C., A. J. Long, F. Bennett, M. J. Whitters, R. Karim, M. Collins, S. J. Goldman, K. Dunussi-Joannopoulos, C. M. Williams, J. F. Wright, and L. A. Fouser. 2007. An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. *J. Immunol.* 179: 7791–7799.
- McKinley, L., J. F. Alcorn, A. Peterson, R. B. Dupont, S. Kapadia, A. Logar, A. Henry, C. Irvin, J. D. Piganelli, A. Ray, and J. K. Kolls. 2008. Th17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J. Immunol.* 181: 4089–4097.
- He, R., H. Y. Kim, J. Yoon, M. K. Oyoshi, A. MacGinnitie, S. Goya, E. J. Freyschmidt, P. Bryce, A. N. McKenzie, D. T. Umetsu, et al. 2009. Exaggerated IL-17 response to epicutaneous sensitization mediates airway inflammation in the absence of IL-4 and IL-13. *J. Allergy Clin. Immunol.* 124: 761–770.e1.
- Park, S. J., K. S. Lee, S. R. Kim, K. H. Min, Y. H. Choe, H. Moon, H. J. Chae, W. H. Yoo, and Y. C. Lee. 2009. Peroxisome proliferator-activated receptor gamma agonist down-regulates IL-17 expression in a murine model of allergic airway inflammation. *J. Immunol.* 183: 3259–3267.
- Wakashin, H., K. Hirose, Y. Maezawa, S. Kagami, A. Suto, N. Watanabe, Y. Saito, M. Hatano, T. Tokuhisa, Y. Iwakura, et al. 2008. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am. J. Respir. Crit. Care Med.* 178: 1023–1032.
- Knott, P. G., P. R. Gater, and C. P. Bertrand. 2000. Airway inflammation driven by antigen-specific resident lung CD4(+) T cells in alpha-beta-T cell receptor transgenic mice. *Am. J. Respir. Crit. Care Med.* 161: 1340–1348.
- Wilder, J. A., D. D. Collier, D. E. Bice, Y. Tesfayigzi, C. R. Lyons, and M. F. Lipscomb. 2001. Ovalbumin aerosols induce airway hyperactivity in naive DO11.10 T cell receptor transgenic mice without pulmonary eosinophilia or OVA-specific antibody. *J. Leukoc. Biol.* 69: 538–547.
- Tanaka, S., T. Yoshimoto, T. Naka, S. Nakae, Y. Iwakura, D. Cua, and M. Kubo. 2009. Natural occurring IL-17 producing T cells regulate the initial phase of neutrophil mediated airway responses. *J. Immunol.* 183: 7523–7530.
- Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCR α thymocytes in vivo. *Science* 250: 1720–1723.
- Billiau, A., H. Heremans, F. Vandekerckhove, and C. Dillen. 1987. Anti-interferon-gamma antibody protects mice against the generalized Shwartzman reaction. *Eur. J. Immunol.* 17: 1851–1854.
- Uyttenhove, C., and J. Van Snick. 2006. Development of an anti-IL-17A auto-vaccine that prevents experimental auto-immune encephalomyelitis. *Eur. J. Immunol.* 36: 2868–2874.
- Uyttenhove, C., R. G. Marillier, F. Tacchini-Cottier, M. Charnoy, R. R. Caspi, J. M. Damsker, S. Goriely, D. Su, J. Van Damme, S. Struyf, et al. 2011. Amine-reactive OVA multimers for auto-vaccination against cytokines and other mediators: perspectives illustrated for GCP-2 in *L. major* infection. *J. Leukoc. Biol.* 89: 1001–1007.
- Azzam, H. S., A. Grinberg, K. Lui, H. Shen, E. W. Shores, and P. E. Love. 1998. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J. Exp. Med.* 188: 2301–2311.
- Hurst, S. D., S. M. Sitterding, S. Ji, and T. A. Barrett. 1997. Functional differentiation of T cells in the intestine of T cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* 94: 3920–3925.
- Saparov, A., L. A. Kraus, Y. Cong, J. Marwill, X. Y. Xu, C. O. Elson, and C. T. Weaver. 1999. Memory/effector T cells in TCR transgenic mice develop via recognition of enteric antigens by a second, endogenous TCR. *Int. Immunol.* 11: 1253–1264.
- Zhou, P., R. Borojevic, C. Streutker, D. Snider, H. Liang, and K. Croitoru. 2004. Expression of dual TCR on DO11.10 T cells allows for ovalbumin-induced oral tolerance to prevent T cell-mediated colitis directed against unrelated enteric bacterial antigens. *J. Immunol.* 172: 1515–1523.
- Alam, S. M., and N. R. Gascoigne. 1998. Posttranslational regulation of TCR Valpha allelic exclusion during T cell differentiation. *J. Immunol.* 160: 3883–3890.
- Elliott, J. I., and D. M. Altmann. 1995. Dual T cell receptor alpha chain T cells in autoimmunity. *J. Exp. Med.* 182: 953–959.
- Heath, W. R., F. R. Carbone, P. Bertolino, J. Kelly, S. Cose, and J. F. Miller. 1995. Expression of two T cell receptor alpha chains on the surface of normal murine T cells. *Eur. J. Immunol.* 25: 1617–1623.
- Padovan, E., G. Casorati, P. Dellabona, S. Meyer, M. Brockhaus, and A. Lanzavecchia. 1993. Expression of two T cell receptor alpha chains: dual receptor T cells. *Science* 262: 422–424.
- Schrum, A. G., and L. A. Turka. 2002. The proliferative capacity of individual naive CD4(+) T cells is amplified by prolonged T cell antigen receptor triggering. *J. Exp. Med.* 196: 793–803.
- Lee, W. T., J. Cole-Calkins, and N. E. Street. 1996. Memory T cell development in the absence of specific antigen priming. *J. Immunol.* 157: 5300–5307.
- Schnyder-Candrian, S., D. Togbe, I. Coullin, I. Mercier, F. Brombacher, V. Quesniaux, F. Fossiez, B. Ryffel, and B. Schnyder. 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* 203: 2715–2725.
- Durrant, D. M., S. L. Gaffen, E. P. Riesenfeld, C. G. Irvin, and D. W. Metzger. 2009. Development of allergen-induced airway inflammation in the absence of T-bet regulation is dependent on IL-17. *J. Immunol.* 183: 5293–5300.
- Li, X. M., R. K. Chopra, T. Y. Chou, B. H. Schofield, M. Wills-Karp, and S. K. Huang. 1996. Mucosal IFN-gamma gene transfer inhibits pulmonary allergic responses in mice. *J. Immunol.* 157: 3216–3219.
- Iwamoto, I., H. Nakajima, H. Endo, and S. Yoshida. 1993. Interferon gamma regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4+ T cells. *J. Exp. Med.* 177: 573–576.
- Fazekas de St. Groth, S., and R. G. Webster. 1966. Disquisitions of original antigenic sin. I. Evidence in man. *J. Exp. Med.* 124: 331–345.
- Ji, Q., A. Perchellet, and J. M. Goverman. 2010. Viral infection triggers central nervous system autoimmunity via activation of CD8+ T cells expressing dual TCRs. *Nat. Immunol.* 11: 628–634.
- Olivares-Villagómez, D., Y. Wang, and J. J. Lafaille. 1998. Regulatory CD4(+) T cells expressing endogenous T cell receptor chains protect myelin basic protein-specific transgenic mice from spontaneous autoimmune encephalomyelitis. *J. Exp. Med.* 188: 1883–1894.
- Sarukhan, A., C. Garcia, A. Lanoue, and H. von Boehmer. 1998. Allelic inclusion of T cell receptor alpha genes poses an autoimmune hazard due to low-level expression of autospecific receptors. *Immunity* 8: 563–570.
- Tuovinen, H., J. T. Salminen, and T. P. Arstila. 2006. Most human thymic and peripheral-blood CD4+ CD25+ regulatory T cells express 2 T-cell receptors. *Blood* 108: 4063–4070.