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Transplant long-surviving induced by CD40–CD40 ligand costimulation blockade is dependent on IFN-γ through its effect on $CD4^+CD25^+$ regulatory T cells

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ABSTRACT

Background: IFN-γ was documented to be commonly associated with acute rejection. In the present study, we investigated the role of IFN-γ in the transplant long-surviving induced by blocking CD40–CD40 ligand (CD40–CD40L) costimulation and its mechanisms.

Methods: IFN-γ expression in cardiac allografts and spleens from syngeneic and allogeneic recipients with or without anti-CD40L monoclonal antibody (MR-1) treatment was examined by real-time RT-PCR. The grafts survival time in Wild type ($IFN\text{-}\gamma^{+/+}$) and IFN-γ deficient ($IFN\text{-}\gamma^{-/-}$) recipients was investigated. Mixed lymphocyte reaction (MLR) of $CD4^+$ T cells and cytotoxic T lymphocyte (CTL) assay of $CD8^+$ T cells were also studied. FoxP3 expression in allografts and spleens from $IFN\text{-}\gamma^{+/+}$ or $IFN\text{-}\gamma^{-/-}$ recipients with MR-1 treatment was examined. Furthermore, FoxP3, IL-10 and CTLA-4 expressions and the suppressive capability of $CD4^+CD25^+$ regulatory T cells were examined.

Results: Rejected allografts showed significantly higher IFN-γ expression than long-surviving allografts. Allograft survival was not prolonged in nonimmunosuppressed $IFN\text{-}\gamma^{-/-}$ mice. Administration of MR-1 induced long-term survival in 90.1% of $IFN\text{-}\gamma^{+/+}$ recipients (98 ± 6.6 days) but failed to do so in $IFN\text{-}\gamma^{-/-}$ group (16.2 ± 4.0 days). $IFN\text{-}\gamma^{-/-}$ recipients facilitated the proliferation and CTL generation of T cells. The allografts and spleens from $IFN\text{-}\gamma^{+/+}$ recipients contained higher FoxP3 expression than $IFN\text{-}\gamma^{-/-}$ recipients. Moreover, $CD4^+CD25^+$ T cells from $IFN\text{-}\gamma^{+/+}$ recipients displayed a higher FoxP3 and IL-10 expression and suppressive capability.

Conclusion: IFN-γ plays an important role in the long-surviving induced by blocking CD40–CD40L through inhibiting the function of activated T cells and increasing suppressive capability of $CD4^+CD25^+$ regulatory T cells.

1. Introduction

Current powerful immunosuppressive treatments can prevent acute transplant rejection, but the life-long administration of non-specific immunosuppressive drugs is accompanied by many side effects. Therefore, induction of a sustained state of donor specific transplant tolerance with minimization or complete withdrawal of immunosuppressants is an ultimate goal [1]. Signaling through the TCR alone in the absence of constimulatory signal leads to a prolonged survival of allograft in transplantation. Especially, targeting the CD40–CD40L (CD154) interaction remains among the most promising approach to induced donor-specific transplant tolerance [2]. Although anergy [2] and deletion [3] have been indicated to participate in the transplant tolerance induced by blocking CD40–CD40L costimulation, the actual mechanism remains to be elucidated.

IFN-γ is a pluripotent proinflammatory mediator that so dominates the tone and character of an immune response that responses involving IFN-γ production are collectively known as Th1 responses [4]. There is an overwhelming body of literature showed increased IFN-γ expression in acutely rejected allografts, and diminished IFN-γ expression in long-term surviving transplants [5–7]. Furthermore, many studies reported that IFN-γ was both necessary and sufficient for graft rejection [8,9]. However, emerging evidence from animal transplant models as well as analysis of human organ transplantation is beginning to indicate that our current thinking regarding the role of IFN-γ as an exclusively proinflammatory cytokine does not accurately reflect the complex properties of IFN-γ. For example, patients with good graft outcome showed higher IFN-γ plasma levels [10,11]. More importantly, IFN-γ was found to be required for the induction of transplant tolerance by some studies [12,13].

Abbreviations: CD40L, CD40 ligand; CTL, cytotoxic T lymphocyte; E/T ratios, effector to target ratios; FACS, fluorescence-activated cell sorter; GVHD, graft versus host disease; i.p., intraperitoneally; i.v., intravenously; mAb, monoclonal antibody; MACS, magnetic-activated cell sorter; MLR, Mixed lymphocyte reaction; WT, Wild type.

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How IFN-γ might play a role in the induction or maintenance of unresponsiveness to nonself antigens has been elucidated more recently with the establishment of links to $CD4^+CD25^+$ regulatory T cells. The IFN-γ may be necessary for the survival, expansion and functional properties of these regulatory T cells [14–16]. However, the influence of IFN-γ on $CD4^+CD25^+$ regulatory T cells in the transplant long-surviving induced by blocking CD40–CD40L costimulation is far from clear.

Taken together, studies which examined the role of IFN-γ in transplantation and its mechanisms have yielded conflicting results. The purpose of this study was to investigate these issues in the transplant long-surviving induced by blocking CD40–CD40L costimulation. Our data provide evidence that IFN-γ is not essential for acute rejection although its expression increased in transplant rejection. Conversely, IFN-γ is required for long-term survival induced by blocking CD40–CD40L costimulation through inhibiting the function of T cells and increasing suppressive capability of $CD4^+CD25^+$ regulatory T cells.

2. Objectives

The aim of this study was to investigate the role of IFN-γ in the transplant long-surviving induced by blocking CD40–CD40L costimulation and its mechanisms.

3. Materials and methods

3.1. Reagents

FITC-conjugated anti-mouse CD4 (L3T4) monoclonal antibody (mAb), PE-conjugated anti-mouse CD25 mAb (7D4), unconjugated anti-CD16/CD32 (2.4G2; Fc block) and purified anti-mouse CD40L mAb (MR-1) were purchased from BD Biosciences (San Jose, CA, USA). Rat IgG mAb (control antibody) was purchased from Sigma (Louis, USA). Anti-CD4 and anti-CD8 microbeads were purchased from Miltenyi Biotec (Auburn, CA, USA). Isogen was purchased from Nippon Gene (Toyama, Japan). Random primer was purchased from Toyobo (Osaka, Japan). SuperscriptII reverse transcriptase was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Foxp3, IL-10 and CTLA-4 20×Mix, and 18s rRNA (20×), Universal PCR Master Mix (No AmpErase UNG) were purchased from Applied Biosystems (New Jersey, USA). RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 1 mM MEM sodium pyruvate, 0.1 mM MEM non-essential amino acids and 5×10^{-5} M 2-ME was used as culture medium. $Na_2^{51}CrO_3$ and thymidine were purchased from Amersham Biosciences UK limited (Buckinghamshire, UK).

3.2. Heart transplantation

Wild type (WT) BALB/c ($H\text{-}2^d$) or WT C57BL6/J (B6, $H\text{-}2^b$) mice were used as donors and WT ($IFN\text{-}\gamma^{+/+}$) or IFN-γ deficient ($IFN\text{-}\gamma^{-/-}$) B6 mice were used as recipients. Intra-abdominal heterotopic heart transplantation was performed according to the technique previously described by Corry et al. [17]. Graft function was monitored daily by trans-abdominal palpation. Rejection was defined as a complete cessation of palpable beat and was confirmed by direct visualization after laparotomy. All mice were housed in specific pathogen-free condition and underwent the experimental procedure at 6–8 weeks in accordance with the protocols for animal experiments approved by the Animal Care Committee of China Medical University.

3.3. In vivo treatment of anti-CD40L mAb (MR-1)

For long-surviving induction, the recipients were intravenously (i.v.) injected with 0.25 mg/mouse of anti-CD40L mAb (MR-1) on day 0 and intraperitoneally (i.p.) injected on days 2 and 4 posttransplant.

3.4. Sorting of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells

Spleens were obtained at indicated time points. After incubation with 2.4G2, splenocytes were sequentially stained with anti-mouse CD4 mAb and anti-mouse CD25 mAb (7D4). Then $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were sorted using a fluorescence-activated cell sorter (FACS Calibur, Becton Dickinson, San Jose, CA, USA). The purity of these cells was >98%.

3.5. Quantitative RT-PCR analysis (real-time RT-PCR)

For analysis of IFN-γ expression, grafts and spleens were harvested from syngeneic recipients on day 100 posttransplant or allogeneic recipients (WT B6 mice) without treatment on day 10 or with MR-1 treatment on day 100 posttransplant. For analysis of FoxP3 expression, grafts and spleens were harvested from syngeneic recipients on day 100 posttransplant or allogeneic recipients (WT B6 mice and $IFN\text{-}\gamma^{-/-}$ B6 mice) with treatment of MR-1 on day 20 posttransplant. For analysis of FoxP3, IL-10 and CTLA-4 of $CD4^+CD25^+$ T cells, they were further sorted from spleens using FACS as described above. FoxP3, IL-10 and CTLA-4 mRNA expressions of $CD4^+CD25^-$ T cells from naive B6 mice were used as negative controls. Total RNA of grafts, spleens and $CD4^+CD25^+$ T cells, $CD4^+CD25^-$ T cells was extracted using isogen according to the manufacturer's instruction. Reverse transcribed cDNA was obtained by the addition of random primer and super Script II reverse transcriptase to RNA according to the manufacturer's instruction. After cDNA synthesis, quantity of mRNA for IFN-γ, FoxP3, IL-10 and CTLA-4 were examined by ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA), normalized with 18s expressions. The 18s-normalized value from syngeneic gene expressions was designated as the calibrator (syngeneic gene expressions were calculated as 1), final relative quantity of mRNA was expressed relative to the calibrator. Formula is as follows: $\text{mRNA expression} = 2^{-\{(\text{determined cycles} - 18\text{s cycles}) - \text{syngeneic cycles}\}}$.

3.6. Mixed lymphocyte reaction (MLR) and cytotoxic T lymphocyte (CTL) assay

Twenty days after heart transplantation, splenocytes was obtained from syngeneic WT ($IFN\text{-}\gamma^{+/+}$) B6 recipients or allogeneic ($IFN\text{-}\gamma^{+/+}$ or $IFN\text{-}\gamma^{-/-}$) B6 recipients with MR-1 treatment. $CD4^+$ and $CD8^+$ T cells were purified with magnetic-activated cell sorter (MACS) (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. The resulting purity was >95% in all experiments. 1×10^5/well $CD4^+$ T cells were cocultured with 2×10^5/well 35 Gy-irradiated splenocytes from BALB/c mice at 37 °C, 5% CO_2 for 4 days, and proliferative responses were measured by [^{3}H] thymidine incorporation of last 8 h culture. $CD8^+$ T cells were cocultured with 35 Gy-irradiated BALB/c splenocytes at 1:2 of cell number ratio in the presence of IL-2 (50 U/mL). Five days later, live cells were collected and cytotoxic activity against P815 cells ($H\text{-}2^d$, 1×10^4/well) at 20:1 of effector to target (E/T) ratios was assessed by a standard ^{51}Cr release assay. Specific lysis was calculated as follows: the percentage of specific ^{51}Cr release = {(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)}×100.

3.7. The suppressive capability of $CD4^+CD25^+$ regulatory T cells

$CD4^+CD25^-$ T cells or $CD4^+CD25^+$ T cells were sorted from naive WT B6 mice or allogeneic recipients ($IFN\text{-}\gamma^{+/+}$ and $IFN\text{-}\gamma^{-/-}$ B6 mice) with MR-1 treatment on day 20 posttransplant. $CD4^+CD25^-$ T cells from naïve WT B6 mice (1×10^5/well) were cocultured with 35 Gy-irradiated splenocytes from naive WT BALB/c mice (2×10^5/well). $CD4^+CD25^+$ T cells from $IFN\text{-}\gamma^{+/+}$ or $IFN\text{-}\gamma^{-/-}$ allogeneic recipients were added at a concentration of 0.5 and 1×10^5/well. The proliferative responses of $CD4^+CD25^-$ T cells were measured after 4 days by assessing the amount of thymidine incorporated in the last 8 h of culture.