Tocilizumab (Anti-IL-6R) Suppressed TNFα Production by Human Monocytes in an In Vitro Model of Anti-HLA Antibody-Induced Antibody-Dependent Cellular Cytotoxicity

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Background. We previously demonstrated that natural killer (NK) cells activated via FcγRIIIa (CD16) interactions with anti-HLA antibodies binding to peripheral blood mononuclear cells (PBMCs) in the in vitro antibody-dependent cellular cytotoxicity (ADCC) assay produced IFNγ. Here we investigate if other CD16 bearing cells are responsive to alloantigen via alloantibody in the in vitro ADCC and if the ADCC-induced cytokine reactions and cytotoxicity can be modified by the anti-interleukin 6 receptor (IL-6R) monoclonal antibody, Tocilizumab (TCZ). Methods. Whole blood from a normal individual was incubated overnight with irradiated allo-PBMCs pretreated with anti-HLA antibody positive (in vitro ADCC) or negative sera (mixed lymphocyte reaction [MLR]), with or without TCZ or control IgG. IFNγ+ TNFα+ IL-6+ or IL-6+ cell% in NK cells, monocytes and CD8+ T cells were enumerated by cytokine flow cytometry. ADCC using PBMCs (effector) and Farage B cells (FB, target) with anti-HLA antibody positive sera, with or without TCZ, was measured by flow cytometry. Results. IFNγ+ and/or TNFα+ cell% in NK cells, monocytes and CD8+ T cells were elevated in the ADCC compared to the MLR condition. IL-6+ cells were significantly increased in ADCC versus MLR (10.2 ± 4.8% vs 2.7 ± 1.5%, P = 0.0003), but only in monocytes. TCZ treatment significantly reduced TNFα+ cell% in monocytes in ADC and had no effect on other cytokine+ cells. TCZ showed no effect on cytotoxicity in ADCC. Conclusions. IFNγ, TNFα, and IL-6 production induced by HLA antibody-mediated CD16 bearing cell activation in NK cells, monocytes, and CD8+ T cells suggests a potential role for ADCC and these inflammatory cytokines in mediation of antibody-mediated rejection. TCZ suppressed TNFα production in monocytes in the ADCC condition, suggesting a role of IL-6/IL-6R pathway in monocytes activation. Inhibition of this pathway could reduce the inflammatory cascade induced by alloantibody, although the inhibitory effect on cytotoxicity is minimal.

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Antibody-mediated rejection (AMR) is a major obstacle to successful transplantation in HLA-sensitized (HS) patients.1 The traditional view of AMR is that of complement-dependent cytotoxicity-mediated injury with characteristic C4d deposition.2-3 However, we and other investigators have suggested that cellular effector pathways including antibody-dependent cell-mediated cytotoxicity (ADCC) also play an important role in the pathogenesis of AMR.4-6 We previously reported that FcγRIIIa (CD16)+ natural killer (NK) cells in HS patient blood responded to alloantigens expressed on alloperipheral blood mononuclear cells (PBMCs) in cytokine flow cytometry (CFC), resulting in IFNγ production, and this NK cell activation was antibody-mediated via CD16 on NK cells, which is an ADCC-like mechanism.7-9 We have also reported that the antibody-mediated NK cell activation was inhibited by calcineurin inhibitors and steroid in the in vitro ADCC and suggested that NK cell activation and their cytokine production via ADCC are likely important in mediating AMR, and may represent a newly recognized opportunity for modification of antibody-mediated allograft injury.10 In addition to NK cells, primary cells for ADCC, other CD16
bearing cells, monocytes and CD8+ T cells, could also be involved in ADCC and cytokine production such as IFNγ, TNFα and/or IL-6.

IL-6 is a pleiotropic cytokine and has proinflammatory and anti-inflammatory properties. It plays central roles in infection, autoimmunity and cancer. IL-6 is directly involved in the initiation and maintenance of inflammatory immune response. Recent clinical and experimental studies suggest that IL-6 contributes to renal injury and is associated with renal allograft rejection. Tocilizumab (TCZ) is a FDA-approved humanized monoclonal antibody to the IL-6 receptor (IL-6R) used for treatment of rheumatoid arthritis, with potential efficacy in other autoimmune diseases. Recent clinical observations and animal studies have shown that anti-IL-6R antibodies reduced graft-versus-host disease and allograft rejection. We have recently reported that anti-IL-6R treatment attenuates de novo DSA production and IL-6 in allosensitized animal model.

In addition, we have shown promising results of TCZ use for reduction of AMR posttransplantation in a phase III clinical trial for desensitization with TCZ and intravenous immunoglobulin followed by kidney transplantation in HS patients. Here, we determined if NK and other CD16+ cells, primarily monocytes and CD8+ T cells, are capable of alloantibody-mediated cell activation, resulting in cytokine production in the in vitro ADCC, and if TCZ is capable of suppressing these activation events and cytotoxicity in ADCC.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board at Cedars-Sinai Medical Center (CSMC) (IRB number Pro00012562). The study was conducted in accordance with the ethical guideline based on federal regulations and the common rule. CSMC also has a Federal Wide Assurance.

In Vitro Antibody-Mediated Cell Activation (In Vitro ADCC)

The in vitro ADCC was performed as previously reported with modification. Briefly, pooled PBMCs isolated from blood of 5 normal individuals and then irradiated (PBMCx) were reinfused with HS (ADCC condition) or normal sera (NS, mixed lymphocyte reaction [MLR] condition), and the antibody-coated PBMCx were used as stimulator cells. HS sera was prepared by pooling sera from 70 end-stage renal disease HS patients with PRA greater than 50% and the same HS sera was used for all experiments in this study. NS from 1 of 6 normal individuals without previous HLA-sensitization events whose serum was previously shown to be negative in the in vitro ADCC (non-HS normal individual) was used for each PBMCx pretreatment. In the standard in vitro ADCC used in this study, 100 μL of whole heparinized blood (responder cells) from a non-HS normal individual were incubated with 100 μL of antibody-coated PBMCx (1 × 10^6 cell/mL, stimulator cells) (PBMC number in stimulator and responder cells, approximately 1:1) at 37°C for 6 hours followed by additional 6 hour incubation with brefeldin A (BFA) (10 μg/mL) (total, 12-hour incubation) to measure accumulated intracellular cytokines (IFNγ, TNFα, and IL-6) by CFC. Blood only without PBMCx was used as another control condition. In a separate study, the blood and PBMCx mixture was incubated for 6, 12, 18, and 24 hours and BFA was added during the last 6 hours to determine the time for the maximal production of each cytokine in each cell type after initiation of the incubation. To investigate the effect of TCZ (Roche, San Francisco, CA) on the in vitro ADCC, the above standard in vitro ADCC (total 12-hour incubation) was performed with or without TCZ at the concentration ranging from 1 to 300 μg/mL. TCZ was added at the beginning of the ADCC culture. Purified human IgG (R&D Systems, Minneapolis, MN) was used as control. The experiments of each study were performed multiple times using blood from different non-HS normal individuals (responder cells).

Measurement of Intracellular IFNγ, TNFα, and IL-6 in NK Cells, Monocytes, and CD8+ T Cells by CFC

CFC analysis was performed as previously described with minor modification. Briefly, after 6-hour incubation with BFA, EDTA (2 mM) was added to the mixture to stop the action and then the cells were stained with fluorochrome-conjugated antibodies to CD3, CD8 (V450), CD14 (V500), CD16 (PerCP Cy5.5) and CD56 (PE Cy7) (BD Biosciences, San Jose, CA). After erythrocytes were lysed followed by permeabilizing the cells, intracellular cytokines were stained with fluorochrome-conjugated antibodies to IFNγ (PE CF594), TNFα (APC) and IL-6 (PE) (BD Biosciences). After cell acquisition by BD LSR Fortessa (BD Biosciences), CD14+ monocytes were gated and then the number of IFNγ+, TNFα+ or IL-6+ cells was enumerated by BD fluorescence activated cell sorting (FACS) Diva (BD Biosciences) (Figure 1). Lymphocytes gated by CD14/side scatter were plotted against CD3 and CD8. CD3- cells were further plotted against CD36 for NK cells, and then cytokine+ cells % in CD56+ NK cells and CD8+ T cells were enumerated (Figure 1).

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

ADCC assay developed in our laboratory as previously described elsewhere with minor modification uses normal individual PBMCs as effector (E) and lymphoma Farage B (FB) cells as target cells (T). In the assay, normal PBMCs were mixed with FB cells prelabeled with FITC-anti-human CD19 at 4°C for 20 minutes at the E:T ratio of 5:1 (0.1 × 10^6 PBMCs + 0.02 × 10^6 FB cells in total volume of 120 μL) together with anti-HLA antibody positive (HS) or negative (NS) sera, and incubated at 37°C for 1 hour in a 5% CO2 incubator. After washing, the cells were resuspended in 100 μL FACS buffer and then 20 μL of 7-AAD was added followed by a 10-minute incubation on ice. After addition of 250 μL of FACS buffer, the cells were submitted for flow cytometry analysis. To examine the effect of TCZ on the ADCC, various concentrations of TCZ (0–100 μg/mL) was added in the ADCC cell mixture. After cell acquisition, CD19+ FB cells separated from nonstained PBMC were plotted against 7-AAD, and then 7-AAD+ FB cell % in total CD19+ FB cells was enumerated.

Statistical Analysis

Cytokine production in the ADCC, MLR, and control conditions in NK cells, monocytes or CD8+ T cells (Figure 3) was compared by repeated-measures ANOVA (RMANOVA) with contrasts. The effect of TCZ on each cytokine production in NK cells, monocytes or CD8+ T cells in the standard in vitro ADCC (Figure 4) was assessed by RMANOVA. When the RMANOVA showed P value less than 0.05, a standard paired
2-tailed $t$ test was performed to identify the effective dose. The effect of TCZ on cytotoxicity in ADCC (Figure 5) was assessed by a standard paired 2-tailed $t$ test. The difference with $P$ less than 0.05 was considered statistically significant.

**RESULTS**

**IFN$\gamma$, TNF$\alpha$, and IL-6 Production in NK Cells, Monocytes and CD8$^+$ T Cells in the In Vitro ADCC**

To detect intracellular IFN$\gamma$, TNF$\alpha$, and IL-6 in antibody-activated NK cells, monocytes, and CD8$^+$ T cells, we modified the original in vitro ADCC assay where only intracellular IFN$\gamma$ in NK cells was detected as previously reported.$^9,10$

We first performed pilot experiments to examine the cytokine production profile in NK cells, monocytes, and CD8$^+$ T cells using CFC at various time points after initiation of in vitro ADCC, MLR, and control (blood only) cultures. BFA was added during the last 6-hour incubation of each culture condition to measure cytokines produced and accumulated. Cytokine+ cells in each cell population of the ADCC and MLR conditions were compared with that in controls at each time point, and the results were expressed as a ratio (Figure 2). The ratios of IFN$\gamma$+ cells at 12 hour incubation in the ADCC condition were 29.2 ± 21.3, 7.1 ± 2.3 and 8.0 ± 9.3 in NK cells, monocytes and CD8$^+$ T cells, and TNF$\alpha$+ cells 7.6 ± 4.1, 2.5 ± 1.1 and 2.9 ± 2.1, respectively, which were much higher than the ratio 1.0 observed at all incubation time points tested in the MLR and control conditions. A similar trend was also observed at 6-hour incubation where BFA was added from the beginning of the cell culture, except for TNF$\alpha$+ monocytes, but the ratios were lower than those at 12-hour incubation. IL-6+ NK cells and CD8$^+$ Tcells in the ADCC condition were minimal and similar to the MLR conditions at all time points. However, IL-6+ monocytes increased after 12-hour incubation although that was minimal at 6-hour incubation.

Because the above pilot experiments showed that 12-hour incubation with BFA added during the last 6-hour incubation was optimal for these cytokine productions in these cell populations in the ADCC condition, similar experiments using only this condition were repeated to determine alloantibody-mediated cytokine production in the in vitro ADCC. As expected, IFN$\gamma$+ and TNF$\alpha$+, but not IL-6+ cells, were significantly elevated in NK and CD8$^+$ T cells in the ADCC condition compared with the MLR and control conditions (Figure 3). TNF$\alpha$+ and IL-6+ monocytes in the ADCC condition were also significantly elevated compared with other conditions, whereas IFN$\gamma$+ monocytes in the ADCC condition were not significantly elevated. This condition was chosen as the standard in vitro ADCC procedure for further experiments.
FIGURE 2. Cytokine production in NK cells, monocytes and CD8+ T cells in the in vitro ADCC and MLR cultures as detected by CFC. Cells were cultured in the ADCC (solid line), MLR (dotted line) and control (Blood only) conditions for 6, 12, 18, and 24 hours, and cytokines produced and accumulated for the last 6-hour incubation with BFA in each culture condition were measured by CFC. The cytokine+ cell% in the ADCC or MLR condition was compared to that in control condition at each incubation time point in each cell population, and the results were expressed as the ratio. Thus, the cytokine+ cell% in the control condition at each time point in each cell population is 1.0. Mean ± standard deviation of 5 experiments using blood from 5 different non-HS normal individuals (responder cells) is shown. The actual cytokine+ cell% in the control condition at 6-, 12-, 18-, and 24-hour incubation was; IFNγ: 0.2 ± 0.1, 0.2 ± 0.2, 0.2 ± 0.1, 0.1 ± 0.1 in NK cells (A), 0.3 ± 0.5, 0.3 ± 0.4, 0.2 ± 0.3, 0.2 ± 0.3 in monocytes (B), 0.1 ± 0.0, 0.1 ± 0.0, 0.1 ± 0.0, 0.1 ± 0.0 in CD8+ T cells (C); TNFα: 0.6 ± 0.3, 0.6 ± 0.4, 0.7 ± 0.5, 0.5 ± 0.3 in NK cells (D), 2.8 ± 2.2, 2.7 ± 1.4, 6.1 ± 4.3, 5.9 ± 4.4 in monocytes (E), 0.6 ± 0.2, 0.7 ± 0.2, 0.9 ± 0.3, 1.0 ± 0.3 in CD8+ T cells (F); IL-6: 0.1 ± 0.1, 0.1 ± 0.0, 0.3 ± 0.4, 0.1 ± 0.0 in NK cells (G), 1.6 ± 2.5, 1.1 ± 1.9, 0.7 ± 1.1, 0.3 ± 0.2 in monocytes (H), 0.1 ± 0.1, 0.1 ± 0.0, 0.2 ± 0.3, 0.0 ± 0.0 in CD8+ T cells (I), respectively.

FIGURE 3. Cytokine production in NK cells, monocytes and CD8+ T cells in the standard in vitro ADCC (black), MLR (slashed) and control (gray) conditions. The standard ADCC and MLR (total 12-hour incubation with BFA added during the last 6-hour incubation) were performed with control (blood only). The results are expressed as the percentage of IFNγ+, TNFα+ and IL-6+ cell% in NK cells, monocytes and CD8+ T cells. Mean ± standard deviation of 9 experiments using blood from 9 different non-HS normal individuals (responder cells) is shown. PBMCxHS and PBMCxNS (stimulator cells) describe irradiated PBMC pretreated with pooled sera from HS patients and a non-HS normal individual, respectively. *P < 0.05 vs control (blood only) condition.
The Effect of TCZ on Cytokine Production in the In Vitro ADCC

We next examined the effect of TCZ on cytokine production in the in vitro ADCC using the standard in vitro ADCC procedure. TCZ significantly suppressed TNFα production in monocytes in a dose-dependent manner, whereas the control IgG showed no effect (Figure 4E); TCZ showed 14.5% reduction at 10 μg/mL, the possible lowest therapeutic concentration, and 32.5%, the maximum reduction, was observed at 50 μg/mL, about half the possible maximum therapeutic concentration. TCZ did not significantly suppress IFNγ+ or IL-6+ monocytes (Figure 4B and 4H). TCZ also had no effect on IFNγ+, TNFα+ or IL-6+ cells in the NK (Figures 4A, D, and G) and CD8+ T cell populations (Figures 4C, F, and I).

The Effect of TCZ on Cytotoxicity in ADCC

We next examined the effect of TCZ on cytotoxicity in ADCC to assess if the reduction of TNFα production in monocytes by TCZ contributes to reduction of cytotoxicity in ADCC. 7-AAD+ FB cell% significantly increased when PBMCs and FB cells were incubated with HS compared with NS (25.6 ± 4.1 vs 7.9 ± 2.1, *P < 0.001) or no serum (25.6 ± 4.1 vs 8.1 ± 2.1, #P < 0.001) (Figure 5). However, the increased

![Figure 4](image)

**Figure 4.** The effect of TCZ on IFNγ (A-C), TNFα (D-F), and IL-6 (G-I) production in NK cells, monocytes and CD8+ T cells in the standard in vitro ADCC. The standard ADCC (total 12-hour incubation with BFA added during the last 6-hour incubation) was performed with or without TCZ or control IgG. The results are expressed as the ratio against each cytokine+ cell% in the ADCC condition without additives in each cell population, and the ratio100 is the basal level. Mean ± standard deviation of 5 experiments from 5 different non-HS normal individuals (responder cells) is shown. Actual mean value of IFNγ+, TNFα+ and IL-6+ cell% in the ADCC condition without additives was 2.9 ± 0.3, 1.9 ± 1.2 and 1.9 ± 2.5 in NK cells, 1.9 ± 1.2, 6.0 ± 3.1 and 7.3 ± 4.8 in monocytes, and 0.9 ± 0.8, 1.2 ± 0.7 and 0.2 ± 0.1 in CD8+ T cells, respectively. Shaded area describes possible therapeutic concentration of TCZ. *P < 0.05 vs control IgG at each concentration. **P < 0.05 vs ADCC condition without additives.

![Figure 5](image)

**Figure 5.** The effect of TCZ on cytotoxicity in ADCC. The ADCC assay was performed using normal PBMCs (E) and FB cells (T) with no serum (control), NS (MLR) or HS (ADCC), with or without TCZ. The results of cytotoxicity are expressed as 7-AAD+ FB cell%. Individual lines describe the result from 7 different normal individual PBMCs (E). Shaded area describes possible therapeutic concentration of TCZ. N.S., not significant.
7-AAD+ FB cell% did not change by addition of TCZ (25.0 ± 4.8 vs 25.6 ± 4.1 at 100 μg/mL, N.S.).

DISCUSSION

We have previously shown that NK cells were activated via CD16 and produced IFNγ in response to anti-HLA antibody-coated allo-PBMC in the in vitro ADCC using CFC.7-10 In this study, we assessed a possibility that other CD16 bearing cells, monocytes, and CD8+ T cells, were capable of antibody-mediated cell activation, resulting in cytokine production in the in vitro ADCC. We found that, IFNγ, TNFα, and IL-6–producing cells were significantly elevated in the ADCC compared to MLR and controls. IFNγ, TNFα, and/or IL-6 production in the ADCC has been reported in other studies using various in vitro ADCC models.24-29 Most studies measured cytokine levels in supernatants from the ADCC culture using purified NK cells, monocytes or T cells,24,28 or PBMCs29 as responder cells, whereas some used CFC to detect cytokines in PBMC.24,29 Most of these studies used antitumor antibody-coated tumor cells as target cells. Our in vitro ADCC used whole blood as responder cells, which might be more biological condition compared to those using isolated single cell type, and alloantibody coated allo-PBMCs as target cells, which might be more appropriate to study AMR in HS transplant patients. In addition, CFC allowed to detect multiple cytokines separately in NK cells, monocytes and CD8+ cells, all at once. Although all studies agreed that IFNγ, TNFα, and/or IL-6 were produced in NK cells, monocytes, and/or T cells in the in vitro ADCC setting, there were some discrepancy in results among studies, such as the type of cytokines detected, the type of cells producing those cytokines, and the time of cytokines detected after the ADCC culture. Overall, most studies were in agreement over IFNγ and TNFα production in NK cells, whereas cytokine production in monocytes and CD8+ T cells was inconsistent among studies. Different responder and target cells, and antibodies used for each ADCC model must constitute one of the reasons for these discrepancies as reports suggest that various factors such as the density of antigens expressed on target cells,30 affinity of antibody to the antigen(s) on the target cells,31 glycosylation of the Fc region of IgG and Fcy receptors,32 and polymorphism of CD16,31 affect interaction between antibody and responder or target cells, resulting in differential effects on responder cell activation, cytokine production and the degree of ADCC.

The methods used for cytokine detection also affect final outcomes of antibody-mediated cell activation and ADCC. Especially, CFC that requires the addition of BFA, a Golgi transport inhibitor, affects the physiology in the ADCC. In our study, cytokine+ cells were elevated in the ADCC condition in NK cells and monocytes at 12-hour incubation or longer compared with 6-hour incubation, whereas this trend was not observed in CD8+ T cells. This might be due to minimal production of cytokines in NK cells and monocytes during the first 6 hours of ADCC culture.24 Another possibility is the lack of soluble growth factors in the 6-hour ADCC culture condition where BFA was added at the initiation of ADCC culture as other reports suggest that addition of IL-12, IL-18, and IL-21 significantly augmented IFNγ and/or TNFα production primarily in NK cells and to a lesser degree in T cells in the ADCC.

In this study, IL-6+ monocytes in the ADCC were significantly elevated and reached approximately 10% of monocytes, but only during 12-hour ADCC incubation or longer. IL-6 production in the responder monocytes in the ADCC condition was not previously reported although elevated IL-6 was detected in supernatant from 3-hour ADCC culture conditions where PBMC and target cells were incubated with the target-specific antibody.39 Significantly elevated TNFα+ monocytes were also found only during 12-hour ADCC incubation or longer in this study. These results suggest that monocytes require soluble factor(s) to be fully activated in the ADCC, resulting in IL-6, TNFα and other cytokine production. Because IL-6 and TNFα are known to act as autocrine and/or paracrine factors, they may represent the soluble factors required for full activation in monocytes and other cells in the ADCC condition.35

We next investigated the effect of TCZ on the in vitro ADCC. In this experiment, we found that TCZ significantly reduced TNFα+ monocytes in a dose-dependent manner and close to maximum 30% reduction was observed at 50 μg/mL, less than the maximum therapeutic blood level, whereas TCZ did not show significant effects on other cytokines and did not have any impact of cytokine production by NK and CD8+ T cells.

TNFα possesses a broad spectrum of proinflammatory properties through its activation of the NFκB pathway, resulting in up-regulation of anti-apoptotic proteins, prolongation of inflammatory cell survival and persistent inflammation.36 It induces the synthesis of proinflammatory cytokines, such as IL-1 and IL-6, and chemokines, such as IL-8, MCP-1, MIP-1α, and RANTES, and activates macrophages.37 In addition, it has been reported that activated monocytes from both healthy controls and patients with rheumatoid arthritis can induce Th17 responses in an IL-1β/TNFα–dependent fashion in vitro and in vivo.37 Another study has also shown Th17 cell differentiation by TNFα with IL-6 and IL-1β produced by monocytes in patients with active rheumatoid arthritis, and IL-17 production was significantly reduced in patients treated with anti-TNFα.38 Suppression of Th17 cells by regulatory T cell through the inhibition of monocyte derived IL-6 in anti-TNFα–treated rheumatoid arthritis patients has been shown in other study.39 In this study, we found that anti–HLA antibody-activated monocytes produced TNFα and IL-6 more than NK and CD8+ cells, and that elevated TNFα production was significantly suppressed by TCZ. This result suggests that proinflammatory cytokines, TNFα and IL-6 produced by anti–HLA antibody-activated monocytes creates an inflammatory environment in the graft, and the blockade of IL-6/IL-6R pathway by TCZ may reduce the TNFα-mediated proinflammatory activity.

TCZ did not show any effect on cytokine production in NK or CD8+ T cells in this study, suggesting that IL-6 and possibly TNFα are not required for cytokine production in NK and CD8+ cells activated by ADCC. We also observed that human IgG used for the control condition tended to increase IL-6+ NK cells, monocytes, and CD8+ T cells in the ADCC condition although this was not statistically significant. A similar trend was observed in another study where IL-6 production increased when PBMCs were incubated with a mouse isotype control antibody.40
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