ORIGINAL ARTICLE

JBMR

Monocytes and γδ T Cells Control the Acute-Phase Response to Intravenous Zoledronate: Insights From a Phase IV Safety Trial

Joanne L Welton,[1] Matt P Morgan,[1] Salvador Martí,[1] Michael D Stone,[2] Bernhard Moser,[1] Andrew K Sewell,[1] Jane Turton,[2] and Matthias Eberl[1]

[1]Cardiff Institute of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom
[2]Bone Research Unit, Cardiff University Academic Centre, Llandough Hospital, Penarth, United Kingdom

ABSTRACT

Aminobisphosphonates (NBPs) are used widely against excessive bone resorption in osteoporosis and Paget's disease as well as in metastatic bone disease and multiple myeloma. Intravenous NBP administration often causes mild to severe acute-phase responses (APRs) that may require intervention with analgesics and antipyretics and lead to treatment noncompliance and nonadherence. We here undertook a phase IV safety trial in patients with osteoporosis to investigate the APR of otherwise healthy individuals to first-time intravenous treatment with the NBP zoledronate. This study provides unique insight into sterile acute inflammatory responses in vivo, in the absence of confounding factors such as infection or cancer. Our data show that both peripheral γδ T cells and monocytes become rapidly activated after treatment with zoledronate, which ultimately determines the clinical severity of the APR. Our study highlights a key role for IFN-γ in the zoledronate-induced APR and identifies pretreatment levels of monocytes and central/memory Vγ9/Vδ2 T cells as well as their responsiveness to zoledronate in vitro as predictive risk factors for the occurrence of subclinical and clinical symptoms. These findings have diagnostic and prognostic implications for patients with and without malignancy and are relevant for Vγ9/Vδ2 T-cell–based immunotherapy approaches. © 2013 American Society for Bone and Mineral Research.

KEY WORDS: AMINOBISPHOSPHONATES; γδ T CELLS; ACUTE-PHASE RESPONSE; OSTEOPOROSIS; IMMUNOTHERAPY

Introduction

There is increasing evidence that γδ T cells play a key role in orchestrating and regulating immune responses in humans and in animal models.[1] Our own recent findings demonstrate that a rapid crosstalk of human γδ T cells and monocytes drives acute inflammatory responses, which may contribute to pathogen clearance and protective immunity but may also lead to tissue damage and poor clinical outcome.[2,3] For reasons not yet understood, human γδ T cells differ fundamentally from those found in nonprimate species, and hence no small animal model replicates the complex interactions between γδ T cells and other immune and nonimmune cells in the human body.[4,5]

Nitrogen-containing bisphosphonates, or aminobisphosphonates (NBPs), are effective drugs against excessive bone resorption in osteoporosis, Paget's disease, metastatic bone disease, and multiple myeloma. Despite their overall safety, NBP therapy is frequently associated with mild to severe inflammatory events, which may require intervention with analgesics and antipyretics and lead to treatment noncompliance and nonadherence.[6,7] Treatment with intravenous NBPs such as pamidronate (Aredia; Novartis, Basel, Switzerland) and zoledronate (Aclasta/Zometa; Novartis) may cause systemic acute-phase responses (APRs) characterized by fever, pain, nausea, and fatigue in up to 50% of all patients within 48 hours after administration. These flu-like symptoms are typically transient, resolve spontaneously, and are accompanied by decreased lymphocyte counts and elevated levels of the pro-inflammatory cytokines IL-6, IFN-γ, and TNF-α.[8–11] The APR upon intravenous treatment with NBPs is most severe in first-time treated patients, whereas subsequent further administration induces no APR

Received in original form June 11, 2012; revised form October 2, 2012; accepted October 8, 2012. Accepted manuscript online Ocober 16, 2012.
Address correspondence to: Jane Turton, MB, ChB, Cardiff and Vale University Health Board, Bone Research Unit, Cardiff University Academic Centre, Llandough Hospital, Penlan Road, Penarth, Vale of Glamorgan CF64 2XX, United Kingdom. E-mail: turtonj@cf.ac.uk. Matthias Eberl, PhD, Cardiff Institute of Infection and Immunity, Henry Wellcome Building, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, United Kingdom. E-mail: eberlm@cf.ac.uk
JT and ME contributed equally to this work.
Additional Supporting Information may be found in the online version of this article.

Journal of Bone and Mineral Research, Vol. 28, No. 3, March 2013, pp 464–471
DOI: 10.1002/jbmr.1797

symptoms at all or an APR with much milder outcome than at first exposure. For instance, in the HORIZON trial, net APR rates were 30%, 7%, and 3% after zoledronate (ZOL) infusions 1 to 3, respectively.[11,12] The immunological basis for this "tolerance" to repeated treatment with NBPs is not known.

Kunzmann and colleagues were the first to ascribe a role for γδ T cells in the NBP-induced APR.[13] Subsequent cell culture–based studies have elegantly demonstrated that NBPs are potent stimulators of Vγ9/Vδ2$^+$ γδ T cells in vitro.[14–18] To act on Vγ9/Vδ2 T cells, NBPs depend on uptake by monocytes and other endocytically active cell types, in which they inhibit farnesyl pyrophosphate synthase (FPPS), a key enzyme in the biosynthesis of sterols, ubiquinones, and other isoprenoids via the mevalonate pathway. Preferential uptake by osteoclasts and subsequent inhibition of FPPS is the prime mechanism of action in the NBP-mediated prevention of bone resorption. However, FPPS inhibition in osteoclasts, monocytes, and other cells also leads to intracellular accumulation of upstream metabolites including dimethylallyl pyrophosphate (DMAPP), isopentenyl pyrophosphate (IPP), and an ATP conjugate of IPP (Apppl), which may function as "danger" signals and be sensed by Vγ9/Vδ2 T cells via a largely unknown mechanism.[19]

Despite the wealth of data from in vitro experiments, there has been a paucity of studies addressing the cellular events in vivo in NBP-treated patients. We here wished to study the physiological consequences of the human γδ T-cell interaction with monocytes in vivo and provide unique insight into purely γδ T cell–mediated responses in the absence of confounding factors, by investigating the immune response of otherwise healthy individuals with osteoporosis to first-time administration of intravenous ZOL. During the revision process of the present study, Kalyan and colleagues reported the presence of circulating monocytes with increased forward scatter in ZOL-treated osteoporosis patients, yet did not characterize these cells further nor give any indication as to the time frame of this response.[20] Our own findings show that both γδ T cells and monocytes become rapidly activated after treatment with ZOL, thus providing proof-of-concept for the crosstalk of both cell types in vivo. Moreover, our study highlights a key role for IFN-γ in the NBP-induced APR and identifies pretreatment levels of monocytes and Vγ9/Vδ2 T cells as well as the proportion of central/memory T_{CM} cells within the Vγ9/Vδ2 T-cell population and their in vitro responsiveness to ZOL as predictive risk factors.

Materials and Methods

Patients

This study was approved by the South East Wales Local Ethics Committee under reference number 10/WSE04/52, EudraCT number 2009-017369-47, and conducted according to the principles expressed in the Declaration of Helsinki and under local ethical guidelines. All patients provided written informed consent. The study cohort comprised 19 healthy nonsmoking adult females with postmenopausal osteoporosis and a bone density *T*-score of −2.5 or worse at either total spine, total hip, or neck of femur when measured by dual-energy X-ray absorptiometry (DXA). The mean age was 68 years (range 57 to 79 years). All study participants were NBP naïve and attended outpatient appointments at Cardiff Royal Infirmary for first-time infusion of 5 mg ZOL (Aclasta). Inclusion criteria included no contraindications to treatment with intravenous NBPs; normal creatinine clearance levels of >35 mL/min; and normal vitamin D levels of 30 to 100 μg/L. Exclusion criteria included a body temperature >38.5°C at first visit; participation in another therapeutic trial within 20 days of consent; history of illness that might compromise participation such as drug or alcohol abuse; hypocalcaemia (<2.2 mmol/L corrected); and current use of oral steroids or other immunosuppressive agents. Vital signs such as resting pulse, resting blood pressure, and oral temperature and APR symptoms were recorded before treatment (day 0) and on days 1, 3, and 9 post infusion; cumulative APR scores (0 to 4) comprised one or several of the following symptoms for at least 24 hours after treatment: fatigue, muscle pain, headache, and/or joint pain. Blood parameters recorded included white blood cell (WBC) counts, erythrocyte sedimentation rates (ESR), and plasma levels of C-reactive protein (CRP) as markers of inflammation. From all patients, 15 mL of blood were drawn on each visit; PBMCs were isolated using Lymphoprep (Axis-Shield, Dundee, United Kingdom) and used directly for multicolor flow cytometric analyses or stored in liquid nitrogen for later stimulation assays.

Flow cytometry

Freshly isolated PBMCs were stained using monoclonal antibodies against CD3 (SK7 and UCHT1), CD4 (SK3), CD8 (HIT8a), CD25 (M-A251), CD27 (M-T271), CD56 (B159), CD69 (FN50), and HLA-DR (L243) from BD Biosciences, Oxford, United Kingdom; Vγ9 (Immu360) and CD40 (MAB89) from Beckman Coulter, High Wycombe, United Kingdom; NKG2D (1D11), CD14 (61D3), CD19 (SJ25C1), CD45RA (HI100), and CD80 (2D10.4) from eBioscience, Hatfield, United Kingdom, together with appropriate isotype controls. Cells were acquired on an eight-color FACSCanto II (BD Biosciences) and analyzed with FloJo 7.6 (TreeStar, Ashland, OR, USA). Leukocyte populations were identified based on their appearance in side scatter and forward scatter area/height, exclusion of live/dead staining (fixable Aqua; Invitrogen, Paisley, United Kingdom), and surface staining: CD3$^-$CD56$^+$ NK cells, CD3$^-$CD14$^+$ monocytes, CD3$^-$CD19$^+$ B cells, and CD3$^+$ T cells. T-cell subsets were identified as CD3$^+$CD4$^+$CD8$^-$Vγ9$^-$ helper T cells, CD3$^+$CD4$^-$CD8$^+$Vγ9$^-$ cytotoxic T cells, CD3$^+$CD56$^+$Vγ9$^-$ NKT cells, and CD3$^+$Vγ9$^+$ γδ T cells.

Plasma analysis

Plasma samples were collected before PBMC separation and stored at −80°C. At the end of the study, all samples were analyzed together on a SECTOR Imager 600 (Meso Scale Discovery, Rockville, MD, USA) for IL-1β, IL-2, IL-6, IL-10, IL-12p70, CXCL8, GM-CSF, IFN-γ, and TNF-α (Human Pro-Inflammatory 9-Plex Assay Ultra-Sensitive Kit; Meso Scale Discovery). In addition, CXCL10 and IL-17 were measured on a Dynex MRX II reader, using conventional ELISA kits (R&D Systems, Abingdon, United Kingdom).

Cell culture

The medium used was RPMI-1640 with 2 mM L-glutamine, 1% non-essential amino acids, 50 μg/mL penicillin/streptomycin, and 10% fetal calf serum (Invitrogen). Frozen PBMC samples were defrosted and cultured for 24 hours in medium or with 10 μM ZOL (Zometa). Activation of Vγ9/Vδ2 T cells was analyzed by flow cytometry using antibodies against CD3, Vγ9, and CD69; levels of IFN-γ were measured on a Dynex MRX II reader, using conventional ELISA kits (R&D Systems).

Statistical analysis

Differences between groups were analyzed using paired Student's *t* tests for normally distributed data or Wilcoxon signed-rank matched pairs for nonparametric data using GraphPad Prism 4.03 software. Advanced statistical analyses were performed using SPSS 18.0. Differences between IFN-γ levels in groups with pretreatment frequencies of Vγ9/Vδ2 T cells above or below the mean were analyzed using independent *t* tests. Pearson's correlations were used to assess any relationships between variables; nonparametric variables not passing the Shapiro-Wilk test were log-transformed as done for plasma levels of IFN-γ and TNF-α on day 1 and all IL-6 levels. Predictive biomarkers were assessed using linear regression; statistically significant ($p < 0.05$) variables from univariate analyses were included in multiple regression analyses based on backward selection. All statistical tests were two-tailed. Box-and-whisker plots depict minimum, 25th percentile, median, 75th percentile, and maximum values; arrows in Fig. 5 and Supplemental Figs. S1 and S2 depict significant correlations as assessed by Pearson's correlations and/or regression analyses as specified in the figure legends. Asterisks indicate statistically significant differences to pretreatment values as indicated in the table and the figures: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Results

First-time intravenous administration of ZOL causes APRs in osteoporosis patients

Upon first-time intravenous treatment with ZOL, the majority of patients experienced at least one APR symptom on day 1 (12/19) and day 3 (15/19) after treatment, two-thirds of which experienced at least two APR symptoms on day 1 (7/19) and day 3 (10/19). Nine days after treatment, 7/19 patients still had an APR score of ≥ 1. In line with the occurrence of APR symptoms, oral temperatures and pulse rates of patients were elevated on day 1 (Fig. 1). These changes were accompanied by a temporary drop in WBC ($p < 0.05$) and a concomitant rise in ESR ($p < 0.01$) on day 3 compared with baseline (not shown). Plasma levels of CRP were elevated on days 1 and 3 but returned to baseline by day 9 in most patients. Of note, all patients (19/19) showed considerable increases in CRP levels by day 3, and increases in CRP levels from baseline to days 1 and 3 correlated well with cumulative APR scores on days 1 and 3 (Table 1). These findings indicate not only that plasma CRP accurately reflected the severity of the APR but also that all patients showed an objective response to ZOL, albeit in many cases subclinically.

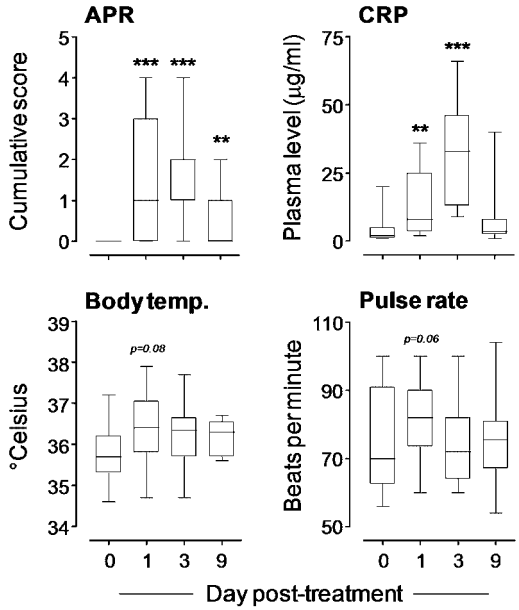

Fig. 1. Acute-phase response to intravenous ZOL in female osteoporosis patients. Cumulative APR scores, CRP plasma levels, resting body temperatures, and pulse rates immediately before and 1, 3, and 9 days after intravenous ZOL administration. Differences were assessed using Student's *t* tests.

ZOL treatment induces systemic activation of γδ T cells

To investigate the immunological basis of the APRs in our patient cohort and to be able to identify biomarkers that may correlate with, or even predict, the increase in CRP levels or the extent of the clinical symptoms experienced, we measured a comprehensive range of humoral and cellular immune parameters of possible relevance in the APR. On the cellular level, we detected a temporary drop in peripheral Vγ9/Vδ2 T cells (measured as proportion of all circulating T cells) from 2.9 ± 0.8% (mean ± SEM) down to 2.1 ± 0.7% on day 3 ($p < 0.05$); these frequencies returned to baseline levels by day 9 (Fig. 2*A*). In contrast, proportions of $CD4^+$ and $CD8^+$ T cells were not affected (data not shown). This transient ~30% decrease in Vγ9/Vδ2 T cells was similar to the findings by Thompson and colleagues[18] in ZOL-treated osteoporosis patients and confirms the specificity of ZOL for Vγ9/Vδ2 T cells. Direct evidence for activation of Vγ9/Vδ2 T cells was obtained by measuring surface expression of CD25, CD69, HLA-DR, and NKG2D. Although CD25 levels remained low throughout the study period, CD69, HLA-DR, and NKG2D showed a significant upregulation on Vγ9/Vδ2 T cells after treatment (Fig. 2*B*). Moreover, the distribution of Vγ9/Vδ2 T-cell memory subsets changed significantly in that the proportion of $CD27^+CD45RA^-$ central memory (T_{CM}) cells dropped, whereas $CD27^-CD45RA^-$ effector/memory (T_{EM}) cells and $CD27^-CD45RA^+$ terminally differentiated effector/memory (T_{EMRA}) cells increased after treatment (Fig. 2*C*). These changes in the distribution of memory subsets were detectable for at least 9 days after treatment and indicated a longer-lasting systemic effect of ZOL on the Vγ9/Vδ2 T-cell compartment beyond the initial APR.

Table 1. Correlations of CRP Values With APR Scores and Immune Biomarkers

		CRP increase	
Parameter		Day 1	Day 3
APR	Day 1	0.510*	0.690*
	Day 3	0.532*	0.806**
IFN-γ	Day 1	0.654**	*0.508*
	Day 3	*0.452*	*0.245*
IL-6	Day 1	0.595*	*–0.135*
	Day 3	*–0.471*	*–0.154*
% Vγ9$^+$	Day 0	0.637**	*0.325*
	Day 1	0.588*	0.706*
	Day 3	*0.400*	*0.464*
	Day 9	0.632**	*0.365*

Note: CRP increases above baseline were calculated by subtracting day 0 values. APR scores were expressed as cumulative symptoms on day 1 (score 0 to 4) or days 1 and 3 (score 0 to 8). Biomarkers analyzed before treatment (day 0) and on days 1, 3, and 9 after ZOL administration included plasma levels of IFN-γ and IL-6 (in pg/mL), and the frequency of Vγ9$^+$ cells within the peripheral T-cell population. Values shown are Pearson's coefficients (*r*). Numbers in italics indicate nonsignificant correlations.

*$p < 0.05$.

**$p < 0.01$.

ZOL treatment induces systemic activation of monocytes

Vγ9/Vδ2 T cells only respond to NBPs after uptake by monocytes or other endocytically active cells.[17,19,21] Because Vγ9/Vδ2 T cells stimulate monocyte survival and activation in vitro,[2,4] we next examined the effects of ZOL administration on the circulating monocyte population in vivo. Of note, we detected a pronounced increase in the proportion of monocytes among total PBMCs on day 1 (Fig. 3). However, it is not clear whether this constituted a specific expansion of monocytes, as we detected a similar increase in B cells and a parallel drop in T cells (data not shown). This notwithstanding, there was a significant increase in the surface expression of CD14, CD40, CD80, and HLA-DR at 1 to 3 days after treatment, indicative of a considerable but transient activation of monocytes in vivo (Fig. 3) and evoking our earlier demonstration of an intimate crosstalk between γδ T cells and monocytes in vitro.[2]

The ZOL-induced APR is characterized by elevated plasma cytokine levels

We next measured a range of soluble mediators in patient plasma, especially those that are associated with Vγ9/Vδ2 T-cell and/or monocyte responses. Among these, a sharp peak on day 1 was observed for IFN-γ, with an average increase of ~50-fold over baseline (Fig. 4). Less pronounced but nevertheless significant changes on day 1 were also detected for TNF-α and IL-6, in accordance with earlier studies.[8,18,22] In addition, changes were seen for IL-2, IL-10, and GM-CSF (Fig. 4) as well as CXCL10 (data not shown). To our knowledge, the latter four factors have not been implicated in the APR to NBPs before. In contrast to these effects, plasma levels of IL-1β, IL-12p70, and CXCL8 (Fig. 4) as well as IL-17 (data not shown) did not change upon treatment with ZOL. These findings suggest that the APR to ZOL, evidenced by the occurrence of clinical symptoms and elevated CRP levels peaking at day 3, is preceded by a rapid and transient production of a distinct set of inflammatory mediators on day 1. Many of these factors were previously shown to play a role in the crosstalk between activated Vγ9/Vδ2 T cells and monocytes (IFN-γ, TNF-α, GM-CSF, IL-6, CXCL10).[2]

IFN-γ takes center stage in the APR to ZOL

In an attempt to delineate the molecular and cellular events after systemic ZOL administration leading to the development of APR symptoms, we performed a detailed statistical analysis of a large range of humoral and cellular parameters, including the proportions of monocytes, B cells, NK cells, Vγ9/Vδ2 T cells, CD4 and CD8 T cells, and their expression of markers associated with activated cells (CD25, CD69, NKG2D), antigen-presenting

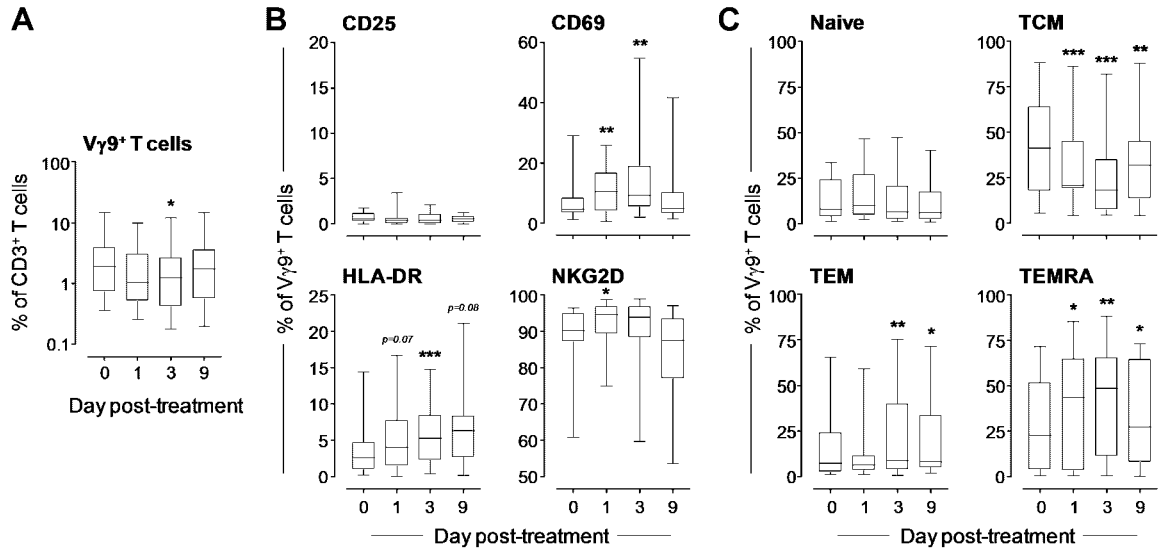

Fig. 2. Activation of peripheral γδ T cells after ZOL treatment. Proportion of Vγ9$^+$ T cells as percentage of all circulating CD3$^+$ T cells (*A*); their surface expression of CD25, CD69, HLA-DR, and NKG2D (*B*); and the relative proportion of CD27$^+$CD45RA$^+$ naive cells, CD27$^+$CD45RA$^-$ T_{CM} cells, CD27$^-$CD45RA$^-$ T_{EM} cells, and CD27$^-$CD45RA$^+$ T_{EMRA} cells amongst circulating Vγ9$^+$CD3$^+$ T cells (*C*). Differences were assessed using Student's *t*-tests.

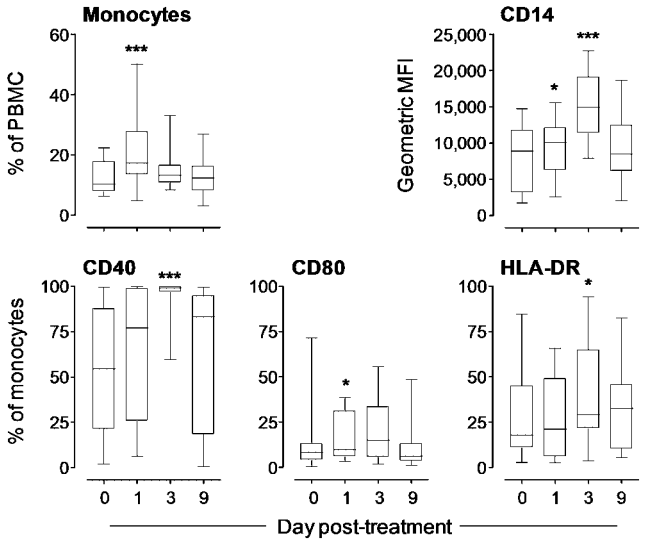

Fig. 3. Activation of peripheral monocytes after ZOL treatment. Proportion of $CD14^+$ monocytes as percentage of all PBMCs as well as surface expression of CD14 (geometric mean fluorescence intensity [MFI]) and CD40, CD80, and HLA-DR on circulating $CD14^+$ monocytes. Differences were assessed using Wilcoxon's signed-rank tests for CD40 levels on day 3 and Student's *t* tests for all other parameters.

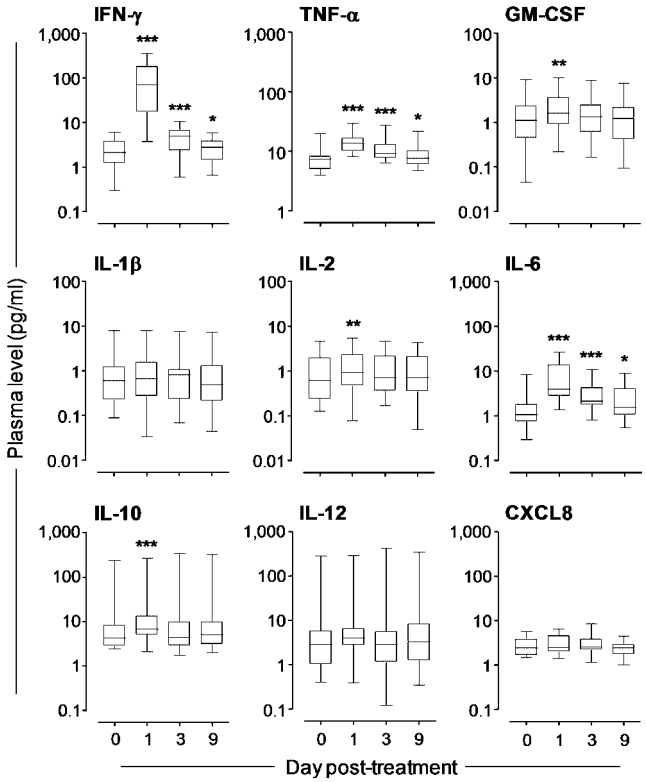

Fig. 4. Increased plasma cytokines and chemokines after ZOL treatment. Plasma levels of the markers indicated as determined by multiplex ELISA. Differences were assessed using Wilcoxon's signed-rank tests for IL-1β, IL-6, IL-10, and IL-12 levels and Student's *t* tests for all other soluble mediators.

cells (CD40, CD80, HLA-DR), and/or memory cells (CD27, CD45RA). Amongst these parameters, we identified a positive correlation between IFN-γ levels on either day 1 or day 3 and the expression of CD69 on Vγ9/Vδ2 T cells on day 1 or day 3 (Supplemental Fig. S1*A–D*). CD69 expression on Vγ9/Vδ2 T cells on day 3 also correlated moderately with plasma TNF-α levels on day 3 (Supplemental Fig. S1*D*). Moreover, pretreatment frequencies of Vγ9/Vδ2 T cells ultimately determined IFN-γ levels on day 1 (Supplemental Fig. S1*A*), and pretreatment proportions of T_{CM} cells within the Vγ9/Vδ2 T-cell population accurately predicted the levels of IFN-γ (Supplemental Fig. S1*A*) as well as TNF-α (Supplemental Fig. S1*E*) detected on day 1. No further post-treatment correlations were found between other cellular parameters and plasma cytokine/chemokine levels, except for a correlation between IFN-γ levels on day 1 and activation on NK cells on day 1 (Pearson's coefficient, $r = 0.640^{**}$) or day 3 ($r = 0.766^{**}$), measured as CD69 expression on $CD56^+$ $CD3^-$ cells (not shown). Taken together, these findings are consistent with the view that activated Vγ9/Vδ2 T cells are the prime source of the elevated levels of IFN-γ in the circulation, and that activated Vγ9/Vδ2 T cells may also induce IFN-γ production by other cell types such as NK cells.[23] A proposed model incorporating the described correlations is shown in Fig. 5. Of note, the depicted associations do not represent the result of a comprehensive path analysis or causal modeling but are derived from nonindependent regression models (individual Pearson's correlations and separate multivariate analyses by backward selection as summarized in Table 1 and Supplemental Figs. S1 and S2) and should thus be interpreted with caution because of the potential inclusion of type I errors.

The occurrence of a specific monocyte-γδ T-cell crosstalk in ZOL-treated patients was supported by the demonstration that pretreatment frequencies of monocytes in PBMCs but no other pretreatment parameters correlated directly with the activation levels of Vγ9/Vδ2 T cells on day 1, day 3, and day 9, expressed as proportion of $CD69^+$ cells (Supplemental Fig. S1*F*). Vice versa, there was a modest correlation between the frequency of Vγ9/Vδ2 T cells on day 1 and the activation status of monocytes on day 1 (Pearson's coefficient, $r = 0.495^*$) and day 3 ($r = 0.557^*$), expressed as CD40 surface levels (not shown).

Of all humoral parameters measured, day 1 levels of IFN-γ (and IL-6) correlated with increased CRP levels on day 1 (Table 1) (Supplemental Fig. S2*A, B*). Using linear regression, only IFN-γ levels on day 1 were predictive of total APR symptoms (Supplemental Fig. S2*A*). No further correlations were found between other plasma cytokines/chemokines and CRP levels or APR symptoms. Taken together, these data emphasize a crucial and previously underestimated role for IFN-γ in the development of a ZOL-induced APR. Of note, CRP levels also correlated with pre- and post-treatment frequencies of Vγ9/Vδ2 T cells, indicating that the extent of the APR upon ZOL administration is influenced by the number of ZOL-responsive Vγ9/Vδ2 T cells (Table 1). In accordance with previous findings,[24] high Vγ9/Vδ2 T-cell frequencies were indeed associated with an increased risk of APRs. Patients with pretreatment Vγ9/Vδ2 T-cell percentages greater than the median of 3% had significantly higher levels of IFN-γ on day 1, with a mean difference of 127 pg/mL (confidence interval 31 to 223 pg/mL; $p < 0.05$).

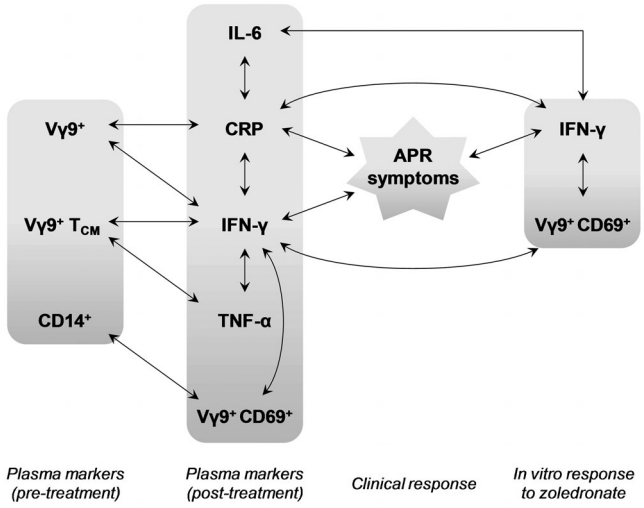

Fig. 5. Proposed relationships of pre- and post-treatment immune parameters, in vitro biomarkers, and clinical response. Biomarkers analyzed before treatment (day 0) and on days 1, 3, and 9 after ZOL administration included plasma levels of IFN-γ, TNF-α, IL-6, and CRP; the proportion of $CD14^+$ monocytes among PBMCs; the frequency of $V\gamma9^+$ cells within the peripheral T-cell population; and the percentage of $CD27^+CD45RA^-$ T_{CM} cells and $CD69^+$ cells within the $V\gamma9^+$ T-cell population. In vitro parameters determined included concentrations of IFN-γ secreted into the culture supernatant and the percentage of $CD69^+$ Vγ9/Vδ2 T cells after overnight stimulation of pretreatment PBMCs with ZOL. APR scores were calculated as cumulative APR symptoms on days 1 and 3 after ZOL administration.

The in vitro responsiveness of pretreatment PBMCs to ZOL predicts the APR in patients receiving intravenous ZOL

Having established the crucial role of Vγ9/Vδ2 T cells, monocytes, and IFN-γ in the APR to ZOL, we finally tested whether the response of pretreatment PBMCs to ZOL in vitro could predict the APR in vivo. Activation markers determined included the expression of CD69 on Vγ9/Vδ2 T cells and levels of IFN-γ in the culture supernatants after overnight stimulation with ZOL. Statistical analyses demonstrated that the in vitro responsiveness of PBMCs before treatment indeed predicted the development of clinical symptoms and the levels of certain blood biomarkers. As such, CD69 expression on Vγ9/Vδ2 T cells after ZOL stimulation in vitro correlated with the IFN-γ concentrations in the culture supernatant of ZOL-stimulated PBMCs as well as with plasma levels of IFN-γ on day 1 in treated patients (Supplemental Fig. S2*C*). Moreover, the IFN-γ production by ZOL-stimulated PBMCs correlated with plasma levels of IL-6 and CRP on day 1 as well as with cumulative APR scores by day 3. These associations between in vitro and in vivo parameters remained significant in multiple regression analyses (Supplemental Fig. S2*D*) and are summarized in the proposed model depicted in Fig. 5. Taken together, our findings demonstrate that a simple screening test to help identify individuals at risk of severe side effects upon intravenous NBP treatment may be feasible.

Discussion

The present phase IV safety trial is the most comprehensive study so far on the cellular immune response after intravenous NBP administration and provides evidence that both Vγ9/Vδ2 T cells and monocytes are involved in mediating the APR in vivo. Moreover, our study identifies a key role for IFN-γ in the NBP-induced APR, which may be of diagnostic and prognostic value and have implications for patient management. Given the proven efficacy of NBPs to reduce the risk of fractures that are associated with high mortality, a simple test predicting the likelihood of a severe APR would allow the attending clinician to tailor medication to the individual and improve adherence to intravenous NBPs.

Our findings identify pretreatment frequencies of peripheral monocytes and Vγ9/Vδ2 T cells as well as the proportion of $CD27^+CD45RA^-$ T_{CM} cells within the Vγ9/Vδ2 T-cell population and their responsiveness to ZOL in vitro as important predictors of the extent of pro-inflammatory cytokine production 24 hours after treatment. Incidentally, the cut-off value of >3% Vγ9/Vδ2 T cells identified by us above which patients showed considerably elevated IFN-γ levels at day 1 is in remarkable agreement with the failure of Rossini and colleagues to observe an APR in ZOL-treated osteoporosis patients who had pretreatment frequencies below 3% γδ T cells.[24] These observations imply that the age and sex bias in peripheral Vγ9/Vδ2 T cells, which are higher in younger individuals and in women, is an important determinant of the APR incidence in different patient groups.[24,25]

Our study also shows that ZOL administration caused a significant (albeit transient) drop in peripheral Vγ9/Vδ2 T cells and a longer-lasting reduction in the percentage of T_{CM} cells among them. Earlier studies reported a similar drop in circulating Vγ9/Vδ2 T cells in osteoporosis patients after receiving a single dose of ZOL[18] and a loss of T_{CM} cells in cancer patients treated repeatedly with ZOL.[26–28] As the loss of T_{CM} cells occurring after a single administration of ZOL may last for at least a year,[29] the qualitative and quantitative long-term effects of NBPs on the Vγ9/Vδ2 T-cell compartment appear to be the underlying basis for the general absence of adverse events in patients receiving repeat treatments.[11,24] However, this correlation between the numbers of T_{CM} cells in the circulation, the plasma levels of IFN-γ reached shortly after ZOL treatment, and the occurrence of APR symptoms is seemingly at odds with the present paradigm in Vγ9/Vδ2 T-cell biology.[30] T_{CM} cells are generally perceived as cells with high proliferative capacity but only modest cytokine production, as opposed to T_{EM} and T_{EMRA} cells, which proliferate poorly but produce copious amounts of cytokines upon restimulation. Our findings suggest that, in fact, activated T_{CM} cells are the major source of the IFN-γ detected in the circulation after ZOL treatment, emphasizing the importance of in vivo studies in patients.

It is known from cell culture experiments that NBP-driven Vγ9/Vδ2 T-cell responses are sensitive to statins.[16] By inhibiting 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme of the mevalonate pathway, statins lower the synthesis of all downstream isoprenoids including cholesterol, which is exploited in the clinic to prevent cardiovascular diseases. Through the same mechanism, statins also counteract

the NBP-induced intracellular accumulation of IPP, DMAPP, and ApppI, and thus inhibit the activation of Vγ9/Vδ2 T cells in vitro.[19] However, studies investigating the efficacy of statins in preventing the APR to intravenous NBPs have failed to demonstrate a clinical benefit,[11,18,22,31,32] most likely because the plasma concentrations required for effective inhibition of IPP/DMAPP/ApppI accumulation may not be reached pharmacologically. Our present findings suggest that interference with key players in the NBP-dependent γδ T cell-monocyte crosstalk such as IFN-γ might be an alternative therapeutic approach to manipulate the APR in patients at risk of severe reactions.

The present study has implications beyond the APR in osteoporosis patients. Recently, a transient drop in Vγ9/Vδ2 T-cell levels was interpreted as extravasation of activated cells into peripheral tissues,[18] evoking similar findings in primate studies upon intravenous treatment with Vγ9/Vδ2 T-cell agonists.[33,34] It is interesting to note that in primates activated Vγ9/Vδ2 T cells accumulate in the lung where they may confer some degree of protection against subsequent infection, which may be mirrored by reports showing that intravenous ZOL treatment has a largely unexplained beneficial effect in reducing pneumonia-related mortality.[35]

Our findings also have implications for current attempts to utilize NBP-based treatment regimes for targeted therapy of cancer patients. Large cohort studies have shown that ZOL has beneficial effects beyond improving bone health and may prolong disease-free and/or overall survival in patients with breast cancer[36] and multiple myeloma.[37] The underlying reason for these direct antitumor properties is not clear but may involve γδ T-cell–mediated effects. ZOL and other NBPs are, therefore, increasingly moving into the focus of novel immunotherapeutic approaches, especially in combination with low-dose IL-2 to boost the expansion and cytotoxicity of Vγ9/Vδ2 T cells.[27,28,38,39] Previous studies identified possible "responders" and "nonresponders" based on the proliferative capacity of a patient's Vγ9/Vδ2 T cells to NBP stimulation in vitro.[14,40,41] However, it remains to be investigated whether the severity of the APR or levels of early immune biomarkers such as those described here correlate with long-term clinical outcomes after administration of NBPs with or without IL-2.

Taken together, our data are consistent with a model in which intravenously administered ZOL is taken up by monocytes, which thereby acquire the ability to "present" isoprenoid metabolites or related structures to Vγ9/Vδ2 T cells.[17,19] Through mutual crosstalk, both monocytes and Vγ9/Vδ2 T cells undergo a series of activation and differentiation steps that ultimately determine the severity of the APR and replicate similar events taking place in acute infection.[2,4] Although the effects on circulating monocytes appear to be only transient (be it because of rapid inactivation, extravasation and/or replenishment from the bone marrow), the Vγ9/Vδ2 T-cell pool shows longer-term changes in that T_{CM} cells disappear and T_{EM} cells and T_{EMRA} cells become more prominent. Our findings imply that the gradual loss of T_{CM} cells and their inability to produce IFN-γ is a major determinant of the reduced risk of experiencing APR symptoms in patients repeatedly treated with NBPs. Future studies will reveal whether the immediate response to first-time NBPs determines clinical outcome in patients with and without malignancy and whether it has diagnostic and/or prognostic value for Vγ9/Vδ2 T-cell–based immunotherapy approaches.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

We are grateful to all patients for their participation in this study and to our nurses Roz Broadbent, Cheryl Culley, Jacqui Dickens, and Sue Day for their help. We thank Sarah James for CRP measurements; Ann Kift-Morgan for technical assistance; Antony Wilkes for statistical advice; and Martin Davey, Francesco Dieli, Keith Thompson, and Massimo Massaia for the stimulating discussion. This research was in part supported by the Cardiff CR-UK Centre Development Fund and CR-UK project grant C28524/A9497.

Authors' roles: Study design: AKS, JT, and ME. Study conduct and data collection: JLW and JT. Data analysis and interpretation: JLW, MPM, JT, and ME. Experimental methods: SMP. Study support and intellectual input: MDS and BM. Drafting manuscript: ME. Approving final version of manuscript: all authors. JT and ME take responsibility for the integrity of the data analysis.

References

1. Bonneville M, O'Brien RL, Born WK. γδ T cell effector functions: a blend of innate programming and acquired plasticity. Nat Rev Immunol. 2010;10:467–78.
2. Eberl M, Roberts GW, Meuter S, Williams JD, Topley N, Moser B. A rapid crosstalk of human γδ T cells and monocytes drives the acute inflammation in bacterial infections. PLoS Pathog. 2009;5:e1000308.
3. Davey MS, Lin CY, Roberts GW, Heuston S, Brown AC, Chess JA, Toleman MA, Gahan CG, Hill C, Parish T, Williams JD, Davies SJ, Johnson DW, Topley N, Moser B, Eberl M. Human neutrophil clearance of bacterial pathogens triggers anti-microbial γδ T cell responses in early infection. PLoS Pathog. 2011;7:e1002040.
4. Eberl M, Moser B. Monocytes and γδ T cells: close encounters in microbial infection. Trends Immunol. 2009;30:562–8.
5. Moser B, Eberl M. γδ T-APCs: a novel tool for immunotherapy?. Cell Mol Life Sci. 2011;68:2443–52.
6. Tanvetyanon T, Stiff PJ. Management of the adverse effects associated with intravenous bisphosphonates. Ann Oncol. 2006;17:897–07.
7. Cramer JA, Gold DT, Silverman SL, Lewiecki EM. A systematic review of persistence and compliance with bisphosphonates for osteoporosis. Osteoporosis Int. 2007;18:1023–31.
8. Thiébaud D, Sauty A, Burckhardt P, Leuenberger P, Sitzler L, Green JR, Kandra A, Zieschang J, Ibarra de Palacios P. An in vitro and in vivo study of cytokines in the acute-phase response associated with bisphosphonates. Calcif Tissue Int. 1997;61:386–92.
9. Buckler HM, Mercer SJ, Davison CE, Hollis S, Richardson PC, Anderson DG. Evaluation of adverse experiences related to pamidronate infusion in Paget's disease of bone. Ann Rheum Dis. 1998;57:572.
10. Dicuonzo G, Vincenzi B, Santini D, Avvisati G, Rocci L, Battistoni F, Gavasci M, Borzomati D, Coppola R, Tonini G. Fever after zoledronic acid administration is due to increase in TNF-α and IL-6. J Interferon Cytokine Res. 2003;23(11):649–54.

11. Reid IR, Gamble GD, Mesenbrink P, Lakatos P, Black DM. Characterization of and risk factors for the acute-phase response after zoledronic acid. J Clin Endocrinol Metab. 2010;95:4380–7.
12. Black DM, Delmas PD, Eastell R, Reid IR, Boonen S, Cauley JA, Cosman F, Lakatos P, Leung PC, Man Z, Mautalen C, Mesenbrink P, Hu H, Caminis J, Tong K, Rosario-Jansen T, Krasnow J, Hue TF, Sellmeyer D, Eriksen EF, Cummings SR. HORIZON Pivotal Fracture Trial. Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis. N Engl J Med. 2007;356:1809–22.
13. Kunzmann V, Bauer E, Wilhelm M. γ/δ T-cell stimulation by pamidronate. N Engl J Med. 1999;340:737–8.
14. Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M. Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood. 2000;96:384–92.
15. Gober HJ, Kistowska M, Angman L, Jenö P, Mori L, De Libero G. Human T cell receptor γδ cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med. 2003;197:163–8.
16. Hewitt RE, Lissina A, Green AE, Slay ES, Price DA, Sewell AK. The bisphosphonate acute phase response: rapid and copious production of proinflammatory cytokines by peripheral blood γδ T cells in response to aminobisphosphonates is inhibited by statins. Clin Exp Immunol. 2005;139:101–11.
17. Roelofs AJ, Jauhiainen M, Mönkkönen H, Rogers MJ, Mönkkönen J, Thompson K. Peripheral blood monocytes are responsible for γδ T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. Br J Haematol. 2009;144:245–50.
18. Thompson K, Keech F, McLernon DJ, Vinod K, May RJ, Simpson WG, Rogers MJ, Reid DM. Fluvastatin does not prevent the acute-phase response to intravenous zoledronic acid in post-menopausal women. Bone. 2011;49:140–5.
19. Riganti C, Massaia M, Davey MS, Eberl M. Human γδ T-cell responses in infection and immunotherapy: common mechanisms, common mediators?. Eur J Immunol. 2012;42:1668–76.
20. Kalyan S, Quabius ES, Wiltfang J, Mönig H, Kabelitz D. Can peripheral blood γδ T cells predict osteonecrosis of the jaw? An immunological perspective on the adverse drug-effects of aminobisphosphonate therapy. J Bone Miner Res. 2012 Sep 18. [Epub ahead of print].
21. Miyagawa F, Tanaka Y, Yamashita S, Minato N. Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human γδ T cells by aminobisphosphonate antigen. J Immunol. 2001;166:5508–14.
22. Silverman SL, Kriegman A, Goncalves J, Kianifard F, Carlson T, Leary E. Effect of acetaminophen and fluvastatin on post-dose symptoms following infusion of zoledronic acid. Osteoporos Int. 2011;22:2337–45.
23. Maniar A, Zhang X, Lin W, Gastman BR, Pauza CD, Strome SE, Chapoval AI. Human γδ T lymphocytes induce robust NK cell-mediated antitumor cytotoxicity through CD137 engagement. Blood. 2010;116:1726–33.
24. Rossini M, Adami S, Viapiana O, Ortolani R, Vella A, Fracassi E, Gatti D. Circulating γδ T cells and the risk of acute-phase response after zoledronic acid administration. J Bone Miner Res. 2012;27:227–30.
25. Caccamo N, Dieli F, Wesch D, Jomaa H, Eberl M. Sex-specific phenotypical and functional differences in peripheral human Vγ9/Vδ2 T cells. J Leukoc Biol. 2006;79:663–6.
26. Dieli F, Gebbia N, Poccia F, Caccamo N, Montesano C, Fulfaro F, Arcara C, Valerio MR, Meraviglia S, Di Sano C, Sireci G, Salerno A. Induction of γδ T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients in vivo. Blood. 2003;102:2310–1.
27. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, Roberts A, Buccheri S, D'Asaro M, Gebbia N, Salerno A, Eberl M, Hayday AC. Targeting human γδ T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res. 2007;67:7450–7.
28. Meraviglia S, Eberl M, Vermijlen D, Todaro M, Buccheri S, Cicero G, La Mendola C, Guggino G, D'Asaro M, Orlando V, Scarpa F, Roberts A, Caccamo N, Stassi G, Dieli F, Hayday AC. In vivo manipulation of Vγ9Vδ2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients. Clin Exp Immunol. 2010;161:290–7.
29. Santini D, Martini F, Fratto ME, Galluzzo S, Vincenzi B, Agrati C, Turchi F, Piacentini P, Rocci L, Manavalan JS, Tonini G, Poccia F. In vivo effects of zoledronic acid on peripheral γδ T lymphocytes in early breast cancer patients. Cancer Immunol Immunother. 2009;58:31–8.
30. Dieli F, Poccia F, Lipp M, Todaro M, Buccheri S, Cicero G, La Mendola C, Guggino G, D'Asaro M, Orlando V, Scarpa F, Roberts A, Caccamo N, Stassi G, Dieli F, Hayday AC. Differentiation of effector/memory Vδ2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med. 2003;198:391–7.
31. Srivastava T, Haney CJ, Alon US. Atorvastatin may have no effect on acute phase reaction in children after intravenous bisphosphonate infusion. J Bone Miner Res. 2009;24:334–7.
32. Makras P, Anastasilakis AD, Polyzos SA, Bisbinas I, Sakellariou GT, Papapoulos SE. No effect of rosuvastatin in the zoledronate-induced acute-phase response. Calcif Tissue Int. 2011;88:402–8.
33. Sicard H, Ingoure S, Luciani B, Serraz C, Fournié JJ, Bonneville M, Tiollier J, Romagné F. In vivo immunomanipulation of Vγ9Vδ2 T cells with a synthetic phosphoantigen in a preclinical nonhuman primate model. J Immunol. 2005;175:5471–80.
34. Ali Z, Shao L, Halliday L, Reichenberg A, Hintz M, Jomaa H, Chen ZW. Prolonged (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate-driven antimicrobial and cytotoxic responses of pulmonary and systemic Vγ2Vδ2 T cells in macaques. J Immunol. 2007;179:8287–96.
35. Colón-Emeric CS, Mesenbrink P, Lyles KW, Pieper CF, Boonen S, Delmas P, Eriksen EF, Magaziner J. Potential mediators of the mortality reduction with zoledronic acid after hip fracture. J Bone Miner Res. 2010;25:91–7.
36. Gnant M, Mlineritsch B, Stoeger H, Luschin-Ebengreuth G, Heck D, Menzel C, Jakesz R, Seifert M, Hubalek M, Pristauz G, Bauernhofer T, Eidtmann H, Eiermann W, Steger G, Kwasny W, Dubsky P, Hochreiner G, Forsthuber EP, Fesl C, Greil R. Austrian Breast and Colorectal Cancer Study Group, Vienna, Austria. Adjuvant endocrine therapy plus zoledronic acid in premenopausal women with early-stage breast cancer: 62-month follow-up from the ABCSG-12 randomised trial. Lancet Oncol. 2011;12:631–41.
37. Morgan GJ, Davies FE, Gregory WM, Cocks K, Bell SE, Szubert AJ, Navarro-Coy N, Drayson MT, Owen RG, Feyler S, Ashcroft AJ, Ross F, Byrne J, Roddie H, Rudin C, Cook G, Jackson GH, Child JA. National Cancer Research Institute Haematological Oncology Clinical Study Group. First-line treatment with zoledronic acid as compared with clodronic acid in multiple myeloma (MRC Myeloma IX): a randomised controlled trial. Lancet. 2010;376:1989–99.
38. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, Tony HP. γδ T cells for immune therapy of patients with lymphoid malignancies. Blood. 2003;102:200–6.
39. Lang JM, Kaikobad MR, Wallace M, Staab MJ, Horvath DL, Wilding G, Liu G, Eickhoff JC, McNeel DG, Malkovsky M. Pilot trial of interleukin-2 and zoledronic acid to augment γδ T cells as treatment for patients with refractory renal cell carcinoma. Cancer Immunol Immunother. 2011;60:1447–60.
40. Mariani S, Muraro M, Pantaleoni F, Fiore F, Nuschak B, Peola S, Foglietta M, Palumbo A, Coscia M, Castella B, Bruno B, Bertieri R, Boano L, Boccadoro M, Massaia M. Effector γδ T cells and tumor cells as immune targets of zoledronic acid in multiple myeloma. Leukemia. 2005;19:664–70.
41. Coscia M, Vitale C, Peola S, Foglietta M, Rigoni M, Griggio V, Castella B, Angelini D, Chiaretti S, Riganti C, Guarini A, Drandi D, Ladetto M, Bosia A, Foà R, Battistini L, Boccadoro M, Fournié JJ, Massaia M. Dysfunctional Vγ9Vδ2 T cells are negative prognosticators and markers of dysregulated mevalonate pathway activity in chronic lymphocytic leukemia cells. Blood. 2012 Aug 29. [Epub ahead of print].