Involvement of CD56^{bright}CD11c^{+} Cells in IL-18–Mediated Expansion of Human γδ T Cells

Junko Tsuda,*†,1 Wen Li,*†,1 Hiromichi Yamanishi,† Hideyuki Yamamoto,* Akico Okuda,* Shuji Kubo,* Zhifeng Ma,* Nobuyuki Terada,‡ Yoshimasa Tanaka,§2 and Haruki Okamura*§2

γδ T cells are considered to be innate lymphocytes that play an important role in host defense against tumors and infections. We recently reported that IL-18 markedly amplified γδ T cell responses to zoledronate (ZOL)/IL-2. In an extension of this finding, we analyzed the mechanism underlying the IL-18–mediated expansion of γδ T cells. After incubation of PBMCs with ZOL/IL-2/IL-18, the majority of the cells expressed γδ TCR, and the rest mostly exhibited CD56^{bright}CD11c^{+} under the conditions used in this study. CD56^{bright}CD11c^{+} cells were derived from a culture of CD56^{dim}CD11c^{+} cells and CD14^{+} cells in the presence of IL-2 and IL-18 without the addition of ZOL. They expressed IL-18Rs, HLA-DR, CD25, CD80, CD83, CD86, and CD11a/CD18. In addition, they produced IFN-γ, TNF-α, but not IL-12, when treated with IL-2/IL-18, and they exerted cytotoxicity against K562 cells, thus exhibiting characteristics of both NK cells and dendritic cells. Incubation of purified γδ T cells with CD56^{bright}CD11c^{+} cells in the presence of ZOL/IL-2/IL-18 resulted in the formation of massive cell clusters and led to the marked expansion of γδ T cells. However, both conventional CD56^{dim}CD11c^{high} dendritic cells induced by GM-CSF/IL-4 and CD56^{dim}CD11c^{−} NK cells failed to support the expansion of γδ T cells. These results strongly suggest that CD56^{bright}CD11c^{+} cells play a key role in the IL-18–mediated proliferation of γδ T cells. The Journal of Immunology, 2011, 186: 2003–2012.

H uman γδ T cells exhibit a rapid response to microbial infections and tumors and serve as a bridge between innate and adaptive immunity. Although the precise mechanism underlying stress-surveillance responses has not been fully clarified, γδ T cells are likely to be activated repeatedly by both common pathogens and autologous stress Ags (1–8). In humans, γδ^{+}V_{γ}^{9}V_{δ}^{62} (also termed γδ^{+}V_{γ}^{2}V_{δ}^{62}) γδ T cells represent a major subset of circulating γδ T cells and constitute 1–10% of total peripheral blood T cells (9). γδ^{+}V_{γ}^{9}V_{δ}^{62} T cells recognize phosphoantigens, like microbial (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate, an intermediate in the 2-C-methyl-d-erythritol-4-phosphate pathway (also known as the 1-deoxy-d-xylulose-5-phosphate pathway) (10) and isopentenyl pyrophosphate (IPP) in the self-mevalonate pathway (11). They are also activated in a NK receptor-mediated manner, but in a MHC-unrestricted manner (12). Recently, it was demonstrated that nitrogen-containing bisphosphonates (N-BPs) can stimulate peripheral blood γδ T cells (13). The pharmacological agents inhibit farnesyl pyrophosphate synthase, leading to the accumulation of IPP in monocyte/dendritic cells (DCs), by which γδ T cells can be efficiently activated (13–15). Moreover, the majority of tumor cells pretreated with N-BPs were demonstrated to stimulate γδ T cells in a species-specific manner (16). Although the molecular mechanism of recognition has not yet been clarified, N-BP–treated monocytes and tumor cells appear to express membrane-associated antigenic determinants on their cell surface. In contrast, (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate and IPP may be presented to γδ T cells in an extracellular pathway that requires neither Ag uptake nor Ag processing (17).

In immune/inflammatory responses, individual immune cells are activated in the context of a complex network, not in isolation. For instance, the activation and regulation of γδ T cells are dependent on signals mediated by TCRs, as well as the costimulatory molecules such as NKGD2, CD28, ICAM, and CD40L (18, 19). These signals are usually provided by accessory cells such as monocytes and DCs and the various cytokines secreted by them (20, 21). Although various subsets of DCs are present in peripheral blood and may play a role in the activation of γδ T cells, DCs involved in γδ T cell responses have not yet been extensively characterized (22, 23).

In murine, a distinct subset of DCs exhibits a phenotype of NK cells and is called NKDCs (24–27). The other subset of murine DCs exerts lytic activity and produces IFN-γ (28). Human cells that correspond exactly to murine NKDCs have not yet been identified. Human DCs with NK-like character and other phenotypes can be generated from PBMCs in vitro (29–33). Recently, it was demonstrated that a subset of DCs exhibiting CD14^{+}CD56^{dim}CD86^{+}HLA-
DRα activated Th1-type γδ T cells in human PBMCs (34), although the ontogeny of these cells (if they originated from NK cells, DCs, or monocytes) was not determined. A number of studies have demonstrated that DCs and innate immune cells activate reciprocally, suggesting that DCs with NK phenotypes may be generated by interaction among DCs, NK cells, and monocytes (35).

The cross-talk between DCs and innate lymphocytes, including γδ T cells, might be carried out through cell–cell interaction and cytokine signaling. Various cytokines are involved in the development of DCs, and growth factors such as IL-1, IL-2, IL-15, and IL-23 are required for the expansion of γδ T cells (36, 37). Other cytokines, including IL-12 (38), TNF-α (39), and TGF-β (40), are also responsible for the activation and regulation of γδ T cells.

Recently, we demonstrated that IL-18 markedly promoted the expansion of γδ T cells in a culture of human PBMCs (41). IL-18 was originally discovered as an IFN-γ–inducing factor (42), and subsequent studies revealed that it also plays essential roles in host defense against infections and tumors, as well as in the pathogenesis of various inflammatory diseases through the upregulation of IFN-γ production (43, 44). Several recent studies have suggested that IL-18 plays a novel role in cellular events such as proliferation, differentiation, and survival (29, 30, 45–47). In these reports, IL-18 was demonstrated to activate various antiapoptotic signals such as PI3K/Akt and Bcl-xL, in both immune and non-immune cells. The molecular mechanisms of IL-18–mediated signaling, however, remain to be clarified in various cells. IL-18 has also been demonstrated to function as an epigenetic regulator that enhances the expression of selected genes by methylation or acetylation of chromatin (48). This may provide an explanation for the apparently paradoxical actions sometimes observed in IL-18.

In the current study, we demonstrate that IL-2/IL-18–efficent generates and expands CD56highCD11c+ cells, which functionally and phenotypically overlap with NK cells and DCs and are essential in the amplification of γδ T cell responses to zolodronate (ZOL) or 2-methyl-3-butenyl-1-pyrophosphate (2M3BPP), an IPP analog. In addition, we characterize CD56highCD11c+ cells and discuss a possible therapeutic use of ZOL/IL-2/IL-18 in the treatment of patients with cancer.

Materials and Methods

Reagents

Recombinant human IL-18 and ZOL were provided by GlaxoSmithKline (Research Triangle Park, NC) and Novartis AG (Basel, Switzerland), respectively. An analog of IPP, 2M3BPP, was chemically synthesized by adding diketene to 1-bromocyclohexane to form a hydrophilic ligand, TDA, in an aqueous mixture. NKG2D–anti–NKG2D-allophycocyanin mAb was purchased from Beckman Coulter (Brea, CA). All of the mAbs were purchased from BD Biosciences, San Jose, CA.

Chemokines were assayed by allowing γδ T cells to pass through a polycarbonate filter with 5 μm pore size in 24-well Transwell chambers (Corning, Lowell, MA). CD3-depleted PBMCs (2 × 10⁶ cells/ml) were precultured in the presence of GM-CSF (5 ng/ml)/IL-4 (20 ng/ml) or IL-2 (10 ng/ml)/IL-18 (100 ng/ml) for 8 d; they were then purified and placed in lower chambers. The purified γδ T cells (2 × 10⁵ cells/ml) were loaded into the upper chambers in the presence of ZOL/IL-2/IL-18 in culture. After incubation overnight at 37˚C, the cell suspension in the upper well was removed. The total number of cells in the lower chamber was counted by the trypan blue dye exclusion method, and the proportion of migrated γδ T cells was calculated as follows: migration (%) = ([migrated γδ T cells]/[total γδ T cells] + [spontaneous release])/[maximum release] × 100 (%).

Cytokine assay by the DELFIA method

Cytotoxic activity was assayed using DELFIA EuTDA cytotoxicity reagents (PerkinElmer, Foster City, CA). NK-susceptible K562 cells were pretreated with a fluorescence-enhancing ligand BiotA, which was hydrolyzed by intercellular esterases to form a hydrophilic ligand, TDA, which was fluorescent. The labeled target cells were placed in 96-well plates at 1 × 10⁵ cells/well. All of the mAbs were purchased from BD Pharmingen (Franklin Lakes, NJ). CD14+, CD56dimCD11c+, CD56lowCD11c+, CD56highCD11c+, and CD56lowCD11c+ cells were purified using a FACSaria cell sorter (BD Biosciences), according to the manufacturer’s instructions.

Statistical analysis

Data are expressed as means ± SD and analyzed using the Student t test or Bonferroni multiple comparisons test. A p value < 0.05 was considered significant.
Results
Accumulation of CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells during IL-18–mediated expansion of γδ T cells

When PBMCs were incubated in the presence of ZOL/IL-2 for 14 d, the number of γδ T cells increased by >1000-fold, consistent with previous reports (1) (Fig. 1A). Moreover, the inclusion of IL-18 amplified the expansion of γδ T cells, as we recently reported (41), whereas the addition of IL-18Rα blocking mAb strongly suppressed the proliferation of γδ T cells, demonstrating the essential role of IL-18 in γδ T cell expansion (Fig. 1A). It is of note that the addition of IL-18 promoted the formation of larger cell clusters compared with those triggered by ZOL/IL-2 (Fig. 1B). In addition, IL-18 also led to augmented γδ T cell responses to 2M3BPP/IL-2 (Fig. 1A). Although there is an intrinsic dichotomy between N-BPs and pyrophosphomonoester compounds in the recognition mechanism (14, 15), these results clearly illustrate that IL-18 can promote the expansion of γδ T cells irrespective of the manner of Ag presentation.

Fig. 1C presents representative dot plot diagrams of PBMCs from a healthy volunteer before and after stimulation with ZOL/IL-2/IL-18. Whereas γδ T cells were only 2% on day 0, a significant proportion of the cells was positive for γδ TCR on day 14. It is intriguing that non-T cells occupied a significant proportion of the non-γδ T cell population. We focused on the characterization of this non-T cell population to explore it further.

Freshly isolated PBMCs from a representative donor were divided into CD11c\textsuperscript{+} and CD11c\textsuperscript{−} cells. The CD11c\textsuperscript{−} cells were further divided into two subpopulations based on the intensity of CD56; one expressing an intermediate level of CD56 (designated as CD56\textsuperscript{int}), and the other lacking the expression of CD56. Thus, freshly prepared PBMCs contained CD56\textsuperscript{int}CD11c\textsuperscript{−}, CD56\textsuperscript{int}CD11c\textsuperscript{+}, and CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells (Fig. 1D, left panel). After culture with ZOL/IL-2, the number of CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells was significantly reduced, and the number of CD11c\textsuperscript{−} cells expressing a high level of CD56 (designated as CD56\textsuperscript{bright}) increased significantly (Fig. 1D, middle panel). When PBMCs were incubated

FIGURE 1. Effect of IL-18 on the growth of γδ T cells and CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells. A, Effect of IL-18 (100 ng/ml) on expansion of γδ T cells. PBMCs derived from a representative healthy adult individual were stimulated by ZOL (1 μM)/IL-2 (10 ng/ml) or 2M3BPP (100 nM−1 μM)/IL-2− (solid bar); ZOL/IL-2/IL-18 or 2M3BPP/IL-2/IL-18 (open bar); or ZOL/IL-2/anti–IL-18Rα mAb (2 μg/ml; gray bar) or 2M3BPP/IL-2/anti–IL-18Rα mAb. The total number of γδ T cells was quantified by trypan blue dye exclusion and flow cytometry. **p < 0.001. B, Massive cell aggregation induced by ZOL/IL-2/IL-18. Cell clusters were photographed using a Nikon Digital Sight TE300-HM-2 (×10) microscope (Nikon, Tokyo, Japan) after 5 d of culture and analyzed by Luminia Vision Software (Mitani, Tokyo, Japan). C, Proportion of γδ TCR-bearing cells in culture on days 0 and 14. PBMCs were incubated in the presence of ZOL/IL-2/IL-18 for 14 d and analyzed for expression of CD3 and γδ TCR. D, Phenotypic analysis of PBMCs in culture on days 0 and 14. PBMCs incubated with ZOL/IL-2 or ZOL/IL-2/IL-18 were analyzed for expression of CD11c, CD3, and CD56 on days 0 and 14. CD56\textsuperscript{int}CD11c\textsuperscript{−}, CD56\textsuperscript{int}CD11c\textsuperscript{+}, CD56\textsuperscript{bright}CD11c\textsuperscript{−}, and CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells were gated, respectively. E, Time course of the expansion of γδ T cells and CD56\textsuperscript{bright} CD11c\textsuperscript{+} cells. PBMCs were incubated with ZOL/IL-2/IL-18, and the numbers of living γδ T cells and CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells were quantified at indicated time points, as described above.
in the presence of ZOL/IL-2/IL-18 for 14 d, the majority of non-γδ T cells exhibited CD56\textsuperscript{bright}, half of which exhibited CD11c\textsuperscript{+} (Fig. 1D, right panel). We designated this population as CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells. It was notable that the CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells increased in number in parallel with γδ T cells after stimulation of PBMCs with ZOL/IL-2 or ZOL/IL-2/IL-18 under the conditions used in this study (Fig. 1E). The addition of blocking mAb instead of IL-18 reduced the expansion of both CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells and γδ T cells (41) (data not shown).

**Effect of depletion of CD56\textsuperscript{*} or CD14\textsuperscript{*} cells on the expansion of CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells and γδ T cells**

We next examined which cell subpopulations are responsible for the proliferation of CD56\textsuperscript{bright}CD11c\textsuperscript{+} and γδ T cells. As illustrated in Fig. 2, the PBMCs used in this experiment contained ∼2.6% γδ T cells, and the non-T cells were composed of CD56\textsuperscript{int}CD11c\textsuperscript{−} (6.5%), CD56\textsuperscript{int}CD11c\textsuperscript{−} (2–4%), and CD56\textsuperscript{−}CD11c\textsuperscript{−} cells (22%) (Fig. 2A). Fifteen percent of the total cells expressed CD14 (data not shown). When PBMCs were stimulated with ZOL/IL-2 in the presence of IL-18, both CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells and γδ T cells expanded markedly compared with those stimulated with ZOL/IL-2 (Fig. 2A). However, the PBMCs depleted of CD56\textsuperscript{*} cells, which contained γδ T cells (∼3.4%) and CD56\textsuperscript{−}CD11c\textsuperscript{−} cells (∼15%), failed to elicit the marked expansion of CD56\textsuperscript{bright} CD11c\textsuperscript{+} and γδ T cells in response to the stimulation of IL-2/IL-18/ZOL (Fig. 2B). Similarly, the depletion of CD14\textsuperscript{*} cells from PBMCs abolished the expansion of both CD56\textsuperscript{bright}CD11c\textsuperscript{+} and γδ T cells (Fig. 2C). The CD14-depleted PBMCs contained γδ T cells (∼3.7%), and non-T cells were composed of CD56\textsuperscript{int} CD11c\textsuperscript{−} cells (∼4–5%), CD56\textsuperscript{−}CD11c\textsuperscript{−} cells (6.6%), and CD56\textsuperscript{−}CD11c\textsuperscript{−} cells (5.5%) (Fig. 2C). Thus, this finding suggests that the presence of either CD56\textsuperscript{int}CD11c\textsuperscript{−}, CD56\textsuperscript{−}CD11c\textsuperscript{−}, or CD56\textsuperscript{−}CD11c\textsuperscript{−} cells alone was not fully sufficient to elicit γδ T cell responses to ZOL/IL-2 or ZOL/IL-2/IL-18. It is therefore considered that CD14\textsuperscript{*} plus CD56\textsuperscript{int}CD11c\textsuperscript{−} cells or CD14\textsuperscript{*} plus CD56\textsuperscript{−}CD11c\textsuperscript{−} cells play key roles in the expansion of γδ T cells in response to ZOL/IL-2/IL-18.

**Derivation of CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells in CD3-depleted PBMCs by IL-2/IL-18**

We next analyzed the mechanism underlying the generation of the CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells that synchronously expanded with γδ T cells in response to ZOL/IL-2/IL-18. First, the effect of the depletion of T cells on the expansion of CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells was examined. When CD3\textsuperscript{*} cells were depleted from PBMCs, 2–4% exhibited the phenotype of CD56\textsuperscript{int}CD11c\textsuperscript{−} (Fig. 3A, left panel). After stimulation with ZOL/IL-2 or ZOL/IL-2/IL-18 for 8 d, CD56\textsuperscript{bright}CD11c\textsuperscript{−} cells occupied ∼60% of the total cells (Fig. 3A, middle and right panels). In addition, CD56\textsuperscript{−}CD11c\textsuperscript{−} cells were almost negligible in the expanded cells, whereas CD56\textsuperscript{bright} CD11c\textsuperscript{−} cells comprised 20–26% of the total. This clearly demonstrates that the expansion of CD56\textsuperscript{bright}CD11c\textsuperscript{+} cells in response to ZOL/IL-2 or ZOL/IL-2/IL-18 does not require the existence of γδ T cells. Because ZOL is known to be a synthetic stimulant of γδ T cells, CD3-depleted PBMCs were treated with various non-ZOL stimulants, including IL-2, IL-2/IL-18, IL-18, and GM-CSF/IL-18.
IL-4. As expected, CD3-depleted PBMCs proliferated strongly in response to IL-2 and IL-2/IL-18 (Fig. 3). In particular, IL-2/IL-18 elicited the vigorous proliferation of CD3-depleted PBMCs, in which the total cell number increased by ∼5-fold (Fig. 3B), and the number of CD56brightCD11c+ cells increased by 150-fold in 8 d (Fig. 3C).

As indicated in Fig. 2, CD14+ plus CD56intCD11c+ and CD14+ plus CD56highCD11c+ cell combinations are likely to play critical roles in the expansion of γδ T cells in response to ZOL/IL-2/IL-18. We thus examined whether CD56brightCD11c+ cells can be derived from these cell combinations. CD14+, CD56int CD11c+, and CD56highCD11c+ cells were purified from PBMCs, and the mixed cell populations comprising CD14+ plus CD56int CD11c+ cells or CD14+ plus CD56highCD11c+ cells were incubated with IL-2/IL-18. As illustrated in Fig. 3D, CD56brightCD11c+ cells were effectively generated in the culture of CD14+ plus CD56int CD11c+ cells, but not in the combination of CD14+ plus CD56high CD11c+ cells. It may be of note that intensity of CD11c was decreased, whereas that of CD56 became brighter during culture (Fig. 3D, middle panel).

We then analyzed CD14-bearing cells during the culture of PBMCs. In the freshly prepared CD3-depleted PBMCs, cells expressing a high level of CD11c also expressed a high level of CD14 (Fig. 3E, left panel). These CD11chighCD14bright cells occupied ∼30% of the initial culture of
CD3-depleted PBMCs. After culture for 8 d in the presence of IL-2 or IL-2/IL-18, however, cells expressing a high level of CD14 disappeared (49) (Fig. 3E, right panels). Thus, in the present experimental system, we failed to detect CD56−CD14+ cells, which were recently reported to play a role in the expansion of γδ T cells (34). Because it has been well established that conventional DCs can be induced by GM-CSF/IL-4, we analyzed the phenotype of conventional DCs as reference cells. When CD3-depleted PBMCs were treated with GM-CSF/IL-4, CD56−CD11c+CD14+ and CD56+CD11c+CD14+ cells, but not CD56+CD11c+ cells, were observed (Fig. 3F). Both CD56+CD11c+CD14+ and CD56+CD11c+CD14+ cells expressed molecules such as CD80, CD83, and CD86, which were commonly observed in conventional DCs (Fig. 3F).

Characterization of CD56brightCD11c+ cells

Freshly isolated peripheral blood CD56intCD11c+ cells were positive for IL-18R α- and β-chains (Fig. 4A). They also expressed NK cell–related molecules such as CD122/IL-2Rβ, but not CD25/IL-2Rα and NKG2D, and DC-related Ags such as HLA-DR, CD11a/CD18 (LFA-1). In addition, they expressed a low level of CD80 and CD83; CD86 expression was negligible (Fig. 4A). CD56brightCD11c+ cells that had been induced by IL-2 with or without IL-18 from CD3-depleted PBMCs expressed IL-18R α- and β-chains (Fig. 4B, 4C). IL-2/IL-18 induced CD25 expression, but CD122 was significantly downregulated compared with freshly isolated CD56intCD11c+ cells. They also expressed molecules commonly displayed in mature DCs, such as CD83, CD80, and CD86, as well as HLA-DR and CD11a/CD18 (Fig. 4B). It is noteworthy that a high level of CD86 was detected in CD56brightCD11c+ cells stimulated with IL-2/IL-18, whereas only a marginal level of CD86 was detected in those incubated in the presence of IL-2 without exogenous IL-18 (Fig. 4B, 4C). As described above, the phenotypes of CD56brightCD11c+ cells were noticeably different from those of DCs induced by GM-CSF/IL-4 (Fig. 3E), which exhibited CD11c+CD14+. It is of note that the CD56brightCD11c+ cells were essentially negative for CD14.
Although IL-2 or IL-2/IL-18 stimulation without ZOL was sufficient for the induction of CD56<sup>bright</sup>CD11c<sup>+</sup> cells, the effect of ZOL on the expression of costimulatory molecules was also examined. As illustrated in Fig. 4D, ZOL enhanced the expression of DC-related molecules, including CD40, CD80, CD83, and CD86 in the cells stimulated by IL-2 alone or IL-2/IL-18, suggesting that it can act directly on CD56<sup>bright</sup>CD11c<sup>+</sup> cells to induce cosignaling molecule expression.

Profiles of cytokine production and cytotoxic activity in CD56<sup>bright</sup>CD11c<sup>+</sup> cells

To further characterize CD56<sup>bright</sup>CD11c<sup>+</sup> cells, the profiles of cytokine secretion were determined. Purified CD56<sup>bright</sup>CD11c<sup>+</sup> and CD56<sup>bright</sup>CD11c<sup>-</sup> cells were restimulated by IL-2 alone or IL-2/IL-18 for 48 h, and the cytokines in the culture supernatants were measured using ELISA. As illustrated in Fig. 5A, IL-2/IL-18 elicited a high level of IFN-γ and TNF-α production compared with other stimulants, although none of the stimulants induced IL-12 production. These results indicate that CD56<sup>bright</sup>CD11c<sup>+</sup> cells produce cytokines that are characteristic of NK cells.

Because the CD56<sup>bright</sup>CD11c<sup>+</sup> cells exhibited NK-like phenotypes, including NKG2D expression (Fig. 4B, 4C) and perforin accumulation (data not shown), we next examined the cells for NK activity. After the incubation of CD3-depleted PBMCs in the presence of IL-2 and IL-18 for 6 d, CD56<sup>bright</sup>CD11c<sup>+</sup> cells were purified and tested for cytotoxicity against K562. As illustrated in Fig. 5B, the cultured cells exerted a high level of cytotoxicity in an E:T ratio-dependent manner, demonstrating that CD56<sup>bright</sup>CD11c<sup>+</sup> cells possess NK cell-like characteristics phenotypically and functionally.

Effect of CD56<sup>bright</sup>CD11c<sup>+</sup> cells on γδ T cell migration and expansion

It is thus clear that IL-2/IL-18 facilitates the expansion of CD56<sup>bright</sup>CD11c<sup>+</sup> cells and that ZOL/IL-2/IL-18 elicits the vigorous expansion of γδ T cells. In this context, we examined the possible role of CD56<sup>bright</sup>CD11c<sup>+</sup> cells in the augmented proliferation of γδ T cells in the presence of IL-18. After treatment with ZOL/IL-2 or ZOL/IL-2/IL-18, CCR5 and CCR7 (the receptors for CCL21) were induced in γδ T cells (Fig. 6A). We then determined whether the chemokine/chemokine receptor system is operative between CD56<sup>bright</sup>CD11c<sup>+</sup> cells and γδ T cells. CD56<sup>bright</sup>CD11c<sup>+</sup> and CD56<sup>int</sup>CD11<sup>int</sup>CD14<sup>+</sup> cells were prepared from CD3-depleted PBMCs and placed in the lower wells, and then γδ T cells were placed in the upper wells together with ZOL/IL-2/IL-18. As depicted in Fig. 6B, γδ T cells migrated toward CD56<sup>bright</sup>CD11c<sup>+</sup> cells generated in the culture with IL-2/IL-18 (Fig. 6B), but not toward CD56<sup>int</sup>CD11<sup>int</sup>CD14<sup>+</sup> cells, which were induced by GM-CSF and IL-4 (Fig. 3F). The migration rate was comparable to a positive control Transwell containing CCL21 instead of CD56<sup>bright</sup>CD11c<sup>+</sup> cells. This finding indicates that it is likely that a chemokine/chemokine receptor system greatly promotes the direct interaction between CD56<sup>bright</sup>CD11c<sup>+</sup> cells and γδ T cells. Consistent with this finding, microscopic analysis revealed that coculture of CD56<sup>bright</sup>CD11c<sup>+</sup> cells with γδ T cells led to the formation of large cell clusters in the presence of ZOL/IL-2/IL-18 (Fig. 6C), both on the size and number of the cell clusters. In contrast, ZOL/IL-2/IL-18 failed to induce the cell aggregation in the culture of γδ T cells plus conventional DCs exhibiting CD56<sup>int</sup>CD11<sup>int</sup>CD14<sup>+</sup> that had been induced by GM-CSF/IL-4. γδ T cells alone also failed to form clusters in the milieu of ZOL/IL-2/IL-18.

We next investigated whether CD56<sup>bright</sup>CD11c<sup>+</sup> cells can support the expansion of γδ T cells through direct interaction.
CD56^{bright}CD11c^{+} cells plus γδ T cells in the absence of ZOL or 2M3BPP. Taken together, these results indicate that CD56^{bright}CD11c^{+} cells strongly facilitate the IL-18–mediated expansion of γδ T cells stimulated by ZOL or 2M3BPP.

Discussion

IL-18 was originally discovered as a factor that induces IFN-γ in immune cells (43). Recent studies have demonstrated that IL-18, like other members of IL-1 family cytokines, is converted to an active form in a stress-induced manner (49), and plays a critical role in the differentiation, proliferation, and survival of various cells (45–48). The molecular mechanisms underlying the IL-18–associated modulation of target immune cells have not yet been fully clarified, however. We previously reported that IL-18 directly activated various antiapoptotic signals in γδ T cells and led to the expansion of γδ T cell population (41). IL-18 is thus likely to be a cellular protective factor in γδ T cells. Because evidence has been accumulating that γδ T cells play a key role in the surveillance and first line of defense against infections and malignancies, IL-18 seems to play an important role in the modulation of stress-surveillance responses against infections and malignancies. Little is known, however, about the cellular and molecular mechanisms by which IL-18 indirectly, rather than directly, amplifies the expansion of γδ T cells in response to phosphoantigens. In the current study, we demonstrated that CD56^{bright}CD11c^{+} cells are closely linked with the IL-18–mediated expansion of γδ T cells.

As reported previously, IL-18 greatly enhanced γδ T cell responses to ZOL/IL-2 (41). It is of note that the addition of anti–IL-18Rα mAb to the culture of PBMCs with ZOL/IL-2 or ZOL/IL-2/IL-18 strongly inhibits the proliferation of γδ T cells. This
clearly shows that IL-18 signaling is essential in the expansion of peripheral blood γδ T cells in response to ZOL even in the absence of exogenous IL-18. Interestingly, the expansion of γδ T cells triggered by a synthetic pyrophosphomonoester Ag, 2M3BP, was also notably enhanced by IL-18, and blockade of the cytokine signaling abrogated the responses. It has been well established that ZOL permeates cell membranes, inhibits farnesyl pyrophosphate synthase, and consequently upregulates intracellular IPP in accessory cells (13). Such accessory cells may serve as APCs for γδ T cells in this culture system. In contrast, pyrophosphomonoester Ags are presented to γδ T cells through an as yet unidentified extracellular pathway even in the absence of third-party cells like monocytes and DCs, but adherent cells might be essential for the sustained expansion of γδ T cells. These findings raise the possibility that IL-18 generates or stimulates CD56<sup>bright</sup>CD11c<sup>+</sup> cells that can efficiently support the expansion of γδ T cells. In fact, the number of CD56<sup>bright</sup>CD11c<sup>+</sup> cells increased between 500- and 1000-fold in 10 d to 2 wk; this was paralleled by the expansion of γδ T cells. In addition, most of the non-γδ T cell populations were composed of CD56<sup>bright</sup>CD11c<sup>+</sup> cells on day 14 in this culture system. It remains unclear whether ZOL-sensitized CD56<sup>bright</sup>CD11c<sup>+</sup> cells can initiate γδ T cell responses.

When CD14-depleted PBMCs were stimulated with ZOL/IL-2/IL-18, a proliferative response was observed in neither CD56<sup>bright</sup>CD11c<sup>+</sup> nor γδ T cells (Fig. 2). This indicates that a relatively small number of naturally occurring or naive CD56<sup>bright</sup>CD11c<sup>+</sup> cells in freshly isolated PBMCs is insufficient for triggering γδ T cell responses. In the current study, CD14<sup>+</sup> cells were demonstrated to play an essential role in the derivation of CD56<sup>bright</sup>CD11c<sup>+</sup> cells (Fig. 3C). Thus, it remains to be determined whether CD14<sup>+</sup> cells indirectly support the proliferation of γδ T cells by generating a more efficient initiator for γδ T cell activation or whether they directly initiate the activation of γδ T cells.

As depicted in Fig. 3E, CD14 is strongly expressed on CD56<sup>+</sup>CD11c<sup>+</sup> cells, whereas freshly prepared CD56<sup>+</sup>CD11c<sup>+</sup> cells and expanded CD56<sup>+</sup>CD11c<sup>+</sup> cells exhibit CD14<sup>+</sup>. Thus, we could not detect a recently reported population of CD56<sup>+</sup>CD11c<sup>+</sup> cells (34) in the present experimental system. Although the exact origin of the mature CD56<sup>+</sup>CD11c<sup>+</sup> cells remains speculative, the present results suggest that CD56<sup>+</sup>CD11c<sup>+</sup> cells develop into CD56<sup>+</sup>CD11c<sup>+</sup> cells. If this is the case, CD14<sup>+</sup> cells may render CD56<sup>+</sup>CD11c<sup>+</sup> cells immunologically active and mature through IL-18 signaling and other as yet unidentified signaling, allowing γδ T cells to proliferate vigorously. As supportive data, IL-18 promoted the expression of costimulatory and adhesion molecules such as CD56 and CD86 in CD56<sup>+</sup>CD11c<sup>+</sup> cells. In addition, CD56<sup>+</sup>CD11c<sup>+</sup> cells produced CCL21 in the presence of IL-18 (data not shown), and γδ T cells expressed its receptors, CCR5 and CCR7. Thus, IL-18 appears to promote γδ T cell proliferation in multiple ways, as follows: forming growth centers, rendering signals through costimulatory molecules, and directly activating survival signals. It is of note that IL-18 can promote the expansion of CD56<sup>+</sup>CD11c<sup>+</sup> cells even in the culture of CD3-depleted PBMCs, suggesting that the proliferation of CD56<sup>+</sup>CD11c<sup>+</sup> cells is apparently independent of γδ T cells. In addition, both CD56<sup>+</sup>CD11c<sup>+</sup> and CD56<sup>+</sup>CD11c<sup>+</sup> cells express IL-18Rs (Fig. 4). It is therefore most likely that IL-18 acted directly on these cells. This finding, however, does not preclude the possible involvement of γδ T cells in the maturation of CD56<sup>+</sup>CD11c<sup>+</sup> cells under other circumstances.

The present finding of the generation of CD56<sup>+</sup>CD11c<sup>+</sup> cells with immunological properties of both NK cells and DCs in humans was unprecedented. There have been several reports of NKDCs in mice that exhibit both NK-like and DC-like characteristics. The murine NKDCs seem to be heterogeneous, and some subsets were shown to be generated in the presence of IL-18 and CpG (25–27). In humans, however, the concept of NKDCs has not been generally accepted because researchers have failed to induce and find human counterparts of murine NKDCs (29–31). The present results unambiguously demonstrated the existence of the cells that phenotypically and functionally overlap with NK cells and DCs in humans and the immunological roles of this subset of cells in the efficient expansion of γδ T cells. Whereas CD56<sup>+</sup>CD11c<sup>+</sup> cells express perforin and Fas (data not shown) and exhibit potent NK-like activity (Fig. 5), the physiological roles of the death molecule and the death receptor remain to be determined.

The mechanism by which accessory cells activate γδ T cells has not yet been clarified (1). Generally, the activation of γδ T cells is independent of conventional MHC class I, MHC class II, CD1a, CD1b, and CD1c molecules, which allow γδ T cells to respond rapidly to infections and malignancies. Recently, CD14<sup>+</sup> monocytes were demonstrated to play crucial roles in the activation of γδ T cells. ZOL specifically inhibited the isoprenoid synthetic pathway and increased the intracellular level of IPP, as mentioned above (13). It was also reported that CD56<sup>+</sup>CD14<sup>+</sup> DCs were essential for the proliferation of Th1-type γδ T cells (34). The CD56<sup>+</sup>CD14<sup>+</sup> cells expressed CD80, CD83, and CD86, and swiftly increased in size. In the current study, however, we failed to identify CD56<sup>+</sup>CD14<sup>+</sup> cells in our culture system. These findings raise the interesting possibility that several different accessory cells may be responsible for the expansion of γδ T cells under different culture or physiological conditions.

Recently, considerable attention has been given to the potent tumoricidal activity of human Vγ9Vδ2-bearing γδ T cells. Several attempts have been made to develop novel cancer immunotherapy using γδ T cells. It has been reported that the adoptive transfer of γδ T cells together with IL-2 and/or ZOL can have beneficial effects in patients with cancer (12). Although the precise mechanism has not been fully clarified, the addition of ZOL to endocrine therapy in the treatment of premenopausal patients with breast cancer significantly improved the disease-free survival rate (50). Because the number of effector cells is critical in cancer immunotherapy, it is imperative that a practical strategy to induce a large number of γδ T cells in vitro as well as in vivo be developed. The present study demonstrates that the use of N-BPs/IL-2/IL-18 greatly facilitates the expansion of γδ T cells. As such, future examination of the precise molecular and cellular mechanisms underlying the IL-18-mediated expansion of γδ T cells is essential in the development of effective cancer treatment regimens.

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Disclosures

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