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Research paper

A simple method for measuring immune complex-mediated, Fc gamma receptor dependent antigen-specific activation of primary human T cells

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ABSTRACT

Immune complex (IC) deposition of IgG containing autologous antigens has been observed in autoimmunity. This can lead to IC-mediated antigen uptake and presentation by antigen presenting cells (APC) driving T cell dependent inflammation. IgG receptors (FcγRs) have been suggested to be involved in this process. Since ICs have been linked to autoimmune diseases, interfering with IC mediated effects on APCs and subsequent autoimmune T cell activation *via* FcγR blockade may be therapeutically beneficial. However, this is currently challenging due to a lack of translatable animal models and specific human *in vitro* assays to study IC-driven T cell responses. Here, we developed a simple cellular assay to study IC-mediated T cell activation *in vitro* using human peripheral blood mononuclear cells and tetanus toxoid as a model antigen. We observed that tetanus ICs led to a strong induction of T cell proliferation and release of pro-inflammatory cytokines, which are hallmarks of chronic inflammation. This process was exacerbated when compared to tetanus toxoid challenge alone. IC-mediated T cell effects were FcγR dependent and inhibited by high-dose intravenous IgG (IVIg), a drug often used for the clinical treatments of autoimmune diseases. Similar effects were also seen using a hepatitis antigen. Consequently, we propose our assay as a rapid yet robust alternative to more labour-intense and time-consuming protocols, for example involving separate maturation of dendritic cells followed by T cell co-culture to study antigen specific primary T cell activation.

1. Introduction

Immune complexes (ICs) play an important role in the removal of foreign pathogens (Theofilopoulos and Dixon, 1979). Cross-linking of antigens by IgG antibodies leads to cell surface Fcγ receptor (FcγR) cross-linking by the protruding Fc parts of antibodies in ICs, leading to leukocyte activation and uptake of the antibody:antigen complex (Baker et al., 2013; Bournazos et al., 2015). Antigens taken up through this mechanism are digested by the antigen presenting cell (APC), leading to its differentiation and subsequent presentation of antigen derived peptides at the cell surface mainly *via* MHC class II molecules to cells of the adaptive immune system (Regnault et al., 1999; den Haan et al., 2014). This step is critical for the efficient activation of antigen specific pro-inflammatory T lymphocytes in an Fc receptor dependent manner *in vivo* and *in vitro* (Moore et al., 2003), and is accompanied by cytokine secretion and lymphocyte proliferation. Naïve antigen can also be taken up by APCs in a non-IC mediated manner through pinocytosis (Lim and Gleeson, 2011). This process is thought to be less effective, probably due to the absence of FcγR-mediated stimulatory effects (Regnault et al., 1999; Bournazos and Ravetch, 2015). Some human autoimmune diseases as well as chronic infections are characterised by T cell activation elicited by autoantigen-specific ICs. Here, pro-inflammatory T cell activity results in inflammation and tissue damage (Bolon, 2012). A drug currently being used in the clinic aimed to treat diseases characterised by the presence of pathogenic auto-antibodies is high-dose intravenous IgG (IVIg) (Hartung et al., 2009). IVIg is a human-derived product. Even though it has been shown to dampen pro-inflammatory antigen-specific T cell responses (Aubin et al., 2010; Trepanier et al., 2014), at very high doses, it can result in side effects (Duhem et al., 1994; Wittstock et al., 2003). Recently, recombinant Fcγ complexed Fcγ fusion proteins targeting FcγRs for blockade were proposed as potential therapeutics with proposed improved effectiveness compared to IVIg (Bazin et al., 2004; Ortiz et al., 2016). However, the effect of such therapeutics on T cell responses to ICs has not been reported.

In order to determine the effects of FcγR targeted drugs on IC-

Abbreviations: IVIg, Intravenous Immunoglobulin; IC, immune complex; TT, tetanus toxoid; FcγR, Fc gamma receptor; APC, antigen presenting cell; PBMC, peripheral blood mononuclear cells; DC, dendritic cell; MOA, mode of action; CTV, CellTrace Violet; IFN-γ, Interferon-γ; SLE, systemic lupus erythematosus; HBS, hepatitis B surface antigen; HBC, hepatitis B core antigen; OVA, ovalbumin

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mediated pro-inflammatory T cell responses, a robust assay is required. Whilst several primary immune cell based protocols to study antigen specific T cell responses have been reported, they either do not use human primary cells (Schuurhuis et al., 2002; Aubin et al., 2010; Trepanier et al., 2014), or use uncoated antigen lacking the IC-component altogether (Cellerai et al., 2007). Human peripheral blood mononuclear cell (PBMC) based IC assay protocols have been published but do not assess FcγR dependent effects on T cell subsets (Jarvis et al., 1999). Consequently, we developed a human PBMC-based assay allowing detailed study of IC-mediated effects on primary human T cell proliferation and cytokine release, using flow cytometry and cytokine detection assays. We used tetanus toxoid (TT) as a model antigen due to high overall vaccination rates (Gergen et al., 1995) in conjunction with a polyclonal anti-tetanus IgG serum (HyperTET) (Tenold, 1985). TT has been shown to elicit pro-inflammatory T cell responses in humans (Cellerai et al., 2007). Here we describe a method that shows highly reproducible, FcγR dependent T cell proliferation and secretion of pro-inflammatory cytokines in PBMCs. We find that an increased response is observed when the antigen is presented as an IC and hence propose that this mode of presentation may provide a more relevant model of responses seen *in vivo* where IC formation is the norm. IVIg at high therapeutic doses efficiently suppresses tetanus toxoid-IC (TT-IC) mediated T cell proliferation and significantly dampens pro-inflammatory cytokine release. We then reproduced the experimental protocol using a hepatitis B antigen IC to prove the robustness of our approach with similar findings, highlighting the potential to use this assay to demonstrate similar mode of action (MOA) and facilitate pharmacokinetics studies for FcγR targeting drug candidates.

2. Materials and methods

2.1. PBMC preparation

Healthy volunteers were recruited in accordance with principles expressed in the Declaration of Helsinki and according to in-house ethical standards. PBMCs were harvested from fresh whole blood from healthy donors using a Ficoll gradient based technique (Corkum et al., 2015) described in detail in the supplementary part of this article.

2.2. Depletion of $CD14^+$ and $CD19^+$ cells

For magnetic depletion of monocyte and B cell populations, 1.0–2.0 × 10^7 PBMCs were incubated with CD14 microbeads (Miltenyi, 130-050-201) or CD19 microbeads (Miltenyi, 130-050-301) or both according to the manufacturer's instructions. Depletion of positively labelled populations was done using LD columns (Miltenyi, 130-042-901) and depletion efficiency was assessed by flow cytometry.

2.3. Tetanus immune complex (IC) preparation and PBMC challenge

All test condition media were prepared in round-bottom 96-well plates (Corning) at 2 × concentrations prior to the addition of PBMCs. The final assay volume per well was 200 µl. To prepare ICs, HyperTet polyclonal human anti-tetanus serum (250 units, 15-18% protein content; Grifols Therapeutics) was diluted in cell culture assay medium ("R-10"; RPMI-1640 containing 10% FCS and 1% L-Glutamate and Penicillin-Streptomycin solutions; gibco) at 1.0–1.2 mg/ml final assay concentration. Tetanus toxoid (TT, Statens Serum Institut) was added at 1.0 µg/ml final concentration to generate TT-ICs. Control conditions included uncoated TT (1 µg/ml final assay concentration), unchallenged cells and TT-IC mixed with test reagents: IVIg (Gamunex-c, Grifols Therapeutics, 1.0–5.0 mg/ml final concentration); FcγR blocking antibodies (anti-CD16 and anti-CD32; AF1330 and AF1597, R&D Systems, each at 20 µg/ml final concentration). The plate was then incubated at 37 °C for 2 h and then transferred to 4 °C for another 2 h. Subsequently, the plate was rested at room temperature for 10 min and pre-warmed (37 °C) CTV labelled PBMCs in "R10" medium were added to the wells and gently mixed by pipetting. 2.5–4.0 × 10^5 total PBMCs were used per assay condition in quadruplicates. The plate was then incubated for 6 days at 37 °C under standard cell culture conditions.

2.4. Preparation of hepatitis B surface antigen and ovalbumin ICs

Hepatitis B surface antigen (HBS, adw variant) was purchased from abcam (ab167754) and re-suspended in water to a working solution of 0.5 mg/ml. HBS immune complexes (HBS-ICs) were then prepared by applying the same protocol as described above for TT-ICs by incubating HBS with Hepatitis B Immune Globulin (HyperHEP B S/D; Grifols) resulting in HBS-ICs with 1.0 µg/ml of HBS in 1.0 mg/ml of HyperHEP B. Ovalbumin (OVA; InvivoGen EndoFit, #vac-pova) was re-suspended in water to a working solution of 1.0 mg/ml and mixed with 4 different, recombinant human OVA specific IgGs produced in-house at UCB (stock concentration: 20 mg/ml each). Final assay concentrations were prepared to yield OVA-ICs with 1 µg/ml of OVA with 250 µg/ml of each antibody equalling a total of 1 mg/ml of IgG in PBS as described above. PBMCs were then incubated with a final concentration of 1.0 µg/ml of HBS or OVA, or corresponding preparations of OVA-IC or HBS-IC with or without IVIg (5.0 mg/ml final concentration). For control experiments, 1.0 µg/ml of HyperHEP B was also incubated with hepatitis B core antigen (HBC; abcam, ab49014).

2.5. Preparation of dendritic cells (DCs) and T cell isolation from PBMCs

In order to generate dendritic cells from PBMC contained monocytes, 5.0 × 10^7 PBMCs from healthy volunteers were incubated with CD14 magnetic microbeads (Miltenyi, pan monocyte isolation kit, 130-096-537) and purified according to the manufacturer's instructions. 5.0 × 10^6 monocytes were then incubated in R-10 assay medium supplemented with IL-4 (50 ng/ml; R&D Systems) and GM-CSF (100 ng/ml; R&D Systems) for 7 days. Medium was replenished at 3 days post-incubation. Subsequently, for the isolation of autologous T cells, T cells were isolated from a fresh PBMC preparation (2.0 × 10^7) of the same initial donor using CD3 magnetic microbeads (Miltenyi, pan T cell isolation kit, 130-096-535). Purified T cells were labelled with CellTrace Violet (ThermoFisher) according to manufacturer's instructions.

2.6. IC challenge of DCs and co-culture with autologous T cells

DCs were seeded in round-bottom 96-well plates (2.0–4.0 × 10^4 cells/well) and challenged with pre-made TT-ICs as described above in 100 µl of R-10 medium for 3 h at 37 °C. Subsequently, 2.0–4.0 × 10^5 cells/well freshly prepared autologous CellTrace Violet labelled T cells were added to the wells in a 1:10 ratio (DC:T cell). The final assay volume/well was 200 µl. After 6 days in culture, T cell proliferation was quantified by flow cytometry.

2.7. Flow cytometry and cytokine detection

Flow cytometry was performed using fluorochrome coupled antibodies using standard protocols. Briefly, T cells were identified using CD3-APCH7 conjugates, and T cell proliferation was quantified as CellTrace Violet (CTV) dilution. Cytokines (IFN-γ, IL-1β, TNF-α and IL-6) were detected using MesoScale Discovery (MSD) kits. Detailed protocols and reagents as well as data analysis methods and statistical considerations are explained in the supplementary methods part of this article.