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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

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Summary

The bisphosphonates are a novel class of drug that have been registered for various clinical applications worldwide. Bisphosphonates, and in particular the aminobisphosphonates (nBPs), are known to have a number of side-effects including a rise in body temperature and accompanying flu-like symptoms that resemble a typical acute phase response. The mechanism for this response has been partially elucidated and appears to be associated with the release of tumour necrosis factor (TNF)α and interleukin (IL)6, although the effector cells that release these cytokines and the mechanism of action remain enigmatic. Here, we show that the nBP-induced acute phase response differs from the typical acute phase response in that $CD14^+$ cells such as monocytes and macrophages are not the primary cytokine producing cells. We show that by inhibiting the mevalonate pathway, nBPs induce rapid and copious production of TNFα and IL6 by peripheral blood γδ T cells. Prior treatment with statins, which inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, blocks nBP-induced production of these proinflammatory cytokines by γδ T cells and may offer a means of avoiding the associated acute phase response. In addition, our findings provide a further mechanism for the anti-inflammatory effects attributed to inhibitors of HMG CoA reductase.

Keywords: bisphosphonates, acute phase response, proinflammatory cytokines, γδ T cells, aminobisphosphonates, statins

Introduction

Bisphosphonates (BPs) are currently in use for the treatment of Paget's disease [1,2], osteoporosis [3], hypercalcaemia [4], multiple myeloma and metastatic bone disease [5–7]. The clinical success of the BPs etidronate and clodronate in the 1970s and 1980s led to trials of BPs with different alkyl chains. In particular, bisphosphonates containing an amino group in their alkyl chain, such as pamidronate and alendronate, were found to be 10- to 100-fold more potent at inhibiting bone resorption than the early nonamino bisphosphonates [8]. The latest generation of bisphosphonates, such as risedronate and zoledronate, contain a nitrogen atom within a heterocyclic ring [9] and have been shown to be up to 10,000-fold more potent than etidronate in experimental systems [10]. Recent mechanistic studies indicate that the bisphosphonates can, broadly speaking, be classified into two groups based on their mode of action: (i) those resembling pyrophosphate (e.g. etidronate, clodronate, and tiludronate) that can be incorporated into cytotoxic ATP analogs; and (ii) the more potent nitrogen-containing bisphosphonates that interfere with other metabolic pathways (e.g. the mevalonate pathway) (reviewed in [11]). Bisphosphonates, and in particular the aminobisphosphonates (nBPs), are known to have a number of side-effects (reviewed in [11]) including a rise in body temperature and accompanying flu-like symptoms that resemble a typical acute phase response; these clinical features occur in over a third of patients receiving treatment for the first time [12,13]. The mechanism for this response has been partially elucidated and appears to be associated with the release of TNFα and IL6 [14–16], although the effector cells that release these cytokines and the mechanism of action remain obscure. nBPs are known to inhibit farnesyl pyrophosphate (FPP) synthase [17–22]. It has recently been shown that this inhibition of the mevalonate pathway leads to an accumulation of metabolic intermediates including isopentenyl pyrophosphate (IPP) [23]. IPP is a potent activator of human peripheral blood γδ T cells [24,25] and nBPs have also been shown to activate these cells [23,25–29]. As the acute phase

response has not been observed with the nonaminobisphosphonates etidronate, clodronate or tiludronate, and is thus a specific feature of the nBPs, it seems feasible that this phenomenon is mediated through γδ T cell activation. Here, we show that nBPs induce rapid and copious production of TNFα and IL6 by peripheral blood γδ T cells. Blockade of HMG CoA reductase by statin pretreatment abrogates this effect.

Materials and methods

Drugs

The following bisphosphonates and statins were used: disodium etidronate (Procter & Gamble Pharmaceuticals Ltd, Staines, UK), disodium clodronate (Roche Products Ltd, Welwyn Garden City, UK), disodium pamidronate (Novartis, Camberley, UK), risedronate sodium (Procter & Gamble Pharm), simvastatin (Ranbaxy Laboratories Ltd, Ealing, UK), pravastatin (Bristol-Myers Squibb Pharmaceuticals, Hounslow, UK) and fluvastatin (Sandoz Pharmaceuticals, Surrey, UK).

γδ T cell culture

γδ T cells were isolated from human peripheral blood using a magnetic bead-based separation kit (TCR γ/δ Microbead Kit and MS columns, Miltenyi Biotech, Surrey, UK). Once isolated, cells were cloned by limiting dilution in T cell medium (RPMI supplemented with penicillin, streptomycin and glutamine, 10% fetal calf serum (FCS), 10% T-STIM (BD Biosciences, Oxford, UK), 200 U/ml Proleukin (Chiron, Cranford, UK)) containing 2×10^6 γ-irradiated human peripheral blood mononuclear cells (PMBC)/ml from at least 3 unrelated donors and 2 μg/ml phytohaemagglutinin (PHA). Clones were maintained with T cell medium, and restimulated with mixed irradiated PBMC and PHA every three weeks. The IMGT system of TCR nomenclature is used throughout this work [30]. Cells that grew were confirmed to be γδ T cells by flow cytometry with TCR chain-specific monoclonal antibodies (mAbs).

Antibodies

The following antibodies were used for flow cytometric analysis: fluorescein isothiocyanate (FITC)-conjugated mouse anti-human Vδ2 TCR mAb clone B6·1 (Pharmingen, Oxford, UK), FITC-conjugated mouse anti-human Vγ9 mAb clone 7A5 (Endogen, Perbio Science UK Ltd, Cramlington, UK), FITC-conjugated mouse anti-human Vδ1 mAb clone TS8·2 (Endogen), FITC-conjugated mouse anti-human CD14 mAb clone UCHMT1 (BD Biosciences), phycoerythrin (PE)-conjugated mouse anti-human Vγ9 mAb clone B3·1 (Pharmingen), PE-conjugated mouse anti-human IL6 clone AS12 (BD Biosciences), peridinin chlorophyll protein (PerCP)-conjugated mouse anti-human CD3 mAb clone SK7 (BD Biosciences), allophycocyanin (APC)-conjugated mouse anti-human IL2 clone MQ1–17H12 (Caltag-Medsystems Ltd, Towcester, UK), APC-conjugated mouse anti-human TNFα clone mAb11 (Pharmingen), and APC-conjugated mouse anti-human IFNγ mAb clone B27 (Pharmingen).

IFNγ ELISpot and TNFα ELISA

γδ T cells were washed in RPMI and incubated overnight in R10 at 37°C. 96-well nitrocellulose plates (Millipore Ltd, Watford, UK) were incubated overnight at 4°C with 15 μg/ml anti-human-IFNγ primary antibody (clone 1-D1K; Mabtech, Nacka Strand, Sweden). The plates were then washed twice with RPMI and blocked with R10 for 3 h at 37°C. R10 was decanted by inversion and assays applied to each well before incubation at 37°C as detailed below. Assays were terminated by washing once in water, followed by 5 washes in PBS. Secondary antibody (anti-human IFNγ-biotin clone 7-B6-1; Mabtech) was applied at 1 μg/ml and the plate incubated for 100 min at room temperature (RT). The plate was washed 6 times with PBS before application of streptavidin-alkaline phosphatase (AP) (1 : 1000 in PBS; Mabtech) for 40 min at RT. After 6 further washes in PBS, spots were revealed by incubation for 15 min at RT with developing buffer (Bio-Rad AP conjugate substrate kit) and counted mechanically using an ELISpot Reader System ELR02 (Autoimmun Diagnostika; Strassberg, France). An ELISA kit (Peprotech EC Ltd, London, UK) was used to quantify TNFα release into the assay supernatant according to the manufacturer's instructions.

Intracellular cytokine staining

10^6 fresh PBMC were incubated in FACS tubes with brefeldin A (10 μg/ml in R10) for 5 h after incubation with relevant antigens for 1 h. The cells were then washed and stained with PE-conjugated anti-Vγ9, FITC-conjugated anti-Vδ2 or anti-CD14 mAbs, and for viability with 7-AAD for 20 min on ice. The cells were then washed again and permeabilized in 10% FACSLyse (BD Biosciences), washed twice in ice cold PBS/0·1% BSA and stained on ice with pretitred APC-conjugated anti-IFNγ, anti-TNFα, anti-IL-2 for 20 min. For single cytokine analysis, cells were stained with PE-conjugated anti-IL6, APC-conjugated anti-TNFα and FITC-conjugated anti-Vγ9 antibodies. In each case, cells were then washed, resuspended in PBS and analysed immediately.

γδ T cell depletion from PBMC

10^8 human PBMC were incubated with 10 μg/ml anti-human pan-γδ antibody for 30 min on ice [25]. Cells were washed once in 20X labelling volume with PBS/0·1%BSA and resuspended at 4×10^7 cells/ml in PBS/0·1%BSA. 2×10^7 anti-mouse IgG1 Dynabeads (Dynal Biotech ASA, Norway) washed in PBS/0·1%BSA were added and the cells incubated

for 1 h at 4°C with gentle rotation. The tube was then placed in a magnetic particle concentrator (Dynal) and left to separate for 30 min. The supernatant was transferred to a fresh tube. Depletion efficiency was determined by FACS analysis with the anti-CD3 and pan-γδ antibodies detailed above, and was > 95% efficient in all cases shown.

Results

nBPs induce rapid and copious production of TNFα by peripheral blood γδ T cells

The dominant subset of human γδ T cells in peripheral blood bears T cell receptors (TCRs) comprising variable regions encoded by the Vγ9 and Vδ2 genes. These cells are known to activate in response to alkylphosphate, alkylamine and some bisphosphonate antigens. It is known that this recognition is a characteristic of cells bearing a Vγ9 chain in conjunction with a JP joining region [31]. Vγ9JP-expressing cells are highly enriched in the peripheral blood when compared to percentages in thymocytes or cord blood [31,32]. The peripheral expansion of Vγ9 cells using JγP appears to be antigen-driven. γδ T cells in cord blood mononuclear cells (CBMC) express a diverse repertoire of γδ TCRs [33]. Stimulation of CBMC with alkylphosphate antigen, but not with PHA, induces the preferential expansion of T cells bearing Vγ9Vδ2 TCRs; 70% of those TCRs expanded with alkylphosphate antigen use a JγP joining region compared to just 20% of those cells expanded with PHA [33].

We grew several Vγ9JPVδ2 T cell clones as described in the Materials and methods. TCR usage in these clones was characterized with a molecular analysis of gene expression [25]. These cells were shown to activate after exposure to the nBPs pamidronate and risedronate, but not the nonaminoBPs etidronate and clodronate, by IFNγ ELISpot (Fig. 1a). Vγ9JPVδ2 T cells also made large amounts of TNFα after exposure to nBPs (Fig. 1c). Production of TNFα was both copious and rapid (Fig. 1c). A Vγ9JPVδ1-expressing clone was unable to recognize these ligands [25], indicating that the δ2 chain also plays an essential role in the response of

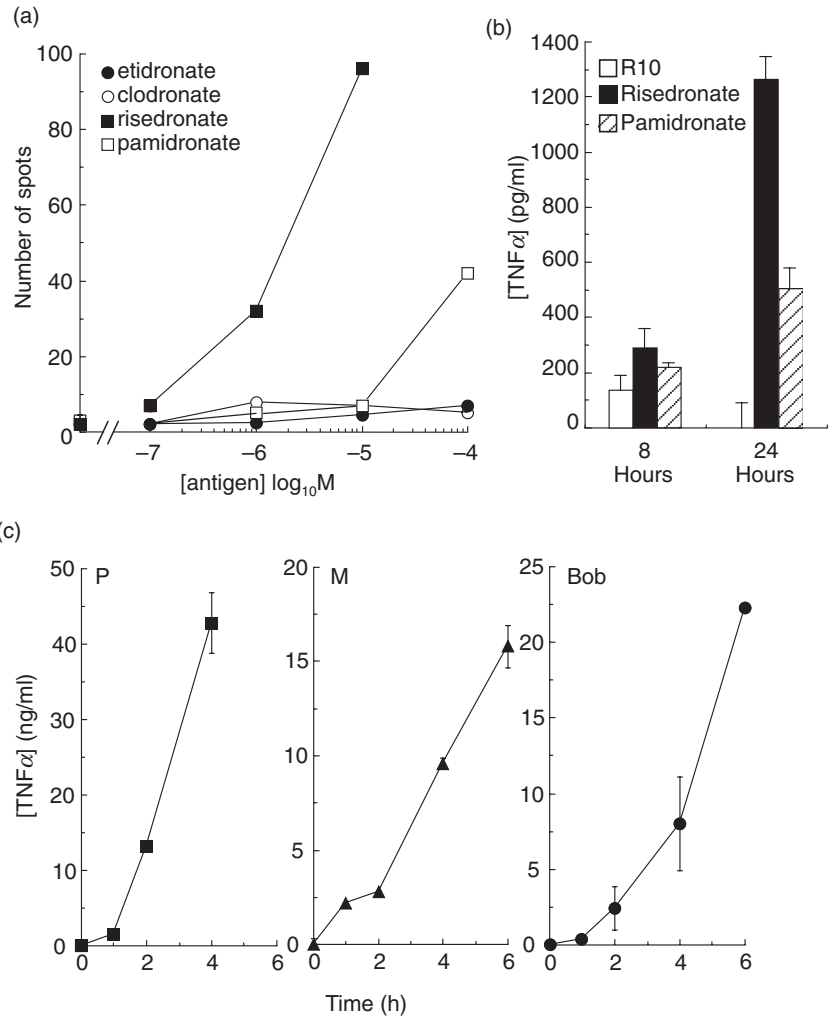

Fig. 1. nBPs induce rapid and copious production of TNFα by peripheral blood γδ T cells. (a) nBPs, but not nonaminoBPs, activate the Vγ9JPVδ2 T cell clone Bob in IFNγ ELISpot. The TCR of this clone has been sequenced and is published elsewhere [25]. ELISpots were performed with 1000 Vγ9JPVδ2 T cells and 25 000 spinner HeLa cells as antigen presenting cells per well and incubated for 6 h prior to development. Standard deviation from the mean of two replicate assays is shown, although in all cases these errors are smaller than the plot symbol. (b) nBPs stimulate TNFα production from direct *ex vivo* human PBMC. 10^6 fresh human PBMC were incubated in 75 × 5mm FACS tubes at 37°C and 5% CO_2 in 1 ml of R10 (RPMI, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin), l ml of R10 + 10 μM risedronate or 1 ml of R10 + 100 μM pamidronate. 60 μl aliquots were removed at the specified times and added to TNFα ELISA assays. Error bars show standard deviation from the mean. (c) Activation of Vγ9JPVδ2 T cell clones by risedronate is extremely rapid. Standard deviation from the mean of two replicate TNFα ELISA assays is shown for three separate clones expressing a Vγ9JPVδ2 TCR. Clone P expresses CD8α; clones M and Bob are $CD8^-$. Clone P appears to make more TNFα and to produce it earlier. 10^6 T cells were activated by 10 μM risedronate in 1 ml R10 in 75 × 5mm FACS tubes at 37°C and 5% CO_2. 60 μl aliquots were removed at the specified times and added to TNFα ELISA assays. Background TNFα production after a similar time period in the absence of risedronate was negligible (data not shown).

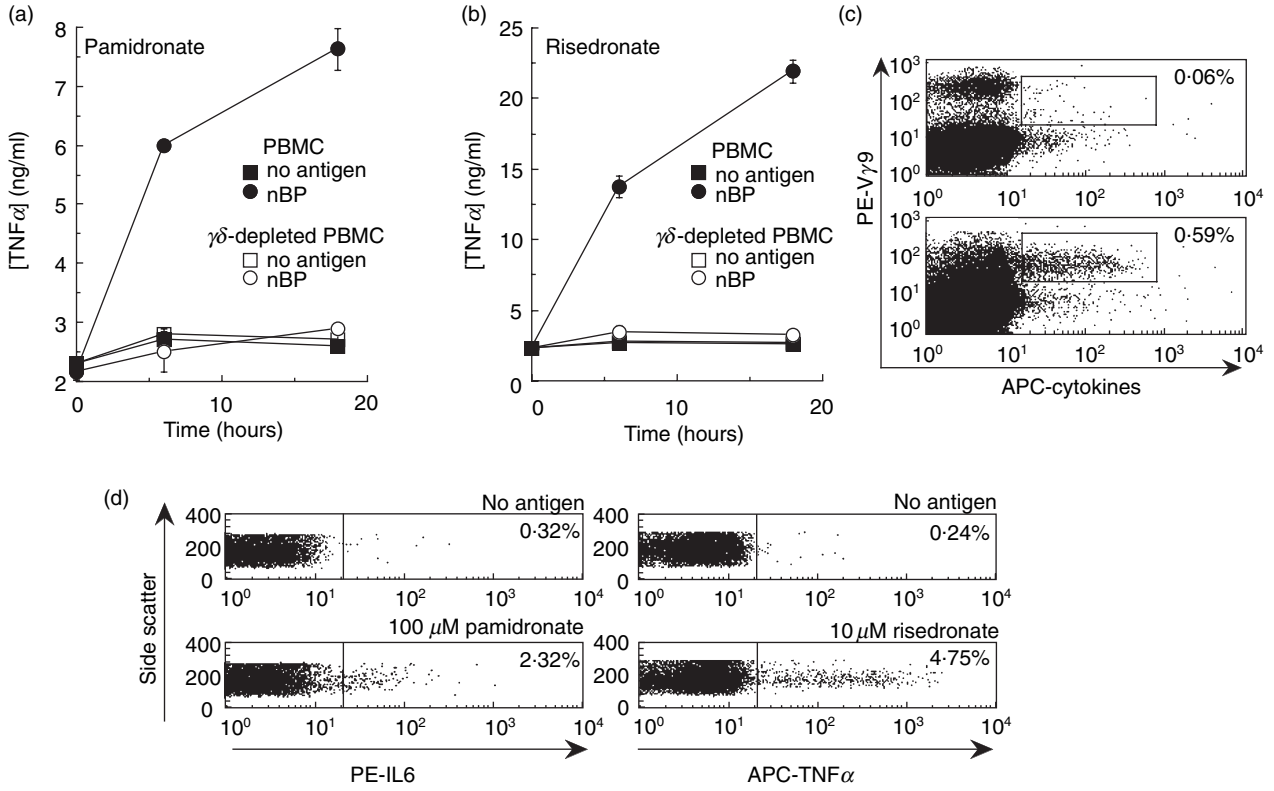

Fig. 2. The TNFα and IL6 produced by PBMC in response to nBPs is derived from γδ T cells. Magnetic depletion of γδ T cells from human PBMC removes their ability to manufacture TNFα in response to 100 μM pamidronate (a) and 10 μM risedronate (b). 5×10^6 PBMC from a healthy donor ± magnetic depletion of γδ T cells were suspended in 1 ml of R10 ± antigen in 75 × 5mm FACS tubes at 37°C and 5% CO_2 for the times shown. 60 μl of cell supernatant was removed, applied to TNFα ELISA plates in duplicate and developed according to the manufacturers' instructions. Standard deviation from the mean of the two replicate ELISAs is shown although in most cases this error is smaller than the plot symbol. (c) Exposure to nBPs activates only lymphocytes that express a Vγ9 receptor. 10^6 fresh human PBMC were exposed to R10 (top panel) or R10 + 10 μM risedronate (bottom panel) for 6 h in an intracellular cytokine staining (ICS) assay. Plots show all the cells in the lymphocyte gate stained for PE-Vγ9 and APC-cytokines (TNFα, IL2, and IFNγ) as described previously [25]. Exposure to risedronate induces cytokine production only in lymphocytes that express a Vγ9 TCR. The percentage of total lymphocytes in the $V\gamma9^+$cytokine$^+$ gate shown is indicated in the upper right of each panel. Almost 10% of the lymphocytes expressing a Vγ9 receptor are activated by exposure to nBPs. Similar results were observed with 100 μM pamidronate (data not shown). It is noticeable that exposure to risedronate lowers the expression of the Vγ9 TCR. (d) Intracellular cytokine staining (ICS) shows that nBPs induce direct *ex vivo* Vγ9-expressing T cells to make TNFα and IL6. Plots are gated to show only Vγ9-expressing lymphocytes. The left hand panels show IL6 production induced by 100 μM pamidronate and the right hand panels show TNFα production induced by 10 μM risedronate. The percentage of cytokine positive cells (fluorescence intensity > 20) is shown in the upper right corner of each panel.

Vγ9JPVδ2 T cells to nBPs. This result is in agreement with those of a previous study that showed Vγ9Vδ1 T cell clones did not respond functionally to monoethyl phosphate [34]. Pamidronate and risedronate were also able to induce the rapid production of large amounts of TNFα from direct *ex vivo* human peripheral blood mononuclear cells (PBMC) (Fig. 1b). The results above suggest that Vγ9Vδ2 T cells in human PBMC might be the source of nBP-induced proinflammatory cytokines. We tested this hypothesis by depleting γδ T cells from PBMC. Magnetic depletion of γδ T cells removed the ability of PBMC to produce TNFα in response to pamidronate (Fig. 2a) and risedronate (Fig. 2b). We used flow cytometry to characterize further the cellular source of acute phase response-inducing cytokines after exposure of human PBMC to nBPs. Intracellular cytokine staining (ICS) showed that exposure of PBMC to nBPs activated only the peripheral blood lymphocyte population bearing a Vγ9 TCR and not other lymphocytes (Fig. 2c). Vγ9-expressing cells were shown to make both IL6 and TNFα in response to pamidronate and risedronate by ICS (Fig. 2d and data not shown). Several recent reports show that nBPs can activate γδ T cells *in vitro* or directly *ex vivo* [23,25–29]. Kunzmann *et al.* [35] found that 4/10 patients given 60 or 90 mg infusions of pamidronate had an acute phase reaction. All of these patients showed a substantial increase in the number of circulating γδ T cells when measured 1 and 3 weeks post-infusion [35]. In one patient, γδ T cells expanded from 4·6% to 70% of $CD3^+$ cells post-infusion. Thus, nBPs are likely to activate γδ T cells *in vivo* to produce acute phase response-inducing cytokines.

Cells of the monocytic lineage are not the primary source of nBP-induced TNFα or IL6

TNFα and IL6 are known to play central roles in the 'typical' acute phase response to infectious tissue injury [36]. While both TNFα and IL6 can be produced by many cell types, monocytes and macrophages at the site of inflammation are the major source of these cytokines during a typical acute phase response [36]. BPs are known to affect monocytic cells and have been observed to depress the accessory function of monocytes in lymphocyte proliferation assays [37]. Makkonen *et al*. [38] showed that pretreatment of RAW 264 macrophages with the nBP alendronate augments LPS-stimulated release of cytokines. However, alendronate itself did not induce cytokine release. These authors also showed that the nBP ibandronate enhances LPS-induced stimulation of IL6 in macrophages [39]. Again, there was no indication that the nBP *per se* induces activation. The fact that monocytes and macrophages are the primary source of TNFα and IL6 in a typical acute phase response, combined with knowledge that these compounds can affect such cells, has lead to the suggestion that cells of the monocytic lineage might be the primary source of nBP-induced cytokines [15]. However, there has been no formal testing of this hypothesis. The fact that γδ T cell-depleted PBMC, which still contain monocytes, do not manufacture TNFα in response to nBPs (Fig. 2a,b) suggests that this hypothesis is incorrect. To exclude monocyte lineage cells formally as the primary cellular source of the nBP-induced proinflammatory response, we positively identified these cells using an anti-CD14 monoclonal antibody to confirm that they do not produce TNFα in our assays (Fig. 3a,b). Parallel stains with a Vδ2-specific monoclonal antibody clearly showed that TNFα is produced by this subset of γδ T cells (Fig. 3c,d).

Statins inhibit nBP-induced activation of γδ T cells

Recent studies indicate that nBPs may exert their effect on the activation of Vγ9JPVδ2 T cells via the mevalonate pathway [23,25] rather than acting as direct ligands for the γδ TCR as had been thought in previous studies [26–29,40]. nBPs inhibit FPP synthase [17–21] and cause an accumulation of IPP within the cell [23] (Fig. 4). IPP is a potent activator of Vγ9Vδ2 T cells [24] and has recently been shown to activate these cells directly *ex vivo* [25]. Blocking the mevalonate pathway above IPP should prevent build up of IPP (Fig. 4). Indeed, inhibitors of HMG CoA reductase, the key enzyme at the beginning of this metabolic pathway, were able to inhibit nBP-induced activation of γδ T cells directly *ex vivo* (Fig. 5). Pravastatin, simvastatin and fluvastatin were all able to inhibit TNFα production by fresh PBMC as measured by ELISA (Fig. 5a). Addition of IPP was able to rescue TNFα production, thus ruling out the possible toxic effects of these statins at the concentrations used. However, statins and bisphosphonates have inhibitory effects on almost all cells as blocking the mevalonate pathway leads to the loss of Ras and other receptor signals due to the blockade of protein prenylation [41]. nBPs block FFP synthase and may act synergistically with inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase to kill cells. Any such effects may be overcome by the replenishment of cells with metabolites in the mevalonate pathway such as IPP. Thus, we felt it was important to examine the combined effects of statins and nBPs using flow cytometry. Pravastatin,

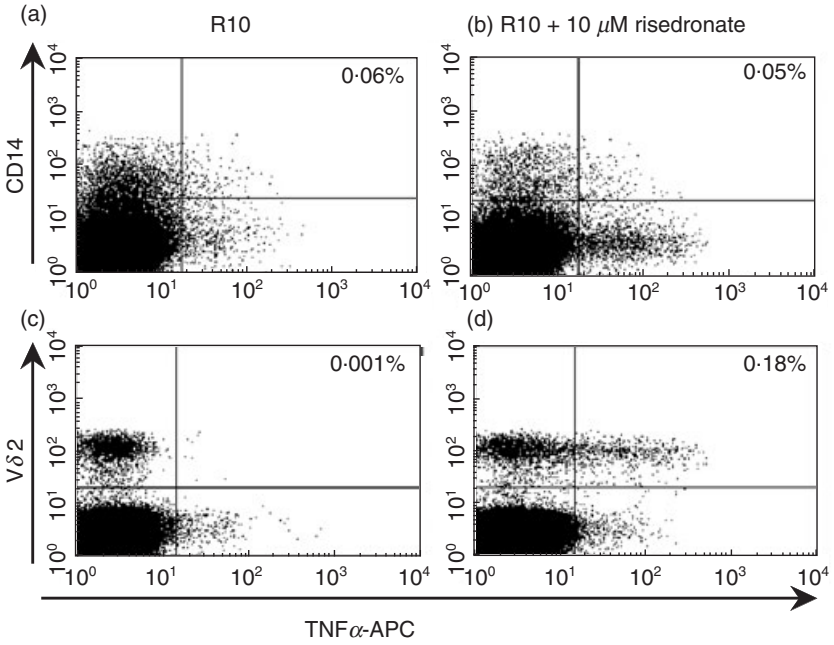

Fig. 3. Incubation with nBPs does not activate cells of the monocytic lineage. Six hour exposure to 10 μM risedronate does not activate $CD14^+$ cells. 10^6 fresh human PBMC were exposed to R10 (a,c) or R10 + 10 μM risedronate (b,d) in an ICS assay. (a,b) show all live cells as determined by dead cell exclusion with 7-amino-actinomycin D (7-AAD; BD BioSciences). $CD14^+$ cells such as monocytes and macrophages do not produce TNFα when exposed to nBP for this duration. The cells that produce TNFα in this assay do not express CD14 (compare bottom right quadrants in (a) and (b)). The cells that do respond in this assay express a Vδ2 TCR (c,d). The number of cells in the upper right quadrant of each plot as a percentage of total live cells is indicated in the upper right corner. There were approximately 30% fewer $CD14^+$ cells after 6 h treatment with nBP compared to treatment with R10 alone as a fraction of the $CD14^+$ cells that became $7\text{-}AAD^+$.

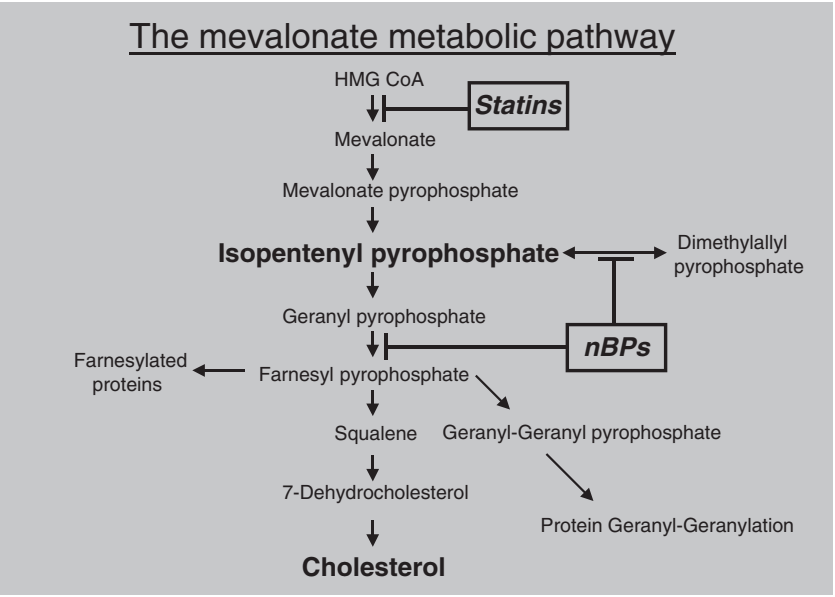

Fig. 4. The mevalonate biosynthetic pathway. nBPs inhibit farnesyl pyrophosphate (FPP) synthase and lead to a build up of IPP [23]. Statins inhibit HMG CoA reductase, a proximal enzyme in this pathway.

simvastatin and fluvastatin were shown to inhibit the activation of Vγ9 cells by ICS (Fig. 5b). Once again, addition of IPP in addition to the statin was able to rescue activation and rule out toxic effects. Flow cytometric analyis using the viability marker 7-AAD showed that the γδ T cell population remained alive throughout the experiment when the statin and nBP were combined without addition of IPP (row 4 in Fig. 5b) (data not shown). Thus, prior administration of a statin is able to prevent the rapid and copious production of acute phase response-inducing cytokines by peripheral blood γδ T cells in response to nBPs.

Discussion

nBPs inhibit bone resorption by inhibiting FPP synthase and preventing protein prenylation of GTP-binding proteins that are necessary for osteoclast function [41–44]. nBPs are known to have a number of adverse side-effects (reviewed in [11]). The principal side-effect, the induction of an acute phase response, was described over 15 years ago [12,13]. The mechanism for this response has only been partially elucidated and appears to be associated with the release of cytokines, in particular TNFα and IL6 [14–16]. The cellular mechanism for the action of nBPs leading to production of TNFα and IL6 has remained unclear. Current thinking has homed in on monocytes and/or macrophages as likely cellular sources of these proinflamatory agents [15]. Here, we challenge this view by showing that nBPs induce rapid and copious production of these proinflammatory cytokines by human peripheral blood γδ T cells (Figs 1, 2 and 5). Monocytes and macrophages can be activated by proinflammatory cytokines and it is possible that these cells are activated at later time points as an indirect result of γδ T cell activation. However, several lines of evidence point against cells of the monocytic lineage being the primary source of acute phase response-inducing cytokines in our experiments. First, γδ T cell-depleted PBMC, which still contain monocytes and macrophages, do not manufacture TNFα in response to nBP treatment (Fig. 2a,b). Second, we do not observe cytokine production by non-Vγ9 expressing cells in our assays (Fig. 2c). Third, only cells expressing a Vδ2 chain produce TNFα in response to risedronate treatment (Fig. 3c,d). Fourth, the cells that do make cytokines in our assays do not express CD14 (Fig. 3a,b). Thus, in contrast to the typical acute phase response, cells of the monocytic lineage are likely not the primary producers of acute phase response-inducing cytokines after exposure to nBPs.

We also demonstrate that statins, which inhibit HMG CoA reductase, are able to block nBP-induced activation of γδ T cells (Fig. 5). This result is important for two reasons. First, it helps to isolate the nBP γδ T cell-activating effects to intermediates in the mevalonate pathway. Second, it suggests that administration of a statin prior to treatment with nBPs might inhibit the acute phase response. Future trials should aim to determine whether this common class of drug can inhibit the nBP-induced acute phase response, an effect that would be useful in many clinical settings given the wide range of applications for nBPs.

Risedronate was a more potent activator of γδ T cells than pamidronate, inducing more TNFα production at a 10 fold lower concentration (Figs 1a,b and 2a,b). This potency correlates with the ability of these drugs to inhibit FPP synthase [18,22]. Risedronate inhibits FPP synthase with an IC_{50} of $0{\cdot}01 \pm 0{\cdot}002$ μM compared to a value of $0{\cdot}2 \pm 0{\cdot}1$ μM for pamidronate [18]. Inhibition of FPP synthase in cellular extracts requires higher concentrations ($0{\cdot}1 \pm 0{\cdot}02$ μM risedronate and $0{\cdot}85 \pm 0{\cdot}34$ μM pamidronate [18]). In most cases, nBP-induced activation of γδ T cells appears to occur

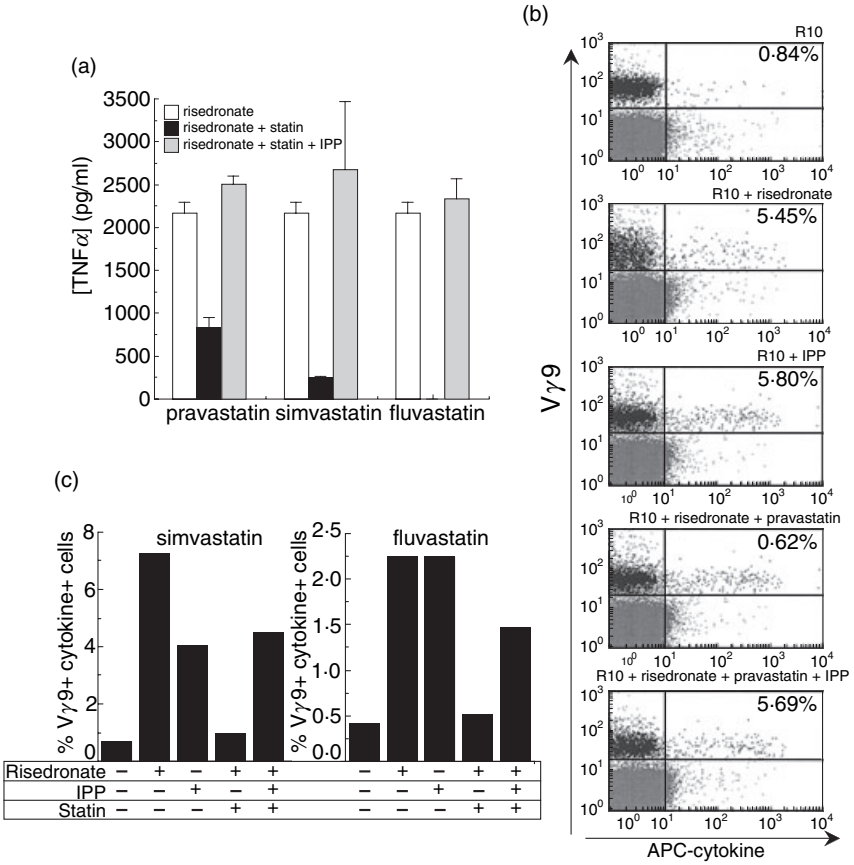

Fig. 5. Statins inhibit nBP-induced TNFα production by human PBMC. (a) Pretreatment of human PBMC with 1 μM pravastatin, 100 nM simvastatin or 100 nM fluvastatin for 2 h inhibits their ability to manufacture TNFα in response to 10 μM risedronate. Addition of IPP restores TNFα production and controls for any toxicity effects of these statins. 5×10^5 statin-treated or untreated cells/well in 200 μl R10 were incubated with nBP for 12 h at 37°C and 5% CO_2 in 96 well U-bottomed tissue culture plates ± 1 μM IPP. 60 μl of supernatant was assayed for TNFα content by ELISA (Peprotech). Assays were performed in duplicate. Bars show standard deviation from the mean of two replicate assays. (b) ICS shows that pretreatment of PBMC with statins inhibits nBP-induced activation of Vγ9-expressing T cells. FACS plots show results with 1 μM pravastatin. Panels show results of incubation for 6 h in R10 only on the top row, R10 + 10 μM risedronate in row 2, R10 + 10 μM IPP in row 3, R10 + 10 μM risedronate + pravastatin in row 4 and R10 + 10 μM risedronate + pravastatin + 10 μM IPP on the bottom row. Pravastatin inhibits nBP-induced activation of Vγ9-expressing T cells (compare rows 2 & 4). Addition of IPP with pravastatin (bottom row) rescues activation and controls for any toxic effect of the statin. The Vγ9-expressing cell population (less than 3% of total lymphocytes) is shown in black. Vγ9 cells expressing cytokines appear in the upper right quadrant. The percentage of Vγ9 cells expressing cytokine is shown in the upper right corner of each plot. (c) Experiments performed as for (b) but with with 100 nM simvastatin or 100 nM fluvastatin instead of pravastatin. Experiments with pravastatin, simvastatin and fluvastatin were performed with different PBMC and show that between 2 and 8% of Vγ9-expressing T cells make cytokines when exposed to 10 μM risedronate. Bar charts show the percentage of $Vγ9^+$cytokine$^+$ cells from flow cytometry plots for PBMC exposed to R10, R10 + 10 μM risedronate, R10 + 10 μM IPP, R10 + 10 μM risedronate + statin and R10 + 10 μM risedronate + statin + 10 μM IPP. Simvastatin and fluvastatin inhibit nBP-induced activation of Vγ9-expressing T cells. Addition of IPP with the statin rescues activation and controls for any toxic effect of the statins. Flow cytometric analysis allowed confirmation that cells remained alive during the experiment and showed that non-Vγ9-expressing cells such as monocytes and macrophages did not make cytokines in response to nBP during the experiment (data not shown).

at clinically relevant concentrations. The serum C_{max} for pamidronate is approximately 10 μM [45]. Activation of Vγ9Vδ2 T cells is known to occur at such concentrations [46]. Zoledronate is the most potent nBP inhibitor of FPP synthase [46] and activates γδ T cells at concentrations as low as 1 μM [46], a concentration exceeded *in vivo* [47]. Risedronate may be different than other nBPs; while it is a very potent inhibitor of FPP synthase [22] and activates γδ T cells at concentrations as low as 1 μM [25,46], maximum serum concentrations have been reported to be below this threshold (C_{max} < 25 nM after oral administration of 30 mg of risedronate [48]). nBPs are currently believed to function by inhibiting FPP synthase [43]. The reported C_{max} value for risedronate (<25 nM) [48] is lower than the IC_{50} for inhibition of FPP synthase in cellular extracts (100 nM) [18]. There may be a number of possible explanations for this discrepancy. First, the C_{max} for risedronate may be greater than claimed. Second, the intracellular, and specifically

intraosteoclast, concentration of risedronate may be significantly higher than that measured in serum. Third, risedronate may function to inhibit bone resorption through an FPP synthase-independent mechanism. A study by Procter and Gamble Pharmaceuticals of 66 subjects taking risedronate concluded that, in contrast to other primary alkyl bisphosphonates, risedronate does not induce an acute phase reaction [48]. Thus it is possible that risedronate concentrations *in vivo* do not exceed the threshold required to trigger activation of γδ T cells and the ensuing acute phase response. However, further comparative, independent studies will be needed to confirm this finding.

Considerable publicity has been given to gastrointestinal disturbances associated with oral administration of the nBP alendronate [49]. Similar problems had earlier led to the discontinuation of oral pamidronate for osteoporosis [50] and the administration of these compounds by intravenous or intramuscular injection is increasing in popularity worldwide. The gastrointestinal tract is the main reservoir of γδ T cells in the body, probably as it is the main portal of entry for bacterial pathogens. The predominant populations of γδ T cells in the gut may also activate in response to nBPs. Thus, it is possible that some of the various complications that have been described after oral administration of nBPs may result from effects mediated through γδ T cells in the gastrointestinal epithelia.

Both simvastatin and fluvastatin inhibited nBP-induced activation of Vγ9Vδ2 T cells at a concentration of 100 nM (Fig. 5). This is close to the concentration of simvastatin found *in vivo* (C_{max} = ~80 nM) and well below the clinically relevant concentration of fluvastatin (C_{max} > 1 μM) [51]. It is further noteworthy that statins have anti-inflammatory properties [52–64] that appear to be mediated through nonsterol mevalonate products [65] and to be paralleled by a dose dependent reduction in IL6 production [57,60,65–67]. Statins also appear to promote a Th2 cytokine bias *in vivo* [68]. The therapeutic relevance of these pharmacologic effects is becoming increasingly apparent. The mechanism(s) by which statins decrease inflammation and autoimmunity are still under debate. Several potential explanations have been put forward (reviewed in [69,70]). First, it has been established that some statins can inhibit interactions between cellular adhesion molecules by binding to leucocyte function antigen-1 [71]. However, it is not clear whether this function is applicable to the majority of statins. Second, it is known that statins block the isoprenylation of molecules such as Ras and Rho that are important for lymphocyte function [72,73]. Third, statins have been shown to decrease IFNγ-induced MHC class II expression [74]. Our work provides a further mechanism by which statins could have anti-inflammatory effects by showing that these drugs inhibit the activation of human peripheral blood γδ T cells in response to endogenous nonsterol mevalonate products.

Several studies have established that γδ T cells play a role in allergic airway inflammation [75–78]. Recent results in an OVA-induced airway inflammation model show that the promotion of airway hyper-reactivity and inflammation is dependent on the Vγ chain these cells express [79]. Curiously, simvastatin has just been shown to modulate airway inflammation in this system [62]. Thus, this model may prove ideal for direct testing of whether the anti-inflammatory effects of statins are mediated primarily by the inhibition of γδ T cell activation *in vivo*. However, it is not yet clear whether some murine γδ T cells activate in response to nonsterol mevalonate derivatives. Thus, future studies in humans, where it is established that statins can inhibit γδ T cell activation *in vitro* in response to nBP-induced accumulation of mevalonate intermediates or endogenous mevalonate metabolites in tumour cells (Fig. 5) [23], should aim to determine whether the anti-inflammatory effects attributed to statins are mediated through γδ T cells.

During the preparation of this manuscript, another group has demonstrated that statins prevent nBP-induced γδ T cell proliferation *in vitro* [80]. While this study did not specifically identify the cellular source of nBP-induced proinflammatory cytokines within *ex vivo* PBMC, it does have a number of complementary findings. First, inhibition of protein isoprenylation with specific protein prenyl transfer inhibitors did not stimulate γδ T cell proliferation [80]. Second, products of the mevalonate pathway downstream of FPP synthase did not prevent nBP-induced γδ T cell proliferation [80]. These findings lend further support to the notion that nBP-induced activation of γδ T cells occurs via mevalonate pathway intermediates upstream of FFP synthase such as IPP. Curiously, Thompson and Rogers did not observe IL6 release after nBP treatment of PBMCs *in vitro*, although it is not clear whether PBMC from several different individuals were examined [80]. In our study, nBP-induced IL6 production by γδ T cells was observed in only one of three donors, while all donors had γδ T cells that made TNFα. As only a minority of patients suffers an nBP-induced acute phase response, it is tempting to speculate that this reflects a particular subset of IL6-producing γδ T cells present only in some individuals. Further work is required to test this possibility.

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