medium. Responder CD4⁺T cells were purified from B6 mice. Enriched splenic CD4⁺T cell suspensions from B6 mice (1×10^6 cells/250 µl/well) were co-cultured with stimulator cells from naïve CBF1 or B6C3F1 mice (1×10^6 cells/250 µl/well) in sterile 96-well flat-bottomed plates at 37°C. Supernatants were harvested after 24 h for analysis of productions of interleukin (IL)-2, IL-6, and IL-8, and after 72 h for analysis of interferon (IFN)-γ, IL-4, IL-5, IL-10, IL-13, and transforming growth factor (TGF)-β production. To stimulate responder T cells, 1 µg/mL/well of anti-CD3 monoclonal antibody (mAb) (145-2C11) was added, and supernatant was harvested after 72 h for analysis of IL-10 production. The cultures were pulsed with 1 µCi/well of ^{3}H-thymidine for the last 12 h, and incorporation of ^{3}H-thymidinewas measured.

Enzyme-linked immunosorbent assay for cytokines

Concentrations of IFN-γ, IL-2, IL-4, and IL-5 in supernatants from MLC were determined by enzyme-linked immunosorbent assay (ELISA) using a pair of anti-cytokine-specific mAb, as described previously [36]. The lower detection limits for IFN-γ, IL-2, IL-4, and IL-5 were 100, 30, 10, and 10 pg/ml, respectively. Concentration of IL-6 was quantified using an ELISA kit (Mouse IL-6 ELISA; BD Biosciences). The lower limit of detection for IL-6 was 15.6 pg/ml. Concentration of IL-8 was quantified using an ELISA kit (IL-8 ELISA kit; Funakoshi Frontiers in Life Science, Tokyo, Japan). The lower limit of detection for IL-8 was 10 pg/ml. Concentrations of IL-10 and IL-13 in the supernatants of MLC were quantified using ELISA kits (Quantikine Mouse IL-10 Immunoassay and Quantikine Mouse IL-13 Immunoassay; R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's recommendations. The lower detection limits for IL-10 and IL-13 were 31.3 and 125 pg/ml, respectively. Concentrations of TGF-β were measured by ELISA kit (Mouse TGF-beta1 Platinum ELISA; eBioscience, Inc., San Diego, CA, USA). Samples were diluted 10-fold, and the lower detection limit for TGF-β was 120 pg/ml.

Serum levels of IL-10 peaked at 4 days after high-dose UV-B irradiation [27]. Peripheral blood samples were obtained at days 1-5 and 7 after UV irradiation. Serum concentrations of IL-10 were measured by ELISA.

Adoptive transfer of CD4⁺T cells

Single splenocytes were isolated from B6 mice treated with immunization and UV irradiation. Four weeks after UV irradiation, splenic CD4⁺T cells were purified and resuspended in PBS. CD4⁺T cells at concentrations of 5×10^5, 1×10^6, 3×10^6, 5×10^6, 1×10^7, and 5×10^7 cells/100 µl/mouse were intravenously injected into age- and sex-matched naïve B6 mice via the lateral tail vein. PBS-transferred mice served as controls.

IL-10 blocking

To block IL-10, culture medium from a hybridoma-secreting anti-IL-10 antibody (JES5-2A5, rat immunoglobulin G [IgG]) was used. The monoclonal antibody (mAb) was diluted in PBS, and a total of 250 µg/100 µl was intravenously injected into B6 mice, on the day before (day −1) and after (day +1) UV irradiation. In preliminary study, we confirmed the effect of this anti-IL-10 antibody in comparisons with control rat IgG (Sigma Chemical, St. Louis, MO, USA).

Statistical analysis

Results are presented as mean ± standard deviation. The *t*-test was used for the comparison of unpaired continuous variables between groups. Survival curves were constructed by the Kaplan-Meier method, and the log-rank test was used for between-group comparisons. Statistical calculations were performed by statistical software (Stat View-J 5.0, SAS Institute Inc., Cary, NC, USA). A p value <0.05 was considered statistically significant, and $p \geq 0.05$ was defined as not significant (NS).

Results

Dose of UV-B irradiation

First, immunosuppressive effects were evaluated at various doses of UV-B. Mice were immunized and received various doses of UV-B at 1 week after immunization. At 1 week after UV irradiation, splenocytes were obtained from immunized and UV-irradiated mice. Proliferation assay by MLR was performed with stimulator cells presenting either the immunizing or irrelevant alloantigen. Proliferation assays by MLR were repeated 4 times (Figure 3A).

Stimulation with immunizing antigen and UV-B irradiation at 10 kJ/m^2 (p=0.0331), 20 kJ/m^2 (p<0.0001), 30 kJ/m^2 (p<0.0001), 40 kJ/m^2 (p<0.0001), 50 kJ/m^2 (p<0.0001), and 60 kJ/m^2 (p=0.0002) showed significant changes in ^{3}H-TdR incorporation compared with control mice receiving 0 kJ/m^2 UV-B irradiation. Stimulation with an irrelevant antigen and UV-B irradiation at 10 kJ/m^2 (p=0.0109), 20 kJ/m^2 (p=0.0009), 30 kJ/m^2 (p=0.0008), 40 kJ/m^2 (p=0.0004), 50 kJ/m^2 (p=0.0032), and 60 kJ/m^2 (p=0.0003) showed significant changes in ^{3}H-TdR incorporation compared with control mice receiving 0 kJ/m^2 UV-B irradiation.

Responses to the immunizing antigen were suppressed according to UV-B dose, although immunosuppressive effects appeared to plateau at >40 kJ/m^2. However, responses to the irrelevant antigen were also suppressed at 1 week after UV irradiation. In addition, a UV-B dose of 60 kJ/m^2 was fatal for mice. Hence, a dose of 40 kJ/m^2 of UV-B was used for all other experiments in this study (Figure 1)

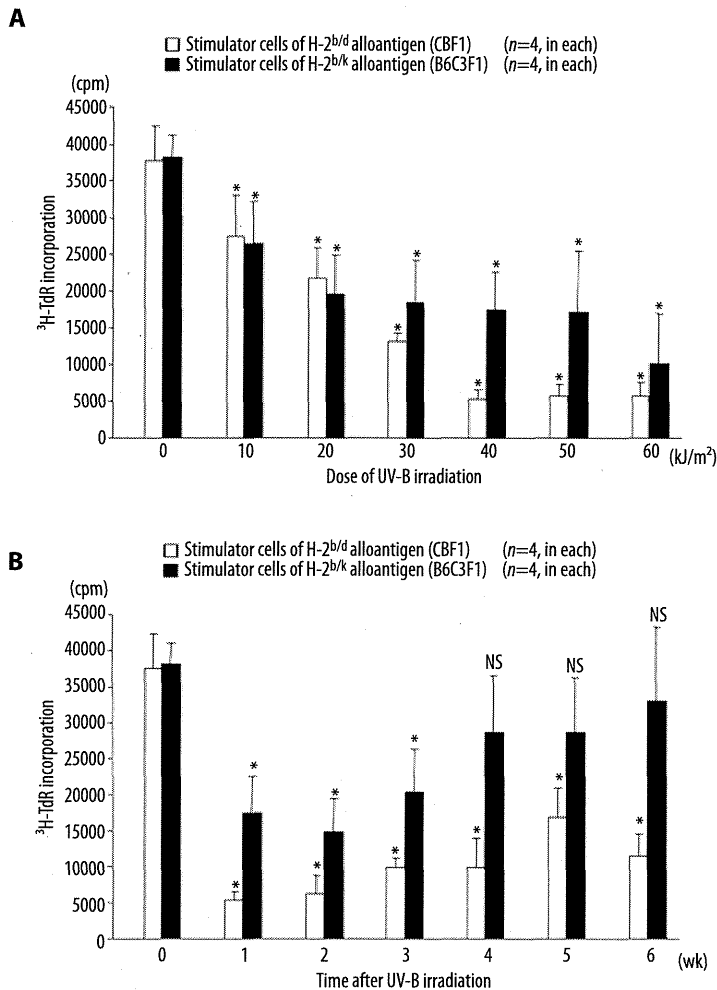

Figure 3. Proliferation assays at various doses of UV-B irradiation and at various time points after UV irradiation (**A**) Proliferation assays at various doses of UV-B irradiation. In each of stimulations with the immunizing or irrelevant antigen, ^{3}H-TdR incorporations at 10-60 kJ/m^{2} were compared with non-UV irradiated mice (0 kJ/m^{2}), respectively. (**B**) Proliferation assays at various time points after UV irradiation. In each of stimulation with the immunizing or irrelevant antigen, ^{3}H-TdR incorporations at 1-6 week(s) after UV irradiation were compared with non-UV irradiated mice (week 0), respectively. *, $p<0.05$.

Temporal immunosuppressive effects of UV-B irradiation for the irrelevant antigen

As described above, responses to an irrelevant antigen were also suppressed at 1 week after UV irradiation. The recoveries of responses to the irrelevant antigen were evaluated at various time points after UV irradiation. Immunized mice received 40 kJ/m^{2} of UV-B at 1 week after immunization. At various time points after UV irradiation, splenocytes were obtained from immunized and UV-irradiated mice and proliferation assays by MLR were performed with stimulator cells presenting either immunizing antigen or irrelevant alloantigen (Figure 3B). Proliferation assays by MLR were repeated 4 times each.

Stimulation with the immunizing antigen induced significant differences in ^{3}H-TdR incorporation at 1 week ($p<0.0001$), 2 weeks ($p<0.0001$), 3 weeks ($p<0.0001$), 4 weeks ($p=0.0001$), 5 weeks ($p=0.0006$), and 6 weeks ($p<0.0001$) after UV irradiation compared with non-UV irradiated mice (week 0).

Stimulation with an irrelevant antigen induced significant differences in ^{3}H-TdR incorporation at 1 week ($p=0.0004$), 2 weeks ($p=0.0001$), and 3 weeks ($p=0.0018$) after UV irradiation compared with non-UV irradiated mice (week 0). However, ^{3}H-TdR incorporation at 4 weeks ($p=0.0630$), 5 weeks ($p=0.0562$), or 6 weeks ($p=0.3695$) after UV irradiation was not significantly different from non-UV irradiated mice (week 0).

Responses to immunization with an irrelevant antigen disappeared at 4 weeks after UV irradiation, whereas all responses to the immunizing antigen were suppressed. Hence, a time point of 4 weeks after UV irradiation was used in this study (Figure 1)

L2 Immunosuppressive effect induced by UV irradiation alone

Transplantations with skin or cardiac allografts were performed at 1 week after UV irradiation. Mice were divided into 4 groups: (i) untreated control, (ii) immunized control (iii), UV-irradiated control, and (iv) immunized/UV-irradiated group. Skin or heart

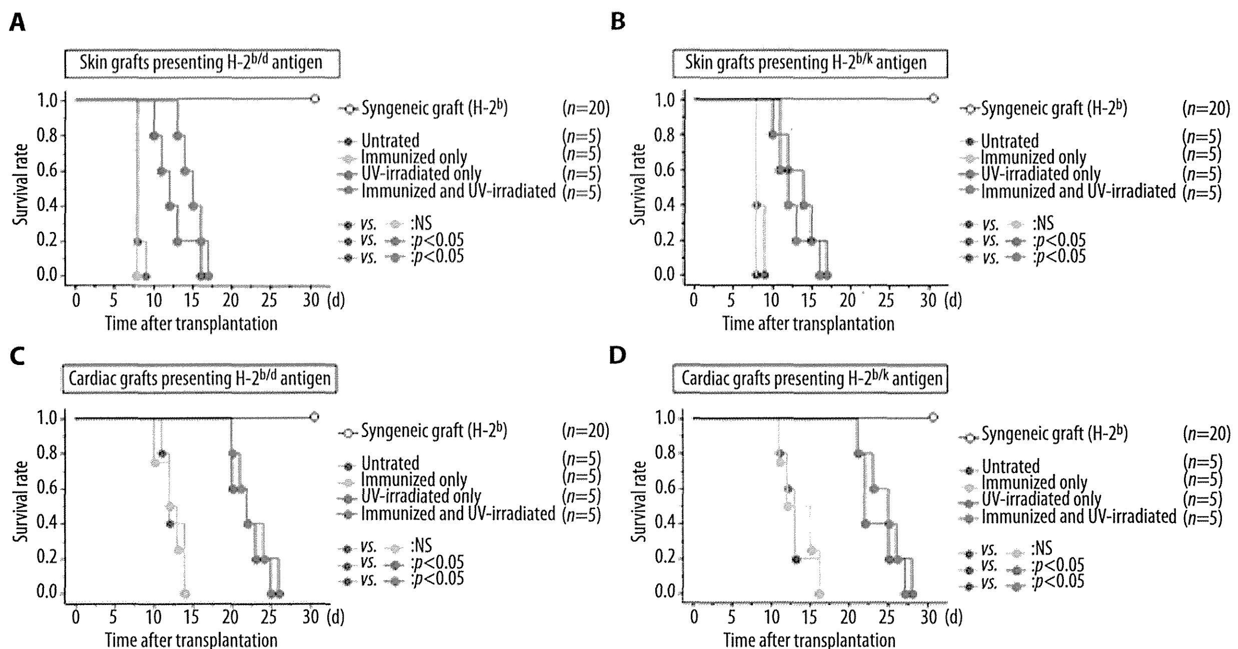

Figure 4. Allograft survival at 1 week after UV irradiation. (**A**) Survival of skin allografts presenting the immunizing alloantigen. (**B**) Survival of skin allografts presenting an irrelevant alloantigen. (**C**) Survival of cardiac allografts presenting the immunizing alloantigen. (**D**) Survival of cardiac allografts presenting an irrelevant alloantigen.

transplantations were performed in 5 mice per group. No rejections were observed following syngeneic grafts. Survival curves are shown in Figure 4.

In skin transplantation with allografts presenting the immunizing antigen, there were significant differences in allograft survival in the UV-immunized control ($p=0.0016$) and immunized/UV-irradiated group ($p=0.0016$) but not immunized controls ($p=0.3711$) compared with the untreated controls (Figure 4A). In skin transplantation with allografts presenting an irrelevant antigen, there were significant differences in allograft survival in UV-immunized control ($p=0.0023$) and immunized/UV-irradiated groups ($p=0.0023$) but not immunized controls ($p=0.1763$) compared with untreated controls (Figure 4B).

In heart transplantation with allografts presenting the immunizing antigen, there were significant differences in allograft survival in the UV-immunized control ($p=0.0062$) and immunized/UV-irradiated groups ($p=0.0062$) but not immunized controls ($p=0.7209$) compared with untreated controls (Figure 4C). In heart transplantation with allografts presenting an irrelevant antigen, there were significant differences in allograft survival in the UV-immunized control ($p=0.0051$) and immunized/UV-irradiated groups ($p=0.0051$) but not immunized controls ($p=0.8585$) compared with untreated controls (Figure 4D).

At 1 week after UV irradiation, UV-B irradiation alone showed immunosuppressive effects in transplantations with allografts regardless of which antigen was used for immunization.

Alloantigen-specific immunosuppressive effects induced by immunization and UV irradiation

Mice were divided into 4 groups: (i) untreated control, (ii) immunized control, (iii) UV-irradiated control, and (iv) immunized/UV-irradiated group. Splenic responder cells at 4 weeks after UV irradiation were cultured with stimulator cells presenting either the immunizing or irrelevant alloantigens (Figure 5). Proliferation assays by MLR were repeated 4 times each.

Immunizing antigen stimulation of the immunized/UV-irradiated group ($p=0.0006$) but not the immunized control ($p=0.2153$) or UV-irradiated control group ($p=0.2459$) showed significant differences in ^{3}H-TdR incorporation compared with the untreated controls. However, there were no significant differences in ^{3}H-TdR incorporation between all groups and unstimulated controls when stimulated with an irrelevant antigen.

Responses to the immunizing antigen were markedly suppressed in immunized and UV-irradiated mice, in an alloantigen-specific manner.

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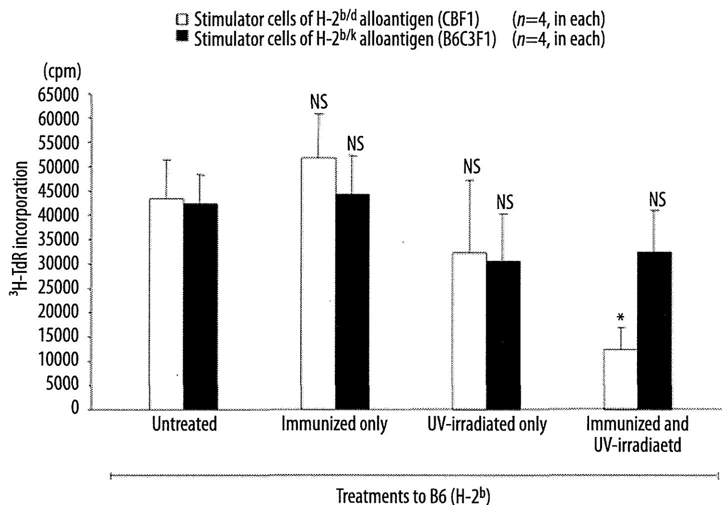

Figure 5. Proliferation assays at 4 weeks after UV irradiation. *, $p<0.05$.

Alloantigen-specific prolongation of allograft survival induced by immunization and UV irradiation

Transplantations with skin or cardiac allografts were performed at 4 weeks after UV irradiation. Mice were divided into 4 groups, as described above. Skin or heart transplantations were respectively performed in 5 mice per group. No rejections were observed in syngeneic grafts. Survival curves are shown in Figure 6.

In skin transplantation with allografts presenting the immunizing antigen, there were significant differences in allograft survival of the immunized/UV-irradiated group ($p=0.0052$) but not the immunized control ($p=0.1763$) and UV-immunized control groups ($p=0.2034$) when compared with untreated controls (Figure 6A). In skin transplantation with allografts presenting the irrelevant antigen, there was no significant difference in allograft survival of all groups when compared with the untreated controls ($p=0.0568$) (Figure 6B).

In heart transplantation with allografts presenting the immunizing antigen, there were significant differences in allograft survival of the UV-immunized control ($p=0.0110$) and immunized/UV-irradiated groups ($p=0.0046$) but not of the immunized control ($p=0.4642$) when compared with the untreated controls (Figure 6C). In heart transplantation with allografts presenting the irrelevant antigen, there were significant differences in allograft survival of the UV-immunized control ($p=0.0120$) and immunized/UV-irradiated group ($p=0.0262$) but not immunized controls ($p=0.3093$) when compared with the untreated controls (Figure 6D).

At 4 weeks after UV irradiation, although immunosuppressive effects by UV-B irradiation alone were still observed in transplantation with cardiac allografts, an alloantigen-specific prolongation of skin allograft survival was confirmed in immunized and UV-irradiated mice.

Immunization by DC

An interesting question arose from these experiments. Allografts were finally rejected, even though immunized and UV-irradiated mice showed an alloantigen-specific prolongation of allograft survival. Therefore, does immunization by mature DC cause stronger immunization and subsequent induction of greater alloantigen-specific immunosuppression in immunized and UV-irradiated mice? Using the transplant model, our preliminary study demonstrated that alloantigen immunization by intraperitoneal injection failed to induce alloantigen-specific immunosuppression or showed only weak immunosuppression (data not shown). Therefore, we used DC to provide a stronger immunization to induce greater immunosuppression.

Proliferation assays stimulated with the immunizing antigen and transplantation with skin or cardiac allografts presenting the same alloantigen were performed at 4 weeks after UV irradiation. Mice were divided into 5 groups: (i) untreated control, (ii) immunized control, (iii) immunized by DC (iv) immunized/UV-irradiated control, and (v) immunized by DC/UV-irradiated group. Proliferation assays by MLR were repeated 4 times each. Five mice were used for skin or heart transplantations. No rejections were observed in syngeneic grafts.

Proliferation assays measuring ^{3}H-TdR incorporation in untreated controls were examined first. There was no significant difference in ^{3}H-TdR incorporation between immunized controls and the immunized DC group ($p=0.2484$) and between the immunized/UV-irradiated control and immunized by DC/UV-irradiated groups ($p=0.7173$).

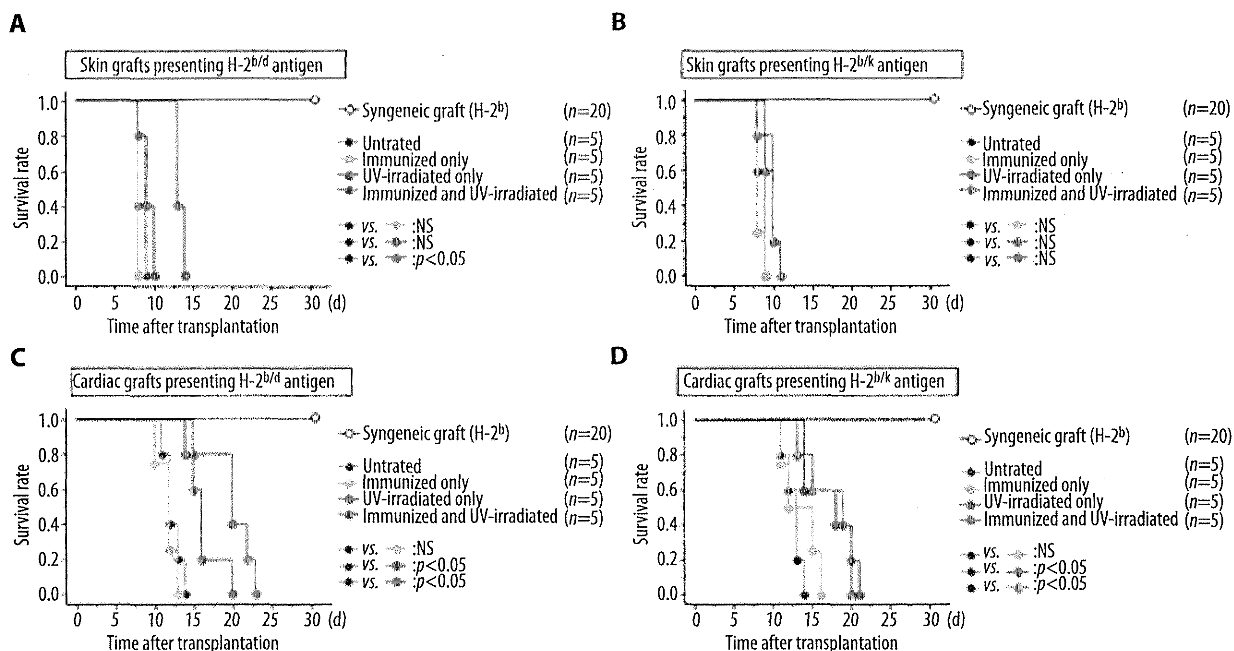

Figure 6. Allograft survivals at 4 weeks after UV irradiation. (**A**) Survival of skin allografts presenting the immunizing alloantigen. (**B**) Survival of skin allografts presenting an irrelevant alloantigen. (**C**) Survival of cardiac allografts presenting the immunizing alloantigen. (**D**) Survival of cardiac allografts presenting an irrelevant alloantigen.

Skin transplantation studies showed no significant difference in allograft survival between immunized controls and the immunized by DC group (p=0.0.8741) and between the immunized/UV-irradiated control and immunized by DC/UV-irradiated groups (p=0.6693). In heart transplantation studies, there was no significant difference in allograft survivals between the immunized group and immunized by DC group (p=0.0.8741) and between the immunized/UV-irradiated group and immunized by DC/UV-irradiated group (p=0.5003).

Although we hypothesized mature DC may have a difference in allograft survival, this was not observed experimentally.

Characterization of Treg induced by immunization after UV irradiation

To characterize whether Treg mediated alloantigen-specific immunosuppression in immunized and UV-irradiated mice, we investigated cytokine production profiles of CD4$^+$T cells by alloantigen stimulation. To prepare CD4$^+$T cells as responders, spleens were harvested from 4 B6 mice (H-2^b) and divided into 4 groups: (i) untreated control, (ii) immunized control, (iii) UV-irradiated control, and (iv) immunized/UV-irradiated group. CD4$^+$T cells were isolated at 4 weeks after UV irradiation. Enriched CD4$^+$T cells from each group were co-cultured with X-irradiated T cell-depleted splenocytes from CBF1 mice (H-2$^{b/d}$) or B6C3F1 mice (H-2$^{b/k}$). These MLCs were repeated 6 times, and supernatants were harvested for ELISA assay (Figure 7).

Proliferation in the MLC plate was measured. There was no significant difference in ^{3}H-TdR incorporation between all groups and the untreated control when stimulated with the immunizing or irrelevant antigens (Figure 7A).

Stimulation with the immunizing alloantigen showed significant differences in IFN-γ production in the immunized/UV-irradiated group (p=0.0010) but not the immunized controls (p=0.3261) or UV-irradiated controls (p=0.0772) compared with untreated controls. There was no significant difference in IFN-γ production between all groups and untreated controls when stimulated with the irrelevant antigen (Figure 7B).

Stimulation with the immunizing alloantigen showed significant differences in IL-2 production in the immunized/UV-irradiated group (p=0.0004) but not the immunized controls (p=0.8274) or UV-irradiated controls (p=0.0924) compared with untreated controls. There was no significant difference in IL-2 production between all groups and untreated controls when stimulated with the irrelevant antigen (Figure 7C).

Concentrations of IL-4 and IL-8 were under the detection limit when cells were stimulated with the immunizing or irrelevant alloantigen (Figure 7D, 7G).

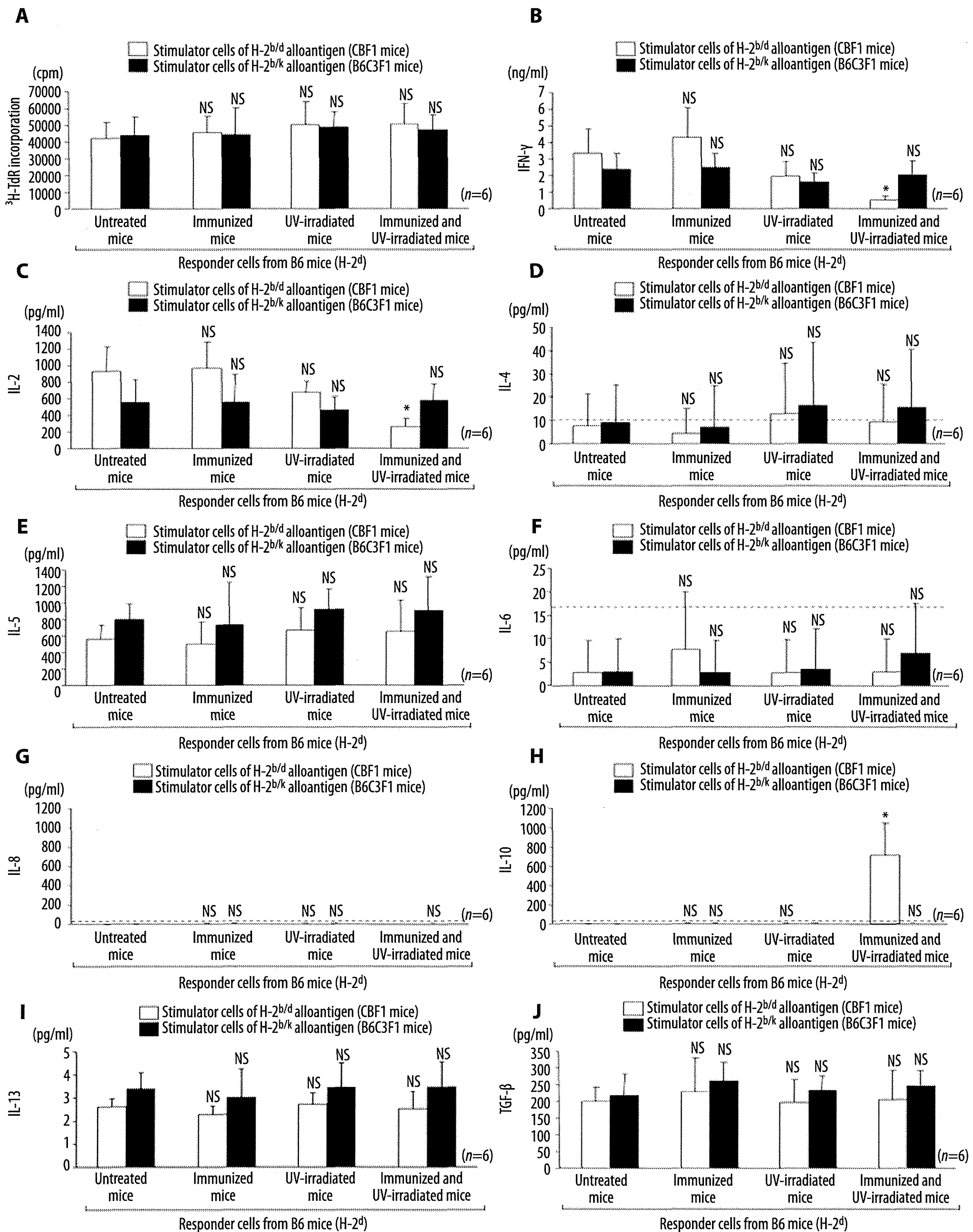

Figure 7. Cytokine profiles in MLC supernatants. (**A**) Proliferation of responder CD4⁺T cells. (**B**) IFN-γ concentration. (**C**) IL-2 concentration. (**D**) IL-4 concentration. (**E**) IL-5 concentration. (**F**) IL-6 concentration. (**G**) IL-8 concentration. (**H**) IL-10 concentration. (**I**) IL-13 concentration. (**J**) TGF-β concentration. *, $p<0.05$; dotted line, the detection limit.

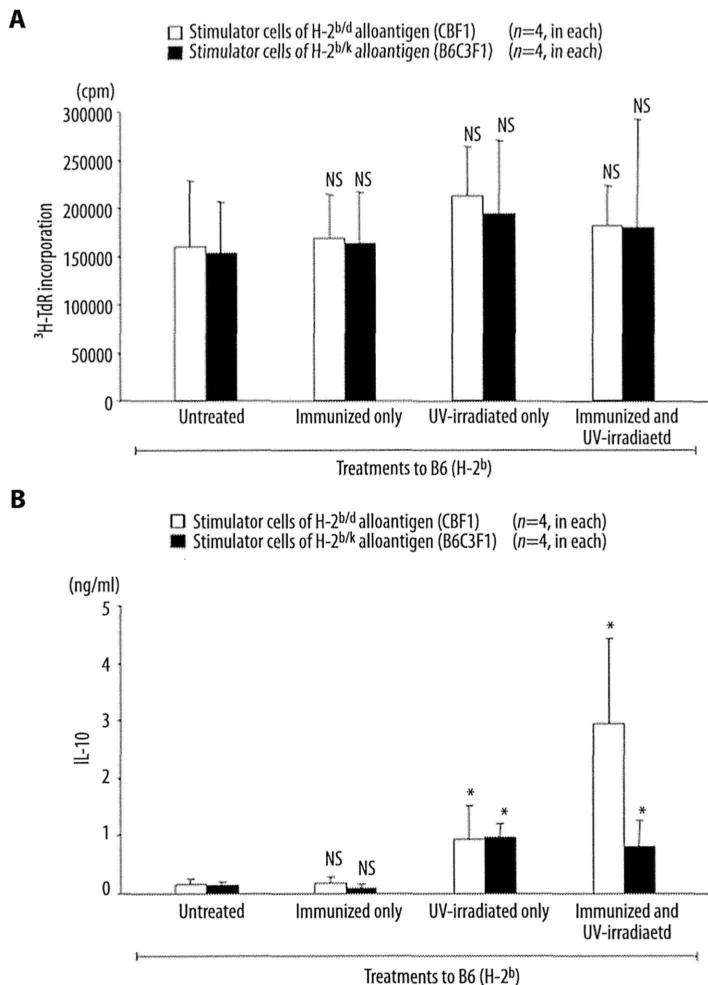

Figure 8. IL-10 production in MLC stimulated with anti-CD3 mAb. (**A**) Proliferation of responder CD4+T cells. (**B**) IL-10 concentration. *, $p<0.05$.

There were no significant differences in the productions of IL-5, IL-6, and IL-13 between all groups when stimulated with the immunizing or irrelevant alloantigen (Figure 7E, 7F, 7I).

Stimulation with the immunizing alloantigen showed significant differences in IL-10 production in the immunized/UV-irradiated group ($p=0.0004$) but not the immunized controls and UV-irradiated controls when compared with the untreated controls. There was no significant difference in IL-10 production between all groups and untreated controls when stimulated with the irrelevant antigen (Figure 7H).

There was no significant difference in TGF-β production between all groups when stimulated with the immunizing or irrelevant alloantigen (Figure 7J).

Data from the current study demonstrated that immunosuppression by immunization and UV-B irradiation did not depend on IL-4 or TGF-β, and suggested that the mechanism involved in immunosuppression was not a simple Th2 shift. CD4+T cells from immunized and UV-irradiated mice produced significantly greater amounts of IL-10 but smaller amounts of IL-2 and IFN-γ compared with untreated controls when stimulated with the immunizing alloantigen but not the irrelevant alloantigen. Thus, antigen-specific Tregs induced by immunization and UV irradiation were characterized as CD4+ T regulatory type 1 (Tr1) cells.

Serum levels of IL-10

Serum levels of collected blood samples were under the detection limit at all time points after UV irradiation (data not shown).

Stimulation of responder T cells by anti-CD3 mAb in MLC

We stimulated cytokine production of CD4+T cells in MLC by anti-CD3 mAb. Mice were divided into 4 groups: (i) untreated control, (ii) immunized control, (iii) UV-irradiated control, and (iv) immunized/UV-irradiated group. MLCs with anti-CD3 mAb were repeated 4 times, and supernatants were harvested for ELISA (Figure 8).

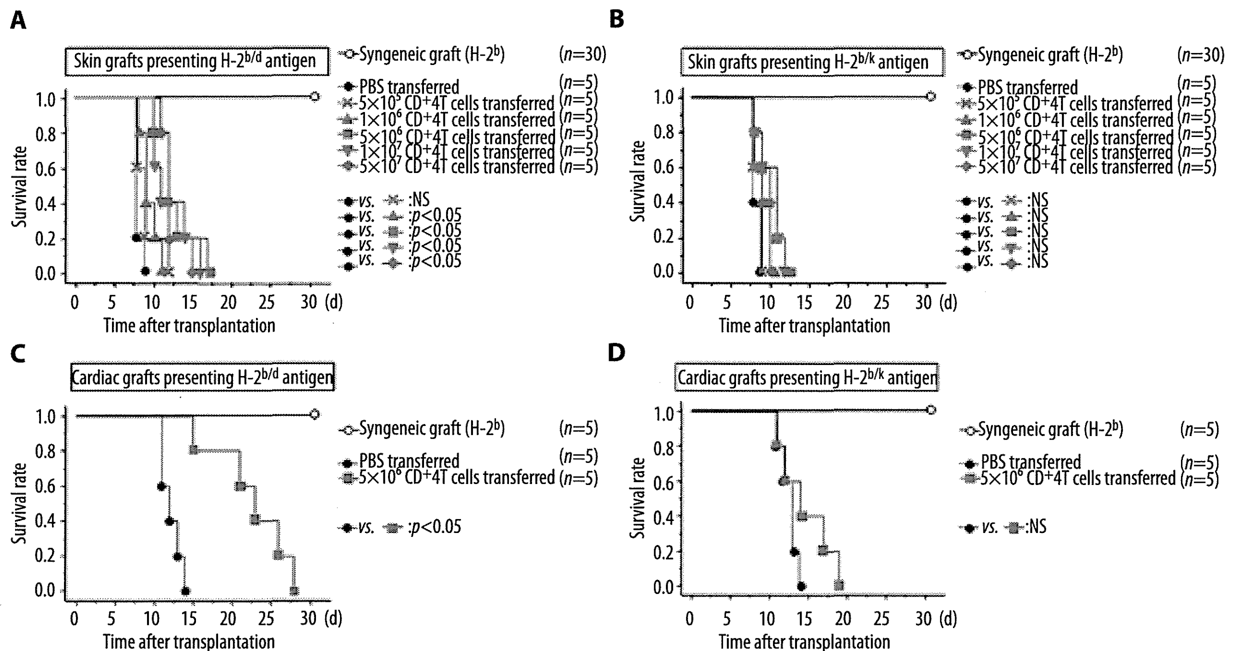

Figure 9. Allograft survivals in CD4⁺T transferred mice. (**A**) Survival of skin allografts presenting the immunizing alloantigen. (**B**) Survival of skin allografts presenting an irrelevant alloantigen. (**C**) Survival of cardiac allografts presenting the immunizing alloantigen. (**D**) Survival of cardiac allografts presenting an irrelevant alloantigen.

There was no significant difference in ^{3}H-TdR incorporation between all groups and untreated controls when stimulated with the immunizing or irrelevant alloantigen (Figure 8A).

Stimulation with the immunizing alloantigen showed significant differences in IL-10 production in the UV-irradiated control (p=0.0389) and immunized/UV-irradiated groups (p=0.0096) but not the immunized controls (p=0.8590) when compared with the untreated controls. Stimulation with the irrelevant stimulator showed significant differences in IL-10 production in the UV-irradiated control (p=0.0004) and immunized and UV-irradiated groups (p=0.0036) but not the immunized controls (p=0.3864) when compared with the untreated controls (Figure 8B).

IL-10 production from CD4⁺T cells in immunized and UV-irradiated mice was clearly enhanced by anti-CD3 mAb stimulation.

Transferable CD4⁺ Tr1 induced by immunization and UV irradiation mediated antigen-specific prolongation of allograft survival

Based on the above results, we investigated whether alloantigen-specific immunosuppression depended on CD4⁺ Tr1 cells induced by immunization and UV irradiation. CD4⁺T cells were purified from pooled splenocytes of immunized and UV-irradiated mice at 4 weeks after UV irradiation (Figure 1). Totals of 5×10^{5}, 1×10^{6}, 5×10^{6}, 1×10^{7}, and 5×10^{7} splenic CD4⁺T cells were transferred into naïve B6 mice. Thereafter, allografts expressing either the immunizing or irrelevant alloantigen were immediately engrafted to the transferred mice. Five transferred mice received each dose. Survival curves are shown in Figure 9.

In skin transplantations, survival of allografts presenting the immunizing alloantigen in CD4⁺T transferred mice were markedly prolonged dose-dependently (p=0.1762, 0.0494, 0.0016, 0.0016, and 0.0016, for CD4⁺T transfer of 5×10^{5}, 1×10^{6}, 5×10^{6}, 1×10^{7}, and 5×10^{7} cells/mouse, respectively) compared with PBS transferred mice (Figure 9A). In contrast, survival of allografts presenting the irrelevant alloantigen showed no prolongation when compared with PBS transferred mice, even in mice administered high-dose cell transfer (p=0.5485, 0.2055, 0.0528, 0.0528, and 0.0993, at CD4⁺T transfers of 5×10^{5}, 1×10^{6}, 5×10^{6}, 1×10^{7}, and 5×10^{7} cells/mouse, respectively) (Figure 9B).

CD4⁺T cells transferred mice from immunized/UV-irradiated mice clearly showed the dose-dependent alloantigen-specific prolongation of skin allograft survival, although the immunosuppressive effects plateaued at a dose of $>5\times10^{6}$ CD4⁺T cells/mouse.

Next, cardiac grafts were engrafted to mice receiving 5×10^{6} CD4⁺T cells. In heart transplantation, survival of allografts presenting the immunizing alloantigen in CD4⁺T transferred mice

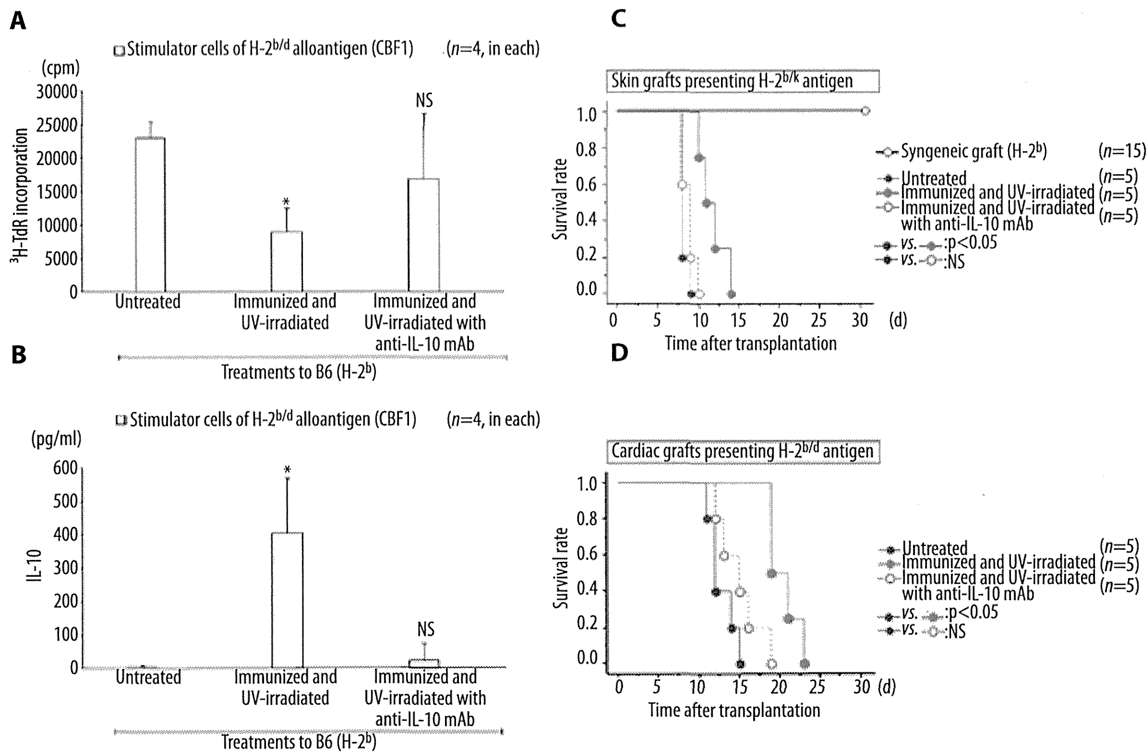

Figure 10. Abrogation of alloantigen-specific immunosuppression by blocking IL-10. (**A**) Proliferation assays. (**B**) IL-10 concentration in MLC supernatants. (**C**) Survival of skin allografts presenting the immunizing alloantigen. (**D**) Survival of cardiac allografts presenting the immunizing alloantigen. *, $p<0.05$.

were clearly prolonged ($p=0.0017$) compared with PBS transferred mice (Figure 9C). In contrast, survival of allografts presenting the irrelevant alloantigen showed no prolongation in $CD4^+T$ transferred mice when compared with PBS-transferred mice ($p=0.2242$) (Figure 9D).

Alloantigen-specific immunosuppression induced by immunization and UV irradiation depended on $CD4^+$ Tr1 cells.

Abrogation of alloantigen-specific immunosuppression by IL-10 blocking

Finally, to clarify a role of IL-10 in the induction of Tregs by immunization and UV irradiation, immunized/UV-irradiated mice were treated with anti-IL-10 mAb intravenously before (day −1) and after (day +1) UV-B irradiation. Mice were divided into 3 groups: (i) untreated control, (ii) immunized/UV irradiated control, and (iii) immunized/UV-irradiated mice with anti-IL-10 mAb treatment. MLRs and MLCs were repeated 4 times. Five mice per group received allograft transplantation, and no rejections were observed in syngeneic grafts (Figure 10).

In proliferation assay by MLRs with stimulator cells presenting the immunizing alloantigen, there was a significant difference in ^3H-TdR incorporation in the immunized/UV-irradiated controls ($p=0.0006$) but not the anti-IL-10 mAb treated group ($p=0.2671$) (Figure 10A).

In MLCs using responder $CD4^+T$ cells and stimulator cells presenting the immunizing alloantigen, there was a significant difference in IL-10 production in the immunized/UV-irradiated controls ($p=0.0027$) but not the anti-IL-10 mAb treated group ($p=0.3559$) (Figure 10B) when compared with the untreated controls. In transplantation with skin allografts presenting the immunizing alloantigen, there were significant differences in allograft survival of the immunized/UV-irradiated control ($p=0.0016$) but not the anti-IL-10 mAb treated group ($p=0.1762$) when compared with the untreated controls (Figure 10C).

In transplantation with cardiac allografts presenting the immunizing alloantigen for immunization, there were significant differences in allograft survival of the immunized/UV-irradiated control ($p=0.0018$) but not the anti-IL-10 mAb treated group ($p=0.1168$) when compared with the untreated controls (Figure 10D).

Treatment with anti-IL-10 mAb markedly abrogated the alloantigen-specific immunosuppression induced by immunization

and UV irradiation. These results suggests that IL-10 plays an important role in the induction of alloantigen-specific CD4+ Tr1 cells in immunized and UV-irradiated mice.

Discussion

UV light is one of the most important environmental factors affecting human health [1–4]. UV-B exposure induces skin tumors by a direct effect on DNA gene mutations and an indirect effect on immune responses [1,3,4]. UV-B exposure can suppress immune responses to various antigens [1,3,4]. In 1974, the first observation of the immunosuppressive effects of UV-B irradiation was reported [37]. UV-B irradiation results in the induction of highly antigenic skin cancers [2,37]. Moreover, immunosuppressive activity of UV-B-induced Tregs is highly selective, and the development of primary UV-B-induced tumors, but not other syngeneic tumors, is suppressed [2,37]. Furthermore, UV-B-induced immunosuppression could be transferred to normal syngeneic hosts by Tregs [38,39] and antigen-specific Tregs were also transferable [11,29,38,39]. Paradoxically, the capacity to modify immune responses by UV irradiation began to be used therapeutically in the 1970s [1,3].

Two models using UV-B irradiation have been developed [1,10,11,27]. Acute low-dose UV-B irradiation induces an inhibition of the local sensitization phase of contact hypersensitivity (CHS) responses to a hapten applied to UV-B irradiated skin [1,2,40]. High-dose UV-B irradiation induces inhibition of the systemic sensitization phase of CHS responses to a hapten and DTH to alloantigens applied to distant non-irradiated skin [1,2,7–11,26–28,33,41]. Both models are associated with the production of transferable antigen-specific Tregs [1,7,10,11,26,28,33]. It was suggested that the mechanisms and pathways involved in the immunosuppression of CHS and DTH induced by UV-B irradiation differ [27]. Our data clearly demonstrated that UV-B irradiation after alloantigen immunization is useful for immunosuppression in a DTH model.

High-dose UV-B irradiation induces the alternation or modulation of antigen-presenting cell (APC) functions for the induction of antigen-specific Tregs [1,3,5,7,10,26,29–31,42]. The early phase of APC functions after high-dose UV-B irradiation has been well studied [3,6,29,43,44]. UV-induced DNA damage has been recognized as the major molecular trigger for photoimmunosuppression [3,4,45]. UV exposure alters the morphology and function of epidermal Langerhans cells (LCs), which play a role in UV-B-induced immunosuppression [46]. LCs were regarded as the most important APC in the epidermis [47–49], and it was formerly believed that LCs were killed by UV irradiation. It is also believed that UV-B exposure triggers the migration of immature LCs from the skin to the draining lymph nodes, where they induce tolerance [46]. UV-B-induced migration of mast cells to draining lymph nodes is also considered an important early step in UV-B-induced immunosuppression [43,50]. Currently, the functional role of LCs has been redefined, and UV-damaged LCs in the regional lymph nodes were required for the Treg induction [45]. Damaged but still-alive LCs will present antigen in a unusual manner, and then, this presentation will induce not effect T cells, but will affect Tregs [4]. UV-B irradiation alters the ability of APCs to activate helper T cells and UV-resistant APCs to induce Tregs [10,26,32]. This might explain the antigen specificity of UV-B-induced immunosuppression, and also why antigen immunization must follow UV-B irradiation and not vice versa [7,10,26].

CD4+ and CD8+ T cells play critical roles during allograft rejection in transplantation [15,33,51–53]. UV-induced antigen-specific immunosuppression is attributable to T cells with suppressive activity (formerly so-called 'suppressor T cells') [54,55], and currently these T cells are renamed as Treg [56–58]. A number of studies have investigated the phenotype and mechanism of UV-B-induced Tregs. UV-induced Tregs express CD4, CD25, and CTLA4 [56,59,60]. Most UV-induced Tregs belong to the CD4+ phenotype [58], and immunosuppressive effects induced by UV irradiation are mediated by CD4+T cells [28,61,62]. These Tregs also express the lymph node-homing receptor (CD62L) and migrate into the lymph nodes [60,63]. Therefore, UV-induced Tregs primarily inhibit sensitization. To detect the phenotype of Tregs induced by UV irradiation after immunization, we had reported that CD4+T cells did not express CD25, CTLA4, or Foxp3 [22–25] and that T cells sorted by only CD4 involved Treg populations with IL-10. Immunosuppressive effects induced by UV-B irradiation were mediated by CD4+T cells [7,11,26,28,33,61], and CD4+T cells induced by UV irradiation after immunization worked well in our DTH model.

CD3+, CD4+, and CD8+ Tregs mediated immunosuppressive effects by releasing IL-4 and IL-10 [11,33,64]. UV-B-induced immunosuppression might also be explained by a shift in the activation of T cells from a Th1 to a Th2 immune response [26,28,65,66]. CD4+ Th2 lymphocytes secrete pro-inflammatory cytokines (IL-4, IL-5, and IL-13) [67,68]. IL-4 is thought to promote the induction of transplantation tolerance and alloantigen-specific Tregs [17]. IL-4 also promotes both regulatory and effector T cells early in the immune response, but once alloimmune tolerance is established, IL-4 promotes the activation of effector cells to mediate rejection and does not support alloantigen-specific Tregs that could transfer specific tolerance [17]. Although IL-10 is a well-known immunosuppressive cytokine [33,69] TGF-β is a growth and differentiation factor that displays multiple functions [70]. It was suggested that the combined use of IL-10 with other inhibitory cytokines such as TGF-β might be have more effect on CD4+ Tregs [33,70,71]. Our results support that immunosuppression induced by UV irradiation after immunization is dependent on

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CD4+ Tregs. However, our results regarding cytokine profiles suggest that alloantigen-specific immunosuppression induced by UV irradiation after immunization does not depend on IL-4, IL-5, IL-13, or TGF-β. As in previous reports, Tregs induced by UV irradiation before immunization showed the differences in IL-4, IL-5, and TGF-β [26,28,52,53], and our preliminary data may also explain immunosuppressive effects induced by UV irradiation before immunization by a Th2 shift (data not shown). Our data support the idea that UV-B-induced immunosuppression is dependent on CD4+ Tregs, although the mechanism of immunosuppression induced by UV irradiation after immunization is not a Th2 shift.

Cytokines are important for UV-B-induced immunosuppression [11,12,27,42,53,64]. Especially, a role of IL-10 in high-dose UV-B irradiation was demonstrated [11,12,27,33,53,72]. High-dose UV-B is almost entirely absorbed within the epidermis [27], and UV-B irradiation causes damage to keratinocytes [27], which produce both IL-10 mRNA and protein *in vitro* [73], and thus are a likely source of IL-10 [27]. Apoptosis may also play an important role in UV-B-induced immunosuppression [1,74]. It is unlikely that UV-B-induced damage has a direct effect on T cells, because UV-B has a low penetration of skin [1]. It is considered that FasL expression on DNA-damaged LCs may stimulate Treg production, which may induce apoptosis in effector T cells [1]. FasL expression on DNA-damaged LCs may directly induce apoptosis of antigen-specific effector T cells [75]. Secretion of IL-4 and IL-10 from UV-B-induced apoptotic cells mediates immunosuppressive effects [1,76] and lymphocyte apoptosis is linked to IL-10 secretion [76]. In our model, possible sources of IL-10 may be apoptotic lymph nodes and/or damaged keratinocytes.

IL-10 is considered necessary for UV-B-induced immunosuppression [12,27,33,77–79]. Transferred IL-10-producing T cells inhibited the priming of alloantigen-specific CD4+T cells, and subsequent induction of alloantigen-specific CD8+T cells [33]. Transferred IL-10 CD4+T cells migrated to the site of allografts and directly inhibited the function of alloantigen-specific cytotoxic CD8+T cells [33]. The inhibitory capacity of UV-induced Tregs crucially depends on IL-10 [60]. Antigen-specific activation of Treg by APC induces the release of IL-10 [59,60] and inhibitory activity of UV-induced Treg is mediated by IL-10 [59,80]. From the viewpoint of the source of IL-10, UV-induced Treg itself [56,77], mast cells [81], and CD11b+ macrophages [82] were listed. Thus, IL-10 is crucial for both the induction and effector phases [11,33,73,76,78], but some researchers suggested that IL-10 is not required for Treg induction by UV irradiation [79]. IL-10 blocking data in the current study supports the importance of IL-10 for CD4+ Treg induction by immunization and UV-B irradiation.

Surprisingly, only CD4+T cells from immunized/UV-irradiated mice produced IL-10 when stimulated with the immunizing alloantigen but not an irrelevant alloantigen. In this study, we used B6 mice as recipients, because B6 mice are Th1 prone [52]. This might explain why serum IL-10 was not detected. However, CD4+T cells from immunized and UV-irradiated B6 mice produced a large amount of IL-10 in an alloantigen-specific manner. High levels of IL-10 and low levels of IL-4 resemble a Tr1 cytokine pattern [83]. The presence of IL-10 gives rise to CD4+T-cell clones with a low proliferative capacity that in turn produce high levels of IL-10, low levels of IL-2, and no IL-4 [69,83]. These antigen-specific T cell clones suppress the proliferation of CD4+T cells in response to antigen [69,83]. Thus, IL-10 drives the generation of a CD4+T-cell subset, designated Tr1, which suppresses antigen-specific immune responses and actively down-regulates pathological immune responses *in vivo* [69,83]. Paradoxically, it may explain why T cells sorted by only CD4 well worked in our model. In our model, CD4+ Treg and a high level of IL-10 are important for alloantigen-specific immunosuppression.

Untreated control and immunized control showed similar survivals of allografts presenting the same alloantigen. Some questions arose. Although UV-irradiated control and immunized/UV-irradiated group showed the differences, does alloantigen immunization work well? Does stronger immunization provide more enhanced results? DC are specialized APCs that monitor the antigenic environment and activate naïve T cells [84]. The role of DC is to sense danger and tolerize the immune system to antigens encountered [84]. If naïve T cells encounter antigens on DC under certain conditions, they can differentiate into Tregs rather than effector T cells [84]. The induction of Tregs by DC in the presence of IL-10 has been documented [53,69,85,86], and bone marrow-derived DC can induce Tr1 differentiation [87,88]. Therefore, we initially expected that alloantigen immunization by bone marrow-derived DC would enhance alloantigen-specific immunosuppression. IL-10 is key for inducing and mediating tolerance [84], and UV-B-induced Tregs released a large amount of IL-10. We failed to enhance the immunosuppressive effect by using bone marrow-derived mature DC in our model, which might suggest the difficulty of memorization of alloantigen to UV-B-induced Tr1. A possible explanation for similar survivals between untreated and immunized controls was that transplanted allografts in this study had strong antigenicity.

Under the stimulation in MLC with anti-CD3 mAb, proliferation of responder CD4+T cells and IL-10 concentration in the supernatants were increased in all UV-irradiated groups. An interesting finding of this study is that high-dose UV-B irradiation alone seemed to result in pan-immunosuppression, although it was documented that UV-B-induced Tregs are highly selective for UV-B-induced cancer [2,37]. Moreover, we showed that the UV-B-induced pan-immunosuppressive effect was related to the time course. An explanation for this is that we used a

higher dose of UV-B (40 kJ/m^2) than previous studies. In addition, the antigenicity of cardiac allografts might be weaker than for skin allografts, and thus some cardiac allografts survived even at 4 weeks after UV-B irradiation alone.

Interestingly, our results revealed that CD4$^+$ Tregs induced by UV irradiation after immunization seemed to produce a subtle IL-10 in the stimulation with the irrelevant alloantigen. A possible explanation for this phenomenon was that UV-induced Tregs will show an unique behavior, so-called 'bystander suppression' [60,77,89]. The antigen specificity appears to be restricted to the activation of UV-induced Tregs and not to the suppressive activity itself, because once activated antigen-specifically, they release IL-10 and thereby suppress also other immune reactions. Further studies were required to explain this phenomenon, and we now are performing advanced studies in this DTH model by using the third-party alloantigen (H-2$^{d/k}$).

Furthermore, high-dose UV-B irradiation after alloantigen immunization, but not before immunization, is a useful tool for transferable Treg induction. We clearly demonstrated beneficial immunosuppression via alloantigen-specific Tr1-like CD4$^+$T cells. UV-B irradiation to the recipient several days before transplantation is impractical, because predicting when a donor organ will be available is difficult. Alloantigen immunization before UV-B irradiation, suggested by the data here, may be clinically advantageous. The view of UV-induced immunology has changed over the past several years [3,4]. Carcinogenesis and immunosuppression due to UV were regarded as detrimental, but now it is thought that a fine-tuned balance is optimal [3,4]. In order to induce alloantigen-specific and transferable CD4$^+$ Tr1 cells, UV is a very useful tool in the DTH model. Alloantigen-specific immunosuppression is ideal for transplant recipients.

Clinically, there is great enthusiasm about the potential to develop strategies that can use Tregs for therapeutic intervention [77].

Conclusions

Induction of alloantigen-specific Tregs by alloantigen immunization and UV-B irradiation may have therapeutic potential. Although we are far from a full understanding of the mechanism involved, beneficial effects of alloantigen immunization and UV-B irradiation via alloantigen-specific Tr1-like CD4$^+$T cells were clearly observed. This transferable Treg may be useful, especially in the field of transplant immunology. We hope that our new insights will help many researchers in the transplant immunology field.

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Conflict of interest

All authors had no financial conflict of interest.

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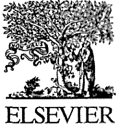

ELSEVIER

Assessment of Four Methodologies (Microparticle Enzyme Immunoassay, Chemiluminescent Enzyme Immunoassay, Affinity Column-Mediated Immunoassay, and Flow Injection Assay-Tandem Mass Spectrometry) for Measuring Tacrolimus Blood Concentration in Japanese Liver Transplant Recipients

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ABSTRACT

Therapeutic drug monitoring (TDM) and subsequent dosage adjustment for individual patients in the treatment with tacrolimus are required after liver transplantation to prevent rejection and over-immunosuppression, which leads to severe infection and adverse reactions including nephrotoxicity. The purpose of this study was to evaluate the analytical performance among commercially available immunoassay methods, which were microparticle enzyme immunoassay (MEIA), chemiluminescent enzyme immunoassay (CLIA), and affinity column-mediated immunoassay (ACMIA), compared with an assay using liquid chromatography-tandem mass spectrometry (LC-MS/MS). In addition, the flow injection assay (FIA-MS/MS) was also evaluated to determine whether it could be available as a new method of analysis in tacrolimus therapy. The blood tacrolimus concentrations in samples from liver transplant recipients (n = 102) were measured using MEIA, CLIA, ACMIA, and LC-MS/MS. Additional blood samples from liver transplant recipients (n = 54) were analyzed using both FIA-MS/MS and LC-MS/MS. Because the assay performance and characteristics of MEIA, CLIA, ACMIA, and FIA-MS/MS are relatively different, the measured data should be carefully considered depending on the methodology.

LIVER transplantation is the treatment of last resort in end-stage liver disease, and tacrolimus is an essential drug to prevent rejection. The dosage adjustment of tacrolimus based on daily therapeutic drug monitoring (TDM) is an important issue to prevent rejection and its severe adverse reactions (eg, central neurotoxicity and nephrotoxicity). In tacrolimus TDM, microparticle enzyme immunoassay (MEIA, IMx system, Abbott, Tokyo, Japan) was the most widely used method for more than 20 years. However, this method was unable to be used since 2009 because of the supply stopping from the company. After that, a chemiluminescent enzyme immunoassay (CLIA, ARCHITECT, Abbott) and an affinity column-mediated immunoassay (ACMIA, Dimension, SIEMENS, Tokyo, Japan) have been introduced and used in Japan. These 2 immunoassay methods use anti-tacrolimus antibodies, which have different properties in the cross-reaction with tacrolimus metabolites. Therefore,

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Table 1. Cross-reactions With Tacrolimus Metabolites of Anti-Tacrolimus Antibodies in MEIA, CLIA, and ACMIA, and the Accuracy of These Assay Methods and FIA-MS/MS

	MEIA	CLIA	ACMIA	FIA-MS/MS
Cross-reactions with anti-tacrolimus antibodies				
Tacrolimus	100	100	100	–
M-I	0.0	8.0	15	–
M-II	54	94	3.0	–
M-III	67	45	1.0	–
ME* (ng/mL)				
<2.0	0.42	0.20	−0.38	−0.29
2.0–5.0	0.35	0.23	−1.42	0.49
5.0–10.0	−0.48	−0.38	−0.65	0.27
≥10.0	−2.32	−2.55	−6.30	0.38
RMSE† (ng/mL)				
<2.0	0.55	0.28	0.76	0.58
2.0–5.0	1.02	1.11	1.79	0.71
5.0–10.0	1.56	1.44	1.62	1.42
≥10.0	3.14	3.27	6.63	0.38

*ME = $1/n\ \Sigma\ (C_{test}-C_{LC\text{-}MS/MS})$.
†RMSE = $(1/n\ \Sigma\ (C_{test}-C_{LC\text{-}MS/MS})^2)^{1/2}$.

different results have been obtained based on the method used from the same clinical sample.

In this study, we evaluated 3 immunoassay systems (MEIA, CLIA, and ACMIA) and flow injection assay–tandem mass spectrometry (FIA-MS/MS) in comparison with liquid chromatography-tandem mass spectrometry (LC-MS/MS) as a reference method.

METHODS

Tacrolimus concentrations were measured in the peripheral blood of 102 patients, who underwent liver transplantation at Kyoto University Hospital, using MEIA, CLIA, ACMIA, and LC-MS/MS. Additional blood samples of 54 patients, who also underwent liver transplantation at Kyoto University Hospital, were analyzed using the newly developed FIA-MS/MS and LC-MS/MS. The immunologic methods were performed according to the instrument manuals supplied by the vendors. The analytical procedures of LC-MS/MS and FIA-MS/MS for blood tacrolimus concentrations have been previously reported [1].

The results were divided into 4 groups according to tacrolimus concentration: <2.0, 2.0–5.0, 5.0–10.0, and ≥10.0 ng/mL. In each group, mean prediction errors (ME) and root mean square prediction errors (RMSE) [2] of the detected values obtained by the 4 methods were calculated against the reference values using LC-MS/MS, as measures of bias and precision, respectively:

$$ME = \frac{1}{n}\sum(C_{test} - C_{LC\text{-}MS/MS})$$

$$RMSE = \left(\frac{1}{n}\sum(C_{test} - C_{LC\text{-}MS/MS})^2\right)^{\frac{1}{2}}$$

This study was approved by the Kyoto University Graduate School and Faculty of Medicine, and Kyoto University Hospital Ethics Committee (E1421).

RESULTS AND DISCUSSION

As shown in Table 1, all assay methods showed small biases and precise values. Notably, CLIA showed excellent accuracy among the 3 immunoassays in the lowest concentration range (<2.0 ng/mL group). On the other hand, the ACMIA method showed negative biases in all concentration groups. Another point to note was that the sample preparation for ACMIA was automated, resulting in the easiest handling. However, in regard to time consumed, this automated preparation was handled 1 sample at a time, and took several minutes for 1 sample. The long waiting time after setting the sample in the instrument of ACMIA could result in negative biases and larger RMSE values because erythrocytes, in which tacrolimus is mainly distributed (about 90%), would be precipitated by gravity. Nonetheless, this automated sample preparation is tandem repeated, thus requiring several minutes for the preparation of 1 sample and causing a difference in the processing time of different samples. During the wait time for the next assay, the cellular components of all blood samples may precipitate by gravity. In this respect, it is important to note that about 90% of tacrolimus is distributed in erythrocytes. Therefore, the differences in the gradients of the blood samples in each batch may affect the data reproducibility, especially in the low concentration group. Hence, this might be the cause of the lower tacrolimus value measured using ACMIA.

Tacrolimus is mainly metabolized by cytochrome P450 (CYP) 3A4 and CYP3A5 [2,3], and its metabolites are excreted into bile [2]. There are 3 primary metabolites: 13-O-demethyl tacrolimus (M-I), 31-O-demethyl tacrolimus (M-II), and 15-O-demethyl tacrolimus (M-III) [1,3]. The test of mixed-lymphocyte reaction has shown that only M-II has immunosuppressive activities and M-I and M-III are nonactive metabolites [1,3]. As summarized in Table 1, the anti-tacrolimus antibody used in CLIA exhibits cross-reactivities for M-I, M-II, and M-III with 8%, 94%, and 45% compared with those for tacrolimus itself, respectively [4,5]. On the other hand, the anti-tacrolimus antibody used in ACMIA has cross-reactivities for M-I, M-II, and M-III with 15%, 3%, and 1% compared with those for tacrolimus itself, respectively, indicating that this antibody in ACMIA is the most specific for unchanged tacrolimus (Table 1). These differences among antibody specificities used in analytical methods might cause discrepancies in the measurement data, even when the same sample has been analyzed [3]. On the contrary, CLIA gives more accurate immunosuppressive activity in the blood because the antibody used in this method has a high cross-reactivity for M-II, which is a pharmacologically active metabolite; it even cross-reacts with a pharmacologically inactive M-III metabolite. Taken together with these results, actual clinical data in TDM for tacrolimus have some margin of error because the assay systems used, such as CLIA, ACMIA, LC-MS/MS, and so on, are different among hospitals.

In conclusion, the assay method should be clearly specified with the TDM data of blood concentration of tacrolimus when the patient transfers to another hospital or clinic. Then, the relationship should be considered between the tacrolimus concentration and the clinical presentations including adverse reactions, infections, and rejections.

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REVIEW ARTICLE

Perioperative nutritional therapy in liver transplantation

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Abstract Protein-energy malnutrition is frequently seen in patients with end-stage liver disease who undergo liver transplantation. This causes a deterioration of the patients' clinical condition and affects their post-transplantation survival. Accurate assessment of the nutritional status and adequate intervention are prerequisites for perioperative nutritional treatment. However, the metabolic abnormalities induced by liver failure make the traditional assessment of the nutritional status difficult. The methods that were recently developed for accurately assessing the nutritional status by body bioelectrical impedance may be implemented in pre-transplant management. Because preoperative malnutrition and the loss of skeletal muscle mass, called sarcopenia, have a significant negative impact on the post-transplantation outcome, it is essential to provide adequate nutritional support during all phases of liver transplantation. Oral nutrition is preferred, but tube enteral nutrition may be required to provide the necessary caloric intake. We herein discuss both bioelectrical impedance and the latest findings in the current perioperative nutritional interventions in liver transplant patients regarding synbiotics, micronutrients, branched-chain amino acid supplementation, the use of immune system modulating formulas, the fluid balance and the offering of nocturnal meals.

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Introduction

The liver orchestrates various physiological processes essential for a well-nourished state. It integrates several biochemical pathways of the metabolism of carbohydrates, fat, proteins and vitamins, as well as the secretion and excretion of bile and the transport of lipids, all of which are involved in muscle and protein metabolism [1–3].

Advances in post-transplantation care and the management of graft rejection have greatly improved the outcomes of patients after orthotopic liver transplantation (OLT). The clinical features of declining liver function tend to normalize following successful organ replacement, but preventing and treating malnutrition in these patients requires more attention [3–7].

Protein-energy malnutrition (PEM) is a common problem in patients with end-stage liver disease (ESLD) awaiting OLT [5, 7]. This applies to nearly every etiology of ESLD with the exception of fulminant hepatic failure. PEM is a risk factor for morbidity and mortality after liver transplantation (LT) [6, 7]. A patient's nutritional status can worsen rapidly during the post-operative period due to preoperative malnutrition, surgical stress, immunosuppressive therapy, post-interventional complications, post-operative protein catabolism and fasting periods [7, 8]. This suggests the need for early nutritional support with liver-adapted formulas containing additional carbohydrates, fat and proteins, especially branched-chain amino acids (BCAAs) [7, 8]. Even in non-cirrhotic and non-diabetic patients, hemihepatectomy results in moderate disturbances in glucose homeostasis, with increases in the

tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 levels [9].

Protein-energy malnutrition predisposes a patient to complications, such as a compromised respiratory function and delayed physical rehabilitation, and it also increases a patient's stay in the hospital and intensive care unit, blood usage and hospital charges [7, 8]. PEM adversely affects both the patient and graft survival, the patient quality of life after OLT and the response to stress, including the stresses of infection and surgery [3–7].

A loss of skeletal muscle mass (SMM), called sarcopenia, reflecting preoperative malnutrition, is the most common complication of liver cirrhosis (LC). Sarcopenia has been found to be closely associated with post-LT mortality in patients undergoing living donor LT (LDLT) [10]. Sarcopenia confers a vulnerability to preoperative infections, including spontaneous bacterial peritonitis and pneumonia due to the deteriorated immune function, as well as post-LT bacteremia, sepsis and wound dehiscence [11, 12].

There is no doubt that ensuring adequate nutritional intake, as well as correcting vitamin and micronutrient deficiencies, is paramount to alleviating some of these complications and reducing the perioperative morbidity and mortality after LT [1–3].

Etiology of Malnutrition

Patients with liver disease have decreased caloric intake due to the anorexia caused by zinc deficiency, hyperglycemia and increased levels of pro-inflammatory cytokines, such as TNF-α, IL-6 and leptin [13–16]. Other causes of the loss of appetite in patients with liver disease are the unpalatable specialized diets; low-salt and low-protein diets for ascites and hepatic encephalopathy (HE), respectively, and altered gustatory sensation due to the hypomagnesemia and autonomic neuropathy in LC, which also causes gastroparesis and a delayed bowel transit time. This, together with bacterial overgrowth and tense ascites, causes nausea, early satiety and increased protein loss [13–17]. In addition, in up to 45 % of patients with cirrhosis, a coexisting infection with *Helicobacter pylori* may cause dyspepsia and a decreased desire for food [14]. Moreover, nil per os (NPO) or limited intake for, during and after diagnostic or therapeutic procedures may further contribute to PEM [15–18].

Patients with liver disease are in a hypercatabolic state. Many cirrhotic patients also have increased basal energy expenditure (BEE), which may be related to increased sympathetic nervous system activity, decreased glycogen stores and impaired glycogenolysis. Although hyperinsulinemia is present, glucose intolerance ensues due to insulin resistance. Increased gluconeogenesis may further exacerbate the muscle wasting due to impaired muscle glucose uptake [19, 20].

Protein malnutrition is the predominant feature of advanced liver disease due to inflammation, impaired liver synthesis and increased protein breakdown, along with low glycogen stores that lead to gluconeogenesis from amino acids [18]. Patients with protein malnutrition have increased protein requirements to maintain a positive nitrogen balance. The low plasma levels of insulin-like growth factor (IGF-1), which mediates most of the growth-promoting effects of growth hormone, also explain the severe growth hormone resistance seen in patients with ESLD [21]. Protein-losing enteropathy has been suggested to play a role in the development of hypoalbuminemia in ESLD patients [22].

Moreover, patients with ESLD may have impaired synthesis of polyunsaturated fatty acids from their essential fatty acid precursors, with increases in the levels of n-6 and n-9 fatty acids and decreases in n-3 moieties in plasma and adipose tissue [23]. Enhanced gluconeogenesis, especially after fasting, with a preference for fat metabolism, lipid peroxidation and lipolysis, also increases upon impaired glycogen storage and utilization [24], further exacerbating the condition.

Other contributing factors include impaired hepato-intestinal extraction, large volume paracentesis, intestinal and drug-induced diarrhea (due to drugs like neomycin, lactulose, diuretics, antimetabolites, cholestyramine) and malabsorption in alcoholic and cholestatic liver diseases, especially sclerosing cholangitis with inflammatory bowel disease or concomitant pancreatic insufficiency [13, 25].

Evaluation of the nutritional status

All patients being prepared for LT should undergo a complete nutritional assessment. Traditional assessment tools are not accurate in patients with liver disease due to fluid retention, which is found in a significant number of patients, and the effects of liver dysfunction on protein synthesis. No gold standard for assessment exists at this time [6, 26].

The initial assessment should begin with a careful history to document weight loss, nausea, anorexia and the use of specialized diets and supplements. A complete physical examination should be performed to search for changes in the oral mucosa, skin and hair, thickness of subcutaneous fat and for muscle wasting, all of which are associated with chronic liver disease. A subjective global assessment (SGA) combines a thorough history-taking and physical examination and rates patients as either "well-nourished", "moderately malnourished", or "severely malnourished" [6].

This test has shown high specificity, but very low sensitivity, for diagnosing malnutrition in patients with alcoholic liver disease. However, it has not been found to be a reliable tool for evaluating the nutritional status in LT patients [6, 26].

Biochemical tests such as evaluations of the serum transferrin levels, retinol binding protein plus creatinine-height index, 24-h urine nitrogen and 3-methylhistidine excretion, as well as the total lymphocyte count and delayed hypersensitivity testing, have been used [27, 28]. However, such parameters have not been shown to be accurate indices of the nutritional status given the fact that their levels correlate with the severity of liver damage, rather than malnutrition due to the catabolic nature of the liver disease and associated protein turnover. Serum albumin frequently serves as an important indicator of the liver function. However, it has a long half-life (17–21 days), and because exogenous albumin supplements are frequently administered in clinical practice, the serum albumin levels cannot sensitively or dynamically reflect early liver damage. The shorter half-life (2–3 days) of prealbumin (transthyretin) renders it a more sensitive indicator of damaged synthetic functioning in the liver and fluctuations in the nutritional status than albumin [29].

Anthropometric measurements, such as the body mass index, mid-arm muscle circumference, triceps skin fold thickness or subscapular skin fold thickness have also been used as part of a nutritional assessment in patients with liver disease [27, 28]. However, these measures have been questioned regarding their reliability in patients with ascites and peripheral edema [27, 30].

The non-protein respiratory quotient (npRQ), a unitless number estimated from the carbon dioxide production, was used to evaluate the nutritional status of patients with LC [31]. The homeostasis model assessment (HOMA), a method used to quantify insulin resistance and β-cell function, has been reported to reflect the nutritional status in patients with nonalcoholic fatty liver disease [31]. The BEE can either be predicted using several formulas, such as the Harris-Benedict equation using ideal body weight plus 20 %, or measured with indirect calorimetry [32].

The body cell mass (BCM) is defined as the sum of the intracellular water and fat-free mass, including skeletal muscle and viscera, without the bone mineral mass. The BCM comprises the metabolically active and protein-rich compartments in the body responsible for the BEE, and is known to be depleted in patients with PEM [9, 33, 34]. The BCM is thus considered to be a highly reliable parameter of the nutritional status, especially for patients undergoing LT, who usually have abundant extracellular fluid, such as edema and ascites. A low BCM in patients with LC suggests a decrease in the skeletal muscle volume, which could interfere with early post-operative mobilization and result in pulmonary complications, including aspiration pneumonia and atelectasis [7, 12].

In the nutritional assessment for patients undergoing LDLT, Kaido et al. [10] used a direct segmental multi-frequency bioelectrical impedance analysis (BIA) with the InBody720 (Biospace, Tokyo, Japan) device, which allowed the body mass index, intra- and extracellular water and body fat percentage to be automatically measured within two minutes. The SMM was measured and shown as a percent against a standard SMM calculated based on the sex and height of each patient. The BCM was automatically calculated by the InBody 720 and displayed against a normal range (e.g., 23.0–28.1 kg).

Bioelectrical impedance analysis measures the body's resistance to flow (impedance) of alternating electrical current at a designated frequency between points of contact on the body. The water in body tissue is conductive; therefore, BIA can indirectly provide information on the body's tissue contents, including the total body water, fat-free mass and SMM, and is accurate in over-hydrated subjects [7, 10]. BIA is increasingly being used because it is easy to perform, portable, non-invasive and quick. It has been highly correlated with hydrostatic weighing, dual energy X-ray absorptiometry, in vivo neutron activation analysis and deuterium isotope dilution, without the radiation exposure hazards [35–38]. BIA is thought to be superior to measuring the psoas muscle cross-sectional area at the L_3 vertebral level by computed tomography (CT) scanning or magnetic resonance imaging (MRI), since it might be more appropriate to evaluate not only the psoas muscle mass, but also the whole body SMM. Other frailty parameters of skeletal muscle function, such as the handgrip strength, walking speed or levels of exhaustion can be used in conjunction with BIA [39, 40].

Considering the feasibility, the current guidelines of the European Society for Clinical Nutrition and Metabolism (ESPEN) recommend only SGA and/or anthropometry parameters to identify patients at risk for poor nutritional status, and recommend that BIA should be used to quantify undernutrition, in spite of the limitations of all techniques in patients with ascitic decompensation [41, 42]. According to the ESPEN, other composite nutrition scores provide no additional prognostic information [41, 42].

Nutritional therapy before liver transplantation

The main goals of pre-LT nutritional therapy are to prevent further nutrient and muscle depletion and to correct any vitamin and mineral deficiencies present to minimize the risk of infections and debility [25]. An early, planned, preoperative nutritional intervention can be performed in most cases of LDLT, since the date of LT is known in