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APCs Expressing High Levels of Programmed Death Ligand 2 Sustain the Development of CD4 T Cell Memory

Jason S. Ellis,* F. Betul Guloglu,* Danielle M. Tartar,* Christine M. Hoeman,* Cara L. Haymaker,* Jason A. Casio,* Xiaoxiao Wan,* Mermagya Dhakal,* Amie VanMorlan,†‡ Seung-Hi Yang,* and Habib Zaghouani*†‡

The role APCs play in the transition of T cells from effector to memory remains largely undefined. This is likely due to the low frequency at which long-lived T cells arise, which hinders analysis of the events involved in memory development. In this study, we used TCR transgenic T cells to increase the frequency of long-lived T cells and developed a transfer model suitable for defining the contribution of APCs to the development of CD4 T cell memory. Accordingly, naive TCR transgenic T cells were stimulated in vitro with Ag presented by different types of APCs and transferred into MHC class II-deficient mice for parking, and the hosts were later analyzed for long-lived T cell frequency or challenged with suboptimal dose of Ag, and the long-lived cells-driven memory responses were measured. The findings indicate that B cells and CD8α+ dendritic cells sustained elevated frequencies of long-lived T cells that yielded rapid and robust memory responses upon rechallenge with suboptimal dose of Ag. Furthermore, both types of APCs had significant programmed death (PD) ligand 2 expression prior to Ag stimulation, which was maintained at a high level during presentation of Ag to T cells. Blockade of PD ligand 2 interaction with its receptor PD-1 nullified the development of memory responses. These previously unrecognized findings suggest that targeting specific APCs for Ag presentation during vaccination could prove effective against microbial infections. The Journal of Immunology, 2010, 185: 000–000.

Immunological memory is the fundamental basis for vaccine development (1–5). An initial encounter with the cognate Ag triggers naive T cells to differentiate into effectors that engage in microbial clearance (1–5). Upon completion of this task, the cells enter a contraction phase during which most effectors cells undergo apoptosis. Very few of the effectors (1 in 10^5–10^6) do not undergo apoptosis but become long-lived microbe-specific memory cells that will respond to future infections (2, 6). Despite the fact that few cells transit from effector to memory, the resulting increase in Ag-specific precursors enables rapid and robust responses against future encounters with the microbe (7–12). Most of the progress made to date on the development of T cell memory has involved the development of CD8^+ T cell memory and late-phase memory responses. Much less is understood about the development and maintenance of CD4^+ T cell memory. Also, little is known on how and when the decision to become a short-lived effector versus a long-lived memory cell is made (2, 13, 14).

The low frequency of effectors that transit to memory and the lack of specific markers to track memory precursors have hindered progress in this field (15, 16). Understanding the events that direct the effector to memory transition will likely aid in the development of effective vaccination strategies (17). We have previously shown that in vivo exposure of TCR transgenic T cells to OVA 323–339 peptide (OVA) yields effector T cells, some of which produce significant IFN-γ, whereas others secrete rather modest levels of IFN-γ (18). Interestingly, the IFN-γ–producing effectors gave rise to memory precursors that sustained rapid and robust memory responses, whereas those with reduced IFN-γ yielded delayed and moderate memory responses. Given that a homogeneous population of naive TCR transgenic T cells was used, the assorted memory responses may reflect differential Ag presentation by various APCs rather than the function of T cell intrinsic factors. The results presented in this paper demonstrate that B cells and the CD8α+ dendritic cell (DC) subset support transition from effector to memory and generate significant memory precursors that sustain rapid and robust responses, the hallmark of memory (19–24). Furthermore, both cells express higher levels of programmed death ligand (PD-L) 2, a ligand for the negative regulator of T cell activation PD-1, in their resting state. This is maintained during presentation of OVA, and blockade of the interaction between PD-L2 and PD-1 drastically reduced memory responses. Therefore, specific types of APCs, such as B cells and CD8α+ DCs, display an intrinsic expression of PD-L2 prior to and during presentation of Ag, thus supporting transition from effector to memory possibly by restraining hyperactivation of T cells.

Materials and Methods

Mice
DO11.10/scid or DO11.10/RAG2^−/− transgenic mice (H-2d) expressing a TCR specific for OVA peptide were described previously (25). BALB/c mice (H-2^d) were purchased from Harlan Sprague Dawley (Indianapolis, IN). MHC II^+ BALB/c mice (cAN 129 S6 [B6] I, tm1 Liz^−/−) (H-2^d)
were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were used in accordance with the guidelines of the University of Missouri Institutional Animal Care and Use Committee.

**Ags**

OVA peptide (SQAVHAAHAEINAGR) encompasses aa residues 323–339 of chicken OVA and is immunogenic in BALB/c (H-2d) hosts. Influenza virus hemagglutinin (HA) peptide as aa residues 110–120 (SFERFEIFPKE) is also immunogenic in BALB/c mice and was therefore used as a negative control (26). Peptides were purchased from EZBioLab (Carmel, IN).

**CFSE**

Naïve splenic DO11.10 CD4+ T cells were isolated using Miltenyi’s magnetic bead positive selection system (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s instructions. The cells were then labeled with CFSE (Molecular Probes, Eugene, OR) as described previously (28, 29). Briefly, spleens were digested in a collagenase solution, then DCs were isolated using a BSA density gradient. These isolated DCs were plated for 1.5 h at 37°C in plain DMEM with no fetal serum. The plates were washed to remove nonadherent cells and incubated further for 12 h at 37°C in complete culture medium containing 10% FBS. Plates were then washed, and the nonadherent mature DCs were collected. Subsets of DCs were obtained through positive selection purification using Miltenyi microbead system (Miltenyi Biotec) and divided into CD8α+, CD8α+ CD4+, and CD8α- CD4- CD11c+ subsets through successive rounds of positive selection.

**B cells.** B cells were purified by panning as described previously (30). In brief, BALB/c spleen cells were incubated for 1 h in flasks coated with anti-κ L chain Ab. Flasks were then washed thoroughly to remove non-adherent cells, and remaining cells were used to infect the captured B cells. The purified B cells were then washed twice with DMEM-10% FCS prior to use.

**Macrophages**. Macrophages (M0s) were purified from the spleen (SP) of BALB/c mice using a standard adherence procedure. Briefly, SP cells were incubated for 2 h at 37°C on a plastic dish. Nonadherent cells were removed by washing, and the remaining cells were incubated for an additional 14 h. Cultures were washed to remove nonadherent cells again, leaving M0s adhering to the plate. M0s were removed using a cell scraper and washed twice with DMEM-10% FCS prior to use.

All purified APCs used in culture were irradiated with 3000 rad using an S5000 Cytocentrifuge (Shandon, Radnor, PA) before use. Purified CD4 T cells (1 × 106 cells/ml) were then incubated with FITC- or PE-labeled (Molecular Probes) Abs, or with PerCP-cyanin 5.5 (PerCP-Cy5.5; Invitrogen, Carlsbad, CA) as described previously (31). The capture Abs were as follows: rat anti-mouse IFN-γ, R4-6A2 and rat anti-mouse IL-5, and TRFK5 (BD Pharmingen, San Jose, CA). The biotinylated anti-CD4 Ab was rat anti-mouse anti-CD4 (BD, San Jose, CA), and the CD11c Ab was rat anti-CD11c (M1/70, BioLegend, San Diego, CA). Flow cytometry was performed using a FACSCanto II and FACS Diva software (BD Biosciences, San Jose, CA). Analysis of the frequency of memory T cell precursors

The spleen was harvested from MHC II-/- mice recipient of effector T cells after 4 mo parking. The frequency of IFN-γ-, IL-4-, and IL-5-producing memory precursors was determined by ELISPOT as described previously (32, 33). Briefly, HA-multiscreen plates were coated with 100 μg/ml 1 M NaHCO3 buffer containing 2 μg/ml capture Ab. After overnight incubation at 4°C, plates were washed three times with PBS and three times with PBS containing Tween 20. Free sites were then saturated with culture medium for 2 h at 37°C. After blocking, the medium was removed, and bulk splenic cells (1 × 106/100 μl/well) and wild-type BALB/c bulk splenic APCs (0.2 × 105/50 μl/well) were incubated with the indicated amounts of OVA peptide or 10 μg HA peptide (30 μl/well) at 37°C and 7% CO2. After 24 h, the plates were washed, and 100 μl of 1 μg/ml biotinylated anti-CD8 Ab was added to each well. The plates were incubated overnight at 4°C and washed, and 100 μl avidin-peroxidase (2.5 μg/ml) was added per well. Plates were incubated for 1 h at 37°C and washed. Spots were visualized by adding 200 μl 3-amino-9-ethylcarbazole substrate in 50 mM acetic acid buffer. Spots were counted on an Immunoscore Series 3B analyzer using Immunoscore version 4.2 software (Shaker Heights, OH). Ab pairs used for ELISPOT were the same used for ELISA.

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**Results**

**B cells and CD8α+ DCs drive induction of IFN-γ-producing memory T cells**

Stimulation of OVA-specific TCR transgenic monoclonal T cells with Ag yields effector T cells heterogeneous at the level of activation and IFN-γ production, leading to assorted memory responses (18). These observations suggest that factors intrinsic to the T cells...
may not account for heterogeneity in effector or memory responses. Rather, presentation of Ag by different APCs could engender different effector or memory responses by the same T cell. Given that M6s, B cells, and DCs, including CD8 and CD4 expressing subsets (34–36), function as major presenting cells, we sought to test for memory development when Ag presentation is performed by these APCs. To this end, a suitable Ag stimulation model was developed and adapted for testing the different APCs for induction of T cell memory (Fig. 1). Accordingly, OVA-specific DO11.10/RAG2−/− T cells were cultured with specific APCs isolated from MHC-compatible BALB/c mice and stimulated with OVA peptide. The T cells were then transferred into MHC class II-deficient (MHC II−/−) BALB/c mice, and 4 mo later, the frequency of long-lived T cells was analyzed ex vivo, and the memory response was determined after immunization with a suboptimal dose of OVA (Fig. 1). In this model, preliminary experiments were carried out to determine the optimal stimulation conditions including the number of presenting cells, the concentration of OVA peptide, and the incubation time for each APC type that yields a common pattern of T cell division in vitro prior to transfer into the host mice. Also, the suboptimal dose of OVA peptide used for rechallenge in vivo was previously determined as the dose of peptide that induces a response in mice recipient of activated but not naive T cells (18). Because the host has no MHC II molecules, CD4 T cell development in the thymus is compromised, and the mouse has very few endogenous CD4 T cells. Therefore, all CD4 T cells recovered at the end of the 4-mo-parking period originate from those introduced during the initial transfer.

To determine the frequency of long-lived T cells that survive for 4 mo after transfer into the host mice, splenic cells were harvested from the MHC II−/− BALB/c hosts and assayed for intracellular IFN-γ and IL-5 by ELISPOT. The data presented in Fig. 2 shows that there are significant numbers of specific long-lived T cells producing IFN-γ in hosts recipient of T cells stimulated with B cells and CD8α+ and CD8α−CD4− DCs (Fig. 2A, 2B, left panels). Cytokine secretion is specific because the control HA peptide did not induce responses above background levels. Interestingly, in addition to the IFN-γ-producing long-lived T cells, the CD8α−CD4− DCs also induced significant numbers of long-lived cells that produced IL-5 (Fig. 2A, 2B, right panels). Overall, development of long-lived T cells, which likely represent memory precursors, occurs when the initial encounter with Ag is mediated by specific types of APCs, such as B cells and CD8α+ and CD8α−CD4− DCs.

To evaluate the response of these potential memory precursors upon rechallenge with Ag, the MHC II−/− host mice were adop-

FIGURE 1. Schematic representation of the animal model used to investigate development of CD4+ T cell memory. Splenic CD4+ T cells from adult DO11.10/Scid mice are plated with irradiated (3000 rad) purified BALB/c APCs and stimulated with OVA peptide. The T cells are then used for expression of costimulatory molecules, cytokine production, or adoptive transfer into MHC II−/− BALB/c mice. For the latter, after 4 mo parking, the hosts are either used to analyze the frequency of memory T cell precursors prior to any rechallenge with OVA peptide or given BALB/c DCs and immunized with a suboptimal dose of OVA peptide in CFA and used to evaluate IFN-γ memory responses.

FIGURE 2. CD8α+ and CD8α−CD4− DCs and B cells induce the generation of memory precursors. MHC II−/− BALB/c mice recipient of DO11.10 CD4+ T cells stimulated in vitro with 0.5 μM (for B cells and M6) or 1.0 μM (for DC subsets) OVA peptide presented by specific APCs were sacrificed 4 mo after transfer. The splenic cells (1×10⁶/well) were then stimulated with graded amounts of OVA peptide presented on MHC II+/+ BALB/c splenic APCs (0.2×10⁶/well), and the frequency of IFN-γ− and IL-5−producing cells was determined by ELISPOT and expressed as SFUs per 10⁶ cells (A). B, Bar graph representing the frequency of cytokine-producing cells obtained with 10 μM OVA peptide stimulation. **p < 0.01; p values represent comparison of IFN-γ production in 10 μM OVA peptide groups to that in 10 μM HA peptide. Data representative of three independent experiments with three mice per group. SFU, spot forming units.
tively transferred with MHC II+ DCs to serve as APCs, and the animals were immunized with a previously determined suboptimal dose of OVA peptide (20 µg peptide/mouse) (18). Five days later, the SP and LN cells were harvested and analyzed for production of IFN-γ by ELISA. The data presented in Fig. 3 show that a dose-dependent IFN-γ memory response developed in both organs when the initial encounter with Ag was carried out by CD8α+ DC subset or B cells (Fig. 3A). The CD8α− subsets, whether CD4+ or CD4−, and MΦs did not support the development of significant memory responses in either organ. In contrast, the CD8α+ DCs yielded long-lived T cell memory precursors similar to B cells, but the memory responses were two to three times higher for B cells than CD8α− DCs in both the SP and LN (Fig. 3B). This is likely reflective of quantitative difference in IFN-γ production by precursor T cells that arise under stimulation by different APCs. Finally, the production of IL-5 was analyzed, but none was found (data not shown). It should be noted that the CD8α+ CD4− DC subset, which yielded significant numbers of long-lived T cells, showed no memory responses. Overall, these results indicate that presentation of Ag by B cells and CD8α− DCs during the initial encounter sustains the development of long-lived T cells that support significant IFN-γ memory response.

**APCs supporting IFN-γ− but not IL-5–producing effectors sustain memory development**

Presentation of Ag by APCs and recognition by T cells involves interaction with costimulatory molecules and production of effector cytokines. One would envision that these events would influence the transition from effector to memory and the development of memory responses (37–40). To determine the type of cytokine produced by the T cell during the initial Ag presentation by the specific APCs, naive T cells were stimulated with OVA peptide presented by the different APCs, and cytokine (IFN-γ and IL-5) production was measured. The results presented in Fig. 4 show that naive T cells were activated and underwent cell division upon stimulation with OVA presented by DC subsets, B cells, or MΦs (Fig. 4A). However, CD8α+ DCs and B cells, which sustained transition to memory, yielded effectors that produce significant amounts of IFN-γ but no measurable IL-5 (Fig. 4B). Stimulation with MΦs, which induced T cell activation and division, did not yield IFN-γ− or IL-5–producing effectors. In contrast, the CD8α− CD4+ and CD8α− CD4− DC subset yielded mixed effector T cells producing IFN-γ and IL-5 cytokines. Given that CD8α+ DCs do not produce IL-5, these results suggest that memory T cells can be sustained by both IFN-γ and IL-5. It should be noted that the CD8α− CD4− DC subset, which yielded high numbers of long-lived T cells, showed no memory responses. Overall, these results indicate that presentation of Ag by B cells and CD8α− DCs during the initial encounter sustains the development of long-lived T cells that support significant IFN-γ memory response.

**FIGURE 3.** CD8α+ DCs and B cells support the development of IFN-γ–producing memory T cells. MHC II− T cell subsets, CD8α+ and CD8α− B cells, and MΦs were given, 4 mo after T cell transfer, for activation by the specific APCs that were then challenged with OVA (2 µg/mouse) in CFA (1/1 v/v). Five days later, the mice were sacrificed, and the SP (9 × 10^5/well) and LN (3 × 10^5/well) were harvested and analyzed for production of IFN-γ by ELISA. The data presented in Fig. 3 show that a dose-dependent IFN-γ memory response developed in both organs when the initial encounter with Ag was carried out by CD8α+ DC subset or B cells. CD8α− subsets, whether CD4+ or CD4−, and MΦs did not support the development of significant memory responses in either organ. In contrast, the CD8α+ DCs yielded long-lived T cell memory precursors similar to B cells, but the memory responses were two to three times higher for B cells than CD8α− DCs in both the SP and LN (Fig. 3B). This is likely reflective of quantitative difference in IFN-γ production by precursor T cells that arise under stimulation by different APCs. Finally, the production of IL-5 was analyzed, but none was found (data not shown).

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**FIGURE 4.** CD8α+ DC- and B cell-stimulated DO11.10 CD4+ T cells produce IFN-γ but not IL-5. CFSE-labeled DO11.10 CD4+ T cells (2 × 10^6 cells/well) were stimulated with OVA peptide (1.0 µM DC subsets, 0.5 mM B cells and MΦs) presented by CD8α+ DCs (0.4 × 10^6 cells/well), CD8α− CD4+ DCs (0.4 × 10^6 cells/well), CD8α+ CD4− DCs (0.4 × 10^6 cells/well), MΦs (0.3 × 10^6 cells/well), or B cells (2.0 × 10^6 cells/well). A shows CFSE dilution by the T cells upon presentation of OVA peptide by the indicated APCs. CFSE-labeled unstimulated T cells (no stimulation) are included as control. B shows IFN-γ and IL-5 production as measured by ELISA for each of the indicated stimulation cultures. *p < 0.05; **p < 0.01. Data representative of four independent experiments. FSC, forward light scatter.
and B cells sustained memory responses whereas CD8α− DCs and Mφ did not, these findings suggest that transition to memory requires Ag presentation by APCs that support the generation of IFN-γ but not IL-5–producing effectors.

**APCs expressing higher levels of PD-L2 sustain induction of T cell memory**

Costimulatory molecules and their ligands affect T cell activation and differentiation (41, 42). Although B7.1 and B7.2 have long been known to exert a stimulatory function on T cell activation, other costimulatory molecules, such as PD-L1 and PD-L2, are rather involved in negative regulation of T cell activation (21–24, 41, 43). The PD-1 molecule, which functions as a receptor for PD-L1 and PD-L2, is expressed in low amounts on resting T cells but has been shown to be upregulated upon long-term stimulation, resulting in exhaustion of the T cells (42). These observations clearly indicate that interaction of costimulatory molecules with their ligands regulates T cell activation and the resulting cytokine production at the effector phase. Because only in vitro primary cultures where IFN-γ but no IL-5 was produced yielded effective memory responses, it is possible that differential expression of specific costimulatory molecules by APCs influences T cell activation, cytokine production, and transition to memory. To address this premise, we began by performing cell surface staining on both the APCs and T cells before and after in vitro stimulation with Ag. Accordingly, purified DC subsets, Mφs, and B cells were stained for surface expression of B7.1, B7.2, CD40, PD-L1, and PD-L2, both before and after incubation with Ag and DO11.10 T cells (Fig. 5). The data presented in Fig. 5A shows that prior to culture with T cells, CD8α+ DCs and B cells have higher expression of PD-L2 (73.6 and 72.1%, respectively) than CD8α− CD4+, CD8α− CD4+, or Mφs. It is interesting to note that the CD8α− CD4+ DCs, which are capable of inducing long-lived T cells that do not mount a memory response have an intermediate level of PD-L2 (41.3%) compared with CD8α+ CD4+ DCs (28.3%) and Mφs (26.1%). B7.1 and B7.2 molecules are highly expressed on CD8α+ DCs but not on B cells and thus may not correlate with memory development. After incubation with Ag and DO11.10 T cells, the expression of B7.1, B7.2, CD40, PD-L1, and PD-L2 on CD8α+ DCs remained at levels comparable to those observed prior to Ag stimulation (Fig. 5B). For B cells, CD40, PD-L1, and PD-L2 expression also remained at levels similar to those observed prior to Ag stimulation, but B7.1 and B7.2 had a slight increase in their expression. Interestingly, although the expression of CD40, B7.1, B7.2, and PD-L1 on CD8α− CD4+, CD8α− CD4+, and Mφs remained similar to levels observed prior to Ag stimulation, the expression of PD-L2 increased on all types of APCs. Overall, PD-L2 expression was high on CD8α+ DCs and B cells prior to and after Ag stimulation but increased on the other APCs after stimulation with Ag, possibly suggesting that PD-L2 affects transition to memory when expressed on the APCs at the beginning of interaction with T cells.

If PD-L2 on APCs plays a role in the induction of T cell memory, its receptor PD-1 should be available on the T cells to facilitate interactions with APCs and transition of T cells from effector to memory precursors. To begin investigation of the role PD-L2/PD-1 interactions may play in the transition of T cell memory, we tested the T cells for expression of PD-1 as well as other markers, such as CCR7, CD28, and IL-7Rα, before and after stimulation with Ag. Fig. 6 shows that prior to Ag stimulation, the T cells express PD-1 significantly but CCR7, CD28, and IL-7Rα were at basal levels (Fig. 6, top panel). Although PD-1 is usually at low levels on naive polyclonal T cells (44), the higher PD-1 expression observed in this study may be related to the fact that the DO11.10 T cells are homogeneous TCR transgenic T cells that come from a lymphopenic environment. Subsequent to stimulation with Ag presented on specific APCs, the expression of PD-1 increased from 78.1 to 95.7% when the APCs were CD8α+ DCs, remained at similar levels as prior to stimulation when the APCs were CD8α+ DCs or B cells, and decreased to 53% with Mφs (Fig. 6, panels 2–6). The expression of CCR7, CD28, and IL-7Rα increased significantly, except when the presenting cells were B cells where CCR7 and IL-7Rα remained at basal levels. These results indicated the naive T cells in this model express PD-1, the ligand for PD-L2, and maintain it at a significant level during Ag stimulation and interaction with APCs.

**PD-1/PD-L2 interactions influence development of T cell memory**

To test the effect the interaction of PD-1 with its ligand PD-L2 might have on the development of memory, DO11.10 T cells were stimulated with OVA peptide presented on different types of APCs in the presence of anti–PD-L2 blocking Ab (24) or a rat IgG isotype control. The cells were then adoptively transferred into MHC II–deficient mice and parked for 4 mo. Subsequently, the hosts were given MHC II+ DCs and immunized with a suboptimal dose of OVA peptide in CFA. Five days postimmunization, the SP and LN were harvested, and production of IFN-γ cytokine was determined by ELISA. As can be seen in Fig. 7, blockade of PD-L2 with anti–PD-L2 Ab during the in vitro stimulation with OVA peptide presented on CD8α+ DCs nullified IFN-γ memory responses in both the SP and LN upon in vivo challenge with a suboptimal dose of OVA peptide. The isotype control Ab had no such effect, and significant IFN-γ responses developed in both the SP and LN. Similar results were observed when B cells were used in the initial presentation of OVA in vitro as memory IFN-γ responses developed when in vitro stimulation was carried out in the presence of isotype control but not anti–PD-L2 Ab (Fig 7, fourth panel from top). No IFN-γ memory response was observed with any of the other APCs whether the in vitro stimulation was carried out in the presence of anti–PD-L2 Ab or the isotype control, indicating that only CD8α+ DCs and B cells support effector memory transition as was observed in Fig. 3. Overall, the results presented in this paper indicate that APCs expressing PD-L2 support the development of memory, and interaction with PD-1 on the T cells is required during the initial encounter with Ag.

**Discussion**

The role APCs might play in the transition of CD4 T cells from effector to memory remains largely undefined. In this study, we developed a model in which naïve CD4 T cells are stimulated in vitro with Ag presented by specific types of APCs and transferred into MHC II−/−–deficient mice for parking, and the hosts were later used to analyze the development of T cell memory (Fig. 1). The findings indicate that transition from effector to memory and the development of rapid and robust memory responses is restricted to T cells that encountered Ag on specific types of APCs during the initial stimulation (Figs. 2, 3). Indeed, CD8α− and CD8α− CD4+ DCs and B cells serving as presenting cells during the initial encounter with OVA peptide yielded significantly greater numbers of long-lived T cells than CD8α+ CD4+ DCs and Mφs (Fig. 2). However, upon rechallenge with a suboptimal dose of OVA peptide, only the precursors generated from stimulation with CD8α+ DCs and B cells sustained rapid and robust memory IFN-γ responses (Fig. 3). The long-lived T cells generated upon stimulation with CD8α− CD4+ DCs developed delayed and weaker responses upon rechallenge with suboptimal dose of OVA peptide (data not shown). The fact that OVA peptide-loaded CD8α+ DCs
yielded IFN-γ-producing T cells during the in vitro stimulation bodes well with earlier observations demonstrating that this subset specifically support the differentiation of Th1 cells (45–47). It is thus not surprising that both the long-lived T cells generated under CD8α+ stimulation and the resulting memory response are of Th1 type T cells.

Lately, it has been suggested that memory development occurs as a result of exposure to low amounts of Ag, such as residual traces of protein leftover after viral clearance (48). Also, late arrival of T cells to local lymph nodes, which subjects the lymphocytes to suboptimal residual Ag, leads to the generation of memory (49). These observations, which suggest that development of T cell memory results from suboptimal Ag stimulation and moderate T cell activation at the initial effector phase, find support in recent studies demonstrating that T cells that undergo restrained activation during the early stages of the effector response yield better memory responses (50). From these observations, it is logical to envision that the type of APCs that favor the development of memory would be endowed with means to control the activation of effector T cells and their transition to memory. In this line of reasoning, we tested the APCs for expression of costimulatory molecules that regulate interactions with and activation of T cells. Surprisingly, PD-L2 was highly expressed on CD8α+ DCs and B cells prior to incubation with T cells and remained at significant levels during presentation of OVA peptide to DO11.10 T cells (Fig. 5).

<table>
<thead>
<tr>
<th>Memory Response</th>
<th>CD8α+ DCs</th>
<th>CD8α+ CD4+ DCs</th>
<th>CD8α+ CD4− DCs</th>
<th>B cells</th>
<th>Mφ</th>
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<tr>
<td>Pre-Stimulation</td>
<td>B7.1: 73.6</td>
<td>B7.2: 28.3</td>
<td>CD40: 41.3</td>
<td>PD-L1: 7.1</td>
<td>PD-L2: 26.1</td>
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<tr>
<td>Post-Stimulation</td>
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<td>B7.2: 59.0</td>
<td>CD40: 61.0</td>
<td>PD-L1: 87.6</td>
<td>PD-L2: 90.1</td>
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</table>

**FIGURE 5.** Pattern of costimulatory molecules expressed on APCs prior to and postculture with DO11.10 T cells. A, Freshly purified CD8α+ DCs, CD8α− CD4+ DCs, CD8α− CD4− DCs, Mφs, or B cells were stained with fluorescently labeled Abs specific for B7.1, B7.2, CD40, PD-L1, and PD-L2 as described in *Materials and Methods* and analyzed by flow cytometry. B, Purified CD8α+ DCs (0.4 × 10^6 cells/well), CD8α− CD4+ DCs (0.4 × 10^6 cells/well), CD8α− CD4− DCs (0.4 × 10^6 cells/well), Mφs (0.3 × 10^6 cells/well), or B cells (2.0 × 10^6 cells/well) were incubated with CD4+ DO11.10 T cells (2.0 × 10^6 cells/well) and OVA peptide. The cells were then stained with fluorescently labeled Abs specific for B7.1, B7.2, CD40, PD-L1, and PD-L2. The numbers indicate percent of marker positive cells relative to isotype control (filled) among cells gated on either CD11c (DCs), CD11b (Mφs), or B220 (B cells). Data representative of three independent experiments.
pressed on the surface of the DO11.10 T cells prior to Ag stimulation and remained highly expressed during presentation of OVA peptide by the APCs (Fig. 6). The interactions of PD-1 with its ligands (PD-L1 and PD-L2) have been viewed as negative regulatory pathways of T cell activation (43, 51). In fact, chronicity of microbial infections was recently attributed to the upregulation of PD-L1/L2 expression on DCs and other APCs during infection, which leads to downregulation of T cell function and the consequent microbial persistence (52–56). Our findings, though, suggest that expression of PD-L2 on CD8α+ DCs and other APCs during infection, supported the development of long-lived T cells that did not respond to suboptimal dose of Ag during rechallenge. Also, the cytokine milieu during the initial encounter with Ag included stimulatory and inhibitory signals depending on the model system used (59, 60) the question remains open as to whether transition to memory involves interaction of PD-L2 with yet undefined molecules beside PD-1. Nevertheless, the observation made in this paper bodes well with reports indicating that heightened activation and proliferation leads to a reduction in the numbers of responding memory cells (50).

The CD8α−CD4+ DCs, despite having reduced PD-L2 expression, supported the development of long-lived T cells that did not yield rapid and robust IFN-γ memory responses. This suggests that a limited threshold of activation needed to be in place at the initial stimulation to generate long-lived memory precursors that respond to suboptimal dose of Ag during rechallenge. Also, the cytokine milieu during the initial encounter with Ag included both Th1 and Th2 effectors. Although this is not surprising as CD8α− DC stimulation can differentiate naive T cells into Th2 (45–47), the presence of a Th2 cytokine during transition to memory may condition the long-lived Th1 cells for minimal memory responses during rechallenge with a suboptimal dose of Ag. Nevertheless, it seems that adequate control of the initial activation by PD-L2/D-1/PD-L2 interactions is required to generate long-lived T cells that sustain robust and rapid memory responses upon challenge with a suboptimal dose of Ag.

Overall, this study has identified CD8α+ DCs and B cells as APCs that support CD4 T cell effector to memory transition and the generation of rapid and robust memory responses upon challenge with suboptimal dose of Ag. Both types of APCs express PD-L2 whose interaction with PD-1 on T cells seems to serve as a rheostat to control the level of T cell activation and thereby

FIGURE 6. Pattern of costimulatory molecules expression on DO11.10 T cells before and after stimulation with Ag. DO11.10 CD4+ T cells (2.0 × 10^6 cells/well) were stained with Abs specific for CCR7, CD28, IL-7Rα, and PD-1 before and after stimulation with OVA peptide presented on CD8α+ DCs (1.0 μM OVA, 0.4 × 10^6 cells/well), CD8α−CD4+ DCs (1.0 μM OVA, 0.4 × 10^6 cells/well), CD8α−CD4− DCs (1.0 μM OVA, 0.4 × 10^6 cells/well), MΦs (0.5 μM OVA, 0.3 × 10^6 cells/well), or B cells (0.5 μM OVA, 2.0 × 10^6 cells/well) as described in Materials and Methods. The histograms show staining of the cells gated on KJ1-26 versus isotype control (filled). The numbers indicate the percentage of positive cells for the indicated markers. Data representative of three independent experiments.

FIGURE 7. Blockade of PD-1/PD-L2 interaction interferes with induction of T cell memory by CD8α+ DC and B cells. DO11.10 CD4+ T cells (2.0 × 10^6 cells/well) were stimulated with OVA peptide presented on CD8α+ DCs (1.0 μM OVA, 0.4 × 10^6 cells/well), CD8α−CD4+ DCs (1.0 μM OVA, 0.4 × 10^6 cells/well), CD8α−CD4− DCs (1.0 μM OVA, 0.4 × 10^6 cells/well) MΦs (0.5 μM OVA, 0.3 × 10^6 cells/well), or B cells (0.5 μM OVA, 2.0 × 10^6 cells/well) in the presence of 5 μg/ml anti-PD-L2 Ab or rat IgG isotype control. After extensive washing, the T cells were adoptively transferred into MHC II−/− host mice. Four months after transfer, the mice were given MHC II+ BALB/c DCs and challenged with a suboptimal dose of OVA peptide (20 μg/mouse) in CFA (1 v/v 1). Five days later, the mice were sacrificed, and the SP (9 × 10^5/well) and LN (3 × 10^5/well) cells were stimulated with OVA or control HA peptide presented on MHC II+ BALB/c APC splenocytes (2 × 10^5/well). IFN-γ responses obtained upon stimulation with graded concentrations of OVA peptide or 10 μM HA peptide were measured by ELISA. MHC II−/− BALB/c mice recipient of unstimulated DO11.10 CD4+ T cells (naive T cells) are included as control. *p < 0.05; **p < 0.01. Data representative of three independent experiments with three to four mice per group.
effector to memory transition. Strategies that target these cells for Ag presentation during immunization could be devised for the development of effective vaccines.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


