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*J Immunol* 2000; 164:2937-2946; doi: 10.4049/jimmunol.164.6.2937
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Induction of Rapid T Cell Activation, Division, and Recirculation by Intratracheal Injection of Dendritic Cells in a TCR Transgenic Model

Bart N. Lambrecht, 2,Romain A. Pauwels, † and Barbara Fazekas de St. Groth 3 *

Dendritic cells (DCs) are thought to be responsible for sensitization to inhaled Ag and induction of adaptive immunity in the lung. The characteristics of T cell activation in the lung were studied after transfer of Ag-pulsed bone marrow-derived DCs into the airways of naive mice. Cell division of Ag-specific T cells in vivo was followed in a carboxyfluorescein diacetate succinimidyl ester-labeled cohort of naive moth cytochrome c-reactive TCR transgenic T cells. Our adoptive transfer system was such that transferred DCs were the only cells expressing the MHC molecule required for presentation of cytochrome c to transgenic T cells. Ag-specific T cell activation and proliferation occurred rapidly in the draining lymph nodes of the lung, but not in nondraining lymph nodes or spleen. No bystander activation of non-Ag-specific T cells was induced. Division of Ag-specific T cells was accompanied by transient expression of CD69, while up-regulation of CD44 increased with each cell division. Divided cells had recirculated to nondraining lymph nodes and spleen by day 4 of the response. In vitro restimulation with specific Ag revealed that T cells were primed to proliferate more strongly and to produce higher amounts of cytokines per cell. These data are consistent with the notion that DCs in the lung are extremely efficient in selecting Ag-reactive T cells from a diverse repertoire. The response is initially localized in the mediastinal lymph nodes, but subsequently spreads systemically. This system should allow us to study the early events leading to sensitization to inhaled Ag.

The Journal of Immunology, 2000, 164: 2937–2946.

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1 B.N.L. was a recipient of a scholarship of the Fund for Scientific Research Vlaanderen (F.W.O.), and was supported by a travel grant from the Horlait Dapsens Foundation, Belgium. B.F.d.S.G. was supported by a Wellcome Trust Senior Research Fellowship. This work was supported by the National Health and Medical Research Council of Australia, the Wellcome Trust, and the Medical Foundation of the University of Sydney.

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*Abbreviations used in this paper: DC, dendritic cell; CFSE, carboxyfluorescein diacetate succinimidyl ester; MCC, moth cytochrome c; PI, propidium iodide; TCM, tissue culture medium; Tg, transgenic.

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0022-1767/00/$02.00
of Ag is restricted to the injected DCs, excluding cross-presentation by endogenous APCs. Using this model, the localization and kinetics of T cell activation, division, differentiation, and reirculation were determined in the primary immune response to an Ag delivered by professional APCs in the lung.

Materials and Methods

**Mice**

Tg mice were bred and maintained under specific pathogen-free conditions at the Centenary Institute animal facility. Appropriate for all animal experimentation was obtained from the Institutional Ethics Committee at the University of Sydney. TCR Tg mice specific for the COOH-terminal epitope of moth cytochrome c (MCC<sub>27-103</sub>) in the context of I-E<sup>b</sup>, I-Eαβ<sup>b</sup>, or I-Eαβ<sup>b</sup> expressed the rearranged Vα11 and Vβ3 genes derived from the 5C.C7 T cell clone under the control of the endogenous TCR 3’ β enhancer, as previously described (21, 22). This Tg line, termed-D, expresses the transgene-encoded β-chain on >95% of CD4<sup>+</sup> T cells, and I-E-mediated thymic positive selection results in expression of the Tg α-chain (Tg<sup>α</sup>), recognized by staining with the mAb R88.1 anti-Vα11 (23)) on 50–80% of peripheral CD4<sup>+</sup> T cells, depending on the age and sex of the mouse. The remainder of CD4<sup>+</sup> T cells express an endogenously rearranged α-chain, paired with the 5C.C7 β-chain.

Mice of the 107-1 line (24), expressing I-E<sup>b</sup> and a transgene under the control of the endogenous MHC class II promoter, and the 3-62 line (24), in which expression of the same I-E<sup>b</sup> transgene is restricted to thymic epithelial cells, were originally the gift of D. Lo (Scirpiss Research Institute, La Jolla, CA). In these lines, the I-E<sup>b</sup> transgene combines with the endogenous I-E<sup>b</sup> chain from the H-2<sup>β</sup> host (C57BL/6, I-E<sup>b</sup>,o rI -E<sup>b</sup>0 chain. The 107-1 line (24), expressing I-E<sup>b</sup> transgene and presentation of MCC<sub>27-103</sub> to the same T cells in the thymus and periphery. Donors of T cells for adoptive transfer into 107-1 or 36-2 recipients, depending on the age and sex of the mouse. The remainder of CD4<sup>+</sup> T cells express an endogenously rearranged α-chain, paired with the 5C.C7 β-chain.

Mice were killed by CO<sub>2</sub>, asphyxiation, and the spleen and superficial cervical, deep cervical, parathyric, mediastinal, subcapsular, brachial, inguinal, and pampetemic lymph nodes were removed and individual cell suspensions prepared as described (25). Cells were washed twice with PBS containing 5% FCS and 5 mM sodium azide (FACS wash). Aliquots of 10<sup>6</sup> cells were stained for five-color immunofluorescence in 96-well round-bottom microtiter plates (ICN, Costa Mesa, CA). All staining reactions were performed for 30 min on ice. As CFSE fluorescence of transferred T cells is detected in the FL-1 channel, no FITC-conjugated Abs were used. For intranasal immunization, 100 µg MCC<sub>27-103</sub> peptide in a final volume of 20 µl was administered to the nares under light ether anesthesia.

**Antigens**

MCC<sub>27-103</sub> peptide, containing residues 87–103 derived from moth cytochrome c (KANERADILAYLKQATK), was obtained from the Queensland Institute of Medical Research (Brisbane, QLD, Australia).

**Isolation and adoptive transfer of TCR Tg T cells**

Pooled peripheral lymph nodes (cervical, mediastinal, brachial, subcapsular, inguinal, paraaortic, and popliteal) were harvested from 107-1 mice and cell suspensions prepared in tissue culture medium (TCM), as previously described (25). Red cell lysis, depletion of B cells and APCs, and labeling with carboxyfluorescin diacetate succinimidyl ester (CFSE) were performed as described (25). Viable cells were enumerated by eosin exclusion before transfer into unirradiated 107-1 or 36-2 recipients. On average, each recipient received 25 × 10<sup>6</sup> labeled cells i.v. via the lateral tail vein.

**Isolation of APCs**

DCs were grown from bone marrow precursors obtained from 107-1 donors using a modification of the Inaba protocol (26). In brief, bone marrow cells were resuspended in Tris-ammonium chloride lysis buffer solution (250 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5), and washed twice with TCM. Cells were incubated for 30 min on ice with a mixture of complement-fixing Abs: anti-B220 (RA3-3A1; Ref. 27), anti-CD4 (RL172.4; Ref. 28), anti-CD8 (3.168; Ref. 29), anti-MHC class II (M5/114; Ref. 30), and anti-Gr-1 (RB6-AC5; PharMingen, San Diego, CA). Prewarmed rabbit complement (C-SIX, Mequon, WI) was added for 30 min at 37°C. Cells were washed twice and resuspended in DC culture medium (TCM supplemented with 2.5 ng/ml of murine rGM-CSF (Soretoc, Kidlington, U.K.) and 0.5 µg/ml of γ-1,2-monomethyl-arginine (Calbiochem, La Jolla, CA)) at a concentration of 0.6 × 10<sup>6</sup> cells/ml in 24-well tissue culture plates (Becton Dickinson, Mountain View, CA). DCs were cultured for 7 days in 5% CO<sub>2</sub>, 37°C, and 80% humidity in 95% air. The purity of low density DCs was assessed by staining cells with antibodies to I-E<sup>b</sup>, I-Eα<sup>b</sup>, CD11c (N418), 33D1, and CD11c (N418), followed by avidin-Quantum Red (Sigma) and analyzing on a FACScan flow cytometer (Becton Dickinson). Abs were acquired from the American Type Culture Collection (Manassas, VA), prepared, and biotinylated in-house or obtained from PharMingen. Cytoxins (Cytopsins I, Shandon, U.K.) of ethanol-fixed cells were stained with M5/114, followed by secondary rabbit anti-rat HRP (Dako, Glostrup, Denmark), and signal was developed using diaminobenzidine substrate (DAB, SigmaFast; Sigma).

**Immunization by intratracheal injection of peptide-pulsed DCs**

After enrichment, DCs were pulsed for 1 h with 5 µM MCC<sub>27-103</sub> dissolved in TCM. Control DCs were incubated in peptide-free TCM. After pulsing, cells were extensively washed to remove unbound peptide, resuspending cells in 12.5 × 10<sup>5</sup> cells/ml in PBS, and adoptively transferred into the trachea of 36-2 mice that had received a cohort of -D 107-1 T cells 2 days earlier (see above). For intratracheal injection, mice were anesthetized by i.p. injection of 2.5% avertin (Sigma), and a volume of 80 µl containing 10<sup>6</sup> DCs was injected under direct vision through the opening of a micropipette. The control group of animals received an injection of 80 µl of PBS containing 100 µg MCC<sub>27-103</sub> peptide.

For intranasal immunization, 100 µg MCC<sub>27-103</sub> peptide in a final volume of 20 µl was administered to the nares under light ether anesthesia. Control animals received 20 µl PBS.

**Flow cytometry**

On days 2, 4, and 7 after the adoptive intratracheal transfer of DCs, mice were killed by CO<sub>2</sub>, asphyxiation, and the spleen and superficial cervical, deep cervical, parathyric, mediastinal, subcapsular, brachial, inguinal, and pampetemic lymph nodes were removed and individual cell suspensions prepared as described (25). Cells were washed twice with PBS containing 5% FCS and 5 mM sodium azide (FACS wash). Aliquots of 10<sup>6</sup> cells were stained for five-color immunofluorescence in 96-well round-bottom microtiter plates (ICN, Costa Mesa, CA). All staining reactions were performed for 30 min on ice. As CFSE fluorescence of transferred T cells is detected in the FL-1 channel, no FITC-conjugated Abs were used. For proliferation assays, six serial 2-fold dilutions, starting at 2 × 10<sup>5</sup> cells/well, were made in TCM in flat-bottom 96-well plates (Falcon;
Becton Dickinson). To provide saturating numbers of APCs in each well, 10^6 irradiated (1500 R) syngeneic spleen cells were added per well. Cells in a final volume of 200 μl/well were stimulated by addition of 5 nM MCC87-103 peptide. After 66 h, cells were pulsed for 6 h by addition of 0.5 μCi/well of [3H]thymidine. Cells were harvested using an automated cell harvester, and [3H]thymidine incorporation was measured in a beta scintillation counter, as described (25).

For measurement of cytokine production, bulk cultures were set up in a final volume of 2 ml in 24-well plates, using 2 × 10^5 lymph node cells, 10^6 irradiated syngeneic spleen cells as APCs, and 10 μM MCC87-103. Supernatants were collected and frozen at 48 h for IL-4 and 72 h for IL-3 and IFN-γ measurement.

Cytokine measurements

IFN-γ was measured in a capture ELISA, as described (22). The limit of detection was 0.78 ng/ml. IL-3 was measured by [3H]thymidine incorporation of the IL-3-dependent cell line R6X, as described (22). One unit/milliliter was defined as the dilution that yielded 50% of maximal [3H]thymidine incorporation. The limit of detection was 0.03 U/ml. IL-4 was measured by [3H]thymidine incorporation of the IL-4-dependent cell line CTAS in response to serial dilutions of culture supernatants (31). After incubation for 24 h, cells were pulsed and harvested as described above. A standard curve was generated using serial dilutions of murine rIL-4; 1 U/ml was defined as the dilution that yielded 50% of maximal [3H]thymidine incorporation. The limit of detection was 0.02 U/ml.

Results

Purification of DCs

DCs were generated in vitro from bone marrow progenitor cells, as described in Materials and Methods. More than 90% of cells had numerous surface extensions or veils and a typical indented or clover leaf-shaped nucleus (not shown). Staining for MHC class II revealed a pool of intracellular MHC molecules (in MHC class II compartments) in addition to surface expression (not shown) (32). On flow-cytometric analysis, cells were invariably positive for MHC class II (I-A^b and I-E^a/b), and expressed costimulatory molecules heat stable Ag, CD80, and CD86. All cells expressed the DC marker CD11c at high levels and the markers 33D1 and 3D1 at lower levels (data not shown). Thus, the cells had a phenotype typical of cultured DCs (26).

An MHC-restricted model for T cell immune responses induced by DCs

The adoptive transfer of Ag-laden splenic DCs into the trachea of a syngeneic host has been used to study T cell activation in the draining mediastinal lymph nodes of the rat (19, 33). However, this methodology carries the risk that Ag may be eluted from the transferred APCs and presented by endogenous APCs (19, 20). To avoid this technical difficulty, Ag presentation can be restricted to a subpopulation of injected cells on the basis of exclusive expression of an MHC allele required for presentation. Our experimental protocol (34, 35), designed to limit Ag presentation to the injected DCs, made use of two MHC Tg lines in which I-E^a was expressed on the H-2^b background, allowing it to pair with numerous I-E^b to form a functional I-E molecule (24, 36) capable of presenting MCC87-103 peptide to naive Tg T cells expressing the 5C7.C7 TCR. Mice from the 36-2 line, expressing I-E^a only in the thymus, sufficient for inducing tolerance to I-E in the T cell compartment, were used as adoptive hosts of purified responder Tg T cells and I-E-positive DCs. As the 36-2 host lacks expression of I-E on peripheral APCs, the injected DCs from the 107-1 donor, expressing I-E with a wild-type distribution, were the only APCs presenting MCC 87–103 peptide to naive Tg T cells expressing the I-E^b transgene, but express no I-E on peripheral APCs. On day ~2, 25 × 10^6 CFSE-labeled MCC-reactive (107-1 × -D) responder T cells were injected i.v. into 36-2 hosts. On day 0, recipients were immunized by intratracheal injection of 10^6 MCC87-103-pulsed or PBS-pulsed DCs. A control group of 36-2 mice received MCC87-103 in the absence of DCs. The response was followed in all accessible nodes and spleen on days 2, 4, and 7 after immunization.

FIGURE 1. A MHC-restricted model in which transferred I-E^b DCs are the only APCs capable of presenting MCC peptide (MCC87-103) to adoptively transferred TCR Tg T cells. Mice from the 107-1 line (in which Tg I-E^a is expressed with a wild-type distribution on all APCs, paired with endogenous I-E^b) served both as donors of I-E^b DCs for adoptive transfer and as parents of (-D) TCR Tg × 107-1F, T cell donors. Mice from the 36-2 Tg host are tolerant of I-E^b cells as a result of thymic expression of the I-E^a transgene, but express no I-E on peripheral APCs. On day ~2, 25 × 10^6 CFSE-labeled MCC-reactive (107-1 × -D) responder T cells were injected i.v. into 36-2 hosts. On day 0, recipients were immunized by intratracheal injection of 10^6 MCC87-103-pulsed or PBS-pulsed DCs. A control group of 36-2 mice received MCC87-103 in the absence of DCs. The response was followed in all accessible nodes and spleen on days 2, 4, and 7 after immunization.

T cell proliferation in draining lymph nodes after intratracheal injection of DCs

On day 0 of the experiment, 10^6 MCC-pulsed or unpulsed DCs were injected into the trachea of 36-2 mice. On days 2, 4, and 7, flow cytometry was used to track cell division of adoptively transferred CFSE-labeled CD4^+ cells in all accessible lymph nodes and spleen. Fig. 2A shows the distribution of the T cell response 2 days after intratracheal injection of MCC-pulsed DCs. In the draining mediastinal lymph nodes, a subpopulation of CD4^+ Tgα^+ T cells (specific for MCC87-103) had already undergone two cell divisions within 2 days of transfer of MCC-pulsed DCs. No division was seen in nondraining lymph nodes, including the superficial cervical, deep cervical, mesenteric, and peripheral (pooled brachial, subscapular, inguinal, and paraaortic) groups. Fig. 2B illustrates the kinetics of T cell division in the mediastinal lymph node after transfer of MCC-pulsed (upper panels) or unpulsed DCs (middle panels). On day 4, up to six cell divisions could be visualized within the CD4^+ Tgα^+ population. By day 7, the total number of divided cells had decreased substantially due, at least in part, to recirculation (see below). Immunization with PBS-pulsed DCs failed to induce a response in CD4^+ Tgα^+ T cells (Fig. 2B, middle panels). Moreover, very few CFSE-labeled CD4^+ Tgα^+ cells (i.e., those not specific for MCC87-103) underwent cell division in response to pulsed or unpulsed DCs (Fig. 2B, upper and middle panels). The presence of divided cells within the Tgα^+ gate on day 7 of the response (Fig. 2B, top right panel) was an artifact due to a poor stain for the Tgα-chain on that day (as indicated by the decrease in the intensity of fluorescence in the Tgα channel), so that divided Tgα^+ cells overlapped the region in which Tgα^+ cells would normally be located. Comparison with a day 7 plot from an experiment with a clearer Tgα stain (e.g., Fig. 4B, top right panel).
indicated that the small degree of spontaneous division within the CD4^{+} Tgα^{+} population gave a CFSE profile distinct from that in Fig. 2B.

The 36-2 host has been shown to express Tg I-E only in the thymus, and thus to be incapable of presenting MCC_{87–103} peptide (24, 36). We also tested whether donor-derived APCs were capable of presenting peptide in the experiment described above by injecting free MCC87–103 into the lower airways. A minor degree of peptide-dependent proliferation of CD4^{+} Tgα^{+} T cells was seen on day 7 (Fig. 2B, lower panels). This is consistent with the previously documented response to donor-derived B cells (34), which in this particular experiment were minor contaminants of the T cell preparation. However, the response was far lower than that to the injected DCs, with no early proliferation on day 2 and recruitment of only a few cells into one to two divisions by day 7. Thus, this response was unlikely to have influenced that to peptide-pulsed DCs, which preceded it, and was of greater magnitude.

Fig. 3 quantitatively summarizes the data derived from mediastinal nodes in the experiment described above. The total number of CD4^{+} Tgα^{+} cells was significantly higher in MCC-DC- than PBS-DC-immunized mice on all days of the response, with the peak at day 4 (Fig. 3A). Although this was in part due to cell division (Fig. 2B), specific recruitment of peptide-reactive cells to the mediastinal node was also apparent, as indicated by the increased CFSE content of the mediastinal node CD4^{+} Tgα^{+} population in the MCC-DC group compared with the PBS-DC controls on all days of the response (Fig. 3C). Calculation of the number of donor-derived cells recruited into cell division indicated that 23% of the cytochrome c-reactive CD4^{+} T cells had undergone cell division as a result of intratracheal immunization with peptide-pulsed DCs, giving rise to progeny that constituted 56% of the CD4^{+} Tgα^{+} cells at that site on day 4 (Fig. 3B). The total number and CFSE content of CD4^{+} Tgα^{+} cells were not significantly different in the MCC-DC group compared with the PBS-DC controls (Fig. 3A and C), confirming the specificity of the response to pulsed DCs.

**Proliferative response to intranasal peptide**

Initiation of a pulmonary immune response by means of intratracheal injection of a bolus of purified bone marrow DCs may not...
reflect the in vivo situation in which Ag is presented by endogenous DCs. To compare Ag presentation by injected DCs with presentation by endogenous DCs, the response to purified MCC87–103 peptide was studied in mice with a wild-type distribution of I-E that had received a cohort of CFSE-labeled MCC-reactive TgT cells. Lymph node T cells were gated based on scatter characteristics, expression of CD4, and exclusion of PI. Distribution of the response 2 days after intranasal administration of 100 µg MCC87–103. B, Kinetics of the response in mediastinal lymph nodes 2, 4, and 7 days after administration of MCC87–103 (upper panels) or PBS (lower panels).

FIGURE 4. Local response of CFSE-labeled T cells after intranasal administration of free MCC87–103 peptide to 107-1 mice with a wild-type distribution of I-E. Two days before immunization, mice received a cohort of MCC-reactive TCR Tg T cells. Lymph node T cells were gated based on scatter characteristics, expression of CD4, and exclusion of PI. A, Distribution of the response 2 days after intranasal administration of 100 µg MCC87–103. B, Kinetics of the response in mediastinal lymph nodes 2, 4, and 7 days after administration of MCC87–103 (upper panels) or PBS (lower panels).

Phenotype and recirculation pattern of T cells activated in draining lymph nodes

To analyze the pattern of T cell activation in draining and nondraining lymph nodes in response to injected DCs, five-color flow cytometry was used to relate cell division pattern (CFSE staining) in CD4+ Tgα− T cells to expression of the lymphocyte activation

FIGURE 5. Quantitative summary of data from the experiment described in the legend to Fig. 4. A, The number of donor-derived CD4+ Tgα− (left panel) or CD4+ Tgα− (right panel) T cells per 103 live mediastinal lymph node cells at various days of the response after intranasal administration of 100 µg MCC87–103 or PBS. Individual points represent the mean ± SEM from the group. B, The percentage of donor-derived CD4+ Tgα− T cells in mediastinal lymph nodes that have divided at least once after intranasal immunization with MCC87–103 or PBS. C, CFSE content, calculated as described in Materials and Methods, within donor-derived CD4+ Tgα− and CD4+ Tgα− populations in mediastinal lymph nodes. Individual points represent the mean ± SEM from the group.

pulsed DCs (Fig. 3) and free peptide presented by host DCs. In contrast to the increased number of CD4+ Tgα− cells during the course of the response to peptide-pulsed DCs, intranasal peptide caused a generalized loss of CFSE+ cells within both CD4+ (both Tgα+ and Tgα−) and CD4− populations in all lymph nodes from mice in the peptide group, as indicated by a decrease in CFSE content (compare Figs. 5C and 3C). This loss suggested a nonspecific toxic effect of the peptide. As a result, the number of CD4+ Tgα− cells in the mediastinal lymph nodes was the same in the peptide and PBS groups 4 days after immunization, despite the substantial amount of cell division in the peptide group (Fig. 5A). Comparison of the quantitative data in Fig. 5B with that in Fig. 3B indicated that the response to this dose of intranasal peptide stimulated earlier cell division and that a larger total proportion of peptide-specific cells had undergone division at each time point. Thus, 45% of Tgα− cells had a CFSE division pattern consistent with at least one cell division by 2 days after immunization with MCC87–103, the figure rising to 82% by day 4. The proportion of mediastinal node CD4+ Tgα− cells recruited into cell division by day 4 was 39%.
markers CD69 (Fig. 6, A and B) and CD44 (Fig. 6C). CD69 can be up-regulated as early as 2 h after Ag encounter (our unpublished findings; Ref. 37). Ag-reactive cells demonstrated enhanced CD69 expression before undergoing cell division, as evident from the high level of expression by undivided cells on day 2 after injection of peptide-pulsed DCs (Fig. 6A, top left panel). At that time, CD69 was also expressed by all the divided CD4+ Tgα−Tgα+ cells, the total representing 76% of the CFSE− CD4+ Tgα− cells in the mediastinal lymph nodes, compared with 1.4% of cells in animals immunized with unpulsed DCs. No significant up-regulation was seen in the inguinal lymph nodes (Fig. 6A, top right panel) or any of the other nondraining nodes (superficial and deep cervical, subscapular, brachial, parathymic, and mesenteric, not shown). By day 4 of the response, a significant number of divided cells no longer expressed CD69, total expression having decreased to 34.4% of donor-derived CD4+ Tgα− cells (Fig. 6B). The pattern of CD69 expression as a function of cell division on days 2 (Fig. 6A), 4 (Fig. 6B), and 7 (not shown) indicated that expression was progressively down-regulated upon each cell division, dividing cells eventually losing expression altogether.

In contrast, expression of the activation/memory marker CD44 (38) was increased with each cell division cycle, cells that had undergone the most divisions having the highest level of expression (Fig. 6C showing the expression of CD44 by CD4+ Tgα− T cells at day 4 of the response). Again, this relationship was observed irrespective of the time after the initiation of the response (not shown).

By day 4, divided CFSE− CD4+ Tgα− T cells were present in nondraining nodes and spleen (Fig. 6, B and C, and not shown). The intensity of CFSE staining in these cells revealed that they had undergone a minimum of four cell divisions, suggesting that they had recirculated to nondraining nodes and spleen after dividing in the draining nodes. Interestingly, cells that had recirculated to distant sites such as the peripheral lymph nodes were uniformly negative for expression of CD69, while retaining high expression of CD44. Recirculating T cells generated by immunization of I-Eφ-positive 107-1 mice with free MCC87−103 peptide (either intranasal, s.c., or i.v.) have the same phenotype (A. L. Smith and B. Fazekas de St. Groth, unpublished data).

In vitro restimulation of lymph node T cells

On day 4 after intratracheal injection of peptide-pulsed or unpulsed DCs, lymphocytes were purified from the draining mediastinal and pooled nondraining lymph nodes and restimulated in vitro with MCC87−103 peptide in the presence of irradiated spleen APC from I-Eφ (107-1) donors (Fig. 7A). In mice primed with peptide-pulsed DCs, enhanced [3H]thymidine uptake was observed in lymphocytes from draining mediastinal lymph nodes compared with nondraining nodes. Enhanced proliferation was also evident in the absence of restimulation with exogenous MCC87−103 (not shown), suggesting that the lymphocyte preparation contained Ag derived from the peptide-pulsed DCs or that proliferation of peptide-specific T cells continued in vitro in the absence of additional peptide. The response of lymphocytes from the nondraining nodes of mice immunized with peptide-pulsed DCs was comparable with that of both draining and nondraining nodes of mice immunized with unpulsed DCs. The magnitude of this response is consistent with that of the primary in vitro response of naive, high affinity peptide-specific T cells (22).

As a further measure of lymphocyte priming by DCs, in vitro secretion of cytokines by lymphocytes from draining and nondraining nodes was determined (Fig. 7B). Production of IL-3 and IFN-γ was specifically increased in the draining lymph nodes of animals immunized with peptide-pulsed DCs compared with unpulsed DCs. In nondraining nodes, there was an increase in the level of IL-3 (but not IFN-γ) production in animals in the peptide-DC group compared with the control DC group. Overall, the level of IL-4 production was very low and no immunization-
dependent increase was seen in this experiment in either draining or nondraining nodes.

Discussion

The activation of naive T cells in vivo is difficult to detect because of the low frequency of high affinity Ag-specific T cells. We have previously generated TCR Tg mice in which a high proportion of the low frequency of high affinity Ag-specific T cells. We have

The adoptive experimental model used in this study (Fig. 1) mimics the initiation of the adaptive immune response in the lung by DCs, the most relevant APCs of the pulmonary immune response (2, 5). Myeloid DCs were grown from bone marrow progenitors, pulsed with the model Ag MCC87–103 in vitro, and injected into the trachea of unirradiated, semiallogeneic mice, avoiding the problems of interpretation related to earlier semiallogeneic DC adoptive transfer models in which the host animals were immunodeficient and therefore had abnormal lymphoid microarchitecture (40). Our approach required a normal host tolerant of semiallogeneic donor DCs, such as the Tg 36-2 line, expressing the I-E molecule only in the thymus, but not on peripheral APCs (33). In further studies, we have confirmed rapid migration of DCs injected into the trachea using green fluorescent protein-transfected mouse bone marrow-derived DCs (B. N. Lambrecht and R. A. Pauwels, manuscript in preparation). Migrated DCs could be detected in the mediastinal nodes 24 h after injection, but were no longer detectable in any lymphoid structure 48 h later. In the current study, we did not observe T cell division or CD69 expression in nondraining lymph nodes or spleen at day 2 of the response, providing further evidence of the highly localized nature of T cell priming by DCs (15, 43, 44).

By day 4 of the response, some Ag-specific T cells in the mediastinal lymph nodes had undergone as many as six cell divisions. Expression of the memory marker CD44 increased with increasing cell division number, whereas expression of CD69 decreased slightly until the fourth, and thereafter showed an abrupt drop to baseline level (Fig. 6). The early induction and subsequent decrease in CD69 expression upon activation of Ag-specific T cells have been described by us and others and occur irrespective of the outcome of T cell activation (tolerance or immunity) (22, 35, 47). When lymphocytes from the mediastinal nodes were restimulated in vitro with MCC87–103 pulsed DCs, this is an illustration of the extraordinary capacity of DCs to specifically select and activate Ag-reactive T cells from a diverse repertoire of T cells, without inducing bystander activation (1, 42). The rapidity of the T cell response in the mediastinal nodes is in agreement with published studies of the kinetics of DC migration into draining lymph nodes (13, 15). Others have shown that spleen DCs injected into the rat trachea reach the T cell area of mediastinal and parathymic lymph nodes within 24 h of transfer (33). In further studies, we have confirmed rapid migration of DCs injected into the trachea using green fluorescent protein-transfected mouse bone marrow-derived DCs (B. N. Lambrecht and R. A. Pauwels, manuscript in preparation). Migrated DCs could be detected in the mediastinal nodes 24 h after injection, but were no longer detectable in any lymphoid structure 48 h later. In the current study, we did not observe T cell division or CD69 expression in nondraining lymph nodes or spleen at day 2 of the response, providing further evidence of the highly localized nature of T cell priming by DCs (15, 43, 44).

FIGURE 7. A. In vitro proliferation of mediastinal (filled symbols) and nondraining (open symbols) lymph node lymphocytes, taken 4 days after immunization with MCC-pulsed (squares) or unpulsed DCs (circles). Cultures of 2 × 10^7 T cells were restimulated with 5 nM MCC peptide in the presence of syngeneic I-E^+^ APCs (lacking in 36-2 hosts; see Materials and Methods). Proliferation was measured 72 h later by [3H]thymidine incorporation. B. Cytokine production during in vitro restimulation (at 10 mM peptide) of the cells described in A, taken 4 days after immunization with MCC-pulsed (filled bars) or unpulsed DCs (open bars). Supernatants were assayed after 48 h for IL-4 and 72 h for IL-3 and IFN-γ using bioassay (IL-3, IL-4) and ELISA (IFN-γ). In groups of peripheral lymph nodes, IFN-γ and IL-4 levels were not detected (N.D.) above the limits of the assay.
The peptide-MHC interaction is involved, such as that between invariant Th2 responses in the airways when a low affinity TCR-previously (48). Our recent data suggest that DCs induce predominately Th1 or Th2 responses of non-Tg and TCR Tg animals. By high affinity TCR-peptide-MHC interactions has been noted (50). Thus, in our model, the bias toward Th1 or Th2 is primarily a function of the TCR affinity, rather than a difference between the responses of non-Tg and TCR Tg animals.

Between days 2 and 4, divided Ag-specific CD4+ T cells recirculated from the mediastinal lymph nodes to the nondraining nodes and spleen. Primary division in the nondraining nodes appeared very unlikely, because the cells detected in blood (not shown) and nondraining sites (Fig. 6) had all divided at least four times. The phenotype of recirculating cells was remarkable in that they expressed uniformly high levels of the memory marker CD44 while lacking expression of the activation marker CD69. This phenotype is consistent with that of differentiated effector T cells (38, 49). Absence of CD69 expression by recently divided, recirculating T cells is seen irrespective of the route of immunization (i.e., s.c., i.v., intratracheal) or the presence of adjuvant at the injection site (unpublished observations and this study). It is striking that thymocytes also down-regulate expression of CD69 before emigrating from the thymus (50), suggesting that the correlation between down-regulation of CD69 and cell recirculation after Ag recognition may be of fundamental importance.

The association between the cell division profile and regulated expression of activation/memory markers (Fig. 6) is reminiscent of the correlation between differentiation of both T and B lymphocytes and the cell cycle (51–54). Thus, the probability of isotype switching to IgG1 (51), IgE (52), IgG2a, IgG2b, IgG3, and IgA (61) is a function of the number of cell divisions, irrespective of the time after stimulation. The production of both Th1 and Th2 cytokines is also a function of cell division rather than time (53, 54). Further in vivo studies will be required to establish whether expression of activation markers, migratory behavior, and pattern of cytokine synthesis are all a strict function of cell division number rather than time after stimulation, and whether this relationship is preserved after activation by various routes, doses, and formulations of Ag administration. It will also be interesting to study whether the program of T cell recirculation following the primary immune response can be modified by interference with the cell cycle, or epigenetic control of gene regulation (54).

The data described in this study strongly support the notion that naive, Ag-specific T cells can be primed in vivo by a single intratracheal injection of DCs. This conclusion is supported by evidence from a number of functional tests, including the use of CFSE to visualize T cell proliferation in situ and measurement of proliferation and cytokine production of T cells in vitro. Although contaminating donor-derived B cells appeared to have stimulated a minor degree of proliferation after direct intratracheal injection of free peptide, the response was both later and smaller than that to pulsed DCs, indicating that they would be very unlikely to serve as efficient presenting cells for peptide eluted from pulsed DCs. The use of a semiallogeneic adoptive transfer also excluded the possibility of elution of peptide to endogenous APCs. It was recently demonstrated that transfer of class II-restricted Ag from injected DCs to endogenous DCs can occur after s.c. injection of apoptotic cells, leading to efficient presentation of Ag by lymph node DCs (20). Our experimental model excludes transfer of Ag from injected to endogenous DCs, because the latter do not express the MHC molecule required for presentation. However, the model does not exclude transfer of already formed peptide-MHC complexes between injected and endogenous DCs, a phenomenon that has recently been demonstrated in vitro (55). As mentioned above, our as yet unpublished experiments have demonstrated direct migration of myeloid DCs expressing green fluorescent protein from the trachea to the mediastinal node (B. N. Lambrecht, manuscript in preparation) and of fluorescein-labeled myeloid DCs from the footpad to the popliteal node (35). However, there is evidence that peptide-MHC complexes from lymphoid DCs can be recognized in the draining node in the absence of viable, donor-derived lymphoid DCs (35), suggesting an alternative route of presentation for Ags associated with nonmammalian or nonviable APCs. It should also be noted that none of the above mechanisms are mutually exclusive, and since all serve to enhance the traffic of T cell antigenic epitopes from the periphery to the site of the primary immune response, they may all be involved in optimizing Ag presentation in vivo.

Previous investigators have induced T cell priming in response to instillation of DCs purified from the spleen (19). Although the functional status of splenic and bone marrow-derived DCs may differ from that of DCs isolated directly from the lung (56, 57), it is not clear whether any differences between lung-derived and spleen- or bone marrow-derived DCs would be retained in the face of conditioning by the microenvironment after intratracheal administration. Moreover, even the behavior of reinstitled ex vivo lung DCs may not provide an exact mimic of that of undisturbed resident lung DCs. For example, previous studies have demonstrated that splenic myeloid DCs home to the splenic T cell zones, rather than the marginal zone in which they are usually located (58), after purification and i.v. administration (59). Their behavior appears to be altered by the purification process itself, which, like administration of adjuvants such as LPS, stimulates maturation and migration (16).

A second difficulty in interpreting the response to injection of a bolus of DCs into the deeper airways is that this mode of administration may not reflect the in vivo situation in which Ag may be intercepted and presented by endogenous DCs in both the upper and lower airways. To examine this aspect, the response to purified MCC87–103 peptide administered intranasally was compared with that to prepulsed intratracheal DCs. The primary proliferative response to intranasal peptide in I-E+ (line 107-1) hosts was more widespread, involving the nodes draining the nose, trachea, lung, oral cavity, and to a lesser extent the gut, presumably as a result of both inhaling and swallowing the peptide. Indeed, it was recently shown that oral administration of MCC87-103 peptide leads to local activation of T cells in the mesenteric lymph nodes of Tg mice expressing the SC.C7 β-chain paired with an endogenous reper- toire of α-chains (46). After intranasal peptide, multiply divided Ag-specific CD4+ CD69− CD44high cells were first seen in non-draining sites on day 4 (not shown), suggesting a very similar pattern of activation, division, and recirculation to that induced by intratracheal peptide-pulsed DCs (Figs. 2 and 6). The primary response to intranasal peptide was somewhat faster and recruited a higher proportion of Ag-specific T cells into cell division, presumably because peptide was presented by a much larger number of host-derived MHC class II DCs migrating from the airways to the draining nodes (6, 13). However, the total number of CD4+ Tg+ cells in the mediastinal lymph nodes at the peak of response was substantially lower than in MCC-DC-immunized mice, as a result of two independent factors. First, a nonspecific toxic effect of soluble peptide (Fig. 5C) was manifest as an Ag-nonspecific decrease in donor-derived T and B cell numbers after administration of peptide by the intranasal route. We have noted a similar decrease after i.v. administration of free peptide (A. L. Smith and B. Fazekas de St. Groth, unpublished data). The mechanism of this effect


