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T Cell–Dendritic Cell Interaction Dynamics during the Induction of Respiratory Tolerance and Immunity

Nadja Bakočević, Tim Worbs, Ana Davalos-Misslitz, and Reinhold Förster

Dendritic cells (DCs) residing in the lung are known to acquire inhaled Ag and, after migration to the draining bronchial lymph node (brLN), to present it to naive T cells in an either tolerogenic or immunogenic context. To visualize endogenous lung-derived DCs, we applied fluorescent latex beads (LXs) intratracheally, thereby in vivo labeling the majority of phagocytic cells within the lung. Of note, LX-bearing cells subsequently arriving in the draining brLN were found to represent lung-derived migratory DCs. Imaging explanted brLN by two-photon laser-scanning microscopy, we quantitatively analyzed the migration and interaction behavior of naive CD4+ T cells and endogenous, lung-derived DC presenting airway-delivered Ag under inflammatory or noninflammatory conditions. Ag-specific naive CD4+ T cells engaged in stable as well as transient contacts with LX-bearing DCs in both situations and displayed similar overall motility kinetics, including a pronounced decrease in motility at 16–20 h after antigenic challenge. In contrast, the comparative analysis of T cell–DC cluster sizes as well as contact durations strongly suggests that lung-derived migratory DCs and naive CD4+ T cells form more stable, long-lasting contacts under inflammatory conditions favoring the induction of respiratory immunity. The Journal of Immunology, 2010, 184: 1317–1327.

The airways are continuously exposed to numerous environmental particles, such as dust, smoke, and pollen, as well as various pathogens of bacterial or viral origin. Representing a common pathogenic consequence of these complex antigenic challenges, allergic airway diseases have been hypothesized to develop during instances when the immune system of the lung fails to correctly discriminate between innocuous and harmful Ags. Over the last four decades of the twentieth century, the prevalence of allergic asthma increased substantially in industrialized countries (1), making a more detailed understanding of the pathogenic mechanisms of this allergic disease highly desirable.

Dendritic cells (DCs) are professional APCs that function as key players during the induction phase of adaptive immune responses, directing the outcome toward either tolerance or immunity (2–5). Airway DCs in particular have been shown to be indispensable for the induction and maintenance of Th2-dependent airway inflammation (6). In the lung, immature DCs are located both within and right beneath the surface of the respiratory epithelium, thereby being ideally positioned for the efficient sampling of airborne Ags (7). Upon capture of innocuous Ag in the absence of so-called pathogen-derived “danger signals,” lung-resident DCs are thought to acquire a “semimature” phenotype that includes intermediate expression of costimulatory molecules, as well as high levels of MHC class II molecules (reviewed in Ref. 4), while migrating toward the draining bronchial lymph node (brLN). Consequently, Ag presentation by these semimature DCs is assumed to result in T cell tolerance, whereas bacterial or viral pathogens induce full maturation of DCs with subsequent generation of strong effector T cell responses. Interestingly, however, proliferation of Ag-specific T cells is occurring in both situations, as confirmed by several in vitro and in vivo studies (4). Furthermore, during the induction of both tolerance and immunity, the trafficking of skin DCs toward the draining lymph nodes (LNs) was shown to crucially require the upregulation of CCR7 (8). In a recent study, we have demonstrated that, in the absence of inflammatory signals, the induction of respiratory tolerance to harmless aerogenic Ags depends on the function of CCR7 as well and is mediated by migratory DCs that transport Ag from the lung to the brLN (9).

Thus, the migratory capacity of DCs is likely to play an important role in the development of both tolerance and immunity (10).

Employing diverse experimental models, several studies have compared the kinetics of early T cell–DC interactions during the development of both tolerance and immunity (11–13). Up to now, no consensual model has been established for the interaction behavior of T cells and DCs during the onset of tolerance and immunity. Furthermore, T cell–DC interactions during the development of immune responses triggered by airway Ags have not been evaluated.

In this study, we provide a detailed analysis of the dynamic behavior of CD4+ T cells within the brLN during the induction of respiratory tolerance and immunity, using two-photon laser-scanning microscopy. The intratracheal (i.t.) application of fluorescently labeled latex beads (LXs) coupled with Ag allowed the simultaneous visualization of endogenous migratory DCs and the characterization of their interactions with T cells within explanted brLN. We found the overall migration behavior of Ag-specific CD4+ T cells being activated in the brLN under tolerance- or immunity-inducing conditions to be highly comparable regarding general motility parameters such as cellular velocity or motility coefficients. In both situations, the T cells underwent a phase of largely reduced motility while interacting with Ag-presenting DCs 16–20 h after Ag challenge. Interestingly, however, the quantitative analysis of the size and stability of T cell–DC clusters revealed that stable, long-lasting contacts were clearly more predominant under conditions leading to immunity. These data...
Materials and Methods

**Mice**

CCR7<sup>+</sup> (14) as well as DO11.10 mice were bred and maintained under specific pathogen-free conditions at the central animal facility of the Hannover Medical School and were used at the age of 8–12 wk. BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All animal experiments were conducted in accordance with local and institutional guidelines.

**i.t. instillation of LXs, OVA-loaded LXs, and OVA-loaded LXs+LPS**

Mice were anesthetized i.p. with 0.2 mg ketamine and 0.02 mg xylazine/g body weight. Subsequently, a blunt cannula (0.7 × 19 mm; Intromed; B. Braun, Melsungen, Germany) was instilled through the larynx into the trachea. The total injected volume was 60 µl for all i.t. applications. Plain yellow-green 0.5 µm LXs (Polyscience, Warrington, PA) were instilled at a concentration of 3.6 × 10<sup>11</sup> particles/ml, with each animal receiving ∼4.3 × 10<sup>6</sup> LX particles. OVA (OVA, Grade VI; Sigma-Aldrich, St. Louis, MO) was passively adsorbed onto 0.5 µm LXs according to the manufacturer’s directions and ∼4.3 × 10<sup>6</sup> OVA-loaded LXs (OVA-LX) were applied i.t. where indicated. All batches of OVA were tested for the presence of LPS (LAL QCL 1000; BioWhittaker-Cambrex, Walkersville, MD), and only batches containing <5 EU/ml were used for adoration loading. Some animals additionally received 10 µg LPS (LPS, Sigma-Aldrich) i.t. where indicated.

**Flow cytometry**

For flow cytometric analysis, lungs and brLNs were cut into small pieces and digested for 30 min (brLN) or 60 min (lung) at 37°C in PBS containing 0.5 mg/ml collagenase A and 20 U/ml DNase I (both from Roche Applied Science, Indianapolis, IN). To obtain single cell suspensions, tissues were subsequently minced through a nylon mesh and washed with PBS supplemented with 3% FCS. Leukocytes isolated from lungs were additionally purified using a Lympholyte M-gradient (Cedarlane, Hornby, ON, Canada). Cells were stained using the following Abs: anti-CD4<sup>+</sup>-Cy5 (clone 5C7; BD Biosciences), anti-CD11b-PECy7 (M1/70), all purchased from BD Pharmingen (San Diego, CA). Biotinylated MHC class II (MHCI [I-A<sup>d</sup>]-biotin (AMS-32.1), and anti–CD11c-APC (HL3), anti–CD62L-mAb (clone MEL-14) i.v. to prevent any further LN homing of lymphocytes. At 16–20 h as well as 36–40 h after i.t. application, the recipient mice were sacrificed and the brLN was explanted. After fixation in a custom-built incubation chamber, using tissue glue (Abbott, Abbott Park, IL), the brLN was cut into thin sections, superfused with oxygenated (95% O<sub>2</sub>, plus 5% CO<sub>2</sub>) RPMI medium containing penicillin/streptomycin (Life Technologies, Rockville, MD; Grand Island, NY). The temperature was monitored directly at the brLN and maintained at 37°C. Two-photon laser-scanning microscopy was performed using an upright Leica Microsystems (Wetzlar, Germany) DM LFSA microscope equipped with a 20 × 0.95 NA water immersion objective (Olympus) and a MaiTai Ti:Sapphire amplified Ti:sapphire laser (Spectra-Physics, Darmstadt, Germany). For simultaneous two-photon excitation of yellow-green LXs and TAMRA, the MaiTai laser was tuned to 858 nm. Green and red fluorescence emission was detected with non-descanned detectors fitted with 535/50 and 610/75 bandpass filters. Two-photon laser-scanning microscopy was performed using an upright Leica Microsystems (Wetzlar, Germany) DM LFSA microscope equipped with a 20 × 0.95 NA water immersion objective (Olympus) and a MaiTai Ti:Sapphire amplified Ti:sapphire laser (Spectra-Physics, Darmstadt, Germany). For simultaneous two-photon excitation of yellow-green LXs and TAMRA, the MaiTai laser was tuned to 858 nm. Green and red fluorescence emission was detected with non-descanned detectors fitted with 535/50 and 610/75 bandpass filters, respectively. To generate time-lapse series, stacks of 11 images (with 1.5–2.5 × electronic zoom and 3.9- to 6-µm z-spacing) were acquired every 15 s, yielding total imaging volumes of 215–360 µm in z and 44–66 µm in z.

**Data analysis**

Imaris (Bitplane, Zurich, Switzerland) was used for four-dimensional image analysis and automated tracking of cells. The accuracy of the automated tracking was manually controlled, and only tracks with durations >60 s were included in the analysis. Average cell velocities and arrest coefficients were calculated in Imaris; mean displacement plots, motility parameters, and turning angle distributions were calculated using Excel (Microsoft, WA). For determination of the arrest coefficient and the parameters of individual spot positions, the arrest coefficient represents the percentage of time points of a given track for which the instantaneous velocity of the cell is <2 µm/min (11); for details on the other motility parameters, see Ref. 16. For the analysis of DC–T cell cluster formation, individual DC–T cell interactions were defined as stable when present over the entire imaging interval (22.5–25 min), whereas contacts with a duration of >75 s (more than five time points) were defined as transient. Statistical analysis was performed with GraphPad Prism 4 (GraphPad Software, La Jolla, CA). All significant values were determined using the unpaired two-tailed t test.

**Results**

Specific labeling of endogenous lung-derived DCs in the brLN after i.t. application of fluorescent LXs

After trafficking toward the draining brLN, lung-derived migratory DCs are assumed to initiate primary T cell immune responses
directed against inhaled Ags. To visualize these DCs during Ag presentation in the brLN by in situ imaging, we applied LX i.t., as it had been previously described that LX-bearing cells, presumably representing lung-derived DCs, arrive in the brLN following i.t. (9) or intranasal application of LX (17). To further characterize this in vivo labeling of endogenous cells, we applied $4.3 \times 10^9$ yellow-green fluorescent LX (0.5-μm diameter) i.t. and phenotypically analyzed LX-bearing cells (LX⁺ cells) of both lung and brLN by flow cytometry. In the lung, the majority of LX⁺ cells were found to be either CD11c⁺CD11b⁻ macrophages (18), CD11b⁺CD11c⁻ granulocytes (17), or CD11c⁺MHCIIhi DCs (Fig. 1A), indicating that all major leukocyte populations with phagocytic capacity take up LX within this tissue. Only a minor population of LX-bearing cells were CD103⁺CD11c⁺ DCs. In contrast, LX⁺ cells in the brLN were mostly confined to the CD11c⁺MHCIIhi DC population (Fig. 1B). In addition, a high proportion of LX⁺ DCs in the brLN was CD103⁺, suggesting that they were actually of lung origin, as CD103 is a surface marker of a large subpopulation of lung DCs (15). To confirm this hypothesis, we applied LX i.t. into CCR7⁻/⁻ recipient mice, which are known to possess a profound defect in the mobilization of DCs from the periphery to the draining LNs. As shown by flow cytometric analysis, LX⁺ DCs were virtually absent from brLNs of CCR7⁻/⁻ animals, confirming the notion that LX⁺ cells present in the brLNs of wild-type (wt) mice are indeed migratory DCs of lung origin (Fig. 1C). Furthermore, LX⁺ cells isolated from the brLN by FACS sorting displayed a typical DC-like morphology when analyzed by cyto spin (Fig. 1D). Thus, the i.t. application of fluorescent LX allows the subsequent identification of lung-derived DCs in the brLN under steady-state conditions.

**Leukocytic infiltration in the lungs draining brLN after i.t. application of LX in the absence or presence of LPS**

To further characterize the i.t. application of LX as a model system for the encounter of airway Ag under inflammatory and non-inflammatory conditions, we analyzed by flow cytometry the amount of leukocytic infiltration as well as the phenotype of Ag-bearing cell populations in the lung after i.t. application of LX with or without LPS. Twenty to twenty-four hours posttreatment, four main subpopulations of phagocytic cells, distinguished by the differential expression of CD11b and CD11c, could be detected in the lungs of recipient mice under both conditions (Fig. 2A). However, indicative for a situation of acute lung inflammation (19), we observed a particularly strong recruitment of CD11b⁺CD11c⁻ neutrophils and CD11b⁺CD11c⁻ monocytes in animals treated with LX+LPS (Fig. 2A, 2B). As both of these populations express CD11b, they were further distinguished by their expression of CX3CR1 (20). CD11b⁺CD11c⁻ neutrophils were found to express intermediate levels of CX3CR1, whereas CD11b⁺CD11c⁻ monocytes expressed high levels of CX3CR1 (data not shown). The increase in these populations under inflammatory conditions was accompanied by a concomitant reduction of CD11c⁺CD11b⁻ alveolar macrophages, whereas the absolute number of DCs increased under these conditions (Fig. 2A, 2B). Similar to the situation in PBS-treated and untreated mice, only a moderate leukocyte recruitment into the lung was observed in animals treated with plain PBS.
LX (Fig. 2A, 2B). The phenotype of LX-bearing cells within the draining brLN was highly similar after treatment with either plain LX or LX+LPS. In both cases, LX-bearing cells in the brLN were, to a large degree, confined to the CD11c+MHCIIhiCD103+ population of migratory DCs (Figs. 1B, 2C). Of note, however, the mean number of DCs migrating into the brLN increased ∼7-fold in the LX+LPS-treated mice compared with mice treated with plain LXs (Fig. 2D), which is in line with a strongly increased DC trafficking that occurs under inflammatory conditions.

In conclusion, the i.t. application of LXs in the presence or absence of LPS represents an antigenic challenge within the airways under inflammatory or noninflammatory conditions, potentially leading to the induction of respiratory immunity or tolerance, respectively. The differing patterns of leukocytic infiltrations in the lung closely mirror the state of inflammation, whereas the phenotype of LX+APCs arriving in the brLN is largely confined to migratory DCs in both cases.

**FIGURE 2.** Differential leukocyte recruitment within lung and brLN of mice treated with LX under inflammatory and noninflammatory conditions. A, Yellow-green fluorescent LX were i.t. applied into BALB/c mice in the presence (LX+LPS) or absence (LX) of LPS as an inflammatory stimulus; 24 h later, leukocytes isolated from the recipients’ lungs were analyzed by flow cytometry for the expression of CD11b and CD11c. B, Number of CD11b+CD11c−/− granulocytes, CD11b+CD11clow monocytes, CD11c+CD11b+ DCs, and CD11c+CD11b− macrophages in the lungs of untreated, PBS, LX-, and LX+LPS-treated animals 24 h after i.t. application. C, Phenotype of LX+cells in the brLN of LX+LPS-treated animals 24 h after i.t. application. D, Number of CD11c+MHCIIhi DCs present in the brLN of untreated, PBS, LX-, and LX+LPS-treated animals 24 h after i.t. application. Data shown are representative of at least two independent experiments.

**Kinetics of LX+ lung-derived DC migration into draining brLN**

Ag uptake by DCs within peripheral tissues is followed by their migration into the paracortical T cell zone of draining LNs, where they are ideally positioned to present Ags to naive T cells. To determine the kinetics of DC migration from the lung toward the draining brLN, we have performed both histological and flow cytometric analyses, addressing the localization as well as the absolute numbers of LX+cells present at various time points after i.t. application of LX. In line with previously published data for intranasal administration of LX (21), the histological analysis showed that single LX+cells can be detected in the subcapsular sinus as early as 12 h after i.t. administration (data not shown). Sixteen hours after i.t. application, the first LX+DCs clearly populate interfollicular regions of the brLN (Fig. 3A). Flow cytometric analysis of both LX+CD11c+MHCIIhi and LX+CD11c−CD103− DC populations confirmed that the first lung-derived migratory DCs arrived in the brLN 12 h after the instillation of LX.

**FIGURE 3.** Lung-derived LX+DCs localize to interfollicular regions of the brLN within 16 h after LX application. A, At 16 h or 38 h after the application of LXs, brLNs were isolated, and cryosections of brLNs were stained as indicated. Scale bars, 200 μm. B, At various time points after i.t. application of LXs, leukocytes from brLNs of wt recipients were isolated and analyzed by flow cytometry. The percentage of LX+ cells within CD11c+MHCIIhi and CD11c−CD103− DCs was calculated.
DO11.10 CD4+ T cells in the brLN following i.t. application of
analyzed the in vivo proliferation of OVA-specific transgenic
efficiently present OVA-derived peptides to naive T cells, we an-
Ag. Therefore, OVA was passively adsorbed to fluorescent LX prior
to the i.t. application of LX. As we further observed a pronounced, almost 15-fold increase in the number of migratory DCs present in the brLN within the following day (not shown), we decided to additionally study the cellular dynamics of Ag-dependent contacts between T cells and migratory DCs at 36–40 h after i.t. application of the particulate Ag.

OVA-LX–bearing DCs are potent APCs
To visualize the Ag presentation of endogenous lung-derived DCs
within the brLN, we aimed to combine the in vivo labeling ap-
proach using fluorescent LX with the targeted delivery of protein
Ag. Therefore, OVA was passively adsorbed to fluorescent LX prior
to their i.t. application. To test whether DCs bearing OVA-LXs
efficiently present OVA-derived peptides to naive T cells, we an-
alyzed the in vivo proliferation of OVA-specific transgenic
DO11.10 CD4+ T cells in the brLN following i.t. application of
OVA-LX. For this purpose, we adoptively transferred MACS-
purified CFSE-labeled CD4+ T cells isolated from DO11.10 donor
mice, which carry a transgenic TCR specific for an MHC class II-
restricted OVA peptide prior to the i.t. application of OVA-LX,
OVA-LX+LPS, or plain LXS. The expansion of DO11.10 CD4+
T cells in the brLN was evaluated 4 d after the i.t. challenge.
As expected, the i.t. application of OVA-LX as well as OVA-LX+LPS,
but not of plain LX, induced a massive proliferation of DO11.10
CD4+ T cells within the brLN (Fig. 4). Together, these data
demonstrate that the i.t. application of fluorescent LX loaded with
OVA results in an efficient activation of Ag-specific T cells in the
draining brLN under both inflammatory and potentially tolerizing
conditions.

Induction of Ag-specific tolerance and immunity after i.t. application of OVA-LX or OVA-LX+LPS
The previous experiment had shown that i.t. application of both
OVA-LX and OVA-LX+LPS leads to a strong proliferation of Ag-
specific T cells in the draining brLN. To further characterize
the nature of the ensuing immune response with regard to the lung as a
target organ, mice received two i.t. applications of either OVA-LX
or OVA-LX+LPS prior to a systemic OVA-ALUM sensitization by
i.p. injection. Two weeks postsensitization, the same animals were
rechallenged i.t. with either OVA-LX or OVA-LX+LPS (Fig. 5A).
Twenty-four hours later, the number and phenotype of infiltrating
cells present in the BAL, including granulocytes, monocytes, DCs,
and lymphocytes, were determined by flow cytometry, and the
lungs were examined histologically. As a negative control, one
group of animals received only plain LX by i.t. application at the
corresponding time points. All animals treated i.t. with OVA-LX
+LPS displayed a strong inflammatory immune response in the
lung, leading to the massive recruitment of infiltrating cells as
evidenced by FACS (Fig. 5B, green columns) and histologic ex-
amination (Fig. 5E, arrow). In contrast, the second i.t. challenge
did not result in the development of overt lung inflammation in
OVA-LX-treated mice (Fig. 5B, red columns; Fig. 5D), despite
the previous systemic sensitization with OVA-ALUM. Whereas the
number of lymphocytes within the lungs is slightly higher in these
animals compared with those receiving plain LX (Fig. 5B, black
columns), the numbers of all inflammatory cell populations are not
significantly increased (Fig. 5B). Furthermore, whereas OVA-LX
+LPS-treated mice display massive peribronchiolar infiltrations
(Fig. 5E), the lung tissues of OVA-LX–treated, and LX-treated
animals compared with those receiving plain LX (Fig. 5B, black
columns), the numbers of all inflammatory cell populations are not
significantly increased (Fig. 5B). Furthermore, whereas OVA-LX
+LPS-treated mice display massive peribronchiolar infiltrations
(Fig. 5E), the lung tissues of OVA-LX–treated, and LX-treated
animals were virtually indistinguishable by histological analysis
(Fig. 5C, 5D). Taken together, these data indicate that i.t. appli-
cation of OVA-LX induces a state of respiratory tolerance re-
garding rechallenge with the same Ag, whereas coadministration
of LPS induces respiratory immunity.

Dynamics of CD4+ T cell migration in brLNs during induction of tolerance and immunity to pulmonary particulate Ags
To study by two-photon microscopy the dynamic behavior of T cells
and DCs during induction of tolerance and immunity to lung-de-
ivered Ags, we adoptively transferred TAMRA-labeled CD4+
DO11.10 T cells prior to i.t. application of either plain LX in the
presence or absence of LPS, of OVA-LX, or of OVA-LX+LPS. As
previously described (23), we injected anti-i-β3-selectin mAb i.v. 3 h
after initial transfer of DO11.10 T cells to prevent further lympho-
cyte homing into LNs, thereby allowing the analysis of LN-
residing T cells with synchronized dwell times (Fig. 6A). Between
16 and 36 h after application of LXs, brLNs of recipient animals
were harvested and placed in a custom-built incubation chamber
for two-photon imaging. Explanted brLNs were kept at a constant
temperature of 37°C and were continually superfused with O2-
enriched RPMI medium.
Adoptively transferred TAMRA-labeled T cells, as well as LX-
bearing migratory DCs, were observed to primarily localize to the
paracortical T cell zone of brLNs (Fig. 3A). Consequently, further imaging analysis was particularly focused on this region ~200–
240 μm below the subcapsular sinus of explanted brLNs. De-
pending on the presence or absence of OVA adsorbed to LXs, the
intranodal migration behavior of the OVA-specific DO11.10
T cells was highly different: Whereas TAMRA+ DO11.10 T cells
exhibited a highly motile migration covering large areas of the
T cell zone after application of plain LXs (Fig. 6B, upper panel),

**FIGURE 4.** LX-bearing DCs induce strong Ag-specific T cell prolif-
eration within brLNs under inflammatory and noninflammatory
conditions. A total of 5 × 10^5 CFSE-labeled MACS-purified CD4+ DO11.10
T cells were adoptively transferred into wt recipient mice. One day later,
mice received i.t. either plain LX or OVA passively adsorbed to LX (OVA-
LX) in the presence or absence of LPS. Five days after the adoptive
transfer of CD4+ DO11.10 cells, brLNs were analyzed by flow cytometry.
Four animals per group were analyzed.
the i.t. application of LXs coupled with Ag resulted in a slower, much more confined movement, being largely restricted to the vicinity of individual LX– Ag-loaded DCs (Fig. 6B, middle and lower panels). At ~16 h after the application of plain LXs, T cells displayed average velocities of 11.7 μm/min (Fig. 6C, Supplemental Video 1), whereas T cells of OVA-LX– or OVA-LX+LPS–treated animals exhibited median cellular velocities of 6.5 μm/min and 6.2 μm/min, respectively (Fig. 6C, Supplemental Videos 2, 3). At 36 h after i.t. application, DO11.10 T cells within the brLNs of OVA-LX– and OVA-LX+LPS–treated animals showed an ~57% and ~71% increase in T cell velocities compared with the 16-h time point: OVA-LX, 10.2 μm/min; OVA-LX+LPS, 10.6 μm/min (Fig. 6C, Supplemental Videos 4, 5). Thus, during this later stage of their interactions with Ag-presenting DCs, DO11.10 T cells had already substantially regained motility. Finally, the mean velocity of DO11.10 T cells migrating in the brLNs of untreated mice (data not shown) was 12 μm/min, equating the value reported for skin-draining peripheral LNs (23). Taken together, analysis of the migration velocity of DO11.10 T cells within brLNs after i.t. application of Abs revealed that neither at early nor at late time points were significant differences in the cellular velocity of DO11.10 T cells observed between conditions leading to induction of tolerance and those leading to immunity. Therefore, to compare in more detail the “character” and directionality of migration, we additionally analyzed the following motility parameters: mean displacement, motility coefficient, arrest coefficient, and distribution of turning angles.

By plotting for all conditions the mean displacement of all tracked cells against the square root of time (Fig. 6D; Ref. 13), we found a clearly more confined motility of Ag-specific T cells at the early compared with late time point, which was again independent of the presence or absence of LPS (Fig. 6D, solid lines). Analysis of the motility coefficient not only provides information about the propensity of a cell to displace from its starting position but also integrates information about cell velocity (24). Consistent with the observation of intense T cell–DC interactions with early arrival of LX– DCs 16 h after i.t. application of OVA-LX or OVA-LX+LPS, the mean motility coefficients dramatically decreased under both tolerance- and immunity-inducing conditions (3.1 μm²/min and 7.8 μm²/min, respectively). Correspondingly, DO11.10 T cells regained higher motility coefficient values at later time points investigated, indicating a change of the intranodal migration behavior toward higher displacement under both conditions (Fig. 6E). To identify pauses in the migration path of individual T cells, we calculated so-called arrest coefficients for each condition analyzed. This parameter expresses the percentage of time a cell is considered to be non-motile (i.e., displaying an instantaneous velocity <2 μm/min; compare Ref. 11). As the initiation of long-lasting stable contacts between T cells and Dcs is known to result in a relative immobility of the participating T cell, a low arrest coefficient indicates that pauses in the T cell tracks and therefore potential stable contacts are rare, whereas high values imply the presence of stable T cell–DC contacts. Compared with animals treated with plain LXs, OVA-LX–treated animals displayed a 2.2-fold increase in the arrest coefficient, whereas OVA-LX+LPS–treated animals showed even a 2.8-fold increase 16 h after Ag challenge (Fig. 6F). Thus, DO11.10 T cells within the brLNs of OVA-LX+LPS–treated animals show the highest propensity to remain immotile during the early phase of T cell–DC interactions, suggesting the predominance of stable T cell–DC contacts. Consistent with the increase in cell velocities and motility coefficients, the arrest coefficient decreased during later stages of T cell–DC interactions (Fig. 6F). Finally, analysis of the turning angle distribution confirmed the existence of subtle differences in T cell–DC interaction dynamics in brLNs during conditions leading to tolerance or immunity. At early time points, DO11.10 T cells displayed higher turning angles under conditions that were observed to induce strong airway inflammation compared with conditions of respiratory tolerance induction (median turning angle value of 76° for OVA-LX+LPS and 67° for OVA-LX; Fig. 6G), again indicating a more confined migration under inflammatory conditions. At late time points after i.t. challenge, the observed turning angle distribution reverted to lower values (Fig. 6H).
To address the question of whether inflammation per se could influence the migration behavior of naive T cells, we also analyzed motility parameters of DO11.10 T cells after i.t. application of plain LX together with LPS. Compared with the situation after application of plain LX alone, the additional application of LPS resulted in a moderate but substantial decrease of T cell velocities (11.7 μm/min versus 8.5 μm/min; Fig. 6C). The observed lower slope of the mean displacement plot (Fig. 6D) and, consequently, the reduced motility coefficient (Fig. 6E) indicated a more confined migration pattern of T cells during inflammation, which is, as well, reflected in changes of the arrest coefficient (Fig. 6F) and the turning angle distribution (median turning angle of 67° for LX+LPS; data not shown).

FIGURE 6. Analysis of CD4⁺ T cell motility during the induction of tolerance and immunity. A, 5 × 10⁶ TAMRA-labeled CD4⁺ DO11.10 T cells were adoptively transferred into wt recipient mice. One hour later, mice received either LX alone or together with LPS or OVA passively adsorbed to LX in the presence (OVA-LX+LPS) or absence (OVA-LX) of LPS. Three hours after the initial transfer of DO11.10 T cells, mice were injected i.v. with 100 μg Mel-14 (anti-α-selectin) mAb to prevent further LN homing of lymphocytes. Between 16 and 40 h after i.t. challenge, brLNs were imaged by two-photon microscopy. B, Migration and interaction behavior of TAMRA-labeled CD4⁺ DO11.10 T cells (red) within the brLNs of LX-, OVA-LX–, or OVA-LX+LPS–treated animals 16 h after i.t. challenge. LX⁺ DCs are depicted in green. Color-coded tracks (blue, start of imaging; white, end of imaging) illustrate the paths of individual DO11.10 T cells (image data left out for clarity in the right panel). Grid, 20 μm. C, Average velocities of DO11.10 T cells. Each dot represents an individual cell; bars indicate median values. Data shown are representative from one of at least three independent experiments. D, Mean displacement plot calculated for DO11.10 T cells migrating within the brLNs 16 h (solid line) and 36 h (dashed line) after the indicated i.t. challenge. E, Motility coefficient. F, Arrest coefficient. Cells with instantaneous three-dimensional velocities of <2 μm/min were considered immotile. Turning angle distribution of DO11.10 T cells within brLNs of LX-, LX+LPS-, OVA-LX–, or OVA-LX+LPS–treated animals at 16 h (G) and 36 h (H) after i.t. challenge. *p < 0.05; **p < 0.01; ***p < 0.001; ns, nonsignificant. Data in D–H are pooled from at least three independent experiments.
shown). Taken together, inflammatory conditions induced by LPS are obviously able to modulate the basal motility level of naive T cells in the brLN also in the absence of cognate Ag. Importantly, however, the T cell motility reduction observed under these conditions is clearly less pronounced compared with the effect of cognate Ag on the migration behavior of naive T cells (Fig. 6). These results extend circumstantial findings of a decreased T cell motility in the presence of LPS (22) not observed in a different experimental setup (11).

In summary, the migration behavior of OVA-specific DO11.10 T cells within brLNs appeared to be largely comparable after i.t. application of OVA-LXs in the presence or absence of LPS. In both cases, Ag-specific T cells frequently engaged in contacts with LX⁺ DCs early (16 h) after Ag challenge, displaying a strongly reduced overall motility and displacement, while clearly regaining cellular motility at 36–40 h after i.t. application. Arrest coefficient and turning angle distribution suggested a slightly higher confinement of DO11.10 T cell movement during the early phase of Ag recognition under immunizing (OVA-LX+LPS) compared with tolerizing (OVA-LX) conditions, warranting further detailed analysis. In addition, our data strongly suggest that inflammatory processes can affect the intranodal migration behavior of naive T cells even in the absence of cognate Ag.

Size and stability of DC–T cell interaction clusters during Ag presentation in brLNs under tolerance- and immunity-inducing conditions

As differences in the interaction dynamics of T lymphocytes and DCs can easily be underestimated when analyzing the migration behavior of Ag-specific T cells solely on the population level, we next addressed the quality of T cell–DC interactions occurring in the brLN 16 h after i.t. challenge on the level of individual cellular contacts. To this end, contacts between TAMRA⁺ DO11.10 T cells and LX⁺ DCs were defined as stable when detectable throughout a complete imaging period (usually lasting 22.5–25 min). All other T cell–DC interactions that were detected for at least five successive time points (contact duration >75 sec) were designated as transient contacts. Using this classification system, we found that under conditions leading to the induction of immunity, LX⁺ DCs predominantly formed large interaction clusters, stably interacting with three or more Ag-specific T cells at the same time (Fig. 7A). By contrast, under tolerance-inducing conditions, LX⁺ DCs were more frequently found to maintain stable contacts with only one or two T cells at the same time (Fig. 7A). An additional analysis of the duration of individual transient T cell–DC interactions showed that, on average, transient contacts lasted substantially longer after i.t. application of OVA-LX with LPS (median contact duration, 6.5 min; Fig. 7B) than without (median contact duration, 4.5 min; Fig. 7B). Collectively, these data indicate that, compared with tolerance-inducing conditions, lung-derived migratory DCs within the brLN form more stable, long-lasting interactions with T cells during presentation of particulate airborne Ag under conditions leading to the induction of immunity.

Discussion

Previous studies (10) as well as our own observations (9) had revealed that tolerance induction to inhaled environmental Ags relies on the migratory capacity of DCs actively transporting Ag from the lung to the draining brLN. Although soluble Ags that reach the brLN via afferent lymph and the conduit system are readily taken up by LN-resident DCs, these cells fail to activate T cells even when carrying large amounts of Ag (8). Thus, at least under tolerance-inducing conditions, Ag presentation and subsequent activation of T cells within the lung-draining brLN is obviously restricted to those DCs that have actually taken up Ag in the lung, subsequently migrating to the draining LN (9). In the current study, we therefore aimed to visualize by two-photon microscopy how specifically these lung-derived migratory DCs interact with T cells during the presentation and recognition of airborne Ag, comparing conditions leading to the induction of either tolerance or immunity. Applying Ag-coated LX i.t., we were able to effectively combine the in situ labeling of endogenous lung-resident DCs with the delivery of the model Ag OVA. The detailed quantitative analysis of the migration and interaction dynamics of CD4⁺ T cells and Ag-loaded LX⁺ DCs in the brLN revealed characteristic differences between the induction of tolerance and immunity toward airway-derived Ags: T cells displayed a slightly more confined migration behavior taking sharper turns while forming more stable long-lasting interaction clusters with Ag-presenting DCs under immunity-inducing conditions.

With a diameter of 0.5 µm, the LX used for Ag loading were significantly below the size range common to pollen of many trees known to induce allergic airway diseases (25). However, the allergen-carrying particles that finally enter the small airways and therefore represent the actual targets for phagocytosis by lung-resident immune cells have been identified as much smaller, ranging from far below 0.5 to 4.5 µm (26–28). We therefore conclude that the model Ag used in this study is actually well suited to model the uptake and transport of particulate Ags potentially involved in allergic sensitization events.

FIGURE 7. Analysis of cluster size and contact duration during Ag-specific T cell–DC interactions in the brLN. T cell–DC contacts lasting for the complete imaging period were defined as stable; otherwise they are designated as transient. A. Size distribution of stable interaction clusters between DO11.10 T cells and LX⁺ DCs 16 h after i.t. application of OVA-LX or OVA-LX+LPS. Clusters were classified based on the number of T cells stably interacting with an individual DC. Only DCs stably interacting with at least one T cell were included in the analysis B. Duration of transient contacts between DO11.10 T cells and LX⁺ DCs 16 h after i.t. application of OVA-LX or OVA-LX+LPS. Transient contacts of individual T cells were grouped into four categories with regard to contact duration. Data are derived from three mice per group analyzed.
Following i.t. application of LX, we found that several subsets of phagocytic cells within the lung were able to take them up, thus becoming stably labeled by the ingested fluorescent particles. Importantly, however, only DCs, but neither neutrophils nor macrophages, were found to subsequently migrate to the lung-draining brLN. Inside the brLN, LX-bearing cells were observed to localize almost exclusively within the paracortical T cell zone, as expected for immigrating tissue-derived DCs. Thus, i.t. application of OVA-coated fluorescent LX allowed for specific visualization of endogenous lung-derived migratory DCs presenting the Ag of interest within the brLN.

We hypothesized that the initial i.t. application of LXs coated with OVA would induce tolerance, whereas the addition of LPS would induce immunity against this model Ag. Indeed, results presented in this study strongly support this hypothesis. Compared with the same treatment without LPS, a massive infiltration of inflammatory cells was observed in mice that received OVA-LX in the presence of LPS prior to i.p. sensitization (Fig. 5).

Recent two-photon studies have analyzed the T cell migration and interaction dynamics in skin-draining as well as mesenteric LNs during the onset of tolerance and immunity (11–13). Hugues et al. (11) have imaged OT-I CD8+ T cells within skin-draining LNs of wt recipients after systemically applying the model Ag OVA coupled to an anti–DEC205-mAb, thereby targeting it to LN-resident DCs. They reported the transient T cells, at all time points analyzed, to primarily form serial brief encounters with CD11c+ DCs under noninflammatory conditions, whereas such T cell–DC interactions were found to be stable and long lasting ~15–20 h after Ag challenge under inflammatory conditions. In contrast to these findings reported for CD8+ T cells, no substantial differences regarding temporal changes in the intranodal migration dynamics of OT-II CD4+ T cells had been observed in another study comparing T cell–DC interactions within skin-draining LNs under tolerance- and immunity-inducing conditions (12). Similar to the previously described study, OVA coupled to anti–DEC205 mAb was injected i.v. for systemic Ag delivery to DCs, with or without the coadministration of anti–CD40-mAb as an adjuvant to induce priming conditions. In both situations, the authors observed a phase of markedly reduced T cell motility due to formation of stable, long-lasting contacts with Ag-presenting DCs. Consequently, the authors conclude that early Ag-dependent T cell arrest on DCs seems to be a shared feature of tolerance and priming associated with activation and proliferation (12). Of note, however, an increased proportion of arrested T cells at 11–17 h after Ag challenge, as well as a slower return to the rapid migration phase, was observed after coadministration of the anti–CD40-mAb, suggesting the formation of relatively more stable initial T cell–DC contacts during priming (12). Analyzing the intranodal motility of adoptively transferred DO11.10 CD4+ T cells within peripheral and mesenteric LNs, Zinselmeyer et al. (13) found a largely similar movement behavior of these transgenic CD4+ T cells ∼20 h after oral application of OVA in the presence or absence of the adjuvant cholera toxin. Interestingly, this group as well reported characteristic differences in T cell clustering behavior: Although fewer clusters were found under immunity-inducing conditions, such clusters were larger and more stable.

Our imaging of explanted brLNs by two-photon microscopy revealed that T cells interacting with lung-derived migratory DCs under inflammatory conditions exhibit a slightly more confined migration behavior compared with tolerance-inducing conditions, potentially resulting from a higher stability of established T cell–DC interactions. Indeed, we found that, under immunity-inducing conditions, the majority of Ag-presenting lung-derived DCs form more stable contacts than under tolerance-inducing conditions, on average recruiting a higher number of Ag-specific T cells into individual interaction clusters. Correspondingly, the formation of transient contacts of shorter duration was more prominent under tolerance-inducing conditions, and the average duration of these transient T cell–DC contacts was also shorter compared with immunity-inducing conditions. Thus, similar to previous reports on the interaction dynamics of T cells and DCs within skin-draining and mesenteric LNs (11–13), data presented in this paper indicate that also in the lung-draining brLN, Ag-dependent contacts between T cells and DCs tend to be more stable under conditions leading to immunity, whereas temporal changes in the T cell migration behavior due to interactions with DCs seem to be largely comparable during the induction of respiratory tolerance and immunity.

The use of OVA-LX in the current study allowed for an unambiguous identification of those DCs that had actually carried the Ag of interest from the lung toward the draining LN. Thus, the type of APC addressed by our approach differs decisively from that of previous studies, which had analyzed the dynamics of T cell–DC interactions within skin-draining LNs by i.v. injected OVA-conjugated anti–DEC205-mAb into either transgenic CD11c+EYFP mice (12) or wt recipients in which endogenous LN-resident DCs had been labeled in vivo by Alexa488-conjugated anti–CD11c-mAb (11). In both cases, delivery of the model Ag OVA as well as distribution of the fluorescent labeling probably affected all (DEC205+ or CD11c+, respectively) DC subpopulations present within the LN under scrutiny, independent of their individual LN dwell time and specific origin as tissue-derived migratory DCs or progeny of blood-borne monocyte progenitors. Importantly, tissue-derived migratory DCs not only differ from monocyte-derived LN-resident DCs regarding their surface marker expression and their capability of Ag presentation and costimulation (29) but have also been shown to display an intranodal migration behavior quite different from that of resident DCs (30). Thus, it seems likely that T cells engaged in Ag-specific contacts with LN-resident DCs might display motility patterns different from those interacting with tissue-derived migratory DCs. Furthermore, the LN dwell time distribution of different DC subpopulations might critically influence the observed kinetics of T cell motility changes.

The observation that short-lasting transient and long-lasting stable T cell–DC contacts predominate at different time points after delivery of the cognate Ag has led to a three-phase kinetic model of T cell priming as initially proposed by Mempel et al. (13). However, recent studies clearly demonstrated that additional factors, such as Ag dose, peptide–MHC potency, as well as T cell and DC frequencies, essentially contribute to the stability of early T cell–DC interactions (31–34). It is therefore not surprising that studies addressing T cell–DC interactions under tolerizing conditions have reported different kinetics of DC–T cell interaction: Hugues et al. (11) found a three-phase kinetic of T cell–DC interactions, whereas Zinselmeyer et al. (13) describe the existence of an early phase, when T cells form transient contacts at 8 h after Ag delivery, and a late phase characterized by stable contact formation ~20 h after Ag delivery. Stable contacts of DCs and T cells 1–6 h after Ag delivery were also reported by Shakhar et al. (12). Obviously, the experimental models employed by different groups to study T cell–DC interaction dynamics during the induction of tolerance and immunity have varied with respect to several of the above-mentioned influencing factors. Considering this, it seems even more likely that the shared observation of an at least slightly higher stability of T cell–DC contacts under priming conditions might indeed represent a general phenomenon associated with the induction of immunity, regardless of the type of LN under investigation.
Several factors might actually have contributed to the characteristic differences in T cell–DC interaction dynamics observed during the induction phase of respiratory immune reactions within brLN. First, when being exposed to inflammatory mediators, DCs have been shown to undergo massive morphological changes, resulting in the up- and downregulation of each of several thousand genes (35). Consequently, a more efficient presentation of exogenous Ags resulting from changes in the metabolism of MHC class II molecules, as well as a higher surface expression of costimulatory molecules on DCs migrating to the brLN under inflammatory conditions, might have contributed to the observed higher interaction stability. Second, it seems possible that under inflammatory conditions in the lung, the composition of migratory APC arriving in the brLN might be different compared with steady-state conditions. However, flow cytometric analysis of LX-bearing cells in the brLN (Figs. 1B, 2C) indicated that lung-derived DCs are clearly the dominant LX+ cell population after i.t. application of OVA-LX in the presence as well as absence of LPS, although they probably differ in their maturation status (see above). Third, changes in the behavior of the LN-resident DCs, which are specifically not visualized in our setup, might have accounted for differences in the movement behavior of Ag-specific T cells under inflammatory versus noninflammatory conditions. If so, this hypothetical influence of LN-resident DCs most probably did not relate to actual Ag presentation, as we observed clusters of TAMRA+ DO11.10 T cells exhibiting a confined migration behavior typical of Ag-specific interactions (22, 23) only in the direct vicinity of LX+ (lung-derived) migratory DCs. Fourth, besides Ag-dependent and -independent interactions between T cells and different DC populations, stroma cells of the T cell zone have been demonstrated to decisively affect the intranodal migration of T cells (36). These fibroblastic reticular cells (FRCs) present key guidance cues for T cells and form a structural network that naive T lymphocytes use during their apparent “random walk” migration within the paracortical T cell zone (36). As FRC undergo substantial remodeling under inflammatory conditions (37), it seems possible that these cells might also shape the intranodal migration behavior of T cells differentially during the event of priming or tolerization. However, such a general change in the migration-modulating influence of the nonlabeled FRC under steady-state versus inflammatory conditions should have been observable throughout the imaged T cell zone, independent of the localization of a T cell relative to the positions of the LX+ migratory DCs. In contrast, most general parameters of T cell movement were largely similar during the induction of tolerance and immunity, and the most pronounced differences in T cell behavior were clearly related to the interaction dynamics of direct cell-to-cell contacts with LX+ DCs, making a critical role of the FRC network rather unlikely.

Finally, the observed differences in cellular dynamics during Ag presentation are in line with the previously proposed concept that long-lasting stable contacts to Ag-presenting DCs actually increase the probability that a T cell will receive those activating signals that lead to the development of immunity (38). In contrast, either the relatively lower stability of T cell–DC interactions observed under conditions leading to the induction of respiratory tolerance may indicate a different quality of DC signaling, or the lower duration of individual T cell–DC contacts itself might be the factor determining the tolerant fate of this specific T cell.

In addition to cognate Ag, the current study also reveals that ligands for Toll-like receptors can profoundly affect the migration behavior of naive T cells independent of the presence of cognate peptide ligands. The application of plain LX together with LPS clearly reduced the velocity, mean displacement, and motility coefficient of DO11.10 cells, although not to the same extent as the delivery of OVA. The underlying mechanisms are currently unknown, but it seems likely that changes in the LN environment induced by LPS, such as upregulation of adhesion molecules (potentially affecting stromal cells and/or resident DCs), might contribute to the effects observed.

In summary, imaging the intranodal migration of DO11.10 T cells within brLNs at different time points after i.t. application of OVA, we find a largely similar kinetic of changes in the intranodal migratory behavior of Ag-specific T cells under conditions leading to tolerance and immunity. However, analyzing the size and stability of T cell–DC interaction clusters, we find large clusters of long-lasting stable contacts to be most prominent under inflammatory conditions, whereas shorter transient contacts prevail under steady-state conditions. Therefore, paralleling the situation within skin-draining LNs of the systemic compartment, the induction of respiratory tolerance and immunity toward particulate airborne Ags might actually be influenced on the level of cellular dynamics of T cell migration and interaction with migratory DCs within brLNs.

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Disclosures

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