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Immune Modulation by Zoledronic Acid in Human Myeloma: An Advantageous Cross-Talk between Vγ9Vδ2 T Cells, αβ CD8+ T Cells, Regulatory T Cells, and Dendritic Cells

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Vγ9Vδ2 T cells play a major role as effector cells of innate immune responses against microbes, stressed cells, and tumor cells. They constitute <5% of PBLs but can be expanded by zoledronic acid (ZA)-treated monocytes or dendritic cells (DC). Much less is known about their ability to act as cellular adjuvants bridging innate and adaptive immunity, especially in patients with cancer. We have addressed this issue in multiple myeloma (MM), a prototypic disease with several immune dysfunctions that also affect γδ T cells and DC. ZA-treated MM DC were highly effective in activating autologous monocytes and dendritic cells (4, 5). The Mev pathway of mammalian cells also generates intermediate phosphorylated metabolites, such as isopentenyl pyrophosphate (IPP), which activate γδ T cells as efficiently as their natural ligands (6, 7). Cell stress and transformation increase Mev activity and accelerate the formation of intracellular phosphorylated metabolites, whose accumulation in excess of physiological levels is detected by γδ T cells and renders the immune system able to sense potential threats such as stressed or transformed cells (6). IPP levels are increased by drugs such as aminobisphosphonates (NBP) that selectively target farnesyl-pyrophosphate synthase (FPPS) in the Mev pathway (6–8). When NBP are used to target the Mev pathway of professional APC, such as monocytes (MC) and dendritic cells (DC), a quick and robust proliferative expansion of γδ T cells with antitumor activity is induced (9–11).

γδ T cells also act as cellular adjuvants bridging innate and adaptive immunity via mutual interactions with DC (12, 13). We have shown in healthy individuals that MHC-restricted antiviral immune responses are improved by the concurrent activation of CD8+ αβ T cells driven by autologous DC copulsed with zoledronic acid (ZA) and influenza virus-derived peptides (11). Similar data have been reported in healthy individuals after copulsing DC with ZA and melanoma-derived peptides (14).

Multiple myeloma (MM) is a disease with several immune dysfunctions that do not spare γδ T cells and DC (9, 15–19). Approximately 50% of MM patients at diagnosis have γδ T cells refractory to stimulation with BrHPP or NBP mediated by MC (9, 15–17). These patients are categorized as nonresponders (NR) and regarded as ineligible for γδ T cell-based immune interventions (16). Cabillic et al. (20) have recently shown that γδ T cell
proliferation can be reinstated in NR patients with solid tumors using ZA-treated DC. However, MM DC are inclined to stimulate γδ T cell proliferation in NR patients, and effectively drive the concurrent activation of Ag-specific αβ T cells and γδ T cells, is an unanswered question not only in MM, but also in patients with cancer.

The aim of this work was: 1) to determine whether MM DC are as effective as normal DC in activating autologous γδ T cells in both responders (R) and NR, and 2) to determine whether immune responses against tumor-associated Ags in MM can be amplified by the mutual interactions between γδ T cells and MHC-restricted CD8+ αβ T cells driven by autologous DC copulsed with ZA and survivin (SRV). SRV was selected as a prototypic tumor-associated Ag on account of its aberrant expression in MM and because SRV-specific cytotoxic CD8+ αβ T cells are tolerized but not deleted from the MM T cell repertoire (23).

Materials and Methods

Cells and reagents

Peripheral blood samples were drawn from MM at diagnosis only. Different series of experiments were performed on a total number of 22 patients. The controls (CTRL) were healthy blood donors kindly provided by the local Blood Bank (Fondazione Strumia). Samples were collected after informed consent and approval by the local Institutional Review Boards.

After isolation on a Ficoll-Hypaque density gradient, PBMC were kept unfraccionated or further processed to purify CD14+ cells and generate different APC preparations. Aliquots of PBMC and CD14-depleted cells (i.e., PBL) were kept at 37˚C or frozen in liquid nitrogen to be subsequently used without further manipulation or as a source of T cells in coculture experiments with autologous APC.

The standard culture medium was RPMI 1640 (Euroclone, Milano, Italy), containing 10% FCS (Euroclone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Flow cytometry


Two- and three-color flow cytometry were performed with the appropriate combinations of FITC, PE–Percoll complex (PerCP), or allophycocyanin-conjugated Abs, a FACScan cell sorter, and CellQuest software (Becton Dickinson, Mountain View, CA).

Regulatory T cell (Treg) cells were identified by incubation with PerCP-conjugated anti-CD4 and PE-conjugated CD25 at 4˚C for 20 min in the dark. After washing, they were fixed and permeabilized at 4˚C for 40–60 min (according to the manufacturer’s instructions), washed twice with permeabilization buffer, and then incubated at 4˚C in the dark for 30 min with 20 μl anti-Foxp3 FITC or isotype CTRL mAb. After washes with Perm/Wash buffer, cells were fixed with 250 μl 1% paraformaldehyde-PBS buffer.

Total 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 two- or three-color flow cytometry and appropriate gating.

APC generation

MC were purified using CD14 MicroBeads and LS columns (Miltenyi Biotec, Bologna, Italy), and incubated for 24 h in 24-well plates at 1 × 10^6/ml in the presence (MCZA+) or absence of 5 μM ZA (MCZA−; kindly provided by Novartis Farma, Origgio, Italy).

Conventional immature DC (iDC) and mature DC (mDC) were generated from CD14+ cells and incubated for 24 h in the presence (iDCZA+ or mDCZA+) by determining total counts of viable cells per well by the percentage of cells of interest, the latter being identified by two- or three-color flow cytometry and appropriate gating.

APC were always washed before mixing with autologous PBL or T cells. In selected experiments, supernatants were collected for quantification of extracellular IP3 levels and to investigate their ability to induce autologous γδ T cell proliferation.

The internalization capability of iDC, mDC, and f-DC was tested by using FITC-conjugated dextran (FITC-dextran; 10 kDa; Sigma Aldrich, Milano, Italy).

γδ T cell proliferation

CTRL and MM patients were classed as R or NR according to their proliferation PBMC responses to 1 μM ZA + 10 IU/ml IL-2 (Eurocetus, Milano, Italy), as previously reported (9).

Cryopreserved or freshly isolated PBL were added at the ratio of 5:1 to autologous iDC/mDC or MC/f-DC, respectively, and cultured at 1 × 10^6/ml with 10 IU/ml IL-2. On day 7, percentages and total counts of viable γδ T cell proliferation

Modulation of SRV-specific immune responses

The HLA-A*0201–restricted, SRV-derived peptide LMLGFLKFL (SRVP; Proimmune) was used to investigate MHC-restricted immune responses mediated by CD8+ αβ T cells. iDC were generated from HLA-A*0201+ CTRL and MM, and left untreated or pulsed with 10 μM SRVP for 2 h at room temperature in serum-free medium. After washing, iDC maturation was induced in the presence or absence of 5 μM ZA as reported earlier. On the same day, CD3+ cells were purified from cryopreserved autologous PBMC (Miltenyi Pan T Cell Isolation Kit), incubated at the DC:T ratio of 1:10 in round-bottom plates, and incubated for 10 d in the presence of 10 IU/ml IL-2, replenished every 3 d. On day 10, T cells were re-stimulated for additional 10 d with a second batch of freshly generated mDC. On days 10 and 20, the frequency of SRV-specific CD8+ αβ T cells was determined by flow cytometry with a commercially available PE-labeled kit (Pro5 MHC Pentamer; Proimmune), according to the manufacturer’s instructions. Because Pro5 Pentamers can bind nonspecifically to B cells, both CD8 and CD19 Abs were added after pentamer staining, and a minimum amount of 500,000 events was acquired after backgating on viable CD8+ CD19+ cells.

CD4+ T cells were also left untreated or pulsed with SRVP, and incubated with f-DCZA+ or without 5 μM ZA (f-DCZA−) as reported earlier for mDC. CD3+ cells were purified from cryopreserved autologous PBMC and incubated for 14 d with f-DC at the DC:T ratio of 1:10 in round-bottom plates in the presence of 10 IU/ml IL-2 that was replenished every 3 d. One round of 14-d stimulation was used based on side-by-side experiments showing that it was equivalent to two rounds of 10-d stimulation (see later and Supplemental Fig. 4).

The cytotoxic activity of CD8+ αβ T cells was tested against the TAP-deficient, SRVP-loaded HLA-A2+ T2 cell line, the HLA-A2+ SRV+ myeloma cell line RPMI 8226, and HLA-A2+ myeloma cells isolated from the peripheral blood of a plasma cell leukemia (PCL) patient, as previously reported (11).

The specificity of anti-SRV immune responses was assessed by using the HLA-A2–restricted CMVp (NLVPMVATL; Proimmune) and HIV-derived (SLYNTVATL; Proimmune) peptides (CMVp, HIVp) to load f-DCZA+ and f-DCZA−, and by determining the frequency and total counts of viable CD8+ αβ T cells specifically reacting against the SRVP, CMVp, and HIVp after 14-d stimulation.

Quantification of the Mey pathway in APC

MC, mDC, and f-DC were incubated at 1 × 10^6/ml for 24 h with 1 μM [H] acetate (3600 mCi/mM; Amersham International, Bucks, U.K.). The intracellular synthesis of farnesyl-pyrophosphate (FPP) and cholesterol was measured with the methanol/hexane extraction method (26). Intracellular and extracellular IP3 levels were measured according to Benford et al. (27) with minor modifications. A total of 300 μl cells lysate or culture supernatant was diluted 1:2 into an ice-cold acetonitrile solution containing 100
mM NaVO₃ and centrifuged at 1200 x g for 5 min at 4°C. After lyophilization under vacuum, samples were resuspended in 20 μl dimethylamine and separated by TLC. Gels were exposed to an iodine-saturated atmosphere for 2 h, spots corresponding to each isoprenoid species were isolated, and radioactivity uptake was measured by liquid scintillation counting (Ultima Gold; Perkin Elmer). The titration curves for each isoprenoids species were performed using three serial dilutions of [³H]cholesterol (Amersham), [³H]arnesyl pyrophosphate (Perkin Elmer), and [¹⁴C] IPP (Perkin Elmer), and counting them with liquid scintillation. The radioactivity (in terms of cpm) of each sample was referred to the respective titration curve to obtain an index of the amount of cholesterol, FPP, and IPP, neosynthesized from the radiolabeled precursor and normalized to the number of cells per milliliter. According to the titration curve, the results are expressed as fmol/1 x 10⁶ cells for cholesterol, FPP, and intracellular IPP, and as pmol/1 x 10⁶ cells for extracellular IPP.

Isolation of Tregs and proliferative assay
Tregs were purified from PBMC by immunomagnetic separation with the CD4⁺CD25⁺ T Regulatory Cells Isolation kit (Miltenyi Biotec, Bologna, Italy), according to the manufacturer's instructions. In brief, CD4⁺ cells were isolated by negative selection after depletion of cells expressing the CD8, CD11b, CD16, and CD56 Ags. CD25⁺ cells were then selected by positive selection with magnetic beads directly conjugated to an anti-CD25 Ab. From 5 x 10⁶ to 1.8 x 10⁵ CD4⁺CD25⁺ cells (purity 76-95%) were obtained from buffy coats containing each from 200–300 x 10⁶ PBMC. Treg function was assessed as previously described (28). Allogenic PBMC were irradiated at 3000 rad and used as accessory cells, and 1 μg/ml soluble anti-CD3 (OKT3 Orthoclone; Janssen-Cilag, Cologno Monzese, Italy) was used to induce polyclonal T cell activation. Tregs (CD4⁺CD25⁺) and CD4⁺CD25⁻ cells were cultured alone (50 x 10⁶/well) or in combination (1:1 ratio) in U-bottom 96-well plates in the presence or absence of 5 μM ZA. Cell proliferation was evaluated on day 3 by adding 1 μCi [³H]thymidine deoxyribose (Amersham) to each well during the last 6 h of culture and measuring its uptake with a scintillation counter.

Statistical analysis
Results are expressed as mean values ± SE. Differences between sample groups were evaluated with the two-tailed nonparametric Mann–Whitney U test for paired samples or with the Fisher exact test for unpaired data. Correlations were assessed by Pearson correlation coefficient. The significance cutoff value was p < 0.05. The p values are reported in the figures.

Results
mDCZA⁺ are potent inducers of γδ T cell proliferation irrespective of R/NR status
The first series of experiments was deliberately confined to CTRL and MM R to identify the most effective APC inducing γδ T cell proliferation in a susceptible population. As expected, no significant proliferation of γδ T cells was observed with IL-2 alone in the absence of ZA (Fig. 1). The highest γδ T cell counts were observed after stimulation with ZA-treated mDC in both CTRL (p < 0.01) and MM (p < 0.3). MM (Fig. 1A, right panel) displayed significantly higher counts than CTRL (Fig. 1A, left panel) after stimulation with both ZA-treated MC (p < 0.05) and ZA-treated mDC (p < 0.005).

A significant increase of central memory (CM) and effector memory (EM) γδ T cells was detected after stimulation with ZA + IL-2 versus IL-2 alone in CTRL and MM R (p < 0.05). In MM, CM and EM γδ T cell counts were significantly higher than in CTRL after both IL-2 and ZA+IL-2 stimulation (both p < 0.05). CM was the predominant subset in CTRL R, whereas EM was predominant in MM R (Fig. 1B).

Approximately 50% of MM patients at diagnosis display minimal or no γδ T cell proliferation after PBMC stimulation with 1 μM ZA + 10 IU/ml IL-2 (NR). Less than 5% of our CTRL were NR. In view of the very effective γδ T cell stimulatory capacity of mDCZA⁺, we performed a side-by-side experiment with PBMCZA⁺ and MCZA⁺ in the two NR groups. mDCZA⁺ restored γδ T cell proliferation in both groups (p < 0.0003; Fig. 1C). Flow cytometry of γδ T cells after stimulation with PBMCZA⁺ and mDCZA⁺ in one representative MM NR is shown in Fig. 1D.

Immunophenotyping showed that mDCZA⁺ induced the expansion of γδ T cells equipped with surface receptors to exert co-stimulatory (HLA-DR, CD86, CD80), but not inhibitory (PD-1, PD-L1), activity on immune responses (Fig. 1E) in MM R and NR. SRV-specific immune responses mediated by CD8⁺ αβ T cells are upregulated by the concurrent γδ T cell proliferation
The baseline frequency of SRV-specific CD8⁺ αβ T cells in the peripheral blood was significantly higher in MM than in CTRL (0.41 ± 0.05 versus 0.07 ± 0.03%; p < 0.0001; Suplemental Fig. 1). The frequency and total counts of SRV-specific CD8⁺ αβ T cells was then investigated after stimulation with mDCSRVP⁻ZA⁻, mDCSRVP⁻ZA⁺, mDCSRVP⁺ZA⁻, and mDCSRVP⁺ZA⁺. Results from representational experiments in the CTRL and MM R and NR groups are shown in Fig. 2A and 2B, and the pooled data are shown in Fig. 2C. Given the equivalent γδ T cell proliferation and number of CD8⁺ αβ T cells induced by mDCZA⁺ R and NR individuals were grouped together in CTRL and MM. Total counts were significantly higher after stimulation with mDCSRVP⁺ZA⁺ compared with stimulation with mDCSRVP⁻ZA⁻ (p < 0.05 in both CTRL and MM), indicating that the concurrent activation of γδ T cells and CD8⁺ αβ T cells was not mutually detrimental.

The cytotoxic activity of SRV-specific CD8⁺ αβ T cells was tested against the HLA-A2⁺ SRV-pulsed T2 cell line (T2SRVP⁺). As expected, the highest cytotoxicity was observed when T cells were stimulated with mDCSRVP⁺ZA⁺ in both CTRL and MM (p < 0.05 compared with cytotoxic values after stimulation with mDCSRVP⁻ZA⁻ in both groups) (Fig. 2D). Similar results were observed with RPMI 8226, a myeloma-derived SRV⁺ HLA-A2⁺ cell line (Suplemental Fig. 2).

f-DCZA⁺ are as effective as conventional mDCZA⁺ in inducing γδ T cell proliferation and upregulating SRV-specific immune responses
The production of conventional mDC is a time-consuming, labor-intensive, and costly process that requires enough PBMC to generate the mDC needed for two rounds of stimulation. To overcome these limitations, we used f-DCZA⁺ to stimulate autologous γδ T cells. f-DC were generated in 48 h from CTRL and MM irrespective of their R/NR status. The immunophenotype and endocytic capacity of immature and mature before (f-DCZA⁻) and after (f-DCZA⁺) ZA treatment are shown in Supplemental Table I and Supplemental Fig. 3. A side-by-side comparison in CTRL R between the ability of f-DCZA⁺ and mDCZA⁺ to stimulate autologous γδ T cells and induce the expansion of SRV-specific CD8⁺ αβ T cells is shown in Supplemental Fig. 4. The total number of γδ T cells was not statistically different either on day 10 (after one round) or 20 (after two rounds), whereas the number of SRV-specific CD8⁺ αβ T cells per well was even higher after stimulation with f-DCZA⁺. We also compared the total numbers of γδ T cells and SRV-specific CD8⁺ αβ T cells per well after two 10-d stimulations as opposed to only one of 14 d without detecting any difference (Supplemental Fig. 4). Based on these results, f-DCZA⁺ were also used to stimulate γδ T cells in MM patients. Indeed, they effectively induced the proliferative expansion of γδ T cells in CTRL R and NR (p < 0.04 and p < 0.03, respectively), and in MM R and NR (both p < 0.04; Fig. 3A). Flow cytometry of reinstated T cell proliferation in representative CTRL and MM NR are shown in Fig. 3B. f-DCZA⁺ also amplified SRV-specific immune responses in both CTRL and MM R and NR. Flow cytometry of representative samples is shown in Fig. 3C and 3D. The specificity of MHC-restricted immune responses generated
by the costimulation of γδ T cells and CD8αβ T cells is demonstrated in Fig. 3E. CTRL T cells were stimulated with f-DCZA+ and f-DCZA+ loaded or unloaded with the HLA-A2-restricted SRVp, CMVp, and HIVp. The frequency of specific CD8αβ T cells was determined with the corresponding PE-labeled pentamers. As expected, only CD8αβ T cells specific for the peptide used to load f-DCZA+ and f-DCZA+ were increased after 14-d incubation. Pooled data reporting total counts of SRV-specific CD8αβ T cells and cytotoxicity against the RPMI 8226 myeloma cell line are shown in Fig. 3F and 3G, respectively. The most effective anti-SRV activity was observed after stimulation with f-DCSRV+ZA+ in both CTRL and MM.

Fig. 3H shows that CD8αβ T cells elicited by f-DCSRV+ZA+ are also able to recognize and kill primary myeloma cells. The specificity of this antitumor immune response is corroborated by the observation that T cells do not exert any cytotoxicity when CMVp is used to load f-DC.

**Accelerated Mev activity in mDC and f-DC, and enhanced IPP production after ZA treatment**

The ability of ZA-treated APC to activate cells is dependent on their capacity to inhibit FPPS in the Mev pathway (10). We have compared the Mev activity rate in MC, mDC, and f-DC at baseline and after treatment with 5 μM ZA (i.e., the conditions used to stimulate T cells). Baseline cholesterol and both intracellular and extracellular IPP levels progressively increased from MC to f-DC, with mDC displaying intermediate values (Fig. 4). Unexpectedly, extracellular IPP concentrations were 1000 times greater than the intracellular concentrations. ZA-induced FPPS inhibition decreased the production of cholesterol and FPP, and further in-
creased intracellular and extracellular IPP levels. All differences were statistically different between untreated and ZA-treated cells. The most striking effect was the increase of extracellular IPP in the supernatants of f-DC to concentrations almost 10 times greater than those in the supernatants of ZA-treated MC and mDC.

To further demonstrate the key role played by the Mev pathway, mDC and f-DC were treated with ZA in the presence or absence of simvastatin (Sim). Sim prevents ZA-induced IPP accumulation by specifically inhibiting hydroxy-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in the Mev pathway acting upstream from FPPS (29, 30). As predicted (9, 22, 30), Sim abrogated the ability of mDCZA+ and f-DCZA+ to induce the proliferation of γδ T cells (data not shown; see also Figs. 5, 6).

The IPP released in the supernatants of f-DCZA+ is sufficient to induce γδ T cell proliferation

PBMC and PBL from CTRL R were incubated for 7 d with the supernatants collected from f-DCZA+ and f-DCZA+, and supplemented with 10 IU/ml IL-2. Final dilutions were 1, 10, and 20% corresponding to extracellular IPP concentrations of 14, 144, and 288 nM, respectively (Fig. 5B, left panel). Standard cultures with IL-2 and ZA+IL-2 served as CTRL (Fig. 5A). As expected, γδ T cell proliferation was induced only in PBMC, because PBL are depleted of MC and cannot generate adequate IPP amounts to stimulate γδ T cells (9, 10). By contrast, γδ T cell proliferation was equivalent in PBMC and PBL after incubation with f-DCZA+ supernatants, indicating that the concentration of extracellular IPP was sufficient to induce γδ T cell proliferation, even in the absence of MC, as previously reported for exogenous IPP (2). In confirmation, PBMC and PBL from CTRL R were stimulated with exogenous IPP at concentrations including those detected in f-DCZA+ supernatants (from 14 to 280 nM; Fig. 5B, right panel). Exogenous IPP induced the proliferation of γδ T cells, but with lower efficiency than f-DCZA+ supernatants, especially if used in the presence of MC (PBMC versus PBL) and at concentrations equivalent to those of f-DCZA+ supernatants (i.e., from 10−8 to 10−7 M). It also induced γδ T cell proliferation in PBMC and PBL as effectively as f-DCZA+ supernatants when used at higher concentrations (>10−6 M). These results suggest that other cytokines and/or growth factors may be present in f-DCZA+ supernatants and promote γδ T cell proliferation in association with the low endogenous IPP concentrations. It is also possible that MC preferentially use exogenous IPP to the disadvantage of γδ T cells, because higher concentrations are required to induce γδ T cell proliferation in PBMC versus PBL.

Extracellular IPP was also measured in the supernatants of f-DCZA+ cultured in the presence or absence of Sim. As predicted,
**FIGURE 3.** f-DCZA+ are as effective as conventional mDCZA+ in inducing γδ T cell proliferation and upregulating SRV-specific immune response. 

A. Total counts of viable γδ T cells after stimulation with untreated (ZA−) or ZA-treated (ZA+) f-DC in CTRL and MM R and NR. Cultures were initiated at 1 × 10⁶/ml with 2 × 10⁵ γδ T cells and autologous f-DC (10:1 ratio) in a final volume of 200 μl/well. Multiple wells were set up as required. Bars represent the mean values ± SE from five CTRL (three R, two NR) and five MM (three R, two NR). ZA-treated f-DC induced γδ T cell proliferation in both CTRL and MM R and NR. Differences between untreated and ZA-treated f-DC are statistically significant in CTRL R and NR (p<0.04 and p<0.03, respectively), and in MM R and NR (both p<0.04), whereas differences between CTRL and MM are not statistically different. 

B. Representative flow cytometry of reinstated γδ T cell proliferation in individual CTRL and MM NR after stimulation with ZA-treated f-DC (f-DCZA+). C and D. Flow cytometry of SRV-specific CD8+ cells by pentamer staining after 14-d stimulation of autologous T cells with f-DCSRVp+ZA− and f-DCSRVp+ZA+ in CTRL and MM R and NR. E. Frequency of pentamer-specific CD8+ αβ T cells after stimulation of CTRL T cells with autologous f-DC±ZA− and f-DC±ZA+ loaded or not loaded with HLA-A2–restricted SRVp, CMVp, and HIVp. Pentamer staining shows that there is no cross-reactivity, and only CD8+ αβ T cells specific for the corresponding peptide used to load f-DC±ZA− and f-DC±ZA+ are increased after 14-d incubation. F. Pooled data reporting total counts of SRV-specific CD8+ αβ T cells. Cultures were set up as described earlier. Bars represent the mean values ± SE from five CTRL and five MM. Given the equivalent γδ T cell proliferation and number of CD8+ αβ T cells induced by f-DC±ZA−, R and NR individuals are grouped together in CTRL (two R, three NR) and MM (two R, three NR). Total counts were significantly higher after stimulation with f-DCSRVp+ZA+ than after stimulation with f-DCSRVp+ZA− in CTRL and MM (both...
Figure 4. Accelerated Mev activity in mDC and f-DC resulting in enhanced intracellular (IPP$_{intra}$) and extracellular IPP (IPP$_{extra}$) production after ZA treatment. Quantification of the Mev activity in MC, mDC, and f-DC at baseline (ZA$^-$) and after treatment with 5 μM ZA (ZA$^+$). Bars represent the mean values ± SE from three CTRL. mDC and f-DC showed an accelerated baseline Mev activity compared with MC, as shown by the increased production of cholesterol, FPP, IPP$_{intra}$, and IPP$_{extra}$. Cholesterol, FPP, and IPP$_{intra}$ levels are shown in the left panels and are expressed as fmol/1 × 10$^6$ cells. IPP$_{extra}$ levels are shown in the right panels and are expressed as pmol/1 × 10$^6$ cells. ZA induced a decreased production of cholesterol and FPP, and an increase of IPP$_{intra}$ and IPP$_{extra}$ levels in MC (p < 0.05), mDC (p < 0.04), and f-DC (p < 0.0001). The highest levels of IPP$_{extra}$ were reached in the supernatants of f-DCZA$^+$ (p < 0.0001 versus MC, p < 0.02 versus mDC). Supernatants of mDCZA$^+$ also contained higher levels of IPP$_{extra}$ than MCZA$^+$ (p < 0.002).

Sim almost set to zero extracellular IPP concentrations (Fig. 5C) and the superantigen of f-DCZA$^+$/Sim$^+$ failed to induce any proliferation of γδ T cells irrespective of whether MC were present in the responding population (Fig. 5D).

Antagonism of ZA and γδ T cells versus Tregs

γδ T cell proliferation can be suppressed by Tregs (17, 31), and their excess over γδ T cells has been proposed as an explanation for defective ZA-induced γδ T cell proliferation in MM NR (17). Treg and γδ T cell counts and ratios were not significantly different in function of R/NR status in the peripheral blood of CTRL and MM patients (data not shown). We also looked for correlations in individual CTRL and MM R between baseline Treg and γδ T cell values, and the increase of γδ T cells after stimulation with ZA+IL-2. The only statistically significant finding was a direct correlation between baseline γδ T cell counts and their fold increase after stimulation in CTRL R, whereas no correlation was detected with Tregs (data not shown).

Total counts of viable Tregs were also calculated after 7-d PBMC stimulation in the presence or absence of IL-2, ZA, or both (Fig. 6A). These are the only culture conditions that discriminate between the R/NR status, which is no longer detectable after mDCZA$^+$ or f-DCZA$^+$ stimulation. IL-2 is a major Treg growth factor (32); therefore, their increase after incubation with 10 IU/ml IL-2 was expected. Notably, the increase was higher in MM R than in MM NR, which rules out enhanced Treg IL-2 reactivity in the latter. The increase was significantly blunted when ZA was added to IL-2 (ZA+IL-2 versus IL-2) in both MM R (p < 0.05) and NR (p < 0.05), even if γδ T cell proliferation occurred in the former only. The fact that Treg counts were also higher after incubation in RPMI 1640 than after stimulation with ZA alone (p < 0.05 in both R and NR), a condition that does not lead to any γδ T cell expansion even in MM R, provides further evidence that ZA restrains Treg expansion via γδ T cell-dependent and -independent mechanisms. Flow cytometry of Treg after PBMC stimulation in representative MM R and NR is illustrated in Supplemental Fig. 5A.

To further investigate the mechanisms exploited by ZA to antagonize Tregs independently of γδ T cells, we tested the ability of Tregs to regulate the proliferation of autologous CD4$^+$CD25$^+$ driven by irradiated accessory cells and OKT3 in the presence or absence of ZA (Fig. 6B). CD4$^+$CD25$^+$ proliferated in response to stimulation with anti-CD3 mAb and irradiated accessory cells, whereas Tregs were anergic. Proliferation was significantly inhibited (p < 0.002) when CD4$^+$CD25$^+$ and Tregs were cultured at 1:1 cell ratio. This inhibition was reverted when ZA was added (p < 0.03). This effect was not dependent on γδ T cells because their proliferation was never observed under these culture conditions (data not shown). Tregs were purified, pretreated with 5 μM ZA for 24 h, and washed before mixing with autologous CD4$^+$CD25$^+$ T cells, to further demonstrate a direct effect. The results in Supplemental Fig. 5B (left panel) indicate that Tregs were still able to suppress the proliferative response of CD4$^+$CD25$^+$ T cells. Sim was also added in two experiments to confirm that the ZA-induced anti-Treg activity did not require any γδ T cell proliferation and was independent of the inhibition of the Mev pathway (Supplemental Fig. 5B, right panel).

Previous reports have shown that MM DC are inclined to stimulate T cells with inhibitory functions (21, 22). Thus, Treg...
**Discussion**

γδ T cells play an important role in immunosurveillance in several ways. Their contribution as effector cells of innate immunity is
very well recognized, and several studies are investigating the possibility of exploiting their effector properties in infections and cancer (1, 5). Much less is known about their ability to act as cellular adjuvants bridging innate and adaptive immunity, especially in patients with cancer. We have addressed this issue in MM, a prototypic disease characterized by multiple immune dysfunctions that also involves \( \gamma \delta \) T cells. mDCZA+ generated from MM R and NR after stimulation with untreated (ZA–) and ZA-treated (ZA+) mDC (left panel) and f-DC (right panel). Cultures were set up as reported in Figs. 2A and 3A. Bars represent the mean values ± SE of side-by-side experiments performed in three MM R and three MM NR. Only mDCZA+ and f-DCZA+ induced \( \gamma \delta \) T cell proliferation, which was uniformly associated with lower Treg counts, even if differences reached statistical significance only after stimulation with mDCZA+ in MM R (p < 0.05). D, Treg counts after T stimulation with mDC and f-DC left untreated, or treated with ZA and Sim alone or in combination (upper panels). Bars represent the mean values ± SE from four MM R. IL-2 was omitted (left) or added (right) to induce \( \gamma \delta \) T cell proliferation. ZA uniformly decreased Treg counts irrespective of any concurrent \( \gamma \delta \) T cell proliferation, which was present only after stimulation with IL-2 in the absence of Sim (see lower panels for corresponding \( \gamma \delta \) T cell counts). The decrease was statistically significant in the presence of IL-2, which is essential for the proliferation of \( \gamma \delta \) T cells, but also the main growth factor of Tregs. Indeed, Treg counts after stimulation with ZA+IL-2 were significantly lower than after stimulation with IL-2 alone (p < 0.005). ZA-induced anti-Treg antagonism was unaffected by Sim.

Approximately 50% of MM patients at diagnosis are classified as NR because \( \gamma \delta \) T cell proliferation is not detected after challenging PBMC for 7 d with ZA+IL-2 (9, 15–17). A similar proportion has been reported in patients with other lymphoproliferative disorders and metastatic solid cancer (17). \( \gamma \delta \) T cells from MM NR produce IFN-\( \gamma \) and exert cytotoxic activity against myeloma cells once activated by ZA+IL-2 (9), but NR patients are typically excluded from \( \gamma \delta \) T cell-based adoptive immunotherapy trials because of the impossibility of increasing the number of cells and establishing a favorable E:T cell ratio in vivo or ex vivo. In view of the high efficiency of mDCZA+, we tested their ability to induce \( \gamma \delta \) T cell proliferation in CTRL and MM NR. Occasionally, healthy individuals (<5% in our experience) are also refractory to ZA stimulation. mDCZA+ were very effective in both CTRL and MM NR, and resulted in the proliferative expansion of

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Antagonism of ZA and \( \gamma \delta \) T cells versus Tregs. A, Total counts of viable Tregs (CD4+ CD25\text{high} FOXP3+) generated after 7-d PBMC stimulation in the presence or absence of IL-2, ZA, or both. Bars represent the mean values ± SE of eight independent experiments performed in two MM R and six MM NR. Cultures were set up as reported in Fig. 1A. The Treg increase was significantly blunted when ZA was added to IL-2 (ZA+IL-2 versus IL-2) in both MM R (p < 0.05) and NR (p < 0.05), even if \( \gamma \delta \) T cell proliferation occurred in the former only. Treg counts were also lower after stimulation with ZA alone compared with those after 7-d incubation in RPMI 1640 (p < 0.05 in both R and NR). B, ZA antagonizes the inhibitory function of Tregs. CD4+CD25+ proliferated in response to stimulation with anti-CD3 mAb and irradiated accessory cells, whereas Tregs (CD4+CD25+) were anergic. These responses were not affected by ZA. Proliferation was significantly inhibited (p < 0.002) when CD4+CD25+ and Tregs were cocultured at 1:1 cell ratio. This inhibition was reverted when ZA was added (p < 0.03). Bars represent the mean values ± SE from three CTRL. C, Treg counts evaluated in MM R and NR after stimulation with untreated (ZA–) and ZA-treated (ZA+) mDC (left panel) and f-DC (right panel). Cultures were set up as reported in Figs. 2A and 3A. Bars represent the mean values ± SE of eight independent experiments performed in two MM R and three MM NR. Only mDCZA+ and f-DCZA+ induced \( \gamma \delta \) T cell proliferation, which was uniformly associated with lower Treg counts, even if differences reached statistical significance only after stimulation with mDCZA+ in MM R (p < 0.05). D, Treg counts after T stimulation with mDC and f-DC left untreated, or treated with ZA and Sim alone or in combination (upper panels). Bars represent the mean values ± SE from four MM R. IL-2 was omitted (left) or added (right) to induce \( \gamma \delta \) T cell proliferation. ZA uniformly decreased Treg counts irrespective of any concurrent \( \gamma \delta \) T cell proliferation, which was present only after stimulation with IL-2 in the absence of Sim (see lower panels for corresponding \( \gamma \delta \) T cell counts). The decrease was statistically significant in the presence of IL-2, which is essential for the proliferation of \( \gamma \delta \) T cells, but also the main growth factor of Tregs. Indeed, Treg counts after stimulation with ZA+IL-2 were significantly lower than after stimulation with IL-2 alone (p < 0.005). ZA-induced anti-Treg antagonism was unaffected by Sim.
γδ T cells phenotypically indistinguishable from that observed in CTRL and MM R (HLA-DR+, CD80+, CD86+, PD-1^2, PD-L1^2). The lack of PD-1 expression sets γδ T cells stimulated by mDCZA+ apart from invariant NKT cells stimulated by synthetic analogs of their natural ligands. Unlike γδ T cells, invariant NKT cells rapidly upregulate PD-1 expression after stimulation and retain this expression for at least 30 d (36). PD-1 expression has been implicated in the induction and maintenance of tolerance in a variety of settings, and interpreted as a marker of T cells unfitted to exert antitumor activity because of their functional exhaustion (37). Cabillic et al. (20) have also reported in solid tumors that γδ T cell proliferation can be reinstated in NR patients using ZA-treated DC.

The next step was to determine whether γδ T cells induced by mDCZA+ could improve immune responses against tumor-associated Ags mediated by CD8^+ αβ T cells not only in R MM, but also in those NR MM in which γδ T cell proliferation was reinstated. SRV was selected as a prototypic tumor-associated Ag because it is expressed by myeloma cells and contributes to their survival (38). SRV-specific T cells have been tolerized but not deleted from the MM T cell repertoire (23), and their effector functions can be reinstated by appropriate SRV presentation and T cell stimulation (39, 40). Moreover, SRV is also expressed by many other tumor cells of hematopoietic and epithelial origin (41). The frequency of SRV-specific CD8^+ αβ T cells in the peripheral blood of MM was higher than in CTRL, suggesting a previous exposure and selective recruitment in the early phases of the disease. Their frequency was significantly enhanced in both CTRL and MM after stimulation with mDCSRV+p+ZA+, leading to enhanced cytotoxicity against the appropriate HLA*0201+ SRV^+ target cells irrespective of the initial R/NR status. These data

![FIGURE 7. ZA downregulates PD-L2 expression in mDC and f-DC. A, Cell surface expression of PD-L2 on untreated (ZA-) or ZA-treated (ZA+) MC, mDC, and f-DC in CTRL (left panel) and MM (right panel). PD-L2 was expressed by a smaller proportion of resting MC and remained unchanged after ZA treatment, whereas it was expressed by the majority of mDC and f-DC under basal conditions, and this expression was uniformly downmodulated by ZA in both CTRL and MM. Downmodulation reached a statistical significance in CTRL mDC (p < 0.001) and f-DC (p < 0.05), and MM mDC (p < 0.05). Bars represent the mean values ± SE from five CTRL and five MM. B, Flow cytometry of PD-L1 and PD-L2 expression after mDC treatment with ZA, Sim, or their combination. Representative data from one of three experiments. PD-L2 expression was not modified by Sim (upper row, left panel), whereas it was downmodulated by ZA (upper row, center panel). Sim did not prevent ZA-induced PD-L2 downregulation (upper row, right panel). Sim and ZA did not affect PD-L1 expression, either alone or in association (lower row).](http://www.jimmunol.org/)

![FIGURE 8. Inhibition of γδ T cell expansion by Sim prevents the amplification of SRV-specific CD8^+ αβ T cells. A, Total counts of viable γδ T cells after 10-, 14-, and 20-d stimulation with untreated (Sim-) or Sim-treated (Sim+) mDC^SRV+p+ZA+. As expected, γδ T cell proliferation was abrogated by Sim treatment. Differences were statistically different on days 14 (p < 0.005) and 20 (p < 0.01). B, Total counts of viable SRV-specific CD8^+ αβ T cells after 10-, 14-, and 20-d stimulation with Sim- or Sim+ mDC^SRV+p+ZA+. As for γδ T cells, the expansion of SRV-specific CD8^+ αβ T cells was abrogated by Sim. Differences are statistically different on days 14 (p < 0.005) and 20 (p < 0.005). Bars represent the mean values ± SEM from three CTRL R.](http://www.jimmunol.org/)
indicate that mDC_{SRVp+ZA+} of CTRL and MM can handle the concurrent activation of autologous γδ T cells and tumor-specific CD8+ αβ T cells with a mutual edge on their immune performances. The final outcome of these reciprocally gainful interactions is the generation of a composite population of innate and adaptive antitumor effector cells, embodying both CM and EM γδ T cells and CD8+ αβ T cells.

The generation of conventional mDC_{ZA+} by standard protocols is a labor-intensive, time-consuming, and costly procedure that requires adequate cell inputs that, in turn, are dependent on sufficient blood samples. To corroborate the translational value of our findings, we conducted a subsequent series of experiments using DC generated according to an alternative protocol yielding mDC from CD14+ cells within only 48 h of in vitro culture (f-DC) (24, 42). This strategy has been adapted and validated for large-scale production under GMP conditions in patients with advanced prostate cancer (43), but the ability of f-DC to induce the proliferation of γδ T cells and concurrently activate MHC-restricted CD8+ αβ T cells is unknown. We have shown that f-DC can be successfully generated from CD14+ cells in both CTRL and MM R and NR, f-DC_{ZA+} were as effective as mDC_{ZA+} in inducing γδ T cell proliferation in CTRL and MM irrespective of their R/NR status. One round of stimulation and a 14-d incubation were sufficient to maximize the frequency of SRV-specific CD8+ cells, as shown by side-by-side experiments comparing mDC_{ZA+} and f-DC_{ZA+}. The cytotoxic function of these cells was confirmed by their ability to recognize and kill the appropriate HLA-A*0102+ SRV+ target cells, including primary PCL cells, after 14 d of stimulation by f-DC_{ZA+}. We selected this time period as the longest affordable with a single round of stimulation because Dauer et al. (42) and Jarnjak-Jankovic et al. (43) have shown that 5–7 d are sufficient for f-DC to induce primary and secondary Ag-specific T cell immune responses, and because the kinetics of SRV-specific CD8+ αβ T cell expansion significantly increased from days 10 to 14, but not from days 14 to 20 (see also Fig. 8B, Supplemental Fig. 4).

As stated earlier, γδ T cells are activated by IPP, an intermediate Mev metabolite mimicking their natural ligands (6, 8–10). Under basal conditions, the highest IPP production was observed in mDC and f-DC, the latter showing significantly higher values. As predicted, IPP concentrations further increased afterZA treatment, and the most striking increase was detected in the supernatants of f-DC_{ZA+} in which extracellular IPP concentrations were 10 times greater than those detected in MC_{ZA+} and mDC_{ZA+} supernatants. Unexpectedly, IPP concentrations in the supernatants were 1000 times greater than the intracellular ones. Thus, IPP is produced and released in the supernatant in sufficient amounts to induce γδ T cell proliferation, even in the absence of MC. These results are in line with previous studies showing that γδ T cell activation by exogenous IPP is TCR mediated and APC independent (2). We have also observed that exogenous IPP induces the proliferation of γδ T cells, but with a lower efficiency than f-DC_{ZA+} supernatants, especially if used at low concentrations and in the presence of MC. So far, most studies have investigated the activity of IPP at high concentrations on purified γδ T cells, γδ T cell lines, or γδ T cell clones, whereas few data are available for IPP at low concentrations on MC-containing populations. The lower efficiency of exogenous IPP compared with f-DC_{ZA+} supernatants, especially in PBMC, could reflect either an MC-mediated IPP sequestration to the disadvantage of γδ T cells or the presence of additional soluble factors in f-DC_{ZA+} supernatants. Studies are currently in progress to address this issue.

The importance of IPP in f-DC_{ZA+} supernatants was confirmed by the experiments in which Sim was used as an irreversibly and selective hydroxy-methylglutaryl-coenzyme A reductase inhibitor to prevent intracellular IPP accumulation (11, 30), and its release in the supernatants. Indeed, Sim treatment blunted the proliferative activation of γδ T cells induced by mDC_{ZA+} and f-DC_{ZA+} by setting almost to zero extracellular IPP concentrations.

Activated γδ T cells are enabled to behave as cellular adjuvants in several ways, including the expression of costimulatory receptors and appropriate cytokine production. The frequency of SRV-specific CD8+ αβ T cells was uniformly higher in cultures in which γδ T cells had proliferated, and this increase was mostly evident after γδ T cells had become the predominant population. These data confirm previous results in healthy individuals indicating that γδ T cell proliferation anticipates and is not detrimental to the subsequent increase of Ag-specific CD8+ αβ T cells (11, 14). Activated CM and EM γδ T cells are enabled to behave as cellular adjuvants in several ways, including the expression of costimulatory receptors and the production of IFN-γ, which promotes the functional maturation of DC and the development of antitumor cell-mediated immune responses (14, 23, 43, 44). mDC_{ZA+} and f-DC_{ZA+} themselves may contribute to Th1 polarization, because we have previously shown that mDC_{ZA+} produce more IL-12 and less IL-6 compared with mDC_{ZA+} (11).

γδ T cells antagonized the IL-2–induced proliferation of Tregs. Low-dose IL-2 is indispensable to sustain ZA-induced γδ T cell proliferation, but it is also the main growth factor for Tregs (32), and mDC from MM patients are intrinsically inclined to promote the growth of Tregs (21, 22). Our results demonstrate that γδ T cell proliferation induced by PBMC_{ZA+}, mDC_{ZA+}, and f-DC_{ZA+} uniformly restrained IL-2–induced Treg expansion. Gong et al. (45) have recently shown a similar anti-Treg activity in PBMC from nonhuman primates after proliferative expansion of γδ T cells induced by BrHPP and IL-2, provided that MC and/or other accessory cells were included in the cultures. A new finding from our experiments was that ZA counteracted Treg independently from γδ T cell proliferation. Lower Treg counts were detected in MM NR after stimulation of PBMC with ZA+IL-2 compared with IL-2 alone, even if γδ T cell proliferation did not occur in these patients. Likewise, PBMC stimulation with ZA alone, which is not sufficient to induce γδ T cell proliferation, yielded significantly lower Treg counts compared with medium alone. ZA also exerted a functional inhibition of Treg activity, as shown by the experiments in which ZA was added to Tregs and autologous CD4+CD25+ T cells stimulated by OKT3 in the presence of irradiated accessory cells. No proliferative expansion of γδ T cells was observed in these experiments because cells were cultured for 3 d only, and IL-2 was not added to the cultures, further supporting the ability of ZA to counteract Treg activity in the absence of γδ T cell proliferation. Moreover, Tregs were functionally impaired even when they were pretreated for 24 h and washed free of ZA before mixing with autologous CD4+CD25+ T cells, suggesting that ZA can directly antagonize Tregs in the absence of γδ T cells. Interestingly, Sim treatment did not abrogate this anti-Treg activity, indicating that FPPS inhibition and intracellular IPP accumulation are unlikely to play a role.

We did not disclose major differences in Treg counts and sensitivity to IL-2 stimulation between MM R and NR, though an excess of Treg over γδ T cells has been proposed as a mechanism determining the NR status of MM and other cancer patients (17). Any role of Treg in this setting is further complicated by the discordant data about their counts and functional status in MM, because both increased numbers of dysfunctional Treg, and normal numbers of immune-competent cells have been reported (46).

An unexpected finding was PD-L2 downregulation on mDC_{ZA+} and f-DC_{ZA+}. Tumor cells and pathogens exploit the PD-1/PD-L
pathway to evade host immunity, and several in vitro and in vivo data indicate that downregulation of the PD-1/PD-L1 pathway may be a profitable way of breaking tolerance and restoring effective Ag-specific immune responses (47). Most studies have focused on the inhibition of PD-L1 solely by means of blocking Abs. To the best of our knowledge, this is the first report of pharmacological downmodulation of PD-L2 ever reported. Like Treg antagonism, this downregulation was not abrogated by Sim, indicating that ZA has immune modulatory activities not solely dependent on its ability to induce intracellular IPP accumulation and γδ T cell proliferation. It has recently been reported that the antiangiogenic activity of ZA can also be independent from its ability to target the Mev pathway (48).

In conclusion, we have deciphered a virtuous cross-talk between γδ T cells, Tregs, DC, and CD8 αβ T cells that is fostered by the proliferative activation of γδ T cells initiated by the large amounts of IPP generated by ZA-treated mDC and f-DC. Ancillary to IPP generation are the ZA’s abilities to antagonize Tregs and downregulate PD-L2 on DC. These ancillary activities are independent from Mev pathway inhibition and γδ T cell proliferation. Altogether, these immunomodulatory events can reestablish γδ T cell proliferation and upregulate SRV-specific immune responses in MM patients irrespective of their initial NR status. These data may help us to further understand the role of γδ T cells as cellular bridges between innate and adaptive immunity, and to fine-tune the development of γδ T cell-based adaptive immunotherapy strategies in MM and other diseases.

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References


Supplemental Table 1: Immunophenotype comparison of mDC and f-DC in CTRL and MM patients

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Frequency of SRV-specific CD8+ αβ T cells in the peripheral blood of CTRL and MM. Detection of SRV-specific CD8+ αβ T cells in the peripheral blood of MM patients and healthy donors, determined by flow cytometry with the PE-labeled A*0201/SRV pentamers gated on viable CD8+/CD19- cells. (A) Analysis of one representative CTRL and MM. (B) Pooled data. Bars represent the mean ± SEM of 8 experiments. The frequency of SRV-specific CD8+ αβ T cells was significantly higher in MM (0.41 ± 0.05% vs 0.07 ± 0.03%, p<.0001).
Cytotoxicity of SRV-specific CD8+ αβ T cells against the HLA-A2+ SRV+ RPMI 8226 myeloma cell line.

Cytotoxicity was evaluated in CTRL and MM after 20 days and 2 rounds of stimulation with autologous mDC^{SRVp-ZA-}, mDC^{SRVp-ZA+}, mDC^{SRVp+ZA-}, and mDC^{SRVp+ZA+}. The highest cytotoxicity was observed when T cells, stimulated with mDC^{SRVp+ZA+}, included the highest frequency of SRV-specific CD8+ αβ T cells.
Supplemental Figure 3

Endocytic capability of \( f-DC \) under basal conditions and after ZA treatment.

Endocytosis was evaluated after incubation with FITC-dextran for 2 hours at 4°C (white histograms) or 37°C (grey histograms). ZA treatment did not affect either CTRL or MM.
**mDC^{ZA+} and f-DC^{ZA+} are equally effective**

(A) Head-to-head comparison between the ability of mDC^{ZA+} and f-DC^{ZA+} to stimulate γδ T cells and induce the expansion of SRV-specific CD8⁺ αβ T cells. Bars represent the mean ± SEM of 3 experiments in CTRL R. The total number of γδ T cells is not statistically different either on day 10 (after one round) or day 20 (after two rounds) (left panel), whereas the number of SRV+ CD8+ cells per well tends to be higher after stimulation with f-DC (the difference on day 10 is statistically significant).

(B) Head-to-head comparison between total numbers of γδ T cells and SRV-specific CD8⁺ T cells per well after two 10-day stimulations as opposed to only one of 14 days. Bars represent the mean ± SEM of 3 experiments in CTRL R. Results indicate that the approaches are superimposable.
Supplemental Figure 5

ZA-induced Treg antagonism. (A) Flow cytometry of Treg cells after PBMC stimulation in representative MM R and NR. (B) The ability of ZA to suppress the inhibitory function of Treg cells is still detectable when they are pretreated with 5 μM ZA for 24 hours and washed free of ZA before mixing with CD4+CD25- cells (ZA-pretreated). For comparison, ZA was also added to cocultures for 3 days as in Figure 6B (see manuscript). Results are from one representative out of two experiments. (C) Sim treatment does not affect the ability of ZA to restrain the inhibitory function of Treg cells. Results are from one representative out of two experiments.