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Intravenous Immunoglobulin (IVIG) Attenuates TNF-induced Pathologic Bone Resorption and Suppresses Osteoclastogenesis by Inducing A20 Expression

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Abstract

Investigations on the therapeutic effects of intravenous immunoglobulin (IVIG) have focused on the suppression of autoantibody- and immune complex-mediated inflammatory pathogenesis. Inflammatory diseases such as rheumatoid arthritis are often accompanied by excessive bone erosion but the effect of IVIG on osteoclasts, bone-resorbing cells, has not been studied. Here, we investigate whether IVIG directly regulates osteoclast differentiation and has therapeutic potential for suppressing osteoclast-mediated pathologic bone resorption. IVIG or cross-linking of Fc γ receptors with plate-bound IgG suppressed receptor activator of nuclear factor-kappa B ligand (RANKL)-induced osteoclastogenesis and expression of osteoclast-related genes such as integrin β 3 and cathepsin K in a dose-dependent manner. Mechanistically, IVIG or plate-bound IgG suppressed osteoclastogenesis by downregulating RANKL-induced expression of *NFATC1*, the master regulator of osteoclastogenesis. IVIG suppressed *NFATC1* expression by attenuating RANKL-induced NF- κ B signaling, explained in part by induction of the inflammatory signaling inhibitor A20. IVIG administration attenuated *in vivo* osteoclastogenesis and suppressed bone resorption in the tumor necrosis factor (TNF)-induced calvarial osteolysis model. Our findings show that, in addition to suppressing inflammation, IVIG directly inhibits osteoclastogenesis through a mechanism involving suppression of RANK signaling. Direct suppression of osteoclast differentiation may provide beneficial effects on preserving bone mass when IVIG is used to treat rheumatic disorders.

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Conflict of interest

The authors declare no financial conflicts of interest.

Keywords

Osteoclasts; IVIG; A20

Introduction

Intravenous immunoglobulin (IVIG) contains pooled immunoglobulins from plasma of thousands of healthy donors, and IVIG therapy has been effective in a variety of autoimmune and chronic inflammatory diseases (Kazatchkine and Kaveri, 2001; Schwab and Nimmerjahn, 2013). IVIG delivers its signals via various receptors including Fc gamma receptors (Fc γ R), which bind to the Fc portion of immunoglobulin G, and CD209 (also known as DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) (Anthony et al., 2011). The mechanisms of anti-inflammatory function of IVIG are complex, and a number of possible mechanisms have been described (Kazatchkine and Kaveri, 2001; Schwab and Nimmerjahn, 2013). However, the exact mechanisms for the immunomodulatory and anti-inflammatory effects of IVIG therapy have not been fully elucidated.

IVIG therapy ameliorates symptoms in rheumatoid arthritis patients (Muscat et al., 1995) and protects mice from developing inflammatory arthritis (Campbell et al., 2014; Lee et al., 2014). IVIG consists predominantly of monomeric IgG, but contains a small fraction of polymeric IgG (immune complexes), both of which are important for anti-inflammatory and immunomodulatory effects in various diseases (Nimmerjahn and Ravetch, 2008; Park-Min et al., 2007; Siragam et al., 2006). However, immune complexes can activate innate immune cells and drive inflammation and thus also have pathogenic properties. Immune complexes in which IgG forms a complex with other proteins or nucleic acids have been implicated in autoimmune and other diseases and are formed during infections, tissue injury and various inflammatory conditions (Hoiby et al., 1986; Waldman and Madaio, 2005; Zvaifler, 1973). Chronic immune complex-mediated inflammatory conditions are, in many cases, associated with bone loss and induce uncoupling of osteoclast and osteoblast function, leading to excessive, pathologic bone resorption (Harre et al., 2012; McInnes and Schett, 2011; Novack and Teitelbaum, 2008; Schett and Gravallesse, 2012). However, the effect of immune complexes on osteoclastogenesis in inflammatory bone diseases remains controversial. While immune complexes are one of the key inducers in promoting bone resorption during inflammatory responses, immune complexes are capable of suppressing osteoclast differentiation in mouse bone marrow cells (Grevers et al., 2013; Seeling et al., 2013). Fc γ receptors play an important role in immune-complex mediated diseases, especially murine activating receptor Fc γ RIV, which has a prominent role in the pathogenesis of autoantibody-induced arthritis (Ji et al., 2002; Mancardi et al., 2011). In murine models of inflammatory arthritis, deficiency of activating Fc γ receptors decreases inflammation (Mancardi et al., 2011), but the role of activating Fc γ receptors for bone destruction remains controversial, illustrating the difficulty of developing therapy for targeting Fc γ receptors.

Activating Fc γ receptors had no direct effect on bone destruction in antigen-induced arthritis models (MacLellan et al., 2011; van Lent et al., 2006), but Fc γ RIV, one of the activating Fc γ receptors, plays a positive role in immune complex-mediated bone destruction in the K/BXN serum-induced arthritis model (Ochi et al., 2007; Seeling et al., 2013). Fc γ R-deficient mice have normal bone phenotype (Seeling et al., 2013), demonstrating that Fc γ receptors minimally modulate bone homeostasis in physiological conditions. Importantly, humans and mice have a different repertoire of Fc γ receptors and Fc γ RIV is not expressed in human cells. Therefore, the receptors responsible for IVIG's action in human cells remain elusive.

Despite extensive studies of the beneficial effects of IVIG on inflammatory diseases, the effects of IVIG on osteoclastogenesis in physiological and pathological conditions are not known. Osteoclasts are multinucleated cells that differentiate from myeloid lineage cells and are responsible for resorbing bone and maintaining bone homeostasis (Lorenzo et al., 2008; Takayanagi, 2007). RANKL (receptor activator of nuclear factor-kappa B ligand), a key inducer of osteoclast differentiation and function, binds to its receptor RANK and activates downstream signaling pathways including NF- κ B (Wada et al., 2006). NF- κ B is a well-known transcription factor that regulates expression of genes important for cancer, autoimmune diseases, and immune responses (May and Ghosh, 1998; Sen and Baltimore, 1986; Tak and Firestein, 2001). NF- κ B pathways play an important role in osteoclast differentiation, function, and survival (Soysa and Alles, 2009), and suppressing NF- κ B signaling pathway blocks osteoclastogenesis (Boyce et al., 2010; Novack, 2011; Wada et al., 2006). The activity of NF- κ B is tightly regulated by interaction with inhibitory I- κ B proteins, multiple negative feedback mechanisms, and regulators including A20 (also known as tumor necrosis factor alpha-induced protein 3, TNFAIP3). In this study, we wished to test whether IVIG directly affects osteoclasts and thus can affect bone resorption independently of its effects on inflammation. We found that IVIG suppressed human osteoclast differentiation in a dose -dependent manner and suppressed pathologic inflammation-induced bone resorption *in vivo*. In addition, IVIG prevented osteoclast precursor cells (OCPs) from responding to RANKL and expressing NFATc1, a master transcription factor of osteoclastogenesis. Crosslinking of Fc γ receptors with plate-bound IgG also inhibited osteoclastogenesis, suggesting that the suppressive effect of IVIG on human osteoclastogenesis is mediated, in part, by polymeric IgG (immune complexes) contained within IVIG preparations. Mechanistically, IVIG suppressed RANKL-mediated NF- κ B activation by inducing A20 expression, resulting in the suppression of RANKL-induced NFATc1 expression. These results identify a new mechanism of action of IVIG with therapeutic implications for suppressing bone resorption in rheumatic diseases.

MATERIALS AND METHODS

Mice and analysis of bone phenotype

Six week-old female C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were approved by the Hospital for Special Surgery IACUC. All animals were randomly assigned into experimental groups. For inflammatory osteolysis experiments, we used an established mouse model, the TNF-induced supracalvarial osteolysis model (Kitaura et al., 2005) with minor modifications. In brief, TNF was

administered daily at the doses indicated in the figure legends to the calvarial periosteum of mice for four to five consecutive days. The mice were then sacrificed for the collection of calvarial bones for sectioning. For histopathological assessment, mice were euthanized, and calvarial bones were harvested and fixed in 4% paraformaldehyde for 2 days. These samples were then decalcified with 10% neutral buffered EDTA (Sigma-Aldrich) and embedded in paraffin. To assess osteoclastogenesis and bone resorption, sections were stained with TRAP (tartrate-resistant acid phosphatase) and hematoxylin for osteoclast visualization, and histomorphometric analysis was performed with the BioQuant image analysis system (Nashville, TN, USA) using standard procedures (Parfitt et al., 1987). Osteoclasts were identified as TRAP⁺ cells that were multinucleated and adjacent to bone.

Reagents

Human M-CSF and sRANKL were purchased from Peprotech (Rocky Hill, NJ). The clinically used IVIG preparation Carimune NF[®] was purchased from Cardinal Health (Dublin, OH). The antibodies used for immunoblotting are as follows: NFATc1 (BD Pharmagen); p38 (Santa Cruz Biotechnology); Lamin B (Abcam); p65, p100/p52, p100/p50, and I- κ B α (Cell Signaling).

Cell culture

Peripheral blood mononuclear cells were obtained from blood leukocyte preparations purchased from the New York Blood Center by density gradient centrifugation with Ficoll (Invitrogen, Carlsbad, CA) using a protocol approved by the Hospital for Special Surgery Institutional Review Board. Monocytes were obtained from peripheral blood, using anti-CD14 magnetic beads, as recommended by the manufacturer (Miltenyi Biotec Auburn, CA). Monocyte-derived osteoclast precursors (OCPs) that express RANK were obtained by culture for one day with 20 ng/ml of M-CSF (Peprotech) in α -MEM medium (Invitrogen) supplemented with 10 % fetal bovine serum (FBS, Hyclone), and purity of monocytes was >97%, as verified by flow cytometric analysis. For ligation of Fc γ receptors, plates were coated with IgG by incubating with IVIG at the indicated concentrations for 1 hr at room temperature and washed with phosphate-buffered saline (PBS) before cells were plated. OCPs were harvested and added to IgG-coated plates for 1 hr. Polymyxin B (14 μ g/ml; Sigma Aldrich), which was verified to not directly stimulate macrophages but essentially completely blocked exogenous LPS up to concentrations of 10 ng/ml in our system, was used to ensure that contaminating endotoxin did not contribute to the observed effects, as previously described (Wang et al., 2010).

Osteoclast differentiation

Human CD14⁺ cells were incubated with 20 ng/ml of M-CSF for one day to generate OCPs. For human osteoclastogenesis assays, cells were added to 96 well plates in triplicate at a seeding density of 5×10^4 cells per well. Osteoclast precursors were incubated with 20 ng/ml of M-CSF and 40 ng/ml of human soluble RANKL for an additional 5 days in α -MEM supplemented with 10 % FBS. Cytokines were replenished every 3 days. On day 6, cells were fixed and stained for TRAP using the Acid Phosphatase Leukocyte diagnostic kit (Sigma) as recommended by the manufacturer. Multinucleated (more than 3 nuclei) TRAP-positive osteoclasts were counted in triplicate wells.

Gene expression analysis

For real time PCR, DNA-free RNA was obtained using the RNeasy Mini Kit from QIAGEN with DNase treatment, and 1 µg of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas, Hanover, MD). Real time PCR was performed in triplicate using the iCycler iQ thermal cycler and detection system (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocols. Expression of the tested gene was normalized relative to levels of GAPDH. Primers used in this study are as follows: hA20 5'-CCGGCTGCGTGTATTTGGGACTC-3', 5'-GGAACCTGGACGCTGTGGGACTGA-3; hNFATc1 5'-CTTCTTCCAGTATTCCACCTAT-3', 5'-TTGCCCTAATTACCTGTTGAAG-3'; hITGB3 5'-GGAAGAACGCGCCAGAGCAAAATG-3' 5'-CCCCAAATCCCTCCCCACAAATAC-3'; hCTSK 5'-CTCTTCCATTTCTTCCACGAT-3', 5'-ACA CCA ACT CCC TTC CAA AG-3'; hBCL6 5'-CCTCGCCAGCCACAAGACCG-3', 5'-CTGGCTCCGCAGGTTTCGCA-3'; hIRF8 5'-TGCGTCCAAACTCATTCTCGTG-3', 5'-GTCTGGCGGCGGCTCCTC-3'; hMAFB 5'-CTCAGCACTCCGTGTAGCTC-3', 5'-GTAGTTGCTCGCCATCCAGT-3'; hFcγRIIa 5'-CATCACTGTCCAAGTGCCCA-3', 5'-CCACAATGATCCCCATTGGT-3'; hGADPH 5'-ATCAAGAAGGTGGTGAAGCA-3', 5'-GTCGCTGTTGAAGTCAGAGG-3'

RNA interference

0.2 nmol of three short interfering RNAs (siRNAs), specifically targeting human FcγRIIa (L-014152-00-0005, Dharmacon), human A20 (TNFAIP3: M-009919-99-0005, Dharmacon), or non-targeting control siRNA (Dharmacon) were transfected into primary human CD14⁺ monocytes with the Amaxa Nucleofector device set to program Y-001 using the Human Monocyte Nucleofector kit (Amaxa), as previously described (Park-Min et al., 2007).

Flow Cytometry

Staining for cell surface expression of various proteins was performed using antibodies against human CD64, CD32, and CD16 (BD Pharmingen). A FACSCalibur flow cytometer with CELLQuest software (Becton Dickinson) was used.

Statistical analysis

All statistical analyses were performed with Graphpad Prism 5.0 software using the 2-tailed, paired *t*-test (two conditions) or one-way ANOVA with Tukey's post *t*-test for multiple comparisons (more than two conditions). *p*<0.05 was taken as statistically significant.

Results

Inhibition of RANKL-induced human osteoclastogenesis by IVIG

IVIG has been applied as a treatment for a broad range of diseases (Kazatchkine and Kaveri, 2001; Schwab and Nimmerjahn, 2013). Here, we wished to test the effect of IVIG treatment on human osteoclast differentiation. Human CD14-positive (CD14⁺) monocytes were cultured with M-CSF and IVIG for 24 hours, and cells were subsequently treated with

RANKL for five additional days. IVIG treatment before RANKL stimulation significantly inhibited osteoclastogenesis in a dose-dependent manner (Fig. 1a and b). Accordingly, IVIG inhibited osteoclast-specific gene expressions such as *CTSK* (encodes cathepsin K) and *ITGB3* (encodes integrin β 3) when it was added before RANKL stimulation (Fig. 1c). The highest dose of IVIG we used (1 mg/ml) is relevant to the therapeutic dose for patients (20 mg/kg of body weight) and completely inhibited *in vitro* osteoclastogenesis. IVIG is endotoxin-free, and we further confirmed that this suppressive effect did not result from LPS contamination (Supplementary Fig. 1). Our results indicate that IVIG directly suppresses osteoclast differentiation of osteoclast precursor cells.

Major receptors for IVIG are Fc γ receptors (Schwab and Nimmerjahn, 2013). In human cells, three different classes of Fc γ Rs (Fc γ RI, Fc γ RII and Fc γ RIII) have been described; Fc γ RII has an activating Fc γ RIIa and inhibitory Fc γ RIIb isoform. Fc γ RIV is only expressed in mouse cells and Fc γ RIIa is only expressed in human cells. In human OCPs, four Fc γ receptors (Fc γ RI, Fc γ RIIa, Fc γ RIIb, and Fc γ RIII) are expressed (19). To test the role of Fc γ receptors in IVIG-mediated inhibition on osteoclastogenesis, we knocked down the expression of individual Fc γ receptor using small interference RNAs (siRNAs). Knock-down of human specific Fc γ RIIa significantly reversed IVIG-mediated suppression of osteoclastogenesis (Fig. 2a and b). Other Fc γ receptors also played a role in IVIG's inhibitory action but the contribution of these receptors was not statistically significant and was not sufficient to rescue IVIG-mediated inhibition of osteoclast differentiation (Supplementary Fig. 2). Decrease of Fc γ RIIa expression increased osteoclastogenesis in the control RANKL-stimulated condition, suggesting that immunoglobulin in serum can be involved in basal suppression in osteoclast differentiation *in vitro*. It has been shown that IVIG utilizes different Fc γ receptors in a disease-dependent manner and thus we measured surface expression of Fc γ receptors during osteoclastogenesis. Consistent with previous observations in murine cells (Seeling et al., 2013), RANKL treatment negatively regulated Fc γ receptor mRNA expression in a time-dependent manner (Fig. 2c). Accordingly, surface expression of Fc γ receptors diminished after RANKL stimulation (Fig. 2d). Our data show that RANKL stimulation decreases Fc γ receptor expression. Next we tested whether the suppressive effect of IVIG correlates with the level of expression of Fc γ receptors. IVIG lost its inhibitory effect on osteoclast differentiation when added after RANKL stimulation (Supplementary Fig. 3a and b). The size of osteoclasts appeared smaller in the IVIG conditions, although total osteoclast number actually increased (Supplementary Fig. 3b, lower panel). Corroborating a lack of suppressive effect on osteoclast differentiation, addition of IVIG after RANKL did not have a significant inhibitory effect on RANKL-induced osteoclast-specific gene expressions (Supplementary Fig. 3c), which contrasts with essentially complete suppression of these genes when IVIG was added prior to RANKL (Fig. 1). Our data suggest that low levels of Fc γ receptor expression may contribute to the inability of IVIG to suppress osteoclastogenesis in RANKL-pretreated cells. Taken together, our data demonstrate that IVIG treatment prior to RANKL suppresses osteoclastogenesis through Fc γ RIIa.

Attenuation of pathologic bone resorption by IVIG in the TNF-induced osteolysis model

To address the biological importance of IVIG-mediated suppression of osteoclastogenesis, we tested whether IVIG could inhibit pathological bone resorption *in vivo*. To minimize the indirect effects that IVIG could exert on osteoclastogenesis by suppressing inflammation, we used the TNF-induced supracalvarial osteolysis model where administration of exogenous TNF allows testing of the effects of IVIG on downstream bone resorption. To test whether IVIG exerts time-dependent effects on *in vivo* osteoclastogenesis, IVIG was administered either at the same time as TNF or 2 days after initial TNF treatment to test preventive and therapeutic efficacy of IVIG on *in vivo* osteoclastogenesis (Fig. 3a, group I versus group II). IVIG attenuated TNF-mediated induction of TRAP-positive osteoclasts and associated bone resorption independent of treatment time (Fig. 3b). The reduction in *in vivo* osteoclastogenesis was corroborated using histomorphometric analysis to quantify osteoclast numbers and surface area; osteoclast numbers per bone surface (N.OC/BS) and osteoclast surface area per bone surface (OC.S/BS) were significantly lower in both IVIG-treated groups (Fig. 3c and 3d). These results show that IVIG effectively suppresses inflammatory bone resorption; the suppression of osteoclastogenesis when IVIG therapy was started after TNF is most likely explained by suppressive effects on osteoclast precursors before they are exposed to RANKL *in vivo*.

IVIG suppress NFATC1 induction

We previously showed that inhibition of distinct signaling pathways, such as Jak-STAT signaling, by IVIG is mediated by soluble polymeric IgGs contained within IVIG preparations (Park-Min et al., 2007). We tested whether IgG-mediated crosslinking of Fc γ receptors could inhibit osteoclast differentiation. We used plate-immobilized human IgG to model crosslinking Fc γ receptors on cells by soluble polymeric IgGs (Ravetch and Bolland, 2001). CD14⁺ cells were plated on IgG-precoated wells to crosslink Fc γ receptors, RANKL was added on the next day, and cells were cultured for five days. Crosslinking of Fc γ receptors (labeled X-linked) by 0.1 mg/ml of IgG strongly suppressed osteoclastogenesis (Fig. 4a) and osteoclast-associated gene expression (Fig. 4b). We next titrated the dose of IgG and tested the effects on osteoclastogenesis. Low avidity crosslinking by small amounts of IgG (0.1 – 1 μ g/ml) slightly, albeit not significantly, increased osteoclastogenesis while the inhibitory effects of crosslinking Fc γ receptors only became clearly apparent at 50 μ g/ml (Fig. 4c). Our data show that crosslinking Fc γ receptors inhibits osteoclastogenesis in a manner parallel to the suppressive effects of IVIG.

We then investigated mechanisms by which IVIG and crosslinking of Fc γ receptors inhibit osteoclastogenesis. A key event in osteoclastogenesis is the induction of the transcription factor NFATc1, a master regulator of osteoclast differentiation. RANKL induced NFATc1 expression, but NFATc1 expression was inhibited by IVIG at both mRNA and protein levels (Fig. 5a and b). Similarly, crosslinking Fc γ receptors suppressed RANKL-induced NFATc1 expression at both mRNA and protein levels (Fig. 5a and b). These results suggest that IVIG and Fc γ receptor crosslinking suppress osteoclastogenesis by inhibiting RANKL-induced NFATc1 induction.

A20 induction by IVIG

Induction of NFATc1 expression requires RANK signaling and concomitant downregulation of transcriptional repressors such as BCL6, IRF8 and MafB that render the *NFATC1* gene unresponsive to upstream signaling (Ivashkiv, 2008; Zhao and Ivashkiv, 2011). We tested the effects of Fc γ receptor crosslinking on expression of transcriptional repressors of osteoclastogenesis whose deficiency in mice accelerates osteoclastogenesis (Zhao and Ivashkiv, 2011) and on RANK signaling. As expected, the transcriptional repressors of osteoclastogenesis BCL6, IRF8 and MafB were downregulated by RANKL (Supplementary Fig. 4). Crosslinking of Fc γ receptors prevented RANKL-induced downregulation of BCL6 and IRF8 (Supplementary Fig. 4). B lymphocyte-induced maturation protein 1 (Blimp1) is a negative regulator of BCL6 and IRF8 (Miyachi et al., 2012; Miyachi et al., 2010) and was induced by RANKL (Supplementary Fig. 5). Crosslinking of Fc γ receptors suppressed RANKL-induced BLIMP1 expression (Supplementary Fig. 5), suggesting that RANK signals could be affected by crosslinking of Fc γ receptors. We performed unbiased transcriptomic analysis to identify IgG-induced negative regulators and found that A20 is induced by IgG (data not shown). We investigated whether A20 is induced by IVIG and crosslinking of Fc γ receptors by IVIG using real-time PCR. IVIG and crosslinking of Fc γ receptors induced the expression of A20 mRNA and protein in a time-dependent manner (Fig. 6a and b), which corroborated previous work (Wang et al., 2010). The deubiquitinating enzyme A20 has been shown to attenuate NF- κ B signaling by suppressing signaling upstream of IKKs (Shembade et al., 2010). We then tested if crosslinking of Fc γ receptors regulates NF- κ B pathways downstream of RANK signaling pathways. RANKL stimulation of human OCPs activated downstream signaling via NF- κ B pathways with robust degradation of I- κ B α (Fig. 6c). Strikingly, prior crosslinking of Fc γ receptors suppressed RANKL-induced degradation of I- κ B α (Fig. 6c). We further analyzed the effect of crosslinking of Fc γ receptors on RANKL-induced NF- κ B signaling. RANKL increased nuclear localization of p50, p52 and p65, whereas crosslinking of Fc γ receptors suppressed p50, p52 and p65 translocation upon RANKL stimulation (Fig. 6d). Taken together, our results show that pretreatment of OCPs with IVIG induces A20 expression and suppresses degradation of I- κ B α and NF- κ B signaling in response to RANKL stimulation.

To test the role of A20 in the inhibitory action of IVIG on osteoclastogenesis, we next knocked down A20 expression using small interfering RNA (siRNA) (Fig. 7a). siRNA-mediated knockdown of A20 increased osteoclastogenesis and partially reversed the suppressive effects of IVIG and crosslinking of Fc γ receptors on osteoclastogenesis (Fig. 7b). Thus, our results show that A20 expression, in part, mediated the suppressive effect of IVIG or crosslinking Fc γ receptors on osteoclastogenesis.

Discussion

IVIG has been used as an effective therapy for a wide range of autoimmune diseases and immunodeficiency disorders (Kazatchkine and Kaveri, 2001; Schwab and Nimmerjahn, 2013). Several mechanisms have been proposed for the immunomodulatory and anti-inflammatory action of IVIG in autoimmune diseases. However, the direct effect of IVIG on osteoclastogenesis has not been mechanistically investigated and signaling pathways that are

important for IVIG's suppressive effect on osteoclast differentiation are not known. In this study, we found that IVIG suppresses osteoclast differentiation and confers partial protection from TNF-induced osteolysis *in vivo*. In our study, IVIG induced a refractoriness of human OCPs to RANKL stimulation, which resulted in diminished NFATc1 induction that prevented osteoclast precursor cells from differentiating into mature osteoclasts. The decrease in NFATc1 expression was explained by attenuated RANKL-induced NF- κ B signaling. Both IVIG and crosslinking of Fc γ receptors induced the expression of A20, an inhibitor of NF- κ B signaling, and knock-down of A20 expression partially reversed IVIG's inhibitory action. Thus, we have identified an inhibitory pathway that plays an important role in the regulation of osteoclast differentiation.

Our study investigated the effect of IVIG on human osteoclast differentiation and supports a new mechanism for the inhibitory action of IVIG. Although IVIG has been used as a beneficial therapy in various inflammatory and autoimmune diseases, the cost, time, and shortage of supply of IVIG poses potential problems for patients (Vaitla and McDermott, 2010). To overcome the limitations of IVIG therapy, several studies have focused on identifying the active component(s) in IVIG preparations, including sialylated IgG and immune complexes (Schwab and Nimmerjahn, 2013). We showed that crosslinking of Fc γ receptors by IVIG inhibits human osteoclastogenesis similarly to the effect of IVIG, suggesting polymeric IgGs serve as an active component for IVIG's inhibitory effect on osteoclastogenesis. In addition, the corresponding receptor for IVIG's inhibitory action has been intensively studied. However, different Fc γ receptors have been implicated in the effect of IVIG, and the role of Fc γ receptors in different diseases is not well understood. Our findings have established that IVIG elicits a suppressive effect on *in vitro* osteoclastogenesis mainly dependent on Fc γ RIIa. Fc γ RIIa contains immunoreceptor tyrosine-based activating motif (ITAM) and has been considered an activating Fc γ receptor. The importance of Fc γ RIIa has been demonstrated in several autoimmune diseases (Tan Sardjono et al., 2005). The classical paradigm of ITAM motifs being activating and ITIM (immunoreceptor tyrosine-based inhibitory motif) motifs being inhibitory has evolved, and it is increasingly appreciated that ITAM motifs can have inhibitory functions as well (Ivashkiv, 2009). This is supported by recent studies demonstrating the inhibitory functions of Fc γ RIIa-ITAM and crosslinking of Fc γ RIIa and IVIG have been shown to ameliorate arthritis in Fc γ RIIa transgenic mice (Ben Mkaddem et al., 2014). In addition, allelic variants in Fc γ RIIa have been associated with differential responsiveness of patients with Kawasaki diseases to IVIG therapy (Shrestha et al., 2012). These studies and our data argue that assessing genetic variation or level of expression of Fc γ RIIa may be useful in evaluating the clinical response to IVIG. However, further study will be needed to demonstrate a conclusive correlation between differences in Fc γ RIIa expression and IVIG treatment in bone diseases.

Previous studies show that ligation of activating Fc γ receptors by immune complexes can generate either negative or positive effects on osteoclastogenesis (Grevers et al., 2013; Negishi-Koga et al., 2015; Seeling et al., 2013). Although the contribution of Fc γ receptors to bone erosion during inflammatory arthritis varies among different models of arthritis (Negishi-Koga et al., 2015; Seeling et al., 2013; van Lent et al., 2006), it has been shown that serum of arthritic mice containing pathological immune complexes directly promotes *in*

vitro osteoclastogenesis (Negishi-Koga et al., 2015), suggesting the diverse function of IgGs in bone metabolism. Depending on the intensity of signals and timing of the ligation, ITAM-mediated signals that are associated with integrin receptors also have a negative role in osteoclast differentiation (Park-Min et al., 2013). Consistently, our findings have established that IVIG elicits a bimodal effect on *in vitro* osteoclastogenesis depending on OCPs' differentiation status – the suppressive effects of IVIG are abrogated by prior RANKL stimulation. One explanation for this time dependent effect of IVIG is likely that downregulation of Fc γ receptor expression by RANK signaling diminishes the ability of IVIG to generate inhibitory signals. This may occur because lower level Fc γ receptor activation may not reach a threshold required to generate an inhibitory signal (for example, a minimal level of A20 required to suppress signaling), or because occupancy of different Fc γ receptors may generate a qualitatively different signal. Because the inhibitory effect of IVIG on osteoclastogenesis, in part, comes from immune-complexes, our findings help to explain the discrepancy between previous studies showing that ligation of activating Fc γ receptors by immune complexes can generate either negative or positive effects on osteoclastogenesis (Grevers et al., 2013; Seeling et al., 2013). However, in the TNF-induced osteolysis model IVIG attenuated *in vivo* osteoclastogenesis regardless of timing of the treatment, suggesting that in this model the response to IVIG of OCPs prior to their arrival at target sites where they are exposed to RANKL plays an important role in suppression of *in vivo* bone destruction.

Although inhibitory functions for Fc γ receptors on osteoclasts have been previously described, signaling pathways that are targeted for suppression are not known. Here, we show that crosslinking of Fc γ receptors mainly targets NF- κ B activation downstream of RANK. NF- κ B signaling is rapidly induced after exposure of cells to various factors including TLR ligands, TNF and RANKL (Ghosh and Hayden, 2008). Both canonical and non-canonical NF- κ B pathways play an important role in osteoclast differentiation, function, and survival (Soysa and Alles, 2009). NF- κ B pathways are tightly regulated by multiple negative feedback mechanisms at different stages of activation to limit detrimental and excessive activation. Two key factors that negatively regulate NF- κ B activation are A20 and I- κ B α (Werner et al., 2008), and IgG crosslinking regulates both A20 and I- κ B α . We have provided evidence supporting the role of IVIG-induced A20 in mediating the suppression of osteoclastogenesis by IVIG, but it is likely that the integrated functions of other signaling pathways downstream of IVIG as well as the maintenance of expression of transcriptional repressors contribute to the IVIG-induced suppression of osteoclast differentiation.

It has been suggested that defective A20 expression or activity is associated with rheumatoid arthritis (Vereecke et al., 2009). A20 deficiency in myeloid cells induces erosive polyarthritis (Matmati et al., 2011), and this induction of arthritis is dependent on Nlrp3 inflammasome (Vande Walle et al., 2014), suggesting that increasing A20 expression would have beneficial effects on inflammatory arthritis. In addition, allelic variants (SNPs) associated with increased risk for rheumatoid arthritis are located in the A20 (*TNFAIP3*) gene locus (Ma and Malynn, 2012; Matmati et al., 2011). In myeloid cells, we have been shown that A20 expression is regulated by ITAM signaling (Wang et al., 2010). Our study demonstrates that A20 is increased by IVIG treatment and plays an important role in the

suppressive effect of IVIG on osteoclast differentiation. We speculate that induction of A20 may have a dual role in erosive arthritis by suppressing both inflammation and osteoclastogenesis. Thus, IVIG would be an efficient tool to induce A20 expression and to provide therapy for pathogenic bone destruction.

Our findings also highlight the importance of negative regulators such as A20 in osteoclastogenesis. Our study and previous reports reveal the negative role of A20 in osteoclast differentiation. Myeloid specific deletion of A20 increased murine osteoclastogenesis (Matmati et al., 2011) and here, we show that increased expression of A20 suppresses human osteoclastogenesis. OCPs express high levels of transcriptional repressors that negatively regulate osteoclast differentiation. RANK signaling decreases these transcriptional repressors, permitting the expression of NFATc1 and osteoclast differentiation. A20 expression is not suppressed by RANK signaling (data not shown) unlike transcriptional repressors in OCPs. Our data suggest that A20 functions as a barrier for uncontrolled activation during osteoclast differentiation but exact mechanisms need to be studied further. Overall, our results support the idea that IVIG changes the balance of negative regulators during osteoclast differentiation towards promoting A20 expression and subsequently suppressing osteoclastogenesis to decrease RANK signaling below the threshold required to induce osteoclast differentiation. In summary, we demonstrate that IVIG has inhibitory effects on osteoclastogenesis and identify the inhibitory signaling pathway that is induced by IVIG. Thus, IVIG may have a beneficial effect on inflammatory bone destruction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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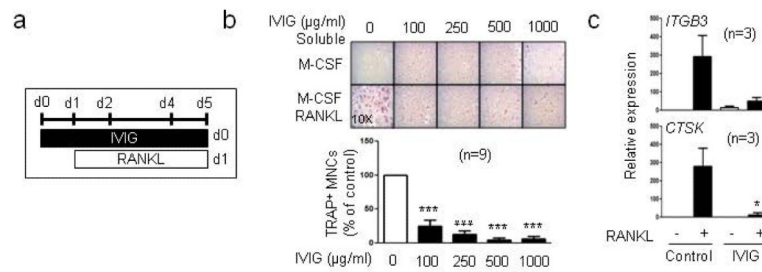


Fig.1. IVIG inhibits RANKL-induced human osteoclastogenesis

(a -c) Human monocytes were cultured with the indicated doses of IVIG (100-1000 $\mu\text{g/ml}$) in the presence of human M-CSF (20 ng/ml) for one day and then 40 ng/ml of human RANKL was added to the culture for 4 days, and TRAP+ multinucleated (more than three nuclei) cells were counted in triplicate on day 5. (a) Experimental scheme, IVIG was added prior to RANKL. (b) *Upper panel*, Representative results obtained from one experiment. *Lower panel*, The number of osteoclasts generated by RANKL alone is set as 100%. Data are shown as mean \pm SEM from 9 independent donors. (c) mRNA was measured using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and normalized relative to the expression of GAPDH. * $p < 0.05$, *** $p < 0.001$ by one-way ANOVA.

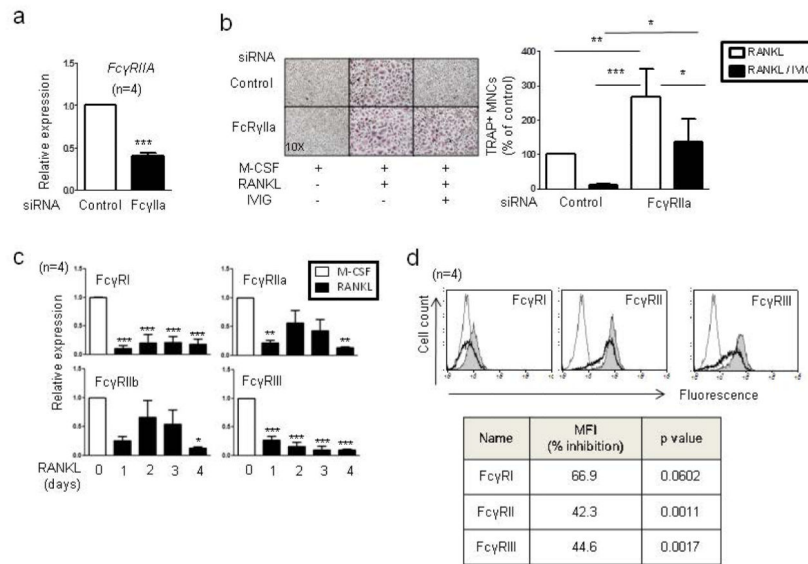


Fig.2. The role of Fc γ RIIa in IVIG-induced suppression of osteoclastogenesis
(a and b) Human monocytes were nucleofected with control or Fc γ RIIa-specific small interfering RNAs (siRNAs). **(a)** Fc γ RIIa knock-down efficiency was measured by RT-qPCR and normalized relative to the expression of GAPDH. *** $p < 0.001$ by paired t -test. **(b)** TRAP-positive, multinucleated osteoclast formation was visualized by TRAP staining. *Left panel*, Representative results. *Right panel*, Values are the mean \pm SEM from three experiments. The number of osteoclasts obtained from control siRNA is set as 100%. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA. **(c-d)** Down-regulation of mRNA and cell surface expression of Fc γ receptors by IVIG. **(c and d)** Human monocytes were cultured for one day with human M-CSF (20 ng/ml). **(c)** Cells were cultured with human RANKL (40ng/ml) for the indicated times and mRNA was measured by RT-qPCR. mRNA of Fc γ receptors was normalized relative to the expression of GAPDH. Values are the mean \pm SEM. *; $P=0.05$, **; $P < 0.01$, *** ; $P < 0.001$. **(d)** RANKL (40ng/ml) was added to the culture for 36 hrs. Surface expression of Fc γ receptors was measured by flow cytometry. Dotted line represents an isotype control. Gray shaded are corresponds to control cells and the thick line represents IVIG treated cells. *Left panel*: Representative histograms from five experiments are shown. *Right panel*: MFI of control cells was set as 100% and mean % of inhibition of surface expression of proteins was shown.

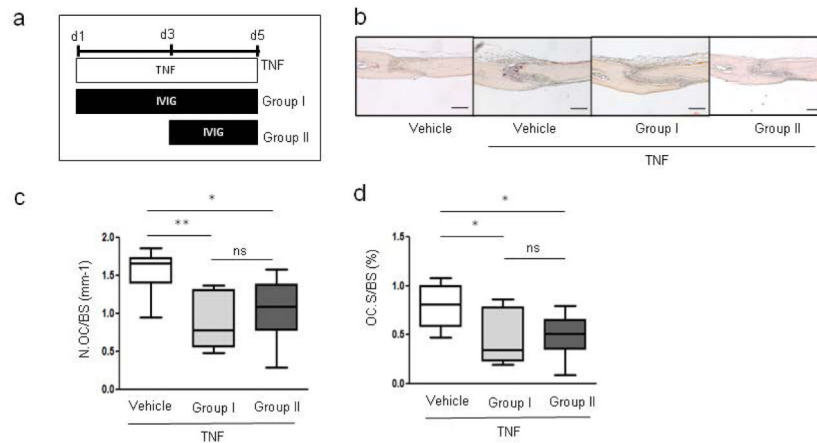


Fig.3. IVIG attenuates *in vivo* osteoclastogenesis in the TNF-induced supracalvarial osteolysis mouse model

(a-d) TNF (1.5 μ g of mouse TNF) was injected over calvarial periosteum of mice (n=6/group) for five consecutive days, and either saline control or IVIG (1 mg/kg body weight) was administered intraperitoneally (i.p.) once per day. (a) Experimental scheme. (b) Representative images of TRAP+ osteoclasts in calvarial sections. Scale bar = 240 μ m (c and d) Histomorphometric analysis; osteoclast number per bone surface (N.Oc/BS) and osteoclast surface area per bone surface (OC.S/BS). * p < 0.05, ** p < 0.01, ns = not significant by unpaired *t*-test.

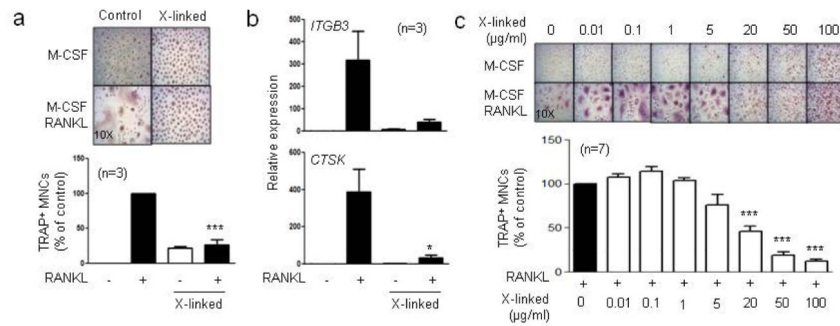


Fig.4. Crosslinking of Fc γ receptors suppresses osteoclastogenesis

(a and b) Human monocytes were cultured for one day with human M-CSF (20 ng/ml) in control wells or wells coated with 100 μ g/ml of IgG (labeled X-linked). After 24 hours, 40 ng/ml RANKL was added for 5 additional days. (a) TRAP⁺ multinucleated cells were counted five days after the addition of RANKL. The number of osteoclasts in control conditions is set at 100%. Values are the mean \pm SEM. (b) Cells were cultured as in (a), and mRNA was measured using RT-qPCR and normalized relative to the expression of GAPDH. (c) Cells were plated in the wells coated with the indicated concentrations of IgG with human M-CSF for 24 hours, and 40 ng/ml RANKL was added for 5 additional days. The number of TRAP⁺ osteoclasts in control condition is set as 100%. Values are the mean \pm SEM. * p < 0.05, *** p < 0.001 by one-way ANOVA.

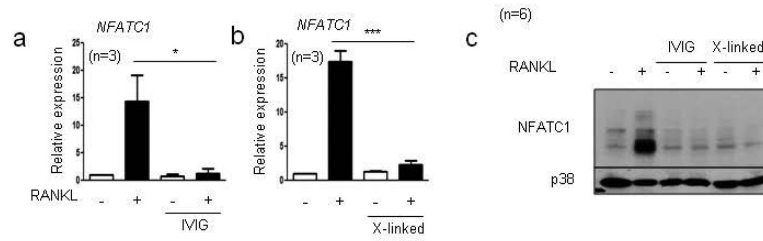


Fig.5. IVIG suppresses RANKL-induced NFATc1 expression

(a-c) Human monocytes were cultured for one day with human M-CSF (20 ng/ml) in control wells, wells with 1 mg/ml of IVIG, or wells coated with 100 μ g/ml of IgG (labeled X-linked). Cells were subsequently cultured with RANKL for 24 hours. (a-b) mRNA was measured using RT-qPCR and normalized relative to the expression of GAPDH. * $p < 0.05$, *** $p < 0.001$ by one-way ANOVA. (c) Immunoblot of whole cell lysates using NFATc1 and p38 antibodies.

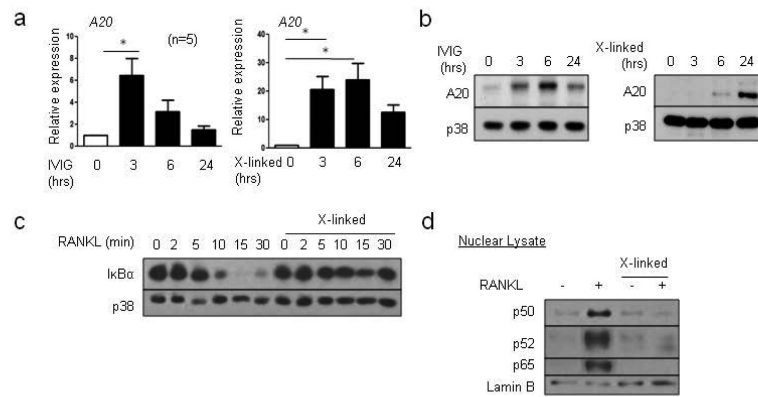


Fig.6. IVIG and plate-bound IgG regulate RANKL-induced signaling pathways, in part, by inducing A20 expression

(a-b) Human monocytes were cultured for one day with human M-CSF (20 ng/ml) in control wells, wells with 1 mg/ml of IVIG, or wells coated with 100 µg/ml of IgG (labeled X-linked). Cells were harvested at 0, 3, 6 and 24 hours after stimulation. (a) mRNA was measured using RT-qPCR and normalized relative to the expression of GAPDH. (b) Immunoblot of whole cell lysates with A20 and p38 antibodies. Representative images from three independent donors. (c) 40 ng/ml RANKL was added for the indicated times, and cells were harvested. Immunoblot of whole cell lysates using I-κBα and p38 antibodies. Representative data from at least three independent experiments are shown. (d) OCPs were treated with 40 ng/ml RANKL for one hour and nuclear lysates were immunoblotted with p50/p105, p52/p100, p65, and lamin B antibodies. Representative data from four independent donors.

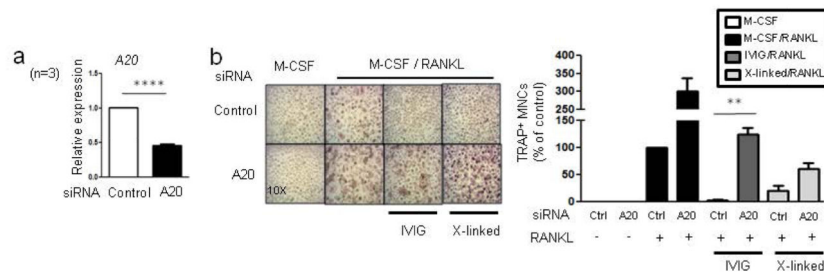


Fig.7. The role of A20 in the inhibitory effect of IVIG on human osteoclastogenesis
(a and b) Human monocytes were nucleofected with control or A20 specific siRNAs. **(a)** A20 knock-down efficiency was measured by RT-qPCR. **** $p < 0.0001$ by paired t -test. **(b)** TRAP-positive, multinucleated osteoclast formation was visualized by TRAP staining. *Left panel*, Representative results. *Right panel*, Values are the mean \pm SEM from three experiments. The number of osteoclasts obtained from control siRNA is set as 100%. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA.