Although effector CD4$^+$ T cells readily respond to antigen outside the vasculature, how they respond to intravascular antigens is unknown. Here we show the process of intravascular antigen recognition using intravital multiphoton microscopy of glomeruli. CD4$^+$ T cells undergo intravascular migration within uninflamed glomeruli. Similarly, while MHCII is not expressed by intrinsic glomerular cells, intravascular MHCII-expressing immune cells patrol glomerular capillaries, interacting with CD4$^+$ T cells. Following intravascular deposition of antigen in glomeruli, effector CD4$^+$ T-cell responses, including NFAT1 nuclear translocation and decreased migration, are consistent with antigen recognition. Of the MHCII$^+$ immune cells adherent in glomerular capillaries, only monocytes are retained for prolonged durations. These cells can also induce T-cell proliferation in vitro. Moreover, monocyte depletion reduces CD4$^+$ T-cell-dependent glomerular inflammation. These findings indicate that MHCII$^+$ monocytes patrolling the glomerular microvasculature can present intravascular antigen to CD4$^+$ T cells within glomerular capillaries, leading to antigen-dependent inflammation.
A growing body of evidence indicates that immune cells can make critical contributions to inflammatory responses while remaining within the vasculature. This concept of “intravascular immunity” is exemplified by the intravascular migration of non-classical monocytes in tissues such as skin, mesentry, muscle, and brain. In vivo imaging studies show that this patrolling function involves prolonged crawling on the endothelium independent of the direction of blood flow. Patrolling Ly6C+ monocytes perform important immune surveillance within the vasculature, internalizing microparticles and soluble material from the bloodstream and responding to microbial infection or tissue injury. Upon detection of these signals, intravascular monocytes are positioned to respond rapidly by inducing recruitment of other immune cells or migrating out of the vasculature. These intravascular activities are not restricted to myeloid leukocytes as in the liver microvasculature, invariant natural killer T (iNKT) cells also constitutively migrate. In this location iNKT cells respond to innate and adaptive signals by modulating their migration and releasing proinflammatory mediators. Whether these intravascular functions also contribute to adaptive immune responses involving conventional T cells is less clear.

Intravital imaging studies have revealed that CD4+ and CD8+ T cells in microvessels of the central nervous system and the renal interstitium can undergo intraluminal crawling and subsequently exit the vasculature. While in contact with the endothelium, effector CD8+ cells can recognize peptide/MHC class I (MHCI) complexes expressed by endothelial cells, leading to T-cell activation and promotion of recruitment and/or tissue retention. Although some endothelial cells can express MHCI class II (MHCII) in inflammatory states, mechanisms of antigen recognition by antigen-specific CD4+ T cells within the vasculature are unclear.

One site where intravascular immunity is crucial is the specialized microvasculature of the glomerulus. Monocytes and neutrophils have been shown to undergo constitutive intravascular adhesion and crawling within glomerular capillaries. During antibody-mediated glomerular inflammation, the duration of the retention of these cells is increased and intravascular neutrophils generate reactive oxygen species (ROS) responsible for glomerular injury. In addition to humoral mediators, CD4+ T cells also have an important function in the development of glomerular injury and dysfunction in severe, rapidly progressive forms of glomerulonephritis. Intraglomerular T cells are detectable in humans with rapidly progressive glomerulonephritis, and functional studies in animal models demonstrate that disease-inducing effector responses can be directed by CD4+ T cells responding to antigen located intravascularly within the glomerulus.

Evidence indicates that glomerular injury mediated by effector CD4+ T cells in glomerulonephritis involves multiple steps, beginning with loss of tolerance to nephritogenic autoantigens in secondary lymphoid organs. This evidence includes the discovery of circulating CD4+ T cells specific for these antigens in patients with autoimmune glomerulonephritis. In patients with autoimmune disease, circulating autoreactive T cells have a memory phenotype, indicating that they have been exposed to cognate antigen and undergone differentiation into effector or memory T cells. However, the existence of circulating, antigen-experienced T cells is insufficient to result in disease. The final steps in the process involve T-cell recognition of antigen in the target tissue, leading to effector T-cell-mediated injury at the site of antigen recognition, the glomerulus. Indeed, analysis of antigen-experienced T-cell responses to antigen in the periphery has shown that these cells can respond within minutes upon recognition of cognate antigen. However, the mechanism whereby CD4+ T cells recognize antigens in the unique microvasculature of the glomerulus is not known. Therefore, the aim of this study is to investigate the mechanisms of intravascular antigen presentation to disease-initiating, antigen-experienced effector CD4+ T cells in the glomerulus, using a validated model of T-cell-mediated glomerulonephritis.

The findings indicate that in the absence of inflammation, MHCII expression in the glomerulus is restricted to subsets of circulating leukocytes. Of these cells, monocytes undergo the most prolonged retention and migration in the glomerular capillaries and are required for CD4+ T-cell-mediated induction of neutrophil-dependent glomerular inflammation.

**Results**

**CD4+ T cells migrate in uninflamed glomerular capillaries.** We first examined whether CD4+ T cells could spontaneously adhere within uninflamed glomerular capillaries. Endogenous CD4+ T cells were visualized using anti-CD4 mAb and intravital multiphoton imaging of the kidney. In uninflamed glomeruli, CD4+ T cells regularly underwent periods of adhesion (defined as retention for >30 s) on the endothelial surface, at a rate of ~5 cells per glomerulus per hour. The majority remained stationary during adhesion (“static” cells), while ~20% underwent crawling (Fig. 1b). Typically, the duration of retention, or dwell time, of CD4+ T cells was ~4 min (Fig. 1c). Crawling CD4+ T cells were retained in glomeruli for twice as long as stationary cells and migrated at ~9 μm min⁻¹ (Fig. 1d).

Detection of these blood-borne CD4+ T cells in this fashion does not allow the differentiation between naive and effector T cells. This is important as for CD4+ T cells to induce disease in the glomerulus in response to local antigen recognition, they require prior activation and differentiation into effector cells in secondary lymphoid organs. Therefore, we next asked whether activated effector T cells also undergo retention in uninflamed glomerular capillaries. To examine this issue, we used OVA-specific T cells from TCR transgenic OT-II mice. OT-II T cells were activated in vitro to a Th1 phenotype, fluorescently labeled and transferred intravenously into recipient mice and subsequently glomeruli were examined via intravital multiphoton imaging. As for endogenous CD4+ T cells, activated OT-II cells underwent retention in glomerular capillaries (Fig. 1e, Supplementary Movie 2). On average, ~1.5 OT-II cells underwent adhesion per glomerulus per hour, of which ~70% remained stationary (Fig. 1f). OT-II cells arrested in glomeruli at a relatively consistent rate during the 90 min imaging period after transfer (Fig. 1g). The dwell time of OT-II cells in glomeruli was ~13 min —this was similar in stationary and crawling OT-II T cells (Fig. 1h) while more than twice that of endogenous CD4+ T cells. The mean migration velocity of crawling OT-II cells was ~9 μm min⁻¹ (Fig. 1i). Together these findings indicate that CD4+ T cells, and specifically effector CD4+ T cells, undergo adhesion and migration in the glomerular microvasculature in the absence of inflammation.

**T-cell adhesion in antigen-bearing glomerular capillaries.** We have previously shown that OT-II cells can trigger antigen-dependent neutrophil recruitment within 4 h in a planted antigen model of CD4+ T-cell-dependent glomerulonephritis. This model, the peptide antigen OVA323–339 (pOVA) is delivered to the glomerular microvasculature via conjugation to 8D1, an mAb that binds to the NC1 domain of the α3 chain of type IV collagen in the glomerular basement membrane without inducing glomerular injury. The 8D1/pOVA construct was transferred into mice intravenously together with fluorescently labeled OT-II cells. In order to focus on initiation of the T-cell-dependent response,
Fig. 1 CD4+ T cells migrate constitutively in the uninflamed glomerular microvasculature. 

**a-d** Retention and migration of endogenous CD4+ T cells in the glomerular microvasculature of untreated mice, as assessed using multiphoton intravital microscopy. 

*Image sequence showing CD4+ T cells (anti-CD4-PE, red) undergoing retention and migration in the glomerular capillaries (vasculature detected via Qtracker® 655—blue). The glomerular border is denoted by a thin dotted line, and the migration paths of two CD4+ T cells are indicated by thick dotted lines (time elapsed shown below images). See also Supplementary Movie 1. Scale bar, 10 μm. 

**b** Quantification of adhesion and migration of endogenous CD4+ T cells in glomeruli of untreated mice (n = 6 mice). Data show number (b) and dwell time (c) of adherent CD4+ T cells, and velocity of crawling cells (d). In b and c, data are shown for total cells, and specifically for static or crawling cells. In b, data are expressed as # per glomerulus per h per mouse. In c, data are expressed per cell (n = 124 total, 96 static and 28 crawling). In d, circles represent individual cells (n = 21). 

**e** Retention and migration of effector CD4+ T cells in the glomerular microvasculature of untreated mice. OT-II cells were activated in vitro, labeled with CFSE (green) and transferred into uninflamed mice. 

*Image sequence showing effector CD4+ T cell (green) migrating within the glomerular capillaries. See also Supplementary Movie 2. Scale bar, 10 μm. 

**f** Quantification of adhesion and migration of activated OT-II cells in glomeruli in the 90 min following transfer (n = 8 mice). 

**g-h** Migration speed of crawling OT-II cells. Dwell time of total, static, and crawling OT-II cells (n = 333 total, 232 static and 101 crawling). 

**i** OT-II cell recruitment to the glomerular capillaries was examined in 10 min intervals during the 2 h after transfer. In mice given 8D1/pOVA, a significant increase in crawling OT-II cells was detectable 71–80 min after transfer (Fig. 2d). 

To identify when the increased retention of crawling OT-II cells occurred, the rate of OT-II cell recruitment to the glomerular capillaries was examined in 10 min intervals during the 2 h after transfer. In mice given 8D1/pOVA, a significant increase in crawling OT-II cells was detectable 71–80 min after transfer (Fig. 2d). 

In contrast, the rate of arrest of static OT-II cells was constant after the first 20 min of the observation period.
Presence of antigen in glomeruli alters recruitment and migration of effector CD4+ T cells. Retention and migration of activated OT-II cells in glomerular capillaries were assessed via intravital multiphoton microscopy after i.v. administration of OT-II cells and either unconjugated control 8D1 mAb or 8D1/pOVA (n = 6 mice per group). Data are shown for the total number of adherent OT-II cells (a), the number of crawling or static OT-II cells (b) and the crawling speed of OT-II cells (c) (8D1, n = 71 cells; 8D1/pOVA n = 83 cells). d, e Rate of arrival of adherent OT-II cells as assessed in 10-min intervals in the 2 h following administration, for crawling cells (d) and static cells (e). f, g Dwell time for OT-II cells, shown for total cells (8D1, n = 226; 8D1/pOVA, n = 423) (f), and for crawling (8D1, n = 132; 8D1/pOVA, n = 257) and static (8D1, n = 90; 8D1/pOVA, n = 148) cells (g). h, i Activated OT-II (pOVA-specific) and SMARTA (LCMV-specific) CD4+ T cells were co-transferred into mice treated with either 8D1-pOVA (h) or 8D1 (i), and glomerular retention of the two types of cells was assessed using multiphoton microscopy. Shown are individual T-cell dwell times 30-120 min after cell transfer, as well as group mean ± s.e.m. Data represent analysis of a total 139 SMARTA and 127 OT-II cells (h) or 90 SMARTA and 125 OT-II cells (i) from n = 3 recipient mice in both experiments. *P < 0.05 for the comparisons shown. a, b Mann–Whitney tests; c, d, f, g, h, i unpaired Student’s t-tests

Antigen-presenting cells patrol glomerular capillaries. Effector CD4+ T cells recognize antigenic peptides presented via MHCII molecules to induce responses in peripheral tissues. However, in non-inflamed glomeruli, it has been reported that resident cells expressing MHCII are rare. We confirmed these findings using MHCII-EGFP mice in which GFP is expressed fused to MHCII, thereby labeling MHCII-expressing cells. Three-dimensional
Multiphoton analysis of fixed tissues confirmed a lack of MHCII expression within uninflamed glomeruli, though many cells of dendritic morphology expressed MHCII in the interstitium and the periglomerular region (Supplementary Fig. 2 and Supplementary Movie 3). To exclude the possibility that projections from periglomerular mononuclear phagocytes extended into the glomerulus, we performed similar analyses of kidneys from CD11c-YFP mice, in which YFP is highly expressed in renal dendritic cells and cellular projections are readily detectable. In agreement with previous studies, these experiments revealed an interdigitated network of dendritic cells in the interstitium, including immediately adjacent to, but not within glomeruli. However, no projections from these cells were detected within glomeruli (Supplementary Fig. 2 and Supplementary Movie 4). These data indicate that mononuclear phagocytes of the renal interstitium are unlikely to contribute to intraglomerular antigen presentation under resting conditions.

We next asked whether the MHCII+ cells responsible for antigen presentation were blood-borne leukocytes migrating within glomerular capillaries. Several populations of MHCII-expressing leukocytes circulate in the bloodstream, including B cells and a subset of monocytes. To determine if MHCII+ cells also undergo retention and migration in glomerular capillaries, we examined kidneys of MHCII-EGFP mice via intravital multiphoton imaging. Experiments in uninflamed MHCII-EGFP mice revealed that numerous intravascular MHCII+ cells migrate within glomerular capillaries (Fig. 3a, Supplementary Movie 5). Approximately 20 MHCII+ cells underwent adhesion in the glomerular capillaries in each glomerulus every hour (Fig. 3b). Nearly 80% of these cells remained static (Fig. 3b), with an average dwell time of ~5 min (Fig. 3c). However, the minority of MHCII+ cells that crawled within glomerular capillaries were retained for more than twice as long (Fig. 3c). We confirmed these findings in glomeruli of intact, non-hydronephrotic kidneys of 3-week-old mice, in which ~15 adherent MHCII+ cells underwent adhesion per glomerulus per hour, the majority of which were stationary (Fig. 3d, Supplementary Movie 6). These experiments demonstrate that MHCII-expressing leukocytes constitutively undergo periods of retention, and in some cases migration, in glomerular capillaries, even in the absence of inflammatory stimuli. Together with our observations of constitutive intraglomerular migration of CD4+ T cells and altered retention of these cells in the presence of cognate antigen, these findings raise the possibility that circulating MHCII+ leukocytes present glomerular antigens to intravascular effectors CD4+ T cells.

T cells interact with intravascular antigen-presenting cells. For intravascular MHCII-expressing leukocytes to present antigens to CD4+ T cells within the glomerulus, these two populations must interact within glomerular capillaries. To determine if this occurs, CMTPX-labeled OT-II cells were imaged 30–90 min after transfer into MHCII-EGFP mice injected with either 8D1/pOVA or unconjugated 8D1. In both groups of mice, OT-II cells and MHCII+ leukocytes consistently underwent interactions in the glomerular microvasculature (Fig. 4a, Supplementary Movie 7). On average, each OT-II cell interacted with one MHCII+ cell during its time within the glomerulus (Fig. 4b), with the number of interactions being similar in mice receiving either 8D1 or 8D1/pOVA. The majority of interactions were no longer than 4 min. However, we noted more prolonged interactions (>10 min) in

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**Fig. 3** MHCII+ immune cells constitutively migrate in glomerular capillaries. Multiphoton microscopy was performed on glomeruli of uninflamed MHCII-EGFP mice. *a* Image sequence showing actions of adherent EGFP+ leukocytes (green) in glomerular capillaries (vasculature detected via Qtracker® 655, blue). Arrow (left panel) indicates a crawling EGFP+ cell and a subset of monocytes. To determine if MHCII+ expressing leukocytes circulate in the bloodstream, including B cells within glomerular capillaries. Several populations of MHCII-expressing leukocytes were blood-borne leukocytes migrating within glomerular capillaries. Several populations of MHCII-expressing leukocytes circulate in the bloodstream, including B cells and a subset of monocytes. To determine if MHCII+ cells also undergo retention and migration in glomerular capillaries, we examined kidneys of MHCII-EGFP mice via intravital multiphoton imaging. Experiments in uninflamed MHCII-EGFP mice revealed that numerous intravascular MHCII+ cells migrate within glomerular capillaries (Fig. 3a, Supplementary Movie 5). Approximately 20 MHCII+ cells underwent adhesion in the glomerular capillaries in each glomerulus every hour (Fig. 3b). Nearly 80% of these cells remained static (Fig. 3b), with an average dwell time of ~5 min (Fig. 3c). However, the minority of MHCII+ cells that crawled within glomerular capillaries were retained for more than twice as long (Fig. 3c). We confirmed these findings in glomeruli of intact, non-hydronephrotic kidneys of 3-week-old mice, in which ~15 adherent MHCII+ cells underwent adhesion per glomerulus per hour, the majority of which were stationary (Fig. 3d, Supplementary Movie 6). These experiments demonstrate that MHCII-expressing leukocytes constitutively undergo periods of retention, and in some cases migration, in glomerular capillaries, even in the absence of inflammatory stimuli. Together with our observations of constitutive intraglomerular migration of CD4+ T cells and altered retention of these cells in the presence of cognate antigen, these findings raise the possibility that circulating MHCII+ leukocytes present glomerular antigens to intravascular effectors CD4+ T cells.
mice that received antigen (Fig. 4c), generally occurring 60–90 min after transfer. These results demonstrate that intravascular OT-II cells are able to interact with antigen-presenting cells within the glomerular capillaries.

**Antigen-dependent activation of OT-II cells in glomerulus.** To assess whether OT-II cells undergo activation within the glomerular capillaries, we made use of a system enabling visualization of translocation of the transcription factor nuclear factor of activated T cells-1 (NFAT1) to the nucleus. In T cells, NFAT1 is a critical transcription factor driving changes in gene expression in response to T-cell activation, and rapidly translocates to the nucleus in response to elevation in cytosolic calcium following TCR engagement. Via use of the fluorescent reporter NFAT1(1–460)-GFP (NFAT-GFP), NFAT1 translocation to the nucleus, and therefore T-cell activation, can be tracked in vivo using multiphoton microscopy. We generated NFAT-GFP-transduced OT-II (OT-II-NFAT-GFP) cells and demonstrated in vitro that the NFAT-GFP reporter readily translocated to the nucleus following T-cell activation (Supplementary Fig. 3). We then transferred these cells into mice 30 min after treatment with either 8D1 or 8D1/pOVA and assessed subcellular localization of the reporter (Fig. 4d–f and Supplementary Fig. 3e–g). Following administration of 8D1-pOVA, NFAT-GFP had either partially or completely translocated to the nucleus in 26% of OT-II-NFAT-GFP cells, whereas in mice that received 8D1, translocation was seen in only 10% of cells (P < 0.05, Fisher’s exact test) (Fig. 4g).

Furthermore, typically T cells with cytoplasmic NFAT-GFP were active and highly migratory, while cells displaying nuclear NFAT-GFP remained static (Supplementary Movie 8), consistent with previous descriptions of T-cell arrest during antigen-dependent activation. These experiments provide direct evidence of T-cell activation occurring within the glomerular capillaries.

To investigate whether OT-II cells in the kidney showed other changes typically associated with antigen recognition, 4 h after transfer kidneys were digested and the T cells analyzed by flow cytometry, assessing IFNγ production. In mice that received 8D1/pOVA, renal OT-II cells had significantly increased de novo production of IFNγ, compared with OT-II cells from mice that received 8D1 control mAb (Fig. 5a–c). This response was not seen in mice that received pOVA conjugated to a control antibody (IgG/pOVA) (Fig. 5b, c), demonstrating that pOVA administered in a non-targeted form was insufficient to induce this response.

We previously observed increased glomerular neutrophil retention in mice 4 and 24 h after receiving OT-II cells and 8D1/pOVA. Here we confirmed that this response was also dependent on glomerular targeting of pOVA. Comparison of neutrophil responses 24 h after administration of OT-II cells plus either 8D1, 8D1/pOVA or non-targeted IgG/pOVA revealed that increased neutrophil dwell time was observed in mice that received 8D1/pOVA but not IgG/pOVA (Fig. 5d). As an additional readout of the neutrophil response, we used the oxidant-sensitive fluorochrome, DHE, to assess ROS production...
by intraglomerular neutrophils\textsuperscript{17,18}. These experiments revealed that induction of neutrophil ROS production also required glomerular targeting of antigen (Fig. 5e). Together these data indicate that glomerular localization of pOVA results in T-cell activation in the microvasculature of the glomerulus and induction of neutrophil-dependent glomerular inflammation.

B cells are not required for T-cell-dependent inflammation. We next sought to identify the MHCII-expressing immune cell in the circulation responsible for intravascular antigen presentation to CD4\textsuperscript{+} T cells in glomerular capillaries. Flow cytometric analysis of blood from MHCII-EGFP mice revealed that the majority (~95%) of MHCII-EGFP\textsuperscript{+} leukocytes were CD19\textsuperscript{+} B cells (Supplementary Fig. 4a). The remainder consisted primarily of a subset of CD115\textsuperscript{+} CD11c\textsuperscript{+} dendritic cells. To determine whether B cells are retained in glomerular capillaries, we used anti-B220 to label B cells in vivo (Supplementary Fig. 4b, Supplementary Movie 9). B cells adhered within glomerular capillaries at a rate of ~13 cells per glomerulus per hour (Supplementary Fig. 4c). The majority of adherent B cells remained stationary (Supplementary Fig. 4c), with a dwell time of ~8 min, while the minor population of crawling B cells had a shorter dwell time of only ~3 min (Supplementary Fig. 4d).

To determine whether B cells were required for induction of T-cell-mediated glomerular inflammation, we next assessed neutrophil retention in B-cell-deficient μMT mice 4 h after transfer of OT-II cells and either 8D1/pOVA or unconjugated 8D1. μMT recipient mice that received 8D1/pOVA had prolonged retention of neutrophils in glomeruli, compared with mice that received 8D1 (Supplementary Fig. 4e), similar to the response described previously in wild-type mice\textsuperscript{17}. These findings indicate that while B cells comprise the majority of MHCII\textsuperscript{+} cells retained in the uninflamed glomerulus, they are not required for induction of CD4\textsuperscript{+} T-cell-mediated inflammation.
**MHCII**+ monocytes have prolonged intraglomerular retention.

We next examined the contribution of circulating monocytes to the MHCII**+** leukocyte population undergoing retention in glomerular capillaries. Flow cytometric analysis of blood from MHCII-EGFP mice revealed that 10–20% of circulating monocytes expressed MHCII (Fig. 6a) similar to previous reports, with the cell being found in both CX3CR1**lo-int** (classical) and CX3CR1**hi** (non-classical) populations (Supplementary Fig. 5). To determine whether monocytes contributed to the MHCII**+** leukocyte population patrolling the glomerulus, we treated MHCII-EGFP mice with clodronate liposomes and analyzed migration of EGFP**+** cells by multiphoton microscopy. Clodronate liposome treatment has recently been shown to almost eliminate patrolling monocytes from the glomerulus. In MHCII-EGFP mice, clodronate liposomes did not significantly affect the number of stationary MHCII**+** cells in glomerular capillaries (Fig. 6b), consistent with the continued retention of B cells.

In the absence of inflammation, monocytes extensively patrol the glomerular microvasculature, being retained in the glomerular capillaries for an average of ~20 min. To determine whether monocytes contributed to the majority of the crawling MHCII**+** leukocyte population within glomerular capillaries, we next examined the contribution of circulating monocytes to the glomerular microvasculature, being retained in the glomerular capillaries for an average of ~20 min. To determine whether monocytes contributed to the majority of the crawling MHCII**+** leukocyte population within glomerular capillaries, we next examined the contribution of circulating monocytes to the glomerular microvasculature, being retained in the glomerular capillaries for an average of ~20 min. To determine whether monocytes contributed to the majority of the crawling MHCII**+** leukocyte population within glomerular capillaries, we next examined the contribution of circulating monocytes to the glomerular microvasculature, being retained in the glomerular capillaries for an average of ~20 min. To determine whether monocytes contributed to the majority of the crawling MHCII**+** leukocyte population within glomerular capillaries, we next examined the contribution of circulating monocytes to the glomerular microvasculature, being retained in the glomerular capillaries for an average of ~20 min. To determine whether monocytes contributed to the majority of the crawling MHCII**+** leukocyte population within glomerular capillaries, we next examined the contribution of circulating monocytes to the glomerular microvasculature, being retained in the glomerular capillaries for an average of ~20 min.
MHCI + monocytes are not recognized as professional antigen-presenting cells. Nevertheless, for them to be important in T-cell-mediated glomerular inflammation, they must be able to induce antigen-dependent responses in T cells. Therefore, we sorted MHCI + monocytes from the blood of MHCI-GFP mice (Fig. 7a) and assessed their capacity to induce OT-II cell proliferation in vitro 39,40. In the presence of OVA-peptide, MHCI + monocytes were able to induce OT-II T-cell proliferation (Fig. 7b-d). T cells did not proliferate in the absence of monocytes and the level of T-cell proliferation was proportional to the number of monocytes in the assay, supporting the specificity of this response. These observations clearly demonstrate that the MHCI-expressing monocyte subset can present antigen to CD4 + T cells. Finally, using electron microscopy, we observed that mononuclear immune cells patrolling glomerular capillaries extended projections in close proximity to fenestrae of glomerular endothelial cells (Supplementary Fig. 8), raising the possibility that these leukocytes were probing the location of the antigen in these experiments. Together these findings indicate that a population of MHCI + monocytes undergoes constitutive patrolling within the glomerular microvasculature, characterized by substantially longer intraglomerular dwell times than any other population previously identified, and that MHCI + monocytes have the capacity to induce CD4 + T-cell proliferation.

Monocytes are required for T-cell-induced inflammation. We next assessed whether the presence of MHCI + patrolling monocytes within glomeruli was associated with a role for monocytes in induction of antigen-dependent glomerular inflammation, using clodronate liposomes to deplete monocytes in the OT-II cell-8D1/pOVA model. Firstly we established that OT-II cell trafficking in the glomerulus was unaltered in clodronate-treated mice (Supplementary Fig. 9). Subsequently we used neutrophil retention and ROS production in glomerular capillaries as readouts of T-cell-initiated glomerular inflammation following clodronate treatment. In mice depleted of monocytes, both neutrophil dwell time and ROS production 24 h after transfer of OT-II cells and 8D1/pOVA were reduced relative to mice treated with control liposomes (Fig. 8a–c and Supplementary Movie 11), with the reduction in ROS-producing neutrophils due to reduced production by crawling cells (Fig. 8d). Together these findings support the hypothesis that induction of glomerular inflammation by intravascular OT-II cells in response to planted antigen requires monocytes.

Discussion

CD4 + T cells specific for nephritogenic autoantigens are present in the circulation during autoimmune glomerulonephritis 26–28, and experimental evidence indicates that CD4 + T-cell-dependent glomerular inflammation can be initiated by T cells responding to antigen located within the vasculature 15,16. However, the cell type that presents antigen to effector CD4 + cells within glomeruli is not known. The present study addresses this by demonstrating that the cellular participants required for a CD4 + T-cell-mediated response—CD4 + T cells and an MHCI-expressing cell—both undergo constitutive periods of intravascular retention and crawling in the glomerular microvasculature. In addition, here we observed that each T cell retained in the glomerulus interacts with on average one MHCI-expressing immune cell. In the presence of antigen, T cells in the glomerular capillaries displayed evidence of antigen recognition including nuclear translocation of NFAT1, prolonged dwell time and reduced migration velocity. The MHCI-expressing cells retained in the glomerulus for the longest duration expressed the monocyte marker CD11b and monocyte

we observed retention of three populations: (1) MHCI + CD11b+ cells, the bulk of which were likely to be B cells; (2) MHCI− CD11b+ cells (neutrophils or monocytes); and (3) MHCI− CD11b+ cells (Fig. 6c and Supplementary Movie 10). Adhesion of these CD11b+ MHCI+ leukocytes occurred at a rate of ~2.5 cells per glomerulus per hour, with the majority undergoing crawling (Fig. 6d). Interestingly, the mean dwell time of CD11b + MHCI + cells in glomeruli was ~40 min, compared to <10 min for CD11b− MHCI + cells, with this applying to both crawling and stationary cells (Fig. 6e). Furthermore, cells with dwell times >35 min were almost exclusively CD11b + MHCI + (Fig. 6f). As B cells typically have a short (~6 min) dwell time in glomeruli (Supplementary Fig. 4d), these findings support the interpretation that the CD11b + MHCI + leukocytes with prolonged dwell times are not B cells.
depletion attenuated inflammation induced by T-cell antigen recognition. Together these findings demonstrate that a CD4+ T-cell-driven immune response in the glomerulus can be mediated by intravascular antigen presentation by an MHCII-expressing subset of circulating monocytes.

We hypothesized that a key step in the development of T-cell-mediated glomerulonephritis occurs when T cells recognize disease-causing antigens within the glomerular microvasculature. Here we provide direct evidence of T-cell activation in the glomerulus during the initial response to locally planted antigen. Using the NFAT-GFP reporter to reveal T-cell activation in vivo at a single cell level, we visualized increased nuclear translocation of this transcription factor in T cells in glomerular capillaries, in the presence of cognate antigen targeted to the glomerulus. These findings clearly demonstrate that antigen-dependent T-cell activation occurs intravascularly within the glomerular capillaries. Glomerular targeting of pOVA was necessary for both T-cell activation and downstream intraglomerular neutrophil activation, as these responses were not seen when pOVA was delivered systemically in a non-targeted fashion. Together these data provide compelling evidence that T cells respond to antigen within glomerular capillaries and that intraglomerular antigen recognition leads to proinflammatory changes in the glomerular microvasculature. In some studies, immunogenic peptides capable of binding directly to MHCII without intracellular uptake or processing have been used to examine the immediate response to antigen presentation.31 As our studies use the peptide recognized by OT-II cells, it is conceivable that a similar response could be at work here. However, as the peptide is covalently conjugated to a much larger immunoglobulin molecule, we anticipate that intracellular processing is the more likely route by which the OVA peptide is loaded onto MHCII.

In this planted antigen model of T-cell-dependent glomerular inflammation, we reasoned that the cell responsible for antigen presentation must either be intravascular or have access to the glomerular capillary lumen. The renal interstitium plays host to an abundant mononuclear phagocyte population, and these cells may access the lumen of the interstitial cortical renal microvasculature via extension of cellular processes. How these cells may access the lumen of the interstitial cortical renal microvasculature via extension of cellular processes. However, results of the present experiments indicate that this is not the case in the glomerulus. Furthermore, no intrinsic glomerular cells, including glomerular endothelial cells, expressed detectable MHCII under resting conditions. Therefore, we next examined circulating immune cells with the potential to present antigen to CD4+ T cells. Circulating B cells expressed MHCII. However, OT-II cells could induce glomerular inflammation in the absence of B cells. Therefore we examined the possibility that a monocyte subpopulation mediated this response.

Fig. 8 Monocytes are required for T-cell-induced neutrophil activation in response to planted antigen. The effect of clodronate liposome-mediated monocyte depletion on glomerular neutrophil retention and activation was assessed 24 h after transfer of OT-II cells and 8D1/pOVA via intravitral multiphoton microscopy. Mice were treated with either control liposomes (white bars) or clodronate liposomes (gray bars) 18 h prior to cell transfer. a Neutrophil dwell time in control and clodronate liposome-treated mice. b–d Production of reactive oxygen species (ROS) by neutrophils, as assessed in intravitral microscopy experiments via the ROS-sensitive fluorochrome, dihydroethidium (DHE). b Multiphoton image sequence showing a neutrophil (arrow) (identified by Gr-1 staining, green, upper panels) positively stained for DHE (red, lower panels). Time elapsed is shown beneath each panel. See also Supplementary Movie 11. c Number of DHE+ neutrophils adherent in the glomerular capillaries shown for the total population (c) and separately for static and crawling neutrophils (d). Data are shown as mean ± s.e.m. from seven mice per group. **P < 0.01 vs Control. a, d Mann–Whitney tests; c unpaired Student’s unpaired t-test.
In mice, monocytes exist in two major subpopulations: classical/anti-inflammatory (CCR2hi CX3CR1lo Ly6C−) and non-classical/patrolling (CCR2− CX3CR1hi Ly6C+). Mounting evidence indicates that the patrolling subset has a specialized role in mediating responses to injury or infection from within the vasculature, including in the glomerulus, and these cells also undergo prolonged retention and migration in the glomerular microvasculature. We observed that a subset of the MHCIIdoxpressing cells that were positive for the monocyte marker CD11b were retained in the glomerulus, often for over 40 min. This prolonged patrolling of the glomerular microvasculature provides opportunity to encounter and internalize antigens while also increasing the probability of this cell encountering intraglomerular T cells. These properties are consistent with an immunosurveillance function for these cells. We also demonstrate that MHCIIdox-expressing cells can induce antigen-specific T-cell proliferation in vitro, providing additional support for the contention that these cells are responsible for antigen-specific T-cell activation in glomerular capillaries. With future technical advances, it may be feasible to isolate or image patrolling MHCIIdoxmonocytes in glomeruli to demonstrate antigen uptake from within the glomerular microvasculature.

To examine a role for monocytes in T-cell-induced glomerular inflammation, we used clodronate to remove intravascular monocytes. Clodronate did not affect retention of the MHCIIdoxexpressing cells that underwent brief, static retention in glomeruli, consistent with our observations that these were B cells. In contrast clodronate markedly reduced the number of migratory MHCIIdox-expressing cells present in glomeruli, providing further evidence that these cells were monocytes. This depletion strategy resulted in a reduction in the downstream inflammatory readouts of neutrophil retention and ROS-generating activity, consistent with the hypothesis that MHCIIdox-expressing monocytes are responsible for intravascular antigen presentation in this model. We recently reported that monocytes promote innate responses of intravascular neutrophils in acute glomerulonephritis. The present findings add to this by showing that monocytes can also perform the function of intravascular antigen presentation to T cells in T-cell-mediated glomerular injury. These findings are of particular relevance to the initial stages of disease. However in established autoimmune glomerulonephritis, further mechanisms of antigen recognition, including antigen presentation by glomerular endothelial cells with upregulated MHCIIdox expression could contribute to activation of CD4+ T cells.

Alterations in migration are characteristic of T cells undergoing antigen recognition. In naïve cells, altered migration occurs over several hours, but effector T cells respond to antigen presentation rapidly via migratory arrest and induction of cytokine production. The T-cell responses in the present study are consistent with previous descriptions of effector T cells. Identification of cells undergoing antigen presentation on the basis of nuclear localization of NFAT-GFP revealed that these cells were uniformly static, while cells with non-translocated NFAT-GFP, and therefore not detecting antigen, continued to migrate. This T-cell response occurred within the first hour after T-cell transfer. Analysis of cytokine production provided further evidence of rapid antigen-specific activation by intrarenal T cells in that many intrarenal effector CD4+ T cells were producing IFNγ 4 h after transfer. Finally, changes in neutrophil retention and activation resulting from this rapid T-cell response were apparent within 4 h. These findings highlight the speed with which effector T cells can respond to antigen in glomeruli and promote downstream inflammation. It should be noted that in these experiments, we used effector T cells generated via a standard ex vivo differentiation protocol. It is conceivable that responses of cells generated in this manner may differ from those of cells differentiated in vivo, a possibility that could be explored in future studies.

The actions of neutrophils observed in glomerular capillaries under resting conditions are similar to those reported for neutrophils in uninflamed pulmonary capillaries. In the lung, this intravascular patrolling may be a form of immune surveillance facilitating rapid responses to local infection. It is possible that this is also an important function of neutrophils in glomeruli. However, microbial infection is less explicitly relevant in the glomerulus than in the lung. In the glomerulus neutrophil patrolling is a double-edged sword as it also underpins the induction of injurious responses after immune complex deposition or in response to other inflammatory stimuli.

In conclusion, these studies have identified a novel mechanism of intravascular antigen recognition in the glomerular microvasculature, in which circulating CD4+ T cells routinely interact with patrolling antigen-presenting cells. Under these circumstances, deposition of antigen within the glomerular vasculature can result in rapid inflammation in the glomerular capillaries. This is the first study to show the coordinated series of events underlying initiation of intravascular CD4+ T-cell responses in the glomerulus.

Methods

Mice. C57Bl/6 wild-type mice were obtained from Monash Animal Research Services and housed in specific pathogen-free conditions. MHC Class II-EGFP knock-in (MHCIIdox-EGFP) mice on a C57Bl/6 background, generously provided by B. Fazekas de St Groth (University of Sydney) were bred in-house. Ifnγ−/−, il2−/−, il12−/−, il10−/−, il6−/− mice, Cxcr4fl/fl mice, and OT-II mice, all on a C57Bl/6 background, were bred in-house. SMARTA-GFP mice were generously provided by S. Mueller (University of Melbourne). Male mice between 3 and 22 weeks of age were used in all experiments. All experimental procedures were approved by the Monash Medical Centre Animal Ethics Committee ‘B’. Sample size calculation was not performed a priori because the effect sizes of our observations and interventions could not be determined before experiments. This study was not randomized and was not blinded. All experiments were included in the analyses.

Antibodies and reagents. For production of ovalbumin peptide (pOVA)- conjugated 8D1, we used 8D1 mAb (grown from hybridoma), chemical linker N-succinimidyl-6-maleimido-caproate (EMCS, Sigma Aldrich), and a custom 8D1 conjugated to Pacific Blue (ThermoFisher Scientific) (see Supplementary Table 1). The following mAbs were used for flow cytometry (see Supplementary Table 1 for information on antibodies used in this study): anti-CD11b PE (clone 205/187, 2C9, BD Biosciences), anti-CD11c APC (HL3), anti-CD11b APC-Cy7 (M1/70), anti-Ly6C-APC (AL-21), and anti-CD45 APC (30-F11) (eBioscience); and anti-CD19 APC-Cy7 (1D3), anti-CD11c-PE (M5/114.15.2), anti-TCR Vβ5.1/5.2 FITC (MR9-1), anti-CD4-PE (RB6-8C5, 24G2), anti-CD8-PE (53-6.7, Biolegend), anti-CD69-PE (H1.2F3, Biolegend), and anti-CD45-PE-Cy7 (30-F11) (eBioscience). Gating strategies used to define the relevant populations are shown in each figure.

In vitro activation of OT-II T cells. OT-II T cells were differentiated into Th1 effector cells as described previously. Briefly, lymph nodes were harvested from OT-II mice, and cells were isolated and cultured at 1×106 cells mL−1. Splenocytes

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from C57BL/6 mice were treated with mitomycin C (50 μg g−1, 20 min at 37 °C), then washed thoroughly and cultured with naïve OT-II cells at a ratio of 10:1 in medium supplemented with 1 μM OVA257-264 peptide. Cells were maintained in media containing rMII-12 (2 ng ml−1) and anti-IL-4 mAb (1B11, 10 μg ml−1). After 48 h, rMII-2 (5 μg ml−1) was added and maintained thereafter. Cells were split once and harvested on day 7. Cell preparations were centrifuged through Histopaque 1083 or Optiprep (1.09 g ml−1) for 30 min at 400 g at room temperature to remove dead cells. 1×106 OT-II T cells were transferred into recipient mice via jugular or tail vein unless otherwise stated. For analysis of migration, OT-II T cells were labeled with 5 μM CFSE or 1 μM CMTX prior to transfer. In some experiments, CD4+ T cells from GFP-expressing SMARTA TCR transgenic mice (specific for the LCMV GP-derived P13 peptide) underwent the same activation protocol, using P13 peptide (1 μM) as the stimulating antigen. In these experiments, SMARTA cells showed comparable levels of activation as OT-II cells (Supplementary Fig. 10).

Antibody peptide conjugation. The 8D1 mAb (of IgG1 subclass) was conjugated to OVA233-339, using a previously published technique. In brief, 8D1 mAb was mixed with tenfold molar excess of EMCS for 2 h at room temperature. Unreacted EMCS was removed by buffer exchange chromatography, and the activated 8D1 was mixed with tenfold molar excess of modified OVA233-339. After incubation for 3 h at room temperature, the reaction was halted by adding 2 μl cytoxane, and the modified 8D1/pOVA antibody was dialyzed in PBS to remove excess OVA233-339. For some control experiments, pOVA was conjugated to an IgG1 control antibody of irrelevant specificity (MOPC-21) using the identical protocol.

Induction of T-cell-dependent glomerular inflammation. In order to specifically localize OVA233-339 to the glomerular vasculature, the 8D1/OVA233-339 conjugate was used, taking advantage of the selective binding of 8D1 to the NC1 domain of α3(IV) collagen in the glomerular basement membrane. To induce glomerular inflammation, 150 μg of 8D1/pOVA was transferred intravenously into recipient mice together with OT-II T cells. Unconjugated 8D1 mAb and pOVA conjugated to MOPC-21 (IgG1/pOVA) served as controls.

Renal intravital multiphoton microscopy. To prepare the kidney for glomerular intravital microscopy, 4–5-week-old mice underwent unilateral ureteric ligation. Mice were housed for 12 weeks to allow the kidney to undergo hydro-nephrosis. Intravital imaging experiments performed on intact kidneys were carried out in 3–4-week-old mice. Multiphoton microscopy was used for intravital imaging studies. Mice were anesthetized and placed in a supine position on a heating pad to maintain the temperature of the mice at 37 °C. The intravital microscope was equipped with a 20× 1.0 NA W plan fluor objective lens and a MaiTai pulsed infrared laser (SpectraPhysics). Experimental images in C57BL6/j and μMT mice were performed at 810 nm excitation, while experiments in MHCII-GFP mice were performed at 900 nm excitation. In most experiments, images were taken every 30 s by collecting z-stacks of approximately 150 μm depth, with 6 μm step size. For 2 h imaging experiments, z-stacks were collected every 60 s. Emitted fluorescence was detected by non-descanned detectors with 432–482 nm, 500–550 nm, 575–605 nm, and 625–675 nm emission filters. Pre-defined settings for laser power and detector gain were used for all experiments. For visualization of the vasculature, either Qtracker 655, rhodamine dextran or Pacific Blue-conjugated 8D1 was used. To label endogenous leukocytes, the following monoclonal antibodies were used as appropriate (i.e., unless otherwise stated): anti-CD4 PE (CD4+ T cells); anti-B220 APC (B220+ B cells); anti-Gr-1 PE or Alex488 (neutrophils); anti-CD11b Nc650 (monocytes – 4.5 μL). OT-II cells were labeled with either CFSE or CMTX according to the manufacturer's instructions. In experiments examining neutrophil ROS production, mice received 2 μg kg−1 of pre-warmed DHE intravenously 20 min prior to imaging and were examined using 810 nm excitation.

Image analysis. Images were analyzed using Imaris software (Bitplane) in a blinded fashion. Leukocytes arrested in glomerular capillaries for at least 30 s (two consecutive frames) were defined as adherent and subsequently categorized as crawling or static. Dwell time was defined as the duration of leukocyte adhesion in the glomerulus. To measure the velocity of crawling cells, images were tracked in three dimensions over time. For neutrophil ROS production, neutrophil DHE positivity was determined after adjusting images using pre-determined thresholds to remove background staining.

Renal leukocyte isolation and analysis. For flow cytometry analysis of OT-II cell cytokine production, OT-II cells were isolated from kidneys using a modification of a previously published technique. OT-II cells were harvested from five to ten mice of OT-II T cells, infused with RPMI 1640 containing collagenase (1 mg ml−1), DNase I (100 μg ml−1) and Brefeldin A (10 μg ml−1) and incubated at 37 °C for 25 min. Kidneys were gently dissociated and incubated at 37 °C for a further 25 min then washed and resuspended in RPMI with 5 μg ml−1 Brefeldin A. The cell suspension was left for 10 min to settle tubular debris. The supernatant was filtered through a 70 μm cell strainer and erythrocytes were lysed. Cells were treated with Fc block, then stained for CD45, CD4, and Vβ 5.1/5.2 TCR. For intracellular cytokine staining, cells were fixed and permeabilized using the BD Cytofix/CytopermTM kit, as per the manufacturer’s instructions, and then stained for IFN-γ.

MHCII+ monocyte isolation and ex vivo T-cell proliferation. Mice were bled from blood of MHCII-GFP donors. Mice (7–11 per experiment) were bled into heparinized syringes and blood lysed using NH4Cl. Leukocytes were resuspended to 5×106 cells in 50μl, then pre-incubated with anti-CD16/CD32 Ab to block non-specific staining. Cells were stained with CD115–APC (clone AF509) to identify monocytes and anti-CD19–APC-Cy7 (clone 1D3) to enable exclusion of B cells. CD115+ monocytes that also expressed MHCII-GFP were sorted by flow cytometry (FACS ARIA Fusion). Post-sort analysis revealed >98% monocytes of which >85% were MHCII+.

OT-II T-cell proliferation was assessed using a modification of a previously published technique. Briefly, CD4+ T cells were positively enriched from LN of OT-II mice using anti-CD4 microbeads (Milteny Biotech) (purity >98%) then labeled with CFSE (5 μM). Cells were subsequently washed twice and resuspended at 1×106 ml−1. Sorted monocytes (ranging from 1.6 to 8×104 per well) were pulsed with 1 μM OVA257-339 and co-cultured with 105 CFSE-labeled OT-II cells. Negative controls included T cells pulsed with peptide alone (no antigen-presenting cells) and unpeled splenocytes (no peptide). A positive control, splenocytes pulsed with antigen as above were used as antigen-presenting cells. Cells were harvested after 65 h and stained with anti-Vα2 APC (clone B20.1) and anti-CD4–APC-Cy7 (clone GK1.5). Dead cells were excluded by propidium iodide staining and viable cells were gated and used using BD Calibrate beads (BD Biosciences). Samples were acquired on an FACS Cantoll flow cytometer and analyzed with FlowJo software (Tree Star), using the level of CFSE staining as an index of proliferation.

NFTAg-GFP vector transduction. T-cell activation was investigated via assessment of nuclear mobilization of NFTAg-GFP, using T cells transduced with retrovirus generated from pMSCVneo–ΔNFTAg-GFP (aa 1–460 of mouse NFTAg) (“NFTAg-GFP”) vector (obtained from Dr. Vigo Heissmeyer), as described previously. Briefly, pMSCVneo–ΔNFTAg-GFP was transplanted into Platinum E packaging cells (Cell Biosci, Inc.) using a calcium phosphate precipitation method. After 48–72 h, retroviral supernatants were collected and filtered through 0.45 μm filters. The virus was concentrated by precipitation using PEG 6000 and sodium chloride, resuspended in RPMI and titrated.

OT-II cells were transduced with 1.5–3.2×104 transducing units of virus on days 1 and 2 of the activation protocol, in six-well plates in the presence of polybrene (4 μg ml−1), using spinfection at 32 °C, 800 g for 1 h. On day 7, transduced NFTAg-GFP-OT-II cells were isolated via flow cytometric sorting on the basis of GFP expression. Typical transduction efficiencies were between 10 and 20%. Preliminary analyses demonstrated that following in vitro activation, the level of IFNγ expression in OT-II-NFTAg-GFP cells was comparable to that in activated non-transduced OT-II cells (Supplementary Fig. 3). Sorted OT-II-NFTAg-GFP cells were labeled with CMTPX and used in in vivo imaging experiments assessing the subcellular localization of GFP.

Statistical analysis. All data presented are shown as mean ± s.e.m. Experimental groups were compared using Student’s unpaired t tests, or if variances were unequal, unpaired t-tests with Welch’s correction or non-parametric Mann Whitney tests (all one-tailed). In experiments involving more than two groups, one-way ANOVA was performed. In experiments involving categorical analysis of T-cell phenotype, Fisher’s exact test was used. The numbers of mice used in groups were based on the expected degree of variability observed in standard kidney imaging experiments, in parameters such as number of adherent cells and dwell time. Typical group sizes ranged from 6 to 8 individual mice examined on multiple different days, assigned randomly to control or treatment groups. Significance was set at P < 0.05.

Data availability. The data that support the findings of this study are available from the corresponding author on request.

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Author contributions
C.L.V.W., M.U.N., S.L.S. and S.L. designed, performed and analyzed the experiments. P.H., M.F., A.L. and Z.H.T. performed and analyzed the experiments. C.L. and S.K.N. provided analytical tools. A.R.K. and M.J.H. designed the experiments and wrote the paper.

Additional information
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