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Invariant NKT Cells Induce Plasmacytoid Dendritic Cell (DC) Cross-Talk with Conventional DCs for Efficient Memory CD8+ T Cell Induction

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A key goal of vaccine immunotherapy is the generation of long-term memory CD8+ T cells capable of mediating immune surveillance. We discovered a novel intercellular pathway governing the development of potent memory CD8+ T cell responses against cell-associated Ags that is mediated through cross-presentation by XCR1+ dendritic cells (DCs). Generation of CD8+ memory T cells against tumor cells pulsed with an invariant NKT cell ligand depended on cross-talk between XCR1+ and plasmacytoid DCs that was regulated by IFN-α/IFN-αR signals, IFN-α production by plasmacytoid DCs was stimulated by an OX40 signal from the invariant NKT cells, as well as an HMGB1 signal from the dying tumor cells. These findings reveal a previously unknown pathway of intercellular collaboration for the generation of tumor-specific CD8+ memory T cells that can be exploited for strategic vaccination in the setting of tumor immunotherapy. The Journal of Immunology, 2013, 190: 5609–5619.

There is a significant clinical need for vaccination strategies that induce immune responses capable of both eradicating existing tumors and preventing their recurrence. Many T cell–based approaches, such as those involving the adoptive transfer of tumor-infiltrating lymphocytes or tumor specific TCR gene-transduced T cells (1), have focused on effector cell populations. Although such CD8+ effector T cells can recognize and directly kill tumor cells, they lack the requisite long-term survival and secondary proliferative capacity that allow memory cells to mediate durable protective immunity (2). Although CD8+ memory T cells have been intensely studied in a range of infectious pathogen models (3), the generation of memory T cells reactive with cell-associated Ags, such as those found in cancer, is less well understood.

The fates of effector T cells depend on several signals. A single, brief TCR stimulus (signal 1) combined with costimulation (signal 2) can induce an extended period of proliferation and the acquisition of effector functions (4). In addition, as extrinsic factors, inflammatory signals (signal 3) provided by type I IFN and IL-12 to T cells may be essential modulators in some models (5). Several groups reported that clonal expansion and memory formation by type I IFN–deficient T cells are reduced during lymphocytic choriomeningitis virus (LCMV) infection but not in vaccinia virus or Listeria monocytogenes infections (6–8). Furthermore, a number of intrinsic factors control effector cell survival and the development of memory T cells postinfection (9). Among cell-intrinsic factors, several transcription factors, including T-bet, Eomes, Id2, and BLIMP-1, have prominent functions in effector and memory T cell differentiation in infection models (9–11). Thus, CD8+ memory T cells have been studied intensely in a range of acute infectious pathogen models (3); however, vaccination therapies to generate memory T cells reactive with cell-associated Ags, such as those found in cancer, are not well understood and have not been established with either intrinsic or extrinsic factors.

We and others showed previously that the maturation of conventional dendritic cells (cDCs) can be induced by the activation of invariant NKT (iNKT) cells in situ, resulting in the generation of effector T cells (12, 13). In fact, after the iNKT cells were activated, cDCs upregulated costimulatory molecules, such as CD80/86 (14) and CD70, produced IL-12 (15, 16), and expressed CCL17 (17). The coadministration of tumor Ag and iNKT ligand effectively generated Ag-specific T cell responses. However, such a coadministration tactic requires a large amount of Ag or irradiated tumor cells, because the Ag must be supplied to host dendritic cells (DCs) prior to their maturation (12, 13, 18). To maximally harness such immunological cascades, we established an efficient system using cells bearing a cell-associated Ag along with the...
iNKT cell ligand, α-galactosylceramide (α-GalCer). For example, we used iNKT ligand–loaded, tumor-associated Ag (TAA)-expressing CD1d+ tumor cells (tumor/Gal) (19–21) or allogeneic fibroblasts loaded with α-GalCer transfect with TAA mRNA (16, 22).

In our previous study, we demonstrated that vaccination with tumor/Gal led to long-term antitumor effects, possibly through the generation of memory CTLs (19). However, details of the mechanism leading to the establishment of this predicted memory T cell response are unknown. In the current study, we characterized the extrinsic signals that act on DCs, resulting in the establishment of memory CD8+ T cells. Our results define a previously undescribed collaboration between iNKT cells and plasmacytoid DCs (pDCs) in the generation of potent CD8+ T cell memory, which can be exploited to optimize vaccine-induced antitumor responses.

Materials and Methods

Mice and cell lines

Pathogen-free, 6- to 8-wk-old C57BL/6 mice were purchased from CLEA Japan. CD40−/− mice and pml-1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IFNα/βR−/− mice were purchased from T. Yano, M. Nobuoka, T. Hirashima, A. Iizuka, K. Sato, Y. Fukuda, T. Yano, M. Nobeuoka, T. Hirashima, A. Iizuka, K. Sato, T. Tanaka, K. Hoshiba, and T. Kaisho, submitted for publication. CD11c-DTR-GFP mice were provided by Dr. D. Littman (New York University, New York, NY). OT-I TCR-transgenic mice (B6 background) were provided by Dr. W. Heath (Walther and Eliza Hall Institute, Victoria, Australia). B6 Js18−/− mice were provided by Dr. M. Taniguchi (Research Center for Allergy and Immunology, RIKEN). All of the mice were maintained under specific pathogen-free conditions and treated in compliance with institutional guidelines. B16 melanoma lines were purchased from the American Type Culture Collection, and NIH3T3 cells were obtained from the RIKEN Cell Bank. High murine CD11c-expressing B16 (CD11c-B16) or NIH3T3 (CD11c-NIH3T3) cells were generated by retrovirus transduction, as previously described (16, 19, 23).

Reagents

The following anti-mouse mAbs were purchased from BD Biosciences, eBioscience, R&D Systems, or BioLegend: anti-CD1d (1B1), CD43 (145-2C11), -CD8 (53-6.7), -CD11c (HL3), -CD122 (TM-b1), -CD125 (PC61), -CD11b (M1/70), -CD25 (PC61), -CD40 (3/23), -CD80 (1B11), -CD86 (GL1), -CD90.1 (OX-7), -CD107a (1D4B), -CD122 (TM-b1), -CD11c (HL3), -CD125 (PC61), -CD40 (3/23), -CD122 (TM-b1), -CD8 (53-6.7), -CD11c (HL3), -CD25 (PC61), -CD40 (3/23), -CD122 (TM-b1), -CD8 (53-6.7), -CD11c (HL3), -CD25 (PC61), -CD40 (3/23), -CD122 (TM-b1), and used as a template. The RNAs were generated under a T7 promoter sequence on the vectors using an mMESSAGE mMACHINE T7 Ultra Kit (Invitrogen), as previously described (22). To load NIH3T3 cells with α-GalCer, the cells were cultured for 48 h in the presence of 500 ng/ml α-GalCer and then washed three times before transfection. The OVA mRNA were transfected into CD11c-NIH3T3 cells with a TransMessenger transfection kit (QIAGEN) following the manufacturer’s instructions, as previously described (16).

Cytokine-secretion assays and intracellular staining

Cytokine expression by CD8+ Thy1.1+ pml-1 T cells was determined using a protocol for intracellular cytokine staining (26). Briefly, spleen cells were incubated in the presence of GolgiPlug (BD Biosciences) for 6 h, with or without hlgp100 peptide25–35, and then incubated with Abs to surface markers. For simultaneous detection of CD107a and IFN-γ, both Golgi-Plug and GolgiStop were added. The cells were then permeabilized in Cytofix/Cytoperm Plus (BD Biosciences) and stained with anti–IFN-γ, anti–IL-2, anti–TNF-α, or anti–CD40L mAbs.

Immunofluorescence microscopy

Spleens were embedded in OCT compound (Sakura Finetek) and snap-frozen in liquid nitrogen. Frozen sections (5 μm thick) were fixed in acetone and blocked in skim milk and 10% goat serum for 10 min at room temperature, and stained with the indicated Abs. The following Abs were used: FITC–anti-CD3, rat anti-Siglec-H (Miltentyi Biotec), rabbit anti-GFP (MBL), and biotinylated rat anti-mouse metallophilic macrophage (MOMA-1; BMA Biomedicals). These Abs were visualized using the appropriate secondary Abs coupled to Alexa Fluor 488 and streptavidin-Cy3. Samples were observed under a fluorescence microscope (BZ-9000; Keyence) or by confocal laser scanning microscopy (Leica Microsystems).

Mixed BM chimeras

The femurs and tibias were flushed with a syringe, and the suspension was passed through a 70-μm nylon mesh. The BRCs were lysed, and T cells and NK cells were removed using biotinylated anti–CD4, anti–CD8, and anti–IFN-γ Abs combined with Streptavidin Particles Plus (BD Biosciences) and a BD IMag Cell Separation System. To generate mice lacking DC-derived IFN-γRβR, B6(Ly5.1) mice were lethally irradiated and reconstituted with 5 × 10^6 IFN-αR−/− BM cells and 5 × 10^6 CD11c-DTR-GFP BM cells. The mice were allowed to “rest” for 6–8 wk before use.

Quantitative PCR assay

For evaluating gene expression in spleen, total RNA was isolated from spleens using an RNeasy Midi Kit (QIAGEN). cDNA was synthesized with 1 μg total RNA and 500 ng Oligo (dT) primer (Invitrogen) by ReverTra Ace (Toyobo) in a 20-μl reaction at 42°C for 30 min. cDNA was diluted appropriately with water and used as a template for subsequent quantitative PCR. Quantitative PCR was performed on an ABI PRISM 7000 (Applied Biosystems) with primer pairs for various chemokine ligands and cytokines (Supplemental Table I) by SYBR Premix Ex Taq II (Takara). Gene expression was measured using the ΔΔCt method, in which HPRT1 expression was used as the internal control.

To evaluate gene expression in iNKT cells, FACS-sorted iNKT cells were directly subjected to cDNA synthesis and preamplification, without purifying RNA, using a CellsDirect One-Step qR-PCR Kit (Invitrogen) with a mixture of pooled gene-specific primers (0.2 μM each; Supplemental Table II). After 18 cycles of the preamplification step (each cycle: 95°C for 30 s, 60°C for 4 min), an aliquot was used as a template for quantitative PCR using FastStart Universal Probe Master (Roche), a gene-specific forward and reverse primer pair (Supplemental Table II), and the corresponding FAM-labeled hydrolysis probe (Universal ProbeLibrary Set; Roche).

Statistical analysis

Differences were analyzed using the Mann–Whitney U test. The p values < 0.05 were considered statistically significant.

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Results

Induction of memory T cells by iNKT cell ligand–loaded tumor cells

We showed previously that tumor/Gal induced both innate and adaptive immunity through enhanced activation of DCs and that the extent of CD1d expression on tumor/Gal correlated with the magnitude of both the innate (NK/iNKT) and adaptive (CD8+ T cell) responses (19, 23). Using this strategy, we demonstrated long-term antitumor effects after rechallenge with tumor cells. We attributed this outcome to the induction of memory T cells, although this hypothesis was not analyzed further in these studies (19). In the current study, we sought to investigate the possible mechanisms in greater detail. Toward this end, we monitored the expansion and development of adoptively transferred CD8+ T cells from pmel-1 TCR-transgenic mice; this TCR is specific for T cells from pmel-1 TCR-transgenic mice; this TCR is specific for melanoma cells. Vaccination with a cell-associated tumor protein Ag and an iNKT cell ligand, such as α-GalCer–loaded tumor cells that highly express CD1d (CD1d-B16/Gal cells; tumor/Gal), was compared with the coadministration of tumor cells with soluble α-GalCer (tumor+Gal) or with DCs loaded with α-GalCer (DC/Gal). As shown in Fig. 1A, vaccination with tumor/Gal induced a much stronger CD8+ T cell–proliferative response than did tumor+Gal, suggesting that having both tumor Ag and the iNKT cell ligand on the same cell resulted in more efficient cross-presentation of the gp100 Ag to CD8+ T cells by DCs, an interpretation that is supported by our previous finding that both molecules can be presented by DCs in vivo (19). Although mice given soluble α-GalCer, DC/Gal showed activation of iNKT cells, it was only when their ligand was associated with the tumor Ag that the optimal proliferative response of CD8+ T cells was observed. We next investigated whether the effect of the iNKT cell Ag on the magnitude and longevity of peptide-specific CD8+ T cell responses could be mimicked by a strong adjuvant (CFA). When tumor, tumor/Gal, or gp100 peptide plus CFA were compared in CD8+ pmel-1 T cell–transferred mice, a robust T cell response was seen on day 7 in the mice given CFA/peptide or tumor/Gal but not in the tumor-only–injected mice (Fig. 1B). Mice immunized with CFA/pep showed a primary expansion of CD8+ T cells that was comparable to that induced by tumor/Gal, but these cells rapidly contracted to a level that was only weakly detectable 2 wk later (Fig. 1B). In significant contrast, the T cells in mice immunized with tumor/Gal showed much greater survival during the same period and beyond.

We previously examined the generation of effector T cells by vaccines consisting of cells bearing two types of Ags: a cell–associated protein Ag and an iNKT cell ligand. These vaccines have included tumor/Gal, as above, and another model vaccine in which tumor Ag–encoding mRNA is transfected into allogeneic cells that are then loaded with α-GalCer (16, 19). In the current study, we evaluated whether these approaches could bring about a memory T cell response. Surprisingly, a single injection of α-GalCer–loaded tumor cells or Ag mRNA–transfected α-GalCer–

![FIGURE 1](http://www.jimmunol.org/content/173/10/5611/F1.1.jpg)

Vaccination of iNKT cell ligand–loaded tumor cells results in the formation of memory T cells after activation-induced cell death. (A) A total of 1 × 10^6 CFSE-labeled CD8+Thy1.1+pmel-1 T (pmel-1 T) cells was transferred into C57BL/6 or Jo18−/− mice. The next day, the mice were given i.v. injections of 5 × 10^5 α-GalCer–loaded high CD1d-expressing B16 tumor cells (CD1d-B16/Gal: tumor/Gal), tumor cells and α-GalCer (500 ng/mouse), 5 × 10^5 α-GalCer–loaded DCs, or CD1d-loaded EL4 cells with α-GalCer (data not shown). CFSE dilution of pmel-1 T cells in the spleen was analyzed after gating CD8+Thy1.1+pmel-1 T cells 5 d later. (B) Mice were adoptively transferred with 1 × 10^6 pmel-1 T cells 1 d before immunization with 5 × 10^5 tumor/Gal i.v., tumor i.v., or gp100pep (100 μg/mouse) plus CFA s.c. The number of pmel-1 T cells was analyzed at the indicated time points. (C) Mice were adoptively transferred with 1 × 10^6 pmel-1 T cells 1 d before immunization with tumor/Gal. (CB) Mice were adoptively transferred with 1 × 10^6 OT-I T cells. Allogeneic CD1d-NIH3T3 cells transfected with 10 μg OVA mRNA that had been loaded with α-GalCer were administered to OT-I–recipient mice (5 × 10^5/mouse). One year later, the frequency of pmel-1 T cells (Ci) or OT-I T cells (Ci) in the vaccinated mice was analyzed. Data are representative of two independent experiments with four mice/group (A), three independent experiments (mean ± SEM of four to eight mice) (B), or two independent experiments (mean ± SEM of four mice) (C).
loaded CD1d-NIH3T3 cells (Fig. 1C) resulted in the generation of long-term memory T cells that could be detected both systemically in nonlymphoid tissues and in lymphoid organs 1 y later. We next analyzed the expression of surface molecules relevant to the functional phenotype of CD8+ T cells 7 d after vaccination. We observed the upregulation of CD122, IL-15Rα, CCR5, and CXCR3, but not CD25 or PD-1, in mice immunized with tumor/Gal (Fig. 2A). To discriminate between the effector and memory subsets of CD8+ T cells, we relied on memory precursor effector cells being enriched in the KLRG1loCD127hi population and short-lived effector cells being enriched in the KLRG1hiCD127lo population (27). As shown in Fig. 2B, the pmel-1 T cells in the tumor/Gal-injected mice contained a high frequency of memory precursor effector cells (KLRG1loCD127hi) and a low percentage of short-lived effector cells (KLRG1hi CD127lo).

**FIGURE 2.** Characteristics of the T cell response induced by the adjuvant activity of iNKT cells. One day after the transfer of $1 \times 10^4$ pmel-1 T cells, mice were vaccinated with CD1d-B16/Gal (tumor/Gal). (A) The expression of IL-15R, CD25, CD122, PD1, CCR5, and CXCR3 on pmel-1 T cells in the spleen was analyzed 1 wk or 1 mo after the vaccination (shaded graph, isotype matched control; blue line, nonimmunized; red line, day 7; green line, day 30). (B) Mice were treated as in (A), but the expression of KLRG1 or CD127 on the pmel-1 T cells was analyzed 7 or 60 d after immunization. (C) The expression of the Eomes and T-bet transcription factors is depicted by flow cytometric contour plots of spleen cells, gated on pmel-1 T cells 30 d after immunization. (D) The absolute number of pmel-1 T cells in the spleen following immunization with tumor/Gal was determined over time. (E) The spleens were harvested at the indicated time points. The expression of CD44 and CD62L is shown in flow cytometric contour plots gated on pmel-1 T cells. (F) The mice were treated as in (E), but the polyfunctional Ag-specific pmel-1 T cells were analyzed at 90 d or 12 mo after immunization. (G) Cytokine-positive cells from (F) were subject to Boolean gate analysis, and the functionality profiles are shown as pie graphs. Data are representative of two independent experiments with four mice/group (A–C, E–G) or three independent experiments (mean ± SEM of 5–10 mice) (D).
The expression of two master transcription factors, T-bet and Eomes, defines the CD8+ T cell effector and memory subsets (10, 11). Memory CD8+ T cells were shown to express Eomes and T-bet in viral infection models (10, 11), and we also found their expression in our memory T cells (day 30) but not in naive pmel-1 T cells (Fig. 2C). We assessed the number of pmel-1 T cells in the spleen over time after immunization with tumor/Gal (Fig. 2D) and found that they were maintained in the spleen for >12 mo.

**Multiple functions of memory T cells demonstrated in the tumor/Gal-injected mice**

The successful elicitation of high-quality antiviral protection by CD8+ T cell vaccines can be correlated with the generation of "polyfunctional" CD8+ T cells capable of expressing multiple effector pathways (28, 29). In fact, detailed flow cytometric analyses of T cell effector functions led to the identification of polyfunctional T cells in infectious disease and vaccine models in mice and in humans (28, 29). We found that the dominant memory T cells were central memory CD8+ T cells transiting over time from the effector T cell–dominant population (Fig. 2E) and assessed their multifunctionality in tumor/Gal-injected mice 90 d and 12 mo after the vaccination. We showed that the central memory T cells expressed TNF-α, IL-2, IFN-γ, and CD107a at day 90 and did so even more clearly at 12 mo (Fig. 2F, 2G), suggesting that the KLRG1loCD127hi central memory phenotype T cells were durably maintained.

**Requirement for type I IFN to generate memory T cells following the adjuvant activity of iNKT cells**

CD40L on CD4+ Th cells is reported to be a licensing signal for the maturation of DCs (30) and, moreover, that iNKT cell CD40L signaling through CD40 on cDCs in situ plays a crucial role in IL-12 production (14, 31) and Ag-specific T cell induction (13, 14). We evaluated the importance of type I IFN and CD40 signaling in T cell responses to tumor/Gal using IFN-α/βR−/− and CD40−/− mice. In the steady state, the number of iNKT cells, DCs, and pDCs in IFN-α/βR−/− mice were comparable to those in wild-type (WT) mice (Fig. 3A). The number of CD8+Thy1.1+pmel-1 T cells in WT and IFN-α/βR−/− mice was higher than in CD40−/− mice on day 7 after immunization (Fig. 3B, 3C). However, despite the efficient effector CD8+ T cell response in IFN-α/βR−/− mice, the number of pmel-1 T cells was decreased by day 30 after immunization (Fig. 3B, 3C). Thus, the type I IFN signal is not essential for initiating adaptive immunity, but it is required for the establishment of memory T cells.

**The type I IFN signal is critical for DC activation that causes memory T cell induction in vivo**

We next examined what types of cells were affected by the type I IFN signal. Because type I IFN production is low in CpG-DNA–treated pDCs from IFN-α/βR−/− mice (32), we initially suspected that the impaired memory T cell response in immunized IFN-α/βR−/− mice was either due to low levels of type I IFN or the lack of direct type I signaling through the IFN-α/βR on T cells. To test this idea, we established CD8+Thy1.1+IFN-α/βR−/−pmel-1 T cells by intercrossing IFN-α/βR−/− mice and pmel-1 mice (Fig. 4A). The frequency and function of the pmel-1 T cells were analyzed 7 and 30 d after transfer of CD8+Thy1.1+IFN-α/βR−/−pmel-1 T cells into WT mice and subsequent immunization with tumor/Gal. After the immunization, the CD8+Thy1.1+pmel-1 T cells showed no significant difference in frequency or function (i.e., they showed the same expression of IFN-γ, TNF-α, IL-2, and CD107a) when restimulated ex vivo with the human gp100 peptide (Fig. 4B).

XCR1+ DCs are exclusively present among the CD8+ subset of cDCs and represent 70–80% of the CD8+ DCs in the spleen (33, 34). The XCR1+ DCs are found in the red pulp, marginal zone, and

![FIGURE 3. Type I IFN is essential for generating memory T cells resulting from the adjuvant activity of iNKT cells. (A) The frequency of iNKT cells, DCs, and pDCs was analyzed by flow cytometry using CD1d-dimer/Gal-PE, CD19-FITC, CD11c-PE, and PDCA-allophycocyanin in WT and IFN-α/βR−/− mice. (B and C) Naive CD8+Thy1.1+ pme1 T cells (1 × 10⁶) were adoptively transferred into WT, CD40−/−, or IFN-α/βR−/− mice, followed by vaccination with tumor/Gal 1 d later. The frequency of pmel-1 T cells was analyzed by flow cytometry (B), and the absolute number of pmel-1 T cells in each group of mice was analyzed 7 and 30 d after vaccination (C). Data are representative of two independent experiments with four mice/group (A) or three independent experiments (mean ± SEM of four to six mice/group) (B, C). *p < 0.05, CD40−/− versus WT and IFN-α/βR−/− (day 7) and WT versus IFN-α/βR−/− (day 30), Mann–Whitney U test.]
central area of the T cell zone and correspond to the distribution of CD8+CD205+ DCs. We recently established XCR1

DTR mice for depleting the XCR1+ DCs (C. Yamazaki et al., submitted for publication). Because a previous report using a tumor/Gal protocol indicated that the T cell response to the adjuvant effect of activated iNKT cells depended on cDCs in situ (19), we confirmed that the CD8+ T cell response occurred in a host XCR1 DC–dependent manner using XCR1-DTRvenus and CD11c-DTR mice (Fig. 4C).

Some reports showed that type I IFN stimulation of DCs can drive T cell activation in an autocrine or a paracrine manner (35–37). For example, both IFN-α and IFN-β from polyinosinic:polycytidylic acid–stimulated stromal cells activate DCs (36). Therefore, to test whether type I IFN could act directly on DCs, we established BM-chimeric mice by transferring the BM of CD11c-DTR and IFN-α/βR2/2 mice into lethally irradiated mice. We verified the presence of IFN-α/βR–deficient DCs after treatment with diphtheria toxin (DT) (Fig. 4D). In addition to the decreased number of pmel-1 T cells in the DT-treated BM chimeras, the multifunctionality of the pmel-1 T cells was also clearly impaired (Fig. 4E). We verified that the number and function of pmel-1 T cells were normal in BM chimeras not treated with DT (data not shown). These findings suggest that CD40L signaling and type I IFN on DCs are required to induce memory T cells.

In vivo cross-talk between pDCs producing IFN-α and cDCs producing IL-12

The production of IL-12p40 by cDCs in situ and in T cell responses depends on the interaction of CD40L on iNKT cells and CD40...
expressed by cDCs, leading the adjuvant activity of iNKT cells for cDCs (12, 19, 38). We verified that CD40L was expressed on iNKT cells and that IL-12 was produced by DCs in tumor/Gal-injected IFN-α/BR−/− mice (Fig. 5A, 5B). There was no statistically significant difference in IL-12p40 production by cDCs or CD40L expression on iNKT cells between WT and IFN-α/BR−/− mice. Although CD40L on pDCs was reported to help cDCs prime T cells in an HSV infection model (39), we did not detect CD40L on the pDCs (data not shown).

The iNKT cells were rapidly activated 2 h after immunization with tumor/Gal (data not shown), whereas we detected type I IFN–producing pDCs 6 h later (Fig. 5C). We detected type I IFN produced by pDCs from mice injected with unbound α-GalCer (Fig. 5C); however, the amount produced by pDCs from tumor/Gal-injected mice was 10-fold greater. Furthermore, IFN-α was detected in tumor/Gal-injected WT or CD40−/− mice but not in IFN-α/BR−/− mice or Jα18−/− mice (Fig. 5D). Taken together, these findings suggest that the IFN-α production by pDCs depends on iNKT cell activation but not on CD40–CD40L signaling. In addition, its production is enhanced in a type I IFN autocrine-dependent manner (32). IFN-α production also requires another factor, possibly danger signals derived from dying cells.

**Chemotactic mechanism for iNKT cell–triggered IFN-α production by pDCs**

In the steady state, pDCs are scattered mainly in the T cell area and red pulp of the spleen (Fig. 5E, upper left panel). We tracked the location of pDCs after immunization and found that, 2 h after immunization with tumor/Gal, most pDCs were still located at the periarteriolar lymphatic sheaths (PALS). However, 6 h later, the pDCs had trafficked to the marginal zone where the iNKT cells reside (40), and they produced type I IFN (Fig. 5C, 5E, upper right panel). The distribution of these cells was analyzed to elucidate the biological mechanism for the induction of effector T cells and memory T cells by the simultaneous production of IL-12 and IFN-α in XCR1+ DCs and pDC cross-talk. Surprisingly, when the interaction between pDCs and XCR1+ DCs was analyzed using XCR1-DTRvenus mice, we found that XCR1+ DCs were clustered...
with pDCs at the marginal zone and red pulp 6 h after immunization (Fig. 5F, middle panel, 5G, 5H); 12 h later, XCR1+ DCs had trafficked to the PALS to stimulate T cells (Fig. 5F, 5H).

To identify the molecules responsible for this interaction, we measured several chemokines known to affect the migration of pDCs and detected an increase in chemokine ligands, including CCL4, CXCL9, CXCL10, and CXCL11, in the spleen (Fig. 6A). By further analyses using real-time PCR, we detected the expression of CCL4, as well as CXCR3 ligands (CXCL9, CXCL10, and CXCL11), by activated iNKT cells (Fig. 6C).

In the steady state, iNKT cells (40) and cDCs (33, 41) reside in the PALS and marginal zones. Taken together, these findings indicate that activated pDCs are recruited from the PALS to the marginal zone, where iNKT cells and cDCs reside and interact with XCR1+ DCs by forming clusters. Therefore, we suspect that such chemokines help to promote the trafficking of pDCs and DCs following iNKT cell activation at the marginal zone. In tumor/Gal-immunized IFN-α/βR−/− mice, we also detected pDC trafficking to the marginal zone (Fig. 5E, lower right panel) and chemokine expression by iNKT cells as well (data not shown). Thus, these findings suggest that type I IFN secretion, as well as both pDC and XCR1+ DC trafficking, is critical.

Molecular mechanism for type I IFN production by pDCs in tumor/Gal-injected mice in vivo

pDCs can be activated through members of the TNF and TNFR superfamilies, including CD40 and OX40L (42). Because we found IFN-α production by pDCs in tumor/Gal-injected CD40−/− mice, we focused on OX40. We found that OX40 was upregulated on iNKT cells in the spleen and liver (Fig. 7A). As shown in Fig. 6C, the level of IFN-α produced by pDCs was higher in mice given tumor/Gal than in mice given free α-GalCer. Thus, we suspected that some danger signals from damage-associated molecular pattern molecules played an additional role in the immunized mice. We evaluated the damage-associated molecular pattern molecules and found that HMGB1 (Fig. 7B), but not Hsp70 or Hsp90 (data not shown), was released into the culture supernatant of tumor/Gal with activated iNKT cells, whereas much less was released into the culture supernatant from tumor cells alone or from activated iNKT cells alone. The blockade of both OX40 and HMGB1 using two Abs resulted in a reduction in IFN-α production by pDCs (Fig. 7C). In addition to OX40 signals, HMGB1 enhanced the production of IFN-α by pDCs. Thus, IFN-α production by pDCs may depend on both the OX40 on activated iNKT cells and HMGB1 from dying cells, whereas the CD40L on iNKT cells stimulates IL-12 production by cDCs. Thus, we showed that the cooperation of HMGB1 from dying tumor cells and OX40 expression on iNKT cells stimulates the pDCs to produce type I IFN. Thus, the synergistic effects of pDCs achieved by their clustering with fully mature cDCs that were initially driven by CD40L on activated iNKT cells are essential for the generation of memory T cells.

Discussion

DCs are known to link innate and adaptive immunity; however, there remained the problem of how to induce memory T cells at the DC level. This study reveals a pivotal role for pDCs, which are

**FIGURE 6.** Chemotactic mechanism for iNKT cell–triggered IFN-α production by pDCs. Quantitative analyses of expression of chemokine genes involved in the migration of pDCs. Expression of each mRNA in the spleen (A) and in isolated iNKT cells (C) was determined by quantitative real-time PCR using the primer sets shown in Supplemental Tables I and II, respectively, and is depicted as the number of transcripts per one copy of the housekeeping gene HPRT. (B) Chemokine receptors on pDCs were analyzed by flow cytometry. Data are shown gated on CD11c+Siglec-H+ cells (shaded graph, isotype-matched control mAb; dashed line, naive; solid line, tumor/Gal). Data are representative of two independent experiments with four mice/group with triplicates (mean ± SEM) (A, C) or two independent experiments with four mice (B).
licensed by activated iNKT cells on cDCs in the development of long-term T cell memory in mice after administration of tumor cells loaded with iNKT cell ligand and bearing a cell-associated Ag. We previously demonstrated that the successful generation of effector T cells using tumor/Gal as the immunogen depends on certain crucial factors, such as CD40L expression on iNKT cells, in addition to the cell type, expression of CD1d, and dose of α-GalCer on the APCs (38). In the current study, we demonstrated that the formation of a cluster composed of two types of DCs in a chemoattractant-dependent manner following the activation of iNKT cells is crucial for the establishment of memory CD8+ T cells. Several studies using viral infection models showed that IFN-α signaling in T cells is required for the induction of T cell immunity (6, 7, 43). Other studies in TLR ligand-admixed models showed that IFN-α signaling in DCs is required for the induction of T cell immunity (35, 36, 44). The current study is consistent with the latter model, but there has not been any report clarifying the interaction between IFN-α–stimulated DCs and memory T cells in conjunction with the adjuvant effects of iNKT cells. In addition, we demonstrated the mechanism of pDC stimulation in vivo in which the cooperation of HMGB1 from dying tumor cells and pDCs are another major DC subset and were reported to have a dual role: they are both immune suppressors and immune stimulators for T cells. The pDCs help to induce effector T cells in some infection models, such as HSV, vesicular stomatitis virus, and Listeria (39, 47, 48). In contrast, with regard to iNKT cell and pDC interactions, one group recently reported, in a LCMV infection type 1 diabetes murine model using Vα14+ iNKT cell–transgenic mice, that virus-stimulated iNKT cells promote tolerogenic pDCs. The pDCs simultaneously produce TGF-β and type I IFN locally in the pancreatic lymph nodes, thereby converting naive T cells into Foxp3+ CD4+ regulatory T cells, which blocked the progression to type 1 diabetes (49, 50). In contrast, we found that type I IFN derived from iNKT cell–stimulated pDCs helped to establish antitumor memory T cells (Figs. 4, 5). The apparent contradiction between these results may be due to several experimental factors. In particular, the two different mouse

FIGURE 7. Mechanism for IFN-α production by pDCs in tumor/Gal-injected mice in vivo. (A) Expression of OX40 on iNKT cells in the spleen and liver was analyzed, gated on CD19−CD1d-dimer/Gal+ cells (shaded graph, isotype-matched control mAb; dashed line, naive; solid line, tumor/Gal). (B) Tumor/Gal (1 × 106) and B cell–depleted spleen cells (5 × 10⁵) from mice that had been immunized with tumor/Gal 6 h previously were cultured in 24-well plates for 48 h. The release of HMGB1 into the supernatants was measured using an HMGB1 ELISA kit (Shino-Test). *p < 0.05, tumor/Gal versus tumor/Gal+B-depleted spleen, Mann–Whitney U test. (C) To block OX40 and/or HMGB1, an anti-OX40 Ab (500 μg/mouse), with or without an anti-HMGB1 Ab (100 μg/mouse), was injected into mice 2 h before tumor/Gal immunization. As shown in Fig. 5C and 5D, pDCs were isolated from immunized mice 6 h after immunization and cultured for 24 h. IFN-α in the supernatants was measured by ELISA. *p < 0.05, control versus anti-HMGB1 or anti-OX40L, **p < 0.001, control versus anti-HMGB1+anti-OX40L, Mann–Whitney U test. Data are representative of two independent experiments with four mice/group (A), three independent experiments (mean ± SEM) (B), or two independent experiments (mean ± SEM of four or five mice/group) (C).
models, LCMV-stimulated iNKT-transgenic mice versus synthetic iNKT ligand–loaded tumor-administered WT mice, might show different iNKT cell–activation kinetics and subsets of activated iNKT cells in different organs, differences that could be related to the subsequent immune response.

Self- or pathogen-derived DNA may activate proinflammatory cytokine pathways by a DNA-sensing mechanism (51). In the engulfment of apoptotic virus-infected cells, viral ssRNA is detected by TLR7 as a pathogenic nucleic acid. In addition, we can identify HMGB1 in our models (data not shown). Therefore, HMGB1 acts as a potent adjuvant for the enhancement of pDCs and anti-OX40. However, pDCs do not show any phagocytic activity in our models (data not shown). Therefore, HMGB1 acts as a potent adjuvant for the enhancement of pDCs. In addition, we can speculate that HMGB1 combines with the self-DNA from dying tumor/Gal cells to form nucleic acid–bound HMGB1, although it remains unclear where the HMGB molecules can bind to nucleic acids and how the HMGB–nucleic acid complexes activate their respective TLR-signaling cascades. Further studies are required to elucidate this mechanism.

Collectively, for the establishment of the memory T cells in our system, CD40L-expressing iNKT-licensed cDCs simultaneously require the pDCs that received signals from both dying cells that express HMGB1 and activated iNKT cells that express OX40. The cross-talk between pDCs and cDCs is tightly regulated by chemokine and IFN-α–IFN-αR signals. Together, this mechanism would enhance the programming of functional memory T cells and, thereby, improve the efficacy of cancer vaccines.

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