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Immune Modulation by Zoledronic Acid in Human Myeloma: An Advantageous Cross Talk between $V_{\gamma}9V_{\delta}2$ T Cells, $\alpha\beta$ CD8+ T Cells, Regulatory T Cells, and Dendritic Cells

Barbara Castella,* Chiara Riganti,* Francesca Fiore,*‡ Francesca Pantaleoni,* Maria Elisa Canepari,* Silvia Peola,* Myriam Foglietta,*‡ Antonio Palumbo,‡ Amalia Bosia,*† Marta Coscia,*‡ Mario Boccadoro,‡ and Massimo Massaia*‡

$V_{\gamma}9V_{\delta}2$ 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 $\gamma\delta$ T cells and DC. ZA-treated MM DC were highly effective in activating autologous $\gamma\delta$ T cells, even in patients refractory to stimulation with ZA-treated monocytes. ZA inhibited the mevalonate pathway of MM DC and induced the intracellular accumulation and release into the supernatant of isopentenyl pyrophosphate, a selective $\gamma\delta$ T cell activator, in sufficient amounts to induce the proliferation of $\gamma\delta$ T cells. Immune responses against the tumor-associated Ag survivin (SRV) by MHC-restricted, SRV-specific CD8+ $\alpha\beta$ T cells were amplified by the concurrent activation of $\gamma\delta$ T cells driven by autologous DC copulsed with ZA and SRV-derived peptides. Ancillary to the isopentenyl pyrophosphate-induced $\gamma\delta$ T cell proliferation was the mevalonate-independent ZA ability to directly antagonize regulatory T cells and downregulate PD-L2 expression on the DC cell surface. In conclusion, ZA has multiple immune modulatory activities that allow MM DC to effectively handle the concurrent activation of $\gamma\delta$ T cells and MHC-restricted CD8+ $\alpha\beta$ antitumor effector T cells.

A key role is played by $\gamma\delta$ T cells in innate immune responses against microbes, stressed cells, and tumor cells (1). Most circulating $\gamma\delta$ T cells use the same TCR V region pair $V_{\gamma}9-V_{\delta}2$ ($V_{\gamma}9V_{\delta}2$ T cells, hereinafter referred to as $\gamma\delta$ T cells) to sense the presence of infectious agents via the recognition of intermediate metabolites produced by the microbial nonmevalonate (non-Mev) pathway of isoprenoid synthesis (collectively termed phosphoantigens) (2, 3). Synthetic phosphoantigen analogs, such as bromohydrin pyrophosphate (BrHPP), have been generated to selectively stimulate $\gamma\delta$ T cells and clinically exploit their effector functions against pathogens and tumor cells (4, 5).

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proliferation can be reinstated in NR patients with solid tumors using ZA-treated DC. However, MM DC are inclined to stimulate T cells with inhibitory functions (21, 22), and whether they can reinstate 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 Sturania). 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 unfractonated 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 t-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Flow cytometry

The following mAbs were used: anti-CD3, anti-CD8, anti-CD4, anti-CD25, anti–TCR-V, anti–TCR-Vγ, anti–TCR-Vδ, anti–CD3ε, anti-CD80, anti–HLA-DR (Becton Dickinson, San Jose, CA); anti-CD3, anti-CD14 (Dako SpA, Milano, Italy); anti-CD38 (BD Pharmingen International, San Diego, CA), anti-CD86 (Chemicon, Chandler Ford, Hampshire, U.K.), anti–HLA-A2 (Proimmune, Oxford, U.K.); anti-CD1a, CD45RA (Valter Occhiena, Torino, Italy), anti-CD27 (Accell, Byport, MN); anti–FOXp3, anti–CD279 (BD Biosciences, San Diego, CA). Two- and three-color flow cytometry were performed with the appropriate combinations of FITC, r–PE– (PE), Tricolor– (Tri), PerCP 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 μg/ml anti-Foxp3 FITC or isotype CTRL mAb. After washes with Perm/Wash buffer, cells were fixed with 250 μl 1% formaldehyde-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 of 5 μM ZA (MC-Za: 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 (iDC-Za+, mDC-Za+) or absence of 5 μM ZA (iDC-Za-, mDC-Za-), as previously reported (11, 22).

Fast DC (f-DC) were generated according to Dauer et al. (24). Purified CD14+ cells were cultured in standard culture medium at 0.5–1.5×10^6 cells/ml supplemented with GM-CSF (1000 U/ml) and IL-4 (500 U/ml) in flat-bottom 6-well plates for 24 h. After washing, they were incubated for a further 24 h with IL-1β (10 ng/ml), TNF-α (1000 U/ml), IL-6 (10 ng/ml), and PGE2 (1 μg/ml) in the presence (f-DC-Za+, mDC-Za+) or absence (f-DC-Za-+) of 5 μM ZA.

APC were always washed before mixing with autologous PBL or T cells. In selected experiments, supernatants were collected for quantification of extracellular IPP 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), as previously reported (9).

Cryopreserved or freshly isolated PBL were added at the ratio of 5:1 to autologous iDC/mDC or MC-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 cells and Tregs were calculated with the trypan blue staining assay and by flow cytometry, as previously reported (11, 25).

In selected experiments, PBMC and PBL from CTRL R were stimulated for 7 d with the supernatants obtained from autologous f-DC-Za+ and f-DC-Za- and 10 IU/ml IL-2 to evaluate their ability to induce γδ T cell proliferation.

Modulation of SRV-specific immune responses

The HLA-A*0201+–restricted, SRV-derived peptide LMLGEFLKL (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 modulation 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), mixed 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 mAbs were added after pentamer staining, and a minimum number of 500,000 events was acquired after backgating on viable CD8+ CD19- cells.

f-DC were also left untreated or pulsed with SRVp, and incubated with f-DC-Za+ or without 5 μM ZA (f-DC-Za-) 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, SRV-p-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-blocked CMV- (NLVPVMATV; Proimmune) and HIV-derived (SLYNTVATL; Proimmune) peptides (CMVp, HIVp) to load f-DC-Za+ and f-DC-Za-, 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 Mev pathway in APC

MC, mDC, and f-DC were incubated at 1×10^6/ml for 24 h with 1 μCi [3H] acetate (3600 mCi/mmol; 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 IPP 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 NaVO₄ and centrifuged at 1200 × g for 5 min at 4°C. After lyophilization under vacuum, samples were resuspended in 20 µl dimethylsulfoxide 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 [¹⁴C]cholesterol (Amersham), [¹⁴C]larnsey pyrophosphate (Perkin Elmer), and [¹⁴C] IPP (Perkin Elmer), and counting them with liquid scintillation. The radioactivity (in terms of cpn) 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 × 10⁶ cells for cholesterol, IPP, and intracellular IPP; and as pmol/1 × 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, CD36, and CD56 Ags. CD25⁺ cells were then selected by positive selection with magnetic beads directly conjugated to an anti-CD25 Ab. From 5 × 10⁶ to 1.8 × 10⁷ CD4⁺/CD25⁺ cells (purity 76–95%) were obtained fromuffy coats containing each from 200–300 × 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 × 10⁶/well) or in combination (1:1 ratio) in U-bottom 96-well plates in the presence or absence of ZA (Fig. 1). The highest proliferation in a susceptible population. As expected, no significance cutoff value was p < 0.05 compared with cytotoxic values after stimulation with mDCSRVp+ZA⁺, mDCSRVp+ZA⁻, mDCSRVP+ZAb-αβ, and mDCSRVP+ZAb+. Results from representative 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 mDCZAb⁺, R and NR individuals were grouped together in CTRL and MM. Total counts were significantly higher after stimulation with mDCSRVp+ZAb compared with stimulation with mDCSRVp+ZAb⁻ (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+ZAb in both CTRL and MM (p < 0.05 compared with cytotoxic values after stimulation with mDCSRVp+ZAb⁻ in both groups) (Fig. 2D). Similar results were observed with RPMI 8226, a myeloma-derived SRV⁺ HLA-A2⁺ cell line (Supplemental Fig. 2).

**Results**

### mDCZAb⁺ 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 PMBC 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 mDCZAb⁺, we performed a side-by-side experiment with PMBCZAb⁺ and MCZAb⁺ in the two NR groups. mDCZAb⁺ restored γδ T cell proliferation in both groups (p < 0.0003; Fig. 1C). Flow cytometry of γδ T cells after stimulation with PBMCZAb⁺ and mDCZAb⁺ in one representative MM NR is shown in Fig. 1D.

Immunophenotyping showed that mDCZAb⁺ 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; Supplemental Fig. 1). The frequency and total counts of SRV-specific CD8⁺ αβ T cells was then investigated after stimulation with mDCSRVp+ZAb⁻, mDCSRVp+ZAb⁺, and mDCSRVP+ZAb⁺. Results from representative 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 mDCZAb⁺, R and NR individuals were grouped together in CTRL and MM. Total counts were significantly higher after stimulation with mDCSRVp+ZAb compared with stimulation with mDCSRVp+ZAb⁻ (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 (T2SRV⁺). As expected, the highest cytotoxicity was observed when T cells were stimulated with mDCSRVp+ZAb in both CTRL and MM (p < 0.05 compared with cytotoxic values after stimulation with mDCSRVp+ZAb⁻ in both groups) (Fig. 2D). Similar results were observed with RPMI 8226, a myeloma-derived SRV⁺ HLA-A2⁺ cell line (Supplemental Fig. 2).

**f-DCZAb⁺ are as effective as conventional mDCZAb⁺ 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-DCZAb⁺ 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-DCZAb⁻) and after (f-DCZAb⁺) 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-DCZAb⁺ and mDCZAb⁺ 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-DCZAb⁺. 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-DCZAb⁺ 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-DCZAb⁺ 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 gd T cells and CD8+ ab T cells is demonstrated in Fig. 3E. CTRL T cells were stimulated with f-DCZA+ and f-DC+zA+ loaded or unloaded with the HLA-A2-restricted SRVp, CMVp, and HIVp. The frequency of specific CD8+ ab T cells was determined with the corresponding PE-labeled pentamers. As expected, only CD8+ ab T cells specific for the peptide used to load f-DC+zA+ and f-DC+zA+ were increased after 14-d incubation. Pooled data reporting total counts of SRV-specific CD8+ ab 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-DC+SRP+zA+ in both CTRL and MM.

Fig. 3H shows that CD8+ ab T cells elicited by f-DC+SRP+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-
Increased 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, the rate-limiting enzyme in the Mev pathway acting upstream from FPPS (29, 30), 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 when used at higher concentrations than those in the supernatants of ZA-treated MC and mDC.

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 when used at higher concentrations than those in the supernatants of ZA-treated MC and mDC.

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 x 10^6/ml with 2 x 10^5 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-DCZA+ and f-DCZA- loaded or unloaded 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-DCZA+ and f-DCZA- 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-DCZA+, 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
Sim almost set to zero extracellular IPP concentrations (Fig. 5C) and the supernatant 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 re straps 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 cocultured 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...
counts were evaluated in MM R and NR after stimulation with ZA-treated and untreated mDC and f-DC (Fig. 6C). As expected, ZA induced the proliferation of γδ T cells, and this was uniformly associated with lower Treg counts in both MM R and NR. To investigate whether ZA could also antagonize Tregs independently of γδ T cell proliferation in these cultures, we evaluated Treg counts on day 7 after stimulation with f-DCZA+ and f-DCZA− in the presence or absence of Sim. Sim alone had no effect on Tregs and did not impair the ability of ZA to antagonize Tregs (Fig. 6D, upper panels) even if Sim completely abrogated γδ T cell proliferation (Fig. 6D, lower panels). Altogether, these results indicate that ZA antagonizes Tregs independently of γδ T cell proliferation and Mev pathway inhibition.

**ZA downregulates PD-L2 expression in mDC and f-DC**

PD-L1 and PD-L2 expression was evaluated in MC, mDC, and f-DC under basal conditions and after ZA treatment. PD-L1 was constitutively expressed on MC, mDC, and f-DC, and its expression was not modified by ZA (data not shown, Fig. 7B). PD-L2 was expressed by a smaller proportion of resting MC and was not changed after ZA treatment (Fig. 7A). By contrast, PD-L2 was expressed by most mDC and f-DC under basal conditions, and this expression was significantly downmodulated by ZA in CTRL mDC (p < 0.001) and f-DC (p < 0.05), and MM mDC (p < 0.05; Fig. 7A).

Sim alone had no effect on PD-L2 expression and did not prevent ZA-induced PD-L2 downregulation (Fig. 7B, upper row). It also had no effect on the expression of PD-L1, whether alone or in association with ZA (Fig. 7B, lower row). These data indicate that ZA modulates the cell surface immunophenotype of mDC and f-DC independently from the Mev pathway.

**Inhibition of γδ T cell expansion by Sim prevents the amplification of SRV-specific CD8+ αβ T cells**

The generation of SRV-specific CD8+ αβ T cells after stimulation with mDCSRVp+ZA+ was evaluated in the absence or presence of Sim. As reported earlier, Sim prevented the IPP-dependent proliferation of γδ T cells (Fig. 8A), which, in turn, was associated with a failed amplification of SRV-specific CD8+ αβ T cells (Fig. 8B). These results provide indirect evidence that activated γδ T cells can improve adaptive immune responses.

**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 and DC (9, 15–19).

The first series of experiments was deliberately confined to CTRL and MM R to identify the APC most capable of inducing the proliferation of autologous \( \gamma \delta \) T cells. mDC\(^{ZA}+\) generated from MM R were even more efficient than their normal counterparts in inducing this proliferation. After 7-d stimulation, there was a preferential expansion of CM and EM \( \gamma \delta \) T cells in MM R patients that is impossible to increase the number of cells and establish a favorable E:T cell ratio in vivo or ex vivo.

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 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 mDC\(^{ZA}+\), 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. mDC\(^{ZA}+\) were very effective in both CTRL and MM NR, and resulted in the proliferative expansion of...
T cells phenotypically indistinguishable from that observed in CTRL and MM (HLA-DR+, CD80+, CD86+, PD-1\(^2\), PD-L1\(^2\)).

The lack of PD-1 expression sets \(\gamma\delta\) T cells stimulated by mDCZA+ apart from invariant NKT cells stimulated by synthetic analogs of their natural ligands. Unlike \(\gamma\delta\) 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 \(\gamma\delta\) T cell proliferation can be reinstated in NR patients using ZA-treated DC. The next step was to determine whether mDCZA+ could improve immune responses against tumor-associated Ags mediated by CD8\(^+\) T cells not only in R MM, but also in those NR MM in which \(\gamma\delta\) 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\(^+\) 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 mDCSRVp+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 \(\pm\) 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 \(\gamma\delta\) T cell expansion by Sim prevents the amplification of SRV-specific CD8\(^+\) T cells. A, Total counts of viable \(\gamma\delta\) T cells after 10-, 14-, and 20-d stimulation with untreated (Sim-) or Sim-treated (Sim+) mDC\(^{\text{SRVp+ZA+}}\). As expected, \(\gamma\delta\) 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\(^+\) T cells after 10-, 14-, and 20-d stimulation with Sim- or Sim+ mDC\(^{\text{SRVp+ZA+}}\). As for \(\gamma\delta\) T cells, the expansion of SRV-specific CD8\(^+\) 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 \(\pm\) SEM from three CTRL R.](http://www.jimmunol.org/)
indicate that mDCSRV+ZAs 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 mDCZAs 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-DCZAs were as effective as mDCZAs 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 mDCZAs and f-DCZAs. 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-DCZAs. 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 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 after ZA treatment, and the most striking increase was detected in the supernatants of f-DCZAs in which extracellular IPP concentrations were 10 times greater than those detected in MCZAs and mDCZAs 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-DCZAs 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-DCZAs 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-DCZAs supernatants. Studies are currently in progress to address this issue.

The importance of IPP in f-DCZAs supernatants was confirmed by the experiments in which Sim was used as an irreversible 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 mDCZAs and fDCZAs 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 maturation of DC and the development of antitumor cell-mediated immune responses (14, 23, 43, 44). mDCZAs and f-DCZAs themselves may contribute to Th1 polarization, because we have previously shown that mDCZAs produce more IL-12 and less IL-6 compared with mDCZAs (11). γδ T cells antagonized the IL-2–induced proliferation of Tregs. Low-dose IL-2 is indispensable to sustainZA-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 PBMCZAs, mDCZAs, and f-DCZAs 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 mDCZAs and f-DCZAs. Tumor cells and pathogens exploit the PD-1/PD-L
pathway to evahe host immunem, and several in vitro and in vivo data indicate that downregulation of the PD-1/PD-L 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 Mvf pathway (48).

In conclusion, we have deciphered a virtuous cross talk between γδ T cells, Tregs, DC, and CD8 γδ 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 Mvf pathway inhibition and γδ T cell proliferation. Altogether, these immunomodulatory events can reinstate γδ T cell proliferation and upregulate SRY-speciﬁc immune responses in MM patients irrespective of their initial NR/R 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 deﬁne the development of γδ T-cell based adaptive immunotherapy strategies in MM and other diseases.

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Disclosures

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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.
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.
**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.