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Activated human blood γδ T cells have also been previously demonstrated to behave as professional APCs, although the processes that control APC function have not been characterized. In this study, we show that the acquisition of potent APC function by human blood γδ T cells is achieved after physical interaction with an Ab-coated target cell, a process that we refer to as licensing. In cancer models, licensing of γδ T cells by tumor-reactive mAbs promotes the uptake of tumor Ags and professional presentation to tumor-reactive αβ T cells. We propose that licensing by Ab is a mechanism whereby the adaptive properties of γδ T cells are induced by their innate functions in a spatially and temporally controlled manner. The Journal of Immunology, 2012, 188: 1708–1716.

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Professional APCs have the hallmark properties of taking up soluble Ag, or of sampling Ags from target cells by phagocytosis, and then presenting processed Ags in the context of MHC class II, or cross-presenting in the context of MHC class I, to induce primary immune responses in naive Ag-specific αβ T cells. Dendritic cells (DCs), the prototypic professional APC, take up Ags at sites of infection, injury, or cancer and undergo a maturation step involving upregulation of costimulatory molecules prior to migrating to sentinel lymph nodes. In this setting, an interaction with CD40L on the surface of CD4-positive T cells results in CD40 signaling in the DC, which is required for acquisition of full Ag presentation function, a process referred to as licensing (1–3).

γδ T cells typically represent 1–5% of T lymphocytes in human blood. In contrast to conventional αβ T cells, γδ T cells express a limited repertoire of TCR V-region genes; the most common subtype in human blood is Vγ9Vδ2, which recognizes a group of nonpeptide phosphorylants, for example isoprenyl pyrophosphate (IPP), associated with infection or cancer (4, 5). Stimulated γδ T cells undergo an activation, which results in a plethora of poorly defined changes including proliferation, proinflammatory cytokine and chemokine secretion, and altered cell surface phenotype (6). Activated human peripheral blood γδ T cells also have properties of innate immunity, including cytokotoxicity mediated by NK receptors (7–11), Ab-dependent cell-mediated cytotoxicity (ADCC) (12, 13), and phagocytosis (14). Notably, we and others have shown FcγRIII (CD16) expressed on at least some human γδ T cells to be a potentially important molecule mediating innate effector functions (15, 16). These changes are likely to be subtype specific along classical T cell divisions; that is, T naive, T central memory, T effector memory, and T effector memory RA+ (16).

At least one subtype of human γδ T cells has been shown to be able to express MHC class II, costimulatory molecules, and lymph node-homing chemokine receptors (e.g., CCL7) and has been characterized as a professional APC (17, 18). Previous studies have thus demonstrated the ability of γδ T APC to present and cross-present Ags from soluble protein (17–19), and we and others have shown that human blood γδ T cells can take up large fragments from intact cells by the processes of phagocytosis (14) or trogocytosis (20). Also, we found that Ags associated with these large (>1 μm) structures can be processed and presented, bound to MHC class II, by γδ T cells to other T cells (14). We therefore hypothesized that when activated γδ T cells are cocultured with transformed cells, the tumor cells would serve not only as targets for γδ T-mediated killing but also as a source of tumor-associated Ags (TAAs) for processing and professional presentation by the γδ T cells to tumor-reactive αβ T cells. In this study, we show that human γδ T cells do indeed present TAAs from tumor cells. Moreover, we have found that the process of APC function is tightly regulated, requiring interaction with cell surface-bound Ab, a requirement we refer to as licensing of γδ T cells for professional APC function.

Materials and Methods

Cell isolation

Fresh peripheral blood was centrifuged over Ficoll-Hypaque to obtain PBMCs according to the local ethical guidelines on experimentation with human samples. Vγ9Vδ2 T cells were isolated by two rounds of purification using anti-γδ TCR (MicroBead Kit, which consists of a hapten-conjugated anti-TCR/γδ Ab and FITC-conjugated anti-hapten microbe-
CD3, anti-CD28, and anti-CD137 stimulating Abs in the presence of 300 IU/ml IL-2 for 3 d. T cell cultures were then transduced as follows: 24-well plates were coated with RetroNectin (Takara, Otsu, Japan), then preloaded with retrovirus according to the manufacturer’s instructions. T cells (2.6 × 10^6) were added to each well in 1.3 ml Mart-1 TCR retroviral supernantant, supplemented with 300 IU/ml recombinant human IL-2, and the plates were centrifuged for 90 min at 1000 × g. The next day, the medium was replaced with fresh retroviral supernatant, and the centrifugation was repeated. Transduction efficiency and TCR expression on T cells were confirmed by flow cytometry using 10 μg/ml anti-TCR Vβ12 (ImmunoTech). The cells were rested for 24 h, after which transduced cells were CFSE labeled and assayed for Ag reactivity by 5-d coculture with DCs or with activated γδ T cells in the presence of opsonized Lan-1 tumor cells and Ag (SK-Mel-23 tumor lysate, Mart-1 peptide [ELAGIGILTV], or irrelevant peptide). IL-2 was added at day 3.

Confluent microscopy

Oxonplated Daudi cells were labeled either with Vybrant DiL (Invitrogen, U.K.) or CFSE and incubated with IPP-activated γδ T cells stained with mouse anti-human TCR-γδ (BD Biosciences, Heidelberg, Germany) and secondary Abs goat anti-mouse Cy5 (CalTag, U.K.), or Alexa Fluor 488 phallloidin (Invitrogen, Grand Island, NY) before being immediately analyzed. Images were captured using LSM 510 confocal microscopy (Zeiss) with Leica confocal software.

Cytotoxicity assays

Freshly isolated or activated γδ T cells were used as effector target. Target cells were coated or not with the opsonizing mAb and labeled with 100 μCi Na^251CrO_4 in cell culture and tested in a standard chromium release assay as previously described.

Activation phenotype of γδ T after coculture

γδ T cells were activated by coculture with lymphoblastoid cell line conditioned media in the presence of 100 IU/ml recombinant human IL-2 and 50 μM IPP and cultured with or without non-opsonized Daudi or Raji cell line for 48 h. γδ T cell phenotype was analyzed by FACS using the following mAbs: monoclonal mouse IgG2 PE-conjugated anti–HLA-DR, clone L243 (BD Biosciences); monoclonal mouse IgG1 PE-conjugated anti-CD86, clone FUN-1; anti-CD11c–allophycocyanin, clone B-ly6; anti-CD40–PE-Cy5, clone 5C3; and CD80–FITC, clone L307.4 (BD Biosciences). To characterize the γδ TCR phenotype, we used anti-Vγ9–PE–Cy5 (Beckman-Coulter) to stain activated Vγ9Vδ2 T cells and a pan anti-Vγ-β–T-biotin clone B1 (BD Pharmingen).

Results

Cross-presentation of TAA by human blood γδ T cells

As a model to study the capacity of human γδ T cells to interact with human cancer cells, we used immunomagnetic positive selection of peripheral blood, using a pan-γδ TCR Ab. Using this method, yields of γδ T cells were more than 98% pure (Fig. 1A), and percentage contamination with CD11c-positive cells was between 0.3 and 0.6% (Fig. 1B). As a tumor model, we initially used the Burkitt lymphoma cell line Daudi because we (Fig. 1C) and others (12) showed that opsonization with anti-CD20 IgG1 mAb (rituximab) enhances ADCC of Daudi cells by γδ T cells, and because we showed that activated human blood γδ T cells could take up fragments from opsonized Daudi cells (Fig. 1D). In addition, we reasoned that opsonized Daudi cells could, in this model, serve as a source of TAA for processing and presentation on γδ T MHC to other T cells in a rituximab-dependent manner. To test this, we investigated cross-presentation of PAX5 Ag, a nuclear transcription factor expressed in B lineage cancer cells including Daudi (22, 23), by γδ T MHC class I. We have previously described a CD8 CTL clone specific for the PAX5 peptide sequence TLPGYPPHV and restricted to HLA-A^*0201 (22). We tested the ability of γδ T cells to present PAX5 Ag from intact tumor cells in a 5-d coculture involving 1) IPP-activated γδ T cells from an HLA-A^*0201–positive donor, 2) rituximab-opsonized HLA-A^*0201–negative Daudi cells, and 3) CFSE-labeled αβ CTL clone restricted to PAX5/HLA-A^*0201 in the presence of
conditioned medium from a B lymphoblastoid cell line (Fig. 2A; see Supplemental Fig. 1 for lymphocytoid cell line detail). Robust proliferation of the αβ clone was individually dependent on γδT cells and opsonized Daudi cells and was Ag- and MHC-restricted as demonstrated through use of an αβ CTL clone specific for HLA-A0201/PRAME (not expressed on Daudi cells) (Fig. 2B) and further demonstrated by lack of proliferation of the PAX5 clone when preincubating the γδT cells with an HLA-A0201 blocking Ab (Fig. 2C). To exclude the possibility that contaminating professional APCs in the γδT cell preparations were responsible for the observed stimulation, we titrated cells from the negative fraction after γδTCR selection back into a coculture involving non-HLA-matched γδT cells. No significant proliferation was observed until the number of negative fraction cells reached 20% (Supplemental Fig. 2). To confirm the interaction between IPP-activated γδT cells and rituximab-opsonized Daudi cells, we used live imaging in which the γδTCR was labeled with a fluorescent Ab and was found to form an aggregate at the immune synapses with Daudi cells. Moreover, a significant number of γδT cells (8–13%) were found to be interacting with Daudi cells at any time point (Fig. 2D). By the end of the 5-d coculture in the presence of IPP, the γδT population was found to be exclusively of the Vγ9 subtype (Fig. 2E). Therefore, we conclude that the coculture of activated Vγ9Vδ2 T cells with intact tumor cells expressing an intracellular Ag can result in the MHC class I-restricted Ag-specific proliferation of an αβ CTL clone.

**Ab-dependent licensing of γδ T APC by direct cell–cell contact**

We wanted to compare the cross-presentation function of human γδ T cells after culture with either Ag-expressing intact tumor cells or with soluble tumor Ag. We also were interested in whether activation of γδ T for cross-presentation could cause bystander activation through release of soluble factors. To this end, we used Transwells with a 1-μm pore size restricting free diffusion to soluble proteins or cell fragments. In the presence of opsonized Daudi cells, γδ T cells could, as expected, cross-present PAX5 Ag to αβ CTL in an upper chamber, but the αβ CTL clone in the communicating lower chamber was only modestly stimulated (Fig. 3A). Similarly, a low level of activation of the CTL clone was seen if Daudi cells were not opsonized (Fig. 3B, upper panel) compared with the negligible background activation seen in the absence of Ag (Fig. 3B, lower panel).

Previous studies have demonstrated convincing Ag presentation to both CD4 and CD8 naive αβ T cells by activated human γδ T cells loaded with soluble whole protein Ags (17–19). We hypothesized that the relatively weak APC function we observed in the absence of opsonized target cell was being greatly enhanced as a result of the interaction with Ab-coated cells. To test this hypothesis, we cultured activated γδ T cells and the PAX5 αβ CTL clone with a lysate from Daudi cells in the presence or absence of a third-party opsonized target cell (Lan-1 neuroblastoma cells, PAX5 nonexpressing, coated with anti-GD2 IgG1 mAb).
Whereas the presence of opsonized target Ag-negative cells induced a robust proliferation, we consistently observed only weak CTL response in the absence of opsonized cells (Fig. 3C, left panel). A control condition, in which Ab was omitted, resulted in loss of the profound APC function to the level seen in the lower chamber with Ag alone (Fig. 3C, middle panel). We refer to this dependency on direct contact with an Ab-coated target cell for induction of full APC function of γδ T cells as “licensing.” Consistent with previous reports (17), proliferation and IFN-γ secretion in the presence of Ag but absence of Ab was consistently greater than the background proliferation (Fig. 3C, middle panel). We excluded the involvement of soluble factors from Daudi cells in licensing by substituting recombinant PAX5 protein for the Daudi lysate (Supplemental Fig. 3).

To demonstrate this licensing concept in a further cell system, we substituted K562 erythroleukemia cells coated with an anti-CD45 IgG1 Ab as the licensing cell and found an equivalent and “opsonized cell”-dependent proliferation and IFN-γ response (Fig. 3D). Notably, γδ T cells that had been licensed by opsonized Lan-1 and then repurified from the licensing conditions and rested overnight had lost the ability to take up and present soluble PAX5 Ag, whereas if these licensed and purified cells were relicensed by coculture with opsonized Lan-1, they fully regained APC function (Fig. 3E, right panel).

**Raji cells fail to license γδ T cells**

Because Daudi cells are well known to be efficiently killed by γδ T cells, we sought to investigate whether another very similar cell line, but one that is resistant to γδ T cell killing, can also license γδ T cells for more potent APC function. To do this, we used Raji cells, which others have described as being more resistant to γδ T cell cytotoxicity (24). We first confirmed that activated HLA-A*0201-positive γδ T cells are poor killers of anti-CD20-opsonized Raji targets. Notably, in contrast to Daudi cells, Raji cells failed to induce any APC function of γδ T cells in a 5-d coculture setting (Fig. 4A). We then sought to determine whether the failure of the induction of APC function of γδ T cells was the consequence of inadequate PAX5 Ag release from Raji cells. To do this, we repeated the 5-d coculture experiment using opsonized Raji cells in the presence of Daudi lysate as a source.

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**FIGURE 2.** Human blood γδ T cells cross-present intracellular Ag from an opsonized tumor cell. A, Cartoon schematic of three-cell, 5-d coculture used in these experiments. B, Ag-specific and MHC-restricted stimulation of an αβ CD8 T cell clone disclosed by CFSE dilution measured by flow cytometry. Data representative of six independent donors. C, Inhibition of αβ T cell clone as defined by CFSE dilution and IFN-γ secretion measured by flow cytometry was inhibited by pretreating γδ T cells with an HLA-A*0201 blocking Ab. D, Confocal imaging of the three-cell coculture. γδ TCR was visualized on γδ T cells with a magenta-labeled anti-pan γδ TCR Ab, opsonized Daudi cells labeled in red, and αβ T cell clone labeled in green. The magenta TCR stain was found to localize to a discrete region of the membrane. The lower panel is a magnified portion of the boxed region of the upper. E, γδ T cells after 5-d coculture stained homogeneously positively with a Vδ9 specific Ab. Results in panels B and C are representative of three independent experiments, and panels D and E are representative data of two separate experiments. Percentages shown in the FACS plots are the means of the percentages of viable positive cells from three experiments yielding similar results.
**FIGURE 3.** Human blood γδ T cells are licensed for Ag cross-presentation by interaction with opsonized cells. A, αβ T cell clone activation determined by CFSE dilution and IFN-γ release in top and bottom chambers of a 1-μm-pore Transwell experiment. Daudi targets were opsonized and repeatedly washed before being added to the top chamber. B, Controls for the three-cell coculture protocol depicted in Fig. 2 in which Daudi cells alone or rituximab alone were used as the licensing agent. C and D, Five-day coculture 1-μm-pore Transwell experiment set up as depicted in the scheme. E, Human γδ T cells were activated with IPP in the absence of PAX5 Ag and then licensed with Lan-1 cells opsonized with anti-GD2 mAb for 2 d before purifying with two rounds of positive selection. Licensed γδ T cells were then rested for 24 h in the presence of IL-2. Twenty-four-hour rested γδ T cells were cocultured with labeled αβ CTL responder clone in the absence (central panel) or presence (right panel) of anti-GD2 opsonized Lan-1 cells. Positive control (left panel) were non-sorted licensed γδ T cells cocultured with responders and Ag. Typical FACS plots are shown from three different experiments performed in triplicate with two different donor cells each, and percentages shown in the FACS plots are the means of the percentages of viable positive cells from three experiments yielding similar results.
of PAX5 Ag. Despite the presence of high amount of soluble PAX5 Ag, opsonized Raji cells failed to license completely γδ T cells for APC function (Fig. 4B). Surprisingly, both opsonized and non-opsonized Daudi and Raji cells induce a strong upregulation of MHC class II (DR), CD40, and costimulatory molecules (Fig. 4C) suggesting that the licensing of γδ T cells for Ag presentation and the upregulation of molecules required for APC function are distinct events. Thus, Raji cells do not only resist γδ T cell killing but also fail to license them, independently of MHC class II and costimulatory molecule induction on the γδ T cells.

Licensed γδ T cells can present TAAs to TCR-transduced naive primary T cells

A hallmark of professional APCs is the ability to stimulate naive CTL. To generate a population of Ag-inexperienced human T cells of a single antigenic specificity, we transduced T cells from an HLA-A*0201–positive donor with a retrovirus encoding a TCR with specificity for an immunodominant HLA-A*0201–restricted peptide from the melanoma Ag MART-1 (Fig. 5A). CFSE-labeled MART-1 TCR-transduced T cells were cocultured with either γδ cells (licensed by the presence of anti-GD2–opsonized Lan-1 cells) or with conventional DCs, both derived from the same HLA-A*0201–positive donor. The Ag was either MEL-23 melanoma cell line lysate or soluble MART-1 peptide. Licensed γδ T cells were found to present either Ag to the same degree as monocyte-derived DCs (Fig. 5B). Opsonization of target cells was mandatory to license γδ T cells for potent APC function (Fig. 5C).

We speculated that interaction of γδ cells with Fc receptors might be critical for the licensing phenomenon and therefore stained activated γδ T cells with Abs against the FcγRIII (CD16) abrogated the licensing
Therefore, the acquisition of full APC function in human peripheral blood γδ T cells requires a licensing event that involves a physical interaction with an Ab-coated target cell, and our data are consistent with a CD16–Fc interaction being essential for licensing.

**Discussion**

We have confirmed the finding of previous studies that, given appropriate stimulation, phosphoantigen-activated human blood γδ T cells, which overwhelmingly express the Vγ9Vδ2 TCR, can take up and cross-present exogenous Ag to Ag-experienced Ag-inexperienced αβ T cells.
or inexperienced αβ T cells. For the first time to our knowledge, we show that this process is subject to regulation, and we have identified one mechanism of likely important physiological significance in the host response to cancer. We have reproducibly seen, using two different antigenic systems and three different “licensing” opsonized cells, that the degree of Ag-specific stimulation of responder αβ T cells is massively increased in the presence of opsonized cells. In the presence of Ab-coated cells, the degree of stimulation is equivalent to that seen with mature Ag-loaded DCs, whereas in the absence of licensing, a more modest degree of stimulation above background is apparent.

Physiologically, we propose that the requirement for Ab coating of target cells provides an important regulatory circuit between innate and adaptive immunity, which will localize functional antigenic sampling of γδ T cells to their site of activation (Fig. 5D). Hence in the cancer models demonstrated here, activation of circulating γδ T cells at the tumor site by TCR ligand will activate their expansion and innate killing. However, full APC function will only be engaged after the binding of Ig to tumor cells. This in turn will both activate γδ T-mediated killing (by ADCC) (Fig. 1C) and will license the cells for Ag presentation. It is not yet clear, however, whether γδ T cells kill their target in the process of being licensed. In this regard, it is of interest that there is a correlation between sensitivity to cytotoxic killing (in Daudi and Raji cells, respectively) and licensing ability. This differential sensitivity to killing has been reported previously, but the precise mechanism is not known although it is speculated to be related to expression of an unknown ligand for Vγ9Vδ2 TCR (25). The differential expression of F1-ATPase related protein (putative ligand for Vγ9Vδ2 TCR) on Daudi and Raji cells might also contribute to the differential sensitivity (26). In support of the physiological significance of this concept in cancer are data documenting infiltration of γδ T cells into a broad array of tumor types (27–29), which when analyzed have been found to be predominantly of the Vγ9Vδ2 subtype (7, 9, 30, 31).

 Licensing of DCs by CD40 ligation is a distinct process from their maturation, the latter involving upregulation of costimulatory molecules, MHC class II, and chemokine receptors. Analogously, for IPP-activated γδ T cells, we found that upregulation of costimulatory molecules and HLA-DR was dependent on interaction with tumor target cells but independent of interaction with Ab. Moreover, activation via ligation of the TCR was not sufficient for full upregulation of costimulatory molecules or HLA-DR (Fig. 4C). The signaling that results in upregulation of costimulatory molecules on γδ T cells is not known, nor is it known whether it requires direct interaction with and/or killing of a target cell.

Cells with combined innate killing and Ag presentation function are well known; for example, macrophages. We have previously demonstrated the capacity of γδ T cells to take up and present Ags, using phagocytosis (14), and γδ T cells also take up Ag by trogocytosis (20). Hence, at the tumor site, γδ T cells have the potential to kill by innate cytotoxic mechanisms releasing Ag into solution and to take up Ags directly by phagocytosis, trogocytosis, or pinocytosis. Ab coating of target cells potentially enhances all these processes but additionally provides a distinct licensing signal for potent activation of tumor-reactive CTL.

It is interesting to speculate how licensing could regulate APC function spatially and temporally within the setting of response to cancer or infection. Previous data have demonstrated that Vγ9Vδ2 TCR activation induces upregulation of secondary lymphoid tissue chemokines CCR7 and CCR4 and enhanced lymphoid tissue-homing capabilities, as well as clustering within germinal centers of B cell follicles (32). However, regardless of the role of γδ T licensing in physiological systems, the availability of clinical reagents for γδ T activation and therapeutic mAbs to effect licensing in the tumor niche suggests new approaches toward Ag-specific vaccination/treatment for cancer and infection.

Disclosures

The authors have no financial conflicts of interest.

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