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Mixed chimerism induction influences cytokine release from chimeric mice cells

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ABSTRACT

Interest in mixed chimerism has evolved from its role in the induction of alloantigen tolerance. However, its precise impact on the host organism remains to be elucidated. In the present work, we analyzed cytokine secretion from chimeric mice cells to assess the influence of different mixed chimerism induction protocols on immune system function in recipient mice. To our knowledge, there have been no reports on using this parameter for the optimization of the mixed chimerism induction method.

B6.SJL-*PtprcaPep3b* or C57BL/6J mice were used as recipients and Balb/c as donors. We utilized four protocols which consisted of: 3 Gy total body irradiation (day −1), the injection of 20–30 × 10^6 bone marrow cells (day 0), and a combination of CD40L (days 0 and 4), CD8 (day −2), and NK1.1 (day −3) blocking antibodies and cyclophosphamide (175 mg/kg — day 2). The concentrations of cytokines (IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF) were evaluated in the supernatants of unstimulated or phytohemagglutinin-stimulated chimeric spleen, bone marrow and peripheral blood cells in the 8th week of experiment. The induction of tolerance to Balb/c mouse antigens was initially tested in chimeric mice by assessing the presence of Vβ5 and Vβ11 TCR-expressing lymphocytes.

The cytokine production was considerably increased, especially in chimeric mice treated by cyclophosphamide. Also the mixed chimerism itself seems to affect IFN-γ, IL-2, IL-4, IL-6, IL-17A, and TNF secretion. Using the optimized induction protocol, we established that chimeric mice cells secreted lower IFN-γ, IL-2, IL-4 and higher IL-6, IL-17A, and TNF levels as compared to control animals. We found that both donor and recipient cells markedly participated in the cytokine production.

In conclusion, our optimization study based on cytokine assessment contributes to establishing an effective protocol of mixed chimerism induction with no cyclophosphamide use and better understanding of the influence of this phenomenon on the recipient organism.

1. Introduction

Transplantation is an irreplaceable treatment method for patients suffering from renal, heart, or liver insufficiency. Unfortunately, there are serious limitations to this treatment, especially the difficulty in obtaining an appropriate donor–recipient match. Consequently, the only option that remains is post-transplantation immunosuppressive therapy, of which effectiveness is unsatisfactory and limits the long-term survival of transplanted organs. Moreover, this chronic immunosuppression is responsible for the side effects such as neoplasms, infections, and metabolic disorders [1,2].

The aforementioned problems could be resolved by the induction of alloantigen tolerance. One of the most promising methods that have been suggested to establish this tolerance is mixed chimerism. This phenomenon is the coexistence of hematopoietic cells from both the donor and the recipient, which could be achieved by the transplantation of hematopoietic stem cells into a properly prepared recipient [2–4]. Mixed chimerism has been broadly studied over recent years, and animal studies have yielded strong evidence for its effectiveness. The tolerance established by mixed chimerism occurs centrally and peripherally. In the thymus of the recipient, transplanted antigen-presenting cells present antigens from the donor to developing T lymphocytes. The lymphocytes that react to the donor antigens are eliminated through negative selection. In the periphery, various populations of regulatory cells play a major role in the maintenance of tolerance [1–4].

The value of the mixed chimerism phenomenon has yet to be proven in human transplantation trials, and an appropriate induction protocol needs to be established. A niche must be created in the bone marrow to facilitate the engraftment of the transplanted hematopoietic cells, and the recipient's immune cells need to be eliminated to prevent graft rejection. The protocol cannot be toxic to the recipient, but it should enable the development of a level of mixed chimerism that is

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high enough to induce tolerance. Current strategies require total body irradiation (TBI) and immunosuppression, factors that are known to cause several side effects. To date, many methods of mixed chimerism induction have been tested, and their toxicity has been reduced [1,5,6]. The dose of TBI has been decreased from 9 gray (Gy) in early studies to 3 Gy, and the conventional immunosuppression dose has been lowered with the introduction of specific anti-lymphocyte antibodies. Although protocols using 3 Gy of TBI have been shown to be less toxic and well tolerated by recipients [7], the precise impact of these "reduced toxicity" protocols on various functions of the recipient organism has not been fully recognized.

2. Objective

Recently, the influence of various protocols of mixed chimerism induction on mouse stem/progenitor cells has been examined in our laboratory[8]. We established four induction protocols that consist of 3 Gy TBI, various combinations of anti-CD40L, anti-CD8, and anti-NK antibodies, and cyclophosphamide (CP) as an immunosuppressant. In this work, we sought to more extensively examine the influence of these protocols on recipient mice. For this reason at the beginning, we assessed the cytokine production capability of spleen cells obtained from chimeric mice in the 8th week of study by analyzing IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF levels in the supernatants of cells that were either unstimulated or stimulated with phytohemagglutinin (PHA). In addition, we initially analyzed the level of tolerance to donor antigens in these four groups of chimeric animals by assessing the Vβ5 and Vβ11 TCR lymphocyte proportions in the peripheral blood and spleen.

In the further part of our study, we selected optimal protocol for mixed chimerism induction (3 Gy, anti-CD8 and anti-CD40Lx2) and compared the cytokine production by chimeric spleen, bone marrow and peripheral blood cells in the 8th week of study with cells of control animals. Moreover, we tried to find out how recipient and donor cells participated in the production of the cytokines.

3. Materials and methods

3.1. Mice

12–16-Week old male Balb/c (H-2K^{d}, I-E^{+}) as donors and B6.SJL-*PtprcaPep3b* (H-2K^{b}, I-E^{-}) as recipient mice purchased from The Jackson Laboratory (Bar Harbor, ME, USA) were used in the first part of experiment. The mice differ in isoforms of the CD45 antigen, CD45.1 (B6.SJL-*PtprcaPep3b*) and CD45.2 (Balb/c), that facilitated an analysis of chimerism by flow cytometry [6,8,9]. C57BL/6J mice (CD45.2, H-2K^{b}, I-E^{-}) were used as recipients in the second part of study. C57BL/6J is a background strain for B6.SJL-*PtprcaPep3b* and the CD45 antigen is the only modification between these mice. In this setting, the difference between the recipient and donor mice is that they vary in MHC class I (H-2) and II (I-E) antigens, that makes them ideal for tolerance studies. They were kept under specific pathogen-free conditions in the BIO.S.A. Blower Unit Vent II (Ehret, Labor- and Pharmatechnik, Germany). All experiments were approved by the Local Ethical Committee and were performed in accordance with the guidelines of laboratory animal care.

3.2. Chimerism induction and analysis

As we have previously described, the conditioning was based on the total body irradiation of B6.SJL-*PtprcaPep3b* mice with gamma rays (60^{Co}) in 3 Gy dose on day −1st of the experiment [8]. Animals were injected intraperitoneally (i.p.) with blocking antibodies against mouse CD40L (anti-CD154 mAb, clone MR1, Becton Dickinson, USA) to block costimulation signal for lymphocytes, and against CD8a (anti-CD8a mAb, clone 53-6.7, Becton Dickinson, USA), and NK1.1 (anti-NK1.1 mAb, clone PK136, Becton Dickinson, USA) to remove cells that could be able to reject transplanted hematopoietic cells. The antibodies were applied in two combinations as indicated in Table 1 for each group of animals (G1–G4) [8,9]. Additionally, cyclophosphamide (Endoxan, Baxter Oncology GmbH, Germany) in dose of 175 mg/kg was given i.p. to mice of first two groups on the 2nd day. A total of 20–30 × 10^{6} of unseparated Balb/c bone marrow cells were given intravenously on day 0 (in 0.2 mL phosphate-buffered saline). The cells were harvested as reported earlier [6,8,9].

Presence of mixed chimerism in the peripheral blood leucocytes was assessed using flow cytometry (FACSAria, Becton-Dickinson, USA) at the 1st, 2nd, 3rd, 4th, 6th and 8th weeks after bone marrow cell infusion based on analysis of CD45.1 (B6.SJL-*PtprcaPep3b*) and CD45.2 (Balb/c) antigens expression as previously described [8,9].

In the extended studies the G3 induction protocol (3 Gy, anti-CD8, and anti-CD40Lx2) was applied on the C57BL/6J mice as recipients (the G3A — additional group). The proportion of mixed chimerism was analyzed by flow cytometry (FACSAria, Becton-Dickinson, USA) in the same time intervals as above. Peripheral blood leukocytes were stained with biotin-conjugated anti-H-2K^{b} (clone AF6-88.5) I^{o} antibody and peridinin-chlorophyll-protein complex-cyanine 5.5 (PerCP-Cy5.5)-conjugated streptavidin as II^{o} antibody and with FITC-conjugated anti-H-2K^{d} (clone SF1-1.1).

3.3. Assessment of cytokine production

A total of 2 × 10^{6} spleen, bone marrow and peripheral blood cells were obtained in the 8th week of the study. After red blood cell lysis with ammonium chloride solution, the cells were incubated in 0.5 mL of Iscove's medium with 10% bovine calf serum for 48 h without/with 20 μL phytohemagglutinin (PHA) [10]. Cell culture supernatants were collected and stored in a −80 °C freezer. The IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF releases were analyzed using BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (FACScan, BD, USA) according to manufacturer instruction [10,11].

The first part of the study was performed on spleen cells. Besides the study groups (G1–G4), we included diverse control groups in the experiment: mice without any treatment (C), mice that were given just two sets of antibodies (CA1 and CA2), mice exposed exclusively to cyclophosphamide (CCP), and mice exposed to 3 Gy only (CTBI). The control animals have not received hematopoietic cells (Table 1). In the second

Table 1
The conditioning strategies.

Group	Dose of radiation	Dose of cyclophosphamide	Dose of antibodies
G1	3 Gy	175 mg/kg	Anti-CD8 0.5 mg i.p. ("−2" day) Anti-CD40L 2 × 0.5 mg i.p. ("0" and "4" days)
G2	3 Gy	175 mg/kg	Anti-NK1.1 0.5 mg i.p. ("−3" day) Anti-CD8 0.5 mg i.p. ("−2" day) Anti-CD40L 0.5 mg i.p. ("0" day)
G3	3 Gy	(–)	Anti-CD8 0.5 mg i.p. ("−2" day) Anti-CD40L 2 × 0.5 mg i.p. ("0" and "4" days)
G4	3 Gy	(–)	Anti-NK1.1 0.5 mg i.p. ("−3" day) Anti-CD8 0.5 mg i.p. ("−2" day) Anti-CD40L 0.5 mg i.p. ("0" day)
CA1	(–)	(–)	Anti-CD8 0.5 mg i.p. ("−2" day) Anti-CD40L 2 × 0.5 mg i.p. ("0" and "4" days)
CA2	(–)	(–)	Anti-NK1.1 0.5 mg i.p. ("−3" day) Anti-CD8 0.5 mg i.p. ("−2" day) Anti-CD40L 0.5 mg i.p. ("0" day)
CCP	(–)	175 mg/kg	(–)
CTBI	3 Gy	(–)	(–)
C	(–)	(–)	Remark: mice from CA1 to CTBI and C groups did not receive Balb C bone marrow cells(–)