

## RESEARCH PAPER

# Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4<sup>+</sup> effector memory T-cells

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**BACKGROUND AND PURPOSE**

In 2006, a life-threatening 'cytokine storm', not predicted by pre-clinical safety testing, rapidly occurred in all six healthy volunteers during the phase I clinical trial of the CD28 superagonist monoclonal antibody (mAb) TGN1412. To date, no unequivocal explanation for the failure of TGN1412 to stimulate profound cytokine release *in vitro* or *in vivo* in species used for pre-clinical safety testing has been established. Here, we have identified a species difference almost certainly responsible for this disparate immunopharmacology.

**EXPERIMENTAL APPROACH**

Polychromatic flow cytometry and intracellular cytokine staining were employed to dissect the *in vitro* immunopharmacology of TGN1412 and other therapeutic mAbs at the cellular level to identify differences between humans and species used for pre-clinical safety testing.

**KEY RESULTS**

*In vitro* IL-2 and IFN- $\gamma$  release from CD4<sup>+</sup> effector memory T-cells were key indicators of a TGN1412-type response. This mechanism of cytokine release differed from that of other therapeutic mAbs, which can cause adverse reactions, because these other mAbs stimulate cytokine release primarily from natural killer cells. In contrast to humans, CD28 is not expressed on the CD4<sup>+</sup> effector memory T-cells of all species used for pre-clinical safety testing, so cannot be stimulated by TGN1412.

**CONCLUSIONS AND IMPLICATIONS**

It is likely that activation of CD4<sup>+</sup> effector memory T-cells by TGN1412 was responsible for the cytokine storm. Lack of CD28 expression on the CD4<sup>+</sup> effector memory T-cells of species used for pre-clinical safety testing of TGN1412 offers an explanation for the failure to predict a 'cytokine storm' in humans.

**Abbreviations**

CCR7, chemokine receptor 7; IFN- $\gamma$ , interferon gamma; IL-2, interleukin-2; IL-8, interleukin-8; mAb, monoclonal antibody; NK, natural killer; PBMC, peripheral blood mononuclear cell; TNF- $\alpha$ , tumour necrosis factor alpha

**Introduction**

TGN1412 is a superagonist CD28-specific monoclonal antibody (mAb) developed as an immunotherapeutic for the purpose of balancing the

immune system (TeGenero, 2005). TGN1412 was called a 'superagonist' because it could activate T-cells without the need for prior engagement of the T-cell receptor complex (Tacke *et al.*, 1997; Lühder *et al.*, 2003). In rodent models, treatment with

CD28 superagonists orthologous to TGN1412 stimulated the release of anti-inflammatory cytokines and the expansion of regulatory T-cells believed to counter autoimmune responses (Rodríguez-Palmero *et al.*, 1999; Lin and Hünig, 2003; Beyersdorf *et al.*, 2005). These CD28 superagonists proved to be an effective treatment of inflammatory autoimmune disease in rodent models (Beyersdorf *et al.*, 2006), and it was believed that a humanized antibody (TGN1412) would have similar potential for human therapy (Elflein *et al.*, 2003). Pre-clinical safety testing of TGN1412 in cynomolgus macaques, at doses of up to 50 mg·kg<sup>-1</sup>·week<sup>-1</sup>, was well tolerated with no signs of toxicity or systemic immune stimulation. However, the first-in-man phase I clinical trial of TGN1412, conducted at a clinical trial unit in London, ended in disaster.

A near-fatal systemic inflammatory immune response, termed a 'cytokine storm', began 1 h after infusion of TGN1412 in all six healthy recipients, and all became critically ill within 12–16 h (Suntharalingam *et al.*, 2006). Subsequent analysis of the TGN1412 used at the trial showed that it was formulated correctly, complied with its product specification and was free from pro-inflammatory contaminants (Duff, 2006; Stebbings *et al.*, 2007). At the request of an Expert Special Group on phase I clinical trials, appointed by the UK Medicines and Healthcare Products Regulatory Agency, an investigation was carried out by the authors into the biological properties of TGN1412. We found that TGN1412 only stimulated a pro-inflammatory cytokine response *in vitro* if appropriately presented to human peripheral blood mononuclear cells (PBMCs) (e.g. by immobilization onto a plastic surface), and that rather than receiving a low safe starting dose, trial volunteers had in fact been given a near maximum immunostimulatory dose (Duff, 2006; Stebbings *et al.*, 2007; 2009). Significantly, PBMCs from cynomolgus macaques were not stimulated *in vitro* by immobilized TGN1412 in agreement with the failure of *in vivo* administration at any dose tested to evoke any adverse response (Duff, 2006; Stebbings *et al.*, 2007).

Here, we address three further questions which arose from the initial investigation. First, we demonstrate that the cytokine release syndrome stimulated by TGN1412 is not mediated by the same mechanism(s) as those underlying adverse reactions to other therapeutic mAbs (e.g. Campath-1H and Mabthera). Second, we show that the new *in vitro* safety procedures are applicable to other therapeutic mAbs if interleukin (IL)-2 and interferon (IFN)- $\gamma$  are used as key markers for a TGN1412-type response. Third, we identify a species difference in CD28 expression on the CD4<sup>+</sup> effector memory T-cell subset

as being most likely responsible for the failure of pre-clinical safety testing of TGN1412 in cynomolgus macaques. This is a CD28-specific, rather than a general, difference affecting applicability of such models to the safety testing of new therapeutic mAbs and immunomodulatory biotherapeutic agents.

## Methods

### *In vitro stimulations with therapeutic mAbs*

Stimulation with therapeutic mAbs was performed in triplicate in 96-well round bottom polypropylene microtitre plates (Corning Inc., Corning, NY, USA) containing TGN1412 (humanized IgG4 $\kappa$ , anti-CD28 superagonist), Mabthera (chimeric mouse/human IgG1, anti-CD20, rituximab), Campath-1H (humanized IgG1, anti-CD52, alemtuzumab), ANC28.1 (murine IgG1, anti-CD28 superagonist), CD28.2 (murine IgG1, anti-CD28 agonist) and humanized IgG4 $\kappa$  isotype-matched control for TGN1412. Therapeutic mAbs were immobilized onto plates by addition of 1  $\mu$ g mAb diluted in 60  $\mu$ L of phosphate-buffered saline (PBS) per well followed by incubation at room temperature for 1–2 h, then washing twice with 200  $\mu$ L of PBS to remove unbound antibody. For analysis of rapid cytokine expression, 96-well round bottom polystyrene microtitre plates (Corning Inc.) were employed with mAbs immobilized by air drying, as previously described (Stebbing *et al.*, 2007; Findlay *et al.*, 2010). Fc immobilization of TGN1412 was accomplished using a polyclonal chicken anti-human IgG Fc that was first air-dried onto plates (Stebbing *et al.*, 2007; Findlay *et al.*, 2010), followed by two washes and a 1 h incubation with 1  $\mu$ g of TGN1412 diluted in 60  $\mu$ L of PBS per well, then washing twice with 200  $\mu$ L of PBS. Human PBMCs, isolated from the heparinized blood of healthy donors by density gradient separation (Lymphoprep, Axis-Shield, Oslo, Norway) according to manufacturer's instructions, were resuspended in RPMI 1640 culture medium supplemented with 15% fetal calf serum, L-glutamine and antibiotics (penicillin and streptomycin). For functional assays, PBMCs were added to therapeutic mAb-coated plates at  $1 \times 10^6$  cells·mL<sup>-1</sup> of culture medium. For the measurement of cytokine release by ELISA, supernatants were harvested after PBMC stimulation for 24, 48 and 72 h in a humidified incubator at 37°C with 5% CO<sub>2</sub>. For cytokine release assays, lectin phytohaemagglutinin (PHA) at 10  $\mu$ g·mL<sup>-1</sup> was used as a positive control. Where intracellular cytokine staining was measured, the secretion inhibitor brefeldin A was added to cell cultures at 10  $\mu$ g·mL<sup>-1</sup>. Early kinetics of cytokine responses to therapeutic mAbs were assessed after 30, 60, 90, 120, 240 and 360 min incubations by

intracellular cytokine staining. The mitogens, phorbol myristate acetate (PMA) at  $0.05 \mu\text{g}\cdot\text{mL}^{-1}$  and ionomycin at  $0.75 \mu\text{g}\cdot\text{mL}^{-1}$ , were used together as a positive control for intracellular cytokine staining. Effector memory cell enrichment and depletion prior to stimulation were performed using MACS human CD4<sup>+</sup> T-cell effector memory isolation kit (Miltenyi Biotech, Surrey, UK), according to the manufacturer's instructions, after first removing sodium azide from all reagents using Amicon Ultra-4 30 kDa centrifugal filter devices (Millipore, Billerica, MA, USA).

### *Cytokine responses to therapeutic mAbs*

Concentrations of IFN- $\gamma$ , tumour necrosis factor (TNF)- $\alpha$ , IL-2 and IL-8 in culture supernatants were measured by ELISA using the following antibody pairs: anti-human IFN- $\gamma$  mAb clone NIB42 and clone 4S.B3, anti-human TNF- $\alpha$  antibody clone 357-101-4 and polyclonal anti-TNF- $\alpha$  antibody (H91), anti-human IL-2 mAb clone 5355 and polyclonal anti-human IL-2, affinity-purified polyclonal sheep anti-human IL-8 antibodies for both capture and detection. Streptavidin-HRP conjugate was used to detect and quantify biotinylated antibody in conjunction with tetramethylbenzidine for IL-2 and IFN- $\gamma$ , and *O*-phenylenediamine for TNF- $\alpha$  and IL-8.

Polychromatic intracellular cytokine staining was performed as described previously (Stebbins *et al.*, 2007). For surface staining, combinations of the following anti-human fluorochrome conjugates were employed: CD4 PE-Cy7 (clone SK3), CD4 APC (clone RPA-TA), CD4 APC-Cy7 (clone OKT4), CD28 PE-Cy7 (clone CD28.2), CD45RO APC (clone UCHL-1), CD95 PE (clone DX2) and CCR7 FITC (clone 150503). For intracellular cytokine staining, combinations of the following anti-cytokine fluorochrome conjugates were used: TNF- $\alpha$  PE (clone MAB11), IL-2 PE (clone 5344.111), IL-2 FITC (clone 5344.111), IL-2 PerCP Cy5.5 (clone MG1-17H12), IFN- $\gamma$  PE (clone 4S.B3), IFN- $\gamma$  Pacific Blue (clone 4S.B3). Acquisition of 30 000 gated lymphocyte events per well was performed using a FACSCanto II flow cytometer equipped with HTS plate reader (BD Biosciences, Oxford, UK) and analysed using FACSDiva software (BD Biosciences). For analysis of memory subsets, differential staining for CD45RO and the chemokine receptor, CCR7, was employed to subdivide human CD4<sup>+</sup> T-cells into CD45RO<sup>-</sup>CCR7<sup>+</sup> naive T-cells, CD45RO<sup>+</sup>CCR7<sup>+</sup> central memory T-cells and CD45RO<sup>+</sup>CCR7<sup>-</sup> effector memory T-cells (Sallusto *et al.*, 1999; 2004). For macaque memory subset analysis, differential staining for CD95 and CD28 was employed to subdivide CD4<sup>+</sup> T-cells into CD95<sup>-</sup>CD28<sup>+</sup> naive T-cells, CD95<sup>+</sup>CD28<sup>+</sup> central memory T-cells and CD95<sup>+</sup>CD28<sup>-</sup> effector memory T-cells (Pitcher *et al.*, 2002; Karlsson *et al.*, 2007). For

cross-species effector memory subset comparison, differential staining for CD28 and CCR7 was employed to subdivide CD4<sup>+</sup> T-cells into CD28<sup>+</sup>CCR7<sup>+</sup> naive and central memory subsets (both species), CD28<sup>+</sup>CCR7<sup>-</sup> human effector memory T-cells, CD28<sup>+</sup>CCR7<sup>-</sup> macaque transitional memory T-cells and CD28<sup>-</sup>CCR7<sup>-</sup> macaque effector memory T-cells (Picker *et al.*, 2006).

Real-time (RT)-PCR was performed as described previously (Burns *et al.*, 2008). For the measurement of cytokine mRNAs, PCR reactions contained intron-spanning primers specific for cytokine or house-keeping gene (GAPDH). Primer sequences for human GAPDH were forward; 5'-GTC AGT GGT GGA CCT GAC CT-3', reverse; 5'-CCC TGT TGC TGT AGC CAA AT-3'. Human IL-2 primer sequences were forward; 5'-AAG TTT TAC ATG CCC AAG AAG G-3', reverse; 5'-AAG TGA AAG TTT TTG CTT TGA GC-3'. Human TNF- $\alpha$  primer sequences were forward; 5'-CAG CCT CTT CTC CTT CCT GAT-3', reverse; 5'-GCC AGA GGG CTG ATT AGA GA-3'. Human IFN- $\gamma$  primer sequences were forward; 5'-GGC ATT TTG AAG AAT TGG AAA G-3', reverse; 5'-TTT GGA TGC TCT GGT CAT CTT-3'. The PCR product size (bp) for TNF- $\alpha$ , IL-2, IFN- $\gamma$  and GAPDH were 123, 113, 112 and 251 respectively. The amplification product of each primer pair was subjected to melting point analysis and subsequent gel electrophoresis to ensure specificity of amplification.

### *Pre-clinical non-human primate model T-lymphocytes*

Macaques were housed and maintained in accordance with United Kingdom Home Office guidelines for the care and maintenance of non-human primates. Blood from four cynomolgus (*Macaca fascicularis*) and four rhesus (*Macaca mulatta*) macaques was obtained under anaesthesia. The following cross-reactive anti-human fluorochrome conjugates were used for whole blood staining; CD4 APC-Cy7 (clone OKT4), CD95 PE (clone DX2), CCR7 FITC (clone 150503) and CD28 PE-Cy7 (clone CD28.2). The extracellular loop of the CD28 molecule is entirely conserved between man and macaques (Bhagal and Combes, 2007), and cross reactivity of CD28.2 for cynomolgus and rhesus macaques is 100% (Sopper *et al.*, 1997; Yoshino *et al.*, 2000). Red blood cells were removed using FACS lysing solution and samples analysed using a FACSCanto II flow cytometer (BD Biosciences).

### *Statistical analysis*

Statistical differences were assessed using the unpaired Student's *t*-test. Significance was assumed at  $P < 0.01$ . Group data are shown as mean  $\pm$  SEM.



## Materials

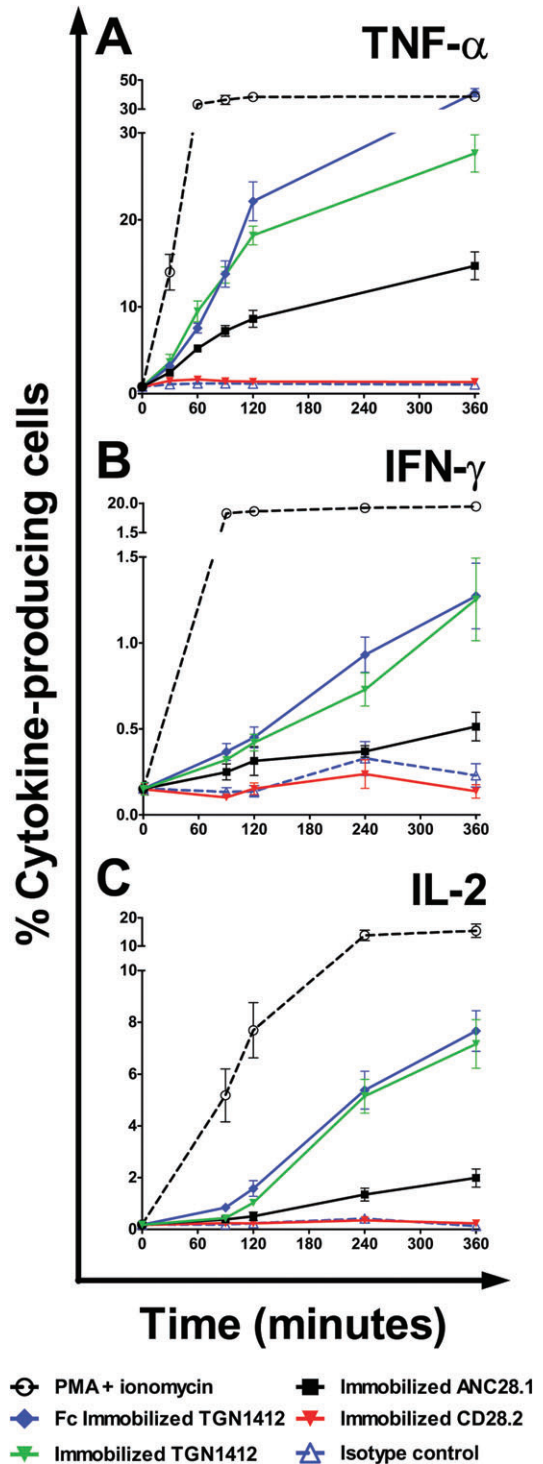
TGN1412 was obtained from TeGenero AG, Würzburg, Germany; Mabthera from Roche Diagnostics Ltd, Burgess Hill, UK; and Campath-1H from GlaxoWellcome plc now GlaxoSmithKline, Brentford, UK. Tysabri, used as a humanized IgG4 $\kappa$  isotype-matched control for TGN1412, was obtained from Elan Pharmaceuticals, Monksland, Ireland; ANC28.1 was purchased from Axxora UK, Cambridge, UK; Pure CD28.2 from BD Biosciences; and polyclonal chicken anti-human IgG Fc from Abcam plc, Cambridge, UK. PHA, brefeldin A, PMA, ionomycin, tetramethylbenzidine, *O*-phenylenediamine, RPMI, L-glutamine, penicillin and streptomycin were purchased from Sigma-Aldrich Ltd, Poole, UK. For ELISA, anti-human TNF- $\alpha$  antibody pairs were a kind gift from Dr A. Meager (Meager *et al.*, 1987); anti-IFN- $\gamma$  antibody pairs were purchased from BD Biosciences; anti-IL-2 antibody pairs from R&D Systems, Abingdon, UK; and affinity-purified polyclonal sheep anti-human IL-8 antibodies were produced in-house. Streptavidin-HRP conjugate was purchased from GE Healthcare Life Sciences, Little Chalfont, UK. For flow cytometry, CD4 PE-Cy7, CD4 APC, CD45RO APC, CD95 PE, TNF- $\alpha$  PE, IL-2 PE, IL-2 FITC, IFN- $\gamma$  PE and FACS lysing solution were purchased from BD Biosciences. IL-2 PerCP Cy5.5 and IFN- $\gamma$  Pacific Blue were purchased from Cambridge Bioscience, Cambridge, UK; CD28 PE-Cy7 from eBioscience Ltd, Hatfield, UK; and CCR7 FITC from R&D Systems.

The drug/molecular target nomenclature follows Alexander *et al.* (2009).

## Results

### TGN1412 stimulates rapid cytokine expression in vitro

To determine if *in vitro* stimulation with immobilized TGN1412 (Stebbins *et al.*, 2007) induces rapid cytokine expression, with kinetics similar to the 'cytokine storm' caused by this mAb in humans, we looked at early responses within the first 6 h following stimulation using intracellular cytokine staining and RT-PCR for cytokine mRNAs. Within 30 min, both immobilized TGN1412 (directly coated onto wells; 3.63%  $\pm$  0.90) and Fc-immobilized TGN1412 (captured from aqueous phase by anti-IgG Fc region-specific mAb directly coated onto wells; 3.19%  $\pm$  0.29) stimulated significant increases (both  $P < 0.0001$ ) above those seen with control antibody (1.08%  $\pm$  0.10), in the frequency of TNF- $\alpha$ -producing lymphocytes (Figure 1A). After 6 h, the frequency of TNF- $\alpha$ -producing lymphocytes obtained with Fc-immobilized TGN1412 was com-



**Figure 1**

Kinetic analysis of cytokine-producing human PBMCs stimulated *in vitro* with 1  $\mu$ g per well Fc-immobilized TGN1412 (IgG4 $\kappa$ ), immobilized TGN1412 (IgG4 $\kappa$ ), immobilized CD28 superagonist ANC28.1 (murine IgG1), immobilized CD28 agonist CD28.2 (murine IgG1) or immobilized isotype-matched control (IgG4 $\kappa$ ). The frequency of cytokine positive cells is expressed as a percentage of total lymphocytes for (A) TNF- $\alpha$ , (B) IFN- $\gamma$  and (C) IL-2, against time in minutes. Data shown are the mean responses of eight donors obtained from two independent experiments.

parable to PMA + ionomycin stimulation, whereas immobilized TGN1412 and ANC28.1 were less stimulatory (Figure 1A). Both CD28 agonist CD28.2 and control antibody failed to stimulate an increase in the frequency of TNF- $\alpha$ -producing cells above background staining (Figure 1A).

Stimulation with Fc-immobilized TGN1412 ( $0.37\% \pm 0.05$ ) and immobilized TGN1412 ( $0.32\% \pm 0.02$ ) both produced significant increases (above those seen with the control antibody,  $0.13\% \pm 0.03$ ) in the frequency of IFN- $\gamma$ -producing lymphocytes within 90 min (both  $P < 0.0001$ ; Figure 1B). After 6 h, Fc-immobilized TGN1412 was no more efficient than immobilized TGN1412 (Figure 1B). However, the frequency of IFN- $\gamma$ -producing cells stimulated by either immobilized TGN1412 or ANC28.1 was very modest compared with PMA + ionomycin stimulation (Figure 1B). Both CD28 agonist CD28.2 and control antibody failed to stimulate an increase in the frequency of IFN- $\gamma$ -producing cells above background staining (Figure 1B).

Within 90 min, both immobilized TGN1412 and Fc-immobilized TGN1412 stimulated significant increases (above those seen with the control antibody) in the frequency of IL-2-producing lymphocytes (both  $P < 0.0001$ ; Figure 1C). After 6 h, the frequency of IL-2-producing cells stimulated by both immobilized and Fc-immobilized TGN1412 was approximately half that achieved with PMA + ionomycin (Figure 1C). The superagonist ANC28.1 did not give a significant increase in the frequency of IL-2-producing cells until 120 min ( $P = 0.004$ ), and the response at 360 min was smaller than seen with immobilized TGN1412 (Figure 1C). Both control antibody and CD28 agonist CD28.2 failed to stimulate an increase in the frequency of IL-2-producing lymphocytes above background staining (Figure 1C).

Rapid expression of the genes for TNF- $\alpha$ , IL-2 and IFN- $\gamma$  was confirmed by RT-PCR following *in vitro* stimulation with immobilized TGN1412 (data not shown). Increased levels of TNF- $\alpha$  gene expression were detected 30 min after stimulation with TGN1412, and increased IL-2 and IFN- $\gamma$  gene expression at 60 min. The cytokine gene expression that showed the greatest increase following TGN1412 stimulation was TNF- $\alpha$ , followed by IL-2 and then IFN- $\gamma$  (data not shown).

### *Substantial IL-2 and IFN- $\gamma$ release is characteristic of a TGN1412-type response*

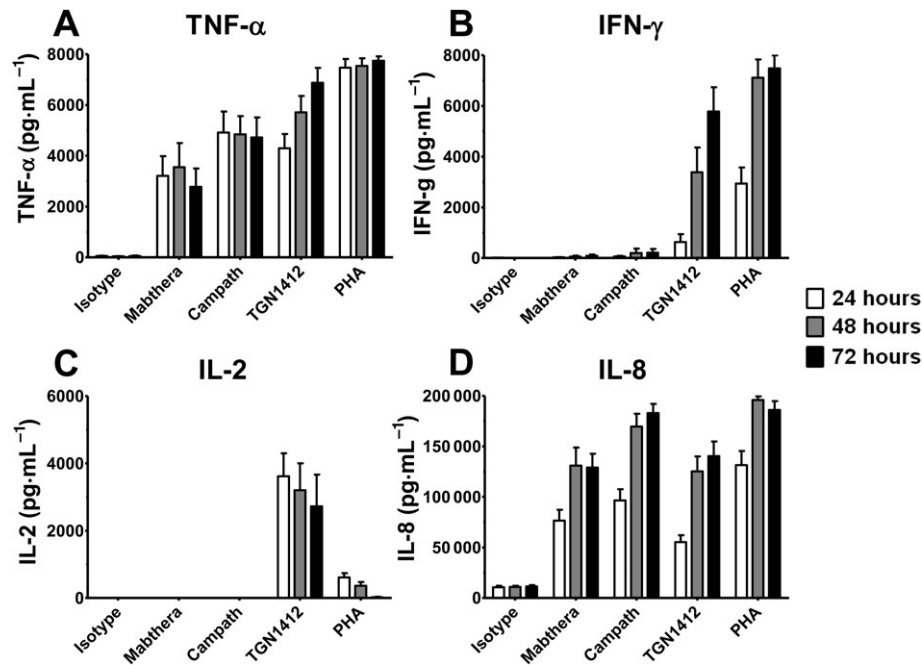
We compared the *in vitro* cytokine release caused by TGN1412 with two other therapeutic mAbs that have been associated with adverse reactions in a proportion of patients (i.e. Campath-1H and Mabthera) (Wing *et al.*, 1995; Chung, 2008). Con-

siderable amounts of TNF- $\alpha$  were measured in culture supernatants, by ELISA, following 24 h *in vitro* stimulation with immobilized Mabthera, Campath-1H and TGN1412, but not an isotype-matched control antibody (Figure 2A). Although concentrations of TNF- $\alpha$  stimulated by the three therapeutic mAbs were not markedly different after 24 h, the order of responses was Campath-1H > TGN1412 > Mabthera. TGN1412 showed a time-dependent increase in TNF- $\alpha$  concentration, such that after 72 h the order of responses changed to TGN1412 > Campath-1H > Mabthera (Figure 2A). After 72 h, TNF- $\alpha$  concentrations in response to TGN1412 were relatively close to those elicited by PHA (Figure 2A).

Substantial quantities of IFN- $\gamma$  were detected in culture supernatants following 24 h *in vitro* stimulation with immobilized TGN1412 and much more after 48 and 72 h (Figure 2B). In contrast, only small amounts of IFN- $\gamma$  were detected following 24 h *in vitro* stimulation with control antibody, Mabthera or Campath-1H (Figure 2B). A small increase in IFN- $\gamma$  concentration was measured following 72 h *in vitro* stimulation with Mabthera and Campath-1H, but not with control antibody (Figure 2B). After 72 h, IFN- $\gamma$  concentrations stimulated by TGN1412 were similar to those in response to PHA (Figure 2B).

Substantial concentrations of IL-2 were detected in culture supernatants following 24 h *in vitro* stimulation with immobilized TGN1412, and these decreased at 48 and 72 h (Figure 2C). In contrast, no IL-2 was detected in culture supernatants following 24, 48 or 72 h *in vitro* stimulation with Mabthera, Campath-1H or control antibody (Figure 2C). After 72 h, IL-2 concentrations stimulated by TGN1412 were substantially greater than responses to PHA (Figure 2C). Consistent with the release of IL-2, only *in vitro* stimulation with TGN1412 resulted in a lymphoproliferative response (data not shown), as previously described (Stebbins *et al.*, 2007).

High concentrations of IL-8 were measured in culture supernatants following 24 h *in vitro* stimulation with immobilized Mabthera, Campath-1H and TGN1412 (Figure 2D). A relatively high background concentration of IL-8 was detected in culture supernatants following 24 h *in vitro* stimulation with control antibody (Figure 2D), and culture medium alone (data not shown). Following 72 h *in vitro* stimulation, increases in IL-8 concentration were measured with all three therapeutic antibodies, but not control antibody. The order of responses at 72 h was Campath > TGN1412 > Mabthera (Figure 2D). After 72 h, IL-8 concentrations stimulated by Campath-1H were similar to responses to PHA (Figure 2D).



**Figure 2**

Cytokine release from human PBMCs stimulated *in vitro* for 24, 48 and 72 h with 1  $\mu\text{g}$  per well immobilized isotype-matched control (IgG4 $\kappa$ ), Mabthera (IgG1), Campath-1H (IgG1) and TGN1412 (IgG4 $\kappa$ ). The lectin PHA at 10  $\mu\text{g}\cdot\text{mL}^{-1}$  was used as positive control. Cytokine release was measured by ELISA of culture supernatants. (A) TNF- $\alpha$  release, (B) IFN- $\gamma$  release, (C) IL-2 release and (D) IL-8 release are expressed in  $\text{pg}\cdot\text{mL}^{-1}$ . Data shown are mean responses of nine donors obtained from two independent experiments.

### *Cytokine release from CD4<sup>+</sup> T-cells is characteristic of a TGN1412-type response*

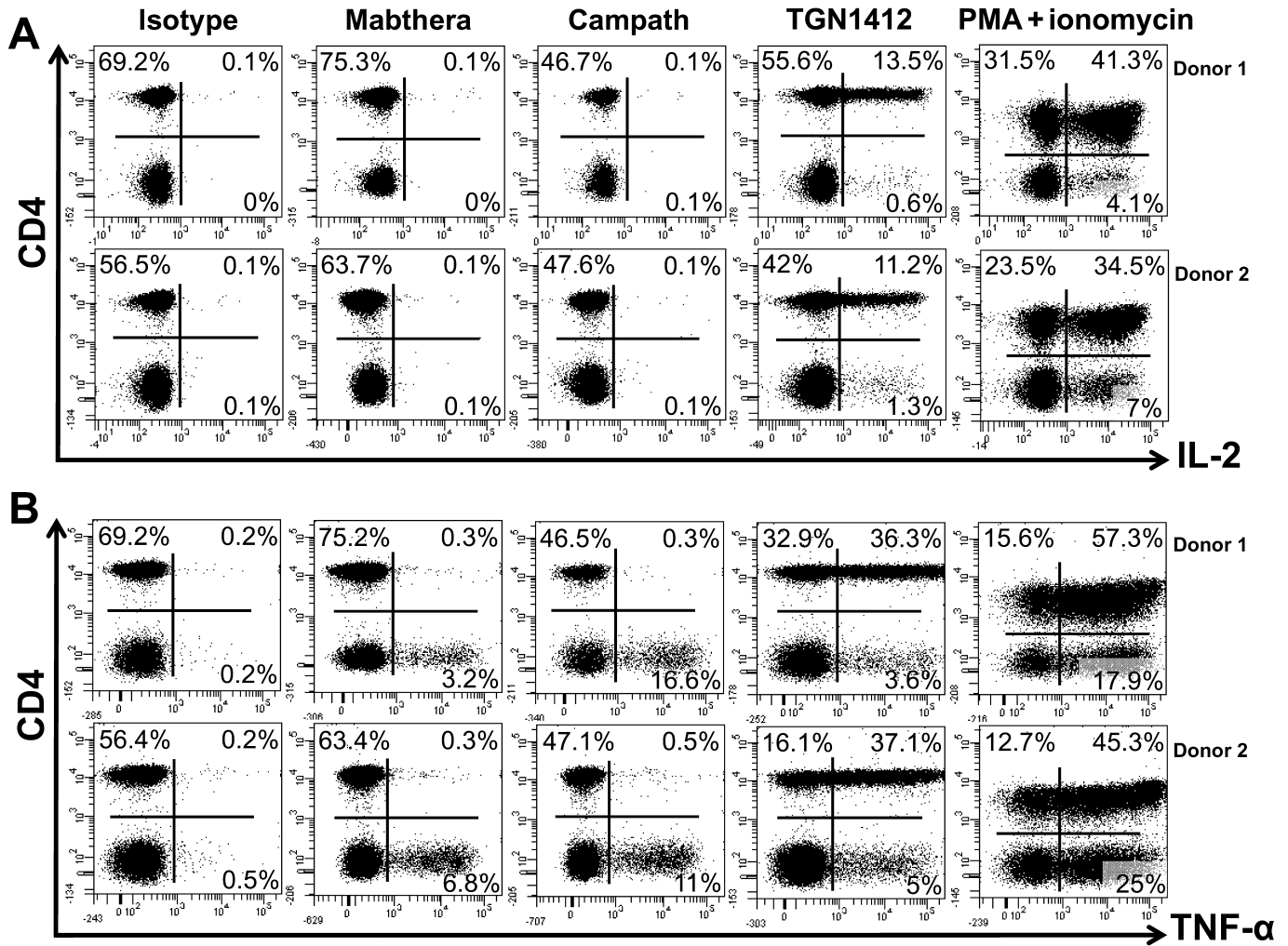
To determine whether or not pro-inflammatory cytokine responses originate from the same cell types following *in vitro* stimulation with immobilized TGN1412, Mabthera and Campath-1H, we used intracellular cytokine staining and immunophenotyping in conjunction with flow cytometry. Following 6 h of *in vitro* stimulation with Mabthera, Campath-1H or control antibody, there was no increase in the frequency of IL-2 producing CD4<sup>+</sup> or CD4<sup>-</sup> lymphocytes (no responses above background staining of 0.1%; data from two representative donors; similar data were achieved with 14 further donors; Figure 3A). In contrast, TGN1412 stimulated a large increase in the frequency of IL-2-producing lymphocytes of which the majority were CD4<sup>+</sup> (Figure 3A; data from two representative donors) and a small proportion were CD4<sup>-</sup>, from the same two representative donors (Figure 3A). Similar to TGN1412, PMA + ionomycin stimulated a large increase in the frequency of IL-2-producing lymphocytes, of which the majority were CD4<sup>+</sup> and a smaller proportion CD4<sup>-</sup> (Figure 3A).

Following 6 h *in vitro* stimulation with control antibody, there was no increase above background staining in the frequency of TNF- $\alpha$ -producing lymphocytes within the CD4<sup>+</sup> (Figure 3B) or CD4<sup>-</sup> frac-

tions (Figure 3B). In contrast, stimulation with Mabthera or Campath-1H stimulated increases in the frequency of CD4<sup>-</sup>, but not CD4<sup>+</sup> TNF- $\alpha$ -producing lymphocytes (Figure 3B). Much greater increases in the frequency of TNF- $\alpha$ -producing lymphocytes were seen with Campath-1H compared with Mabthera. TGN1412 stimulated a large increase in the frequency of TNF- $\alpha$ -producing lymphocytes, but the majority of these were CD4<sup>+</sup> and a small proportion CD4<sup>-</sup> (Figure 3B). In comparison, PMA + ionomycin stimulated a large increase in the frequency of CD4<sup>+</sup> and CD4<sup>-</sup> TNF- $\alpha$ -producing lymphocytes (Figure 3B).

### *TGN1412 stimulates multiple cytokine release from CD4<sup>+</sup> effector memory T-cells*

Following stimulation with immobilized TGN1412, we identified that human CD4<sup>+</sup>CD45RO<sup>+</sup> memory T-cells, rather than CD4<sup>+</sup>CD45RO<sup>-</sup> naive T-cells, were the predominant source of TNF- $\alpha$ , IFN- $\gamma$  and IL-2 (data from two representative donors; similar data were achieved with six further donors; Figure 4A, B and C respectively). The frequency of TNF- $\alpha$ , IFN- $\gamma$  and IL-2 producing CD4<sup>+</sup> memory T-cells was greater compared with CD4<sup>+</sup> naive T-cells (Figure 4A, B and C respectively). Moreover, the overall intensity of intracellular cytokine staining was greater for CD4<sup>+</sup> memory T-cells compared to



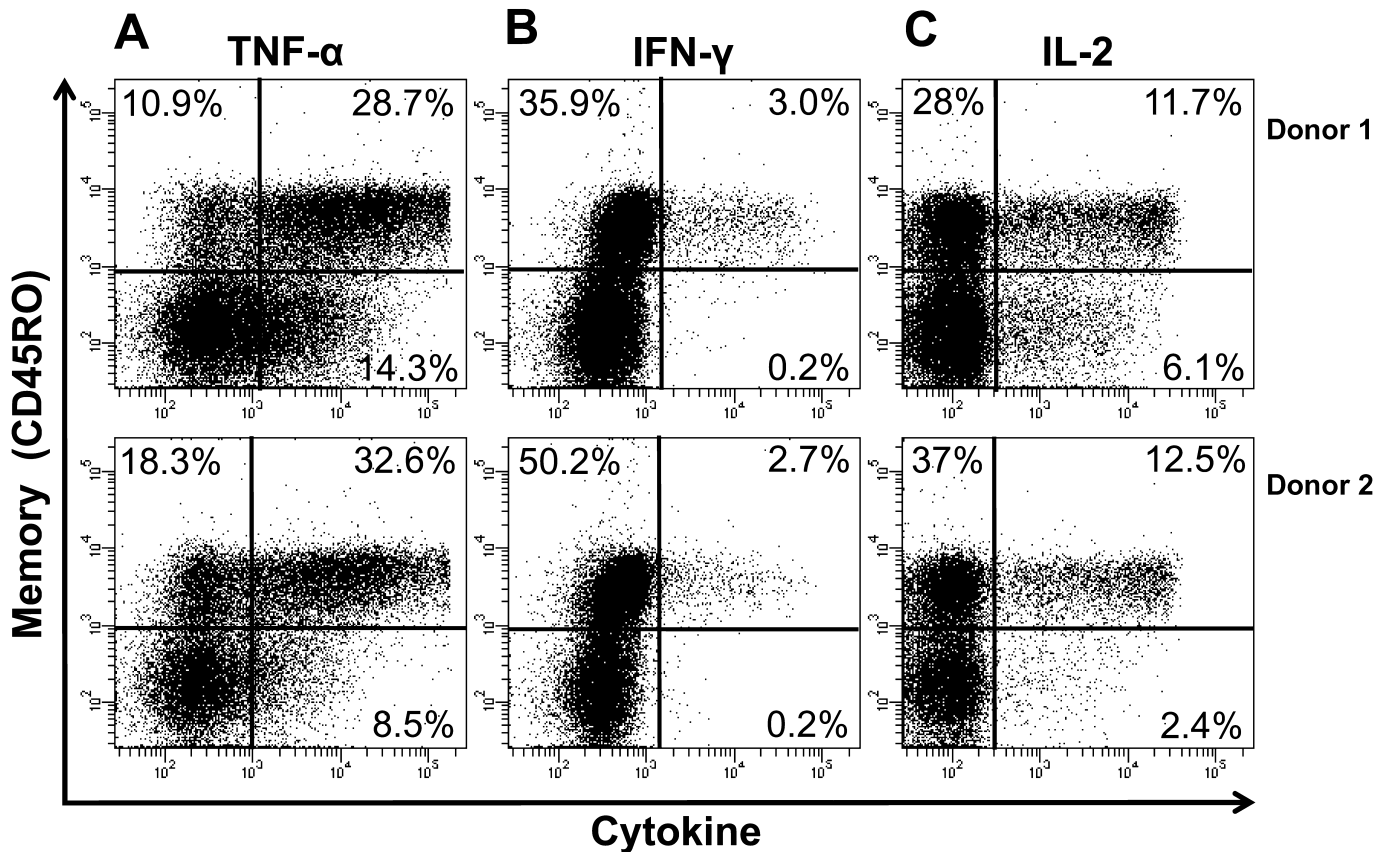
**Figure 3**

Intracellular cytokine staining of human PBMCs for (A) IL-2 and (B) TNF- $\alpha$ . Prior to staining, PBMCs were stimulated with 1  $\mu$ g per well immobilized isotype-matched control (IgG4 $\kappa$ ), Mabthera (IgG1), Campath-1H (IgG1) or TGN1412 (IgG4 $\kappa$ ) for 360 min. PBMCs were counterstained for surface CD4 expression. The frequency of cytokine positive cells is expressed as a percentage of total lymphocytes. Cells in the right-hand quadrants are positive for cytokine expression. Cells in the upper quadrants are positive for CD4 expression. The mitogens PMA + ionomycin were used as positive control. Data from two representative donors are shown (similar data were obtained with 14 additional donors from three independent experiments).

naive T-cells (Figure 4A, B and C). Further subdivision of CD4<sup>+</sup> memory T-cells into effector and central memory T-cells, based upon differential CCR7 staining, could only be achieved prior to stimulation as CCR7 expression changes upon activation (Sallusto *et al.*, 1999; 2004; Amyes *et al.*, 2005). We therefore prepared PBMC cultures that were enriched in CD4<sup>+</sup> effector memory T-cells and compared them with those that were depleted in CD4<sup>+</sup> effector memory T-cells following *in vitro* stimulation with TGN1412. Human CD4<sup>+</sup> effector memory T-cell enrichment produced cells that were on average 88.4%  $\pm$  0.64 CD4<sup>+</sup>CCR7<sup>-</sup>. Staining for TNF- $\alpha$ , IL-2 and IFN- $\gamma$  from two representative donors is shown; similar data were obtained with

four further donors (Figure 5A,B). Following stimulation with TGN1412, intracellular cytokine staining revealed that few (0.8 and 0.9%) CD4<sup>+</sup> T-cells in effector memory-depleted PBMCs produced IFN- $\gamma$  (Figure 5A) compared with substantial frequencies (14.3 and 16.3%) in effector memory-enriched PBMCs (Figure 5B). The majority of IFN- $\gamma$ -producing CD4<sup>+</sup> T-cells in effector memory-enriched PBMCs also produced IL-2 and TNF- $\alpha$  (Figure 5B). Similarly, the proportion of CD4<sup>+</sup> T-cells producing IL-2 in effector memory-enriched PBMCs (55.4 and 49.4%; Figure 5B) was far greater compared with effector memory-depleted PBMCs (both 10.2%; Figure 5A) following stimulation with TGN1412. A less marked difference was seen with the frequency of TNF- $\alpha$ -





**Figure 4**

Intracellular cytokine staining of human CD4<sup>+</sup> memory T-cells for (A) TNF- $\alpha$ , (B) IFN- $\gamma$  and (C) IL-2, following stimulation with 1  $\mu$ g per well immobilized TGN1412. Analysis shown is gated on CD4<sup>+</sup> T-cells. Cells in the upper quadrants are CD45RO<sup>+</sup> memory T-cells, and cells in the lower quadrants are CD45RO<sup>-</sup> naive T-cells. Cells in the right-hand quadrants are positive for the indicated cytokines. The frequency of cytokine positive cells is expressed as a percentage of CD4<sup>+</sup> T-cells. Data from two representative donors are shown (similar data were obtained with six additional donors from two independent experiments).

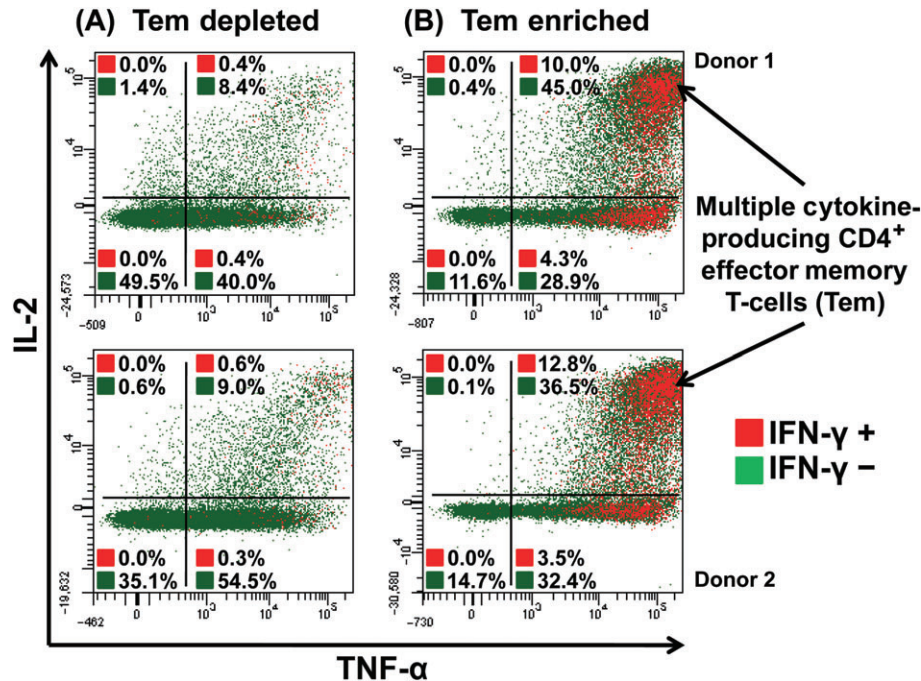
producing CD4<sup>+</sup> T-cells in the effector memory-enriched PBMCs (88.2 and 85.2%; Figure 5B) compared with (49.2 and 64.4%; Figure 5A) in effector memory-depleted PBMCs.

#### *CD28 is expressed by human, but not macaque, CD4<sup>+</sup> effector memory T-cells*

Immunophenotyping of human, cynomolgus and rhesus macaque peripheral blood CD4<sup>+</sup> T-cell memory subsets was performed using a range of markers (CD95 and CD28 for macaques, CD45RO and CCR7 for humans) as previously described (Sallusto *et al.*, 1999; 2004; Pitcher *et al.*, 2002). Anti-human CD45RO mAbs are not cross-reactive with macaque T-cells, so CD95 expression was used to distinguish CD4<sup>+</sup> memory from naive T-cells in macaques (data not shown). For comparative analysis of human and macaque effector memory subsets, the markers CCR7 and CD28 were utilized. Dot plots from two representative individuals from each species are shown (Figure 6A). All human CD4<sup>+</sup>

T-cell memory subsets (naive, central and effector) expressed high levels of CD28 with the effector memory subset defined by a lack of CCR7 expression (Sallusto *et al.*, 1999; 2004; Figure 6A). Similarly, the naive and central memory CD4<sup>+</sup> T-cells of both cynomolgus and rhesus macaques expressed CD28 (Figure 6A). However, we found that the CD4<sup>+</sup> effector memory T-cell subsets of both cynomolgus and rhesus macaques did not express CD28, as previously reported for rhesus macaques (Pitcher *et al.*, 2002; Moniuszko *et al.*, 2004; Figure 6A). As for human cells, we found that both cynomolgus and rhesus macaque CD4<sup>+</sup> effector memory T-cells lack CCR7 expression; however, a population of CD28<sup>+</sup>CCR7<sup>-</sup> were noted that have previously been described as a transitional central memory subset in rhesus macaques (Picker *et al.*, 2006). When stimulated with a CD28 independent agent such as PMA and ionomycin for 4 h, both cynomolgus and rhesus macaque CD4<sup>+</sup>CD28<sup>-</sup>CCR7<sup>-</sup> effector memory T-cells produced pro-inflammatory cytokines, such





**Figure 5**

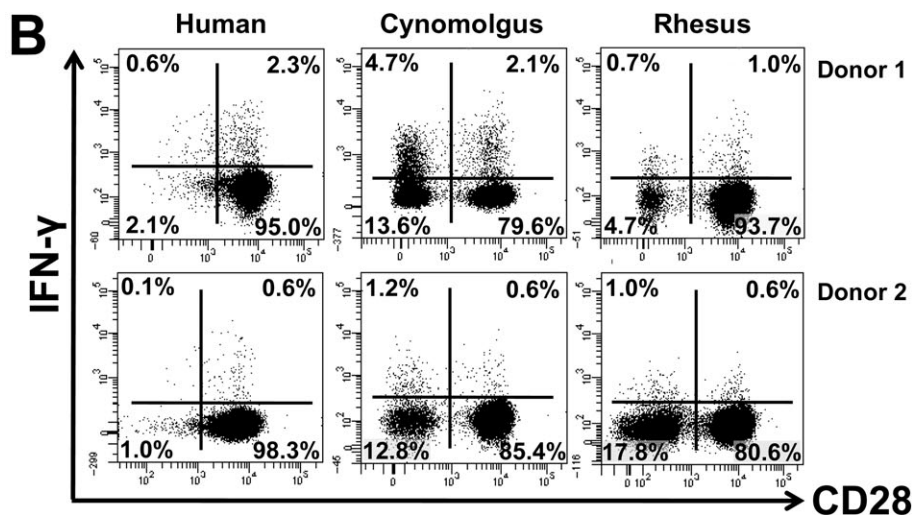
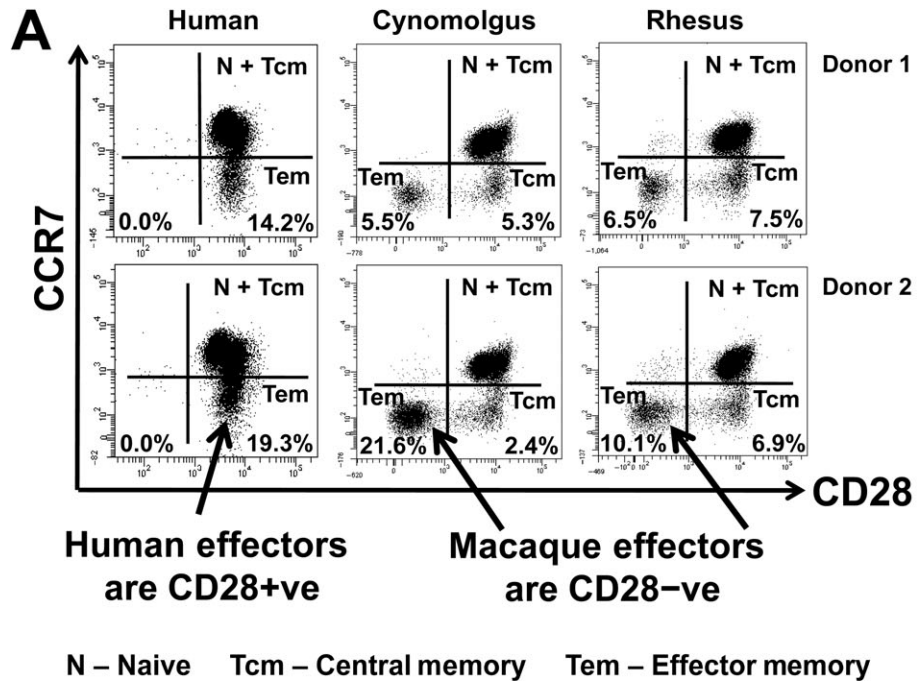
Intracellular cytokine staining of human CD4<sup>+</sup> effector memory T-cell (Tem)-depleted (A) and -enriched (B) PBMCs for TNF- $\alpha$ , IL-2 and IFN- $\gamma$ , following stimulation with 1  $\mu$ g per well immobilized TGN1412. Tem-depleted (left panels) and Tem-enriched (right panels) PBMCs were surface stained for T-cell markers and intracellular cytokines; analysis shown is gated on CD4<sup>+</sup> T-cells. Cells in the right-hand quadrants are positive for TNF- $\alpha$ , cells in the upper quadrants are positive for IL-2, cells shown in red are positive for IFN- $\gamma$  and cells shown in green are negative for IFN- $\gamma$ . Cells in the upper right corner in red are positive for TNF- $\alpha$ , IL-2 and IFN- $\gamma$ . The frequency of cytokine positive cells is expressed as a percentage of CD4<sup>+</sup> T-cells. Data from two representative donors are shown (similar data were obtained with four additional donors from two independent experiments).

as IFN- $\gamma$ , demonstrating their functional ability (Figure 6B).

## Discussion

Following the TGN1412 incident, we developed the new pre-clinical *in vitro* procedures using human PBMCs which can be applied to therapeutic mAbs during development, in order to prevent a repeat of the TGN1412 incident (Stebbing *et al.*, 2007; Findlay *et al.*, 2010). However, at the time, we were unaware of whether or not the cytokine release syndrome caused by TGN1412 was stimulated by the same mechanism(s) as the adverse effects caused by other therapeutic mAbs in a proportion of recipients. We also did not understand why TGN1412 bound to human and macaque CD28 with equal affinity (TeGenero, 2005), yet caused a 'cytokine storm' only in humans. This difference not only questioned the suitability and validity of existing pre-clinical non-human primate models for the safety testing of therapeutic mAbs, but also for other immunomodulatory biological agents.

When we investigated other therapeutic mAbs, known to be capable of causing adverse reactions, Mabthera and Campath-1H (Wing *et al.*, 1995; Chung, 2008), using our new *in vitro* procedures, we did not detect lymphoproliferative responses or IL-2 release, the cardinal features of TGN1412 stimulation (Stebbing *et al.*, 2007). Thus, the 'cytokine storm' caused by TGN1412 appeared to be mediated by a mechanism of action different from that responsible for the adverse reactions seen with other therapeutic mAbs. Interestingly, very low *in vitro* levels of IFN- $\gamma$  were detected with both Mabthera and Campath-1H, compared with TGN1412, which may prove a good correlate for the likelihood and severity of adverse reactions. In contrast, TNF- $\alpha$  and IL-8 responses were less discriminatory as substantial release was seen with TGN1412, Mabthera and Campath-1H. The significance of substantial IL-8 and TNF- $\alpha$  release with Mabthera and Campath-1H in these assay systems is unclear because cross-linking of Campath-1H-opsionized CD4<sup>+</sup> T-cells failed to stimulate TNF- $\alpha$  release (Wing *et al.*, 1996). Such release could be caused by an Fc region-mediated mechanism such



**Figure 6**

Comparative immunophenotyping of human, cynomolgus and rhesus macaque CD4<sup>+</sup> T-cells. All analysis shown is gated on CD4<sup>+</sup> T-cells. (A) Analysis of memory markers using CD28 and CCR7 staining. Human, cynomolgus and rhesus macaque naive CD4<sup>+</sup> T-cells (CCR7<sup>+</sup>CD28<sup>+</sup>) and central memory T-cells (CCR7<sup>+</sup>CD28<sup>+</sup>), macaque transitional memory T-cells (CCR7<sup>-</sup>CD28<sup>+</sup>) and human effector memory CD4<sup>+</sup> T-cells (CCR7<sup>-</sup>CD28<sup>+</sup>) all express CD28. In contrast, both cynomolgus and rhesus effector memory T-cells (CCR7<sup>-</sup>CD28<sup>-</sup>) do not express CD28. Data from two representative donors are shown (similar data were obtained with two additional rhesus, two cynomolgus and six human donors from two independent experiments). (B) Intracellular IFN- $\gamma$  staining versus CD28 expression on CD4<sup>+</sup> T-cells following PMA and ionomycin stimulation for 4 h. Human CD4<sup>+</sup> T-cells constitutively express CD28, so IFN- $\gamma$  staining stems only from the CD28<sup>+</sup> population. Macaque CD4<sup>+</sup> T-cells can be subdivided into CD28<sup>+</sup> (naive, central and transitional memory T-cell) and CD28<sup>-</sup> (effector memory T-cell) populations, both of which produce IFN- $\gamma$  following PMA and ionomycin stimulation for 4 h. A greater fraction of macaque CD4<sup>+</sup>CD28<sup>-</sup> T-cells produce IFN- $\gamma$  compared to CD4<sup>+</sup>CD28<sup>+</sup> T-cells following mitogen stimulation (similar data were obtained with two additional rhesus, two cynomolgus and two human donors).

as ligation of CD16 on natural killer (NK) cells in the case of Campath-1H (Wing *et al.*, 1996), as orientation of immobilized mAb in wells is random. However, it is unlikely that TGN1412-mediated

IL-8 and TNF- $\alpha$  release *in vitro* was predominately an Fc region-mediated mechanism as no responses were seen with the IgG4 $\kappa$  isotype-matched control.

TGN1412-mediated cytokine release predominantly originated from CD4<sup>+</sup> memory T-cells, which produced high levels of TNF- $\alpha$ , IL-2 and IFN- $\gamma$ . In contrast, other therapeutic mAbs that we tested only stimulated CD4<sup>+</sup> lymphocyte cytokine release that comprised high levels of TNF- $\alpha$ , but very low levels of IFN- $\gamma$  and no IL-2. Thus, it can be concluded that the cytokine release stimulated by TGN1412 was mediated via a mechanism distinct from that underlying cytokine release stimulated by the other two therapeutic mAbs tested here, because different cell types were involved. We also noted that a small proportion of TGN1412-mediated cytokine release originated from CD4<sup>+</sup> lymphocytes, but at present it is unclear whether this occurs via the same mechanism underlying the cytokine release mediated by other therapeutic mAbs or not. Within blood, multiple cell types and their subsets can release different cytokines with overlapping repertoires which are not discriminated by ELISA assessment of culture supernatants from mixed cell populations stimulated with mAbs, whereas phenotypic analysis of cytokine-producing cells using flow cytometry, as is shown here, has allowed the dissection of different effector mechanisms. The likely candidates for cytokine release from the CD4<sup>+</sup> leucocytes triggered by different mAbs were CD8<sup>+</sup> T-cells, NK cells, neutrophils and B-cells. Overall, these findings demonstrate that the *in vitro* safety procedures described by Stebbings *et al.* (2007) are applicable to other therapeutic mAbs, if IL-2 and IFN- $\gamma$  release are used as biomarkers for a TGN1412-type adverse response.

One of the most striking features of the TGN1412-mediated 'cytokine storm' observed in humans was substantial TNF- $\alpha$  release within the first hour of infusion followed by dramatic increases in IFN- $\gamma$ , IL-2, IL-6, IL-8 and IL-10 plasma concentrations within 4 h (Suntharalingam *et al.*, 2006). The rapid nature of this response suggested direct stimulation of pro-inflammatory cytokine-producing cells, rather than a cascade of cytokine release (with each cytokine produced stimulating the release of another cytokine or cytokines). Similarly, within the first hour of *in vitro* TGN1412 stimulation, we observed a rapid induction of TNF- $\alpha$  mRNA and protein expression, consistent with the rapid release of this cytokine in the trial volunteers. Concentrations of TNF- $\alpha$  measured by ELISA in culture supernatants collected 24 h after *in vitro* TGN1412 stimulation were also consistent with the peak concentrations reported in trial volunteers, with the considerable caveat that a comparison is being made between an *in vitro* system and an *in vivo* response. However, despite similar *in vitro* expression kinetics for IFN- $\gamma$  following TGN1412 stimulation, the concentrations of IFN- $\gamma$  were far lower than

TNF- $\alpha$  responses *in vitro*, and an order of magnitude lower than concentrations of IFN- $\gamma$  predicted from *in vivo* responses in trial participants (Suntharalingam *et al.*, 2006). Only after prolonged *in vitro* stimulations for 48–72 h could high concentrations of IFN- $\gamma$ , equivalent to levels measured in the plasma of trial volunteers at 4 h, be achieved. This delayed release of high levels of IFN- $\gamma$  was presumably due to a requirement for expansion of low initial numbers of IFN- $\gamma$  producing T-cells through differentiation of stimulated T-cells and/or cell proliferation. Similarly, the expression of IL-2 following *in vitro* TGN1412 stimulation showed similar kinetics to its appearance in humans, but the concentrations in cell supernatants were lower than expected. These apparently disparate results did not seem to support our hypothesis that *in vitro* TGN1412 stimulation is a model of *in vivo* responses seen in man. Moreover, it appeared that *in vitro* TGN1412 stimulation greatly underestimated some *in vivo* cytokine responses compared with those reported in humans. If the explanation were as simple as differences in cell numbers, then all cytokine responses would have been equally lower, but they were not.

To understand why *in vitro* levels of IFN- $\gamma$  in response to TGN1412 stimulation were at least an order of magnitude lower than plasma concentrations reported in humans, we immunophenotyped the cytokine-producing cells. Initial data showed that human CD4<sup>+</sup>CD45RO<sup>+</sup> memory T-cells were the predominant source of cytokines following TGN1412 stimulation, but this did not explain why *in vitro* IFN- $\gamma$  levels were low when compared with TNF- $\alpha$  levels. However, when we subdivided CD4<sup>+</sup> memory T-cells into CCR7<sup>+</sup> central memory and CCR7<sup>-</sup> effector memory cells, we were able to identify a difference in TGN1412-stimulated cytokine release that could account for low *in vitro* IFN- $\gamma$  release. PBMCs depleted of CD4<sup>+</sup> effector memory T-cells and stimulated with TGN1412 produced mainly TNF- $\alpha$ , some IL-2 and very little IFN- $\gamma$  (i.e. responses very similar to the responses of whole PBMCs). In marked contrast, PBMCs enriched for CD4<sup>+</sup> effector memory T-cells produced high levels of TNF- $\alpha$ , IL-2 and IFN- $\gamma$ , when stimulated with TGN1412, with a large proportion of cells simultaneously producing all three cytokines. Differences in the proportions of CD4<sup>+</sup> effector memory T-cells producing TNF- $\alpha$ , IL-2 and IFN- $\gamma$  combinations following TGN1412 stimulation may reflect the heterogeneous nature of this population which contains multiple functional subsets with different cytokine repertoires (e.g. Th1, Th2, Th9, Th17 and Th22) (Annunziato and Romagnani, 2009; Sallusto and Lanzavecchia, 2009). PBMCs would also become enriched in effector memory T-cells follow-



ing prolonged stimulation as activated naive and central memory T-cells matured into effector memory T-cells gaining their expanded cytokine-secreting potential, including IFN- $\gamma$ . This would explain why *in vitro* stimulation with TGN1412 for 24 h initially produced only low levels of IFN- $\gamma$  compared to very high levels at 72 h.

This finding suggests that the 'cytokine storm' observed in trial participants likely involved TGN1412 stimulation of effector memory T-cells in peripheral tissues such as the lungs and gut, resulting in secretion of high levels of cytokines into the blood. This notion is also consistent with the known capability of CD4<sup>+</sup> T-cell subsets to produce cytokines (i.e. naive and central memory CD4<sup>+</sup> T-cell subsets both have limited potential for cytokine expression, whereas the effector memory subset has a considerable capability for cytokine production, with IL-2 being a major component of this) (Sallusto *et al.*, 1999; Amyes *et al.*, 2005). Importantly, IFN- $\gamma$  expression is largely limited to the CD4<sup>+</sup> effector memory T-cells (Sallusto *et al.*, 1999; Amyes *et al.*, 2005). Because CD4<sup>+</sup> effector memory T-cells are only a minor subset of peripheral blood lymphocytes compared with CD4<sup>+</sup> naive and central memory T-cells, their low numbers in peripheral blood could have explained why *in vitro* IFN- $\gamma$  responses were low compared with plasma concentrations observed in trial participants. Conversely, central and effector memory T-cells show a different pattern of distribution within the tissues compared with peripheral blood (Campbell *et al.*, 2001; Picker, 2006). The lymph nodes and tonsils are enriched in central memory CD4<sup>+</sup> T-cells, whereas the lungs and gastrointestinal mucosa contain mainly effector memory CD4<sup>+</sup> T-cells (Campbell *et al.*, 2001; Picker, 2006). Moreover, peripheral blood contains just 2–5% of all lymphocytes, while the lungs and gastrointestinal mucosa harbour up to 90% of the body's lymphocytes (Cerf-Bensussan and Guy-Grand, 1991; Smit-McBride *et al.*, 1998).

If infusion of TGN1412 caused high concentrations of cytokines to be rapidly released into the blood by the large numbers of CD4<sup>+</sup> effector memory T-cells within the tissues, then this would explain why *in vitro* procedures using peripheral blood containing few of these cells greatly underestimated some cytokine responses such as IFN- $\gamma$  in comparison with responses observed *in vivo*. However, it should be noted that immobilizing mAb to a plastic surface is a highly artificial means of stimulation that is unlikely to simulate what is happening inside the body. Nevertheless, this is the only published method currently available to induce substantial cytokine release with TGN1412, and the conclusion that it is the CD4<sup>+</sup> effector

memory T-cells which make the pro-inflammatory cytokines in response to TGN1412 stimulation, but not in the case of other therapeutic mAbs studied, is likely to be correct.

Identification of the central role of CD4<sup>+</sup> effector memory T-cells in TGN1412-mediated cytokine release provides an explanation for the failure of pre-clinical testing using non-human primate models to predict human responses. This is because we and others (Pitcher *et al.*, 2002; Moniuszko *et al.*, 2004) have shown that cynomolgus and rhesus macaque CD4<sup>+</sup> effector memory T-cells do not express CD28; therefore, they cannot be stimulated by CD28 superagonists including TGN1412. In contrast, the CD4<sup>+</sup> effector memory T-cells of humans express high levels of CD28 on their surface (Sallusto *et al.*, 1999; 2004), so therefore can be stimulated by TGN1412. It should also be noted that this species difference was only identified through careful dissection of the cellular components of the immune system to look at the individual subsets stimulated by TGN1412. The evolutionary reason for this subtle comparative immunological difference in receptor expression is unclear, but retention of CD28 expression on human CD4<sup>+</sup> effector T-cells would allow the required co-stimulatory signals to reduce activation thresholds (Fraser *et al.*, 1991). Effector memory CD4<sup>+</sup> T-cells lacking CD28 expression have been reported in humans, but only in association with autoimmune diseases such as rheumatoid arthritis, Crohn's disease or multiple sclerosis (Markovic-Plese *et al.*, 2001; Fasth *et al.*, 2004; Garcia de Tena *et al.*, 2004), and were not observed with the healthy donors used in this study. Similar to human CD4<sup>+</sup> effector memory T-cells, macaque CD4<sup>+</sup> transitional memory T-cells lack CCR7 expression and express CD28 (Picker *et al.*, 2006). However, macaque CD4<sup>+</sup> transitional memory T-cells are functionally different to macaque CD4<sup>+</sup> effector memory T-cells; IL-2 preferentially induces macaque transitional memory T-cells to proliferate, whereas macaque effector memory T-cells are poorly responsive and *vice versa* for IL-15 (Picker *et al.*, 2006). Both macaque and human CD4<sup>+</sup> central memory T-cells and macaque CD4<sup>+</sup> transitional memory T-cells express CD28, so why did TGN1412 not stimulate these subsets in macaques as well as in humans? Central memory CD4<sup>+</sup> T-cells are characterized by strong lymphoproliferative responses (Sallusto *et al.*, 1999; 2004), which TGN1412 stimulates from human, but not macaque PBMCs (Stebbing *et al.*, 2007). However, we previously reported that immobilized TGN1412 primed macaque CD4<sup>+</sup> T-cells and that they underwent marked lymphoproliferation only when



exogenous IL-2 was added (Stebbing *et al.*, 2007). Because macaque T-cells do not secrete IL-2 when stimulated with TGN1412 (Stebbing *et al.*, 2007), it could be speculated that production of IL-2 by stimulated CD4<sup>+</sup> effector memory T-cells is a missing factor required for *in vitro* lymphoproliferative responses by macaque CD4<sup>+</sup> memory T-cells. Alternatively, it has been suggested that differences in human- and macaque TGN1412-driven responses are due to the expression of inhibitory Siglecs on non-human primate T-cells, which have been lost during human evolution, rendering them less responsive to *in vitro* stimulation (Nguyen *et al.*, 2006; Waibler *et al.*, 2008). However, it should be noted that macaque T-cells are not 'less responsive' to *in vitro* stimulation with TGN1412, but 'non-responsive'.

The commercially available antibody ANC28.1 was used as a comparative antibody because it, like TGN1412, is a superagonist anti-human CD28 antibody. However, ANC28.1 is a superagonist *in vitro* in both aqueous phase and when immobilized, whereas we found that TGN1412 was only a superagonist when immobilized. Therefore, it is likely that ANC28.1 binds to a different region of CD28 from that binding TGN1412, which may explain why lower responses were observed. Moreover, it has been previously published that ANC28.1 induces greater or similar *in vitro* cytokine release than TGN1412 (Waibler *et al.*, 2008), which is in contrast to our findings here that TGN1412 is far more potent than ANC28.1. Nevertheless, ANC28.1 does stimulate release of the same pattern of cytokines as TGN1412 so is likely to activate the same pro-inflammatory pathways.

The new *in vitro* procedures described here show that TGN1412-mediated cytokine release evokes a unique mechanism that can be discriminated from the cytokine release observed with the other therapeutic mAbs tested. While much of the immunopharmacology of cytokine release syndrome in humans remains largely unknown, pre-clinical safety testing in non-human primate models will still be useful, but needs to be interpreted in conjunction with subtleties in antigen expression on target cells in pre-clinical models. We now offer an explanation for why pre-clinical testing in non-human primate models failed to predict a 'cytokine storm' in humans that is based upon a species difference in CD28 expression on CD4<sup>+</sup> effector memory T-cells and that is specific for TGN1412 and other CD28-specific therapeutic mAbs. The likelihood that another mAb or immunomodulatory biotherapeutic agent could stimulate a TGN1412-like adverse response can now be linked to the ability to activate CD4<sup>+</sup> effector memory T-cells.

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## Conflict of interest

The authors have no conflicts of interests.

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