Intravenous immunoglobulin suppresses the polarization of both classically and alternatively activated macrophages

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ABSTRACT

Intravenous immunoglobulin (IVIG) is one of the widely used immunotherapeutic molecules in the therapy of autoimmune and inflammatory diseases. Previous reports demonstrate that one of the anti-inflammatory actions of IVIG implicates suppression of macrophage activation and release their inflammatory mediators. However, macrophages are highly plastic and depending on the microenvironmental signals, macrophages can be polarized into into pro-inflammatory classic (M1) or anti-inflammatory alternative (M2) type. This plasticity of macrophages raised additional questions on the role of IVIG towards macrophage polarization. In the present report we show that IVIG affects the polarization of both classically and alternatively activated macrophages and this process is $F(ab')_2$-independent. Our data thus indicate the lack of reciprocal regulation of inflammatory and non-inflammatory macrophages by IVIG.

Introduction

Macrophages are heterogeneous and ubiquitous cell populations. They are the principal mediators of immune response to pathogens and inflammation. In addition, macrophages also play a pivotal role in tissue homeostasis and repair. In order to exert these diverse functions, macrophages acquire distinct features in response to local microenvironmental signals.[1] Thus, macrophages are broadly divided into two subtypes with opposing functions: classically activated macrophages or M1 and alternatively activated or M2 macrophages.[2,3] M1 macrophages are induced by type I cytokines like interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα), or following recognition of pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS). These macrophages contribute to inflammatory processes and macrophage-mediated tissue injury. In contrast, M2 macrophages are induced by Th2 cytokines like IL-4 and IL-13 and play a role in the resolution of inflammation.[1]

Intravenous immunoglobulin (IVIG), a therapeutic preparation of human normal IgG purified from pooled plasma of thousands of donors is extensively used in the therapy of diverse autoimmune and inflammatory conditions including immune thrombocytopenic purpura, Guillain-Barré syndrome, Kawasaki disease, inflammatory myopathies, chronic inflammatory demyelinating polyneuropathy and many others.[4,5] The current evidences indicate that the therapeutic benefits of IVIG implicate several mutually nonexclusive mechanisms targeting the diverse arms of the inflammatory responses.[6-8]

Previous data show that IVIG suppresses the activation of monocytes/macrophages leading to inhibition of their inflammatory mediators. However, plasticity of macrophages raises additional questions on the role of IVIG towards macrophage polarization. In the present report we show that IVIG affects the polarization of both classically and alternatively activated macrophages thus indicating the lack of reciprocal regulation of inflammatory vs non-inflammatory macrophages by IVIG.

Results

We first investigated whether IVIG could modulate M1 and M2 macrophage polarization. Human peripheral blood monocyte-derived macrophages were cultured with LPS and IFN-γ to obtain M1 macrophages. IVIG was added at two concentrations (10 and 25 mg) during this polarization process. We found that IVIG significantly down modulated the proportion of M1 macrophages as demonstrated by the reduced percentage of cells positive for M1 markers CD80 (Fig. 1A-C) and CCR7 (Fig. 1A, B and D). However, the intensity of expression of both CD80 and CCR7 was not significantly modified by IVIG (Fig. 1C and D). Although polarized M1 macrophages were heterogeneous regarding the expression of CD80 and CCR7 ($CD80^{+}CCR7^{+}$ and $CD80^{+}CCR7^{-}$ cells; Fig. 1A and B), IVIG did not display preferential action on these populations. The proportions of both these populations were reduced by IVIG. As expected, M2 markers CD209 and CD206 were minimally expressed on M1 macrophages and were further reduced by IVIG.

We then explored whether the inhibitory effects of IVIG on macrophage polarization are restricted only to M1 or whether IVIG suppresses the polarization of M2 macrophage as well. Monocyte-derived macrophages were cultured with IL-13 and IL-4 for M2 macrophage polarization and IVIG was added at two concentrations during this polarization process. Although M2 macrophages are associated with anti-inflammatory process, we found that IVIG at high concentrations (25 mg) inhibited M2 macrophage polarization, shown by abrogated expression of CD206 (mannose receptor) (Fig. 2A-C) and CD209 (DC-SIGN) (Fig. 2A, B and D).

Next, we then examined if suppressive effect of IVIG on macrophage polarization is also reflected on the expression of their cytokines. M1 macrophages express predominantly IL-12 while M2 macrophages display more of IL-10. A small proportion of cells (M1 or M2) expressed both the cytokines. However, the proportion cells expressing IL-10 and IL-12 are

low in M1 and M2 macrophages respectively (Fig 3). The low percentage of polarized macrophage populations positive for either IL-12 or IL-10 was possibly due to exhaustion of cells to produce cytokines ('ex-producers').

Our data revealed that IVIG dampens the expression of cytokines from both classically activated M1 macrophages as well as alternatively activated M2 macrophages as shown by reduced expression of IL-12 (Fig. 3A and B) and IL-10 (Fig. 3C and D) respectively. All together, these results suggest that irrespective of inflammatory or anti-inflammatory macrophage phenotype, IVIG hampers both M1 and M2 macrophage polarization.

We explored the mechanism by which IVIG inhibits macrophage polarization. IgG contains Fab region that recognizes specific antigen while Fc exerts effector functions by binding Fcγ receptors. Certain mechanisms of IVIG were reported to be $F(ab')_2$-dependent while others were Fc-dependent.[6] Therefore, we investigated if $F(ab')_2$ fragments of IVIG were able to inhibit macrophage polarization similar to that of intact IVIG. Flow cytometric analyses of the expression of M1 (CD80; Fig. 4A) and M2 macrophage (CD206; Fig. 4B) surface markers revealed that $F(ab')_2$ fragments were dispensable for the IVIG-mediated inhibition of macrophage polarization thus indicating that this effect is mediated either by Fc-domain or by whole IVIG (Fig. 4). Human serum albumin (HSA), used as a protein control for IVIG did not affect polarization of macrophages, thus confirming the specificity of IVIG action on macrophages (Fig. 4).

Discussion

Although monoclonal antibodies and recombinant proteins have revolutionized the management of autoimmune diseases, and several other novel immunotherapies are in pipeline,[9-18] immunotherapy with IVIG is still attractive and has several advantages because of broad-spectrum action and safety profiles. These mechanisms of IVIG represent functions

of circulating normal IgG in the regulation of immune tolerance and immune homoeostasis. The mechanisms of action of IVIG vary depending on the pathologies. Several reports have demonstrated that IVIG imparts tolerogenic properties to innate cells and effector T cells. In addition, IVIG regulates the functions of B cells and neutralizes pathogenic autoantibodies, inflammatory cytokines and complements.[6-8,19-28]

Previous data from several laboratories including ours have demonstrated that IVIG targets monocytes/macrophages to suppress the inflammation both in humans and mice models. IVIG suppresses the production of several inflammatory mediators from the monocytes and macrophages, and enhances the anti-inflammatory cytokines like IL-1RA.[29-32] The suppressive effects of IVIG on the activation of monocytes/macrophages are associated modulation of intracellular signaling events and in particular reduced ERK1/2, P38 MAPK and NF-κB pathways.[33] The microarray data from the monocytes of IVIG-treated patients also indicate suppression of inflammatory genes.[34,35]

The reciprocal regulation of cells implicated in inflammation and anti-inflammation has been reported with IVIG. Thus, IVIG reciprocally regulates immunoprotective regulatory T cells and pathogenic Th1-Th17 cells.[19,21,24-28] Our current data with macrophages however show that this reciprocal regulation is not applicable to all immune cells. Thus, under specific *in vitro* polarization conditions, IVIG suppresses the polarization of both inflammatory and anti-inflammatory macrophages. Data from previous reports both *in vitro* and *in vivo* have demonstrated that the phenotype and cytokine profiles of polarized M1 and M2 macrophages are reversible.[36,37] By suppressing both M1 and M2 polarization, IVIG thus ensures global suppression of macrophage-mediated inflammatory responses. Although M2 macrophages are implicated in eliciting Th2 responses and thus suppress effector Th1 or Th17 cytokines in autoimmune and inflammatory conditions, our data suggest that enhanced Th2 responses

observed following IVIG therapy in autoimmune pathologies does not implicate regulation of cytokine profiles of macrophages.[38]

Of interest, we have observed variations among the individual donors regarding the expression of macrophage markers. For example, the expression of M2 marker CD206 was varying from 33% to 60.4%. It is well recognized that genetic, epigenetic and environmental factors influence the ability of immune cells to respond to stimuli. This individual variation is also reflected among the patients treated with IVIG wherein all the patients treated with this immunotherapy do not respond in a similar way.[39]

A recent report shows that IVIG prevents infiltration of M1 macrophages in a rat model of chemotherapy-induced peripheral neurotoxicity.[40] Another report also found significant reduction in inflammatory cytokines in M1 macrophages upon IVIG exposure thus validating our observations.[41] Similar to this report, we did not observe enhancement of anti-inflammatory cytokine IL-10 in M1 macrophages upon IVIG treatment. Moreover, we have extended our investigation by analyses of surface markers and confirm suppressive effect of IVIG on M1 macrophages based on the expression of CD80 and CCR7. However, in contrast to the report of Dominguez-Soto et al.[41] we did not notice enhancement of M1 macrophage features in IVIG-treated M2 macrophages. These discrepancies are mainly due to experimental conditions. We have used specific polarizing cytokines to explore the effect of IVIG on macrophage polarization while Dominguez-Soto et al. have used LPS stimulation in their experiments.

In our experimental conditions, the uniform expression of CD80 or CD209 indicates differentiation of cells into M1 or M2 macrophages. However, the expression of CCR7 and CD206 in corresponding macrophage populations was not uniform. Thus, we observed $CD80^{+}CCR7^{+}$ and $CD80^{+}CCR7^{-}$ M1 macrophages, and $CD209^{+}CD206^{+}$ and $CD209^{+}CD206^{-}$ M2 macrophages. These data suggests heterogeneity in polarized M1 or M2 macrophages.

Whether cells double positive for the indicated markers are functionally potent compared to single positive cells remain to be explored in the future.

We found that suppressive effects of IVIG on macrophage polarization does not implicate $F(ab')_2$ fragments indicating that either Fc-fragment or entire IgG molecules are required for these effects. These data also indicate that the suppressive effects of IVIG on macrophage polarization are not due to passive neutralization of polarizing cytokines used in the experiments. Though controversy,[42] Fc-mediated effects of IVIG are reported to be mediated mainly by α(2,6)-sialic acid-linkages[43] that are recognized by lectin receptors such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), dendritic cell immunoreceptor (DCIR) and CD22.[20,44-46] M2 macrophages are positive for DC-SIGN (CD209) and further work is necessary to identify the nature of receptor(s) on M1 macrophages that mediate anti-inflammatory action of IVIG.

Methods

Buffy coats of healthy donors were purchased from Centre Necker-Cabanel (Etablissement Français du Sang, Paris, France). Ethical approval (Institut National de la Santé et de la Recherche-EFS Ethical Committee Convention N° 15/EFS/012/ and 18/EFS/033) was obtained for the use of such materials. Monocytes were isolated from peripheral blood mononuclear cells (PBMC) of buffy coats by using CD14 microbeads (Miltenyi Biotec, Paris, France), and subsequently cultured for 6 days in complete RPMI 1640 supplemented with recombinant M-CSF (1000 IU/10^6 cells, Miltenyi Biotec). The obtained nonpolarized macrophages were stimulated with LPS (200 ng/10^6 cells, *Escherichia coli*, Sigma-Aldrich, St. Quentin Fallavier, France) and IFN-γ (40 ng/10^6 cells) (Immunotools, Friesoythe, Germany) for 72 hours to polarize M1 macrophages. Alternatively, nonpolarized macrophages were cultured with IL-4 (500 IU/10^6 cells, Miltenyi Biotec) and IL-13 (400

ng/10^6 cells, Immunotools) to obtain M2 macrophages. IVIG (10 or 25 mg/ml/0.5x10^6 cells; Tegeline®, LFB Biomedicaments, Les Ulis, France) or equimolar concentrations of $F(ab')_2$ fragments of IVIG (16 mg) or HSA (10 mg; LFB Biomedicaments, France) were added during this 72 hours of polarization of nonpolarized macrophages into M1 or M2.

IVIG and HSA were dialysed before their use. $F(ab')_2$ fragments of IVIG were obtained by pepsin digestion (2% wt/wt; Sigma Aldrich). The digested antibodies were passed through protein G Sepharose column to remove intact IgG and SDS-PAGE analysis was done to confirm the purity of $F(ab')_2$ fragments.

Following 72 hours of culturing the cells in the presence or absence of IVIG, the phenotype of macrophages was analyzed by flow cytometry (LSR II, BD Biosciences, Le Pont de Claix, France) using fluorochrome-conjugated MAbs and data were analyzed by BD FACS DIVA software (BD Biosciences). For intracellular staining of cytokines, macrophages following 72 hours of culturing under polarizing conditions were stimulated with phytohaemagglutinin-L (10 µg/ml, Sigma-Aldrich) at 37°C for 18 hours and with golgistop (BD Biosciences) for additional 2 hours. Cells were fixed and permeabilized using Foxp3 Fixation/Permeabilization kit (eBioscience, Paris, France) and incubated at 4°C with fluorochrome-conjugated mAbs.

The antibodies used for flowcytometry were PE-conjugated MAbs to CD80, CD206, IL-10, FITC-conjugated MAbs to IL-12, APC-conjugated MAb to CD209 (all from BD Biosciences), and APC-conjugated MAbs to CCR7 and IL-12 (all from eBioscience).

Data were analyzed by one-way ANOVA with Tukey's multiple comparison test (*P <0.05, ** p< 0.01) using Prism 6 software (GraphPad Software, Inc, La Jolla, USA).

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Disclosure of potential conflicts of interest

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Author contributions

CS, PK, SVK, JB were involved in the study design. All authors participated in the acquition and interpretation of the data. All authors had full access to the data and gave final approval before submission.

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Figure Legends

Figure 1. IVIG suppresses the polarization of classically activated (M1) macrophages. Human monocyte-derived macrophages were treated with LPS and IFN-γ either alone (Ctr) or along with IVIG (10 and 25 mg/ml) for 72 hours and analyzed for the expression of CD80 and CCR7 by flow cytometry. Representative dot-blots (A), scatter plots displaying the relative expression of markers (B), and the expression levels (% positive cells and mean fluorescence intensity, MFI) of (C) CD80 and (D) CCR7. Data are presented as mean ± SEM from 4 independent donors. Statistical significance (*, $p< 0.05$, **, $p<0.01$) as analyzed by One-way ANOVA Tukey's multiple comparison test. ns, not significant.

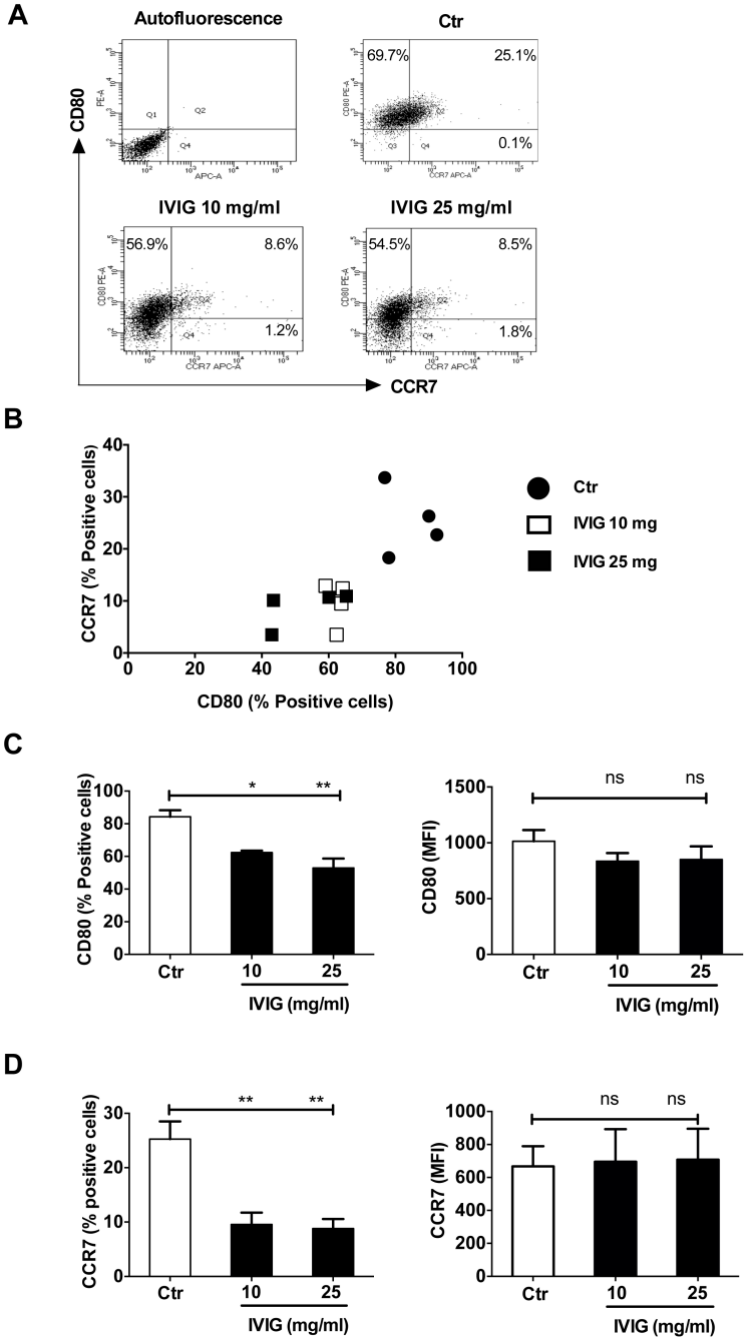

Figure 2. IVIG down regulates the surface markers of alternatively activated (M2) macrophages. Monocyte-derived macrophages were cultured for 72 hours with IL-13 and IL-4 either alone or along with IVIG (10 and 25 mg/ml). The expression of CD206 and CD209 was analyzed by flow cytometry. Representative dot-blots (A), scatter plots displaying the relative expression of markers (B), and the expression levels (% positive cells and mean fluorescence intensity, MFI) of (C) CD206 and (D) CD209 was analysed by flow cytometry. Data are presented as mean ± SEM from 4 independent donors. Statistical significance (*, $p< 0.05$, **, $p<0.01$) as analyzed by One-way ANOVA Tukey's multiple comparison test. ns, not significant.

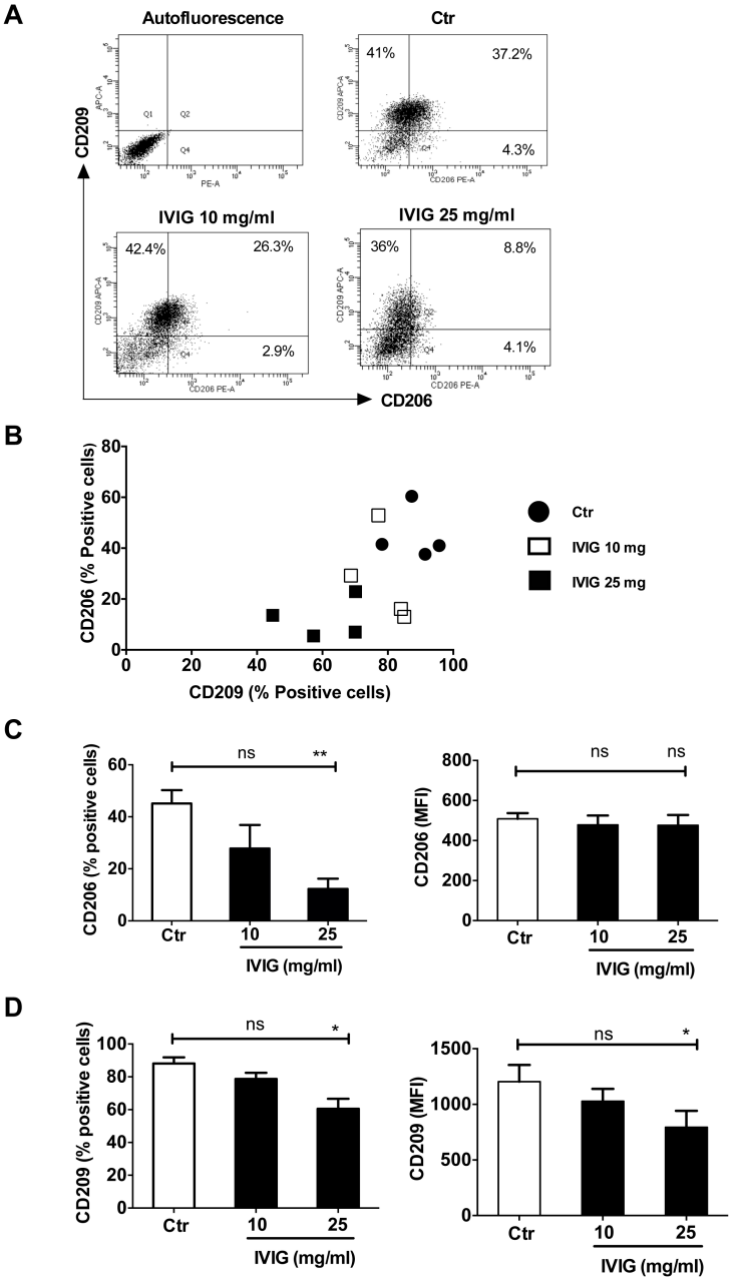

Figure 3. Inhibition of macrophage polarization by IVIG is associated with reduced intracellular expression of (A and B) IL-12 (M1 macrophages) and (C and D) IL-10 (M2 macrophages). Human monocyte-derived macrophages were treated either (A and B) with LPS and IFN-γ either alone (Ctr) or along with IVIG (10 and 25 mg/ml) for 72 hours (M1), or (C and D) with IL-13 and IL-4 either alone or along with IVIG (10 and 25 mg/ml) for 72 hours (M2). The cells were then stimulated with phytohaemagglutinin-L for 18 hours and with golgistop for additional 2 hours. Representative dot-blots are presented in panels A and C. Data are presented as mean ± SEM from 3-4 independent donors. Statistical significance (**, *p<0.01*) as analyzed by one-way ANOVA Tukey's multiple comparison test. ns, not significant.

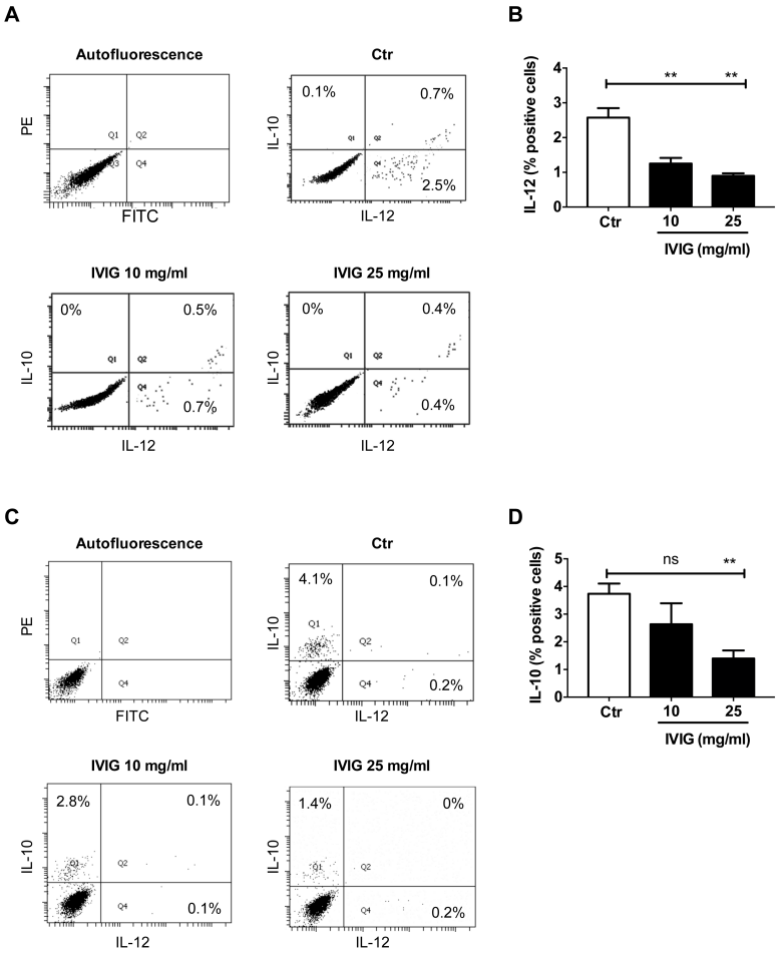

Figure 4. Inhibition of M1 and M2 macrophage polarization by IVIG is F(ab')$_2$-independent. Monocyte-derived macrophages were cultured under either (A) M1 or (B) M2-polarizing conditions for 72 hours along with equimolar concentrations of IVIG, F(ab')$_2$ or HSA. The expression (% positive cells, mean ± SEM from 3 independent donors) of (A) CD80 and (B) CD206 was analysed by flow cytometry. Statistical significance (*, $p < 0.05$) as analyzed by One-way ANOVA Tukey's multiple comparison test. ns, not significant.