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*J Immunol* published online 4 November 2009

http://www.jimmunol.org/content/early/2009/11/04/jimmunol.0901144

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2009/11/04/jimmunol.0901144.4.DC1

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Intranasal Immunization Promotes Th17 Immune Responses

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Th17 cells are a lineage of CD4^+ T cells characterized by IL-17 secretion, which plays a crucial role in immune responses against important respiratory pathogens, such as *Mycobacterium tuberculosis*. In this study, we demonstrated that intranasal (i.n.) immunization leads to Th17-biased immune responses, regardless of the adjuvant used. The activated CD4^+ T cells also showed an up-regulated expression of the chemokine receptor CCR6, which is a marker for murine Th17 cells. These results have important implications in the context of optimizing rational vaccine design, since i.n. immunization appears to be the strategy of choice for situations where the induction of a Th17 phenotype would be beneficial. *The Journal of Immunology*, 2009, 183: 0000 – 0000.

Mucosal vaccination is an attractive strategy for Ag administration. This approach is not associated with pain or stress, has an extremely easy and cost-efficient administration logistics, and does not require highly trained health personnel. In addition, it is not associated with any risk of cross contamination (1). The immune responses stimulated following mucosal administration differ from those elicited after parenteral vaccination. Parenteral immunization mainly leads to the stimulation of systemic responses, whereas mucosal vaccination promotes both systemic and local immune responses (2). Although many features of the immune responses stimulated after mucosal immunization have been dissected, our current knowledge on the structure and function of the mucosal immune system is still fragmentary. Therefore, it is critical to unravel the mechanisms operating during the elicitation of immune responses following vaccination by the mucosal route.

Th responses were traditionally classified as either Th1 or Th2. However, recent studies using murine models of experimental autoimmune encephalitis and collagen-induced arthritis resulted in the discovery of the Th17 lineage. This led to a new understanding of the nature of Th immune responses (3, 4). Studies on human cells have also shown an association between the expression of the chemokine receptor CCR6 and the Th17 phenotype. CD4^+ memory cells producing IL-17 also express this chemokine receptor. Cytokines responsible for Th17 cells differentiation (i.e., TGF-β, IL-6, and IL-1β) also induce CCR6 expression in vitro (5).

Different Th subsets can be preferentially stimulated during the course of natural infections caused by specific pathogens, such as Th1 for the influenza virus (6), Th2 for *Trichuris trichiura* (7), and Th17 for *Candida albicans* (8). Thus, the stimulation of the proper Th phenotype after natural infections or vaccination can be crucial for a successful clearance, as demonstrated in the *Leishmania major* infection model (9). Therefore, it would be an asset to develop strategies to simulate specific response patterns at will. In this study we showed that intranasal (i.n.) immunization leads to the induction of a Th17 phenotype and that this phenomenon does not depend on the kind of adjuvant used. This discovery is of great importance in the field of vaccinology, since it is critical to stimulate the adequate type of response to promote efficient protection, minimizing the risk of side effects. In this context, the data presented here demonstrate that the exploitation of the i.n. route would allow to selectively expand Th17 cells.

**Materials and Methods**

**Mice**

Naïve C57BL/6 animals were purchased from Harlan and were used at the age 8–16 wk. B6-PL-Thy1a/CyJ (Thy1.1) mice were purchased from The Jackson Laboratory. Thy1.2 OTII animals were bred at the animal facilities of Helmholtz Centre for Infection Research under specific pathogen-free conditions. All animal experiments have been performed in accordance with institutional guidelines and have been approved by the local government.

**Adoptive transfer**

Cells were isolated from lymph nodes and spleen (Sp) of Thy1.2 OT-II animals. B and CD8^+ T cells were depleted using PanB and CD8 magnetic beads (Dynal Biotech), according to manufacturer’s protocol. Subsequently, cells were stained with 10 μM CFSE in PBS for 5 min. The reaction was stopped by adding equal volume of FCS and further incubation for 5 min. After extensive washing, cells were resuspended in PBS and transferred to recipient Thy1.1 C57BL/6 animals by i.v. injection into the tail vein. Each animal received equal number of cells. This number varied in different experiments from 1 × 10^7 to 2 × 10^7. The following day, animals received the OVA 323–339 peptide (100 μg) of a pegylated derivative of the TLR2 agonist macrophase-activating lipopeptide 2 kDa (BPPcysPEG) by i.n. or i.p. route. Control animals did not receive Ag. Five days after immunization, animals were euthanized, and the expression of surface markers and proliferation (CFSE dilution) was analyzed by FACS on transferred cells.

**Cell isolation**

In all the experiments, mice were euthanized by CO2 inhalation. Sp were dissected and single-cell suspensions were obtained by mincing the tissue through a 100-μm nylon mesh. For isolation of lymphocytes from lungs,
mice were first perfused with 20 ml of PBS by heart puncture, and tissues were then mechanically disrupted. Erythrocytes were lysed when necessary using the ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA). For isolation of peritoneal cells, the peritoneal cavity (PerC) was flushed with 10 ml of PBS. Nasal cavity (NC) lymphocytes were isolated as described before (10) with modifications. In brief, the lower jaw and tongue were removed. Then, the upper palate together with the nasal-associated lymphoid tissue (NALT) was also removed with scalpel and tweezers. NC lymphocytes were prepared from the remaining portion of the NC, which did not contain the NALT. Epithelial cells were scratched from the NC walls and single-cell suspensions were prepared by mincing the tissue through a 100-μm nylon mesh.

**Antibodies**

The following Abs have been used for flow cytometry: anti-CD4-FITC, anti-CD4-PE, anti-CD4-PerCP, anti-CD4-allophycocyanin, anti-CD4-allophycocyanin-Cy7, anti-IFN-γ-PE, anti-CCR6-allophycocyanin and anti-CD11c-PE from BD Biosciences; anti-IL-17-FITC, anti-IL-17-allophycocyanin, anti-IL-4-allophycocyanin, anti-IL-4-PE-Cy7, anti-IFN-γ-allophycocyanin, and anti-Thy1.2-PE-Cy7 from eBioscience; and anti-CCR6-PE from R&D Systems.

**Flow cytometry and cell sorting**

Cells suspended in PBS supplemented with 5% FCS were stained with Abs and analyzed using a FACScanto (BD Biosciences). Data were analyzed using FACSdiva (BD Biosciences) or FlowJo (Tree Star). Cells were sorted on a MOFlo (DakoCytomation) or a FACSAria (BD Biosciences).

**Results**

CD4⁺ cells activated after i.n. but not i.p. immunization up-regulate CCR6

To compare differences in the expression of the chemokine receptor on CD4⁺ cells activated after mucosal or parenteral immunization, an adoptive transfer model was established. In this model, CD4⁺ cells were isolated from Thy1.2 OTII animals, stained with CFSE, and transferred to naïve Thy1.1 C57BL/6 mice. Animals were then immunized with an OVA peptide (323–339) coadministered with BPPcysPEG by either i.n. or i.p. route. Mice were euthanized after 5 days, and the expression level of CCR6 on Thy1.2⁺ CD4⁺ cells isolated from NC, lungs, PerC, and Sp was then analyzed by flow cytometry (Fig. 1). The obtained results showed that there is specific up-regulation of CCR6 on CD4⁺ cells activated after i.n. but not i.p. immunization. Approximately 10% of the adoptively transferred cells isolated from NC or lungs, which were activated after i.n. administration of the Ag express this chemokine receptor on their surface. In contrast, only 3.7, 1.6, and 1.2% of cells isolated from NC, lungs, or PerC, which were activated after i.p. immunization were positive for this molecule, respectively. We also analyzed the expression of CCR6 on Thy1.2⁺ CD4⁺ cells isolated from lymphoid organs draining the NC (i.e., CLN) and the PerC (i.e., mesenteric lymph nodes); however, we did not observe up-regulation of this molecule (data not shown). These results suggest that the analysis of CCR6 expression in the course of primary immune responses requires cells isolated from non lymphoid organs.

**Murine Th17 cells express CCR6**

Data from humans showed that there is an association between CCR6 expression by CD4⁺ cells and production of IL-17, suggesting that CCR6 is a marker for Th17 cells (5). To evaluate if this also holds true in the murine system, we evaluated IL-17 expression by CCR6⁺ or CCR6⁻ CD4⁺ cells. Since treatment with brefeldin A leads to down-regulation of CCR6 expression (data not shown), CD4⁺ cells positive or negative for CCR6 were sorted from the Sp of C57BL/6 mice, and their IL-17 expression levels were assessed by intracellular staining (Fig. 2A). The obtained results demonstrated that in the murine system an association of CCR6 with IL-17 expression also occurs. Almost 20% of CCR6 positive cells also express IL-17, whereas <1% of the CCR6⁻ cells express this cytokine. To determine whether the up-regulation of CCR6 expression by CD4⁺ cells activated after i.n. immunization is also associated with up-regulation of IL-17 expression, we performed an adoptive transfer experiment.

Thy1.2⁺CD4⁺
CFSE\textsuperscript{low}CCR6\textsuperscript{+} and CCR6\textsuperscript{−} cells generated after i.n. immunization were sorted and restimulated with ionomycin and PMA. The level of expression of IL-17 by these two different cell populations was then assessed by ELISA. The obtained results showed that the production of IL-17 by CD4\textsuperscript{+} cells generated after i.n. immunization is restricted to the CCR6\textsuperscript{+} subpopulation (Fig. 2B).

**Immunization by i.n. route leads to increased production of IL-17 by CD4\textsuperscript{+}**

To determine whether i.n. immunization is associated with specific up-regulation of IL-17 expression, we performed an intracellular staining of cytokines on adoptively transferred CD4\textsuperscript{+} Thy1.2 OTII cells activated after immunization by different routes. In these experiments, we also concentrated on CD4\textsuperscript{+} cells isolated from non-lymphoid organs since, as we observed and in agreement with data reported in the literature (11), during the course of primary immune response CD4\textsuperscript{+} cells start to produce detectable levels of IL-17, IL-4, and IFN-\(\gamma\) only after egress from lymphoid organs. The results obtained in this experiment showed that the expression levels of IL-17 are tightly dependent on the route of immunization. In lungs and NC of animals that received Ag by the i.n. route 10% of the activated cells produce IL-17. In contrast, no IL-17-producing cells were detected in the NC and only 4% in the lungs of i.p. immunized animals. Only 2.5% of adoptively transferred cells isolated from the PerC of animals that received the Ag by i.p route express this cytokine (Fig. 3A). Vaccination by both routes led to expansion of IFN-\(\gamma\)-producing cells (Fig. 3B). Nevertheless, it is difficult to compare the level of expression of this cytokine because of differences in its distribution throughout different organs. In fact, there is an increased proportion of IFN-\(\gamma\)-producing cells in lungs and PerC of i.p. immunized animals, whereas an opposite trend is observed in the NC. An increased proportion of Th2 cells was also observed in NC and lungs after i.n. immunization (Fig. 3C). This is in agreement with previous reports (12). However, this increase was not as marked as the observed increase in the number of IL-17-producing cells.

The expression of IL-17 after i.n. immunization is not dependent on the kind of adjuvant used

Additional immunization studies were performed to assess if the observed phenotype is a general characteristic associated to immunization by the i.n. route or rather related to the specific adjuvant used (i.e., BPPcysPEG). In contrast to the adoptive transfer...
experiments mentioned above, in which primary immune responses were analyzed, we examined in this set of experiments the memory cells generated after immunization. Vaccinated animals received 50 μg of the model Ag OVA mixed with different adjuvants by either i.n., i.p., or s.c. route. The third route of administration was chosen as model parenteral delivery route, because it is used to administrate vaccines to humans. The selected moieties were 1) CpG ODN, a typical Th1 inducer adjuvant (13), 2) BPPcysPEG, which was shown to induce Th2 immune responses (14), and 3) β-glucan curdlan, which is known to be a strong inducer of Th17 responses (8). Control animals received PBS or OVA alone. The numbers of IL-17, IL-4, and IFN-γ-producing cells present in splenocytes isolated from immunized animals were then assessed by ELISPOT. The obtained results demonstrated that all tested adjuvants induced strong Th17 immune responses after i.n. immunization (Fig. 4A). Neither BPPcysPEG nor CpG ODN was able to induce IL-17 production after i.p. or s.c. immunization. As expected, immunization with curdlan resulted in the induction of a Th17 phenotype regardless of the administration route. However, a higher number of IL-17-producing cells was observed after i.n. than i.p. administration. The differences in the induction of IFN-γ and IL-4 after different routes of immunization were not statistically significant, with the exception of the increased production of IFN-γ observed after s.c. immunization using either CpG ODN or curdlan (Fig. 4, B and C). These results clearly demonstrated that despite differences in the Th1/Th2 profiles dependent on the adjuvant use, i.n. immunization in presence of different adjuvants always leads to the stimulation of a Th17 phenotype. Even a prototypic stimulator of Th17 responses, such as curdlan, promoted a stronger production of IL-17 when administrated by i.n. than by i.p. route. The preferential induction of IL-17 after i.n. immunization was also observed when analyzing cells isolated from lungs of immunized animals (supplemental Fig. S1).

Induction of CCR6 expression after i.n. immunization is independent on the type of adjuvant used

To assess if the expression of CCR6 after i.n. immunization is also independent on the kind of adjuvant, we sorted CD4+ CCR6+ cells from animals vaccinated with OVA coadministered with different adjuvants (i.e., CpG ODN, BPPcysPEG, and curdlan) by either i.n. or i.p. route. Cells were then stimulated with OVA for 24 h in the presence of naive splenocytes. The level of expression of IL-17 was determined by ELISA. The obtained results showed that induction of CCR6 expression in CD4+ cells is adjuvant independent. CCR6+ cells (Fig. 5A) produce vastly more IL-17 than CCR6− cells (Fig. 5B). This holds true regardless of whether the animals were immunized by i.n. or i.p. route. However, the level of expression of IL-17 by cells isolated from the i.p. group was considerably lower than in animals receiving the Ag by i.n. route. This can be explained by the fact that in the case of i.p. immunization smaller numbers of Th17 cells are generated, which in turn results in OVA-specific CD4+ CCR6+ cells becoming a smaller portion of the whole CD4+ CCR6+ population than in case of i.n. immunization.

DC from NALT produce increased levels of IL-6

In an attempt to dissect the mechanistic events leading to a preferential Th17 induction after i.n. immunization, we assessed the levels of IL-6 produced by DC from different lymphoid organs because previous studies have demonstrated that this cytokine is involved in the induction of IL-17 (15). To this end, we sorted DC defined as CD11c+ cells from NALT, cLN, and Sp. These cells

4 The online version of this article contains supplemental material.
were stimulated with ionomycin and PMA in the presence of BPPcysPEG for 24 h. The amount of IL-6 in the culture supernatants was then measured by ELISA. The obtained results showed that DC from NALT produced more IL-6 than those from cLN or Sp (Fig. 6), thereby suggesting a role for this cytokine in preferential induction of Th17 after i.n. immunization. We then assessed the levels of TGF-β production, because this cytokine is also involved in Th17 differentiation (15). However, we did not observe an increased production of this molecule by DC from NALT in comparison to DC isolated from the other tested lymphoid organs (data not shown).

Discussion

The data emerging from this study have demonstrated that i.n. immunization leads to the induction of a Th17-biased immune response and up-regulated expression of CCR6 on the surface of CD4+ T cells and that this phenotype is independent on the kind of adjuvant used. In the last years, it was shown that IL-17 is crucial for successful immune response against certain respiratory pathogens. The administration of a neutralizing anti-IL-17 Ab to mice reduces the protective effect of vaccination against *Bordetella pertussis* (16). IL-17 also plays a crucial role in the establishment of protective pulmonary CD4+ T cell responses after vaccination or infection with *Mycobacterium tuberculosis* (17). Furthermore, taken together with the other Th17 cytokine, IL-22, IL-17 appears to be critical for maintaining local control of *Klebsiella pneumoniae*, because a reduced survival rate after pulmonary infection was observed in mice deficient in the IL-17R (18).

The only pathogen for which i.n. vaccine formulations are available is the influenza virus (1). There are numerous studies indicating differences in the protective efficacy of the immune response against the influenza virus depending on the route of vaccine administration (19, 20). However, these publications do not or only poorly explain the mechanisms leading to these differences. Moreover, it was shown that the Th phenotype is important for the outcome of infections caused by the influenza virus (6) and that in experimental flu infection models the administration of IL-17 blocking Abs results in increased weight loss and reduced survival of animals following challenge (21). These data suggest
that it would be highly desirable to stimulate a Th17 phenotype following immunization. Whether i.n. immunization against influenza indeed leads to a desired Th phenotype in humans still remains to be established, and the putative positive effect, if any, should be ascribed to serendipity rather than to a rational design. Nevertheless, additional studies are required to elucidate the role played by IL-17 in both the clearance of influenza infection and the induction of Th17 memory cells after vaccination.

Recent studies have identified cells from the innate immune system that are responsible for the production of IL-17 in the respiratory tract (22). These are NK1.1-negative NK T cells, which are especially abundant in the lungs and produce high amounts of IL-17 during the first 24 h post-LPS administration. Existence of these cells should be carefully taken under consideration in functional studies addressing the role of IL-17 in the immune responses against different pathogens. In fact, the use of knockout animals or blocking Abs would not allow discriminating between the IL-17 produced by cells from the innate or adaptive immune systems.

In conclusion, a considerable body of experimental and clinical evidence suggests that certain infections require a Th17 immune response to achieve a proper clearance and that immunization by the i.n. route could be beneficial in case of certain pathogens. Thus, our observation that a Th17 response pattern is specifically promoted by i.n. immunization has important implications in the context of optimizing rational vaccine design. However, it is critical to consider that there are also reports showing a negative influence of IL-17 during the first 24 h post-LPS administration. Existence of such a Th17 response pattern is specifically promoted by i.n. immunization has important implications in the context of optimizing rational vaccine design.

Disclosures

The authors have no financial conflict of interest.

References

FIGURE S1. Induction of IL-17 production after i.n. immunization is independent of the kind of adjuvant used. Lung cells were isolated from animals which were previously immunized with OVA in the presence of different adjuvants and re-stimulated with OVA for 24 h. For the last 6 h brefeldin A was added to the cultures. Cells were then stained for IL-17 production and analyzed by FACS. From the presented data background was subtracted.