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J Immunol 2011; 186:2148-2155; Prepublished online 17 January 2011;
doi: 10.4049/jimmunol.1002917
http://www.jimmunol.org/content/186/4/2148

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Presentation of Acquired Peptide-MHC Class II Ligands by CD4+ Regulatory T Cells or Helper Cells Differentially Regulates Antigen-Specific CD4+ T Cell Response

Gang Zhou,*+1 Zhi-Chun Ding,∗ Jie Fu,‡ and Hyam I. Levitsky‡

Activated T cells can acquire membrane molecules from APCs through a process termed trogocytosis. The functional consequence of this event has been a subject of debate. Focusing on transfer of peptide-MHC class II (MHC-II) complexes from APCs to CD4+ T cells after activation, in this study we investigated the molecule acquisition potential of naturally occurring regulatory T cells (Tregs) and CD4+ Th cells. We show that acquisition of membrane molecules from APCs is an inherent feature of CD4+ T cell activation. Triggering of the TCR enables CD4+ T cells to acquire their agonist ligands as well as other irrelevant membrane molecules from the interacting APCs or bystander cells in a contact-dependent manner. Notably, trogocytosis is a continuous process during cell cycle progression, and Th cells and Tregs have comparable capacity for trogocytosis both in vitro and in vivo. The captured peptide–MHC-II molecules, residing in sequestered foci on the host cell surface, endow the host cells with Ag-presenting capability. Presentation of acquired peptide–MHC-II ligands by Th cells or Tregs has either stimulatory or regulatory effect on naive CD4+ T cells, respectively. Furthermore, Th cells with captured peptide–MHC-II molecules become effector cells that manifest better recall responses, and Tregs with captured ligands exhibit enhanced suppression activity. These findings implicate trogocytosis in different subsets of CD4+ T cells as an intrinsic mechanism for the fine tuning of Ag-specific CD4+ T cell response.

The Journal of Immunology, 2011, 186: 2148–2155.

Intercellular molecule transfer among immune cells has long been recognized (1, 2). Studies in recent years have firmly established that lymphocytes can capture membrane components from APCs in a process termed trogocytosis (3). T, B, and NK cells have been shown to acquire from their respective target cells a variety of surface molecules, including MHC class I (MHC-I) and MHC class II (MHC-II) proteins, costimulatory molecules, adhesion molecules, and other membrane components (4, 5). Whereas significant progress has been made toward understanding the underlying mechanisms involved during the molecule transfer process, the physiological significance of this phenomenon remains largely unknown (6).

Particularly, the functional consequence of acquisition and subsequent presentation of peptide–MHC ligands by CD4+ T cells is unclear and may be highly diverse. There is accumulating evidence that acquired TCR ligands (peptide–MHC-I and peptide–MHC-II) displayed on CD4 Th cells can lead to sustained T cell activation and memory cell formation for CD8+ and CD4+ T cells (7–9). In contrast, these acquired ligands may serve as a regulatory mechanism to modulate immune response by making the host cells susceptible to apoptosis or CTL killing (10, 11). Furthermore, Ag presentation by CD4+ T cells can induce anergy and apoptosis in responding T cells (10, 12, 13). Of particular interest, specialized regulatory T cells (Tregs), including induced Tr1 cells (14) and CD4+ CD8– CD3– double-negative Tregs (15), have been shown to be able to use acquired peptide–MHC-I ligands to target Ag-specific CD8+ T cells and subsequently suppress their activation and function. Few studies, however, have examined trogocytosis in naturally occurring Tregs under steady-state conditions (16). In particular, no previous studies have investigated whether natural Tregs use acquired peptide–MHC-II ligands to attract and suppress Ag-specific CD4+ T cells.

In this study, we focused on the transfer of peptide–MHC-II ligands from APCs to CD4+ T cells during activation and examined the molecule acquisition potential of Tregs and Th cells both in vitro and in vivo. We demonstrate that continuous acquisition of peptide–MHC-II molecules from APCs is a fundamental feature of CD4+ T cell activation. Th cells with captured ligands respond to restimulation more vigorously, and Tregs with captured ligands display stronger suppression potency. Furthermore, Ag presentation by Th cells bearing acquired ligands results in CD4+ T cell priming, which can be abrogated by Tregs presenting the same acquired ligands. These findings provide novel insight into the process of trogocytosis and suggest its role in regulating CD4+ T cell response.

Materials and Methods

Mice

BALB/c mice 4–6 wk old were purchased from the National Cancer Institute (Frederick, MD). TCR transgenic mice on a BALB/c background expressing an αβ TCR specific for amino acids 110–120 from influenza hemagglutinin (HA) expressed by MHC-II molecule IE3 were originally
generated in the laboratory of Dr. H. von Boehmer (Harvard Medical School, Boston, MA). DO11.10 mice expressing TCR for amino acids 323–339 from chicken OVA presented by MHC-II molecule IA<sup>α</sup> were purchased from Taconic. The Foxp3<sup>GFP</sup> mice (17) generated in the laboratory of Dr. Alexander Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY) were inbred nine generations onto the BALB/c background. HA-TCR transgenic (Tg) mice were crossed with Foxp3<sup>GFP</sup> mice to generate HA-TCR Foxp3<sup>GFP</sup> double-Tg mice. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine and the Medical College of Georgia.

**Cell lines**

The A2O cell line is a B cell lymphoma line derived from a spontaneous reticulum cell neoplasm found in a BALB/c mouse (18). The I-E<α> construct (19), provided by Dr. Ira Mellman (Genentech, San Francisco, CA), was inserted in front of GFP in EF.GFP LV vector provided by Dr. Linzhang Cheng (Johns Hopkins University, Baltimore, MD). A20<sup>IEGFP</sup> was made by transfecting A20 cells with the EF.I-E<α>GFP construct and selected for GFP<sup>+</sup> clones by limiting dilution. EL4 is a carcinogen-induced T cell lymphoma cell line derived from a C57BL/6 mouse. EL4 cells do not express MHC-II molecules due to defective transcription of the MHC-II transactivator (CZTA) (20). Introducing functional CZTA into EL4 (EL4.CZTA) enables these cells to express MHC-II molecules (IA<sup>α</sup>).

**Abs, flow cytometry, and cell imaging**

The following fluorochrome-conjugated (FITC, PE, PerCP, and allophycocyanin) Abs were used for flow cytometry: anti-mouse CD4 (RM4-5), anti-Thy-1.1 (OX-7), KJ1-26, streptavidin–PE, anti–MHC-II (M5/114.15.2), anti-IA<sup>α</sup> (AMS-32.1), anti-IA<sup>β</sup> (25-9-3), anti–H-2<sup>D<sub>b</sub></sup> (28-14-8) (purchased from BD Biosciences). Flow cytometry was performed on a FACS Calibur (BD). For immunofluorescence microscopy, CD<sub>4</sub><sup>+</sup> T cells were labeled with SNARF (Molecular Probes) and incubated with peptide-pulsed A20<sup>IEGFP</sup>. After overnight culture, cell mixture was transferred onto poly-L-lysine-coated cover glasses for 30 min followed by brief fixation with 3% paraformaldehyde. Cells were mounted with Vectashield mounting medium containing DAPI (Vector Labs) and viewed by oil immersion fluorescence microscopy. Digital imaging was performed on an ImageStreamX system (Amnis). Ten thousand cells were imaged for each sample and analyzed using the IDEAS software (Amnis).

**Cell isolation and culture**

CD<sub>4</sub><sup>+</sup> T cells from the spleens of specified Tg mice were enriched using CD<sub>4</sub><sup>+</sup> isolation kit from Miltenyi. Further purification was conducted by FACS sorting (FACS Aria) to collect CD25<sup>+</sup> CD<sub>4</sub><sup>+</sup> cells and CD25<sup>−</sup>CD<sub>4</sub><sup>+</sup> subsets when needed. CD11c<sup>+</sup> dendritic cells (DCs) were isolated using MACS CD11c beads. For T cell–APC culture, 1 × 10<sup>5</sup> purified CD<sub>4</sub><sup>+</sup> T cells were mixed with 1 × 10<sup>5</sup> peptide-pulsed DCs or tumor cells as indicated. For peptide loading, HA110–120 (10 µg/ml) or OVA257–264 peptide (0.5 ng/µl) was added to APCs to incubate at 37°C for 2 h before washing. For cell–cell mixing, more than 1:1 tumor cells were irradiated at 10,000 rads or fixed with 0.05% glutaraldehyde on ice for 1 min and washed with ice-cold 0.2 M glycine–PBS buffer. Cell proliferation was evaluated either by CFSE dilution of the responder cells or by [3H]thymidine incorporation (1 µCi/well, 0.037 MBq). For T–T cell culture, purified CD25<sup>−</sup> CD<sub>4</sub><sup>+</sup> T cells (Th) or CD25<sup>+</sup> CD<sub>4</sub><sup>+</sup> T cells (Treg) were cultured overnight with irradiated A20 cells in the presence or absence of the cognate peptide. CD4<sup>+</sup> T cells were repurified to high purity (>99%) by FACS sorting. Newly isolated naive CD25<sup>−</sup> CD<sub>4</sub><sup>+</sup> T cells were labeled with 1 µM CFSE (Invitrogen) and used as responder cells. Fifty thousand responder cells were cultured either alone or in mixture with equal number of the indicated Th cells or Tregs (distinguishable by Thy1.1 marker). At indicated time after culture, cell division of the responder cells was evaluated by flow cytometry.

**In vitro APC-free suppression assay using preactivated Tregs**

Live Tregs from HA-TCR Foxp3<sup>GFP</sup> double-Tg mice were sorted to high purity (>99%) based on GFP(Foxp3) expression. These Tregs were expanded in vitro in 24-well plates precoated with anti-CD3 and anti-CD28 Abs (2 µg/ml each). rHIL2 (2000 U/ml) was added to the culture. On day 6, expanded Tregs were harvested and mixed with either HA peptide-pulsed or unpulsed A20 cells for 12 h before CD4<sup>+</sup> T cell isolation by MACS beads. HA-specific naive CD25<sup>−</sup> CD<sub>4</sub><sup>+</sup> T cells were labeled with CFSE and used as responder cells. Fifty thousand responder cells, either alone or in mixture with equal number of Tregs (distinguishable by Thy1.1 marker), were plated to wells that have been precoated with 0.5 µg/ml anti-CD3 Ab.

**In vivo Ag capture by Th cells and Tregs**

Next, 0.5 × 10<sup>5</sup> purified CD25<sup>−</sup> CD<sub>4</sub><sup>+</sup> cells (Th) or CD25<sup>+</sup> CD<sub>4</sub><sup>+</sup> cells (Treg) derived from Thy1.1<sup>+</sup> HA-TCR Tg mice were adoptively transferred to BALB/c mice via tail vein injection. The next day, mice were immunized with a recombinant vaccinia virus encoding hemagglutinin (vaccHA). The use of vaccHA was previously described (21). Briefly, mice were primed by i.p. inoculation with 1 × 10<sup>7</sup> PFU vaccHA suspended in 0.1 ml HBSS. Six days after immunization, mouse spleen cells were harvested and subjected to FACS analysis.

**Statistical analysis**

The significance of the results was determined using the Student t test. A p value <0.05 was considered statistically significant.

**Results**

**Acquisition of membrane molecules from APCs is an inherent feature of CD4<sup>+</sup> T cell activation**

T cells can acquire a variety of membrane molecules, including MHC-II, from APCs through trogocytosis (5). To facilitate monitoring the transfer of peptide–MHC-II complex from APCs to CD4<sup>+</sup> T cells, A20 B cells transfected with GFP-tagged I-E<α> (A20<sup>IEGFP</sup>) were used as APCs. To examine activation-induced intercellular molecule transfer, CD4<sup>+</sup> T cells purified from HA-TCR Tg mice were mixed with A20<sup>IEGFP</sup> in the presence or absence of HA<sub>110–120</sub> peptide. After overnight stimulation, a fraction of CD4<sup>+</sup> T cells became enlarged (Fig. 1A, left panel); this coincided with the emergence of a distinct subset of CD4<sup>+</sup> T cells with intermediate GFP intensity (Fig. 1A, middle panel), and these GFP<sup>+</sup> CD4<sup>+</sup> T cells were predominately newly activated CD25<sup>+</sup> T cells (Fig. 1A, right panel). Immunofluorescence microscopy confirmed the presence of acquired IE-GFP molecules on the surfaces of CD4<sup>+</sup> T cells, which were either in contact with (Fig. 1B) or separated from (Fig. 1Bii) A20<sup>IEGFP</sup> cells, excluding the possibility that the observed GFP signal on CD4<sup>+</sup> T cells was due to cell conjugates.

To determine if the acquired MHC-II molecules contained the cognate peptide, CD4<sup>+</sup> T cells were incubated with A20 cells that had been prepulsed with biotinylated HA peptide. After extensive washing, cells were stained with streptavidin–PE to detect biotin-conjugated HA peptide in association with I-E<α>. Fig. 1C shows that there was a significant shift in the streptavidin–PE profile of CD4<sup>+</sup> T cells cultured with biotin-HA peptide aa 110–120 (HAp)-pulsed A20 cells compared with that of T cells cultured with A20 cells that were pulsed with unmodified HA peptide. This result strongly indicates the presence of the cognate peptide within the acquired MHC-II molecules.

To exclude the possibility that the readily detectable intercellular molecule transfer was due to a peculiarity of the engineered A20<sup>IEGFP</sup> cell line, purified HA-specific CD4<sup>+</sup> T cells were stimulated with HA peptide-loaded CD11c<sup>+</sup> DCs. By high-throughput imaging flow cytometry, PE-conjugated Ab specific for I-A/I-E<sup>α</sup> was detected on the surfaces of CD4<sup>+</sup> T cells (Fig. 1D). Notably, these acquired MHC-II molecules formed sequestered foci on CD4<sup>+</sup> T cell surfaces.

The above results, consistent with published data (22), demonstrate that acquisition of the peptide–MHC-II complex from interacting APCs is a fundamental feature of CD4<sup>+</sup> T cell activation. The use of A20<sup>IEGFP</sup> cells as APCs affords the convenience of monitoring the transfer of peptide–MHC-II complexes.

**Activated CD4<sup>+</sup> T cells can acquire molecules from bystander APCs in a contact-dependent manner**

In the preceding experiments, the HAp–IE<α> complex acquired by CD4<sup>+</sup> T cells is the exact agonist ligand for the T cells. To ex-
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FIGURE 1. CD4+ T cell activation is accompanied by concomitant acquisition of peptide–MHC-II complex from APCs. Purified HA-specific CD4+ T cells were incubated with HA peptide-pulsed or unpulsed A20EGFP cells overnight. The cell mixtures were subjected to flow cytometry analysis. A, Live cells were gated and examined for cell size and GFP fluorescence. CD4+ T cells and A20EGFP cells can be distinguished by size (marked by dashed circles). Upon antigenic stimulation, some CD4+ T cells increased in size (forward scatter and side scatter) and acquired GFP signal. Gated on CD4+ T cells, coexpression of GFP and activation marker CD25 is shown. The arrowheads mark the indicated cell populations. Results shown are representative of at least three independent experiments. B, Presence of captured MHC-II molecule on CD4+ T cells confirmed by immunofluorescence microscopy. SNARF-labeled CD4+ T cells (red) were incubated with peptide-pulsed A20EGFP. After overnight culture, the cell mixture was examined by fluorescence microscopy (original magnification ×100). The images show individual CD4+ T cells either in contact with (i) or apart from (ii) an APC. C, Presence of cognate peptide on the surface of CD4+ T cells. A20 cells preloaded with unmodified or biotinylated HA peptide were incubated overnight with purified HA-specific CD4+ T cells. Cells were washed extensively and stained with streptavidin–PE. Histogram shows the streptavidin–PE expression profiles of gated CD4+ T cells. D, Fluorescence images of CD4+ T cells with acquired MHC-II molecules from DCs using the high-throughput ImageStream system. HA-specific CD4+ T cells were incubated with peptide-pulsed DC11c+ DCs for 3 d and then stained with Abs against CD4 (RM4-5–allophycocyanin, red) and MHC-II (M5/114.15.2–PE, yellow). Shown are representative T cells with single or multiple foci of captured molecules from two independent experiments (original magnification ×100).

amino the transfer of membrane molecules that are nonagonist ligands for the interacting CD4+ T cells, DO11.10 CD4+ T cells, which bear a TCR-recognizing OV A323–339–IAd complex, were incubated with OVA peptide-loaded A20EGFP. Fig. 2A shows that OVA-specific CD4+ T cells acquired IA^b, the TCR-restricting MHC-II molecules, as well as the irrelevant MHC-II molecules IEd(GFP) from the same APCs, and the linear correlation of IA^b and IEd indicates that the same CD4+ T cells acquired both molecules. To test if molecules can be acquired from neighboring APCs that do not present the cognate Ag to T cells, DO11.10 CD4+ T cells were incubated with peptide-pulsed A20 and unpulsed A20EGFP cells. After overnight culture, a fraction of IA^b-restricted OVA-specific CD4+ T cells acquired GFP(IE) molecules from bystander A20EGFP cells (Fig. 2B, middle plot). This intercellular molecule transfer was apparently contact dependent (or at least required close physical co-localization), because GFP acquisition by CD4+ T cells was abolished when A20EGFP cells were separated from the mixture of CD4+ T cells and OVA peptide aa 323–339 (OVAp)/A20 by Transwell (Fig. 2B, right plot).

In the preceding experiment, there existed the possibility that OVA peptide may dissociate from IA^b on A20 cells and then bind to IA^a on bystander A20EGFP cells. To exclude this possibility, EL4 cells were used as bystander cells. EL4 cells, a thymoma cell line derived from a C57BL/6 (H-2b) mouse, do not express MHC-II molecules due to impaired C2TA transcription (20). IA^a molecule is expressed, however, when functional CIITA is introduced into EL4 cells (EL4.C2TA). It is true that the same OVA peptide (OVA323–339) could bind both IA^a and IA^b molecules (23). However, DO11.10 CD4+ T cells only recognize OVAp–IA^b presented on A20 cells, excluding the possibility of antigenic recognition between CD4+ T cells and bystander EL4.C2TA cells. As has been shown in other studies (24), down-modulation of TCR (recognizable by clonotypic Ab KJ1-26) upon activation was evident in DO11.10 CD4+ T cells cultured with OVA-pulsed A20 cells, and these activated CD4+ T cells acquired IA^a molecules from EL4.C2TA cells but not the parental EL4 cells (Fig. 2C, upper panel). In contrast, activated CD4+ T cells were equally capable of acquiring MHC-I molecules (Dp) from either EL4 or EL4.C2TA cells (Fig. 2C, lower panel). Some degree of selectivity existed for molecule acquisition by CD4+ T cells because certain membrane molecules, such as B220 and CD19, were not transferred (data not shown). Collectively, these data demonstrate that activated CD4+ T cells can acquire membrane molecules from adjacent APCs in a contact-dependent but Ag-independent manner. This feature of trogocytosis may result in antigenic spreading of the targets of Th cells and Tregs during an evolving immune response.

Continuous molecule acquisition during cell cycle progression marks a population of highly activated CD4+ effector cells

It is unclear if the molecule acquisition shown above is a one-time event or a continuous process during T cell activation. To address this, naive HA-specific CD4+ T cells were labeled with CFSE before incubation with peptide-pulsed DCs. Fig. 3A shows that 4 d after stimulation, CD4+ T cells have undergone at least five cell divisions, and a fraction of cells in each generation has acquired MHC-II molecules, suggesting continuous molecule acquisition by dividing cells. This raised the question whether the two subsets of activated CD4+ T cells, distinguishable by MHC-II acquisition, have different functionalities. To address this issue, divided CD4+ T cells were sorted into MHC-II positive versus negative subsets. Upon antigenic restimulation, CD4+ effector cells with acquired MHC-II molecules (MHC-II^+ Th) exhibited stronger reactivity than their MHC-II^- counterparts as manifested by more vigorous proliferation and higher IFN-γ production (Fig. 3B).

A similar pattern of continuous molecule acquisition was observed for DO11.10 CD4+ T cells stimulated with OVA-pulsed, irradiated A20 cells (Fig. 3C, upper panel), and also for non-TCR Tg CD4+ T cells stimulated with soluble anti-CD3 plus irradiated A20 cells (data not shown). These results suggest that this is a general phenomenon, not peculiar to the types of CD4+ T cells, APCs, or stimuli. However, a fluidic APC membrane is a prerequisite for this intercellular molecule transfer because fixed APCs, though able to drive CD4+ T cells into cell cycling, could not contribute MHC-II molecules to activated T cells (Fig. 3C, lower panel).

Next, we wanted to test whether preactivated CD4+ T cells, as opposed to naive CD4+ T cells used in preceding experiments, can
acquire molecules from APCs. To this end, purified CD4+ T cells were CFSE-labeled and stimulated with plate-bound anti-CD3 and anti-CD28 Abs. Four days after stimulation, CD4+ T cells had undergone at least five cell divisions but were devoid of molecule acquisition in this APC-free culture condition. These T cells were recovered from culture, washed extensively, and then incubated with A20 cells in the absence of cognate peptide. After overnight culture, CD4+ T cells in each cell division appeared to have acquired MHC-II molecules even when stimulation had ceased (Fig. 4D). It has been shown previously that molecule acquisition by activated CD4+ T cells may occur in vivo (10, 13). One study reported that Tregs display slightly more MHC-II than their non-Treg counterparts in steady state (16). Yet, no study has directly compared the molecule-acquiring potential between Tregs and Th cells recovered from unimmunized mice followed by immunization of the recipients with vacHA. Six days after immunization, both types of donor cells expanded significantly in vivo as previously reported (21). Regardless of their origins, the two donor cell populations had largely overlapping MHC-II staining profiles, which exhibited a significantly higher level of MHC-II than that in Th cells recovered from unimmunized mice (Fig. 4C).

**Ag presentation by effector cells or Tregs can lead to either activation or suppression of naive T cells through direct T–T interactions**

Acquisition of peptide–MHC-II ligands from APCs may endow CD4+ T cells’ Ag presenting capability. We show that activated Tregs and non-Tregs were roughly comparable in MHC-II acquisition (Fig. 4). However, it was unclear whether they exert different functions when acting as APCs. FACS-sorted HA-specific CD25+CD4+ and CD25−CD4+ cells were separately incubated with HAp-pulsed A20 cells. After overnight culture, both populations contained acquired MHC-II molecules, which were most likely loaded with the cognate peptide (Fig. 1C), and were thus referred to as pMHC-Th and pMHC-Treg, respectively (Fig. 5A). CD4+ T cells were reisolated by FACS sorting to high purity (>99%) and were subsequently used as APCs to culture with CFSE-labeled naive responder CD4+ T cells. Fig. 5B shows that pMHC-Th cells were able to drive activation (CD25 upregulation) and cell division of the responder cells. In contrast, pMHC-Tregs did not lead to activation of the responder CD4+ T cells, but rather, the presence of pMHC-Tregs completely prevented responder cell activation driven by pMHC-Teff cells, suggesting an active suppression. Addition of sorted Th cells or Tregs without captured peptide–MHC-II to the coculture did not lead to activation or suppression. The Journal of Immunology 2151

**FIGURE 2.** Activated CD4+ T cells can acquire nonagonist ligands from interacting APCs or bystander cells in a contact-dependent manner. A. Purified DO11.10 CD4+ T cells were incubated with OVA peptide-pulsed or unpulsed A20EGFP cells. After overnight culture, appearance of IAα and IIAε molecules on CD4+ T cells were examined by FACS (A), B and C. Contact-dependent acquisition of MHC-II molecules by activated CD4+ T cells from bystander cells. DO11.10 CD4+ T cells were incubated with OVA peptide-pulsed A20 cells. A20EGFP cells were added to this mixture directly or in a Transwell that prevented A20IEGFP cells from contacting T cells. After overnight culture, capture of GFP by CD4+ T cells was evaluated by FACS (B). To exclude any possible antigenic recognition between CD4+ T cells and bystander cells, EL4 cells (MHC-II negative) or EL4.C2TA cells (IAα positive) were added to the culture containing DO11.10 CD4+ T cells and OVA-pulsed A20 cells. After overnight incubation, capture of EL4.C2TA cell-derived MHC-II (IAα) and class-I (Dβ) by CD4+ T cells was assessed (C). CD4+ T cells in culture with A20 cells were included as staining controls. Numbers in plots represent percentage of the indicated population in CD4+ T cells. One representative experiment of two is shown.

**Th cells and Tregs have comparable capacity for trogocytosis**

Using acquisition of GFP from A20EGFP cells as the readout, we next tested whether naturally occurring Tregs and non-Tregs execute molecule acquisition differently upon stimulation in vitro. FACS-sorted CD25+CD4+ (Th) and CD25−CD4+ (Treg) cells, both specific for HA, were stimulated with HA-pulsed A20EGFP cells. These two populations, cultured either alone or in mixture (distinguishable by congenic marker Thy1.1), were monitored for acquisition of GFP (IE) 12 h and 96 h after stimulation. Fig. 4A shows that singly cultured Th cells and Tregs had comparable levels of GFP acquisition at each time point, suggesting that these two populations are equally capable of acquiring molecules from APCs. The extent and kinetics of GFP acquisition by Tregs was not altered in the presence of Th cells. In contrast, GFP acquisition was initially evident but subsequently abolished in Th cells that were in coculture with Tregs, suggesting that ceasing of molecule acquisition is a feature of suppressed CD4+ T cells.

It is known that antigenic stimulation plus exogenous IL-2 allows Treg proliferation in vitro (25, 26). It was therefore of interest to compare molecule acquisition between Th cells and Tregs during cell cycle progression. We reasoned that a small number of Tregs may be driven to proliferate in the presence of Ag and a large number of Th cells, which can provide sufficient amount of endogenous IL-2. To this end, unfractionated CD4+ T cells (containing 5–10% Foxp3+ Tregs) derived from HA-TCR Tg mice were CFSE-labeled and stimulated with peptide-pulsed APCs. Fig. 48 shows that both Tregs and Th cells had considerable cell divisions while retaining a stable relative ratio, suggesting a parallel proliferation of both populations. Notably, continuous MHC-II acquisition occurred in both divided Tregs and Th cells. There was a tendency that the frequency of molecule acquisition in divided Tregs was higher than that in Th cells, but the difference did not reach statistical significance.
CD4+ T cells that have undergone trogocytosis represent a population of highly activated effector cells. A, Cycling CD4+ T cells continuously acquire MHC-II molecules from APCs. Purified HA-specific CD25+ CD4+ T cells were incubated with peptide-pulsed or unpulsed DCs for 4 d. Profile of MHC-II on CD4+ T cells relative to cell cycle progression is shown. Divided CD4+ T cells were FACS sorted into MHC-II+ versus MHC-II− subpopulations as indicated. B, Recall response of Th cells with or without captured molecules. Sorted cells (1 × 10^5) of each population were restimulated with 3 × 10^5 irradiated BALB/c spleen cells in the presence of HA peptide for 3 d. Supernatants were collected for IFN-γ ELISA, and cells were pulsed with [3H]thymidine overnight before harvesting and measuring scintillation counts. Data represent mean ± SE of one of three independent experiments performed in triplicate. C, Cross-linking of APC membrane diminishes intercellular molecule transfer. A20 cells were preloaded with OVA peptide. Half cells were irradiated, and half of the cells were fixed with glutaraldehyde. These APCs were incubated with CFSE-labeled, purified DO11.10 CD4+ T cells for 5 d. Shown are the profiles of clonotypic T cells (KJ1-26) and IAα relative to cell division. Numbers in plots represent percentage of the indicated population in CD4+ T cells. Results shown are representative of two independent experiments.

Activated Tregs with captured ligands exhibit enhanced suppression activity

We further explored whether acquisition of peptide–MHC-II ligands alters the suppression potency of Tregs. It has been shown that preactivated Tregs can subsequently mediate suppression in an Ag-independent manner (26). We hypothesized that activated Tregs with captured peptide–MHC-II ligands can exert enhanced suppression toward their target CD4+ T cells. To test this, live Tregs isolated from HA-TCR Foxp3GRp mice were expanded in vitro with plate-coated anti-CD3 and anti-CD28 in the presence of high concentration of exogenous rIL2. The resulting activated Tregs were either mixed with HA peptide-pulsed or unpulsed A20 cells to allow the transfer of Ag-loaded or unloaded MHC-II to Tregs, respectively. After overnight incubation, there was equivalent amount of MHC-II acquisition by Tregs under each of the culture conditions (Fig. 6A). The suppression activities of these activated Tregs were assessed using an APC-free assay in which immobilized anti-CD3 Ab was used as stimulus. In this assay, Tregs with or without captured ligands were mixed with CFSE-labeled, Ag-specific naive CD25+ CD4+ responder cells, and the level of suppression was assessed by the extent of cell division of the responder cells. Under this experimental setting, Tregs have already been extensively activated before acquisition of Ag-loaded or “empty” MHC-II, thus any difference in suppression should be attributed to the presence of captured ligands on Tregs, but not due to different activation status of Tregs. Fig. 6B shows that by day 6 after stimulation, responder cells had extensive cell divisions (>90% of cells have divided) in the absence of Tregs. As predicted, the presence of preactivated Tregs, regardless of whether they had previously acquired peptide–MHC-II ligands from APCs, led to reduced cell cycle progression in responder cells. However, Tregs that captured peptide–MHC-II ligands (pMHCTreg) were notably more suppressive (49% responder cells have divided) than Tregs that acquired “empty” MHC-II (76% of responder cells have divided).

Discussion

In this report, we provide the first evidence to our knowledge that CD4+ T cells constantly acquire molecules from surrounding APCs during cell cycle progression, and Tregs and Th cells are comparable in trogocytosis both in vitro and in vivo. These results may have important implications for understanding the role of trogocytosis in regulating Ag-specific immune responses. Our study suggests that trogocytosis may lead to multiple layers of immune regulation. The first layer of immune regulation can occur via modulating the APCs. It has been shown that effector T cells compete for access to Ag-bearing APCs (27, 28), and Tregs directly alter the immunogenicity of APCs (29). Deprivation of Ag from APCs by Th cells and Tregs through trogocytosis may be involved in these processes. The second layer of immune regulation lies in the Ag-presenting capability of Tregs and Th cells that have captured Ag from APCs. In this regard, we show for the first time to our knowledge that Ag presentation by naturally occurring Tregs can suppress naive T cell activation driven by cognate Ag or potent, nonspecific stimuli (Figs. 5, 6). Whereas our study and others (14, 15) illustrate the inhibitory effect of Ag presentation mediated by various types of Tregs, the outcome of Ag presentation by Th cells may be rather diverse and context
Tg mice were CFSE-labeled and mixed with peptide-pulsed or unpulsed BALB/c splenocytes as APCs. Four days after culture, CD4+ T cells were assessed for Foxp3 expression profile relative to cell cycle progression. Gated on Foxp3+ Tregs and Foxp3+ Th cells, profiles of MHC-II relative to cell cycle progression are shown. Numbers in plots represent percentage of the indicated population. Dot plots shown are representative of three independent experiments. Frequencies of MHC-II+ cells in the divided populations from all experiments are summarized in the bar graph. The horizontal dotted line represents the basal level of GFP on CD25+ CD4+ cells in culture with A20IEGFP cells without HA peptide. Comparable level of trogocytosis by Th cells and Tregs during cell cycle progression. Unfractionated total CD4 T cells derived from HA-TCR Tg mice were CFSE-labeled and mixed with peptide-pulsed or unpulsed BALB/c splenocytes as APCs. Four days after culture, CD4+ T cells were assessed for Foxp3 expression profile relative to cell cycle progression. Gated on Foxp3+ Tregs and Foxp3− Th cells, profiles of MHC-II relative to cell cycle progression are shown. Numbers in plots represent percentage of the indicated population. Dot plots shown are representative of three independent experiments. Frequencies of MHC-II+ cells in the divided populations from all experiments are summarized in the bar graph. In vivo evidence of trogocytosis by Ag-specific CD4+ effector cells and Tregs. After the procedures depicted in the experimental schema, HA-specific Th cells or Tregs were adoptively transferred to BALB/c mice. After immunization, mouse spleen cells were harvested and subjected to FACS analysis. The histogram shows the overlay of the IA/IE profiles of donor CD4+ T cells in each group. Results shown are representative of three independent experiments with similar results.

**FIGURE 4.** CD4+ effector cells and Tregs have comparable capability for trogocytosis. A, Trogocytosis by CD25+ CD4+ cells is diminished in the presence of Tregs in vitro. A total of 0.5 × 10⁶ purified HA-specific CD25+ CD4+ cells (Th) or CD25−CD4+ cells (Treg) differing in Thy1.1 congenic marker, either singly or in mix, were cultured with equal number of irradiated A20IEGFP cells in the presence of HA peptide. At the specified time, acquisition of GFP by each CD4+ T cell population was accessed by flow cytometry. Percentage of GFP+ CD4+ T cells in the respective population under each culture condition was plotted. The horizontal dotted line represents the basal level of GFP on CD25+ CD4+ cells in culture with A20IEGFP cells without HA peptide. B, Comparable level of trogocytosis by Th cells and Tregs during cell cycle progression. Unfractionated total CD4 T cells derived from HA-TCR Tg mice were CFSE-labeled and mixed with peptide-pulsed or unpulsed BALB/c splenocytes as APCs. Four days after culture, CD4+ T cells were assessed for Foxp3 expression profile relative to cell cycle progression. Gated on Foxp3+ Tregs and Foxp3− Th cells, profiles of MHC-II relative to cell cycle progression are shown. Numbers in plots represent percentage of the indicated population. Dot plots shown are representative of three independent experiments. Frequencies of MHC-II+ cells in the divided populations from all experiments are summarized in the bar graph. C, In vivo evidence of trogocytosis by Ag-specific CD4+ effector cells and Tregs. After the procedures depicted in the experimental schema, HA-specific Th cells or Tregs were adoptively transferred to BALB/c mice. After immunization, mouse spleen cells were harvested and subjected to FACS analysis. The histogram shows the overlay of the IA/IE profiles of donor CD4+ T cells in each group. Results shown are representative of three independent experiments with similar results.

Our observation that activated CD4+ T cells promiscuously acquire molecules from APCs provides a novel perspective on the controversy of Ag specificity of Treg-mediated suppression. We show that activated CD4+ T cells not only can acquire the agonist ligands but also may collaterally obtain irrelevant peptide–MHC-II molecules that might be the ligands for other CD4+ T cells of different specificity (Fig. 2A). Besides acquiring molecules from interacting APCs, activated CD4+ T cells have the ability to acquire peptide–MHC-II complexes from irrelevant APCs that happen to be in close proximity (Fig. 2B, 2C). These features may broaden the suppression repertoire of Tregs that are activated by specific pathogens. The Ag-specific suppression mediated by acquired ligands on Tregs may explain some of the results reported in the literature showing seemingly nonspecific suppression by activated Tregs (26, 31–34).

Tregs may suppress target cells through multiple mechanisms, among which suppression is shown to be contact dependent in many cases (35). In an APC-free experimental setting, our data suggest that acquired ligands displayed on Tregs may serve as the molecular basis for the contact dependency of T–T interactions (Figs. 5, 6). One notable feature of captured MHC-II molecules is that they form sequestered foci on host CD4+ T cell surfaces (Fig. 1B, 1D), suggesting that they are acquired as a patch of membrane fragment from APCs and not readily dispersed after relocating onto the surface of their host cells. Clustered localization of the acquired molecules, such as peptide–MHC-II ligands, may facilitate engagement of Ag-specific CD4+ T cells. It is conceivable that acquired ligands on Tregs can attract target cells and facilitate subsequent suppression executed by other effector molecules such as cAMP (36) and membrane-bound TGF-β (37). Therefore, acquired ligands may contribute to the specificity or selectivity of Treg suppression, but Tregs do not rely on them to exert suppressive activity. The enhanced suppression exhibited by ligand-bearing Tregs may be due to rapid T–T engagement and thereby more efficient suppression (Fig. 6). Although demonstrable in vitro, the contribution of acquired ligands to Treg suppression is difficult to be dissociated from other contributing factors, such as Ag deprivation and alteration of APC immunogenicity by Tregs, under physiological conditions, because these events may occur simultaneously and are interdependent. The involvement of acquired ligands in Treg suppression is more complicated in humans due to the fact that human CD4+ T cells can both acquire foreign MHC-II molecules and express endogenous MHC-II upon activation. Further investigations are required to determine the role of acquired peptide–MHC-II ligands in Treg suppression under physiological conditions in both human and mouse systems.
In summary, we demonstrate that both Tregs and Th cells are inherently capable of acquiring peptide–MHC-II complexes from APCs upon activation. Ag capture by Th cells or Tregs is associated with enhanced effector activity or suppression potency, respectively. Moreover, presentation of acquired peptide–MHC class-II ligands by Th cells or Tregs differentially regulates Ag-specific CD4+ T cell response. These findings provide novel insight into the role of trogocytosis, a fundamental, intrinsic cellular process, in regulating adaptive immune response and may help interpret experimental results concerning the specificity of Treg-mediated suppression.

FIGURE 5. Ag presentation by Th cells or Tregs leads to either priming or suppression of naive CD4+ T cells. Purified CD25+ CD4+ cells or CD25−CD4+ cells derived from Thy1.1+ HA-TCR Tg mice were incubated with HA peptide-pulsed A20 cells overnight and analyzed for acquisition of IA/IE by flow cytometry (A). CD4+ T cells with captured peptide–MHC ligands, designated as pMHCTh or pMHCTreg, were reisolated by FACS sorting to high purity (>99%) and used as APCs to mix with equal number (1 × 10^5) of CFSE-labeled naive CD4+ T cells purified from HA-TCR Tg mice on Rag2−/− background. Six days after culture, responder cell activation (CD25) and division (CFSE) were evaluated by FACS (B). Plots shown are gated on Thy1.1+ Thy1.2+ responder cells. Numbers in plots represent percentage of the indicated population in CD4+ T cells. Data shown are representative of two separate experiments with similar results.

FIGURE 6. Activated Tregs with captured peptide–MHC-II ligands exhibit enhanced suppression activity. A, Preactivated Tregs effectively acquire MHC-II molecules from APCs. Highly purified live Tregs derived from HA-TCR Foxp3GFP double-Tg mice were expanded for 6 d in vitro with plate-coated anti-CD3 and anti-CD28 in the presence of exogenous rIL2. Expanded Tregs were harvested and mixed with either HA peptide-pulsed or unpulsed A20 cells for 12 h before reisolation of CD4+ T cells by MACS beads. Plots shown are expression profiles of MHC-II on activated Tregs before and after overnight incubation with APCs. Numbers in plots represent percentage of the indicated population. B, Assessing the impact of captured ligands on Treg suppression activity. CFSE-labeled, HA-specific naive CD25− CD4+ responder cells, either alone or in mixture with the indicated Tregs (distinguishable by Thy1.1 marker), were plated to wells precoated with 0.5 μg/ml anti-CD3 Ab. Six days after culture, cell division of the responder cells was assessed by flow cytometry. Histograms shown are gated on responder CD4+ T cells. Numbers in each plot represent percentage divided and undivided cells. Data shown are representative of three independent experiments.
Acknowledgments
We thank R.L. Blosser and A. Tam at the Johns Hopkins Medical Institution’s flow cytometry core facility for assistance in cell sorting and William King at the Medical College of Georgia flow cytometry core facility for collecting data using the Amnis ImageStream® system.

Disclosures
The authors have no financial conflicts of interest.

References