



A novel prothrombotic pathway in systemic sclerosis patients: possible role of bisphosphonate-activated $\gamma\delta$ T cells

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Objectives: Infusions of aminobisphosphonates (ABP) activate $V\gamma9\delta2$ T cells *in vivo* and induce an acute inflammatory response in 30% of patients treated for osteoporosis. Following the observation of digital thrombosis in a systemic sclerosis (SSc) patient after treatment with an intravenous ABP, zoledronate (Zol), we evaluated whether patient and control peripheral blood (PB) mononuclear cell (MC, PBMC) acquire a prothrombotic phenotype in response to Zol.

Results: $V\gamma9\delta2$ T cells of both patients and healthy donors (HD) upregulated the CD69 activation antigen and secreted tumor necrosis factor (TNF) α in response to Zol *in vitro*. In addition, exposure to either Zol or lipopolysaccharide (LPS), or to both additively, induced expression of the highly procoagulant, tissue factor (TF)-1 on CD14+ monocytes. Importantly, only Zol-induced TF-1 was blocked by a monoclonal antibody to TNF α . Interestingly, we found that SSc, but not HD, $V\delta1+$ T cells were concurrently activated by Zol to produce interleukin (IL)-4. Addition of plasma from the blood of the SSc patient who developed critical digital ischemia after infusion of Zol, but neither plasma from a second patient with no adverse clinical response to Zol infusion nor of a HD, strongly enhanced Zol-induced monocyte TF-1, which could still be blocked by anti-TNF α .

Conclusion: Aminobisphosphonates induced secretion of TNF α by $V\gamma9\delta2+$ T cells may lead to TNF α -dependent induction of procoagulant TF-1 induction on monocytes. In certain clinical settings, e.g., SSc, TF-1+ monocytes could play a role in triggering clinically relevant thrombosis.

Keywords: T cells, $\gamma\delta$ T cell, scleroderma, tissue factor, thrombosis, aminobisphosphonate, $V\gamma9\delta2$ T cells

INTRODUCTION

$\gamma\delta$ T cells are a subset of T cells combining innate and adaptive functions (1). In Caucasians, 50% of the circulating $\gamma\delta$ T cells express the $\gamma9$ and $\delta2$ genes in the Variable (V) region of the $\gamma\delta$ T cell receptor (TCR) $V\gamma9\delta2$ T cells (2). $V\gamma9\delta2$ TCR recognize metabolites produced in the classical (isopentenyl pyrophosphate, IPP) and alternative [(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)] mevalonate metabolic pathways. These antigens are presented for TCR-mediated recognition by CD277, a ubiquitously expressed cell surface membrane antigen presenting molecule (APM) (3). Together with co-stimulatory signals delivered by antigen presenting cells (APC), $V\gamma9\delta2$ TCR-CD277/IPP cognitive interactions activate the $V\gamma9\delta2$ T cells to secrete cytokines and exert cytotoxic effects. A second major subset of $\gamma\delta$ T cells expresses the $V\delta1$ gene in the TCR structure,

among which a major portion recognize phospholipid antigens (e.g., sulfatide) presented by CD1 family molecules (4).

$V\delta1$ $\gamma\delta$ T cells have been shown to expand oligoclonally in the PB of certain systemic sclerosis (SSc) patients, infiltrate the skin in early phases of the disease, and may secrete factors enhancing collagen production (5). $V\gamma9\delta2$ T cells are also functional in SSc patients, since their circulating $V\gamma9\delta2$ T cells secrete tumor necrosis factor (TNF) α and IFN γ and induce fibroblast apoptosis in the presence of exogenously added IPP (6). However, the immunopathogenic significance of these cells concerning the clinical manifestations in SSc patients remains largely unknown.

In this regard, interactions of $\gamma\delta$ T cells with bisphosphonate-activated CD14+ monocytes may play a critical role. Bisphosphonates block farnesylpyrophosphate synthase (FPPS) downstream

of IPP in the mevalonate pathway in circulating CD14⁺ monocytes, increasing intracellular IPP, which is presented to circulating V γ 9 δ 2 T cells leading to their activation (7, 8). As a consequence, these cells produce TNF α and IFN γ , the central mediators of the acute phase response (APR) following infusion of zoledronate (Zol) to patients (8). Accordingly, upon administration of a bisphosphonate drug for osteoporosis or to decrease bone metastasis in cancer, an APR characterized by fever, chills, and arthralgia occurs in up to 30% of patients (8). Zol also activates dendritic cells and natural killer (NK) cells at least in part dependent upon V γ 9 δ 2 T cell activation (9, 10). CD86 and other stimulatory molecules, which enhance activation of other T cell subsets are also upregulated by Zol on dendritic cells (11). Recently, Zol was also shown to stimulate B cells directly (12). Our recent observation of the rapid onset of gangrene of fingers and toes in a patient with SSc after Zol administration prompted the experiments in this study that were designed to evaluate how bisphosphonate-activated $\gamma\delta$ T cells could play a pathogenic role in SSc.

MATERIALS AND METHODS

PATIENTS

The study was approved by the Institutional Review Board (Helsinki Committee) of the Sheba Medical Center, Ramat Gan, Israel. All patients participating in the study were seen in the Rheumatology and Pulmonary clinics at Sheba or Rambam medical centers. Patients fulfilled the criteria of the American College of Rheumatology for systemic sclerosis (SSc), also named herein scleroderma (SCL) (13). Controls included six healthy donors (HD), two patients with idiopathic pulmonary fibrosis (IPF), and one patient with polymyalgia rheumatica (PMR).

ISOLATION OF PBMC AND CHARACTERIZATION OF CELL SUBSETS

PBMC were isolated by Lymphoprep (AXIS-SHIELD, Oslo, Norway) density centrifugation and cultured in growth medium as previously described (14). Cells were stained with fluorochrome conjugated monoclonal antibodies (mAb) specific to CD4, CD3, CD142, CD14, CD69, or isotype control (BD Biosciences), or to human V γ 9, V δ 2 (Immunotech), and V δ 1 (Endogen, Pierce) and analyzed by flow cytometry (Calibur, Beckton Dickinson, CA, USA).

TISSUE FACTOR INDUCTION AND INHIBITION

PBMC were incubated and stimulated either with 2 μ M of Zol (Novartis) overnight (ON) or with 50 ng/ml of *E. coli* 0111:B4 lipopolysaccharide (LPS) (InvivoGen) for 3 h. For double LPS + Zol stimulation, cells were cultured with Zol ON then LPS was added for an additional 3 h. Cells were harvested, washed, and stained with CD14 and CD142 specific antibodies. For inhibition experiments, PBMC from HD were cultured in medium with increasing dilutions of either anti-TNF α antibody (Infliximab, Janssen Biologics) or control IgG mix (Gammaplex, Bio Products Laboratory, Herts, UK) prior to stimulation with Zol or/and LPS.

CYTOKINE DETECTION

Intracellular cytokine detection was performed as previously reported (14). IFN γ detection in supernatants was done using

the ELISA max Deluxe Sets (Biolegend, CA, USA) according to the manufacturer's instructions.

RESULTS

SSc-DISEASE SPECIFIC RESPONSE OF V δ 1+ T CELLS TO ZOL

Activation of $\gamma\delta$ T cells in SSc patients was compared to that of healthy individuals and patients with other chronic inflammatory diseases quantitating percentage of cells producing a panel of relevant intracellular cytokines. Thus, PBMC were incubated ON with Zol and secretion of cytokines was measured by intracellular staining of PB T cell subsets. Percentage of cells among the CD4⁺, V γ 9 δ 2⁺, and V δ 1⁺ T cell subsets in PBMC of 3 SSc patients (RP0-2), 3 non-SSc patients with IPF or PMR (Pt 1-3), and 6 HD that were induced to secrete IFN γ , TNF α , IL-4, or IL-9 after 4.5 h of incubation in medium alone, or with Zol or PMA (20 ng/ml) and ionomycin (0.8 μ M) (P/I) are shown in **Figure 1**. A significantly higher percentage of IL-4 producing cells was observed among SSc patient's V δ 1⁺ cells exposed to Zol compared to either non-SSc patients ($p < 0.03$, Student T test) or HD ($p < 0.003$, Student T test). In contrast, there was no significant difference in the mean percentage of cells secreting any of the other cytokines in SSc compared to HD in the remaining T cell subsets. SSc patient IFN γ production was, however, significantly lower than in the non-SSc patients among Zol-activated CD4⁺ T cells, and P/I-activated V δ 1⁺ T cells. Among SSc V δ 1⁺ T cells, the percentage of Zol-activated IL-9 producers were also significantly lower than in non-SSc patients ($p < 0.05$). These results point to a unique pattern of production of cytokines of SSc V δ 1⁺ $\gamma\delta$ T cells in response to Zol, relative to HD and patients with other chronic inflammatory and fibrotic diseases, characterized by increased production of IL-4 (relative to both HD and disease controls), and decreased production of IL-9 relative to the disease controls.

ACTIVATION OF PATIENT RP2 V γ 9 δ 2+ T CELLS BY ZOL

During the course of these studies, only one of our SSc patients (RP2) developed an unusually dramatic APR after receiving intravenous Zol. Since TNF α and IFN γ produced by V γ 9 δ 2 $\gamma\delta$ T cells are thought to be the mediators of the APR and IFN γ was weakly produced in SSc patients in response to Zol (**Figure 1**), we examined in further detail how Zol had affected TNF α production by this patient's (RP2) PB T cell subsets. As expected, CD4⁺, V γ 9 δ 2 as well as V δ 1⁺ T cells in PBMC of RP2, RP1, and a healthy blood donor all increased their intracellular TNF α in response to P/I, an activating stimulus for T cells that bypasses signals dependent upon cognitive TCR-antigen interactions. Zol potently induced RP2 V γ 9 δ 2 T cells (but not CD4⁺ or V δ 1⁺ T cells) to produce TNF α , similar to its effect on a HD PBMC (**Figure 2**) whereas those of RP1 an SSc patient who had received Zol but no clinical APR did not secrete TNF α in response to Zol application *in vitro*. Production of TNF α was linked to Zol-dependent activation of V γ 9 δ 2 + $\gamma\delta$ T cells. Thus, a markedly increased expression of CD69 on the surface of V γ 9 δ 2 cells (but not V δ 1⁺ cells or CD4⁺ T cells) was concomitantly noted in the presence of Zol on RP2 and HD but not on RP1 V γ 9 δ 2 + T cells. In contrast, P/I stimulation increased CD69 expression in all T cell subsets in all individuals tested (except in HD V δ 1⁺ cells).

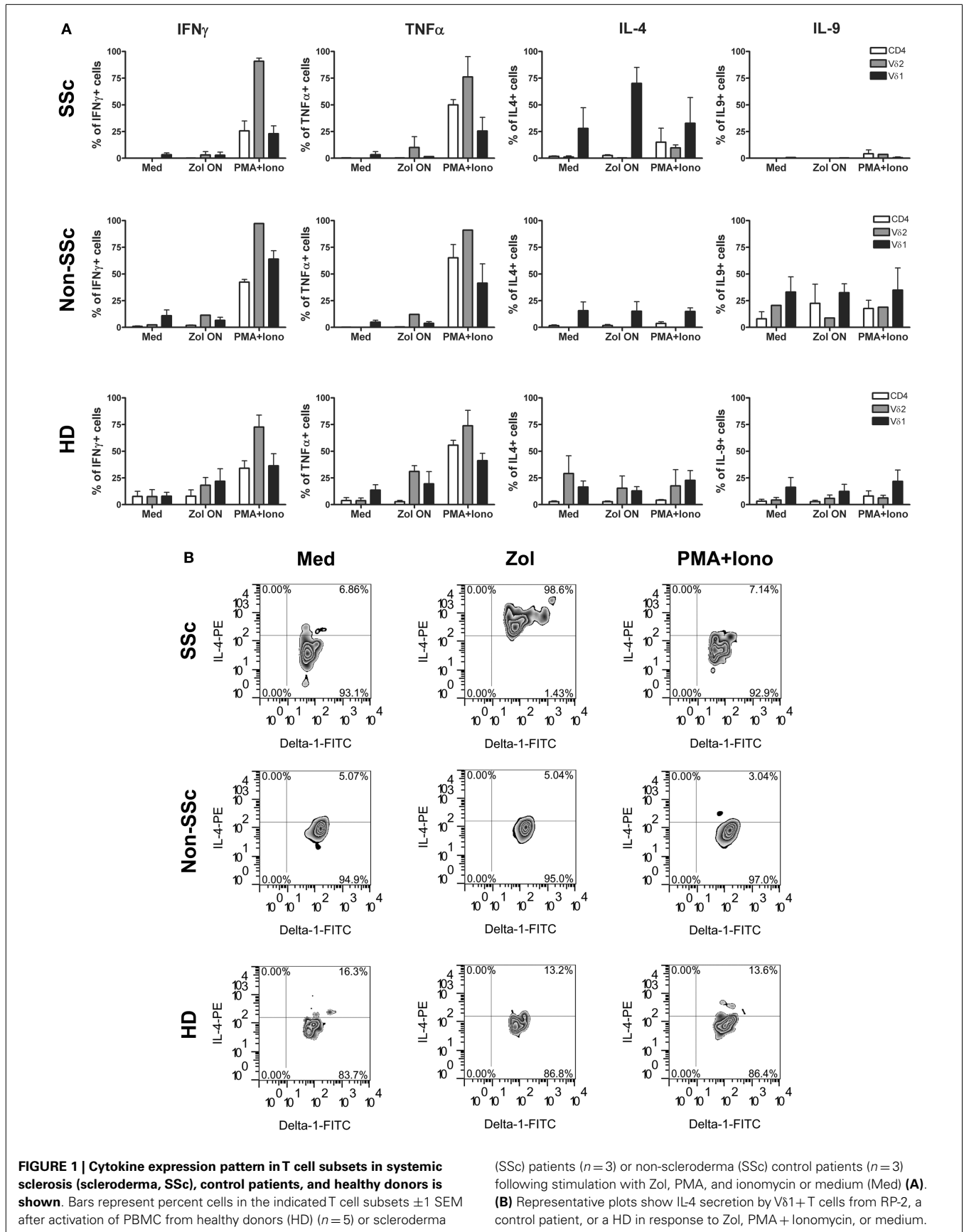


FIGURE 1 | Cytokine expression pattern in T cell subsets in systemic sclerosis (scleroderma, SSC), control patients, and healthy donors is shown. Bars represent percent cells in the indicated T cell subsets \pm 1 SEM after activation of PBMC from healthy donors (HD) ($n = 5$) or scleroderma

(SSc) patients ($n = 3$) or non-scleroderma (SSc) control patients ($n = 3$) following stimulation with Zol, PMA, and ionomycin or medium (Med) (A). (B) Representative plots show IL-4 secretion by V δ 1+ T cells from RP-2, a control patient, or a HD in response to Zol, PMA + Ionomycin, or medium.

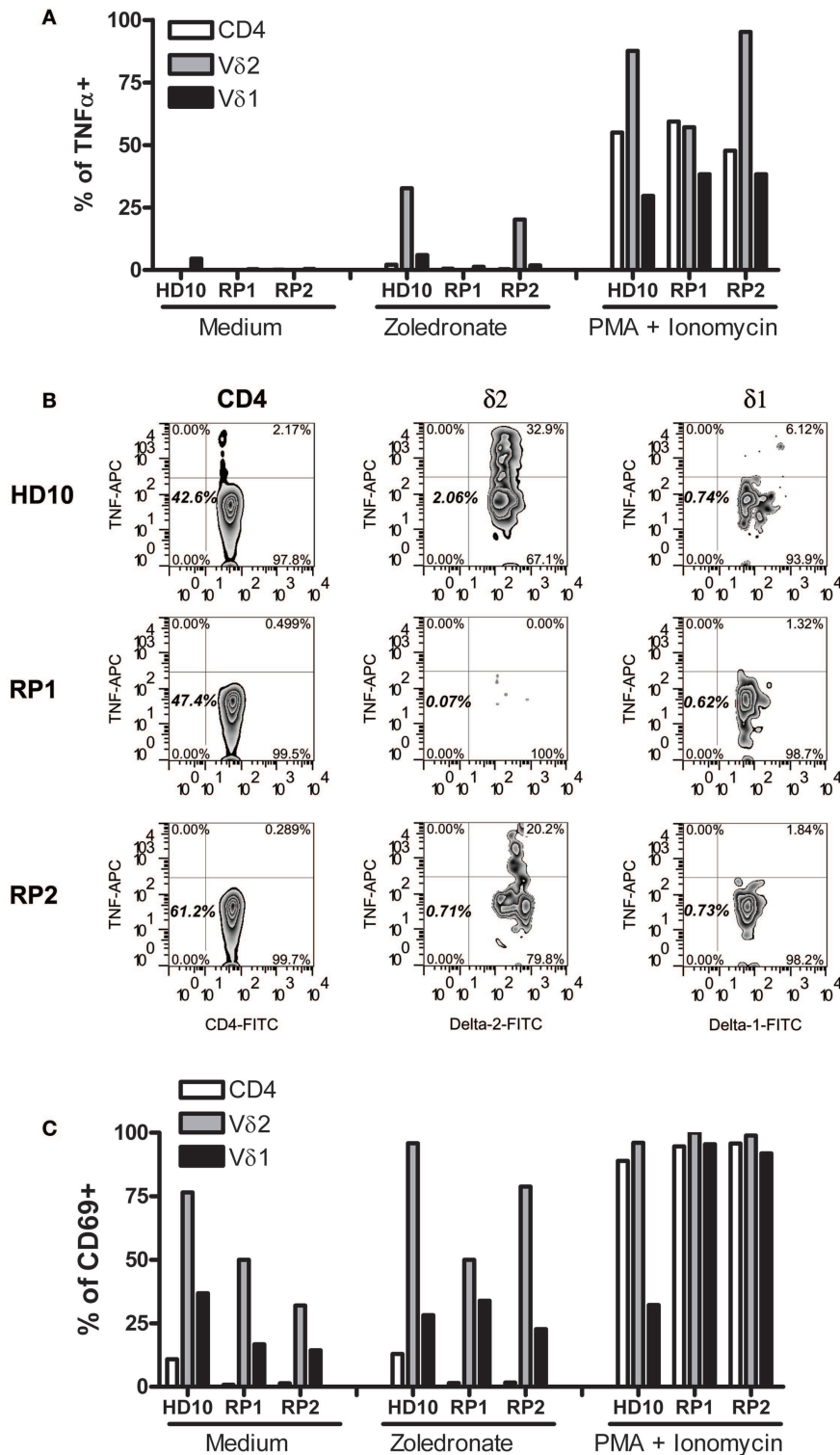


FIGURE 2 | V γ 82 T cells of patient RP2 produced TNF α in response to zoledronate. Bars represent percent cells of HD10, and of Ssc patients RP2 and RP1, within the indicated T cells subsets expressing intracellular TNF α (A) or surface CD69 (C) after stimulation with medium, Zol or PMA, and

iononycin. (B) Representative FACS plots of TNF α staining in the corresponding T cells from patients indicated on the left after Zol stimulation. Numbers indicate percentages of the particular cell population in the respective rectangle.

INDUCTION OF TNF α -DEPENDENT TISSUE FACTOR ON CD14+ MONOCYTES BY ZOL

The occurrence of digital ischemia during the APR in RP2 prompted us to examine whether TNF α produced by V γ 9 δ 2+ cells in response to Zol is sufficient to induce tissue factor (TF)-1, a potent procoagulant factor, on the cell surface membrane of monocytes present in PBMC (15). We utilized HD PBMC to address this issue, because we could obtain only two PB samples from patient RP2 and the patient's response to Zol with respect to TNF α secretion was similar to that of HD (Figure 2). Thus, HD PBMC were incubated ON in medium alone or medium containing Zol. Subsequently, LPS, a known inducer of TF-1 on

monocytes, was added to the medium for an additional 3 h. The expression of the CD142 antigen, which identifies TF-1 on the cell surface, was assessed by FACS analysis after gating on CD14+ monocytes. A marked and significant upregulation of CD142 on HD-derived monocytic CD14+ cells cultured with either Zol alone ON or after the brief 3 h LPS stimulation was observed (Figure 3). There was no upregulation of CD142 in these experiments, indicating that upregulation of CD142 was not due to non-specific elevation of surface membrane molecules on monocytes (data not shown). Furthermore, an additive effect on CD142 levels of expression was noted in PBMC cultured in the presence of both reagents (Figure 3).

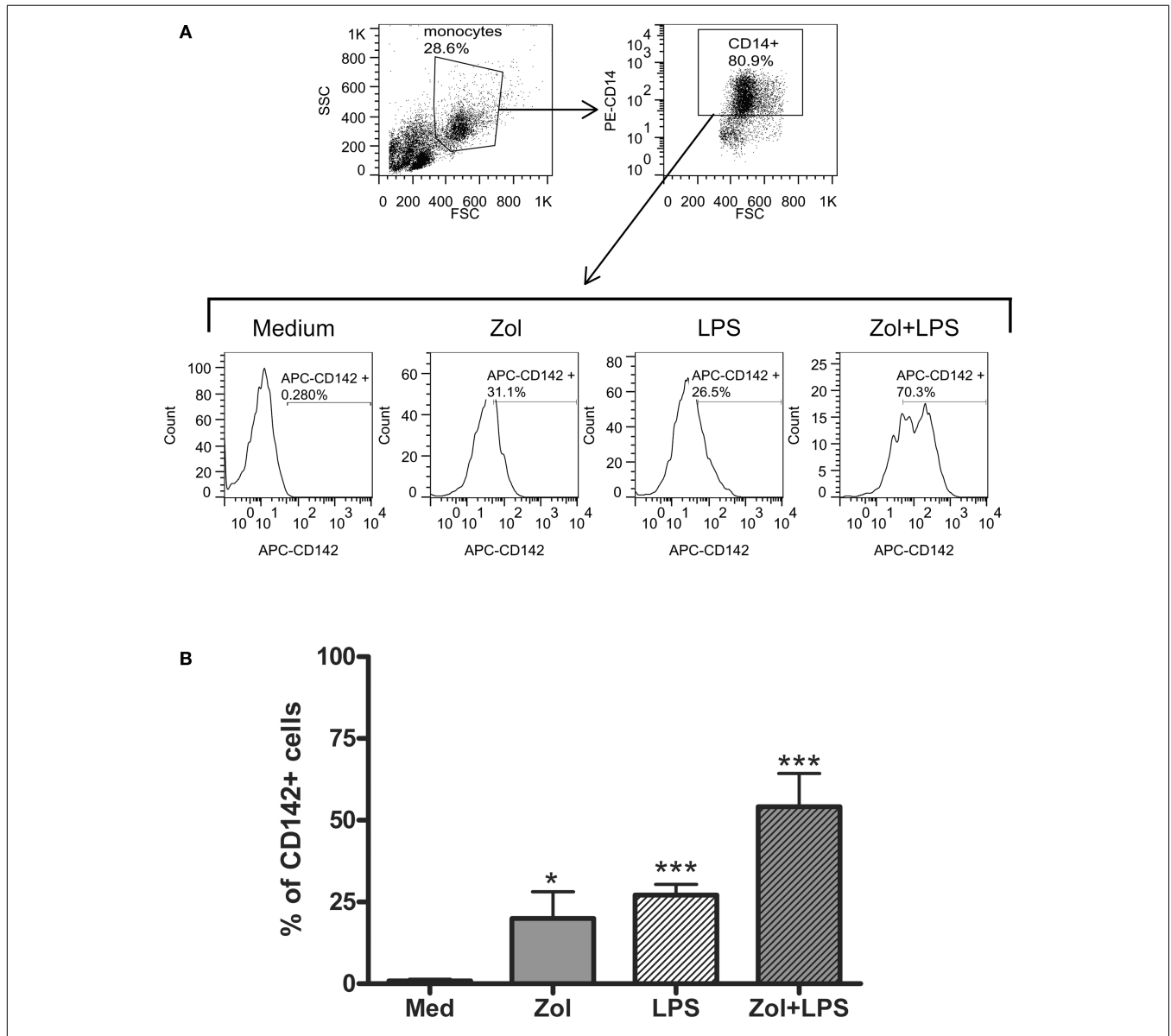


FIGURE 3 | Zoledronate induces tissue factor-1 (TF-1) surface expression on CD14+ monocytes. (A) Histograms in the second panels depict TF-1 expression as percent CD142+ among CD14+ monocytes in PBMC cultured in medium or after stimulation with Zol, LPS, or with both, as indicated (see Materials and Methods). The panel above shows

forward (FSC) and side scatter (SSC) dot plots of the PBMC gating used to identify monocytes and expression of CD14 in the gated monocyte subset. (B) Summary of five independent experiments showing %CD142+ monocytes in cultures of HD PBMC as above. * $p < 0.05$, *** $p < 0.0001$.

We next performed similar experiments and included infliximab, a $\text{TNF}\alpha$ neutralizing humanized mAb, or similar concentrations of non-specific human IgG, in parallel cultures. Infliximab abolished, in a dose-dependent manner, induction of TF-1 on the surface membrane of CD14+ monocytes in response to Zol but not to LPS (Figure 4). Although control IgG also slightly reduced TF-1 expression induced by Zol, inhibition was incomplete even at high concentrations of IgG. Together, these results indicate that Zol, when added to PBMC, induces $\text{TNF}\alpha$ secretion by the $\text{V}\gamma 9\delta 2+$ T cells in the PBMC and $\text{TNF}\alpha$ -dependent expression of TF-1 on CD14+ monocytes.

EFFECT OF PATIENT PLASMA ON TF-1 INDUCTION

Zoledronate has not been previously reported to induce digital necrosis despite its widespread use in patients. Furthermore, in our experience, which included four SSc patients, only RP2 developed an APR and critical digital ischemia, in which TF-1 may have contributed. It was thus of interest to determine the role of RP2 patient specific factors in the development of the unusual response to Zol. We found that addition of RP2-plasma (RP2-P) markedly increased TF-1 induction on CD14+ monocytes in response to Zol as well as LPS, whereas control plasma (RP1-P) from SSc patient RP1 who had received Zol with no toxic effect did not enhance TF-1 induction (Figure 5). Importantly, addition of anti- $\text{TNF}\alpha$ mAb but not of non-specific IgG still completely abolished TF-1 upregulation in response to Zol even in the presence of RP2-P. Anti- $\text{TNF}\alpha$ mAb did not reduce TF-1 upregulation in response to LPS or the combination of LPS and Zol in the presence of RP2-P. These results suggest that the patient’s plasma specifically contained factor/s that enhance Zol-induced $\text{V}\gamma 9\delta 2+$ produced $\text{TNF}\alpha$ -dependent TF-1 expression on monocytes *in vitro*, suggesting that a similar effect may have taken place following IV infusion on her circulating monocytes.

DISCUSSION

This paper shows, for the first time, an SSc specific effect of Zol, i.e., the enhancement of IL-4 secretion by PB $\text{V}\delta 1+$ T cells, coupled with diminished ability of these cells to secrete IL-9 relative to two

patients with another form of fibrosing disease, IPF. Furthermore, Zol induced secretion of $\text{TNF}\alpha$ by $\text{V}\gamma 9\delta 2+$ T cells from some SSc patients similar to healthy individuals, which in turn induced TF-1 on CD14+ monocytes. Finally, plasma of an SSc patient who suffered a clinically detrimental inflammatory response strongly augmented this $\text{TNF}\alpha$ -dependent TF-1 expression on monocytes. These data suggest that activation of both major $\gamma\delta$ T cell subsets – $\text{V}\delta 1+$ and $\text{V}\gamma 9\delta 2+$ by Zol could activate pathogenic mechanisms, e.g., fibrosis (via IL-4) and thrombosis (via TF-1) – relevant to clinical manifestations in SSc patients.

The bisphosphonate-induced APR in patients receiving intravenous therapy for osteoporosis or cancer differs from a typical APR. Thus, CD14+ monocytes and macrophages are the primary cytokine producing cells in the latter, whereas bisphosphonates induce rapid and copious production of $\text{TNF}\alpha$, IFN γ , and IL-6 primarily by PB $\text{V}\gamma 9\delta 2$ T cells (16). Activation of the $\text{V}\gamma 9\delta 2+$ T cells triggered by upregulation of IPP in monocytes in turn enhances CD14, CD40, CD80, and HLA-DR on circulating monocytes (8). Zol also enhances TNF -related apoptosis-inducing ligand (TRAIL) in $\gamma\delta$ T and NK cells, and release of high mobility group box 1 (HMGB1) from $\gamma\delta$ T cells and monocytes (17). Furthermore, soluble factors released by activated $\text{V}\delta 2$ /monocytes cocultures induce granulocyte migration and activation (18). Activated $\text{V}\gamma 9\delta 2$ T cells also trigger granulocyte functions via MCP-2 release during bacterial infection (9).

The novel finding shown here, i.e., induction of TF-1 on monocytes, in a manner dependent upon $\text{TNF}\alpha$ produced by $\text{V}\gamma 9\delta 2+$ T cells stimulated with Zol, adds an additional dimension to the role of monocyte- $\text{V}\gamma 9\delta 2$ interactions, that may play a critical role in clinical medicine, since TF-1 expressing monocytes play an important role in thrombotic diseases (19). For example, patients with cardio- and cerebro-vascular disease have increased TF-1 expression on circulating monocytes and TF-1-positive monocyte-derived circulating microparticles in the blood (20, 21). Furthermore, circulating monocyte-derived microparticles expressing TF-1 are associated with acute recurrent deep venous thrombosis (22). In experimental hypercholesterolemic mice, the associated prothrombotic state is caused by oxidized low density lipoprotein

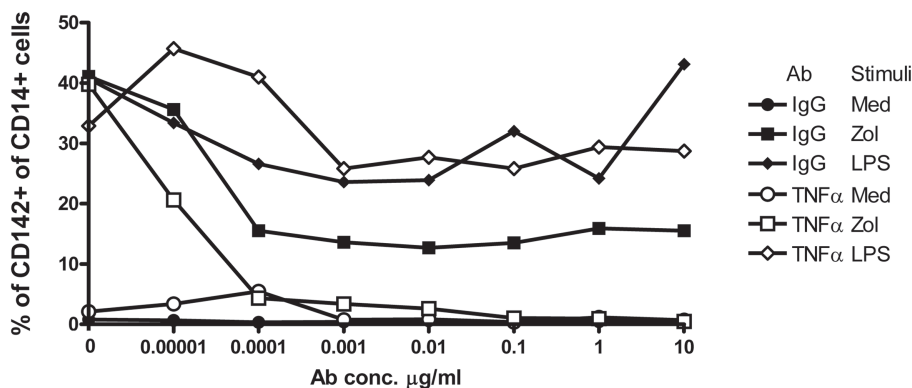
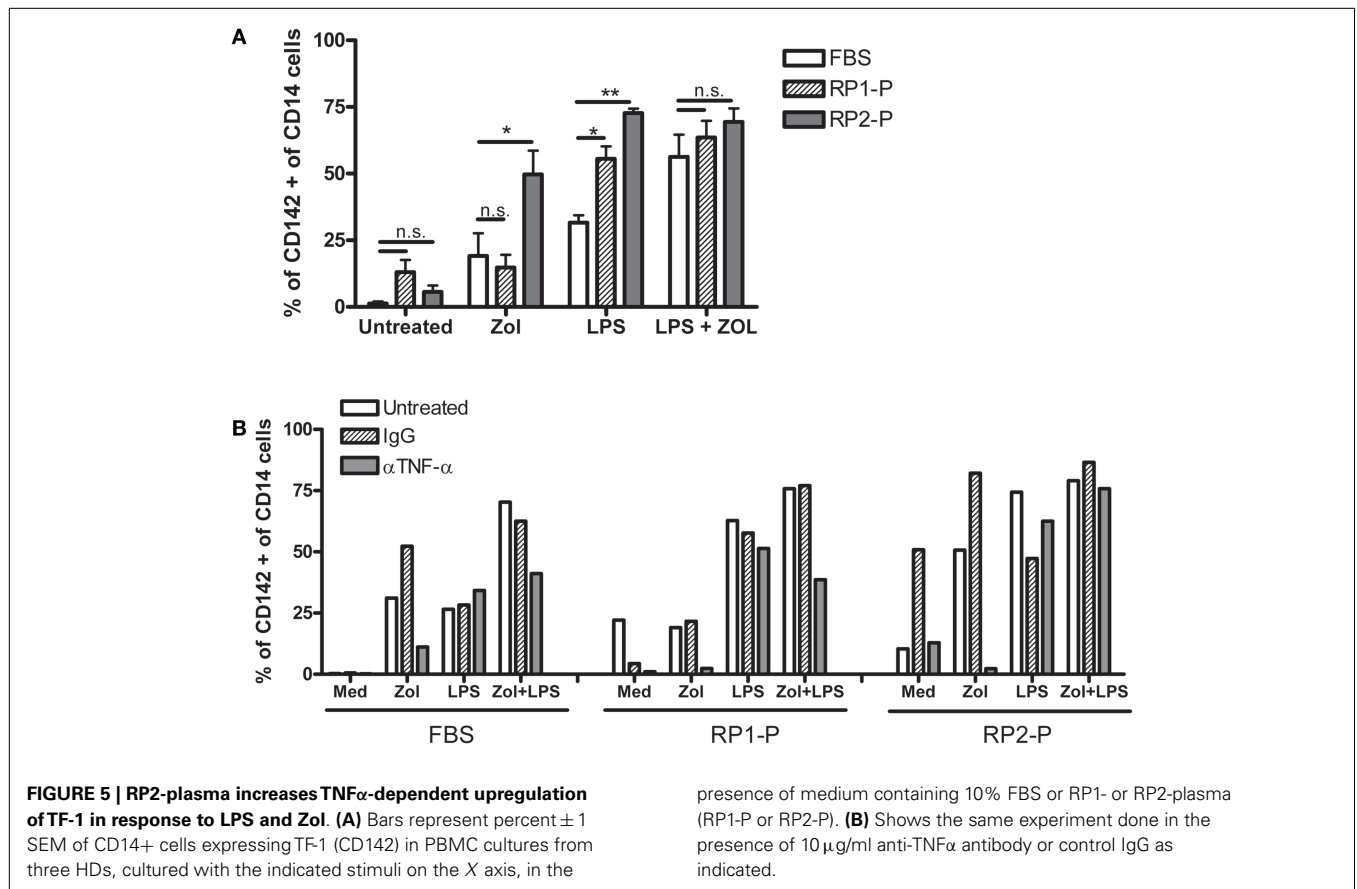


FIGURE 4 | Upregulation of TF-1 in response to Zol is $\text{TNF}\alpha$ -dependent. PBMC from a healthy donor were cultured in medium with graded dilutions (indicated on the x axis) of either anti- $\text{TNF}\alpha$ mAb or control IgG. Cells were

stimulated with Zol, with LPS or not stimulated (medium), and TF-1 was measured by flow cytometry as described in Figure 3. Results are representative of three experiments.



engagement of a toll-like receptor (TLR)4/TLR6 complex, leading to induction of TF-1 in monocytes (23). In addition, in rats, monocytes in blood vessels of kidneys undergoing acute rejection express high levels of TF-1 (24). These data suggest that Zol infusion, which achieves concentrations of zoledronate in the plasma similar to those we have used in our experiments, may lead to induction of TF-1 on circulating monocytes resulting in a prothrombotic state, which may have contributed to development of ischemic digits in SSc RP2 patient described here (25). Our data, furthermore, show that Zol-induced TF-1 on monocytes is at least partly dependent upon $V\gamma 9\delta 2$ cell produced TNF α (Figures 1 and 5). In contrast, a combination of mAb to TNF α and IL-1 β , but neither alone, was required to inhibit high molecular weight kinogen induced monocyte TF-1 (26). TNF α may also upregulate monocyte TF-1 activity indirectly via its effects on endothelial cells (27, 28).

Although Zol induced increase of TF-1 on monocytes, overt thrombosis in the absence of additional factors is rare. Nevertheless, inclusion of zoledronic acid in the treatment protocols for multiple myeloma (MM) and breast cancer significantly increases venous thrombosis (29–32). In this regard, $V\gamma 9\delta 2$ T cells may be playing a role, since in both MM and breast cancer patients, these cells are known to become activated by Zol (33–36). These data suggest that Zol-induced thrombosis is dependent upon disease and/or patient specific factors, which may include Zol responsive $V\gamma 9\delta 2$ T cells in the PB and additional factors.

Our data suggest that SSc may constitute a risk for severe prothrombotic $\gamma\delta$ T cell-mediated Zol-induced reactions. In this regard, the already diseased endothelium in SSc may play a role, since activated $\gamma\delta$ T cells of SSc patients, in particular, adhere to and damage endothelial cells, creating a substrate for enhancing thrombosis (37). The current data suggest that at least two additional factors could play a role in Zol-induced thrombosis in SSc patients. First, SSc patient’s plasma may contain factors that enhance TF-1 expression in response to Zol [Figure 5 and Ref. (16)]. Indeed, SSc plasma has been shown previously to contain increased levels of circulating TNF α , platelet microparticles, and soluble CD40 ligand, which could collaborate in the induction of TF-1 (38). That TF-1 induction in the setting of exposure to Zol can be blocked by an anti-TNF α antibody even in the presence of enhancement by patient’s plasma (Figure 5) suggests that TNF α released by Zol-activated $V\gamma 9\delta 2$ T cells plays a major role in induction of TF-1 and that anti-TNF α mAb could be used to prevent thrombosis in high risk SSc patients treated with Zol. In addition, our study is the first, to our knowledge, to describe a disease-specific IL-4 response of SSc patients $V\delta 1$ T cells to Zol (Figure 1). A bias toward IL-4 secretion by SSc patients CD4 $^{+}$ TCR $\alpha\beta$ T cells in response to non-specific stimulation has already been observed (39). Moreover, $V\delta 1$ $\gamma\delta$ T cells often predominate in the context of a Th2-biased environment, e.g., in the broncho-alveolar lavage fluid obtained from allergic individuals (40, 41). In addition, the majority of

phosphatidyl-ethanolamine CD1d-restricted $\gamma\delta$ T clones in allergic individuals are V δ 1+ and secrete high levels of IL-4 (42). We hypothesize that Zol stimulation may upregulate co-stimulatory monocytes CD40, CD80, and CD1d molecules, which in turn, enhance IL-4 secretion by CD1d-restricted lipid antigen responsive V δ 1+ T cells in the PB of SSc patients (43, 44). The combined effects of the TNF α secreted by V γ 9 δ 2 cells, together with the IL-4 produced by V δ 1+ T cells increases vascular cell adhesion molecule 1 (VCAM-1) expression on endothelial cells in digital arteries and VCAM-1-mediated adhesion of TF-1+ monocytes to the endothelium could then activate local thrombosis and gangrene (45).

In summary, the hitherto described disastrous consequences of a seemingly innocuous and highly utilized drug, Zol, in an SSc patient, which prompted these investigations, led to the discovery of a novel prothrombotic pathway involving V γ 9 δ 2 $\gamma\delta$ T cells and CD14+ monocytes and a disease-specific activation of IL-4 producing V δ 1 $\gamma\delta$ T cells. Future studies into this pathway may lead to new insights into the immunopathogenic mechanisms of thrombotic diathesis in immune-mediated, infectious, and malignant diseases, in which $\gamma\delta$ T cells play a role.

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