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

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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 microbiota. 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.
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 μg OVA (chicken-egg OVA grade V, Sigma-Aldrich) with 70 μl alum (Alum Injekt; Thermo-Scientific) in PBS to reach 200 μl 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; Systm). 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-μm nylon filters. RBCs were lysed by osmotic shock. For R2 (CD5(+)KJ1-26(+) and R3 (CD5(+)KJ1-26(+) sorting or Vα2+ sorting, CD4+ spleen cells were isolated using magnetic beads (MACS; Miltenyi Biotec), stained with anti-CD5 Ab (BD Pharmingen; final concentration, 2 μg/ml) and the KJ1-26 anti-D011.10 TCR Ab (Caltag Laboratories; final concentration, 2 μg/ml), and sorted with a FACSVantage instrument. For spleen cell transfer, 80 × 106 cells (BALB/c or DO11.10 donors) and 10 × 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 × 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−/− mice 24 h before the sensitization with OVA/alum and transferred with CD4+ spleen cells from donors (RAG2+/− mice). RAG2−/− mice 1 wk before starting aerosol protocol, without any sensitization. For Vα2+ cell transfer, 1 × 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 μg/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 μg/ml and incubated for 1 h at 4°C. For BAL analysis, 5 × 106 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, -Vα2, -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 MM17F8-SEP5 mouse anti-mouse IL-17F (24). Three hundred to 500 μg each Ab was injected 24 h before starting the lung inflammation protocol.

T cell stimulation assays

In vitro stimulation of sorted R2 and R3 populations, 1.5 × 106 cells per condition were seeded in 24-well plates coated with anti-CD3 (clone 145.2C11, 5 μg/ml). Cells were stimulated for 24 h in Iscove Dulbecco’s medium supplemented with 5% heat-inactivated FBS, 0.24 mM ascoragine, 1.5 mM glutamine, 0.55 mM arginine, 50 μM 2-ME, and 2.5 μg/ml anti-CD28 (clone 37.51). A total of 4 × 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 μg/ml OVA or 1 μg/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, RORγt, 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: IL-4, 5′-GAGCAGGGTCA-GAGGAAAGG-3′ (forward) and 5′-GGACATTTCTAGGTTGAACGT- TA-3′ (reverse); IL-5, 5′-GAAATGTGTCCTGCAGTGTG-3′ (forward) and 5′-GAGGAAAGGCTTCTGGTACGT-3′ (reverse); IL-17A, 5′-GGCACCATCAGCAG-3′ (forward) and 5′-TCGAGGATATTCA-3′ (reverse); IL-17F, 5′-CTGGTACACATGTTGAC-3′ (forward) and 5′-TCCGAGGATATTCA-3′ (reverse); RORγt, 5′-CGGCTGTACCATGCATT-3′ (forward) and 5′-TGGCAGGATATTCA-3′ (reverse); T-bet, 5′-CATAAG-CAAGGAGAGAAAG-3′ (forward) and 5′-GTACACATCAGCAG-3′ (reverse); GATA3, 5′-GAGACATCTGCAAGCAG-3′ (forward) and 5′-ATCCCAGTCAAGGAGAAGT-3′ (reverse); β-actin, 5′-CTCTAGGTCGTCGTCGTCGTCG-3′ (reverse) and 5′-GCTGGAAGGGTACATGTTGACG-3′ (reverse).

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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 ± 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>+</sup> 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<sup>−/−</sup> 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 DO11.10 mice, we sorted cells corresponding to the R2 and R3 subsets from DO11.10 spleen cells. Cells were adoptively transferred into RAG2<sup>−/−</sup> 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<sup>−/−</sup> DO11.10 T cells. Taken together, these results indicate that the neutrophilic response of DO11.10 mice is mediated by a CD5<sup>high</sup> KJ1-26<sup>dull</sup> (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
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\(\alpha\)2 domain or for the DO11.10 receptor. As the transgenic receptor contains the V\(\alpha\)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\(\alpha\)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\(^{\text{high}}\) 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 naïve 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\(\alpha\)2\(^{+}\) cells from DO11.10 mice. In this population, all OVA-responsive cells should therefore express a dual TCR. BALB/c V\(\alpha\)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\(\alpha\)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 naïve 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 naïve T cells to this response is less clear. To address this question, we 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 naïve T cells became CD44\(^{\text{high}}\), reflecting their antigenic stimulation (Fig. 6A). By contrast, we could not observe any increase in the percentage of CD44\(^{\text{high}}\) when gating on KJ1-26\(^{\text{high}}\) T cells (corresponding to R3 cells) from OVA-sensitized DO11.10 mice.
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 upregulated 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. 7A, 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, whereas 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 ontogeny 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 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 expression in dual TCR cells from both donor mouse strains was significantly downregulated by minus-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. 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.

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**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).

**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).

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**FIGURE 6.** Restimulation of OVA-specific T cells results in the generation of dual TCR cells both in vitro and in vivo. A, Vα2⁺KJ1-26⁺ cells were purified and stained with 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. 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.

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suggested 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 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 stimulation 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
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 RORγt 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<sup>−/−</sup> 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

![Figure 8](http://www.jimmunol.org/)

**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 with DO11.10 OVA 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. 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. **p < 0.01. Data are representative of three experiments. 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. *p < 0.05, unpaired t test with Welch correction. 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. *p < 0.05, unpaired t test. Data are representative of three experiments.
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.

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Disclosures

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