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Early Events in Peripheral Regulatory T Cell Induction via the Nasal Mucosa

Wendy W. J. Unger,† Femke Hauet-Broere,‡ Wendy Jansen, Lisette A. van Berkel, Georg Kraal, and Janneke N. Samsom

Nasal application of soluble Ags leads to Ag-specific suppression of systemic immune responses. This tolerance can be transferred to naive mice by CD4+ regulatory T cells (T_{R} cells) from the spleen, but little is known about the induction of mucosal T_{R} cells in vivo. To investigate the induction of T_{R} cells in the nose-draining cervical lymph node (CLN), CD4+ T cells from DO11.10 OVA TCR transgenic mice were transferred to BALB/c recipients. Within 48 h after nasal OVA application, CD4+ DO11.10 T cells in CLN, but not in the peripheral lymph node, had divided. Similarly, nonmucosal (i.m.) OVA application also induced CD4+ DO11.10 T cells to proliferate in the draining inguinal lymph node (ILN), yet more vigorously and with different kinetics than the CD4+ DO11.10 T cells in CLN. Functional analysis revealed that only proliferating CD4+ DO11.10 T cells from CLN, and not ILN, could transfer tolerance to naive recipients. CD4+ DO11.10 T cells from CLN were phenotypically similar to CD4+ DO11.10 T cells from ILN, however, in CLN a higher percentage of CD25+ proliferating CD4+ DO11.10 T cells were detected compared with ILN. CD25 is not a discriminative marker for mucosal T_{R} cells because both CD25+ and CD25− CD4+ DO11.10 T cells from the CLN could suppress delayed type hypersensitivity responses in adoptive transfer. These findings demonstrate that although striking similarities exist between the differentiation of T_{R} and effector T cells, this does not include their function. We are the first to demonstrate that functional T_{R} cells, which reside within both CD25+ and CD25− subsets, can be isolated from CLN as early as 3 days after nasal OVA application. The Journal of Immunology, 2003, 171: 4592–4603.

However, little is known about the earliest events in the induction of mucosal T_{R} cells in vivo and the factors that control their differentiation from naive T cells. In a series of transplantation experiments, we have previously shown that the nasal draining lymph nodes, the cervical lymph nodes (CLN), are essential for the induction of tolerance via the nasal mucosa (6). Tolerance could no longer be induced when these lymph nodes were removed; moreover, replacement of the CLN by peripheral lymph nodes (PLN) could not restore the capacity to induce nasal tolerance. Evidently, the local microenvironment of the CLN is critical for the induction of nasal tolerance. As nasal tolerance induction is strictly dependent on the presence of the CLN (6), we postulate that T_{R} cells are generated in these CLN.

There is indirect evidence that T_{R} cells may arise in the CLN, since Hoyne et al. (7) observed a 3- to 4-fold increase in the cellularity of the CLN after nasal peptide administration, which peaked 4 days after peptide treatment and declined over time. Moreover, application of Ag to other mucosal sites, such as the intestinal and conjunctival mucosa, has been shown to induce clonal expansion of CD4+ Ag-specific T cells in the appropriate draining lymph nodes (8–11), although simultaneous T cell expansion in local and peripheral lymphoid tissue has also been reported (12, 13). Phenotypic analysis of these proliferating CD4+ T cells revealed that they were activated, as reflected by high expression of CD69 and low expression of CD45RB and CD62L (9, 10, 12). However, direct evidence for this and more detailed information on the kinetics, phenotype, and function of the proliferating T cells at these early time points after mucosal Ag application is lacking.

Therefore, in the present study we focused on the induction of T_{R} cells resulting from nasally administered Ag, using the DO11.10 OVA TCR transgenic adoptive transfer system, and compared this with a nonmucosal response, induced by i.m. application of OVA.
We have assessed the kinetics of cell division, phenotype, and cytokine production at different time points after OVA administration and determined the functionality of the OVA-specific CD4+ DO11.10 T cells.

Materials and Methods

Mice

Eight- to 12-week-old female BALB/c mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany) and kept under standard animal housing conditions. DO11.10 OVA-TCR transgenic mice on the BALB/c background, kindly provided by Prof. Dr. F. Prowier (University of Oxford, Oxford, U.K.), were bred at our own facilities and used at 8–12 wk of age. All experiments were approved by the Animal Experiments Committee of the Vrije Universiteit Medical Center (Amsterdam, The Netherlands).

Abs and Ag

The following unlabeled Abs were used: anti-CD28 (clone 37.51), anti-CD62L (clone MEL14), anti-CD38 (clone 90), anti-CD11b (clone M1/70, MAC-1), anti-MAC2 (clone M3/83), anti-MHC class II (clone M5/114), anti-F4/80, anti-CD8 (clone 53–6.7), anti-CD45R (clone H1.2F3), anti-CD45RB (clone GK1.5), anti-CD8 (clone 53–6.7), anti-CD25 (clone PC61), and clonotypic anti-TCR (KJ1-26, a gift from P. van Kooten, University, Netherlands).

Measurement of DTH responses by ear swelling

Mice were sensitized for a DTH response as described earlier (3). In short, mice were sensitized i.n. followed by 400 μg of OVA in 25 μl of saline, in the auricle of each ear. Directly before challenge, the initial thickness of the ear was determined with an engineer’s micrometer (Mitutoyo, Tokyo, Japan). DTH responses were expressed as the mean increase in ear thickness of both ears 24 h postchallenge following subtraction of ear thickness before challenge. In all experiments the ear thickness was measured in a blinded fashion.

Cytokine secretion assay and ELISA

To determine both the number of cytokine-secreting cells and the amount of cytokine produced, 72 and 96 h after i.n. or i.m. OVA administration, LN cells (5 × 10^6) were collected. LN cells (5 × 10^6) were cultured in 1 ml of DMEM (Life Technologies, Breda, The Netherlands) supplemented with 10% heat inactivated FCS (BioWhittaker, Verviers, Belgium), 50 U/ml sodium-penicillin-G (BioWhittaker), 50 μg/ml streptomycin (BioWhittaker), 2 mM L-glutamine (BioWhittaker), and 50 μg/ml 2-ME (Merck, Darmstadt, Germany) in the presence or absence of 0.5 μg/ml of OVA. After 18 h of culture, the supernatants were harvested and used for detection of cytokines. At this time the cells were labeled with a cytokine-specific high affinity capture matrix (Miltenyi Biotec, Bergisch Gladbach, Germany) for cell surface detection of secreted cytokines. After a secretion period of 45

cosal response, mice were injected with 400 μg of OVA in 20 μl of saline i.m. in each hind limb. The draining inguinal lymph node (ILN), PLN, and spleen were isolated at indicated time points and analyzed for cell division and phenotype by flow cytometry.

To induce mucosal priming, mice received 25 or 250 μg of LPS (011:B4 Escherichia coli) i.v. or i.m. followed by 500 μg of OVA in 10 μl of saline 15 min later. The draining CLN and PLN were isolated at indicated time points and analyzed for cell division and phenotype using flow cytometry.

Transfer experiments

To obtain as much T cells as possible for transfer of tolerance, the draining lymph nodes (CLN or ILN) were collected and pooled on day 3 after OVA or saline administration. Single cell suspensions were obtained by mincing the lymph nodes and incubating them in a collagenase solution (type IV; Sigma-Aldrich) for 30 min at 37°C.

For enrichment of CD4+ T cells, lymph node cells were depleted of B220+, MAC-1+, MHC class II+, and CD8+ by negative selection with Dynabeads as described above. The resulting cell population contained between 50–60% CD4+ T cells, as determined by flow cytometric analysis (FACStar; BD Biosciences). The enriched CD4+ T cells were washed and resuspended in saline, and 2 × 10^6 cells in 200 μl of saline were transferred to naive BALB/c acceptor mice by i.v. injection via the lateral tail vein.

For transfer of purified dividing CD4+ DO11.10 T cells from CLN or ILN, the enriched CD4+ T cells were stained with PE-conjugated anti-CD4 and biotinylated KJ1-26 Ab, followed by CyChrome-labeled streptavidin, and divded CD4+KJ1-26+ T cells were sorted by three-color sorting on a FACSVantage (BD Biosciences). The dividing CD4+KJ1-26+ T cell population from CLN was ~95% pure as determined by reanalysis. The purity of the dividing CD4+KJ1-26+ T cell population from ILN was ~97%, as determined by reanalysis. After washing, the cells were resuspended in saline and 10^6 cells in 200 μl were transferred to naive acceptor mice by i.v. injection via the lateral tail vein.

For transfer of CD4+ DO11.10 T cell subsets, the enriched CD4+ T cells were stained with PE-conjugated anti-CD25 Ab and biotinylated KJ1-26 Ab, followed by CyChrome-labeled streptavidin, and divided CD4+KJ1-26+ and undiveded KJ1-26- T cell fractions by three-color sorting on a FACSVantage (BD Biosciences). The dividing CD4+KJ1-26+ T cell population was ~85% pure (the remaining 15% consisted of 5% dividing CD4+KJ1-26- T cells and 10% CD25+KJ1-26− cells), as determined by reanalysis. The purity of the dividing CD4+KJ1-26+ T cell population was ~97%, and of the undiveded KJ1-26- T cells ~90%, as determined by reanalysis. After washing, the cells were resuspended in saline, and 10^6 cells in 200 μl were transferred to naive acceptor mice by i.v. injection via the lateral tail vein.

Adaptive transfer and 5,6-CFSE labeling of CD4+ DO11.10 T cells

Adaptive transfer of CD4+ DO11.10 T cells was performed as previously described (14). Briefly, lymph nodes and spleens were collected from DO11.10 mice, and single cell suspensions were obtained by mincing the spleens and lymph nodes and straining them through a 100-μm gauge. Erythrocytes were depleted from this cell suspension by incubation in lysis buffer (150 mM NH4Cl; 1 mM NaHCO3, pH 7.4) for 5 min on ice. CD4+ DO11.10 T cells were obtained by negative isolation: spleen white cells were incubated with an excess amount of anti-CD45R (anti-B220), -CD8, -MHC class II, -CD11b and -F4/80. Subsequently, positively stained cells were depleted using sheep-anti-rat coated Dynabeads (Dynal, Oslo, Norway). The resulting enriched CD4+ T cell population contained ~35–50% CD4+ DO11.10 T cells (the remaining 50–65% consisted of 20% CD4+ non-Tg T cells, 3% CD8+ T cells, 30% B cells, and 3% macrophages) as determined by flow cytometric analysis (FACStar; BD Biosciences, Mountain View, CA). Labeling of cells with CFSE (Molecular Probes) was performed as previously described (15). In short, after washing, the enriched CD4+ DO11.10 T cell population was resuspended in PBS at 10^5/ml and incubated in a final concentration of 5 μM CFSE for 10 min at 37°C. Labeled cells were washed twice with ice-cold PBS/2% FCS before being resuspended in saline and transferred to naive acceptor mice. Each recipient received 10^7 CD4+ DO11.10 T cells in 200 μl by i.v. injection via the lateral tail vein.

Tolerance induction, immunization, and mucosal priming in recipient mice

One day after adoptive transfer of CD4+ DO11.10 T cells, mice were either tolerized or immunized. For tolerization, mice received a single application of 400 μg of OVA in 10 μl of saline intranasally (i.n.). Control mice received 10 μl of saline i.n. The CLN, PLN, and spleen were isolated at indicated time points and analyzed for cell division and phenotype using flow cytometry (FACScan; BD Biosciences). For induction of a nonmucosal response, mice were injected with 400 μg of OVA in 20 μl of saline i.m. in each hind limb. The draining inguinal lymph node (ILN), PLN, and spleen were isolated at indicated time points and analyzed for cell division and phenotype by flow cytometry.
min, cells were stained with PE-labeled anti-cytokine mAb (Miltenyi Biotec) and biotin-labeled streptavidin and 7-aminoactinomycin D (7-AAD) to exclude dead cells. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences).

Quantitative ELISAs for IL-2, IFN-γ, IL-4, and IL-10 were performed using Ab pairs and recombinant cytokines from BD PharMingen according to manufacturer’s instructions.

Statistics
Data are expressed as the mean ± 1 SD. Groups are compared with control mice using one-way ANOVA followed by Student’s t test for ear swelling responses. A p value of <0.05 was considered significant.

Results
Proliferation of T cells in draining lymph nodes following a single exposure to OVA
To assess where differentiation of T<sub>R</sub> cells occurs during nasal tolerance induction, the kinetics and organ distribution of CD4<sup>+</sup> DO11.10 T cell proliferation was established. The induction of the CD4<sup>+</sup> DO11.10 T cell response in the CLN was visualized by adoptive transfer of CFSE-labeled CD4<sup>+</sup> DO11.10 T cells to naive BALB/c mice, thus increasing the frequency of OVA responder cells. We first assessed whether the higher frequency of Ag-specific T cells affected tolerance induction in recipient mice. Therefore, recipient mice that received a single OVA instillation were sensitized for a DTH response. Clearly, these mice could become tolerant as nasal OVA treatment induced only marginal ear swelling (mean increase in ear thickness was 4 ± 0.47 mm<sup>2</sup> compared with 9.38 ± 0.85 mm<sup>2</sup> in control mice treated nasally with saline).

On days 1, 2, 3, and 4 after a single application of 400 µg of OVA i.n., flow cytometry was used to track cell division of the transferred CFSE-labeled CD4<sup>+</sup> DO11.10 T cells. We compared this to the division of T cells in the thigh draining ILN elicited by an i.m. injection of 400 µg of OVA, denoted a nonmucosal response (effector response), to assess whether the results obtained are specific for a mucosal response.

A subpopulation of CD4<sup>+</sup> DO11.10 T cells had already undergone four cell divisions in the CLN by 48 h after nasal OVA administration (Fig. 1B), but no division was seen in any other PLN. One day later, some cells had undergone five cell divisions and on day 4 up to six cell divisions could be visualized within the CD4<sup>+</sup> DO11.10 T cell population (Fig. 1, C and D). Nevertheless, up to 70% of the CD4<sup>+</sup> DO11.10 T cells remained undivided, dependent on the time point assessed. In the spleen and in the PLN, no T cell division was detected during the first 3 days. On day 4, in the PLN of mice that had received OVA i.n., a subpopulation of the CD4<sup>+</sup> DO11.10 T cells had undergone five cell divisions (Fig. 1P) and in spleen three cell divisions were detected (data not shown). Intranasal saline administration failed to induce proliferation of the CD4<sup>+</sup> DO11.10 T cells in CLN at any of the time points indicated (Fig. 1, I-L).

In contrast, the injection of OVA i.m. induced a more vigorous proliferation of the CD4<sup>+</sup> DO11.10 T cells than nasal application of OVA. In the draining ILN, up to five cell divisions could be detected within 48 h (Fig. 1F). By now, division of CD4<sup>+</sup> DO11.10 T cells was also detected in PLN and spleen (Fig. 1R). On day 3, some cells in ILN had undergone six cell divisions, and the majority had undergone at least four (Fig. 1G) and by day 4,
most cells had undergone eight cell divisions (Fig. 1H). Moreover, on days 3 and 4, neither undivided nor cells that had divided once could be detected in the ILN. In contrast, the CLN contained up to 40% of undivided cells at these time points.

In sum, expansion of Ag-specific T cells in response to OVA was detected in the draining lymph nodes in both the mucosal and nonmucosal response within 2 days. Interestingly, the proliferation of CD4⁺ DO11.10 T cells in the ILN was more vigorous than of the CD4⁺ DO11.10 T cells in the CLN as demonstrated by the proliferation of all cells in ILN by day 3, whereas in CLN only a proportion of cells underwent proliferation.

Functional Treg cells can be isolated from the CLN by 3 days after nasal OVA application

We determined whether the observed difference in the kinetics of proliferation between T cells from a nonmucosal and a mucosal response also reflected a functional difference by analyzing the regulatory capacity of the proliferating CD4⁺ DO11.10 T cell subsets.

Therefore, BALB/c mice that had been adoptively transferred with CFSE-labeled CD4⁺ DO11.10 T cells, received either 400 μg of OVA i.n. or i.m. in each hind limb. Control mice received saline i.n. Three days later, CLN or ILN were collected and CD4⁺ DO11.10 T cells that had clearly divided, based on CFSE intensity, were purified using flow cytometric cell sorting. CLN cells from control mice were enriched for CD4⁺ T cells, as no division was detected in these LNs (as shown in Fig. 1, I–L). Upon transfer of each population, the naive acceptor mice were sensitized and challenged with OVA for a DTH response. As shown in Fig. 2, only dividing CD4⁺ DO11.10 T cells from CLN of nasally tolerized mice were able to significantly suppress the DTH response, indicating that they had become Treg cells. Dividing CD4⁺ DO11.10 T cells from ILN of mice that received OVA i.m. failed to induce tolerance in the recipient, as ear swelling responses were similar to those in acceptors of CD4⁺ T cells from nontolerized donors.

Thus, by 3 days after nasal OVA application, T cells with regulatory capacity reside in the proliferating CD4⁺ DO11.10 T cell population in the CLN.

Phenotypic analysis of CD4⁺ DO11.10 T cells in CLN and ILN over time

We then assessed the phenotype of the responding CD4⁺ T cells of a mucosal response by analyzing the expression of activation and memory markers in relation to cell division. We compared this to the phenotype of the CD4⁺ T cells of a nonmucosal (effector) response, which we defined as the control for normal T cell activation. The phenotype of the cells is represented either as the mean percentage of surface marker-positive CD4⁺ DO11.10 T cells, or as the mean fluorescence intensity (MFI) of surface marker expression per cell division, as analyzed by flow cytometry. For the latter, the MFI of the peak of nondividing cells is set to 100%.

CD69

A marker for early activation of T cells is CD69, which becomes up-regulated as soon as 2–3 h after Ag encounter and is gradually down-regulated after stimulation withdrawal (16, 17). The nonmucosal response was taken as a control for normal T cell activation. As expected, these cells rapidly up-regulated CD69 expression on day 1 (Fig. 3B), and also the highest frequency of CD69⁺ cells in ILN was found (Fig. 3C). Thereafter, the percentage of CD69⁺ T cells rapidly decreased, which is in line with decreasing levels of CD69 expression on positive cells with advancing cycles of cell division (Fig. 3B). T cells in CLN lagged 1 day behind T cell ILN as an increase in the frequency of CD69-positive cells was detected on day 2 compared with day 1 for those in ILN. This ties in with the accelerated rate of cell division of T cells in ILN relative to T cells in CLN (Fig. 1, F–H vs B–D).

CD44

The expression of the activation/memory marker CD44 was increased on T cells that are activated by Ag (18). In contrast to CD69, the frequency of CD44⁺ cells increased up to day 3 and remained constant to day 4 (Fig. 3E). This was mirrored by the expression levels of CD44 on positive cells (Fig. 3D). Throughout the early cycles of cell division, the CD44 expression increased. However, the expression level remained high on T cells in CLN but decreased per day on T cells in ILN. Again, similar to the proliferation pattern, the kinetics of CD44 expression on T cells in CLN lagged behind that on T cells in ILN.

CD25

Another indicator for early activation of T cells is the expression of CD25 (or IL-2Rα), although more recently it has also frequently been used as a marker to discriminate the naturally existing Treg cells (19–22). The highest proportion (40%) of dividing CD4⁺ DO11.10 T cells expressed CD25⁺ on day 2 (Fig. 3G). Thereafter, the number of positive cells declined, although much more rapidly in the ILN than in the CLN. Analysis of the pattern of CD25 expression as a function of cell division indicated that, on days 2 to 4 in both the CLN and ILN, the population could be divided into CD25high- and CD25low-expressing cells (Fig. 3, A and F). On day 2, the CD25low-expressing cells progressively up-regulated CD25...
FIGURE 3. Nasal and i.m. OVA administration rapidly activates CD4$^+$ DO11.10 T cells in the draining lymph nodes. BALB/c mice that were adoptively transferred with CFSE-labeled CD4$^+$ DO11.10 T cells, either received 400 μg of OVA i.n. or i.m., as described in the legend to Fig. 1. The CD4$^+$ DO11.10 T cells were assessed for both the frequency of expression (C, E, and G) and the MFI per cell division peak (B, D, and F), of CD69 (B and C), CD44 (D and E), and CD25 (A, F, and G) in the draining lymph nodes at the time points indicated after Ag exposure using flow cytometry. Gates were drawn to define the population in terms of CFSE intensity and surface marker expression. Next, the MFI per individual cell division peak was assessed. For each

(Figure legend continues)
expression with each cell division, while on days 3 and 4, these cells had lost CD25 expression. The pattern of CD25 expression on the CD25high-expressing cells mirrored the expression on CD25low-expressing cells. Thereafter, the expression of CD25 was down-regulated by the CD25high-expressing cells although earlier by the cells in ILN (by day 3) than in the CLN (by day 4).

Changes in CD69, CD44, and CD25 expression in control, saline-treated animals were not significant (data not shown).

CD45RB

The CD4+ DO11.10 T cells in the CLN and ILN were then examined for the expression of the memory markers CD45RB and CD62L, which are highly expressed by naive T cells and become down-regulated following T cell activation (23–25). As depicted in Fig. 4A, the CD4+ DO11.10 T cells in both CLN and ILN rapidly progressed to a more memory-like phenotype as the expression levels of CD45RB declined with cell division from day 2. This is in line with the frequency of CD45RBhigh-expressing cells, which also declined but more rapidly in ILN (from day 2) than in CLN (from day 3, Fig. 4B). On days 3 and 4 after OVA application, the majority of the CD4+ DO11.10 T cells in both CLN and ILN expressed low levels of CD45RB. Again, the decrease in both the expression of CD45RB and number of CD45RBhigh cells in CLN lagged 1 day behind that of cells in ILN, which might be the result of the accelerated rate of cell division of T cells in ILN relative to T cells in CLN.

CD62L

As expected, the frequency of CD62Lhigh-expressing cells also declined with time, both in the CLN and ILN (Fig. 4D). However, the expression levels presented a more complex picture: the expression of CD62L on the CD4+ DO11.10 T cells in CLN was down-regulated with each cell division, except for the cells that had gone through five or more divisions, which began to up-regulate CD62L expression again (Fig. 4C). On day 3, also the CD4+ DO11.10 T cells in ILN up-regulated the expression of CD62L; however, in contrast to the cells in CLN, they had not down-regulated CD62L before this (day 2). This up-regulation of CD62L expression in ILN is clearly apparent on day 3 until division 5. Thereafter, the
expression of CD62L was slightly down-regulated and on day 4 all cells expressed such levels of CD62L that are characteristic of naive cells.

**CTLA-4**

CTLA-4 is a immunoinhibitory receptor, which is highly expressed on activated T cells (26). It has been reported that signaling via CTLA-4 plays an important role in the induction of TR cells (27) and is essential for their effector function (22, 28). A large proportion of CTLA-4 is present intracellularly, and so cells were fixed and permeabilized with saponin to enhance CTLA-4 detection. The number of CTLA-4+ T cells in both CLN and ILN increased with time, peaked on day 3 (Fig. 5A), and then declined. This is reflected in the increased expression per cell division for T cells in CLN on days 2 and 3 and lower levels of expression on day 4 (Fig. 5A). In contrast, the expression of CTLA-4 on CD4+ DO11.10 T cells in the ILN is only increased on cells that had divided four or more times on days 2 and 3. On day 4, these cells displayed, similar to those in CLN, lower levels of CTLA-4. Interestingly, this is the first time the kinetics of a surface marker in the CLN precedes that of the ILN.

**CD38**

Several recent reports on TR cells demonstrated the specific expression of CD38 (29, 30). Indeed, the proportion of CD38+ T cells in the CLN increased over the time sampled, whereas we detected a decrease in the frequency of CD38+ T cells in ILN (Fig. 5D). The expression levels showed a clear subdivision of dividing cells, both in CLN and ILN, into CD38low- and CD38high-expressing cells (~75% of T cells in CLN and 90% in ILN are CD38low, whereas 25% in CLN and 10% in ILN are CD38high-expressing cells). Both, the expression levels of CD38 per cell division as well as the total percentage of CD38+ cells were increased in CLN. Despite the decrease in the percentage of CD38+ T cells in ILN, the level of expression on these cells increased with each cell division from day 3, with highest levels expressed on day 4.

Changes in CD45RB, CD62L, CTLA-4, and CD38 expression in control, saline-treated animals were not significant (data not shown).

Together, these expression data clearly show that i.n. and i.m. OVA administration rapidly activates CD4+ DO11.10 T cells in the draining LN as revealed by the rapid up-regulation of CD69, CD44, and CD25 on days 1 and 2. Both TR and effector T cells acquire a memory phenotype by day 3 as shown by the decreased expression of CD45RB and CD62L. Thus, the TR cells are phenotypically similar to normal activated T cells, with the exception that after i.m. Ag application this is induced more rapidly. However, the major phenotypic differences between mucosal and non-mucosal Ag application are the higher percentages of CD25+ and CD38+ proliferating CD4+ DO11.10 T cells in CLN from nasally tolerized mice and the prominent expression of CTLA-4 on all of these cells, which corresponds with the phenotype of some subsets of TR cells, as reported in literature.

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**FIGURE 5.** CTLA-4, but not CD38, is differentially expressed on TR cells. BALB/c mice that were adoptively transferred with CFSE-labeled CD4+ DO11.10 T cells, received 400 μg of OVA i.n. or i.m., as described in the legend to Fig. 1. The MFI per cell division peak (A and C) was assessed for CTLA-4 (A) and CD38 (B) on CD4+ DO11.10 T cells in the draining lymph nodes at the indicated time points after Ag exposure, using flow cytometry. The proportion of CTLA-4+ (B) and CD38+ (D) cells was also assessed. For MFI, the nondividing cell population is set to 100% and shown in black; adjacent gray bars represent separate cell division peaks. CD38 is heterogeneously expressed, and so CD38low-expressing cells are shown as bars, and CD38high-expressing cells are represented by squares (Fig. 5C). Graphs show the mean of four separate experiments.


**T<sub>R</sub> cells are present in both CD25<sup>+</sup> and CD25<sup>-</sup> dividing T cell subsets**

We were keen to determine whether the functional T<sub>R</sub> cells identified (Fig. 2) could be discriminated by surface marker phenotype. Two candidate markers from literature are CD25 and CD38 (19). We showed that the percentage of CD25<sup>+</sup>CD4<sup>+</sup> DO11.10 T cells in CLN was significantly higher than in ILN on day 3 after OVA application (Fig. 3G). However, this difference was less prominent for CD38 (Fig. 5D). Therefore, we further investigated whether CD25 could distinguish T<sub>R</sub> cells.

The CD4<sup>+</sup> DO11.10 T cells from CLN were separated into three subsets based on CD25 expression and CFSE intensity (dividing CD25<sup>+</sup>CD4<sup>+</sup> DO11.10 T cells, dividing CD25<sup>-</sup>CD4<sup>+</sup> DO11.10 T cells, and undivided CD4<sup>+</sup> DO11.10 T cells from tolerized mice, see Fig. 6A) and adoptively transferred to naive acceptor mice. As a control, CD4<sup>+</sup>-enriched CLN T cells from saline-treated mice were transferred. Upon transfer, the acceptor mice were sensitized and challenged for a DTH response against OVA to assess whether they had become tolerant after such cell transfer.

Clearly, the dividing CD25<sup>+</sup>CD4<sup>+</sup> DO11.10 T cells were able to suppress the OVA-specific DTH responses whereas the undivided CD4<sup>+</sup> DO11.10 T cells failed to induce tolerance in the recipient (Fig. 6B). As few as 0.85 × 10<sup>5</sup> dividing CD25<sup>+</sup>CD4<sup>+</sup> DO11.10 T were also able to suppress ear-swelling responses in recipient mice, although the suppression observed was not as homogeneous as that by the dividing CD25<sup>+</sup> subset. However, adoptive transfer of a higher number of dividing CD25<sup>+</sup>CD4<sup>+</sup> DO11.10 T did not result in an enhanced or more homogeneous suppression by this subset.

These data demonstrate that T<sub>R</sub> cells reside in both CD25<sup>+</sup> and CD25<sup>-</sup> subsets from dividing CD4<sup>+</sup> DO11.10 T cells in the CLN from nasally tolerized mice, and that, therefore, CD25 cannot be used as a marker to positively discriminate mucosal T<sub>R</sub> cells.

**Secretion of cytokines**

IL-2 and several other cytokines are known to drive the proliferation and differentiation of naive T cells. Therefore, we determined the cytokine production profile of the CD4<sup>+</sup> DO11.10 T cells in the draining lymph node after both i.n. and i.m. OVA instillation. On days 3 and 4, cell suspensions were prepared from CLN and ILN and restimulated in vitro with OVA for 18 h. Thereafter, we determined both the percentage of cytokine-secreting Ag-specific T cells per cell division using a bispecific Ab, and the cytokine production by the total LN cells using ELISA.

As shown in Table I, Cytokine-Secreting CD4<sup>+</sup> DO11.10 T Cells columns, 47% of the CD4<sup>+</sup> DO11.10T in the CLN on day 3 produced IL-2 in response to OVA, compared with 79% of the CD4<sup>+</sup> DO11.10 T cells in ILN. We detected a decline in the number of IL-2-secreting effector T cells in the ILN 1 day later to 58%, in contrast, the number of IL-2-secreting T<sub>R</sub> cells in the CLN increased to 59%. For IFN-γ, no differences were detected between the percentage of secreting OVA responders in the CLN and ILN on day 3 (~26%). However, on day 4 the percentage of IFN-γ-secreting effector T cells in the ILN subsided to control levels (7%), whereas it remained unchanged in the CLN (25%). No IL-4- or IL-10-producing CD4<sup>+</sup> DO11.10 T cells above background could be detected in CLN and ILN. Control cells did not produce any of these cytokines.

The data obtained by ELISA for IL-2 and IFN-γ production by whole LN cell suspensions revealed the same kinetics as was observed for the percentage of cytokine-secreting T cells. On day 3, cells in ILN and in CLN produced both IL-2 and IFN-γ. Only the production of IFN-γ by the cells in the ILN was decreased to control levels 1 day later, whereas the cells in the CLN maintained to produce either cytokine. On day 3, the ILN cells produced significant amounts of IL-4, despite no IL-4<sup>+</sup> cells being detected (A), but not on day 4. In the total cell suspensions from both CLN and ILN, high amounts of IL-10 were found, which

![FIGURE 6](http://www.jimmunol.org/) Both CD25<sup>+</sup> and CD25<sup>-</sup> dividing T<sub>R</sub> cells in CLN can suppress an OVA-specific DTH response. BALB/c mice were adoptively transferred with CFSE-labeled CD4<sup>+</sup> DO11.10 T cells, as described in the legend to Fig. 1, and tolerized by i.n. instillation of 400 μg of OVA. Control mice received saline i.n. A. Flow cytometric profile of the expression of CD25 on CFSE-labeled DO11.10 T cells on day 3 after i.n. OVA administration. Gates drawn indicate the sorted populations. B. On day 3, CLN were collected and highly purified dividing CD25<sup>+</sup>CD4<sup>+</sup> DO11.10 T cells, CD25<sup>-</sup>CD4<sup>+</sup> DO11.10 T cells, or undivided CD4<sup>+</sup> DO11.10 T cells from tolerized mice were adoptively transferred to naive recipients. Control mice received CD4<sup>+</sup>-enriched T cells from saline-treated mice. One day later, the recipient mice were sensitized for a DTH response by a s.c. injection of 100 μg of OVA in IFA, followed 5 days later with an injection of 10 μg of OVA in each ear. Each point represents the mean increase in ear thickness of both ears per mouse 24 h after challenge. Bars indicate the mean of each group. *, A significant difference between acceptors of tolerant cells and control mice (p < 0.001). One representative experiment of two is shown.
was comparable to the amounts produced by control cells, and therefore not Ag-specific. Together these results show that on day 3, a high percentage of IL-2- and IFN-γ-secreting Ag-specific T cells reside in the draining LN. These cells subside on day 4 in the ILN, but not in the CLN. The ELISA data mirror the data obtained by flow cytometry: whereas total cells in ILN and in CLN produce high amounts of IL-2 and IFN-γ, this is down-regulated on day 4 by cells from ILN,
but not by those from the CLN. Again, the cytokine production by cells in CLN lagged behind that of cells in ILN.

Nasal OVA administration in the presence of LPS abrogates tolerance

Several studies have reported on the prevention or abrogation of mucosal tolerance induction by applying the Ag in the presence of an adjuvant, such as cholera toxin (12, 31, 32). In this study, we have examined the induction of effector T cells by Ag application in the absence of an adjuvant, because the dosage and type of adjuvant used may have a profound effect on the outcome of the subsequent response. However, considering this we wished to determine whether mucosal priming induced similar T cell responses as immunization via the thigh muscle, and elicited mucosal priming by applying OVA in the presence of LPS. First, we assessed whether nasal OVA application in the presence of LPS abrogated tolerance induction. Indeed, as shown in Fig. 7A, the presence of 25 μg of LPS partially reversed nasal tolerance as ear swelling responses in these mice were significantly increased compared with those in tolerant mice which received OVA i.n. This low dose LPS (25 μg) did not alter the rate of cell division by the DO11.10 T cells in the CLN when compared with no LPS (Fig. 7B). However, instillation of high dose (250 μg) LPS drastically enhanced the kinetics of cell division. Moreover, the percentage of cells that remained undivided or that had divided once was significantly decreased when compared with low dose or no LPS. Until now, we were unable to identify significant changes in known surface marker expression on the dividing cells due to the addition of either dose of LPS (data not shown). In contrast, we did detect significant changes in the cytokine secretion by the dividing T cells in the CLN after instillation of OVA in the presence of 250 μg of LPS. On day 2, we found a high percentage of IFN-γ- and IL-4-producing DO11.10 T cells in the CLN after OVA instillation in the presence of LPS, whereas we did not detect any IFN-γ- or IL-4-secreting DO11.10 T cell in the absence of LPS (data not shown). To determine whether the abrogation of nasal tolerance by LPS is due to the induction of primed T cells, opposed to T R cells, we analyzed the regulatory capacity of the dividing CD4+DO11.10+ T cells that are present in the CLN after nasal administration of OVA/LPS using an adoptive transfer. As shown in Fig. 7C, dividing CD4+ DO11.10+ T cells from CLN of mice treated with OVA/LPS failed to induce tolerance in the recipient, as ear swelling responses were significantly different from those in acceptors of dividing CD4+ DO11.10+ T cells from CLN of tolerized mice.

Thus, nasal administration of OVA in the presence of LPS abrogates tolerance by inhibiting the differentiation of T R cells in the CLN, and depending on the concentration of LPS used, induces the enhanced kinetics of DO11.10 T cell division as seen in the effector T cell response.

Discussion

In the current study, we have investigated how T R cells differentiate from naive T cells in a tolerogenic mucosal response and compared this with the differentiation of naive T cells in an immunogenic response. We are the first to show that within 72 h of Ag administration via the nasal mucosa, a population of functional T R cells is induced in the nose-draining CLN. Functional T R cells were not observed in the ILN, following Ag administration via the muscles of the hind limb. Upon detailed phenotypic analysis and comparison with the nontolerogenic T cell response in the ILN, we observed that differentiation of these T R cells strongly resembles the differentiation of an effector T cell, as revealed by a similar pattern of expression of activation and memory markers. However, the kinetics of T cell division in the CLN was slower, with fewer cells undergoing fewer cell divisions than T cells in the ILN during a nonmucosal response. Furthermore, the response in the CLN was heterogeneous, as demonstrated by the segregated expression of CD25 and CD38, suggesting that a mixture of effector and T R cells may differentiate in the CLN. Nevertheless, both dividing CD25+ and CD25− CLN T cell subsets were shown to possess regulatory capacity in adoptive transfer experiments.

The T cells in the CLN had differentiated such that they possessed regulatory activity, by 72 h after mucosal Ag application. This is clearly demonstrated by their ability to suppress other T cells in nontolerant surroundings upon adoptive transfer. Possibly, functional T R cells might already be present in these LN on day 2. However, attempts failed to collect enough dividing cells from these LN that are necessary for adoptive transfer.

As a result of the differentiation of the T R cells, a characteristic pattern of cell division arises in the mucosa-draining lymphoid tissue, and is followed by division in the periphery at later time points. Interestingly, the differentiating T cells in CLN showed a delay in proliferation compared with effector T cells. This may be due to differences in Ag load or may reflect differences in activation signals from DC. Similarly to the effector T cells, mucosal priming induced by treatment of mice with OVA + LPS enhanced the kinetics of division at the highest dosage. Lower dosages, although not clearly showing this effect, did inhibit the induction of T R cells and subsequent tolerance. Recent studies suggest that APC residing in different tissues are capable of inducing distinct immune responses either due to differential expression of surface receptors or response to incoming signals (33–35). This may also be related to the differences in microenvironment, influencing the regulation and expression of surface molecules and cytokine production.

Interestingly, the phenotypic analysis per cell division peak over time revealed striking similarities between the differentiation of both effector and T R cells. As demonstrated by the up-regulation of the early activation markers CD25 and CD69, both T cell subsets acquired an activated phenotype, which was subsequently converted into a memory-like phenotype, as indicated by the decreased expression of CD45RB and CD62L. However, CTLA-4 was expressed on the T R cells during their differentiation, but only on effector T cells that had undergone four or more divisions. This may suggest that CTLA-4 may be involved differently in the induction of T R cells than in the induction of effector T cells. CTLA-4 may be required to down-regulate the T cell response irrespective of tolerogenic or immunogenic nature. However, CTLA-4 has also been reported to control suppressive function by T R cells (28).

The percentage of CD25-expressing cells in CLN is quite different from that of cells in ILN. Nevertheless, the expression of CD25 did not discriminate T R function, which is in line with our previous finding on T R cells from the spleen (5). Although the heterogeneous CD25 expression did not reflect functional heterogeneity among the T R cells from the CLN, it may distinguish a difference in specificity as observed for the splenic CD25+ and CD25− T R subsets. Whereas both splenic subsets from OVA-tolerized mice were able to suppress an OVA-specific DTH response in acceptor mice, only the CD4+CD25+ T cell subset was specific for the Ag used for tolerization (5).

In contrast to some subsets of T R cells that have been reported (36–38), these mucosal T R cells display high proliferative capacities and produce large amounts of IL-2 and IFN-γ. Why the T R cells in the CLN continue to produce these cytokines is unclear. It has been shown that during the induction of oral tolerance the
production of IFN-γ is transient (39, 40), but that it may not be essential for the induction of tolerance (40).

Again, in contrast to many reports on T<sub>REG</sub> cells (22, 29, 41), the mucosal T<sub>REG</sub> cells described here do not produce IL-10 during their induction. Upon in vitro restimulation, high levels of non-T cell-derived IL-10 were released by cells in the CLN, but also by cells in the nonmucosal ILN. The IL-10 may create a microenvironment that is conducive to the generation of T<sub>REG</sub> cells, as has been reported for T<sub>REG</sub>1-like cells (41–43). However, the production of similar levels of IL-10 by ILN cells suggests that IL-10 may be needed to down-regulate the T cell response, irrespective of tolerogenic or immunogenic nature.

The observation that functional T<sub>REG</sub> cells differentiate within 72 h after mucosal Ag application implies that within this time Ag is acquired at the mucosa by dendritic cells (DC) that migrate to the CLN and present Ag to naïve T cells. These T cells become activated and start to differentiate into T<sub>REG</sub> cells. It is likely that a complex series of events, occurring during the interaction between T cells and DC, mediates the induction of T cell tolerance. CLN are essential for the induction of nasal tolerance and cannot be replaced by PLN (6), and so the local CLN microenvironment may provide decisive signals to the DC to induce a tolerogenic form of Ag presentation. In this regard, soluble factors may be involved, in conjunction with cell surface markers such as inducible costimulator or programmed death-1, both of which have been shown to be associated with T<sub>REG</sub> induction (44, 45).

The mechanisms used by these T<sub>REG</sub> cells to suppress naïve T cells in the periphery remain to be elucidated, but may involve the infectious tolerance pathway as shown for the splenic T<sub>REG</sub> cells (5). Upon encounter and recognition of Ag in the context of an APC the T<sub>REG</sub> cell will be activated and may regulate the naïve T cell via multiple pathways. Regulation may involve either direct signaling, such as via programmed death-1 (44) or Notch (46, 47), or occur through secretion of immunosuppressive cytokines as TGF-β (48, 49) by either the T<sub>REG</sub> cells or the APC. Furthermore, mechanisms that appeared not to play a role during the induction may now be required by the T<sub>REG</sub> cells to exert their immunosuppressive function, such as the secretion of IL-10 (50). Contribution of each of these pathways is the subject of current investigation.

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References