Enhanced ability of dendritic cells to stimulate innate and adaptive immunity on short-term incubation with zoledronic acid

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Vγ9Vδ2 (γδ) T cells play a major role in innate immunity against microbes, stressed, and tumor cells. They represent less than 5% of peripheral blood lymphocytes but can be activated and expanded in vitro by aminobisphosphonates (ABP)-treated monocytes. The aim of this work was to determine whether ABP-treated dendritic cells (DCs) can also activate γδ T cells and regulate immune responses mediated by conventional αβ T cells. Highly purified immature (iDC) and mature DC (mDC) were generated from peripheral blood monocytes of healthy donors and incubated with zoledronic acid (Zol) for 24 hours. Zol-treated iDC and mDC retained their immunostimulatory properties and induced the vigorous expansion of central memory and effector memory γδ T cells. γδ T cells displayed antitumor activity and appropriate cell surface antigens to target secondary lymphoid organs and exert costimulatory activity. Antigen-specific MHC-restricted immune responses, mediated by conventional αβ T cells, were improved by the concurrent γδ T-cell activation. In conclusion, large numbers of γδ T cells with effector and costimulatory activities are rapidly generated by Zol-treated iDC/mDC. This strategy is worthy of further investigation to improve adoptive cell therapy and vaccine interventions against tumors and infections. (Blood. 2007;110:921-927)

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Introduction

γδ T cells are unconventional T cells playing a major role in innate immune responses against microbes, stressed cells, and tumor cells. Most of circulating γδ T cells use the same T-cell receptor (TCR) V region pair Vγ9-Vδ2, which enables them to recognize unprocessed nonpeptide compounds, which are referred to as phosophoantigens and produced via the mevalonate or the 1-deoxy-D-xylulose-5-phosphate pathway in mammalian and nonmammalian cells. γδ T cells also recognize induced self ligands like stress-inducible MHC class I-related MICA/MICB molecules or complexes comprising ATP-synthese subunits, which are induced or upregulated on the surface of stressed cells or some tumor cells. This peculiar antigen recognition ability is the basis of the putative role of γδ T cells in tumor immunosurveillance.

Aminobisphosphonates (ABP) are synthetic compounds commonly used to treat bone disease and hypercalcemia in patients with multiple myeloma and breast and prostate cancer. Interestingly, ABP have also been shown to activate γδ T cells in vitro and in vivo. We and others have demonstrated that ABP increase the activation of γδ T cells via monocytes (Mo). One interpretation is that ABP share the chemical features of γδ T-cell ligands, but the prevailing view is that they specifically target the mevalonate pathway of Mo and induce the accumulation of phosphorylated metabolites naturally recognized by γδ T cells. Dendritic cells (DCs) are much more efficient than Mo as professional antigen-presenting cells to activate conventional αβ T cells and act as cellular bridges between innate and adaptive immunity. A reciprocal and positive influence has recently been described between DC and innate effector cells, including γδ T cells. However, whereas a few articles have investigated the effects of activated γδ T cells on DCs, very limited data are available on the opposite interaction with special regard to the ability of DCs to modulate immune responses mediated by conventional αβ T cells. To this end, we have generated highly purified immature DCs (iDCs) and mature DCs (mDCs) from peripheral blood Mo of healthy donors, and evaluated the effect of short-term incubation with zoledronic acid (Zol) on their phenotypic and functional properties. Our results demonstrate that Zol improves the immunostimulatory ability of iDC and mDC toward unconventional γδ and conventional αβ T cells.

Materials and methods

Blood samples were drawn from healthy donors with informed consent in accordance with the Declaration of Helsinki. The study and the consent form were approved by the Comitato Etico Regionale (Piedmont Ethical Committee).

iDC and mDC generation

iDCs were generated from positively isolated CD14+ cells in 18 healthy donors, as previously reported. Briefly, CD14+ cells were purified using CD14 MicroBeads and LS columns, according to the manufacturer’s instructions (Miltenyi Biotec, Bologna, Italy; purity > 90%). The standard culture medium was RPMI 1640 (Euroclone, Milano, Italy), containing 10% fetal calf serum (Euroclone), 2 mM l-glutamine, 100 U/mL penicillin,


The online version of the article contains a data supplement.
eral blood lymphocytes (allo-PBL) at a ratio of 1:5. On day 5, proliferation of mDCZol/H9251 (100 ng/mL; Alexis Biochemicals, Vinci, Italy) and TNF-α (25 ng/mL) were added to the culture. On day 6, the resulting cells were harvested and confirmed as iDC by flow cytometry. Fresh cytokines were added on day 3.

24 hours in the presence (mDCZol/H9251) or absence of Zol (iDCZol/H9251), kindly provided by Novartis Pharma, Origgio, Italy.

mDCs were generated by 24-hour stimulation of iDCZol/H9262 with IL-1β (100 ng/mL; Alexis Biochemicals, Vinci, Italy) and TNF-α (50 ng/mL; Peprotech, Rocky Hill, United Kingdom) in the presence (mDCZol/H9262) or absence of Zol (mDCZol/H9251). In some experiments, iDCs or mDCs were incubated for 24 hours in the presence of Zol and 25 μM mevastatin (Mev) (Sigma-Aldrich, Milan, Italy).

iDCZol/H9262, iDCZol/H9251, mDCZol/H9251, and mDCZol/H9262 were scored for cell number and viability by trypan blue staining. Supernatants were collected and stored at −80°C until cytokine contents were assessed.

Cytofluorimetric analyses

Immunophenotyping of DCs and cell subsets was performed with the following monoclonal antibodies: anti-CD3, anti-CD8, anti-CD54, anti-CD62L, anti-CXCR4 (Caltag Laboratories, Burlingame, CA), anti-pan-TCR-γδ (Endogen, Woburn, MA); anti-TCR-αβ, anti-CD56, anti-CD80, anti-HLA-DR (Becton Dickinson, San Jose, CA); anti-CD3, anti-CD14 (Dako SpA, Milano, Italy); anti-Vy9TCR, anti-CD83 (BD Pharmingen International, San Diego, CA), anti-CD86 (Chemicon, Chinders Ford, Hampshire, United Kingdom), anti-HLA-A2 (Proimmune, Oxford, United Kingdom), anti-CD1a (Valter Occhiena, Torino, Italy), anti-CD11a (Diaclone, Besançon, France), anti-CD27 (AnCell, Byport, MN), anti-CCR7 (R&D Systems, Minneapolis, MN), and anti-DC-LAMP (Immunictech, Marseille, France). Appropriate combinations of fluorescein isothiocyanate (FITC), r-phycocerythrin–conjugated, Tri color-conjugated, or allophycocyanin-conjugated antibodies were used.

Quantification of apoptotic and necrotic cells was performed by annexin V and propidium iodide staining with the MEBCYTO-Apoptosis Kit (MBL Medical and Biological Laboratories, Naka-ku Nagoya, Japan). The internalization capability of iDCZol/H9262 and iDCZol/H9251 was tested using FITC-conjugated dextran (FITC-dextran; 10 Kda; Sigma-Aldrich), as reported. On day 7, counts of specific cell subsets per well were determined by multiplying total counts of viable cells per well by the percentage of cells of interest, the latter being identified by 2- or 3-color cytofluorimetric analysis and appropriate gating.

Quantification of cytokine production

Quantification IL-4, IL-6, IL-10, and IL-12p70 in the supernatants of iDCZol/H9262, iDCZol/H9251, mDCZol/H9251, and mDCZol/H9262 was performed with a multiparametric cytokine bead immunoassay using the Basic FlowCytomix and the Simplex kits (Bender MedSystems GmbH, Vienna, Austria).

Allostimulatory capacity of iDCZol/H9262 and mDCZol/H9251

iDCZol/H9262 and mDCZol/H9251 were incubated in triplicate with allogeneic peripheral blood lymphocytes (allo-PBL) at a ratio of 1:5. On day 5, proliferation was evaluated by pulsing cells with 1 μCi of methyl-3H]thymidine ([3H]Tdr) (47 Ci/mmol specific activity; Amersham, Milano, Italy) per well and harvesting 4 hours later with an automated sample harvester (Packard Instrument) using UNIFilter plates (Perkin Elmer, Wellesley, MA).

[3H]Tdr uptake was measured with a TopCount microplates scintillation counter (Perkin Elmer).

γδ T-cell stimulatory activity of iDCZol/H9262, mDCZol/H9251, and MoZol/H11001

Autologous PBL were added to iDC/mDC subsets at the DC:PBL ratio of 1:5, and cultured at 1 × 10^5/mL, in standard medium supplemented with IL-2 (10 U/mL; Eurocetus, Milan, Italy). After 7 days, percentages and total counts of γδ and γδ T cells were determined by flow cytometry. Side-by-side experiments were also performed to compare Zol-treated Mo (MoZol/H11001) with iDCZol/H9262 and mDCZol/H9251. To this end, CD14+ T cells were magnetically purified from cryopreserved peripheral blood mononuclear cells (PBMCs) of the individuals from whom iDC or mDC were generated, and incubated with autologous PBL as described.

In 3 experiments, PBL were labeled with 5 μM carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), according to the manufacturer’s instructions. CFSE-labeled PBL were washed once and incubated with iDCZol/H9262 or mDCZol/H9251 as reported. On day 7, cells were surface-stained with anti-TCRγδ mAb and proliferation was determined by evaluating the logarithmic decrease of CFSE fluorescence intensity after gating on γδ T cells.

Modulation of adaptive immune responses

iDCs were generated from CD14+ cells of HLA-A*0201+ healthy donors and pulsed with the HLA-A*0201+ restricted influenza matrix protein-derived peptide GILGFVFTL (MP; Proimmune, Oxford, United Kingdom) at 10 μM final concentration for 2 hours at room temperature in serum-free medium (indicated). After washing, aliquots of iDCMP/H9251 or unpulsed iDC (iDCMP/H9250) were induced to fully mature by incubation for 18 hours with TNFα + IL-1β in the presence or absence of 5 μM Zol. Thus, 4 subsets of mDC were available on day 7: mDCMP/Zol−, mDCMP/Zol+, mDCMP/Zol−, and mDCMP/Zol+. On the same day, CD3+ cells were isolated from autologous cryopreserved PBMCs (Miltenyi Pan T Cell Isolation Kit), according to the manufacturer’s instructions. Purity of CD3+ cells (always >90%) and percentages of γδ T cells were determined by flow cytometry, mDC subsets and autologous T cells were incubated for 10 days at the DC:T ratio of 1:10 in 96-well round-bottom plates, in the presence of 10 IU/mL IL-2, which was replenished every 3 days. On day 10, T cells were restimulated for additional 10 days with a second batch of freshly generated appropriate mDC subset.

On days 10 and 20, the frequency of MP-specific CD8+ T cells was determined by flow cytometry with the r-phycocerythrin–labeled A*0201/GILGFVFTL (MP) PentaMere (Proimmune, Oxford, United Kingdom), according to manufacturers’ instructions.

On day 20, the cytotoxic activity of T cells generated by 2 rounds of stimulation with mDCMP/Zol−, mDCMP/Zol+, mDCMP/Zol−, or mDCMP/Zol+ was tested against the TAP-deficient HLA-A2+ T2 cell line. This cell line is susceptible to the cytotoxic activity mediated by γδ T cells. To this end, by preliminary experiments using PBMCs stimulated for 7 days with Zol plus IL-2.6 Both MP-loaded (T2MP/H9251/H9252) and unloaded (T2MP/H9253/H9254) T2 cells were used as target cells. The former served as target cells for MP-specific, MHC-restricted, conventional cytotoxic γδ T cells, whereas both T2MP+ and T2MP− cells served as target cells for cytotoxic γδ T cells, which are neither MP-specific nor MHC-restricted. The cytotoxic activity of γδ and γδ T cells was evaluated by flow cytometry based on CFSE staining of T2MP− and T2MP+ target cells and identification of dead cells by propidium iodide staining of CFSE-labeled target cells as previously reported.6.16 This method works as a single-platform assay and does not require cell counting to determine the percentage of killed cells in each well.6

In 2 experiments, T2MP− cells were incubated with anti-MHC class I A*0201 antibody for 30 minutes at 4°C before washing and mixing with effector cells as reported above.

Statistical analysis

Results are expressed as mean ± SEM. Differences between sample groups were evaluated with the 2-tailed nonparametric Mann-Whitney U test for paired samples or with Fisher exact test for unpaired data. P < .05 was the significance cut-off.
Results

Viability, phenotype, and function of iDCZol+ and mDCZol+
are preserved

The first series of experiments were aimed at determining any toxic effect of short-term incubation with Zol on viability, phenotype, and basic immunostimulatory functions of iDC and mDC. Exposure to Zol up to 10 μM for 72 hours did not affect the viability and total cell counts of iDC (Figure 1A). Based on these and previous results, 6 5 μM Zol was selected as the standard treatment concentration. Zol alone did not induce any significant change in the immunophenotype of iDC (Table 1). Rather, a significant increase in the expression of HLA-DR and CD80 was observed in mDC matured with TNF-α plus IL-1β and Zol (mDCZol+) versus TNF-α plus IL-1β alone (mDCZol−) (Table 1).

The ability of iDC to internalize FITC-dextran was not affected, confirming that Zol alone is unable to induce the maturation of iDC (Figure 1B). As expected, mDC downregulated their ability to internalize FITC-dextran, and this downregulation was unaffected by Zol (Figure 1B), indicating that mDC can fully mature and maintain this status in the presence of Zol. The allostimulatory capacity of iDCZol+ and mDCZol+ was slightly increased compared with iDCZol− and mDCZol−, even if the differences were not statistically different (Figure 1C).

The release of IL-6, IL-10, IL-4, and IL-12 in the supernatants by iDCZol+ and mDCZol+ is shown in Figure 1D. IL-10 production was decreased in iDCZol+, whereas IL-12 was increased, and IL-6 decreased, in mDCZol+. None of these differences was statistically significant. More importantly, these changes did not invalidate the intrinsic ability of iDCs and mDCs to drive Th1 immune responses, as shown by the results of pentamer and cytokotoxic experiments reported (Figure 5A-C).

Altogether, these data indicate that Zol has no toxic effect and does not hamper the basic phenotype and immunostimulatory functions of iDC and mDC.

iDCZol+ and mDCZol+ are strong inducers of autologous γδ
T-cell proliferation

Next, we evaluated the ability of iDCZol+ and mDCZol+ to activate autologous γδ T cells. Neither iDCZol− nor mDCZol− induced the expansion of autologous γδ T cells, whereas both iDCZol+ and mDCZol+ were strong inducers (Figure 2A). MoZol+ also induced the proliferation of autologous γδ T cells as previously reported 6 (Figure 2A). Total counts of viable γδ T cells per well were significantly increased in iDCZol+ versus iDCZol−, mDCZol+ versus mDCZol− and MoZol+ versus MoZol− (Figure 2B). When the comparison was restricted to side-by-side experiments (n = 4), iDCZol+ and mDCZol+ were confirmed to act as better inducers than MoZol+, although the differences were not statistically significant.

Total counts of viable αβ T cells were unaffected by the proliferative expansion of γδ T cells (Figure 2B), indicating that neither a shortage of nutrients nor the production of cytokines, such as IFN-γ, or the expansion of γδ T cells with effector activity had a detrimental effect on conventional αβ T cells. Total γδ and αβ T-cell counts also indicate that the former only accounted for the increased overall cellularity observed after stimulation with iDCZol+, mDCZol+, and MoZol+ (Figure 2B), further confirming that active proliferation, and not a relative enrichment, was the main cause of the selective γδ T-cell expansion. Experiments with CFSE-labeled T cells confirmed the selective proliferation of γδ T cells induced by iDCZol+ and mDCZol+ (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article).

To further demonstrate the ability of Zol to specifically recruit γδ T cells, iDC were incubated with 25 μM Mev immediately before treatment with Zol. iDCZol+Mev− were then incubated with autologous T cells and total counts of γδ and αβ T cells determined on day 7. The increase of γδ T cells induced by iDCZol+ was blunted by Mev treatment (Figure 3). Mev treatment did not affect αβ T-cell counts. These data further confirm that the immunostimulatory ability of iDCZol+ and mDCZol+ is specifically directed toward γδ T cells and highly dependent on the mevalonate pathway.

Zol-treated iDCs and mDCs preferentially induce the expansion of central memory and effector memory γδ T cells with enhanced expression of specific homing and costimulatory receptors

These experiments were performed to determine γδ T-cell subset distribution after stimulation with iDCZol+ and mDCZol+ γδ T cells
whereas effector memory (EM: CD45RA
+ display high proliferative capacity, but low effector functions, well; mDC
+T cells/well; Mo
+T cells/well; Mo
- with iDC
+H9253/H9254
(A) Flow cytometry of representative /H11001
and /H11006
determined after 48 hours of incubation.

Table 1. Immunophenotype of iDC
+ and mDC
+

|            | iDC
+ | iDC
+ | mDC
+† | mDC
+‡ |
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<tr>
<td>HLA-DR</td>
<td>680 ± 283</td>
<td>622 ± 249</td>
<td>1.287 ± 586</td>
<td>1.698 ± 910‡</td>
</tr>
<tr>
<td>CD86</td>
<td>78 ± 25</td>
<td>142 ± 55</td>
<td>113 ± 30</td>
<td>121 ± 30</td>
</tr>
<tr>
<td>CD80</td>
<td>235 ± 57</td>
<td>246 ± 55</td>
<td>263 ± 57</td>
<td>349 ± 85‡</td>
</tr>
<tr>
<td>CD54</td>
<td>1.617 ± 341</td>
<td>1.685 ± 487</td>
<td>2.219 ± 780</td>
<td>2.168 ± 520</td>
</tr>
<tr>
<td>CD83</td>
<td>82 ± 23</td>
<td>59 ± 15</td>
<td>139 ± 69</td>
<td>121 ± 46</td>
</tr>
<tr>
<td>CD11a</td>
<td>116 ± 58</td>
<td>113 ± 55</td>
<td>121 ± 48</td>
<td>619 ± 517</td>
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<tr>
<td>CCR7</td>
<td>367 ± 35</td>
<td>368 ± 13</td>
<td>356 ± 36</td>
<td>317 ± 25</td>
</tr>
<tr>
<td>CXCR4</td>
<td>132 ± 58</td>
<td>128 ± 57</td>
<td>136 ± 66</td>
<td>137 ± 72</td>
</tr>
<tr>
<td>DC-LAMP</td>
<td>58 ± 2</td>
<td>54 ± 5</td>
<td>78 ± 10</td>
<td>88 ± 2</td>
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Results are mean fluorescence intensity (MFI) plus or minus the SEM from 10 (HLA-DR) to 3 (DC-LAMP) experiments.

‡ Difference statistically significant between mDC
+ and mDC
- (both P < .05).

Table 1. Immunophenotype of iDC
+ and mDC
+

HPLA-DR CD86 CD80 CD54 CD83 CD11a CCR7 CXCR4 DC-LAMP
iDC
+ 680 ± 283 78 ± 25 235 ± 57 1.617 ± 341 82 ± 23 116 ± 58 367 ± 35 132 ± 58 58 ± 2
iDC
+ 622 ± 249 142 ± 55 246 ± 55 1.685 ± 487 59 ± 15 113 ± 55 368 ± 13 128 ± 57 54 ± 5
mDC
+† 1.287 ± 586 113 ± 30 263 ± 57 2.219 ± 780 139 ± 69 121 ± 48 356 ± 36 136 ± 66 78 ± 10
mDC
+‡ 1.698 ± 910‡ 121 ± 30 349 ± 85‡ 2.168 ± 520 121 ± 46 619 ± 517 317 ± 25 137 ± 72 88 ± 2

can be divided into 4 subsets according to their phenotype, proliferative capacity and effector functions. Naive (N; CD45RA
+, CD27
+) and central memory (CM; CD45RA
-, CD27
+) γδ T cells display high proliferative capacity, but low effector functions, whereas effector memory (EM; CD45RA
-, CD27
-) and terminally differentiated late effector (TEMRA; CD45RA
-, CD27
+) γδ T cells display the opposite pattern.17 Immunophenotyping on day 7 showed that total counts of viable CM and EM γδ T cells per well were significantly increased after stimulation with iDC
+ compared with iDC
- (Figure 4A,B).

iDC
+ and mDC
+ stimulation also increased the number of γδ T cells with appropriate cell surface receptors to target secondary lymphoid organs (CD62L)17 and exert costimulatory activity (HLA-DR, CD80)18 (Figure 4C,D).

![Image](image-url)

Figure 2. Proliferative expansion of γδ T cells by iDC
+ , mDC
+, and Mo
+. (A) Flow cytometry of representative γδ T-cell expansions after 7 days stimulation with iDC
+ (upper quadrants), mDC
+ (middle quadrants), and Mo
+ (lower quadrants). mDC
+ were matured with TNF-α and IL-1β. Results are from 1 representative out of 11 (iDC) to 4 (Mo) experiments. (B) Total counts of viable cells, γδ T cells, and αβ T cells per well after 7 days of stimulation with different DCs, mDCs, and Mo subsets. Bars represent the mean (± SEM) of 11 (iDCs) to 4 (mDCs) experiments. Side-by-side experiments (n = 4) and unpaired experiments are pooled together. Total counts of γδ T cells are significantly higher in iDC
+ versus iDC
- (133 000 ± 44 000 versus 14 000 ± 4200; n = 11, P < .001), mDC
+ versus mDC
- (124 000 ± 41 000 versus 21 700 ± 6000; n = 6, P < .005), and Mo
+ versus Mo
- (104 000 ± 40 000 versus 21 000 ± 9000; n = 4, P < .05). When the comparison is restricted to side by side experiments, differences are not statistically different between iDC
+ (285 000 ± 96 000 γδ T cells/well), mDC
+ (250 000 ± 67 000 γδ T cells/well) and Mo
+ (103 000 ± 40 000 γδ T cells/well). Differences among total counts of αβ T cells are also not statistically different (iDC
+: 208 000 ± 36 000 αβ T cells/well; iDC
+: 188 000 ± 53 000 αβ T cells/well; mDC
+: 189 000 ± 43 000 αβ T cells/well; mDC
+: 177 000 ± 33 000 αβ T cells/well; Mo
+: 152 000 ± 54 000 αβ T cells/well; Mo
+: 146 000 ± 39 000 αβ T cells/well, P > .05).
Finally, 2 experiments were performed in which T2MP+ cells were incubated with anti-MHC class I antibody before mixing with effector cells stimulated with mDCMP+ Zol+. One representative experiment is shown in Figure 5C, showing that the cytotoxicity against T2MP+ cells was partly abrogated by anti-MHC class I antibody. Thus, MP-specific CD8+ T cells are generated under these conditions and contribute to the cytotoxic response against T2MP+ cells.

In conclusion, iDC can be concurrently loaded with antigens specific for ß and γ T cells and effectively induce the activation of both subsets. The largely predominant activation of γ T cells does not overwhelm, but rather it has beneficial effects on the generation of antigen-specific cytotoxic responses mediated by ß T cells.

**Discussion**

The aim of this work was to determine whether short-term incubation with Zol had any impact on the immunostimulatory properties of iDC, with special regard to their ability to activate autologous γ T cells and modulate antigen-specific immune responses mediated by ß T cells. Cell count and viability of iDC as well as their subsequent differentiation into mDC, induced by TNFα and IL-1β, were not affected by Zol exposure up to 10 μM for 72 hours. Similar results have been reported by Wolf et al. On the contrary, Zol did affect the generation of iDC, as previously shown by von Lilienfeld-Toal et al and Wolf et al. However, these experiments were performed with Mo incubated with Zol for 3 days during their differentiation process into iDC, and not with freshly generated iDC as in our case.

Zol alone was unable to induce iDC maturation, as shown by the very similar immunophenotype and ability of iDCZol− and iDCZol+ to internalize FITC-dextran. The immunostimulatory phenotype of mDCZol+ was slightly improved, as shown by the increased cell surface HLA-DR and CD80 expression, whereas both mDCZol− and mDCZol+ equally downregulated their ability to internalize...
FITC-dextran, according to their full maturation status. Curiously, the increased HLA-DR and CD80 expression of mDC\(^{Zol+}\) did not translate into an increased allostimulatory activity, as already reported by Wolf et al.\(^{19}\)

Last, the pattern of cytokine production by iDC\(^{Zol+}\) and mDC\(^{Zol+}\) was not statistically different from that of iDC\(^{Zol-}\) and mDC\(^{Zol-}\), even if a trend toward a more favorable cytokine production to drive Th1 immune responses could be envisaged in mDC\(^{Zol+}\) (eg, higher and lower production of IL-12 and IL-6, respectively). Altogether, these data indicate that Zol has no toxic effect and does not hamper the basic phenotype and immunostimulatory functions of iDCs and mDCs.

Previous reports have shown that exogenous phosphoantigens, like isopentenyl pyrophosphate and bromohydrin pyrophosphate, mimic the natural ligands of \(\gamma\delta\) T cells and do not induce any phenotypic change or cytokine modulation in iDCs.\(^{12,20,21}\) However, phosphoantigens and ABP act by very different mechanisms. The former mimic the natural ligands of \(\gamma\delta\) T cells and are not internalized by iDCs or mDCs, whereas ABP target the intracellular mevalonate pathway by blocking the farnesyl pyrophosphate synthase. Among ABP, Zol is approximately 100-fold more potent than pamidronate in blocking the farnesyl pyrophosphate synthase.\(^{5,7}\) These data have been validated in vivo with CD8\(^+\) cells only, but recent findings indicate that \(\gamma\delta\) T cells, too, can be subdivided in the same subsets (N, CM, EM, TEMRA) with similar phenotypic and functional properties.\(^{5,7}\) Indeed, iDC\(^{Zol+}\) preferentially induced the expansion of CM and EM \(\gamma\delta\) T cells, which are well-fitted to exert antitumor activity, as shown by their cytotoxic activity against the T2 cell line and other tumor cell lines, as previously reported.\(^{6}\)

Zol was the main immunostimulatory function of iDC\(^{Zol+}\) and induced the expansion of CM and EM \(\gamma\delta\) T cells, which are more abundant than iDC\(^{Zol-}\). So far, only von Lilienfeld-Toal et al.\(^{20}\) have investigated and enabled the increased HLA-DR and CD80 expression of mDC\(^{Zol+}\) to drive Th1 immune responses mediated by conventional \(\alpha\beta\) T cells. iDC\(^{Zol+}\) and mDC\(^{Zol+}\) very efficiently induced the proliferative expansion of autologous \(\gamma\delta\) T cells, Zol was the main cause of \(\gamma\delta\) T-cell activation because: (1) iDC\(^{Zol-}\) and mDC\(^{Zol-}\) were totally ineffective; (2) iDC and mDC subsets were extensively washed before adding autologous PBL or purified T cells; (3) both PBL and T cells were devoid of CD14\(^+\) cells potentially targetable by residual Zol, if any; and (4) the immunostimulatory ability of iDC\(^{Zol+}\) and mDC\(^{Zol+}\) was abrogated by Mev, indicating that targeting the mevalonate pathway of iDC and mDC is an essential prerequisite to recruit \(\gamma\delta\) T cells, as previously reported in Mo and tumor cells.\(^{5,9,10}\)

So far, only von Lilienfeld-Toal et al.\(^{20}\) have investigated and failed to show that ABP-treated iDCs induce the proliferation of \(\gamma\delta\) T cells. However, these experiments were performed with positively selected \(\gamma\delta\) T cells, fully activated by anti-CD3 mAb and high doses of IL-2 and IL-1\(\beta\), and proliferation was evaluated 3 days only after coculturing with iDC. Given the strong \(\gamma\delta\) T-cell baseline activation status and the short incubation time, it is not surprising that ABP-treatment of iDC did not significantly affect the proliferation of \(\gamma\delta\) T cells. Devilder et al.\(^{13}\) did not observe any proliferation after incubation of \(\gamma\delta\) T-cell clones with iDC or mDC in the presence of bromohydrin pyrophosphate. These results further confirm the inferiority of phosphoantigens to ABP in the ability to specifically prime DCs to induce \(\gamma\delta\) T-cell activation.

iDC\(^{Zol+}\) were slightly better than mDC\(^{Zol+}\), and both subsets were better than Mo\(^{Zol+}\) in inducing the expansion of \(\gamma\delta\) T cells. This is the first time that these subsets are side-by-side compared. Previous experiments were performed with PBMCs, rather than purified Mo, and cultures were never washed free of Zol.\(^{6,10}\) Devilder et al.\(^{13}\) have shown that pamidronate-treated iDCs are superior to mDCs in inducing the expression of intracellular TNF\(\alpha\) in clonal or polyclonal \(\gamma\delta\) T cells. To the best of our knowledge, this is the only other demonstration that ABP specifically prime iDCs and mDCs to activate \(\gamma\delta\) T cells.

The vigorous \(\gamma\delta\) T-cell expansion induced by iDC\(^{Zol+}\) and mDC\(^{Zol+}\) indicates that this strategy is worthy of further investigation as a platform for adoptive cell therapy. It has recently become clear that the successful outcome of adoptive cell therapy very much depends on the differentiation status of the cells infused. CM T cells have more effective antitumor activity in vivo than EM or TEMRA T cells, even though the latter are more potent in vitro.\(^{22,24}\) These data have been validated in vivo with CD8\(^+\) cells only, but recent findings indicate that \(\gamma\delta\) T cells, too, can be subdivided in the same subsets (N, CM, EM, TEMRA) with similar phenotypic and functional properties.\(^{5,7}\) Indeed, iDC\(^{Zol+}\) preferentially induced the expansion of CM and EM \(\gamma\delta\) T cells, which are well-fitted to exert antitumor activity, as shown by their cytotoxic activity against the T2 cell line and other tumor cell lines, as previously reported.\(^{6}\)

iDC\(^{Zol+}\) and mDC\(^{Zol+}\) induced the expansion of \(\gamma\delta\) T cells expressing homing receptors for secondary lymphoid organs (CD62L\(^+\)) and costimulatory molecules (HLA-DR\(^+\), CD80\(^+\)). The former are especially expressed at the stage of CM cells\(^{25}\) and enable \(\gamma\delta\) T cells to migrate to lymph nodes where they interact with DC and other cells to improve the outcome of adaptive immune responses. The expression of costimulatory molecules has been related to the capacity of \(\gamma\delta\) T cells to act as APC themselves,\(^{18}\) even if these data are controversial and we were not able to confirm the endocytic function of activated \(\gamma\delta\) T cells. Whether \(\gamma\delta\) T cells can act as antigen-presenting cells, the phenotype expressed by activated \(\gamma\delta\) T cells, the well-preserved immunostimulatory functions of iDC\(^{Zol+}\) and mDC\(^{Zol+}\), and the preserved numbers of conventional \(\alpha\beta\) T cells prompted us to investigate whether iDC could be primed with the appropriate antigens to simultaneously activate both \(\alpha\beta\) and \(\gamma\delta\) T cells. To gain insights into this issue, we pulsed iDCs with MP in the presence or absence of Zol and then induced their differentiation into mDC with TNF-\(\alpha\) + IL-1\(\beta\). Then, we determined the frequency of MP-specific CD8\(^+\) T cells and cytotoxic responses against T2\(^{MP+}\) and T2\(^{MP-}\) cells. Notably, the highest frequency of MP-specific CD8\(^+\) T cells and the highest cytotoxicity against T2\(^{MP+}\) cells were observed after stimulation with mDC\(^{MP+Zol+}\). These results indicate that iDC can be concurrently loaded with antigens specific for \(\alpha\beta\) and \(\gamma\delta\) T cells and effectively induce the activation of both subsets. The largely predominant activation of \(\gamma\delta\) T cells has beneficial effects on the generation of MP-specific CD8\(^+\) \(\alpha\beta\) T cells as shown by the higher frequency of MP-specific CD8\(^+\) \(\alpha\beta\) T cells and higher cytotoxic activity against T2\(^{MP+}\) cells detected after stimulation with mDC\(^{MP+Zol+}\) compared with mDC\(^{MP+Zol-}\). Blocking experiments with anti-MHC class I antibody also indicate that: (1) MP-specific CD8\(^+\) \(\alpha\beta\) T cells are generated after stimulation with mDC\(^{MP+Zol+}\); (2) these cells are not overwhelmed by the much more abundant activated \(\gamma\delta\) T cells; and (3) they contribute to the bulk cytotoxic activity detected against T2\(^{MP+}\) cells.

The generation of MHC-restricted antigen-specific cytotoxic \(\alpha\beta\) T cells seemed to draw more benefit from the concurrent \(\gamma\delta\) T-cell activation than vice versa, as shown by the similar cytotoxic activity against T2\(^{MP+}\) cells induced by mDC\(^{MP+Zol+}\) and mDC\(^{MP+Zol-}\). Given the very different frequencies, it is not surprising that the much more abundant activated \(\gamma\delta\) T cells
improve the efficiency of the much less represented MHC-restricted antigen-specific γδ T cells. This is the first report in humans showing that iDCs can be simultaneously primed to activate both γδ and αβ T cells, and that the former act as cellular adjuvants for the development of adaptive immune responses. These data further support the notion that γδ T cells are endowed with more sophisticated functions than simply providing a first-line defense against pathogens or self-induced stress antigens in the peripheral tissues. Mouse models have confirmed that γδ T cells do provide help to adaptive immune responses by promoting the formation of germinal centers, the somatic hypermutation of B cells,26 and by positively regulating superantigen-specific immune responses mediated by αβ T cells.27

In conclusion, our results indicate that it is possible to quickly generate large numbers of activated γδ T cells with effector and costimulatory activities by short-term incubation with iDCs/mDCs pulsed with the appropriate antigens. This strategy is worth of further investigation to improve adoptive cell therapy and vaccine interventions against tumors and infections.

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References


Authorship

Contribution: M.M. and F.F. designed the study, analyzed the data, and wrote the paper. F.F., B.C., S.M., F.P., and M.F. performed the research and analyzed the data. B.N. assisted in experimental analyses. R.B., B.B., and M.B. helped to design the study and provided critical suggestions.

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Enhanced ability of dendritic cells to stimulate innate and adaptive immunity on short-term incubation with zoledronic acid

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