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Alloantigen-specific CD4$^+$ regulatory T cells induced *in vivo* by ultraviolet irradiation after alloantigen immunization require interleukin-10 for their induction and activation, and flexibly mediate bystander immunosuppression of allograft rejection ☆

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ABSTRACT

Ultraviolet (UV) irradiation prior to antigen immunization is employed to induce antigen-specific regulatory T cells (Tregs). UV-induced Tregs demonstrate unique bystander suppression, although antigen-specific activation is required initially. We previously reported the phenotype of alloantigen-specific transferable Tregs induced by UV-B irradiation after immunization was the same as T regulatory type 1-like CD4$^+$ T cells, with antigen-specific interleukin (IL)-10 production. Here, by using semi-allogeneic transplantation models *in vivo*, we investigated the role of IL-10 in the induction and activation of these Tregs, and the possibility of bystander suppression of third-party allograft rejection. Naïve mice (H-2^b) were immunized with alloantigen (H-2$^{b/d}$), and received UV-B irradiation (40 kJ/m^2) 1 week later. Four weeks afterwards, splenic CD4$^+$ T cells were purified from the UV-irradiated immunized mice, and were transferred into naïve mice (H-2^b). Allografts expressing the same alloantigen as T-cell donors were immunized against (H-2$^{b/d}$) or an irrelevant alloantigen (H-2$^{b/k}$) were transplanted to CD4$^+$ T-cell-transferred mice, and an alloantigen-specific prolongation of allograft survival observed. Experiments where IL-10 was neutralized by monoclonal antibody in the induction or effector phase revealed that IL-10 is critical, not only for induction but also for immunosuppressive function of CD4$^+$ Tregs induced by UV irradiation after alloantigen immunization. Third-party allografts (H-2$^{d/k}$) were transplanted to CD4$^+$ T-cell-transferred mice, and graft survival was also prolonged. Even a graft only partially compatible with immunized alloantigen worked well *in vivo* to activate CD4$^+$ Tregs induced by UV irradiation after alloantigen immunization, which resulted in the bystander suppression of third-party allograft rejection.

1. Introduction

Ultraviolet (UV) light of the mid-wave range, UV-B, is an important environmental factor that affects human health [1]. In addition to UV irradiation representing one of the major environmental threats to human heath as a carcinogen [2], it also impairs immune responses to oncologic and infectious antigens [3,4]. Paradoxically, UV-induced immunosuppression may have therapeutic potential [5–8]. UV-induced immunosuppression is mediated via antigen-specific regulatory T cells (Tregs) [9,10], which can exert immunosuppressive functions upon adoptive transfer [11]. Immunosuppressants have revolutionized clinical transplantation, but also cause pan-immunosuppression [12]. Infectious complications are the highest cause of death owing to complications for transplant recipients [13]. Thus, after organ transplantation, when deciding immunosuppressant dosage, clinicians face a dilemma between infectious morbidity and graft rejection. Therefore, alloantigen-specific immunosuppression is an ideal therapy for transplant recipients [14,15].

UV irradiation alters antigen-presenting cell (APC) function [16], with UV-induced DNA damage being recognized as the major molecular trigger for photoimmunosuppression [9,10,17]. Langerhans cells (LCs)

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are regarded as the most important APC in the epidermis [18–20], and recently, the functional role of the LC was redefined. Damaged but still viable LCs will present antigens in a nonprofessional manner, which will induce not effector T cells, but Tregs [10]. Currently, the concept that LCs, mast cells, and natural killer T (NKT) cells may serve in an unconventional manner is accepted in the photobiology and immunology communities [9,10]. This phenomenon may explain the theory that antigen immunization must follow UV-B irradiation and not *vice versa* [21–23]. Therefore, almost all researchers focused on antigen-specific Tregs induced by high-dose UV-B irradiation before antigen immunization [21–23], because it was considered that functional modulation of APCs by UV irradiation is required for the induction of antigen-specific immunosuppression [16]. However, our previous studies demonstrated that mice exposed to UV irradiation at 1 week after immunization exhibited reduced Th1- and Th2-driven immune responses, and had prolonged allograft survival in an antigen-specific manner [24–29]. UV irradiation of the graft recipient several days before transplantation is impractical, because predicting when a donor organ will be available is impossible [21]. Therefore, a procedure of alloantigen immunization prior to UV irradiation that induces an alloantigen-specific immunosuppressive status in patients receiving transplantation therapies represents a more practical approach [21].

Antigen-specific immunosuppression is mediated by regulatory T-cell populations that included $CD3^+$ $DX5^+$ NKT cells, $CD4^+$ $CD25^+$ T cells co-expressing cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor (TNF)-related protein and neuropilin-1, and $CD4^+$ $Foxp3^+$ T cells [30–37]. We have previously demonstrated that alloantigen-specific immunosuppression induced by UV irradiation after immunization depends not on interleukin (IL)-4, IL-5, IL-13, or transforming growth factor (TGF)-β, but on IL-10 [25–29]. Furthermore, we have also demonstrated that the phenotype of transferable Tregs induced by UV irradiation after immunization is a T regulatory type 1 (Tr1)-like $CD4^+$ T-cell phenotype [25–29], because these $CD4^+$ Tregs had a cytokine profile with high IL-10 production, low levels of IL-2 and no IL-4, resembling a Tr1 cytokine pattern [38,39].

Whether IL-10 is crucial for the induction and effector phases of UV-induced Tregs or not is controversial [33,40–43]. Additionally, UV-induced Tregs demonstrate a unique behavior, so-called 'bystander suppression'. The antigen specificity thus appears to be restricted to the activation of UV-induced Tregs [33,44]. However, once activated suppression is nonspecific [45].

2. Hypothesis

Here, in our delayed-type hypersensitivity (DTH) model *in vivo* accompanied by UV irradiation after alloantigen immunization, we investigated the role of IL-10 in inducing and activating Tregs, and the possibility of their immunosuppressive effects on third-party allografts that are only partially compatible with the alloantigen used in immunization.

3. Materials and methods

3.1. Animals

Female C57BL/6 (B6, H-2^b), (BALB/c × C57BL/6)F_1 (CBF1, H-$2^{b/d}$), (C57BL/6 × C3H/He)F_1 (B6C3F1, H-$2^{b/k}$), and (BALB/c × C3H/He)F_1 (CC3F1, H-$2^{d/k}$) mice were obtained from Japan SLC (Hamamatsu, Japan). Mice were cared for in accordance with the institutional guidelines for animal welfare. The mice were 6 weeks old at the time of the first experimental procedure. The study design is summarized in Fig. 1. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine (No. 3106).

3.2. Immunization with alloantigen

Spleens were removed from naïve CBF1 mice (H-$2^{b/d}$). Splenocytes were rapidly isolated, and resuspended in phosphate buffered saline (PBS). Next, single-cell splenocytes at 2×10^7 cells in 0.5 mL were slowly injected intravenously into age- and sex-matched naïve B6 mice (H-2^b).

3.3. UV-B irradiation after alloantigen immunization

One week after alloantigen immunization, immunized B6 mice received UV-B irradiation at a dose of 40 kJ/m^2. The UV source was a bank of three unfiltered UV lamps (UVP Inc., Upland, CA, USA) with an emission spectrum in the UV-B range (280–320 nm). The average UV-B irradiation dose was 2372 mJ/cm^2/h. A 10-cm^2 area of the ventral skin was carefully shaved. To prevent unevenness of UV-B irradiation, mice were anesthetized during UV exposure, and their feet were fixed to a metallic halftone plate by threads. Thus, the shaved abdominal wall was sufficiently extended and equally exposed to the UV lamps.

3.4. Preparation of $CD4^+$ T cells

Single-cell splenocyte suspensions were incubated with CD4 microbeads (CD4 [L3T4] MicroBeads; Miltenyi Biotec Inc., Auburn, CA, USA) and positively selected over separation columns according to the manufacturer's recommendations (AutoMACS program; Miltenyi Biotec Inc.). The purities of sorted $CD4^+$ T-cell preparations were routinely >98% $CD4^+$ T cells.

3.5. Adoptive transfer of $CD4^+$ T cells

Splenocytes were isolated from UV-irradiated immunized mice. Four weeks after UV irradiation, splenic $CD4^+$ T cells were purified and resuspended in PBS. A total of 5×10^6 enriched splenic $CD4^+$ T cells in 100 μL PBS were injected intravenously into age- and sex-matched naïve B6 mice. PBS-injected mice served as a control. In our previous studies, $CD4^+$ T-cell transfers of greater than 5×10^6 cells/mouse seemed to plateau for alloantigen-specific prolongation of allograft survival [25,28].

3.6. Skin transplantation

Skin grafts were transplanted onto graft beds on the backs of B6 mice by microsurgery under ×4.5 magnification (Panoramic-XL; Keeler Ltd., Windsor, UK). Full-thickness tail skin grafts were harvested from age- and sex-matched donor mice (B6, CBF1, B6C3F1, or CC3F1 mice). All grafts were adjusted to an area of 10 × 5 mm to allow a quantitative uniformity of alloantigen. Syngeneic and allogeneic grafts were bilaterally engrafted, and surgical procedures and post-transplant care are described in detail elsewhere [25]. Graft rejection was defined as >90% necrosis of graft epithelium [46].

3.7. Heart transplantation

As the second model to confirm the immunosuppressive effects *in vivo*, heterotopic heart transplantation was performed. Cardiac grafts were harvested from age- and sex-matched donor mice (B6, CBF1, B6C3F1, or CC3F1 mice). Cardiac grafts were ectopically transplanted into recipient mice. Surgical procedures including ultra-microsurgery are described in detail elsewhere [47]. Ultra-microsurgical procedures were performed under ×20 magnification (Surgical Scope M680, Type 10445496; Leica Microsystems Inc., Bannockburn, IL, USA). Graft rejection was defined as no palpable pulsation of the heterotopic graft [48].