Brief report

Blockade of CD154-CD40 pathway induces interleukin-10 dependent T regulatory type 1 like cells

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Several subsets of T-regulatory (Tr) cells with distinct phenotypes and distinct mechanisms of action have been identified. These include Tr type 1 (Tr1) cells; Th3 cells, which primarily secrete transforming growth factor (TGF)-β; and CD4⁺CD25⁻ T cells, which inhibit immune responses through cell to cell contact. It has been shown that CD4⁺CD25⁻ immunoregulatory T cells induced by the blockade of CD154-CD40 pathway are tolerant to alloantigen, resulting in secondary mixed lymphocyte reaction (MLR) hyporesponsiveness in vitro and tolerance to alloantigen in vivo. Previous studies mainly paid attention to CD4⁺CD25⁺ immunoregulatory T cells induced by CD154-CD40 blockade, but it was unclear whether CD154-CD40 blockade might induce Tr1 or Tr1-like cells.

In this study, we investigated the possibility of Tr1 cells induced in vitro by the blockade of CD154-CD40 pathway and the in vivo effect on prolonging allograft survival by transferring Tr1 cells.

METHODS

Mice
Male BLAB/c (H-2ᵇ) and C3H/HeJ (H-2ᵇ) mice, aged 8 weeks to 12 weeks, were purchased from the Experimental Animal Centre of Chinese Academy of Science (Shanghai, China) and were maintained in a dedicated, pathogen free mouse facility.

Cell purification
BALB/c spleen was harvested and single cell suspension was prepared. After red cell lysis, the cells were suspended in PBS/1% BSA and isolated CD4⁺ T cells via Mouse CD4⁺ T Cell Isolation Kit (R&D Systems, USA) according to the manufacturer's instructions. Cultures of BALB/c responder CD4⁺ T cells mixed with 30 Gy γ-irradiated C3H/HeJ stimulators at a 1:1 ratio at a final concentration of 0.5 × 10⁶ cells per millilitre were plated in 24-well plates in complete Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Anti-CD154 mAb 50 µg/ml (clone MR1, BD PharMingen, USA) was added to MLR cultures and incubated at 37°C and 5% CO₂. On day 7 of culture, cells were harvested and washed to remove Ab and cytokines. Anti-CD154 induced CD4⁺ cells were depleted of CD25⁺ cells by incubation with anti-CD25 mAb (Clone 3C7; BD PharMingen) and sheep anti-rat Dynal beads, and were 95% pure as analysed by FACS Vantage (Becton Dickinson, USA).

Cytokine production
Anti-CD154 induced CD4⁺CD25⁻ cells (1 × 10⁶ /ml) were stimulated with phorbol-12-myristate-13-acetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml). The amounts of IL-2, IL-4, IL-10 and TGF-β1 in supernatants collected at 48 hours or 72 hours of culturing were measured by ELISA (R&D System, USA) according to the manufacturer's instructions. For analysis of intracellular IL-10 production, induced CD4⁺CD25⁻ cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 6 hours. Two µmol/L monensin (Sigma-Aldrich, USA) was added for the last 5 hours of culturing. Cells were collected, fixed, and saponine permeabilized (fix/perm solution; BD PharMingen) and stained with PE-conjugated specific anti-IL-10 Ab.

Proliferative assay
For determination of proliferative effect, 2 × 10⁵ anti-CD154 induced CD4⁺CD25⁻ cells and 2 × 10⁵ γ-irradiated

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stimulator were cultured with or without 10 U/ml IL-2 (R & D System, USA). After 5 day incubation at 37°C in a humidified 5% CO₂ atmosphere, cells were pulsed with 37 kBq/well §H-TdR, and proliferation was measured during the last 12 hours. The cells were harvested onto glass filter paper and analysed by a scintillation counter (Beckman LS6500, USA).

**Transwell experiments**

Transwell experiments were performed in 24-well plates. Naïve CD4⁺ T cells from BALB/c mice (1 × 10⁶) were stimulated with irradiated C3H/HeJ splenic stimulators (1 × 10⁶) at the 1:1 ratio in the bottom compartment of a transwell system for 3 days. These naive T cells were cocultured with anti-CD154 induced CD4⁺CD25⁻ cells (2 × 10⁵) contained in the upper transwell chambers (1 µm; BD Biosciences, USA). After 3 days the basket was removed and cocultured T cells were transferred to 96-well plates (1 × 10⁵ cells per well) in triplicates. §H-TdR incorporation was measured during the last 12 hours and analysed by a scintillation counter.

**Transplantation and treatment protocols**

All recipient mice were irradiated at a dose of 3.0 Gy to adapt to [this use of 'accommodate' is doubtful as the word is given as obsolete by Oxford] the adoptively transferred cells. According to the method described by Hancock,⁴ heterotopic abdominal cardiac allografting is done using C3H/HeJ donors and 3.0 Gy γ-irradiated BALB/c recipients. Anti-CD154 induced CD4⁺CD25⁻ cells were intravenously injected into the recipient mice immediately after transplantation. Rapamycin (Wyeth Pharmaceutical Company, USA) was given to recipient mice (1 mg · d⁻¹ · kg⁻¹, beginning on day 1 after transplantation), and rapamycin therapy was stopped on day 14 after transplantation. Mice were divided into five groups for allograft survival: untreated; rapamycin therapy; injected with anti-CD154 induced CD4⁺CD25⁻ cells (1 × 10⁷); injected with induced CD4⁺CD25⁻ cells (1 × 10⁷) given rapamycin therapy; injected with the same number (1 × 10⁷) of control CD4⁺CD25⁻ cells (cultured without antibody) and given rapamycin therapy. Furthermore, additional recipients from each group were killed on day 14 and cardiac allografts were harvested for pathological analysis.

**Graft pathology**

On day 14 after cardiac transplantation, cardiac allografts were harvested, sliced and then preserved in 10% buffered neutral formalin. Tissue samples were embedded in paraffin, cut into 5 µm sections, and then assessed by routine staining with haematoxylin and eosin for myocyte damage and infiltrated cells.

**Statistical analysis**

Graft survival in different experimental groups was compared using the log rank test. For other data, group comparisons were made by Student’s t test. P values less than 0.01 were considered statistically significant. All statistical calculations were performed by the SPSS 10.0 software.

**RESULTS**

**Anti-CD154 blocking induced anergic and high IL-10-producing CD4⁺CD25⁻ T cells**

Anti-CD154 induced CD4⁺CD25⁻ cells separated from anti-CD154 induced CD4⁺ cells by Dynal beads were anergic and could not proliferate following alloantigen stimulation [(5865 ± 703) vs (6199 ± 1311) count per minute, P = 0.562]. Compared with control CD4⁺CD25⁻ cells (cultured without antibody), these cells had low proliferative responses by antigenic rechallenge in the presence of IL-2 [(4.36 ± 0.81) folds vs (1.67 ± 0.24) folds, P < 0.01].

We analysed the supernatant of anti-CD154 induced CD4⁺CD25⁻ cells for different cytokines after PMA and ionomycin stimulation. It was found that the levels of IL-10 produced by induced CD4⁺CD25⁻ cells were higher than those of control CD4⁺CD25⁻ cells [(1313 ± 245) pg/ml vs (212 ± 45.6) pg/ml, P<0.01], whereas the levels of IL-2 [(10.6 ± 7.6) pg/ml vs (424 ± 73.7) pg/ml, P<0.01] and IL-4 [(18.8 ± 9.5) pg/ml vs (219.6 ± 52.9) pg/ml, P<0.01] were lower. In addition, increased production of TGF-β1 was not observed in the cultures [(511 ± 73)pg/ml vs (543 ± 102) pg/ml, P=0.382]. Intracellular FACS revealed that there was an increased number of IL-10 producing cells observed in induced CD4⁺CD25⁻ cells [(23.75 ± 3.62)% vs (8.22 ± 2.09)%, P<0.01].

**Anti-CD154 induced CD4⁺CD25⁻ cells suppress proliferation of MLR in an IL-10 dependent manner**

To determine if IL-10 was implicated in the regulatory
activity of anti-CD154 induced CD4\(^+\)CD25\(^-\) cells or if cell to cell contact was a requirement for the downregulation, transwell culture was used. It was found that the diffusion of IL-10 from anti-CD154 induced CD4\(^+\)CD25\(^-\) cells in the transwell had significant effect on the proliferation of the naïve cells in response to alloantigen, which indicated cell to cell contact was not essential for inhibition of a naïve alloresponse. Furthermore, addition of neutralizing anti-IL-10 antibodies to the transwell systems augmented the proliferative responses of naïve CD4\(^+\) T cells in the presence of Tr1 cells (Fig. 1). Therefore, the high IL-10 production of anti-CD154 induced CD4\(^+\)CD25\(^-\) cells leads to secondary suppression of other T cells.

Anti-CD154 induced CD4\(^+\)CD25\(^-\) cells transfer and short-term rapamycin therapy prolongs allograft survival

To test whether anti-CD154 induced CD4\(^+\)CD25\(^-\) cells could prevent allograft rejection in vivo, these cells were injected intravenously into irradiated BALB/c mice immediately after transplantation of C3H/HeJ cardiac allograft. Untreated irradiated animals showed acute rejection of the allografts with a median survival time (MST) of 7 days. Recipients transferred with anti-CD154 induced CD4\(^+\)CD25\(^-\) cells alone or rapamycin-therapy showed MST of 15 and 16 days, respectively. It was found that anti-CD154 induced CD4\(^+\)CD25\(^-\) cells transfer and short-term rapamycin therapy prolonged allograft survival significantly (MST 45 days, \(P<0.01\)). Allograft rejection in the mice injected with control CD4\(^+\)CD25\(^-\) cells (cultured without antibody) and given rapamycin therapy (MST 10 days) occurred earlier than in those mice with rapamycin therapy alone. It meant that control cells were primed T cells which accelerated rejection. Cardiac allografts were harvested on day 14 after cardiac transplantation for histopathological evaluation. Combined treatment with induced CD4\(^+\)CD25\(^-\) cells injection and rapamycin therapy largely preserved cardiac architecture and markedly depressed mononuclear cell (MNC) infiltration. In contrast, allograft from other groups showed dense areas of MNC infiltration, myocyte necrosis, severe interstitial edema and vasculitis (Fig. 2).

**DISCUSSION**

Previous reports have noted that CD4\(^+\)CD25\(^-\) T cells via CD154-CD40 blockade can act as potent Immuno-
Fig. 2. Anti-CD154 induced CD4^{+}CD25^{-} cells transfer and rapamycin therapy preserve graft histology. Comparison of intragraft events on day 14 after transplantation showed that other therapies had no effect on graft leukocyte recruitment and myocyte injury, whereas anti-CD154 induced CD4^{+}CD25^{-} cells transfer and rapamycin therapy preserved essentially normal cardiac morphology and lessened leukocyte recruitment. 

A: Rapamycin therapy. B: Anti-CD154 induced CD4^{+}CD25^{-} cells transfer + rapamycin therapy (HE, original magnifications ×400).

regulatory cells in vitro and in vivo. This report is the first demonstration of anergic and high IL-10-producing CD4^{+}CD25^{-} T cells by anti-CD154 blocking. First, these CD4^{+}CD25^{-} T cells resemble CD4^{+} T cells with low proliferative capacity generated in the presence of IL-10, which have been termed Tr1. Classical Tr1 cells show a state of functional unresponsiveness to stimulation and a seriously impaired IL-2 production, i.e. anergy. In our experiments, anti-CD154 induced CD4^{+}CD25^{-} T cells were also anergic. Furthermore, the presence of mAbs in the primary MLR might explain the hyporesponsiveness. To exclude this possibility, cultured cells were harvested, washed and recuperated in fresh medium without mAbs. Hyporesponsiveness was not due to cell death because antigen stimulation in the presence of exogenous IL-2 led to a response. Thus, anti-CD154 blocking, not cell death, induces anergic CD4^{+}CD25^{-} T cells. Second, anti-CD154 induced CD4^{+}CD25^{-} T cells produced high level of IL-10 and low levels of IL-2 and IL-4, which was also similar to classical Tr1 cells. Because of a lack of cellular surface or intracellular markers to identify Tr1 cells until now, we prefer to describe these anti-CD154 induced CD4^{+}CD25^{-} T cells as Tr1-like cells.

To understand whether high level of IL-10 produced by anti-CD154 induced Tr1-like cells to have an effect on T cell proliferation, we used the transwell culturing system to allow the free exchange of IL-10 and prevent cell contact. It was demonstrated that Tr1-like cells significantly suppressed the proliferation of syngenic CD4^{+} T cells, and addition of anti-IL-10 antibody eliminated the suppressive effects of Tr1-like cells. The results indicate that IL-10 is essential for the suppressive function of anti-CD154 induced Tr1-like cells. This is consistent with other Tr1 cells experiments in which IL-10 is also the pivotal immuno-suppressive cytokine.

In our experiments, Tr1-like cells transfer alone only slightly prolonged cardiac allograft survival because Tr1-like cells might not be sufficient to block the expansion of effector T cells in vivo after transplantation. Postoperative IL-2 produced at the transplant site can markedly stimulate the proliferation of effector T cells, but only weakly activate Tr1-like cells due to their low proliferative capacity. Thus, this imbalance eventually leads to the majority of mice undergoing graft rejection after adoptive transfer of Tr1-like cells. It might be feasible to inhibit the effector phase of immune response and prolong allograft survival by a combination of Tr1 cells with an immuno-suppressive drug. Rapamycin is an immuno-suppressive drug with promising immunomodulatory properties to prevent expansion of activated T cells. Moreover, rapamycin does not interfere with tolerance induction. For this purpose, we combined transferring Tr1-like cells with rapamycin therapy to prolong allograft survival. It was found that such a combined therapy manifested a synergistic effect on prolonging allograft survival and reducing rejection. The exact mechanism of such synergistic effect needs to be elucidated further.

In conclusion, the results of the present study indicate that the blockade of CD154-CD40 pathway induces interleukin-10 dependent T regulatory type 1 like cells and these cells transfer combined with rapamycin therapy prolongs allograft survival significantly. This procedure might be clinically useful for prolonging allograft survival if optimal protocols are developed.

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