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### **Research Article**

The administration of the immunosuppressive humanized monoclonal antibody CAMPATH 1-H, which recognizes CD52 on lymphocytes and monocytes, is associated with a first-dose cytokine-release syndrome involving TNFα, IFNγ, and IL-6 clinically. In vitro models have been used to establish the cellular source and mechanism responsible for cytokine release, demonstrating that cytokine release is isotype dependent, with the rat IgG2b and human IgG1 isotype inducing the highest levels of cytokine release, which was inhibited with antibody to CD16, the low affinity Fc-receptor for IgG (FcγR). Cross-linking antibody opsonized CD4 T lymphocytes failed to stimulate TNFα release, which together with the observation that TNFα release by purified natural killer (NK) cells stimulated by fixed autologous CAMPATH 1-H-opsonized targets was inhibited with anti-CD16, indicates that cytokine release results from ligation of CD16 on the NK cells, rather than Fc-receptor (FcR)-dependent cross-linking of CD52 on the targeted cell. Since the hierarchy of isotypes inducing cytokine release in these cultures matches that seen clinically, we conclude that ligation of CD16 on NK cells is also responsible for cytokine release after injection of CAMPATH 1-H in vivo.

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# Mechanism of First-Dose Cytokine-Release Syndrome by CAMPATH 1-H: Involvement of CD16 (Fc $\gamma$ RIII) and CD11a/CD18 (LFA-1) on NK Cells

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## Abstract

The administration of the immunosuppressive humanized monoclonal antibody CAMPATH 1-H, which recognizes CD52 on lymphocytes and monocytes, is associated with a first-dose cytokine-release syndrome involving TNF $\alpha$ , IFN $\gamma$ , and IL-6 clinically. In vitro models have been used to establish the cellular source and mechanism responsible for cytokine release, demonstrating that cytokine release is isotype dependent, with the rat IgG2b and human IgG1 isotype inducing the highest levels of cytokine release, which was inhibited with antibody to CD16, the low affinity Fc-receptor for IgG (Fc $\gamma$ R). Cross-linking antibody opsonized CD4 T lymphocytes failed to stimulate TNF $\alpha$  release, which together with the observation that TNF $\alpha$  release by purified natural killer (NK) cells stimulated by fixed autologous CAMPATH 1-H-opsonized targets was inhibited with anti-CD16, indicates that cytokine release results from ligation of CD16 on the NK cells, rather than Fc-receptor (FcR)-dependent cross-linking of CD52 on the targeted cell. Since the hierarchy of isotypes inducing cytokine release in these cultures matches that seen clinically, we conclude that ligation of CD16 on NK cells is also responsible for cytokine release after injection of CAMPATH 1-H in vivo. (*J. Clin. Invest.* 1996. 98:2819–2826.) Key words: Fc receptor • serotherapy • humanized • monoclonal antibody • adverse reaction

## Introduction

CAMPATH 1-H is a humanized IgG1 monoclonal antibody which recognizes the abundant glycolipid anchored CD52 antigen on lymphocytes and monocytes (1). After its infusion, lymphocyte and monocytes counts fall rapidly over the first hour, and a prolonged period of lymphopenia ensues for over 2 yr. CAMPATH 1-H has been extensively used for the serotherapy of leukemias and lymphomas because of its ability to debulk circulating tumor cells (2, 3), and to prevent renal allograft rejection (4). Recently CAMPATH 1-H has also found

use in the treatment of autoimmune diseases, including rheumatoid arthritis (5) and vasculitis (6, 7).

We have administered CAMPATH 1-H to a small group of multiple sclerosis patients, where it was shown to reduce the rate of new lesion formation and stabilize disease for between 2–4 yr (8) and unpublished observations. Shortly after antibody infusion the patients displayed a reversible exacerbation of existing neurological symptoms and activation of certain asymptomatic lesions (9), which was associated with elevated serum levels of TNF $\alpha$  and IFN $\gamma$ , shortly followed by IL-6. Cytokine release is also observed after the first dose of CAMPATH 1-H in other clinical settings (Isaacs, J., P. Rebello, and N. Rapson, personal communication), but without the neurological side effects, indicating that the more severe side effects seen in the MS patients are dependent on a demyelinating lesion. Given the encouraging medium term benefit of this treatment to MS patients in terms of reduced disease activity, we have used in vitro models to study the stimulus responsible for the release and cellular source of these cytokines after antibody infusion, which could include complement intermediates C3a and C5a (11, 12), engagement of Fc-receptor for IgG (Fc $\gamma$ R)<sup>1</sup> (13–15), or ligation of CD52 (16) on different leukocyte populations.

## Methods

**Antibodies.** An anti-CD32 Fab fragment (clone number: IV.3) was purchased from Medarex, (West Lebanon, NH) and MOPC21 from Serotech (Oxford, UK). CAMPATH 1-M and 1-G are rat anti-human CD52 monoclonal antibodies of the IgM and IgG2b isotype, respectively. CAMPATH 1-G was used to produce the humanized IgG1 antibody CAMPATH 1-H by CDR grafting (10). The rat anti-human CD4 antibody was also humanized using the same human IgG1 framework as CAMPATH 1-H. The therapeutic grade antibodies were made in Chinese hamster ovary cells and purified on protein A. The proposed sites for C1q or CD64 interaction on the CH2 region of CAMPATH 1-H were inactivated by site directed mutagenesis (17). Human IgG was purified from serum on a PROSEP<sup>R</sup>-A column (Bioprocessing LTD., Co., Durham, UK) in accordance with the manufacturer's instructions. Murine monoclonal antibodies were purified from 50% NH<sub>4</sub>SO<sub>4</sub> cuts on a PROSEP<sup>R</sup>-A column. Antibodies were biotinylated using NHS-biotin (Pierce Chem. Co., Rockford, IL), removing free biotin by dialysis against PBS + 10 mM NaAzide. Antibody Fab fragments were made by reducing pepsin-derived F(ab')<sub>2</sub> fragments with DTT, followed by alkylation with iodoacetamide, then dialysis against PBS. All the antibodies were passed over a Detoxi-Gel<sup>TM</sup> (Pierce) column according to the manufacturer's instructions to ensure they were free of endotoxin, which was confirmed using the Limulus crab amoebocyte lysate assay (BioWhittaker, Walkersville, MD).

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1. Abbreviations used in this paper: FcR, Fc-receptor; Fc $\gamma$ R, Fc-receptor for IgG; NK, natural killer cell.

**Ex vivo whole blood assays.** 0.5-ml cultures of heparinized blood were incubated with antibodies to CD52, CD4, or human IgG at a final concentration of 10 µg/ml for up to 6 h at 37°C. The cells and plasma were then separated by centrifugation and the concentration of cytokines in the plasma was determined as described below.

**Separation of cell populations.** Mononuclear cells were obtained after centrifugation over Lymphoprep (Nycomed LTD., Birmingham, UK) and the lymphocytes purified from the monocytes by allowing the latter to adhere to plastic flasks for 1 h, after which the nonadherent lymphocytes were pipetted from the flasks. Alternatively the monocytes were purified from mononuclear cell preparations by adherence to 24-costar wells at 37°C for 1 h, after which the nonadherent lymphocytes were vigorously washed off the plates. Granulocytes were prepared by flash lysing the pellet which passed through the Lymphoprep, then readjusting the osmolality with  $\times 10$  PBS, before resuspending the cells in RPMI + 10% FCS. T lymphocyte and natural killer (NK) cells were enriched by incubating the mononuclear cells on 0.5-g nylon wool columns in RPMI + 10% FCS for 1 h at 37°C. The nonadherent cells were eluted from the column with warm RPMI + 10% FCS, washed, and resuspended in fresh medium. In some experiments, these cells were stained with anti-CD3<sup>FITC</sup> (Sigma Chem. Co., St. Louis, MO), then sorted in sterile PBS by flow cytometry into the CD3<sup>-</sup> NK fraction and CD3<sup>+</sup> T lymphocyte fraction. CD4 lymphocytes were purified from nylon wool nonadherent mononuclear cells by incubation with 10 µg/ml anti-CD4 microbeads (Eurogenetics UK Ltd) washing, then pipetting them onto a MiniMacs Separation Column (Miltenyi Biotec, Bergisch Gladbach, Germany). Bound CD4 positive cells were recovered after removal of the column from the separation unit.

**Flow cytometry.** The cell preparations were analyzed by flow cytometry, using combinations of anti-CD45<sup>PE</sup> and anti-CD14<sup>FITC</sup> or anti-CD19<sup>PE</sup> and anti-CD2<sup>FITC</sup> (Eurogenetics), and anti-CD56 (Eurogenetics) plus polyclonal anti-mouse IgG1<sup>FITC</sup> (Caltag Labs., South San Francisco, CA). Nonspecific binding was determined using isotype matched irrelevant controls conjugated with PE (Sigma Chem. Co., St. Louis, MO) or FITC (Caltag). Antibodies to CD2<sup>PE</sup> (Sero-tech), CD3<sup>FITC</sup> (Sigma), and CD16<sup>Biotin</sup> plus Streptavidin<sup>Tricolour</sup> (Caltag) were used for three-color analysis.  $1 \times 10^5$  cells were incubated with PBS + 10 mM NaAzide + 5% heat-inactivated mouse serum for 30 min on ice, then the cells washed and incubated with primary antibodies for 1 h on ice in PBS + 10 mM NaAzide + 1% BSA (PBA). The cells were washed three times, and incubated with the secondary reagents where appropriate for 30 min on ice in PBA. The cells were washed once, fixed with 100 µl of 1% formaldehyde in PBS, and analyzed on a Becton Dickinson FACScan<sup>®</sup> interfaced with a Hewlett Packard 310 computer using FACSmate<sup>®</sup> and Lysis II software. A FACSmate was used for automated data acquisition. A live FCS/SSC gate was set for data acquisition which included all peripheral blood leukocyte populations, 10,000 events being collected.

**Leukocyte cultures.**  $1 \times 10^6$  purified lymphocytes, monocytes, granulocytes, or monocytes and lymphocytes were incubated at 37°C in 0.5 ml of RPMI + 10% FCS in a 24-well plate, either with 10 µg/ml anti-CD4, CD52, 10 nM PMA (Sigma) plus 1 µg/ml A23187 (Sigma), or 1 µg/ml LPS (Sigma). After 4 h, the cell-free supernatants were tested for TNFα or IFNγ as described below. In some experiments Fab fragments of neutralizing antibodies to CD16, 32, 11a or 11b were added to the cells for 30 min on ice at a final concentration of 10 µg/ml before their stimulation with CAMPATH 1-H as above. Alternatively nylon-wool nonadherent mononuclear cells were opsonized with or without 10 µg/ml CAMPATH 1-H for 1 h on ice in RPMI + 10% FCS. The targets were washed, fixed with freshly made PBS + 0.25% paraformaldehyde and left on ice for 1 h, before washing in PBS + 10% FCS, and adjusted to the required cell concentration in RPMI + 10% FCS.  $1 \times 10^6$  unfixed nylon-wool nonadherent cells or  $4 \times 10^5$  NK cells or T lymphocytes, which had been pretreated for 30 min on ice with or without 10 µg/ml anti-CD16 (Fab), were incubated at 37°C with an equal number of fixed targets for 4 h then the supernatants tested for TNFα as below.

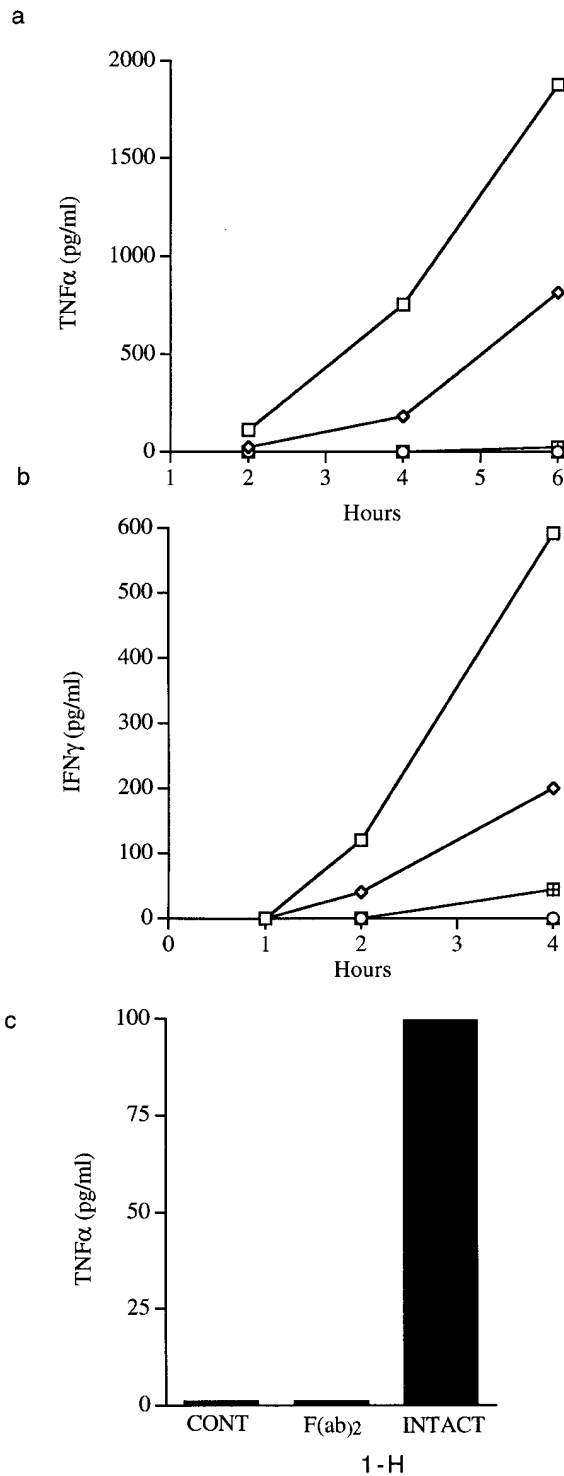
**Cytokine assays.** Plasma TNFα and IFNγ were measured using a sandwich ELISA sensitive to 4.4 pg/ml (R&D Systems, Abingdon, UK), and an IRMA sensitive to 0.1 NIH U/ml (Centocor, Malvern, PA), respectively. An in-house ELISA (sensitivity 10 pg/ml IFNγ), was used to assay culture supernatants for IFNγ, using a capture monoclonal antibody (ECACC number 92030610), which was developed with a polyclonal rabbit anti-human IFNγ (Genzyme Corp., Cambridge, MA). Plasma IL-6 was measured by proliferation of the IL-6-dependent B-lymphocyte cell line MH60, as previously described (9), in comparison to recombinant human IL-6 (R&D Systems), and confirming specificity with a neutralizing antiserum to IL-6 (R&D Systems). The concentration of TNFα in tissue culture supernatants was determined as previously described (18) using the TNF sensitive L929 bioassay by comparison to a standard curve generated using recombinant human TNFα (R&D Systems) and the specificity confirmed using 10% hybridoma supernatant which neutralizes TNFα (ECACC number 92030602).

The data presented in the figures were derived from a single representative experiment which was repeated at least twice, and while the absolute levels of cytokine measured varied between experiments, the same trend was always observed. Cytokine measurements were determined over the linear range of the standard curves.

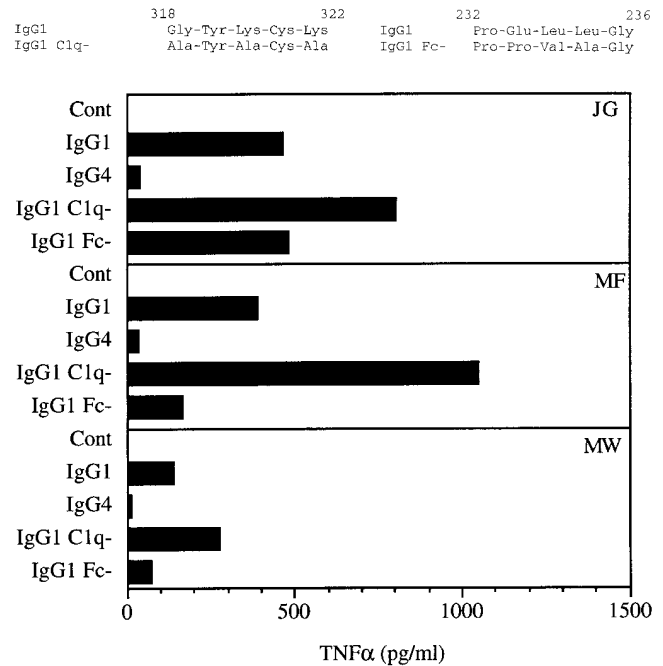
## Results

**Cytokine release is dependent on antibody specificity as well as isotype.** Ex vivo whole blood cultures were incubated with rat or humanized monoclonal antibodies to CD52. TNFα, IFNγ could be detected 2 h after the addition of CAMPATH 1-G, the rat IgG2b or CAMPATH 1-H, its humanized IgG1 derivative (Fig. 1, a and b). 2–3.8 ng/ml of plasma IL-6 was observed after incubation with CAMPATH 1-G or 1-H for 4 h, at all other time points the concentration of IL-6 was below detection. The rat IgM or humanized IgG4 antibody failed to stimulate cytokine release. The kinetics of cytokine release was similar to that seen in vivo (9), with TNFα and IFNγ detected as early as 2 h after incubation with the therapeutic antibodies, IL-6 in contrast, was not detected until 4 h. Despite being the same isotype as CAMPATH 1-H, the humanized antibody recognizing CD4 failed to stimulate cytokine release, thus both the specificity and isotype of the antibody are important in determining cytokine release. The importance of the Fc region was further emphasized by the inability of a F(ab')<sub>2</sub> fragment of CAMPATH 1-H to stimulate cytokine release in the ex vivo cultures (Fig. 1 c).

**Cytokine release is not a consequence of complement activation.** Mutation of CAMPATH 1-H which prevented activation of the classical pathway of complement were still able to stimulate TNFα release (Fig. 2). Indeed, in all the individuals tested, the IgG1 C1q<sup>-</sup> mutant induced higher levels of cytokine release than the wild-type antibody. One explanation for this may be that the fixation of complement intermediates on the constant region of the complement-fixing antibodies either interferes with Fc/FcR interactions or there is competition between complement receptors and FcRs. In contrast, using the CAMPATH 1-H Fc<sup>-</sup> mutant, the level of TNFα release was reduced by 40–50% compared to the parental antibody in two of the three individuals. These results confirmed that complement intermediates were unlikely to be responsible for cytokine release, consistent with the inability of the complement fixing IgM antibody to stimulate cytokines release. While the pattern of TNFα release was similar for the three normal individuals tested, i.e., the IgG4 antibody induced modest TNFα



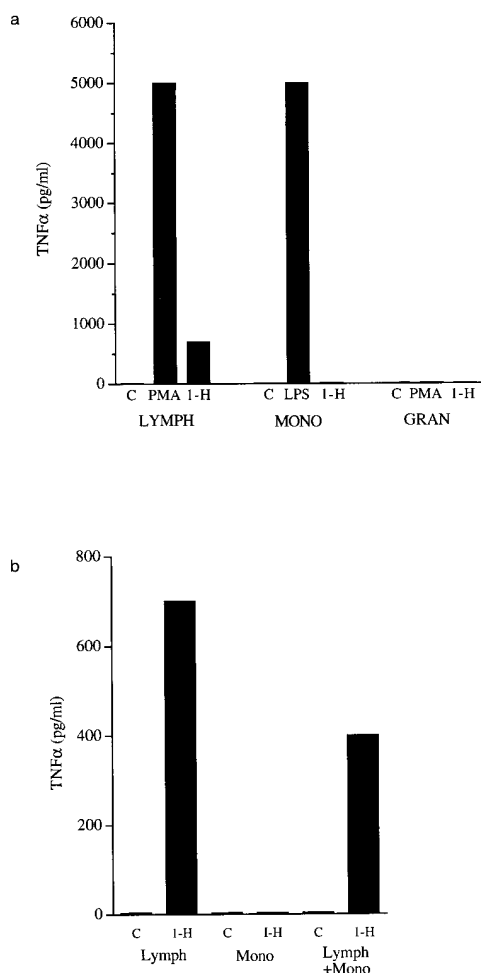
**Figure 1.** Ex vivo whole blood cultures were incubated for up to 6 h with rat and humanized antibodies to CD52 at a final concentration of 10  $\mu$ g/ml. At selected time points the plasma and cells were separated by centrifugation, and the plasma levels of (a) TNF $\alpha$  and (b) IFN $\gamma$  determined by ELISA and IRMA, respectively. (Squares) CAMPATH 1-G (IgG2b); (diamonds) CAMPATH 1-H (IgG1); (crossed squares) CAMPATH 1-M (IgM); (circles) CAMPATH 1-G4 (IgG4); (triangles) CD4 (IgG1). (c) Ex vivo whole blood cultures were incubated with 10  $\mu$ g/ml of either intact CAMPATH 1-H or a pepsin digested F(ab')<sub>2</sub> fragment for 4 h. Plasma TNF $\alpha$  was measured by ELISA after separation of the cells and plasma by centrifugation.



**Figure 2.** Ex vivo whole blood cultures were incubated for 4 h with the humanized antibodies to CD52 of the IgG1 and IgG4 isotype. In addition the wild-type IgG1 antibody, mutants which were unable to bind to C1q, IgG1 C1q- or the high-affinity Fc receptor CD64, IgG Fc- were added to these cultures. The mutated sequences are shown together with the wild-type molecule. Plasma TNF $\alpha$  release from three healthy individuals is shown.

release compared with the humanized IgG1 antibody and its derived mutants, the absolute concentration of cytokine release varied both between and within individuals. The mean interindividual and intraindividual variation in the level of TNF $\alpha$  release in response to 10  $\mu$ g/ml CAMPATH 1-H in these assays was 210 pg/ml (range 79–545 pg/ml,  $n = 8$ ), and 160 pg/ml (range 55–380 pg/ml,  $n = 8$ ), respectively. The intraindividual assays were conducted over a 2-yr time period. As a typical responder, MW was used as a source of cells for subsequent assays.

*Nonadherent mononuclear cells are responsible for TNF $\alpha$  and IFN $\gamma$  release.* To determine which population(s) of cells in the whole blood cultures are responsible for cytokine release, lymphocytes (plastic nonadherent mononuclear cells), monocytes (adherent mononuclear cells) and granulocytes were incubated with CAMPATH 1-H or a positive control for 4 h and the presence of TNF $\alpha$  in the supernatants determined using the L929 bioassay. Although the positive controls resulted in good TNF $\alpha$  release from the lymphocyte and monocyte populations, TNF $\alpha$  was not detected in the granulocyte cultures in response to PMA and the calcium ionophore A23187 (Fig. 3 a). The supernatants from the control and activated granulocytes contained 4 and 36.5  $\mu$ g/ml lactoferrin, respectively, indicating elevated secondary granule release after activation and hence functional activity of these cells. The only cell population which secreted TNF $\alpha$  in response to CAMPATH 1-H was the nonadherent lymphocytes, which produced  $\sim 20\%$  of the TNF $\alpha$  seen with the positive control, suggesting that a subpopulation of the lymphocytes was responding to



**Figure 3.** Cultures of (a)  $1 \times 10^6$  lymphocytes (LYMPH), monocytes (MONO) or granulocytes (GRAN), or (b) lymphocytes and monocytes were prepared by differential adhesion of mononuclear cells to plastic or flash lysis of cells passing through Lymphoprep. The cells were incubated in 0.5 ml RPMI + 10% FCS with 10 nM PMA plus 1  $\mu$ M A23187, 1  $\mu$ g/ml LPS, or 10  $\mu$ g/ml CAMPATH 1-H. The cell-free supernatants were harvested after 4 h and TNF $\alpha$  measured using the L929 bioassay in conjunction with a neutralizing mAb to TNF $\alpha$  to confirm specificity.

CAMPATH 1-H stimulation. To establish if cytokine release was due to contaminating monocytes binding to opsonized lymphocytes, lymphocytes were added to the monocyte cultures and stimulated as before. TNF $\alpha$  was only observed in the lymphocyte or lymphocyte plus monocyte cocultures (Fig. 3 b). Since there was not an increase in the level of TNF $\alpha$  detected in the cocultures compared to the lymphocyte cultures, it seems unlikely that the TNF $\alpha$  was derived from contaminating monocytes. Culture supernatants of nonadherent mononuclear cells stimulated with or without CAMPATH 1-H for 4 h were tested for IFN $\gamma$ , which revealed 5.6 ng and less than 0.01 ng/ml, respectively, demonstrating that the IFN $\gamma$  and TNF $\alpha$  producing cells both resided within the nylon-wool nonadherent mononuclear cells.

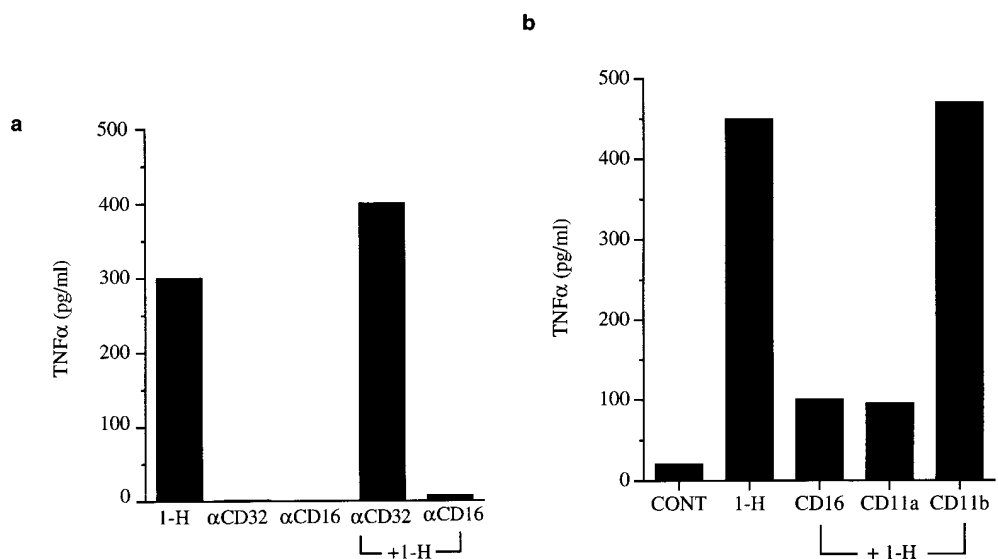
**Involvement of CD16 (Fc $\gamma$ RIII) and CD11a/CD18 (LFA-1) in antibody-dependent TNF $\alpha$  release by nylon-wool nonadherent cells.** The nylon-wool nonadherent mononuclear cells were typically > 95% CD2 $^{+}$ 45 $^{+}$  lymphocytes, being largely

depleted of B lymphocytes (< 1% CD19 $^{+}$ ) and monocytes (< 1% CD14 $^{+}$ 45 $^{+}$ ). These cells responded to the different antibodies in the same way as the cells in the whole blood assays, i.e., the rat IgG2b and the humanized IgG1 anti-CD52 stimulated TNF $\alpha$  release, while the IgM, IgG1 F(ab') $_2$ , humanized IgG1 anti-CD4 and polyclonal human IgG did not (data not shown). While prior incubation with a Fab to CD32 failed to inhibit CAMPATH 1-H-dependent TNF $\alpha$  release in nylon-wool nonadherent mononuclear cell cultures, the anti-CD16 fragment was able to inhibit cytokine release by 85% (78–97%,  $n = 4$ ) (Fig. 4 a). Fab fragments of an anti-CD11a, but not CD11b, were also able to inhibit 74% (70–78%,  $n = 2$ ) of the CAMPATH 1-H mediated TNF $\alpha$  release by the nylon-wool nonadherent cells (Fig. 4 b).

**Ligation of CD16 not CD52 results in cytokine release by NK cells.** CD4 $^{+}$  lymphocytes were purified from nylon-wool nonadherent peripheral blood mononuclear cells using a superparamagnetic anti-CD4. The cells were incubated for 4 h with 10  $\mu$ g/ml CAMPATH 1-H with or without a cross-linking second antibody. In contrast to stimulation with PMA plus the calcium ionophore A23187, ligation of CD52 by CAMPATH 1-H failed to stimulate TNF $\alpha$  release (Fig. 5). Nylon-wool nonadherent mononuclear cells were incubated in medium with or without 10  $\mu$ g/ml CAMPATH 1-H, washed, then fixed with paraformaldehyde. Incubation of fresh nonadherent mononuclear cells with fixed opsonized, but not control cells resulted in TNF $\alpha$  release which could be inhibited with a neutralizing Fab anti-CD16 (Fig. 6 a). This experiment was repeated with T lymphocytes (99.3% CD3 $^{+}$ ) or NK cells (0.2% CD3 $^{+}$ , 80% CD16 $^{+}$ ) which had been sorted by flow cytometry, revealing that NK cells were responsible for the observed CD16-dependent TNF $\alpha$  release (Fig. 6 b). In contrast no TNF $\alpha$  could be detected when the T lymphocytes were stimulated with antibody opsonized targets, however their capacity to secrete TNF $\alpha$  was demonstrated after stimulation with PMA and calcium ionophore. To exclude the possibility that cytokine release was being released by CD16 $^{+}$  cells other than NK cells, nylon-wool nonadherent mononuclear cells were stained with anti-CD3 $^{FITC}$ , anti-CD2 $^{PE}$ , and anti-CD16 $^{Biotin}$ , followed by streptavidin $^{Tricolour}$ . The CD2 $^{+}$ 3 $^{+}$  positive T lymphocytes were distinguished from the CD2 $^{+}$ CD3 $^{-}$  NK cells, demonstrating that CD16 expression was confined to the NK cells (data not shown). This together with the data shown in Fig. 6 b, confirms that NK cells were the source of cytokine release. The number of CD2 $^{+}$ CD3 $^{-}$ CD16 $^{+}$  cells (10.5%) was in good agreement with the number of CD56 $^{+}$  NK cells (13.2%), since it is known that between 80–90% of human NK cells express CD16 (19).

## Discussion

The availability of monoclonal antibodies able to specifically target and deplete cell populations in vivo has had a significant impact in the fields of oncology, transplantation, and more recently in the management of autoimmune disease. However monoclonal antibodies in addition to binding antigen, possess additional biological activities through the interaction of the Fc region of the molecule with the complement system and FcRs, properties that are not necessarily beneficial when used clinically. CAMPATH 1-H, like the immunosuppressive rodent monoclonal antibody OKT3, is associated with the sequential release of TNF $\alpha$  and IFN $\gamma$  followed by IL-6, 2–4 h after infusion of the first dose of antibody, which can be inhib-



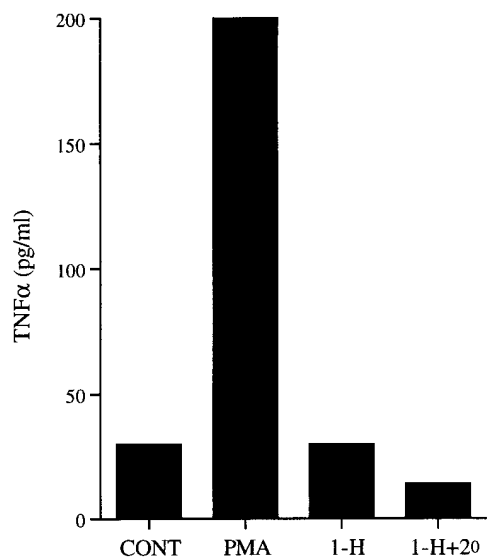
*Figure 4.*  $1-2 \times 10^6$  nylon wool nonadherent lymphocytes were preincubated with or without Fab fragments of neutralizing antibodies to (a) CD32 or CD16 and (b) CD16, CD11a or CD11b for 30 min on ice before incubation with 10  $\mu$ g/ml CAMPATH 1-H for 4 h at 37°C in RPMI + 10% FCS. The concentration of TNFα in the cell-free supernatants was then measured using the L929 bioassay in conjunction with a neutralizing mAb to TNFα to confirm specificity.

ited by the administration of methylprednisolone immediately before antibody infusion (9, 20). Since steroids are known to inhibit cytokine synthesis at the level of transcription and translation (21–23), this together with their sequential release, implies that they are de novo synthesized in a coordinated response.

We initially chose ex vivo whole blood cultures as a model to study cytokine release after therapeutic antibody administration because it reproduced as far as possible the cellular composition which these antibodies would encounter immediately after infusion. We believe both the ex vivo whole blood and nonadherent mononuclear cell cultures are good models since the hierarchy of antibody isotypes which initiate a first dose response clinically, CD52 hIgG1 = rIgG2b > hIgG4 > hCD4 IgG1 (24), is preserved in vitro. Furthermore using clinically relevant concentrations of therapeutic antibody in vitro, the kinetics of cytokine release was similar to that seen with the use of CAMPATH 1-H in multiple sclerosis (9), rheumatoid arthritis, transplant recipients, and patients undergoing cancer serotherapy (Isaacs, J., P. Rebello, and N. Rapson, personal communication). Cytokine release was not due to endotoxin contamination since addition of polymyxin B, an antibiotic able to neutralize the biological properties of LPS (25, 26) did not reduce CAMPATH 1-H induced TNFα release in the whole blood cultures (24). The reason for the modest cytokine release induced by the humanized IgG1 anti-CD4 antibody, despite having an isotype capable of ligating FcγRs is unclear, however since there is ~ 20 times more CD52 expressed per cell than CD4, antigen density may play a role in cytokine release. This is supported by the observation that the ability of rat IgG2b or human IgG1 antibodies to lymphocyte surface antigens to induce cytokine release in whole blood cultures is in approximate relationship to their cell surface expression (24). An additional variable that could critically determine the extent of cytokine release is the accessibility of the antibody Fc region to FcRs after antigen binding. Thus the combination of antigen specificity and antibody isotype may both be important in FcR-dependent cytokine release.

OKT3, which binds to CD3 on T lymphocytes, has been extensively used to prevent graft rejection as well as graft versus

host disease (27, 28). Though still controversial, it is thought that the cytokine release associated with OKT3 results from the transient activation of T lymphocytes after FcR-dependent CD3 cross-linking (29–32). Like OKT3, the involvement of FcγRs in CAMPATH 1-H-mediated cytokine release was also demonstrated by the 40–50% reduction in TNFα release in two individuals using the IgG1 Fc– mutant, in which the IgG1 CH2 hinge-link sequence had been mutated to FcR non-binding IgG2 sequence (17). Since it is believed that there is some overlap in the site on the antibody for interaction with both the high- and low-affinity CD64 and CD16 FcγRI and III, respectively (33, 34), it is likely that mutations designed to inhibit CD64 interactions may to some extent also affect CD16. However the ability of an antibody to CD16 to inhibit cytokine release confirmed the involvement of this receptor. The failure to inhibit cytokine release using the IgG1 Fc– antibody in one of the individuals was consistent with their ability to mediate ADCC reactions using different isotypes of CAMPATH 1-H (17). Since the cytokine release induced by CAMPATH 1-H opsonized cells is CD16-dependent, this indicates that the stimulus for cytokine release is mediated by CD16 rather than CD52. Thus in contrast to OKT3, which transiently activates T lymphocytes through the antibody Fab, CAMPATH 1-H induced cytokine release appears to be a consequence of ligation of CD16 on NK cells. Because previous studies have demonstrated that removal of CD56<sup>+</sup> NK cells, but not CD14<sup>+</sup> monocytes abolishes the ability to mediate autologous antibody dependent cell cytotoxicity in vitro using CAMPATH 1-H (Tite, J., personal communication), it appears that in addition to playing a role in lymphocyte cytotoxicity, NK cells are also responsible for the first dose response via the secretion of cytokines. That the cytokine producing cells only represented 13% of the nonadherent mononuclear cell cultures was consistent with the observation that CAMPATH 1-H-induced TNFα release was only a fraction of the total inducible by a nonspecific stimulus such as PMA and the calcium ionophore A23187. While modest levels of IL-6 were detected in the whole blood cultures, this cytokine was below the limits of detection in nonadherent mononuclear cell cultures stimulated with CAMPATH 1-H (data not shown). The delay in the appearance of

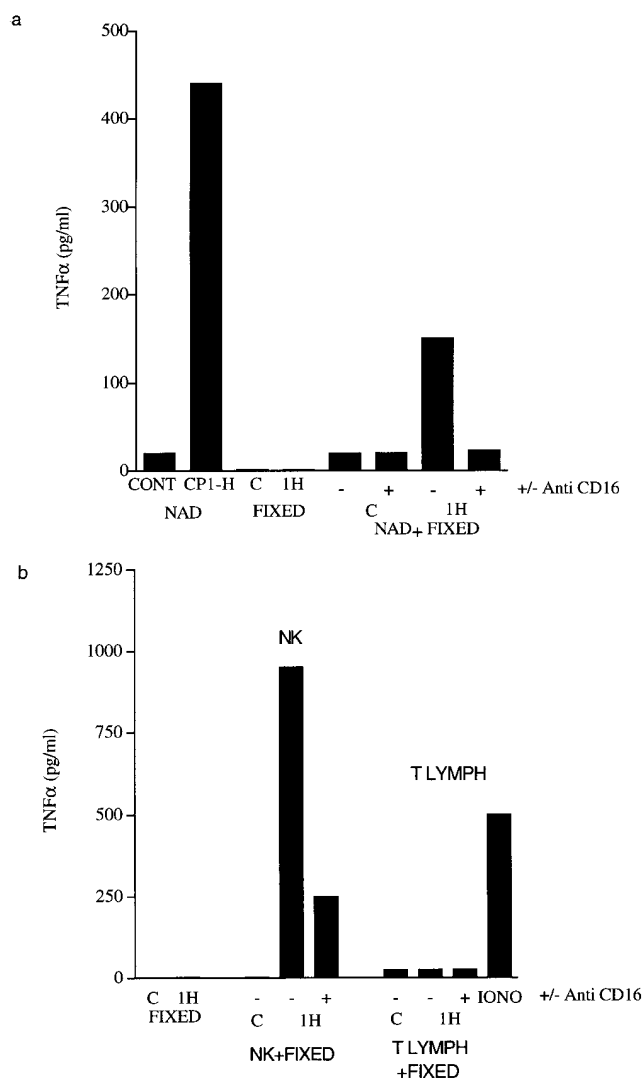


**Figure 5.**  $2 \times 10^6$  CD4 positive lymphocytes purified from nylon wool nonadherent cells by magnetic sorting were incubated in 0.5 ml RPMI + 10% FCS for 4 h with 10 nM PMA plus 10  $\mu$ M A23187 or 10  $\mu$ g/ml CAMPATH 1-H plus or minus a rabbit anti-human IgG second antibody. The concentration of TNF $\alpha$  in the cell-free supernatants was then measured using the L929 bioassay in conjunction with a neutralizing mAb to TNF $\alpha$  to confirm specificity.

IL-6 compared to TNF $\alpha$  and IFN $\gamma$  after administration of CAMPATH 1-H *in vivo* (9 and Rapson, N., personal communication), suggests that IL-6 may be released from other cell(s) in response to the above cytokines, which *in vivo* could include monocytes (35, 36) and endothelial cells (37).

In spite of its abundance on the surface of lymphocytes and monocytes, a physiological role for CD52 has yet to be found, though recently it has been demonstrated by Rowan et al. (16) that CAMPATH 1-H plus cross-linking second antibodies are mitogenic only in the presence of sub-optimal concentrations of PMA, in highly purified CD4 and CD8 cultures which had been exhaustively depleted of CD16<sup>+</sup> NK cells. While modest levels of TNF $\alpha$  and IFN $\gamma$  were detected in these cultures, they were not apparent until 96 h of culture, indicating that the acute release of these cytokines seen clinically and *in vitro* in our study within 2–4 h of stimulation with CAMPATH 1-H, are derived from a different cellular source to that described by Rowan et al. (16). In contrast to the lack of evidence for acute cytokine release being mediated after ligation of CD52, IFN $\gamma$ , and TNF $\alpha$  mRNA was detected within 2 h of cross-linking CD16 on NK cells, which was followed shortly by cytokine secretion (13). CD16 on NK cells consists of a noncovalent complex with at least two additional proteins; the T lymphocyte receptor  $\zeta$  chain (38) and Fc $\epsilon$ R1  $\gamma$  chain (39), which by analogy to the signal transduction pathways described after T lymphocyte activation through the CD3  $\zeta$  chain dimers (40), may ultimately result in cytokine transcription by NK cells.

The ability of a Fab fragment of an antibody to CD11a (LFA-1) to also inhibit TNF $\alpha$  secretion following CAMPATH 1-H stimulation is not without precedent, because this molecule, which is constitutively expressed at high levels by NK cells (41), has been shown to be important in promoting effector–target interactions by these cells (42). Furthermore it has



**Figure 6.** (a)  $1 \times 10^6$  nylon wool nonadherent lymphocytes (NAD) or (b)  $4 \times 10^5$  flow cytometry purified NK cells or T lymphocytes were incubated either alone (CONT), with 10  $\mu$ g/ml CAMPATH 1-H (CP1-H), or alternatively with (+) or without (–) a Fab fragment of a neutralizing mAb to CD16 before the addition of an equal number of nylon wool nonadherent cells which had been fixed with paraformaldehyde (FIXED) after incubation with CAMPATH 1-H (1H) or without (C). The concentration of TNF $\alpha$  in the cell-free supernatants was measured after 4 h using the L929 bioassay in conjunction with a neutralizing mAb to TNF $\alpha$  to confirm specificity.

been demonstrated that some antibodies to CD11a are able to synergize with CD16 in the acute release of TNF $\alpha$  by NK cells (43). Because there was little evidence of cytokine release in the absence of CD16 ligation, this argues that the involvement of LFA-1 is secondary to CD16 ligation. Thus while it is probable that CAMPATH 1-H is responsible for establishing the initial bridge between CD52 on the antibody-coated targets and CD16 on the NK cells, other molecules such as CD11a on the NK cell and its ligand ICAM, may subsequently contribute to this interaction.

In conclusion, we feel that the chain of events initiated after injection of CAMPATH 1-H resulting in a cytokine-related first dose response, is a consequence of antibody-coated tar-

gets interacting with CD16<sup>+</sup> NK cells. Because the rapid and profound lymphopenia that results from the injection of this antibody appears to be dependent on FcγR positive cells (3), engineering-out FcR interactions by site-directed mutagenesis to reduce the side effects may create an antibody which is less efficient at lymphocyte depletion. Since we have previously demonstrated that pretreatment of the patients with a single dose of methylprednisolone before the start of antibody infusion largely prevented the side effects, without obviously affecting the rate or length of lymphocyte depletion (9), this may represent a better approach for the management of patients receiving this form of immunosuppressive treatment.

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