The Kidney-Renal Lymph Node-System Contributes to Cross-Tolerance against Innocuous Circulating Antigen

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The Kidney-Renal Lymph Node-System Contributes to Cross-Tolerance against Innocuous Circulating Antigen

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Soluble Ags devoid of inflammatory stimuli, derived for example from self-serum or food proteins, induce T cell tolerance, predominantly in the spleen. In this study, we describe an additional role of the kidney-renal LN (rLN) system in tolerogenic presentation of circulating soluble Ags. Protein below albumin molecular mass constitutively passed the kidney glomerular filter and was concentrated in the tubular compartment. Enriched filterable Ag was endocytosed by kidney dendritic cells (kDCs). Simultaneously, it was transported cell independently within 2 min to DCs resident in rLNs. These DCs phagocytosed Ag that was then transported by kDCs to rLNs. DCs presented this Ag to naive T cells in rLN and induced T cell tolerance, as evidenced by the absence of Ag-specific CD8+ T cell proliferation and effector cytokine production. These findings demonstrate that the kidney-renal LN system is important for tolerizing T cells via cross-tolerizing DCs. This mechanism may contribute to avoid immunity against innocuous circulating protein Ags below albumin size.

Dendritic cell (DC)3 capture Ags in nonlymphatic organs and transport them to draining lymph node (LN) for presentation to naïve T cells (1–4). DC migration can be triggered by microbial stimuli and requires the chemokine receptor CCR7 (5–7). Activation of cytotoxic CD8+ T cells is facilitated by specialized CD8α+ DC capable of presenting endocytosed Ag to CD8+ T cells (cross-presentation) (8, 9). Recent findings showing that migratory DC transferred viral Ag to CD8α+ DC resident in cutaneous or pulmonary LNs (10–12) have challenged the traditional view that migratory DC directly activate CD8+ T cells in draining LNs.

In the absence of infection, low-level CCR7-dependent migration of immature DC has been observed, for example, in the skin, gut, and lungs (4, 6, 7, 13–16). Such “steady-state” migratory DC, which presumably carry peripheral tissue autoantigens, are believed to directly tolerate autoreactive T cells in draining LNs (4, 13). Although tolerance of CD8+ T cells could be induced by LN-resident non-DC-expressing autoantigens themselves (17), tolerance against exogenous autoantigens (cross-tolerance) has been shown to be induced by DC presentation, for example, pancreatic, renal, and intestinal Ag (9, 18–20). Cross-tolerizing DC in pancreatic LNs expressed CD8α+ (21), whereas those in the mesenteric LN were CD8α− (20) and those in renal LNs could be either (22). However, in these sites, steady-state DC migration has not been reported. Thus, it remains to be clarified whether cross-tolerance is induced by migratory DC that carried Ag into draining LNs, or by LN-resident DC that received organ-derived Ag by other means, for example, from other, migratory DC, as reported in cutaneous or pulmonary herpes virus infection (10–12).

Cross-tolerance may be required also against certain nontoxic proteins, such as innocuous self-serum or food proteins or orally administered Ag. Systemic injection of soluble Ag devoid of inflammatory stimuli has been widely used to study such T cell tolerance (23–25). Cross-tolerizing blood-borne soluble Ag is thought to be induced in the spleen, because cross-presentation of such Ag is particularly efficient in this organ (26–28), but not in LNs (29). Experimental evidence also suggested a role of the liver in tolerogenic presentation of soluble Ag (30).

The kidney is a site of extensive turnover of circulating soluble protein Ags. Ags below albumin molecular size (68 kDa) can pass the glomerular filter and reach the tubular lumen (31). To prevent their loss with the urine, they are reabsorbed by tubular epithelial cells (32) and released in degraded form into peritubular capillaries. Recently, it was shown that filterable dextran molecules are not only taken up by tubular epithelial cells, but also by kidney DCs (33). The kidney contains remarkably abundant numbers of DC (34), whose function is largely unresolved. Murine kidney DCs (kDCs) were characterized by CD11c, MHC class II (MHC II) and CD11b expression and an immature phenotype, and elicited weak T cell responses in vitro (34). In vivo, they suppressed pathogenic CD4+ T cells in experimental crescentic glomerulonephritis and attenuated disease (35). Their location in the tubulointerstitium may allow capture of pathogens that ascend through the tubular system or of circulating Ags that entered this system via glomerular filtration (36). In the present study, we demonstrate selective enrichment of circulating filterable Ag in the kidney. This

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Abbreviations used in this paper: DC, dendritic cell; LN, lymph node; rLN, renal LN; kDC, kidney DC; MHC II, MHC class II; aOVA, Alexa647-labeled OVA; 7AAD, 7-aminoactinomycin D; cLN, cutaneous LN; MFI, mean fluorescence intensity; NX, nephrectomy; MR, mannose receptor; FSC-w, forward scatter pulse width.

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phenomenon allowed studying transport of kidney-derived Ag to rLNs, and consequences for T cell activation. We provide evidence for a complementary mechanism of cross-tolerance induction that is unique to the kidney and independent of steady-state DC migration.

Materials and Methods

Reagents and mice

All reagents were obtained from Sigma-Aldrich, if not specified otherwise. A total of 500-kDa FITC-dextran and 10-kDa Cascade-Blue dextran were obtained from Molecular Probes. Alexa488-labeled OVA (aOVA) was prepared using a labeling kit (Molecular Probes) according to the manufacturer’s guidelines. Dextrans diluted in PBS were injected i.v. in a total volume of 400 l. C57BL/6, OT-I.RAG−/− mice were isolated from M. Nussenzweig (New York, NY); CCR7−/− mice were obtained from M. Lipp (Berlin, Germany). For all experiments, female mice between 8 and 12 wk of age were used in accordance with local animal experimentation guidelines. For priming mice with soluble protein, OVA (grade V) was redissolved in PBS and run over Sephadex G-25 (PD10 column; Amersham) to remove peptide contaminations. A total of 10 μg of soluble OVA per gram of body weight in a total volume of 400 μl was injected i.v. For priming under immunogenic conditions, an emulsion of 200 μl of CFA was injected s.c into the flank.

Abs and flow cytometry analysis

Cells were stained using the following fluorochrome-labeled Abs: anti-CD8, Vα2-TCR, Vβ-5 TCR, CD11c, CD44-biotin, CD62L-biotin, CD25-biotin (BD Pharmingen), B7-DC, B7-H1, and CD90.2 (Thy1.2) (eBioscience). FcRs were blocked with rat anti-FcR from 24G2 hybridoma supernatant; dead cells were excluded by Hoechst-33342 dye or 7-aminoactinomycin (7AAD). Flow cytometry was performed on a LSR II using FACS Diva software (BD Biosciences) and analyzed using FlowJo software (Tristar Software).

Intracellular cytokine staining

Forty-eight hours after priming, single-cell suspensions were prepared and restimulated with SIINFEKL for 4 h in the presence of GolgiPlug (BD Biosciences). Cells were fixed at 15 min for 4°C with 2% paraformaldehyde in PBS, then washed and incubated for 20 min in PBS 0.1% BSA, 0.5% saponin, followed by an additional 30 min of incubation with fluorescein-conjugated Ab IL-2 PE and IFN-γ allophycocyanin (BD Biosciences) in PBS 0.1% BSA, 0.5% saponin, and unconjugated rat IgG.

Purification of OT-I cells

OT-I cells were isolated from OT-I.Rag−/− mice as described (9). Briefly, spleen and LN cells were treated with erythrolysis buffer (146 mM NHeCl, 10 mM NaHCO3, 2 mM EDTA) to remove RBC. Cells then were labeled with CFSE (Invitrogen Life Technologies) as described (18) by resuspension at 10–20 × 106 cells/ml in PBS 0.1% BSA (GERBU), followed by addition of CFSE to a final concentration of 5 μM for 10 min at 37°C. Purity of OT-I cells was usually >75–85% of viable lymphocytes.

Isolation of DC from mice

DC were isolated from kidney, spleen, and LN by digestion with collagenase (Roche Diagnostic) and DNase I (Sigma-Aldrich) as described (34). Tubular fragments from digested kidneys were removed by sedimentation. For determining expression of DC subtype markers such as CD8α, only forward scatter pulse width (FSC-w) single events were analyzed as shown in Fig. 2F.

In vivo cytotoxicity assay

In vivo cytotoxicity assays were performed as described (12). In brief, spleen cells were either pulsed with SINFEKL (1 μg/ml, 45 min at 37°C) and labeled with 1 μM CFSE (CFSE^e^b^e^ cells), or were not pulsed with peptide and labeled with 0.1 μM CFSE (CFSE^e^m^e^ cells). A total of 1 × 107 of both target cell types were injected i.v. After 4 h, the survival of target cells in spleen and LN was analyzed by flow cytometry. Specific lysis was calculated using the formula: percent-specific cytotoxicity = 100 – (100 × (CFSE^e^b^e^-CFSE^e^m^e^-)/CFSE^e^m^e^-). For FC measurements (FSC-m), the donor's lymphocytes were labeled 500-kDa dextran i.v. into C57BL/6 mice. Indeed, 1 h after injection, kDCs carried more filterable than nonfilterable dextran, but reproducibly little small dextran for unknown reasons (Fig. 1A).

Apoptosis detection

The FLICA apoptosis detection kit (Axxora) was used to detect caspase 3/7 activity according to the manufacturer’s guidelines. As the FAM reagent was fluorescent at the same wavelength as CFSE, OT-I cells were labeled by resuspension in PBS containing 4 μM Far-red (Molecular Probes) at 37°C for 15 min. To determine caspase 3/7 activity, single-cell suspensions from mice injected with Far-red labeled OT I cells at a concentration of 2 × 106 cells/200 μl were incubated with 5 μl of FAM reagent at 37°C for 45 min. Late apoptotic/dead cells were labeled with 7AAD.

Statistical analysis

Results are expressed as mean ± SD; n indicates the number of animals per group. Comparisons were drawn using a two-tailed Student t test (Prism 4; Graphpad Software).

Results

kDCs preferentially endocytose filterable Ag

Molecules below albumin molecular size (68 kDa) constitutively pass the kidney glomerular filter and are reabsorbed by tubular epithelial cells (37, 38). kDCs have recently been shown to take up filterable 40-kDa and nonfilterable 500-kDa dextran molecules (33). We speculated that the concentration of the glomerular filtrate in the tubular system might cause enrichment of filterable dextran in kDCs, but not in DCs in other sites such as the spleen or the cutaneous LNs (cLNs). To test this hypothesis, we investigated the simultaneous uptake of filterable and nonfilterable dextrans by coinjecting Cascade-Blue-labeled 10-kDa and FITC-labeled 500-kDa dextran i.v. into C57BL/6 mice. Indeed, 1 h after injection, kDCs carried more filterable than nonfilterable dextran, whereas DC from cLNs had internalized both markers with similar efficiency (Fig. 1A). Splenic DC took up high amounts of large dextran, but reproducibly little small dextran for unknown reasons (Fig. 1A).

We decided to investigate whether this led to immunological consequences. To this end, we replaced the 10-kDa dextran with the model Ag, OVA, in an aOVA form, and coinjected it together with 500-kDa dextran as marker for nonfilterable molecules, which could be acquired only from the blood. Splenic DC endocytosed both molecules with high efficiency exceeding the uptake by cLNs DCs, but the ratio was similar (Fig. 1B). In contrast, kDCs efficiently endocytosed aOVA, but little FITC-dextran (Fig. 1B), again indicating superior uptake of filterable molecules and inferior uptake of blood-borne molecules. Analysis of control mice not injected with fluorescent tracers confirmed absence of autofluorescent DCs (Fig. 1B). Uptake of aOVA was very rapid and could be detected already after 30 s (Fig. 1, C and D). It was even larger in kidney cells deficient for the common leukocyte marker CD45 (Fig. 1, C and D). Histology identified these cells as tubular epithelial cells (data not shown), consistent with their established function of reabsorbing filterable protein (32, 39).

kDCs acquired i.v.-injected aOVA very rapidly. Already 30 s after injection, ~12% of the kDC had accumulated some aOVA (Fig. 1C). After 10 min, the mean fluorescence intensity (MFI) had risen >5-fold (Fig. 1, C and D), and Ag was detectable within nearly half of the kDCs (Fig. 1C), equivalent to 30–50 × 105 DCs per kidney. All these DCs showed the CD11b+/MHC class II− phenotype, whereas Gr-1− plasmacytoid and CD8α− DCs were absent (Fig. 1E). Endocytosis of aOVA by kDC did not result in DC maturation as evidenced by lack of CD80 or MHC II up-regulation (Fig. 1F).
Rapid cell-independent transport of filterable Ag to rLN DCs

Kidney autoantigens have been reported to be presented to CD8\(^+\) T cells in the rLN (9, 18). To test whether also filterable Ag reached this LN, we injected mice i.v. with aOVA and determined its uptake by rLN cells. A cLN that did not drain the kidney was used as a control to assess the amount of aOVA taken up from the blood. Indeed, more cells captured aOVA in the rLNs than in cLNs (Fig. 2, A and B), and higher Ag uptake was detected (Fig. 2, A and B). Importantly, aOVA accumulation was evident already 90 s after aOVA injection and was further elevated after 5 min (Fig. 2, A and B). Left NX before aOVA injection abrogated Ag accumulation in the left rLN, while that in the right rLN remained unaffected (Fig. 2C). Thus, aOVA accumulation in rLNs depended on its enrichment in the kidney, and was not due to particularities of rLNs.

Most rLN cells that contained filterable (kidney-derived) aOVA were CD11c\(^-\)CD11b\(^+\) cells, and thus most likely DCs, while CD11c\(^+\)CD11b\(^-\) macrophages represented a minority (Fig. 2, A and D). Determining the absolute numbers of aOVA\(^+\) DCs showed 20-fold less DCs in one rLN than in one kidney (Fig. 2E). Interestingly, in rLNs aOVA was detected mostly in CD11c\(^{\text{high}}\) cells (Fig. 2, A and D), whereas aOVA\(^+\) DCs in the kidney were predominantly CD11c\(^{\text{low}}\) (Fig. 1C). The rLN differed from the kidney also by containing some CD8\(\alpha\)^+ DCs that had captured aOVA (Fig. 2F).

These phenotypic differences and the rapid kinetics of aOVA accumulation suggested cell-independent Ag transport into rLNs. To test this possibility, we took advantage of our recent discovery that endocytosis of soluble OVA by DCs in lymphatic tissues was facilitated by the mannose receptor (MR) (29). Indeed, this receptor was used by rLN DCs for uptake of aOVA, because no uptake was seen in MR\(^{-/-}\) mice (Fig. 3A). In contrast, kDCs internalized aOVA in a MR-independent fashion and at somewhat less efficiency (Fig. 3A). The absence of kidney DCs containing high amounts of MR-endocytosed aOVA argued against this organ as origin of DCs carrying such Ag in rLNs.

To confirm this interpretation, we used CCR7\(^{-/-}\) mice, in which DC trafficking from nonlymphoid organs to their draining LN is incapacitated (6, 7). Despite this defect, rLN DCs of CCR7\(^{-/-}\) mice showed strong uptake of aOVA 5 min after injection, while again little uptake was noted in the cLN (Fig. 3B). These findings verified that rapid Ag transport to the rLN must have occurred cell independently.

In addition, we noted that DCs with high CD11c\(^{+}\) expression were fewer in the rLNs of CCR7\(^{-/-}\) mice. These DCs had captured only little amounts of aOVA within this short time (Fig. 3B), and therefore did not appear to belong to the subset of rLN DCs that capture filterable Ag. However, it is possible that these cells represented steady-state migratory DCs that continuously carry Ags at a low rate to the rLN, reminiscent of the situation in the lung draining LNs (15).

Efficient cross-presentation of filterable Ag in the rLN

To investigate whether filterable Ag was presented to T cells, we injected mice with OVA and CFSE-labeled transgenic OVA-specific CD8\(^+\) T cells (OT-I cells), which have been widely used for detecting cross-presentation of OVA (18). Indeed, OT-I cells proliferated more vigorously in rLNs than in cLNs (Fig. 4, A and B). After injection of 10 \(\mu\)g of OVA/g body weight, the division index was three times higher in rLN than in cLN (Fig. 4, A and B). Also higher aOVA doses were presented more efficiently in rLNs, whereas doses lower than 5 \(\mu\)g/g body weight did not cause significant T cell activation in any LN (Fig. 4, A and B). OT-I cell proliferation in the
spleen was always higher than in rLNs (Fig. 4, A and B), consistent with the efficient OVA uptake by splenic DCs (Fig. 1A).

We next investigated whether the proliferation of OT-I cells in rLNs had been induced by filterable Ag rapidly conveyed there in a cell-independent fashion, or by Ag shuttled there by steady-state migratory DCs at later time points, as suggested by Fig. 3B. To distinguish between these two possibilities, we examined proliferation of OT-I cells in mice unilaterally nephrectomized after injection of OVA. NX was conducted 90 min after Ag injection, because this time had been reported to suffice for clearing the murine plasma of a single bolus injection of filterable molecules (40). OT-I cell proliferation was similar in the ipsilateral and contralateral rLN of nephrectomized mice, and in sham-operated animals (Fig. 4C), indicating that rapidly transported Ag was sufficient to drive T cell proliferation. Hypothetical DC-mediated steady-state Ag transport at later time points was not required.

Filterable Ag activates T cells in a tolerogenic fashion

We next investigated the phenotype of OT-I cells activated by filterable Ag. The activation marker CD44 was increased, but CD62L only moderately decreased when compared with naive OT-I cells (Fig. 5A). However, these activation signs were much less pronounced than those displayed by OT-I cells proliferating in the draining cLN of mice s.c. injected with OVA in CFA (OVA/CFA), which served as control for immunogenic priming (Fig. 5A). In particular, immunogenic priming resulted in strong up-regulation of the high-affinity IL-2 receptor α-chain, CD25, whereas OT-I cells in the rLN showed levels hardly above those of naive cells (Fig. 5A). This CD44-CD25-CD62L- expression pattern was consistent with previous studies on the phenotype of tolerized T cells (9, 18, 19, 30, 41–43). The few OT-I cells proliferating in response to soluble OVA in non-draining cLNs also showed this phenotype, albeit less pronounced (Fig. 4, A and B).

Consistent with this surface phenotype, OT-I cells activated by filterable Ag produced little IFN-γ and hardly any IL-2 upon re-stimulation with the antigenic OVA peptide, whereas OT-I cells responding to OVA/CFA produced both cytokines well (Fig. 5, B and C). OT-I cells produced low cytokine amounts also when mice were unilaterally nephrectomized 90 min after OVA injection (Fig. 2).
Thus, rapidly transported Ag was sufficient for imprinting OT-I cells for defective cytokine production, demonstrating once more that hypothetical DC-mediated Ag transport at later time points was not required.

Next, we determined whether OT-I cells developed OVA-specific cytotoxic effector function after priming by filterable Ag. Cytotoxicity was evident neither in rLNs, nor in any other location (Fig. 6). In contrast, cytotoxicity was detected in the draining cLN of mice s.c. injected with OVA/CFA, and to a lower extent in other LNs and in the spleen (Fig. 6), presumably due to recirculation of activated OT-I cells. In summary, OT-I cells primed by filterable Ag expressed reduced activation markers, lacked cytokine production and cytotoxic activity, consistent with tolerance induction.

**T cells activated by filterable Ag possess a curtailed lifespan**

To provide more evidence for tolerance induction, we determined the lifespan of T cells activated by filterable Ag. To this end, OT-I cells activated in the rLN of OVA-injected mice had to be separated from those activated in other locations and from Ag. This was achieved by adoptive transfer into RAG$^{-/-}$ secondary recipient mice to examine survival of OT-I cells 2 wk after transfer (experimental plan in Fig. 7A). The very small cell numbers in the tiny rLN precluded isolation from other cell types. Therefore, total rLN cells pooled from several mice were transferred, and subsequently OT-I cell proportions within transferred CD8$^+$ T cells were examined. As a control for survival after immunogenic priming, draining cLN cells from OT-I cell-injected mice injected with s.c. with OVA/CFA were transferred. A further control for survival of naive OT-I cells was necessary, because rLNs contained not only OT-I cells activated by filterable Ag, but also nonactivated OT-I cells (Figs. 4 and 5) that presumably had not yet encountered OVA-presenting DCs after entering the node. Such naive OT-I cells may survive, resulting in a background level below which OT-I cell levels cannot fall even if all activated cells were deleted. To define this background, we also transferred rLN cells from mice injected with OT-I cells but not with OVA.

**FIGURE 3.** Ag uptake in rLNs depends on the MR, but not on CCR7. A, MR$^{-/-}$ or wild-type mice were injected i.v with 6 μg/g body weight aOVA. After 5 min, CD11c$^+$ cells in kidney and rLN were analyzed for aOVA MFI ($n = 3$ mice). Background fluorescence intensity detected in DCs from noninjected mice was subtracted from the MFI values. B, CCR7$^{-/-}$ or wild-type mice (two per group) were injected i.v with 6 μg/g body weight aOVA. After 5 min, total rLN and cLN cells were analyzed for CD11c expression and Ag uptake. Results are representative for two individual experiments.

**FIGURE 4.** Enrichment of filterable Ag in rLN DCs increases specific CD8$^+$ T cell activation. A, A total of $2 \times 10^6$ CFSE-labeled OT-I cells were injected into C57BL/6 mice. Eighteen hours later, different doses of OVA were i.v. injected. Forty-eight hours after OVA injection, the proliferation of OT-I cells was analyzed in spleen, cutaneous, and rLN. Histograms show the proliferation profiles of CFSE$^+$ CD8$^+$ cells. The numbers indicate the division index ± SD ($n = 3$ mice). B, Statistical analysis of A. The ratio between the division indices in the spleen or in rLNs divided by that of the cLN was given as a quantitative parameter for the superiority of spleen and rLNs in uptake of soluble Ag at different doses. C, Division indices of proliferating OT-I cells were determined in the left and right rLN after left NX, and in the left rLN of sham-operated animals ($n = 6$ mice/group, rLNs from two mice were pooled for analysis). Shown are results typical of two individual experiments.
Before this secondary transfer, OT-I cells were more abundant in mice primed with soluble OVA or with OVA/CFA than in unprimed animals (Fig. 7B), as a consequence of proliferation in response to Ag (Figs. 4 and 5). Two weeks after transfer into secondary recipients, the frequency of OT-I cells primed by filterable Ag was reduced to the background level of naive OT-I cells (Fig. 7B). In contrast, the frequency of OT-I cells activated by OVA/CFA had not declined, but showed a tendency toward expansion (Fig. 7B), indicating that immunogenically primed OT-I cells had been programmed to survive, as opposed to those primed by filterable Ag. Their loss was Ag specific, because the frequency of $\alpha2^+\beta5^-$ CD8$^+$ cells, which were examined as examples for

FIGURE 5. T cells activated by filterable Ag in rLN show a tolerized phenotype. A, A total of $2 \times 10^6$ CFSE-labeled OT-I cells were injected into C57BL/6 mice. After 18 h, either 10 $\mu$g body weight of OVA was injected i.v. or OVA-CFA s.c. Forty-eight hours later, expression of CD44, CD25, and CD62L on OT-I cells proliferating in response to these two Ag forms or in unprimed control mice was determined. Quantitative analysis of activation marker expression, expressed as MFI for each cell division, is shown on the right ($n = 3$ mice/group). B, Intracellular IL-2 and IFN-$\gamma$ staining of CFSE-labeled OT-I cells proliferating in response to filterable OVA or to OVA/CFA as described in A. C, Quantitative analysis of B, showing the proportions of cytokine-producing OT-I cells in different organs (three to four mice per group). D, Same experimental setup as in B, except that left NX was performed 90 min after injection of OVA. Shown are the intracellular IL-2 and IFN-$\gamma$ content of OT-I cells proliferating in the left and right rLN and in the left rLN of sham-operated animals. E, Quantitative analysis of D, showing the proportions of cytokine-producing OT-I cells (six mice per group, rLNs from two mice were pooled for analysis). Shown are results typical of two individual experiments.
non-OVA-specific CD8+ T cells, was not significantly altered in any experimental group (unprimed 6.6 ± 2.8, filterable Ag 3.5 ± 1.4, OVA/CFA 3.3 ± 0.3% of total CD8+ T cells, n = 3, p > 0.17 for all comparisons).

We also followed the fate of OT-I cells activated by filterable Ag in RAG-competent secondary recipients, in which they had to compete with endogenous T cells for entry into the pool of recirculating lymphocytes. As we expected this to be far more difficult, we used as first and secondary recipients Thy1.1 congenic mice, in which OT-I cells expressing Thy.1.2 are easier to track. In addition, we transferred pooled rLN cells from six primary recipients into a single secondary recipient (precluding statistical analysis) and analyzed already on day 5 (experimental protocol in Fig. 7C). In this experiment, we recovered from the secondary recipient only 2.2% from the transferred OT-I cells primed by filterable Ag, as compared with the background of 6.1% recovery of transferred naive OT-I cells from unprimed controls (Fig. 7D). In contrast, 16% of the OT-I cells primed by OVA/CFA were retrieved (Fig. 7D), consistent with better survival after immunogenic priming as compared with naive OT-I cells. OT-I cells from the spleen were almost completely lost (0.15% recovery, Fig. 7D), confirming its dominant role in tolerance induction against soluble Ag. When we examined IFN-γ production as a functional parameter, 41% of the OT-I cells primed by OVA/CFA produced this cytokine, as opposed to only 1.9% of the cells primed by filterable Ag in the rLN (Fig. 7E). A total of 12.5% of OT-I cells primed in the spleen did so, but this corresponded to a very low absolute number of cells, due to their almost complete loss (Fig. 7E).

Next, we examined OT-I cells primed by filterable Ag for direct evidence for apoptosis. To this end, we used the fluorescence reagent, FAM, which covalently binds to active caspase 3 and 7. Indeed, after priming with soluble OVA more apoptotic OT-I cells were present in the rLN than in the spleen, or in the cLN after immunogenic priming with OVA/CFA (Fig. 8). The number of apoptotic cells detectable at one time point was small, consistent with rapid clearance of apoptotic cells in vivo, as opposed to the in vitro situation, in which apoptosis mechanisms are often studied. Taken together, the tolerated phenotype, lack of cytotoxicity, reduced lifespan and the higher proportion of apoptotic OT-I cells supported tolerance induction by filterable Ag in the rLN.

Finally, we studied whether DCs in the rLNs that had captured filterable Ag showed any particularities that may be related to tolerance induction. B7-H1 (PD-L1) expression was unchanged in any site (data not shown), whereas aOVA+ DCs in the rLN and the
spleen expressed more B7-DC (PD-L2), a negative regulator of T cell activation (44, 45), than other DCs (Fig. 9). Analysis of B7-DC on Ag-bearing DCs in other LNs was not possible be-

cause only very few DCs had captured aOVA there (Fig. 2, gray areas).

Discussion

Systemic injection of soluble Ag devoid of inflammatory stimuli has been widely used to study mechanisms of T and B cell tolerance (23–25). This system is thought to mimic presentation of innocuous self-serum proteins and possibly also orally administered Ag, against which immunity is not required or may even be harmful. It is generally assumed that such tolerance is induced in the spleen, which presents soluble Ags to T cells particularly well (26–28). However, splenectomy usually does not result in autoimmunity against otherwise ignored soluble Ags, suggesting that further sites may exist where tolerance can be induced. Here, we propose the kidney-rLN system as one such additional site.

Liquid reabsorption from the glomerular ultrafiltrate causes enrichment of filtrated molecules below albumin size in the kidney tubular system (31). Unlike metabolic waste products, such proteins are not excreted with the urine, but instead are reabsorbed by tubular epithelial cells. It has recently been reported that filterable dextran molecules also reached DCs in the kidney (33). Our approach of simultaneous injection of small and large fluorescent molecules allowed demonstration that uptake from the tubular filtrate was far more efficient than that from the circulation. Furthermore, it revealed selective enrichment of filterable protein Ag in rDC and rLN DCs. Although the functional relevance of Ag accumulation in rDC remains to be addressed, the enrichment in rLNs could be shown here to result in efficient activation of naive CD8+ T cells at concentrations too low for priming in any other LN. The cells that acquired such Ag were most likely DCs, because in our experiments, they could activate naive OT-I cells. These DCs may be related to a recently described MR+ DC subset located in the paracortex of peripheral LNs (46). Also the spleen enriched soluble protein Ag, and even more efficiently so than rLNs. This observation was consistent with the previous identification of a splenic conduit system that efficiently targeted soluble small molecules to DCs in the splenic paracortex (28). Also, LNs (47, 48) and the thymus (49) contain conduit systems. Such a system may have conveyed filterable Ag arriving with the lymph flow toward resident MR+ DCs within rLNs. The concentration mechanism in the kidney may complement these anatomical structures at improving presentation of soluble Ag to T cells in the LN.

Presentation of filterable Ag induced T cell tolerance, as evidenced by low activation markers, lack of significant cytokine production, absence of cytotoxic activity, higher apoptosis-indicating caspase 3/7 activity, and a curtailed lifespan of OT-I cells, all of which are characteristic for tolerized T cells (9, 18, 19, 30, 41–43). The few OT-I cells proliferating in nondraining cLNs in response to nonenriched circulating Ag did not possess a reduced lifespan, but showed a tolerized phenotype, suggesting that also other LNs may contribute to tolerization, albeit to a lesser extent because they were not supplied with concentrated Ag by the kidney. Determining the lifespan of OT-I cells primed in individual LNs was technically demanding, because not only activated, but also naive OT-I cells that had not yet encountered filterable Ag were present in these nodes. The extremely low cell numbers in the rLN precluded separation of naive and activated OT-I cells for determining their individual lifespans. Nevertheless, transfer of unseparated OT-I cells allowed demonstration of their decline to levels below those observed after transfer of naive OT-I cells both in RAG-deficient and -competent recipient mice. This demonstrated that OT-I cells activated by filterable Ag had been deleted, while the naive ones in the rLNs survived. In contrast, the high proportions of immunogenically activated OT-I cells had increased to some

FIGURE 8. Apoptosis of T cells activated by filterable Ag in rLNs. A. A total of 2 × 10^6 Far-red-labeled OT-I cells were injected into C57BL/6 mice. After 18 h, mice were injected with either 10 µg/g OVA i.v. or OVA-CFA s.c. After 48 h, single-cell suspensions from the rLNs, the cLNs, and the spleen were prepared and caspase 3/7 activity was determined on OT-I cells identified by gating for CD8+ Far-red+ events. FAM+7AAD+ represented early apoptotic and FAM+7AAD+ late apoptotic/dead OT-I cells. Statistical analyses of apoptotic cells are shown (n = 3 mice/group). B. A representative dot plot is shown.

FIGURE 9. Expression of B7-DC by T cells activated by filterable Ag. C57BL/6 mice were injected i.v with 6 µg/g body weight aOVA. After 45 min, CD11c+ DC in the rLN, cLN, mesenteric LN (mLN), spleen, and kidney were analyzed for Ag uptake and B7-DC expression. The black line indicates unstained cells, the black shaded line total CD11c+ DCs, and the gray line with gray area the aOVA+ and CD11c+ cells.
extent. Induction of anergy as an alternative explanation is unlikely, because others showed that transfer into RAG-/- mice rescued anergic T cells, and enabled vigorous homeostatic proliferation (50). Furthermore, if OT-I cells had been anergized, then by definition, proliferation should have ceased. However, after transfer of CFSE-labeled OT-I cells into RAG-/- recipients, we did not observe any cells that had retained fluorescence (V. Lukas-Kornek, unpublished observations). Thus, these cells either had continued to divide or, alternatively, had been deleted. If proliferation had continued, then not a decline, but instead expansion of OT-I cells would have resulted. Although an additional effect of anergy cannot be formally excluded, these results demonstrate that the observed decline of OT-I cells activated by filterable Ag resulted to a great extent from deletion. The mechanisms inducing such tolerance remain to be identified, but may be related to the selective expression of B7-DC (PD-L2), which negatively regulates T cell activation (44, 45), on DCs carrying filterable Ag. Alternative to deletion, it is possible that regulatory CD4+ T cells were induced and contributed to tolerance against filterable Ag. Regardless of the underlying mechanism, this type of peripheral tolerance can apply only to molecules too small to represent infectious pathogens, because the intact glomerular filter can be passed only by molecules below albumin size (31).

The selectivity for enrichment of filterable molecules in kDC and in rLN DCs allowed for study of how tolerizing Ag was transported from the kidney to the rLN. Several lines of independent evidence indicated that this did not occur via steady-state DC migration. First, enrichment of filterable Ag was detectable already 90 s after Ag injection. Moreover, we found that 5 min after injection, 40–50 × 10^6 of DCs carrying filterable Ag were present in the left kidney, whereas the left rLN contained 2–3 × 10^5 DCs (Fig. 2E). If all these rLN DCs had arrived from the kidney within this short time span, then >500 × 10^3 DCs would migrate per day. In this case, kDCs would turn over more than five times and rLN DCs >200 times per day, which is clearly incompatible with reports by others showing DC half-lives of 1.5–2.9 days in the spleen (51) and >2 wk in the kidney (33). This estimation of DC turnover, together with the rapid kinetics of Ag relocation, is incompatible with low-level steady-state DC migration. The observed phenotypical differences between DCs carrying filterable Ag in the kidney and in the rLN provide the second argument against DC migration. Down-regulation of CD11c and up-regulation of CD8α in such short time appears unlikely. Besides, CD8α is considered a lineage marker that is believed not to be expressed de novo on conventional DCs (4, 52), such as those residing in the kidney (34). The absence of CD8α+ DCs from the kidney implied that the CD8α+ DCs carrying filterable Ag in the rLN cannot have immigrated from this organ. Third, rLN DCs rapidly took up large Ag amounts via the MR, which we have recently described to mediate large scale uptake of OVA in lymphatic tissue (29, 53). Also these DCs cannot be kidney-derived, because this organ contained no such DCs either. Fourth, the rapid accumulation of filterable OVA in rLN DCs occurred also in the absence of CCR7, which is required for DC trafficking to draining LNs (5–7, 15, 16) and which is expressed by kDC (54). These four findings together can only be explained by cell-independent Ag transport to rLNs and subsequent uptake by LN-resident DCs.

Cell-independent Ag transport from peripheral tissues to draining LNs differs from previously described peripheral T cell tolerance via steady-state migratory DCs (4, 13, 15, 16, 21). Various approaches have been used to track migratory DCs, for example, local labeling by FITC-painting, injection of fluorochromes or latex beads, or the injection of labeled DCs (55). It is difficult to exclude that these methods themselves caused DC maturation and triggered migration. Our approach of i.v. injection of tracers bona fide allowed physiologic labeling of DCs and reproducibly did not result in any detectable maturation. Under these conditions, tolerogenic Ag was relocated without the need for DC migration. These findings do not exclude DC-mediated Ag transport to LNs at later time points, but our NX experiments demonstrated that such hypothetical steady-state migration was not required for tolerance in our system.

Others have previously shown that LN-resident DCs could obtain viral Ag from skin- or lung-derived migratory DCs (10–12). Apart from the organ studied, these systems differ from ours by the presence of an infectious agent that could mature DCs and induce their migration. In contrast, innocuous Ag injected into the skin reached the LN through lymph vessels before skin-derived DCs had immigrated (56, 57). Also the kidney is connected with its draining LN by lymph vessels, as demonstrated by cannulating experiments in sheep (58). As cannulation of the tiny renal lymph vessels of mice has not been achieved yet, we were unable to directly prove that rapid cell-independent relocation of filterable Ag occurred through lymph vessels. Nevertheless, our results demonstrate continual DC-independent relocation of innocuous circulating Ag from the kidney to the rLN and local uptake by tolerogenic resident DCs. This mechanism may contribute to preventing unwanted immunity against innocuous foreign or self proteins in the serum.

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Disclosures
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