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T Cell Dynamics during Induction of Tolerance and Suppression of Experimental Allergic Encephalomyelitis

Rohit D. Divekar,* Cara L. Haymaker,* Jason A. Cascio,* Betul F. Guloglu,* Jason S. Ellis,** Danielle M. Tartar,* Christine M. Hoeman,* Craig L. Franklin,† Bernd H. Zinselmeyer,‡ Jennifer N. Lynch,‡ Mark J. Miller,‡ and Habib Zaghouani*§

The cell dynamics associated with induction of peripheral T cell tolerance remain largely undefined. In this study, an in vivo model was adapted to two-photon microscopy imaging, and T cell behavior was analyzed on tolerogen-induced modulation. FcγR-deficient (FcγR<sup>−/−</sup>) mice were unable to resist or alleviate experimental allergic encephalomyelitis when treated with Ig-myelin oligodendrocyte glycoprotein (MOG) tolerogen, an Ig carrying the MOG35–55 peptide. However, when FcγR<sup>+/+</sup> dendritic cells (DCs) are adoptively transferred into FcγR<sup>−/−</sup> mice, uptake and presentation of Ig-MOG occurs and the animals were able to overcome experimental allergic encephalomyelitis. We then fluorescently labeled FcγR<sup>+/+</sup> DCs and 2D2 MOG-specific TCR-transgenic T cells, transferred them into FcγR<sup>−/−</sup> mice, administered Ig-MOG, and analyzed both T cell–DC contact events and T cell motility. The results indicate that tolerance takes place in lymphoid organs, and surprisingly, the T cells do not become anergic but instead have a Th2 phenotype. The tolerant Th2 cells displayed reduced motility after tolerogen exposure similar to Th1 cells after immunization. However, the Th2 cells had higher migration speeds and took longer to exhibit changes in motility. Therefore, both Th1 immunity and Th2 tolerance alter T cell migration on Ag recognition, but the kinetics of this effect differ among the subsets. The Journal of Immunology, 2011, 187: 000–000.

Peripheral T cell tolerance represents a safeguard system that prevents self-reactive T cells, which have escaped negative selection in the thymus, from causing autoimmunity (1). The molecular mechanisms that underlie peripheral tolerance are diverse and include T cell deletion (2), anergy (3, 4), cytokine bias (5, 6), and suppression by T regulatory (Treg) cells (7). For these mechanisms to function, APCs and potentially autoreactive T lymphocytes must interact under circumstances that dictate the fate of the T cells. Although the cellular and signaling mechanisms underlying peripheral T cell tolerance are well established, the cell dynamics that manifest while T cells are undergoing tolerance are poorly understood (8). In this study, an Ag-induced T cell tolerance model was developed and used to analyze the characteristic T cell behaviors and dendritic cell (DC) interactions that lead to T cell tolerance.

FcγR-deficient (FcγR<sup>−/−</sup>) mice are unable to reverse experimental allergic encephalomyelitis (EAE) when treated with tolerogenic Ig-myelin oligodendrocyte glycoprotein (MOG), an Ig carrying the MOG35–55 peptide (9). However, when FcγR<sup>+/+</sup> mice are transferred with FcγR<sup>+/+</sup> DCs, they became able to take up Ig-MOG through FcγRs and EAE subsides (9). This model is advantageous in that tolerance can be initiated by injection of aggregated (agg) Ig-MOG, providing a highly sensitive readout system for analysis of T cell motility and shape in vivo (10). Moreover, the cell dynamics displayed during Ag recognition reflect abundance of Ag and the strength of the Ag signal (11, 12). Our hypothesis was that tolerance would be associated with transient DC–T cell interactions and reduced T cell velocity, similar to early T cell Ag recognition events (13–15) and to transient interactions observed by others in models of tolerance (16, 17) or as a result of Treg function (18, 19). To test this hypothesis, we imaged adoptively transferred MOG35–55–specific 2D2 TCR transgenic (Tg) T cells (CFSE, green) and C57BL/6 FcγR<sup>+/+</sup> DCs (5-[and-6]-carboxy seminaphthorhodafluor-1 [SNARF], red) on exposure to Ig-MOG in the C57BL/6 FcγR<sup>−/−</sup> hosts. Initially, the experiments were focused on determining where contact between T cells and DCs occurs. Surprisingly, a significant number of T cell–DC contacts were observed in lymphoid tissues but not in nonlymphoid organs such as the lung, intestine, and liver. Moreover, only lymphoid organs in close proximity with the i.p. portal of entry of Ig-MOG displayed significant T cell–DC contacts. Two-photon microscopy and cell tracking showed decreased T cell displacements and reduced

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speeds but straighter trajectories relative to T cells that were not exposed to agg Ig-MOG. This pattern is similar to immune-activated T cells (13, 14) and raised the question whether tolerant T cells had activated phenotypes. Analyses of these premises indicated that the tolerant T cells had an activated phenotype and produced both IL-4 and IL-5 Th2 cytokines. Moreover, when compared with immunogen-induced, IFN-γ-producing 2D2 TCR Tg Th1 cells, the decrease in velocity appeared delayed in time but otherwise similar to the Th1 counterparts that moved at a slower pace with a more directed trajectory than naive T cells. These results indicate that tolerance during EAE occurs in lymphoid organs and displays cell dynamics that are remarkably similar to those associated with inflammatory immunity.

### Materials and Methods

#### Animals

C57BL/6 mice were purchased from The Jackson Laboratory. 2D2 TCR Tg mice have been described previously (20). FcεRII mice deficient for FcεRI and FcεRIII were previously described (9). Mice were bred and maintained in our animal care facility for the duration of the experiments. All experimental procedures were performed according to the guidelines of the University of Missouri institutional animal care committee.

#### Ags

**Peptides.** The peptides used in this study were purchased from Metabion (Munich, Germany) and were HPLC purified to >90% purity. MOG peptide encompassing amino acid residues 35–55 of MOG is encephalitogenic for C57BL/6 mice (21). Proteinpeptide 1 (PLP1) peptide encompasses an encephalitogenic sequence corresponding to aa 139–151 of PLP (22).

**Ig chimeras.** The Ig chimeras used in this study, Ig-W and Ig-MOG, have been previously described (9, 23). In brief, Ig-MOG incorporates MOG peptide inserted within the H chain complementarity determining region 3. Ig-W is the parental IgG2b,κ molecule not encompassing any myelin or other peptide. All chimeras transfectants were grown up in large-scale culture of DMEM containing 10% iron-enriched calf serum (HyClone). The chimeras were purified from culture supernatant on affinity chromatography columns made of rat anti-mouse κ-chain coupled to CNBr-activated Sepharose 4B (Amersham Biosciences). To avoid cross-contamination, we used separate columns to purify each chimaera. Aggregation of the Ig chimeras was done using 50%–saturated (NH₄)₂SO₄ as described previously (24). All the Ig chimeras were derived from the same Ig backbone and thereby comprise identical IgG2b isotype.

#### Isolation of T cells and DCs

CD4+ T cells were isolated from spleens of 2D2 Tg mice by positive selection using the MACS cell separation system (Miltenyi Biotec). APCs in this study, including DCs, were purified from culture supernatant on affinity chromatography columns made of rat anti-mouse κ-chain coupled to CNBr-activated Sepharose 4B (Amersham Biosciences). To avoid cross-contamination, we used separate columns to purify each chimaera. Aggregation of the Ig chimeras was done using 50%–saturated (NH₄)₂SO₄ as described previously (24). All the Ig chimeras were derived from the same Ig backbone and thereby comprise identical IgG2b isotype.

#### Induction of EAE

**Active EAE.** Induction of EAE has been previously described (9, 24). In brief, FcεRII knockout mice (6–8 wk old) were induced for EAE by s.c. injection in the footpads and at the base of the limbs of a 200 μl aqueous solution containing 300 μg MOG and 200 μg Mycobacterium tuberculosis H37Ra (Difco). Six hours later, the mice were given i.v. 500 ng purified Bordetella pertussis toxin (List Biological Laboratories). A second injection of B. pertussis toxin was given after 48 h. The mice were then scored for clinical signs of EAE as follows: 0, no clinical score; 1, loss of tail tone; 2, hind-limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund or death.

**Passive EAE.** Splenocytes were purified from 2D2 TCR Tg mice and activated in vitro with 30 μg/ml MOG peptide for 72 h in the presence of 10 ng/ml rIL-12. Six to 8 wk-old female FcγR−/− mice were irradiated (500 rad) and reconstituted with 6 × 10⁶ C57BL/6 FcγR−/− DCs and 10 × 10⁶ activated 2D2 cells. The mice were observed daily for disease according to the scale described earlier.

**Treatment of EAE with Ig-MOG.** For tolerance of T cells before disease induction, the mice were given a single i.p. injection of 600 μg agg Ig-MOG. For tolerance of T cells after induction of EAE, the mice were given i.p. 600 μg agg Ig-MOG three times every other day.

#### Cell labeling with fluorescent dyes

Purified 2D2 CD4+ T cells were labeled with CFSE as previously described (26). In brief, CD4 T cells (10 × 10⁶ cells/ml) were incubated with 10 μM CFSE at 37°C for 10 min. The labeled cells were then washed twice with ice-cold DMEM-10% FCS and PBS before transfer into mice. A similar protocol was used for labeling the cells with SNARF (Molecular Probes).

#### Tissue preparation and cryosections

Mice that received the adoptively transferred fluorescently tagged cells were sacrificed at respective time points. The organs were fixed internally by perfusion with 2% paraformaldehyde after perfusion with PBS. The fixed tissues were dissected and placed in a biopsy cassette filled with freezing medium (OCT; Ted-Pella). The tissues were frozen at −80°C, and cryosections 8 mm thick were obtained using Leica CM 1850 cryostat.

#### Imaging

Cryosections were imaged using the Zeiss Axiohot microscope fitted with an Olympus DP-70 camera. Images were acquired using DP controller software. Further analysis of images including overlaying, contrast, picture quality adjustment, and so on were done using Photoshop software (Adobe).

#### Measurement of cytokines by ELISA

IFN-γ, IL-4, and IL-5 were detected by ELISA according to BD Pharmingen’s (San Jose, CA) standard protocol. The capture Ab was rat anti-mouse IL-4 (RA-6-82) and the biotinylated Ab was rat anti-mouse IFN-γ (XMGl2). For IL-4 detection, the capture Ab was rat anti-mouse IL-4 (11B11) and the biotinylated Ab was rat anti-mouse IL-4 (BVD6-24G2). anti-IL-4Rα mAb (M1) was provided by Amgen (Seattle, WA). For detection of IL-5, the capture Ab was rat anti-mouse IL-5 (TRFK5) and the biotinylated Ab was rat anti-mouse IL-5 (TRFK4). The OD₅₄₀ was read on SpectraMax 190 counter (Molecular Devices, Sunnyvale, CA) and analyzed using SOFTmax PRO 3.1.1 software. A graded amount of recombinant IL-5 was included for construction of a standard curve. The cytokine concentration in culture supernatants was extrapolated from the linear portion of the standard curve.

#### Measurement of cytokines by ELISPOT

IFN-γ, IL-4, and IL-5 were detected by ELISPOT using an Immunospot analyzer as described previously (27). In brief, HA-multiscreen (Millipore) plates were coated with 100 μl/well 1 M NaHCO₃ buffer containing 2 μg/ml capture Ab. After an overnight incubation at 4°C, the plates were washed three times with sterile PBS and free sites were saturated with DMEM culture media containing 10% FCS for 2 h at 37°C. Subsequently, the blocking medium was removed and 1 × 10⁶ splenic cells were added per well together with 30 μg MOG peptide or 15 μg PLP1 peptide. After 24-h incubation at 37°C in a 7% CO₂ humidified chamber, the plates were washed and incubated overnight. The nonadherent cells that are enriched for bulk DCs were collected and used in experiments. CD11c⁺ purified DCs were obtained from spleens of C57BL/6 mice by positive selection of bulk DCs using MACS separation system.

#### Flow cytometry

SNARE+ MOG Tg 2D2 CD4+ T cells were adoptively transferred to the FcγR knockout mice. The cells were harvested from spleens and lymph nodes (LN) 48 h after treatment with Ig-MOG. Subsequently, these cells were incubated with FITC-conjugated anti-CD25 (PC61), anti-CD62L (MEL-14), or anti-CD44 (IM7) at 4°C for 30 min. The cells were then washed and analyzed using FACS Vantage flow cytometer and Cell Quest software (BD Biosciences). Dead cells were excluded based on their forward and side-scatter profiles. For staining of intracellular caspase in T cells, FITC-conjugated ZVAD FMK (BD Pharmingen) was used according to the protocol available.
**Two-photon imaging and analysis**

Mice were sacrificed by CO₂ asphyxiation at various times after T cell/DC transfers. LN were removed and analyzed by two-photon microscopy as described previously (14, 15). In brief, LN were secured to coverslips with a thin film of VetBond (3M) and placed in a flow chamber maintained at 37°C by perfusion with warm, high-glucose DMEM bubbled with a mixture of 95% O₂ and 5% CO₂. Time-lapse imaging was performed using a custom-built two-photon microscope, fitted with two Chameleon Ti:sapphire lasers (Coherent) and an Olympus XLUMPlanFI 20× objective (water immersed; numerical aperture, 0.95), and controlled and acquired with ImageWarp (A&B software). For imaging of CFSE, the excitation wavelength was set to 915 nm. Signals from fluorescent dyes were separated by dichroic mirrors (490 and 560 nm). To create time-lapse sequences, we scanned the volumes of tissue at 100 × 120 × 75 μm at 2.5-μm Z-steps at ~30-s intervals for up to 60 min.

Image acquisition, morphometric analysis, and cell tracking were performed using Metamorph software (Universal Imaging). T cell–DC contacts were visualized in three dimensions at different times using a fully rendered multidimensional data set (Imaris, Bitplane). Cells were tracked over time manually using Velocity (Improvement). Instantaneous velocities were calculated from the distance moved between successive time points.

**Statistical analysis**

Statistical analyses were done using either an unpaired, two-tailed Student t test or one-way ANOVA as indicated.

**Results**

In vivo presentation of agg Ig-MOG by DCs modulates EAE

To investigate the cellular and molecular mechanisms of T cell tolerance, we devised an in vivo model where Ag-induced interactions between T cells and APCs could be visualized. In our model, 2D2 Tg T cells carrying the MOG35–55 peptide-specific TCR are the target of tolerance induction (20). The tolerogen is Ig-MOG, an Ig chimera carrying MOG35–55 peptide within the H chain complementarity determining region 3 (9). Ig-MOG is internalized by APCs mainly via FcγRs and drives efficient peptide presentation to T cells (9). Because DCs were reliable for Ag presentation and imaging (28, 29), we used FcγR⁷/⁺ DCs to drive presentation of Ig-MOG. Accordingly, 2D2 T cells and FcγR⁷/⁺ C57BL/6 DCs were labeled with the green fluorescent dye CFSE and the red fluorescent stain SNARF, respectively, and adoptively transferred i.v. into FcγR⁷/⁻ C57BL/6 recipient mice. Subsequently, Ig-MOG was given i.p. to initiate Ag uptake and presentation to T cells by the exogenous FcγR⁷/⁺ DC. Colocalization of cells in the spleen of the host mice can be readily visualized, indicating that the approach is feasible (Supplemental Fig. 1). This model was then used to evaluate Ig-MOG–induced contact between the two cells and to analyze the cellular dynamics that govern tolerance.

The initial experiments were conducted to determine whether Ig-MOG treatment could support tolerance of T cells in this model. Accordingly, FcγR⁷/⁻ C57BL/6 mice were adoptively transferred with FcγR⁷/⁺ DCs and naive 2D2 T cells, and tolerance was induced with agg Ig-MOG before induction of EAE with MOG peptide (MOGp) (Fig. 1A). The results show that mice recipient of Ig-MOG developed clinical signs of EAE with a mean maximal score (MMS) of 1 ± 0.0, whereas those given Ig-W, the Ig backbone without MOGp, had more severe disease with a MMS of 2 ± 0.3. The disease course remained unchanged for both groups of mice for the duration of the monitoring period. These results indicate that exogenous DCs are able to take up Ig-MOG and sustain tolerogenic contact with 2D2 T cells through presentation of MOGp. To further substantiate this phenomenon, we tested whether similar tolerogenic contacts could occur when the 2D2 T cells are activated before exposure to DCs and Ig-MOG. Indeed, the transfer of disease by activated 2D2 T cells is inhibited when the mice received both FcγR⁷/⁺ DC and Ig-MOG treatment, but not when the recipients were given Ig-MOG without DC transfer (Fig. 1B). The MMS decreased from 1.75 ± 0.5 in the mice without DC transfer to 0.7 ± 0.5 in those receiving DC transfer.

In addition, Ig-W treatment had no effect (MMS: 1.8 ± 0.3) when given with DC transfer. Finally, to determine whether the DC transfer and Ig-MOG treatment system is effective with endogenous polyclonal T cells after induction of disease, we induced FcγR⁷/⁻ C57BL/6 mice for EAE with MOGp; then the animals were given FcγR⁷/⁺ DCs and treated with agg Ig-MOG. Fig. 1C shows that treatment with Ig-MOG suppressed paralysis, whereas treatment with Ig-W did not. Indeed, the MMS was reduced from 2.5 ± 1.7 in the control mice treated with Ig-MOG without DC transfer to 0.3 ± 0.5 in those recipient of both Ig-MOG and DC transfer. Moreover, the disease remained severe (MMS: 2.5 ± 0.5) when Ig-W was used instead of Ig-MOG. These results indicate that FcγR⁷/⁺ DCs are able to take up Ig-MOG and establish tolerogenic contact with T cells, thus providing a unique model to study the cell dynamics associated with tolerance in vivo.

**FIGURE 1.** Agg Ig-MOG treatment modulates both active and passive EAE. A, Groups of adult FcγR⁷/⁻ mice were given i.v. 6 × 10⁶ purified FcγR⁷/⁺ DCs and 10 × 10⁶ naive 2D2 T cells. The next day, the mice were given i.p. 600 μg agg Ig-MOG (circles) or control Ig-W (squares). One day later, the hosts were induced for active EAE with 300 μg MOG peptide and monitored daily for signs of paralysis. B, Groups of FcγR⁷/⁻ mice were given 6 × 10⁶ purified FcγR⁷/⁺ DCs and induced for passive EAE by adoptive transfer of 10 × 10⁶ MOG-activated 2D2 TCR Tg splenocytes. The next day, the mice were treated i.p. with 600 μg agg Ig-MOG (circles) or control Ig-W backbone without MOG peptide (squares) and monitored for signs of paralysis for the duration of the experiment. Groups of mice that did not receive any FcγR⁷/⁺ DCs (triangles) were included for comparison purposes. C, Groups of adult FcγR⁷/⁻ mice were induced for active EAE with 300 μg MOG peptide and 4 d later were transferred i.v. with 6 × 10⁶ purified FcγR⁷/⁺ DCs. The next day, the hosts were treated i.p. with 600 μg agg Ig-MOG (circles) or the control Ig-W (squares). The mice were then monitored for reduction in disease severity for the duration of the experiment. Groups of mice that did not receive any FcγR⁷/⁺ DCs (triangles) were included for comparison purposes. Each point represents the mean clinical score of six to eight mice and is representative of four independent experiments.
Kinetics of T cell contact with APCs

FcγR−/− mice received SNARF-labeled DC followed 6 h later by 10 × 10^6 CFSE-labeled naive 2D2 TCR Tg CD4 T cells. The next day, the mice received i.p. 600 μg agg Ig-MOG or Ig-W, and 36 h later, specimens from the liver, lung, intestine, spleen, and LN were harvested. Cryosections were then prepared and examined under UV microscope. Images acquired in red and green channels were overlaid and analyzed for pattern of cellular migration. Data are representative of three independent experiments.

Kinetics of T cell contact with APCs

FcγR−/− mice received SNARF-labeled DC followed 6 h later by CFSE-labeled 2D2 T cells to determine the site where APCs and naive T cells interact after administration of Ig-MOG. Ig-MOG was then administered, and cryosections from lymphoid and non-lymphoid organs were examined for T cell–DC interactions as an indication of Ag recognition. Few T cells and DCs were observed in the nonlymphoid organs such as the liver, lung, or intestine, and these would presumably be transient migrants (30) (Fig. 2). However, T cells and DCs were found abundantly in the spleen and peripheral LN (Fig. 2). Moreover, there was no apparent difference in the homing of T cells and DCs to these tissues whether the mice received Ig-MOG, control Ig-W, or did not receive Ag at all. Thus, although these results suggest that Ag may not influence trafficking between the lymphoid organs, they do not exclude effects on retention and on contact between specific T cells and APCs.

Given that Ig-MOG reversed both active and passive EAE, we postulated that a characteristic interaction behavior between T cells and APCs might be associated with T cell tolerance. Mice were given dye-labeled T cells and APCs, and injected with agg Ig-MOG to test the kinetics of this premise. Cryosections of the spleen and LN were cut, and contact between T cells and DCs was assessed by fluorescence microscopy at 12, 36, 60, and 96 h after Ag exposure. A contact was defined as the physical apposition of cell membranes as determined by the presence of contiguous pixels from T cells (green channel) and DCs (red channel; Fig. 3A, inset). Although the cells appeared randomly distributed in the control inguinal LN (InLN), they were more clustered in Ig-MOG–treated mice by 36 h (Fig. 3A). The findings indicate that T cell–APC contacts were apparent by 12 h in both the spleen and LN (Fig. 3B). However, the frequency of T cell–DC contacts was significantly higher 36 h after Ag injection for the spleen (p = 0.0028), axillary LN (AxLN; p = 0.007), and InLN (p = 0.0005) as compared with the “no Ag” control group. In contrast, the cervical LN did not show an increase in contact frequency (p = 0.35). Contact events decreased by 60 and 96 h in all LN. These results indicate that Ag recognition as evidenced by T cell–DC contacts occurs 36 h after Ag exposure in organs proximal to the site of injection.

**Tolerized T cells display characteristic cell dynamics during Ag recognition**

We performed two-photon microscope analyses to evaluate the cell dynamics underlying T cell tolerance. Accordingly, unlabeled DCs served in the nonlymphoid organs such as the liver, lung, or intestine, and these would presumably be transient migrants (30) (Fig. 2). However, T cells and DCs were found abundantly in the spleen and peripheral LN (Fig. 2). Moreover, there was no apparent difference in the homing of T cells and DCs to these tissues whether the mice received Ig-MOG, control Ig-W, or did not receive Ag at all. Thus, although these results suggest that Ag may not influence trafficking between the lymphoid organs, they do not exclude effects on retention and on contact between specific T cells and APCs.

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**Tolerized T cells display characteristic cell dynamics during Ag recognition**

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FIGURE 2. Assessment of T cell and DC trafficking on induction of tolerance with agg Ig-MOG. FcγR−/− mice were given i.v. 6 × 10^6 SNARF-labeled FcγR+/+ DCs followed 6 h later by 10 × 10^6 CFSE-labeled naive 2D2 TCR Tg CD4 T cells. The next day, the mice received i.p. 600 μg agg Ig-MOG or Ig-W, and 36 h later, specimens from the liver, lung, intestine, spleen, and LN were harvested. Cryosections were then prepared and examined under UV microscope. Images acquired in red and green channels were overlaid and analyzed for pattern of cellular migration. Data are representative of three independent experiments.

FIGURE 3. Analysis of T cell–APC contacts by fluorescence microscopy. FcγR−/− mice were given i.v. 6 × 10^6 SNARF-labeled FcγR+/+ DCs followed 6 h later by 10 × 10^6 CFSE-labeled naive 2D2 TCR Tg CD4 T cells. The next day, the mice received i.p. 600 μg agg Ig-MOG. Cryosections were examined for T cell–DC contact under a UV microscope. Images were acquired in red and green channels and overlaid for analysis of the proximity of red DCs in conjunction with green CD4 T cells. Such proximity events were quantified manually. Untreated mice were included as control. A, Histology images are shown for a section from the InLN at 36 h to illustrate T cell–APC contacts when the mice are given agg Ig-MOG (lower panel) versus no Ag (upper panel). Scale bars, 100 μm. B, At 12, 36, 60, and 96 h after Ag exposure, a group of mice was sacrificed, and specimens from spleen (SP), cervical LN (CxLN), AxLN, and InLN were collected as indicated in the pictogram at the top of the figure. Cryosections prepared from these organs were examined for T cell–DC contact as described earlier from untreated (control) or agg Ig-MOG (Ig-MOG) recipient mice. Each point represents the number of contacts ± SD of two different experiments. **p < 0.05 as analyzed by unpaired Student t test.
and CFSE-dyed T cells were adoptively transferred into FcγR−/− mice, and the hosts were given agg Ig-MOG as a tolerogen. Forty-eight hours after Ig-MOG injection, LN were explanted and the T cell motility was visualized by two-photon microscopy (14, 31). Cell tracks were normalized to their starting positions and plotted in Fig. 4A (top panels). Under tolerizing conditions, T cell tracks were substantially shorter and had reduced displacement over time compared with naive T cells. The reduced motility of tolerized T cells is also evident in the snapshot of representative T cells (green) and their nonnormalized tracks (yellow) shown in Fig. 4A (lower panels). The velocity of tolerized T cells (3.93 ± 0.312 μm/min) was significantly lower than that of naive T cells (8.30 ± 0.777 μm/min; p < 0.001; Fig. 4B, left panels, Supplemental Videos 1 and 2). The motility coefficient, which is a measure of how rapidly cells disperse from a given starting point, was also lower in tolerized versus naive T cells (56.75 ± 7.69 versus 110.2 ± 14 μm²/min; p = 0.0011; Fig. 4B, right panels). These results indicate that tolerization is associated with reduced T cell motility. The change in T cell motility is consistent with Ag recognition but contrasts with the cell arrest and clustering observed for activated T cells in other systems (13, 14, 32, 33).

Tolerization similar to immunization induces expression of activation markers on T cells

The fact that the T cell dynamics were quantitatively different between tolerance and naive conditions suggests that the tolerization protocol may be leading to an apoptotic process or eliciting a state of anergy. To test these premises, we injected FcγR−/− mice given FcγRα/α DCs and SNARF-labeled 2D2 T cells with agg Ig-MOG and analyzed their LN T cells for apoptosis and expression of activation markers. As indicated in Fig. 5A, the level of active caspase as measured by Z-VAD binding (26) was not significantly different in naive versus tolerized T cells (Fig. 5A), indicating that apoptosis may not be the mechanism underlying T cell tolerance. Surprisingly, however, expression of the activation markers CD25 (IL-2Rα-chain) and CD44 were significantly increased in tolerized versus control T cells. Indeed, CD25 expression increased from 4% in the control cells to 34% in tolerized T cells, whereas CD44 expression increased from 11 to 34% in control versus tolerized T cells (Fig. 5B). Finally, the T cells displayed reduction in the expression of CD62L, further indicating transition from naive to an active state (Fig. 5B). This was rather surprising and we sought to compare the activation marker expression of tolerized T cells with those activated by immunization. To test the level of activation, we conducted the same experimental design described earlier using 600 μg soluble (sol) Ig-MOG plus 33 μg CPG-oligodeoxynucleotides (ODN) as an immunizing rather than

![Image](http://www.jimmunol.org/)

**FIGURE 4.** In vivo imaging analysis of tolerized T cells. A, FcγR−/− mice were adoptively transferred with 10 × 10⁶ CFSE-labeled 2D2 CD4⁺ T cells and 6 × 10⁶ FcγRα/α DC. The next day, the hosts were given i.p. 600 μg agg Ig-MOG tolerogen (TOL) in saline or saline without Ig-MOG (NIL). The AxLN and InLN were harvested at 48 h after tolerogen injection and subjected to two-photon imaging. The behaviors of the 2D2 T cells were analyzed in detail using the Imaris software program (Bitplane). Two-dimensional vectors of T cell movement are depicted with normalization of starting point to center of an XY grid (top panels) of images (original magnification ×200) showing cell tracks (bottom panels). B, Graphs illustrate the velocity and motility coefficient of live cells with movement tracks of eight frames or higher. Scatter plots (top panels) show representative LN for each condition. Bar graphs (bottom panels) show the cumulative events collected from the LN of three mice per condition. The line represents the mean of analyzed cells. *p < 0.05 in comparison with NIL mice as analyzed by unpaired Student t test.

**FIGURE 5.** Tolerized T cells express activation markers and do not undergo apoptosis. A, FcγR−/− mice recipient of 6 × 10⁶ unlabeled FcγRα/α DC and 10 × 10⁶ SNARF-labeled 2D2 CD4⁺ T cells were given PBS (Nil) or 600 μg agg Ig-MOG (tolerized) the next day. Forty-eight hours later, LN cells were stained with ZVAD-FITC. ZVAD binding was analyzed on SNARF⁺ cells. B, FcγR−/− mice recipient of 6 × 10⁶ unlabeled FcγRα/α DC and 10 × 10⁶ CFSE-labeled 2D2 CD4⁺ T cells were given PBS (no Ag), 600 μg agg Ig-MOG (tolerized), or 600 μg sol Ig-MOG plus 33 μg CPG-ODN the next day (immunized). Forty-eight hours later, LN cells were stained with Abs to the activation markers CD25, CD62L, and CD44. Activation marker expression was assessed on CFSE⁺ cells. C, Both the DC and 2D2 cells were unlabeled, and analysis of marker expression was made on Vα3.2⁺ T cells under both immunizing and tolerizing conditions. Data are representative of three independent experiments with two mice per condition.
Interestingly, the tolerized T cells had minimal IFN-γ production (Fig. 6A). The immunized T cells produced significant amounts of IFN-γ by ELISA and ELISPOT. The results depicted in Fig. 6 indicate that functional tolerance was perhaps the result of a switch in cytokine expression profile. To test this hypothesis, we injected FcγR−/− mice given FcγR+/+ DCs with or without 2D2 T cells with agg Ig-MOG (tolerogen) or sol Ig-MOG plus CPG-ODN (immunogen), and their LN T cells were harvested, stimulated with MOG peptide, and analyzed for cytokine production by ELISA and ELISPOT. The results indicated that immunized T cells produced significant amounts of both IL-4 and IL-5 cytokines. Cytokine production is due to exposure to Ig-MOG because the mice recipient of saline solution did not produce measurable cytokines. The cytokines are produced by the transferred 2D2 cells rather than endogenous T cells because there was no detectable cytokine production in the control group recipient of DC and agg Ig-MOG but no 2D2 T cells (Fig. 6, left panels). Also, cytokine production, whether detected by ELISA or ELISPOT, is induced by Ig-MOG because mice recipient of 2D2 T cells and DCs but no Ig-MOG did not mount significant cytokine responses. Finally, cytokine production is specific because only in vitro stimulation with MOG, but not PLP1, peptide triggered cytokine production (Fig. 6, right panels). Given the specificity of cytokine production and the concordance of the results among the two techniques (ELISA and ELISPOT), we are confident that the immunizing conditions induce a Th1-type response, whereas tolerizing conditions result in Th2 responses.

**Tolerized T cells produce Th2 cytokines**

Because the tolerized T cells expressed activation markers to the same degree as immunized T cells, we investigated the possibility that functional tolerance was perhaps the result of a switch in cytokine expression profile. To test this hypothesis, we injected FcγR−/− mice given FcγR+/+ DCs with or without 2D2 T cells with agg Ig-MOG (tolerogen) or sol Ig-MOG plus CPG-ODN (immunogen), and their LN T cells were harvested, stimulated with MOG peptide, and analyzed for cytokine production by ELISA and ELISPOT. The results depicted in Fig. 6 indicate that the immunized T cells produced significant amounts of IFN-γ (Fig. 6A) and little or no IL-4 or IL-5 cytokines (Fig. 6B, 6C). Interestingly, the tolerized T cells had minimal IFN-γ but produced significant amounts of both IL-4 and IL-5 cytokines. Cytokine production is due to exposure to Ig-MOG because the mice

**FIGURE 6.** Agg Ig-MOG treatment induces Th2 cytokines, whereas sol Ig-MOG induces Th1. FcγR−/− mice recipient of 6 x 10^6 unlabeled FcγR+/+ DC alone or in combination with 10 x 10^6 unlabeled 2D2 CD4^+ T cells were on the next day given saline, 600 μg agg Ig-MOG (agg), or 600 μg sol Ig-MOG (Sol) plus 33 μg CPG-ODN (Cpg). Forty-eight hours later, LN cells were stimulated in vitro with 30 μg/ml MOG peptide (filled bars) or 15 μg/ml PLP1 peptide as control (open bars) in the absence or presence of 20 μg/ml anti-IL-4R Ab (anti-IL-4R; left column). In this case, anti-IL-4R is used to prevent reabsorption by T cells. Cytokine production was measured by ELISA (left column) and ELISPOT (right column) for IFN-γ (A), IL-5 (B), and IL-4 (C) as described in Materials and Methods. Each bar represents the mean ± SD of triplicate wells from three to four mice. N.D., not done.

**FIGURE 7.** Differential motility kinetics between immune Th1 and tolerant Th2 cells. FcγR−/− mice were adoptively transferred with 10 x 10^6 CFSE-labeled 2D2 CD4^+ T cells and 6 x 10^6 FcγR−/− DC. The next day, the hosts were given i.p. 600 μg agg Ig-MOG tolerogen in saline (TOL), 600 μg sol Ig-MOG plus 33 μg CPG-ODN in saline (IMM), or saline without Ig-MOG (NIL). The AxLN and InLN were harvested at 36 h after Ag injection and subjected to two-photon imaging. The behaviors of the 2D2 T cells were analyzed in detail using the Imaris software program (Bitplane). Graphs illustrate the (A) velocity, (B) motility coefficient, and (C) MI of live cells with movement tracks of eight frames or higher. Scatter plots show representative LN for each condition. Bar graphs show the cumulative events collected from the LN of three mice per condition. The line represents the mean of analyzed cells. *p < 0.05 as analyzed by one-way ANOVA.
velocity relative to the naive control ($p = 0.0476$), suggesting that they may be involved in short-duration contacts with DCs, similar to what has been previously demonstrated for tolerogenic T cell responses (16, 17). Also, the motility coefficient was significantly lower for the immunized T cells ($32.7 \pm 5.9 \mu m^2/min$) as compared with the tolerized ($75.4 \pm 8.2 \mu m^2/min$; $p = 0.0004$) and naive ($161.8 \pm 31.23 \mu m^2/min$; $p = 0.0132$; Fig. 7B). Moreover, there was a significant decrease in the meandering index (MI) of immunized T cells (0.2909 ± 0.026), as compared with tolerized T cells (0.6194 ± 0.0313; $p < 0.0001$) and the naive T cells (0.5372 ± 0.0224; $p < 0.0001$; Fig. 7C), indicating that immunized T cells make fewer turns and migrate with straighter paths. Representative image recordings of T cell motility presented in Supplemental Videos 3–5 clearly show reduced T cell velocity postimmunization. These results indicate that the T cell dynamics related to tolerance are distinct from those that occur during T cell priming with immunization. Specifically, tolerized cells display intermediate velocity between primed and naive cells. Moreover, because T cells exposed to the immunogen displayed a Th1 phenotype, whereas those exposed to the tolerogen had a Th2 phenotype, the results suggest that there are previously unappreciated differences in the cell dynamics associated with Th cell subsets (Figs. 6, 7).

**Discussion**

Presentation of Ag by nonprofessional APCs residing in non-lymphoid organs was for long viewed as a major mechanism for peripheral T cell tolerance (34). The study presented in this report shows that exposure to tolerogen in vivo leads to contact between the target T cells and APCs rather in lymphoid tissues with minimal interactions in nonlymphoid organs such as the liver, lung, or intestinal wall (Fig. 2). Moreover, such interactions lead to modulation of naive T cells and suppression of EAE (Fig. 1). Also, the tolerogenic contacts, like immunogenic interactions, take place in LN (INLN and AxLN) close to the portal of tolerogen entry and had defined kinetics beginning at 12 h, maximizing at 36 h, and weaning by 96 h after tolerogen administration (Fig. 3). Although it takes longer than usual for the DCs and T cells to establish contact, which is probably related to FcyR internalization and delayed processing (35), the observation was intriguing and prompted us to examine the dynamics underlying tolerogenic T cell–APC contacts. Two-photon microscopy revealed differences in T cell behavior during immunization and tolerization, perhaps reflecting the strength or the quality of the Ag recognition event. Tolerized T cells displayed lower velocity and traveled shorter distances in comparison with cells that have not been exposed to tolerogen, but were generally more motile than cells stimulated under immunization conditions (Figs. 4, 7). Initially, this was not surprising because the cells could be undergoing a state of anergy or apoptosis. However, a follow-up investigation indicated that the T cells were neither apoptotic nor anergic but expressed markers indicative of T cell activation (Fig. 5). Despite the expression of CD25, these activated T cells could not be Tregs because they did not express Foxp3 (data not shown) and did not preserve CD62L (Fig. 5). However, cytokine production analysis indicated that they produced both IL-4 and IL-5 in picogram quantities despite similar frequency in cytokine-producing cells (Fig. 6). The findings are consistent with earlier reports indicating T cells that traffic to the T zone boundary display increased motility (38).

Overall, the study presented in this article demonstrates that T cell tolerance takes place within lymphoid organs and is performed by professional DCs in our EAE model. Moreover, Th2 cells display distinct motility characteristics compared with immunized Th1 cells despite having similar activation states.

Recognition of Ag, whether under immunogenic or tolerogenic regimens, induces slower T cell motility (13–15, 32, 33, 39). Although the T cell dynamic may depend on the APC phenotype, the APC activation state, chemokine environment, or the mode of Ag delivery, decreased motility was commonly observed during Ag recognition (17, 28, 38–40). Even during thymic education a slow motility ensued for thymocyte selection to occur (41). The findings reported in this article extend these observations to Th2 T cell dynamics. Although Th2 cells displayed a reduction in motility after Ag recognition, Th2 cells had higher overall migration speeds and took longer to exhibit changes in their motility than Th1 cells induced by immunization. Both Th1 immunity and Th2 tolerance alter T cell migration on Ag recognition, but the kinetics of this effect differ among the subsets. To which extent this difference in subset behavior might be related to alterations in the chemotactic environment, the strength of cell–cell contact as a result of differential costimulation, the specific Th cell function, or epigenetic characteristics of the T cell (19) is an avenue for future studies.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplemental Figure 1. Model for visualization of T cell-DC interactions. CFSE-labeled MOG-specific 2D2 TCR transgenic CD4 T cells were transferred i.v. into FcγR−/− C57BL/6 mice (10 x 10^6 cells per mouse) followed 6h later by 6 x 10^6 SNARF-labeled splenic FcγR+/+ C57BL/6 DC. The next day the mice received 600 μg agg Ig-MOG i.p. to initiate presentation and trigger contact between the T cells and DCs. The lower panel
shows a splenic section illustrating the presence of green-labeled CD4 T cells and red-stained DCs.

**Supplemental Video 1. Kinetics of naïve T cells.** FcγR−/− mice were given 10 x 10^6 CFSE-labeled 2D2 CD4+ T cells and 6 x 10^6 unlabeled FcγR+/+ DCs. The next day, the hosts were given i.p. saline without Ig-MOG (Nil). The axillary and inguinal LNs were harvested at 48h post injection and subjected to two-photon imaging. (926 KB)

**Supplemental Video 2. Kinetics of tolerized T cells.** FcγR−/− mice were given 10 x 10^6 CFSE-labeled 2D2 CD4+ T cells and 6 x 10^6 unlabeled FcγR+/+ DCs. The next day, the hosts were given i.p. 600 µg agg Ig-MOG tolerogen in saline (Tol). The axillary and inguinal LNs were harvested at 48h post injection and subjected to two-photon imaging. (1.5 MB)

**Supplemental Video 3. Kinetics of naïve T cells at 36h.** FcγR−/− mice were given 10 x 10^6 CFSE-labeled 2D2 CD4+ T cells and 6 x 10^6 unlabeled FcγR+/+ DCs. The next day, the hosts were given i.p. saline without Ig-MOG (Nil). The axillary and inguinal LNs were harvested at 36h post injection and subjected to two-photon imaging. (541 KB)

**Supplemental Video 4. Kinetics of tolerized T cells at 36h.** FcγR−/− mice were given 10 x 10^6 CFSE-labeled 2D2 CD4+ T cells and 6 x 10^6 unlabeled FcγR+/+ DCs. The next day, the hosts were given i.p. 600 µg agg Ig-MOG tolerogen in saline (Tol). The axillary and
inguinal LNs were harvested at 36h post injection and subjected to two-photon imaging.

(422 KB)

Supplemental Video 5. Kinetics of immunized T cells. FcγR⁻/⁻ mice were given 10 x 10⁶ CFSE-labeled 2D2 CD4⁺ T cells and 6 x 10⁶ unlabeled FcγR⁺/+ DCs. The next day, the hosts were given i.p. 600 μg sol Ig-MOG and 33 μg CPG-ODN immunogen in saline (Imm). The axillary and inguinal LNs were harvested at 36h post injection and subjected to two-photon imaging. (885 KB)