Batf3-Dependent Dendritic Cells in the Renal Lymph Node Induce Tolerance against Circulating Antigens

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ABSTRACT

Although the spleen is a major site where immune tolerance to circulating innocuous antigens occurs, the kidney also contributes. Circulating antigens smaller than albumin are constitutively filtered and concentrated in the kidney and reach the renal lymph node by lymphatic drainage, where resident dendritic cells (DCs) capture them and induce tolerance of specific cytotoxic T cells through unknown mechanisms. Here, we found that the coinhibitory cell surface receptor programmed death 1 (PD-1) on cytotoxic T cells mediates their tolerance. Renal lymph node DCs of the CD8+ XCR1+ subset, which depend on the transcription factor Batf3, expressed the PD-1 cognate ligand PD-L1. Batf3-dependent DCs in the renal lymph node presented antigen that had been concentrated in the kidney and used PD-L1 to induce apoptosis of cytotoxic T cells. In contrast, T cell tolerance in the spleen was independent of PD-1, PD-L1, and Batf3. In summary, these results clarify how the kidney/renal lymph node system tolerizes the immune system against circulating innocuous antigens.

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Cytotoxic CD8+ T cells (CTLs) mediate host defense against tumors and intracellular pathogens like viruses. However, they may cause diseases if they are specific for self-antigens (e.g., in type 1 diabetes mellitus). The first checkpoint for preventing autoimmunity is the thymus that elimi- nates autoreactive T cells. However, T cells specific for innocuous foreign antigens, like food antigens, or for self-antigens that are not expressed in the thymus escape central tolerance. Such T cells can be silenced by peripheral tolerance mechanisms in secondary lymphatic tissues. The spleen possesses a conduit system for concentrating soluble antigens, which enables splenic dendritic cells (DCs) to endocytose them for inducing T cell tolerance.1-3 Liver sinusoidal endothelial cells can capture antigens from the portal vein and anergize CTLs.4 We recently identified the kidney as the third organ involved in T cell tolerance against innocuous circulating antigens: Antigens below albumin size constitutively pass the kidney glomerular filter and are concentrated when fluid is reabsorbed in the tubular system. Concentrated low molecular weight (LMW) antigens reach the renal lymph nodes (RLNs) by bulk lymph drainage, where they are endocy- tosed by resident DCs. The RLN is the only lymph node showing such antigen concentration (Figure 1, A and B), illustrating the unique role of the kidney in handling LMW antigens.

We previously showed that CTLs responding to LMW antigen in the RLN or in the spleen lacked effector function and possessed a curtailed life-span.5 The molecular mechanisms of this CTL deletion are unclear. Ligation of pro- grammed death 1 (PD-1; CD279), a member of the family of CD28-like costi- mulatory molecules,6 by one of its two ligands, PD-L1 (B7.1H, CD274) and PD-L2 (B7-DC, CD273), can induce apoptosis in T cells.7,8 We recently showed that regulatory T cells use PD-1 ligands to directly tolerize B cells specific for glomer- ular autoantigen.9 The role of PD-1 in renal disease is unclear.

To study whether PD-1 ligands are involved in CTL cross-tolerance against LMW antigens, we used our adoptive transfer protocol where ovalbumin (OVA)–specific CD8+ T cells (OT-1 cells), OVA antigen, and antibodies...
blocking PD-L1 and/or PD-L2 were intravenously injected into mice. Without antibody blockade, OT-I cells in the RLN produced low levels of IFN-γ compared with an immunogenic challenge (OVA/CpG subcutaneously) (Figure 1C), indicating lack of effector function, and >20% of them were apoptotic (Figure 1D). Blockade of PD-L1, but not of PD-L2, increased IFN-γ production and inhibited apoptosis of OT-I cells in the RLN (Figure 1, C and D and Supplemental Figure 1A). Combined blockade of PD-L1 and PD-L2 improved OT-I cell proliferation in this lymph node (LN) (Figure 1E and Supplemental Figure 1A). In the spleen, PD-L1 blockade had little effect, and PD-L2-blockade had no effect on IFN-γ production and apoptosis of OT-I cells (Figure 1, F and G). Generally, the proportion of apoptotic OT-I cells was lower in the spleen than the RLN (Figure 1, D and G and Supplemental Figure 1B). Interestingly, PD-L2 blockade slightly reduced OT-I cell proliferation in the spleen, albeit not significantly, whereas PD-L1 blockade was ineffectual (Figure 1H). These results suggest that PD-L1 was required for CTL tolerization in the RLN, but not in the spleen.
To determine whether CTL apoptosis in the RLN resulted in their deletion, we first transferred OT-I cells and OVA into recipient mice and after 2 days transferred splenocytes or RLN cells into separate recipient mice, to follow the fate of OT-I cells (experimental protocol in Figure 2A). Again, we used OT-I cell–injected mice immunized with OVA/CpG subcutaneously as a control for immunogenic CTL activation. After 5 days, a substantial number of immunogenically activated OT-I cells were recovered from pooled spleen and LNs of secondary recipients (Figure 2B), and these produced robust amounts of IFN-γ (Figure 2C). OT-I cells from mice that had not been injected with OVA were recovered in lower numbers and produced no IFN-γ, indicating their retained naïve phenotype (Figure 2, B and C). Very few OT-I cells responding to OVA in the RLN or in the spleen were recovered from the secondary recipients and these produced hardly any IFN-γ (Figure 2, B and C), indicating deletion and functional incapacitation, respectively. When we transferred OT-1.PD1−/− cells activated in the RLN, almost 10× more cells were recovered and these produced IFN-γ (Figure 2, B and C). In contrast, OT-1.PD-1−/− cells activated in the spleen were still deleted (Figure 2B), but nevertheless produced IFN-γ (Figure 2C), suggesting that an additional PD-1–independent deletion mechanism operated in the spleen that did not switch off cytokine production. Nonactivated OT-1.PD-1−/− cells survived, but produced no IFN-γ (Figure 2, B and C).

Figure 2. PD-1 mediates CTL deletion in the RLN, but not in the spleen. (A) Experimental scheme for OT-I survival analysis. The 5×10⁶ CFSE-labeled OT-I (white) or OT-I.PD1−/− (black) cells are transferred into wild-type recipients, which are then immunized with 8 μg/g body weight endotoxin-free OVA intravenously or OVA/CpG subcutaneously (pos ctrl; gray). One day later, single cell suspensions from RLN, spleen, or draining cutaneous LNs are analyzed for absolute OT-I cell numbers by flow cytometry. Suspensions are transferred into separate secondary recipient mice, and the proportion of transferred OT-I cells that survive (B) and produce IFN-γ (C) are determined in pooled spleen and LNs after 5 days. Data are representative of four independent experiments. CFSE, carboxyfluorescein succinimidyl ester. These findings indicated that CTLs responding to LMW antigen in the RLN were deleted through PD-1 engagement, whereas CTLs responding in the spleen were deleted by other mechanisms.

We next aimed to identify the DC subset that tolerized CTLs in the RLN. To this end, we intravenously injected fluorescent OVA and used flow cytometry to search for DCs that captured this filterable 45-kd antigen, expressed PD-L1, and were capable of cross-presentation. This term denotes extracellular antigen uptake, processing, and presentation capabilities necessary for activation of CTLs. Only some DCs are capable of cross-presentation, and these can be identified by the lack of CD11b and by the expression of CD8, CD103, and/or the chemokine receptor XCR1. Cross-presentation is required both for immunogenic activation of CTLs (cross-priming), such as against viruses or tumors, and for the deletion of CTLs specific for autoantigens, a process known as cross-tolerance.

Among the RLN DCs that had captured OVA, there were both CD11b+ non-cross-presenting DCs and CD8+ CD103+ XCR1+ cross-presenting DCs (Figure 3A). In the steady state, the development of cross-presenting DCs depends on the basic leucine zipper transcription factor (Batf3; ATF-like 3). Mice deficient for this factor lack cross-presenting DCs and show severely compromised CTL responses. When we injected OVA into Batf3−/− mice, the abundance of OVA+ cross-presenting DCs in the RLN was more than 80% lower than in Batf3-competent controls (Figure 3B). Immature DCs can express PD-1 ligands. We found selective PD-L1 expression on CD8+ DCs, but not on CD11b+ RLN DCs (Figure 3C), supporting a role of the former DC subset in cross-tolerance in this node. Such a role would be consistent with a previous report showing that CD8+ DCs tolerized CTLs against islet autoantigen in the pancreatic LN, and OVA+ DCs of the CD8+ and the CD11b+ subset were also found in the spleen (Figure 3D). Spleens of Batf3-deficient mice contained far less OVA+ cross-presenting.
DCs, reminiscent of the RLN (Figure 3, B and E). However, no selective PD-L1 expression on any splenic DC subset was noted (Figure 3F), supporting our conclusion that other tolerance mechanisms operate in this organ (Figures 1 and 2).

To address the functional necessity of Batf3-dependent DCs, we injected OT-I cells and OVA into Batf3−/− and control mice and determined CTL apoptosis. Indeed, less apoptotic OT-I cells were seen in the RLN, but not in the spleen of Batf3−/− mice compared with Batf3-competent controls (Figure 4A), indicating that Batf3-dependent DCs are important for cross-tolerance in the RLN, but not in the spleen.

Finally, we asked whether PD-L1 expression by Batf3+ DCs was required for CTL cross-tolerance. To this end, we created mixed bone marrow chimeras by combining 50% Batf3-deficient and 50% PD-L1-deficient bone marrow. In these chimeras, Batf3-dependent DCs were PD-L1 deficient, whereas the other DCs expressed PD-L1 (Figure 4B and Supplemental Figure 2A). When we transferred OT-I cells and OVA into these chimeras, apoptosis induction in the RLN was abrogated but remained intact in the spleen (Figure 4C). Apoptosis occurred in the RLN of Batf3/wild-type mixed bone marrow chimeras that served as a positive control for the integrity of the PD-1 pathway (Figure 4C and Supplemental Figure 2B). These findings verified that PD-L1 expression of Batf3-dependent DCs was required for CTL cross-tolerance against LMW antigens in the RLN. We also created such mixed bone marrow chimeras using PD-L1−/− mice as recipients. Again, CTL apoptosis in the RLN, but not in the spleen, was attenuated (Figure 4D), excluding a role for PD-L1 on radioresistant host cells for cross-tolerance.

In summary, we showed that Batf3+ DCs in the RLN capture LMW antigen filtrated and concentrated in the kidney and present it together with PD-L1 to CTLs, resulting in cross-tolerance. This is consistent with a previous study showing that immature DCs can tolerize autoreactive CTLs through PD-1.23 This mechanism is unique to the kidney, because the spleen, the only other organ in which antigen concentration occurs, uses other immune tolerance mechanisms that remain to be identified. The scenario that kidney DCs, which are tolerogenic under homeostatic conditions,24–28 capture LMW antigens and carry them to the RLN for tolerance induction is unlikely to be relevant because few DCs migrate to draining LNs in the steady state, whereas bulk drainage transports large antigen amounts very rapidly toward RLN-resident DCs.5

Batf3-dependent DCs are necessary for CTL cross-priming against various pathogens and tumors.19 To our knowledge, this report is the first to imply these DCs in peripheral cross-tolerance. It is also the first report linking Batf3-dependent DCs for CTL cross-tolerance. To this end, we created mixed bone marrow chimeras by combining 50% Batf3-deficient and 50% PD-L1-deficient bone marrow. In these chimeras, Batf3-dependent DCs were PD-L1 deficient, whereas the other DCs expressed PD-L1 (Figure 4B and Supplemental Figure 2A). When we transferred OT-I cells and OVA into these chimeras, apoptosis induction in the RLN was abrogated but remained intact in the spleen (Figure 4C). Apoptosis occurred in the RLN of Batf3/wild-type mixed bone marrow chimeras that served as a positive control for the integrity of the PD-1 pathway (Figure 4C and Supplemental Figure 2B). These findings verified that PD-L1 expression of Batf3-dependent DCs was required for CTL cross-tolerance against LMW antigens in the RLN. We also created such mixed bone marrow chimeras using PD-L1−/− mice as recipients. Again, CTL apoptosis in the RLN, but not in the spleen, was attenuated (Figure 4D), excluding a role for PD-L1 on radioresistant host cells for cross-tolerance.

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with functional PD-L1 expression. PD-1 signaling has been shown to induce the proapoptotic molecule Bim in vitro,\textsuperscript{29} which might have caused CTL apoptosis in our system. Blockade of PD-1 or PD-L1 is currently under clinical investigation for improving inefficient CTL responses against tumors\textsuperscript{30,31} or in chronic hepatitis (ClinicalTrials.gov identifier: NCT010703469). Our results imply that such blockade will not compromise cross-tolerance against LMW antigens, provided that the spleen remains operational.

The tolerance mechanism described here may help prevent unwanted immunity against LMW antigens that lack molecular patterns indicative of danger or infections, and that are too small to represent pathogens (e.g., food antigens or hormones that are not expressed in the thymus).\textsuperscript{32} Thus, PD-L1\textsuperscript{+} Batf3-dependent DCs of the kidney/RLN system purge the T cell repertoire of reactivity against innocuous LMW antigens. Future studies may clarify whether this mechanism induces tolerance against tumor antigens and whether tolerance against autoantigens fails in inflammatory kidney disease.

**CONCISE METHODS**

**Reagents and Mice**

All reagents were obtained from Sigma-Aldrich, if not specified otherwise. OVA-Alexa647 was prepared using EndoGrade OVA (Hyglos) and the succinimidyl ester of AlexaFluor647 carboxylic acid (Invitrogen) according to the manufacturers’ guidelines. All mice were bred under specific pathogen-free conditions at the central animal facility of the University Clinic of Bonn (House for Experimental Therapy) and were used in accordance with local animal experimentation guidelines at 8–12 weeks of age. All animal studies were approved by an external review board (Bezirksregierung Köln, Cologne). For immunization of mice with soluble protein, OVA (grade VII) was dissolved in PBS and run over Sephadex G-25 (PD10 column; Amersham) to remove peptide contaminations. Eight micrograms of soluble OVA per gram body weight in a total volume of 300 \( \mu \)l was injected intravenously. For immunogenic immunization, 50 \( \mu \)g of OVA and 10 \( \mu \)g of CpG ODN1668 in a total volume of 200 \( \mu \)l were injected subcutaneously into the flank. PD-L1 and PD-L2 were blocked as recently described, by intraperitoneally injecting 250 \( \mu \)g of MIHS or TY25,\textsuperscript{9,33} respectively, on the day before and on the day of immunization.

**Flow Cytometry**

Cells were stained on ice with the following fluorochrome-labeled Abs: anti-CD8, CD11b, CD11c, CD45.1, CD90.1 (Thy1.1), CD90.2 (Thy1.2), CD103, F4/80, MHC-II, PD-L2 (CD273), PD-L1 (CD274), Va2-TCR, VB-5 TCR (ebioscience), and XCR1.\textsuperscript{17} OT-I cells were identified as CD8\textsuperscript{+} Va2\textsuperscript{+} VB5\textsuperscript{+} cells or by the congenic markers Thy1.1 or CD45.1. Fc receptors were blocked with 2.4G2 hybridoma supernatant; dead cells were excluded by Hoechst-33342 dye or Violet Dead Cell Stain (LIVE/DEAD Fixable Dead Cell Stains; Invitrogen). Apoptosis was determined using the FLICA caspase 3/7 activity detection kit (Immunochemistry Industries) according to the manufacturer’s instructions. Intracellular IFN-\( \gamma \) staining was performed as described.\textsuperscript{9} Cytometry was performed on a FACS Canto II using FACS Diva software.
(BD Biosciences) and analyzed using FlowJo software (TriStar Software). Proliferation analysis was performed by cell cycle analysis using FlowJo software. Absolute cell numbers were determined using 1 × 10⁶ CaliBRITE APC beads.

**Statistical Analyses**

Statistics are given as the mean ± SEM. Comparisons were made using ANOVA with the Bonferroni post test using Prism 5 software (GraphPad Software).

**REFERENCES**


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