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Induction of CD4⁺ T Cell Apoptosis as a Consequence of Impaired Cytoskeletal Rearrangement in UVB-Irradiated Dendritic Cells

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Low dose UVB irradiation of dendritic cells (DC) dose-dependently decreases their allostimulatory capacity and inhibits alloreactive T cell proliferation. The reduction of the stimulatory capacity is not associated with a perturbation of CD28 costimulation. To examine the underlying mechanism, cell cycle analysis of T cells from cocultures with UVB-irradiated DC (UVB-DC) was performed, revealing no cell cycle arrest, but an increased number of apoptotic T cells in sub-G₀ phase. We confirmed T cells to undergo apoptosis after coinoculation with UVB-DC by TUNEL staining and DNA laddering. To analyze whether T cell apoptosis requires the Fas/Fas ligand (FasL) pathway, MLRs were performed with Fas-, FasL-deficient, and wild-type DC and T cells. No differences were found on comparison of wild-type DC with Fas-/FasL-deficient DC or T cells. Likewise, addition of a neutralizing anti-TNF-α mAb to cocultures could not overcome inhibition of T cell proliferation by UVB-DC, excluding involvement of the TNF-α/TNF-αR pathway. FACS analysis of CD69 and CD25 revealed no up-regulation on T cells cocultured with UVB-DC, suggesting a perturbation of early T cell activation. Analysis of UVB-DC by confocal microscopy demonstrated impaired filamentous actin bundling, a process critical for T cell stimulation. To investigate the functional relevance of these observations, time lapse video microscopy was performed. Indeed, calcium signaling in CD4⁺ T cells was significantly diminished after interaction with UVB-DC. In conclusion, UVBR of DC impairs their cytoskeletal rearrangement and induces apoptosis in CD4⁺ T cells by disruption of early DC-T cell interaction, resulting in a reduced Ca²⁺ influx in T cells. The Journal of Immunology, 2003, 171: 776–782.

Dendritic cells (DC)³ are the most potent APC for the initiation of primary T cell-mediated immune responses by naive CD4⁺ and CD8⁺ T cells (1, 2). Complete T cell activation requires a first, Ag-specific, and a second, costimulatory signal by APC resulting in a proliferative T cell response (3). Fully activated T cells will up-regulate the activation marker CD69, IL-2 and the IL-2 receptor, CD25 (4). DC-T cell interactions in the absence of a costimulatory signal lead to a state of unresponsiveness in T cells, that means they become tolerant toward the presented Ag (5, 6).

UVB irradiation (UVBR) not only has carcinogenic potential by inducing skin tumors but also has immunosuppressive effects, in that UVBR alters the APC function of the principal epidermal DC, the Langerhans cell (LC), in vitro and in vivo (7–9). Specifically, low dose UVBR of LC in vivo suppresses the development of contact hypersensitivity. Furthermore, UVBR interferes with the APC function of LC in vitro and induces Ag-specific tolerance in CD4⁺ T cells by inhibition of costimulatory molecule up-regulation (10–13).

Recently, we have shown that the stimulatory capacity of DC is abrogated by low dose UVBR (14). UVB-irradiated DC (UVB-DC) lose their capacity to induce primary and secondary T cell responses. However, UVB-DC only induced Ag-specific tolerance in primed Ag-specific CD4⁺ T cells from the Th1 clone AE7 but were not capable to tolerate naive allogeneic and naive Ag-specific CD4⁺ T cells (14).

Here, we wanted to examine possible mechanisms underlying the proliferation-inhibiting effects of UVB-DC on naive CD4⁺ T cells. We show that UVBR of DC interferes with their cytoskeletal rearrangement and thereby causes apoptosis of CD4⁺ T cells by disruption of early DC-T cell interaction, resulting in a reduced Ca²⁺ influx in T cells.

**Materials and Methods**

**Animals**

Wild-type BALB/c (H-2b), wild-type C57BL/6 (H-2b), Fas ligand (FasL)-deficient gld-C57BL/6 (H-2b), Fas-deficient ly-1-C57BL/6 (H-2b) mice (15), and DO11.10 mice (H-2d) (all 6–12 wk old), which carry a transgenic TCR for aa 323–339 of OVA peptide SIINFEKL (16), were obtained from the animal facility of the Max-Planck-Institute for Immunobiology (Freiburg, Germany).

**Media and reagents**

Complete RPMI 1640 (cRPMI) was supplemented with 10% (v/v) heat-inactivated FCS and 1% (v/v) penicillin-streptomycin (100×) (all from Life Technologies, Eggenstein, Germany). Recombinant murine IL-4 and...
mAbs with specificity for the following murine Ags were used: anti-I-A\(^d\) (AF6-120.1, mouse IgG2a (mlgG2a)); anti-CD3 (145-2C11, hamster IgG (hlgG)); anti-CD4 (RM-4-5, rat IgG2a (rIgG2a)); anti-CD8 (53-6.7, rIgG2a); anti-CD11c (HL3, hlgG); anti-CD16/CD32 (2.4G2, rIgG2b); anti-CD25 (7D4, rat IgM); anti-CD28 (37.51, hlgG); anti-CD40 (3/23, rIgG2a); anti-CD45R/B220 (RA3-6B2, rIgG2a); anti-CD54 (3E2, hlgG); anti-CD69 (H.12F3, hlgG); anti-CD80 (1G10, rIgG2a); anti-CD86 (GL1, rIgG2a); anti-CD95 (Jo2, hlgG); anti-CD95L (Kay-10, mlgG2b); anti-Ly-6G\(^-\)Gr-1 (RB6-8C5, rIgG2c); anti-MAC-3 (M3/84, rIgG1); and control mlgG2b, rIgG, and hlgG mAb (all BD Pharmingen, Hamburg, Germany). Polyclonal Dynabead-conjugated Ab with specificity for murine CD4, CD8, and CD45R/B220 were purchased from Dynal (Hamburg, Germany).

**Cell culture**

DC were generated from bone marrow cell suspensions cultured in rPMA, GM-CSF (40 ng/ml), and IL-4 (10 ng/ml) as described previously (15). For the experiments, DC were used on day 6 after initiation of culture. Bulk lymph node cells containing 80–90% CD5^+^ T cells and 10–20% CD5^-^ B cells were freshly isolated from lymph nodes and responded only poorly to PHA (2.5 μg/ml; Sigma-Aldrich, Munich, Germany).

**UVBR**

Low dose UVBR of DC, resuspended in PBS, was conducted with four unfiltered FS20 fluorescent tubes (band broad spectrum, 250–400 nm; peak at 365 nm; Cathay, Pittsburgh, PA) placed 46 cm above the target as described (15). UVBR was administered as a single dose.

**Clostridium difficile toxin B treatment**

DC were harvested on day 6 and preincubated with 100 mM endotoxin-tested *C. difficile* toxin B (kindly provided by Dr. Pfleifer, Department of Pharmacology, University of Freiburg, Freiburg, Germany) in PBS on ice for 1 h as described previously (17). Cells were then washed with PBS and cocultivated with T cells as described.

**T cell proliferation assay (MLR)**

DC (C57BL/6; 1 × 10^5^) were irradiated with 200 mJ/m^2^ UVB or left untreated. After being washed with PBS, cells were fixed with 4% paraformaldehyde-PBS for 30 min at 25°C and then permeabilized and blocked with 0.1% Tween 20 and 4% BSA in PBS. F-actin was fixed in 4% paraformaldehyde-PBS for 30 min at 25°C. Cells were stained with FITC-conjugated mAb against CD4 or CD3 as described between DC and T cells, cells were resuspended in MACS buffer mixed with cRPMI. After further preparation of the samples, cell surface Ags were stained with FITC-conjugated mAb against CD4 or CD3 as described above. Cells were then fixed with 70% (v/v) ethanol under permanent vortexing using 1 ml of ethanol for up to 1 × 10^6^ cells. After a centrifugation step and addition of RNase A (10 μg/100 μl), PI was added to the pellet in a concentration of 1/100. Cells were then analyzed with a FACScan and CellQuest software.

**Filamentous actin (F-actin) staining and laser-scanning confocal microscopy**

Bone marrow-derived DC were cultured on a coverslip and irradiated with 2000 mJ/m^2^ UVB or left untreated. After being washed with PBS, cells were fixed in 4% paraformaldehyde-PBS for 30 min at 25°C and then permeabilized and blocked with 0.1% Tween 20 and 4% BSA in PBS. F-actin was stained with 0.5 μg/ml rhodamine-conjugated phalloidin (Sigma-Aldrich) for 30 min at 25°C. Microscopy was performed with a LSM 510 laser-scanning confocal microscope and captured images were processed and superimposed by using the LSM Image Examiner software package (both from Zeiss, Jena, Germany). Twelve-bit images of 20 cells of 5 randomly selected areas were acquired at their greatest diameter and averaged over 8 scans at a pixel scaling of 60 × 60 nm.

**Time lapse video microscopy**

DO11.10 CD4^+^ TCR-transgenic T cells were labeled for 20 min at 37°C in 2 ml of medium containing 1 μM 1-[(2-(5-carboxyoxoazol-2-yl)-6-amino-nobenzofuran-5-oxyl)-2-(2′-aminomethyl)phenoxy]ethan-N,N,N′,N′,N′-tetraacetate, pentacetoxymethyl ester (fura 2-AM; Molecular Probes, Leiden, The Netherlands), and washed twice in PBS. Time lapse video microscopy was performed using a TILL-Photonics digital video imaging system consisting of a TILL-imago CCD camera and a Polychrome II monochromator (TILL Photonics, Munich, Germany) connected to an IMT-2 inverted microscope (Olympus, Hamburg, Germany). The cells were maintained in a heated (37°C) incubation chamber under sterile conditions in RPMI 1640. Ca^{2+} influx was calculated using the TILLvision V3.03 software deducting the OD_{355} from the OD_{380} (free fura anions) at an excitation wavelength of 480 nm.

**Statistical analysis**

Values are expressed as the mean ± SEM. The significance of differences between values was assessed using ANOVA followed by Bonferroni’s post test.

**Results**

**Low dose UVB-DC inhibit T cell proliferation independent of B7/CD28 costimulation**

It is well established that low dose UVBR of human and murine LC, the principal DC of the epidermis, inhibits proliferation of allogeneic and Ag-specific T cells (12). This inhibition of T cell proliferation could be linked to a perturbed up-regulation of the B7-costimulatory molecules, CD80 and CD86, on LC, because exogenous addition of a stimulatory anti-CD28 mAb restored T cell proliferation (10, 12, 13, 19, 20). Recently, we have shown low dose UVB-irradiated murine bone marrow-derived DC to inhibit allogeneic T cell proliferation. However, the surface expression of the B7-costimulatory molecules on DC was not affected by UVBR (15). Therefore, we asked now whether B7 expression on DC was functionally perturbed by UVBR. To address this issue,
primary, one-way MLR were performed using unirradiated DC or low dose UVB-DC as stimulators and allogeneic CD4$^{+}$ lymph node cells as effectors. As shown in Fig. 1, low dose UVBR of DC inhibited T cell proliferation considerably when compared with unirradiated DC. Furthermore, exogenous triggering of CD28 by addition of a stimulatory mAb for CD28 to cocultures of UVB-DC and T cells did not reverse the functional inhibition of the stimulatory capacity of DC. Comparable results were obtained when purified CD4$^{+}$ lymph node T cells were used as effectors (data not shown). Thus, murine DC respond differently to low dose UVBR than LC because their reduced stimulatory capacity is independent of B7/CD28 costimulation.

Low dose UVBR DC induce apoptosis in T cells

To analyze the underlying mechanism for the T cell unresponsiveness, apoptosis induction in T cells was examined after coculture with UVB-DC. First, T cell apoptosis was tested by TUNEL (21). Time course experiments from 0 to 96 h were performed on CD4$^{+}$ T cells from cocultures with unirradiated or UVB-irradiated DC (Fig. 2). After 72 h, the number of TUNEL-positive T cells was peaking, and no further increase could be detected after 96 h (Fig. 2). In detail, after coculture with unirradiated DC for 72 h, 21% of T cells were TUNEL positive. Importantly, depending on the dose of UVBR administered, cocultures with UVB-DC induced 40–44% TUNEL-positive T cells, suggesting that the abrogated proliferation in CD4$^{+}$ T cells relates to the induction of apoptosis. As a positive control, MLR were coincubated with 10 $\mu$g/ml of the proapoptotic anti-Fas mAb Jo2 overnight, inducing 42% TUNEL-positive T cells (Fig. 2) (22). Because the process of apoptosis leads to DNA fragmentation, T cells from cocultures were analyzed for DNA fragmentation by gel electrophoresis (Fig. 3). T cells cocultured with unirradiated DC showed no DNA laddering, in contrast to T cells cocultured with UVB-DC, confirming the induction of T cell apoptosis by UVB-DC. Furthermore, cell cycle analysis was performed on T cells from UVB-DC cocultures. Proliferating T cells are in M-S phase, resting T cells in G0-G1 phase, and apoptotic T cells in sub-G0 phase. As shown in Fig. 4, after 96 h of coculture with unirradiated DC, 8.5% of T cells were apoptotic in sub-G0 phase, whereas 24

FIGURE 1. UVBR of DC abrogates the strong proliferative response of allogeneic T cells independent of B7/CD28 costimulation (Stim.). Bulk lymph node cells ($1 \times 10^6$) were cocultured in a MLR with $1 \times 10^5$ cells of either unirradiated DC or UVB-DC, irradiated with 200 J/m$^2$, ± a neutralizing CD28 mAb as detailed in Materials and Methods. MLR were pulsed with 1 $\mu$Ci of [3H]Tdr on day 5 and harvested 16–24 h later. Proliferation was measured in a scintillation counter. A representative of five independent experiments is shown. *, p < 0.001 compared with the untreated control.

FIGURE 2. Dose dependence of T cell (TC) apoptosis during coculture with UVB-DC. T cells were cocultured with untreated, 100 J/m$^2$, and 200 J/m$^2$ UVB-irradiated DC at a DC:T cell ratio of 1:20 for the time points indicated. Alternatively, DC and T cells were coincubated 16 h before harvesting with 10 $\mu$g/ml of the proapoptotic anti-Fas mAb Jo2 as a positive control. The cells were TUNEL labeled as described in Materials and Methods and analyzed by FACS for the percentage of FITC-dUTP-labeled, apoptotic T cells. A representative of three experiments is shown. FL-1, fluorescence-1.
and 33% were apoptotic after coculture with UVB-DC irradiated with 100 or 200 J/m², respectively. Similar observations were made after 16 h of coculture of the MLR with 10³ H9262 g/ml of the proapoptotic anti-Fas-Ab Jo2 (Fig. 4).

Fas/FasL and TNF-α/TNFFR signaling pathways are not involved in T cell apoptosis induced by UVB-DC

A major apoptosis pathway is activation-induced cell death (AICD). AICD is mediated by Fas-FasL and TNF-α/TNFFR interactions and is an important mechanism to avoid uncontrolled immune responses (23). In a series of experiments, MLR were performed with Fas-, FasL-deficient, and wild-type DC and T cells (14) (Fig. 5A). Unirradiated wild-type DC stimulated wild-type T cells irrespective of their genetic background which was significantly reduced when DC were UVB irradiated. When gld-DC or gld-T cells both lacking FasL function were used, similar results were obtained (Fig. 5A). Comparable findings were also made using lpr-T cells or lpr-DC both lacking Fas function (Fig. 5A), suggesting UVBR to inhibit the allostimulatory capacity of DC independent of the Fas-FasL pathway. To
confirm that T cell apoptosis induction by UVB-DC was independent of the Fas-FasL pathway, cell cycle analysis of wild-type T cells from cocultures with FasL-deficient DC was performed (Fig. 5B). After 72 h of coculture with unirradiated FasL-deficient gld-DC, ~20% of T cells were in sub-G1 phase compared with almost 70% after coculture with UVB-DC deficient in FasL function. Similar results were obtained after 96 h of coculture with gld-DC, and when FasL-deficient T cells from cocultures with wild-type DC were analyzed (Fig. 5B). To explore the influence of the TNF-α-TNFR pathway in UVB-dependent T cell apoptosis, a neutralizing anti-TNF-α mAb was added to the cocultures of UVB-DC and T cells. However, addition of this Ab could not overcome inhibition of T cell proliferation by UVB-DC, indicating T cell apoptosis not to be mediated by the TNF-TNFR pathway (Fig. 6). Taken together, AICD mediated by Fas-FasL and TNF-α-TNFR pathways is not involved in T cell apoptosis induced by UVB-DC.

**UVBR of DC interferes with early activation of CD4+ T cells**

CD69 and CD25, the IL-2α receptor, are surface markers on T cells that are involved in the process of early T cell activation. CD69, a C-type lectin, is up-regulated as early as 30 min after T cell activation. Cross-linking induces secretion of IL-2 and up-regulation of its receptor, CD25 (24). Therefore, we wished to know whether the up-regulation of CD69 and CD25 on T cells is affected after coculture with UVB-DC. To address this issue, FACS for CD69 and CD25 expression was performed on T cells cocultured with unirradiated DC and UVB-DC. As shown in Fig. 7A, unirradiated DC induced on 52% of T cell CD25 expression after 72 h of coculture, whereas UVB-DC failed to up-regulate the early activation marker CD25 on T cells to this extent. Fig. 7B depicts a decrease of CD69 expression on TC from 43% after coculture with unirradiated DC to 21% with DC UVB 100 J/m² and 25% after incubation with DC UVB 200 J/m². We conclude that these essential signals for a proliferative response after an IL-2 stimulus can no longer be given after UVBR of the APCs. A very early event occurring within the first seconds during DC-T cell interactions is an increase in the Ca²⁺ concentration inside the T cell (25). Ca²⁺ influx in CD4+ T cells was investigated by a time lapse video microscopy system using OVA peptide-specific TCR-transgenic naïve CD4+ T cells from DO11.10 mice that respond similarly to UVBR as allogeneic naïve CD4+ T cells (Ref. 15 and 26 and data not shown). Three different types of Ca²⁺ influx profiles of T cells are observed after contact with peptide-loaded DC (Fig. 8, left). Preapoptotic massive calcium influx (Fig. 8A), null events (Fig. 8B) and normal Ca²⁺ signals were found (Fig. 8C). The proportion of these events with respect to the total number of interactions was quantified for T cell contacts with UVB-DC (Fig. 8, right, ■) and unirradiated DC (Fig. 8, right, □). After coculture with UVB-DC, the number of contacts resulting in normal Ca²⁺ signaling is strongly reduced (Fig. 8C). Moreover, some representative results are shown. Stim., Costimulation.

**FIGURE 6.** UVB-irradiated DC inhibit T cell proliferation independent of TNF-α. UVB-irradiated (200 J/m²; ■) or unirradiated DC (□) were cocultured with allogeneic T cells in a DC:T cell ratio of 1:10. Then, 100, 500, or 2500 μg/ml of an anti-TNF-α mAb was added to the MLR as indicated. After 96 h, the cells were pulsed with 1 μCi [3H]Tdr for the final 18 h of coincubation, and the T cell proliferation was determined in a scintillation counter. Mean values were taken of triplicate measurements. A representative experiment of three is shown. Stim., Costimulation.

**FIGURE 7.** UVB-DC failed to induce expression of the early activation marker CD25 and CD69 on T cells (TC). The expression levels of the activation markers CD25 (A) and CD69 (B) on T cells cocultured for 72 h with unirradiated or 100 or 200 J/m² UVB-irradiated DC were analyzed by FACS. CD25 or CD69*CD3* T cells were gated as indicated in the dot plot. Dead cells were excluded by 7-aminoactinomycin staining. A representative of three independent experiments is shown. FL, Fluorescence.

**FIGURE 8.** UVB irradiation of DC shifts the Ca²⁺ responses in CD4+ T cells to preapoptotic signals. CD4+ TCR-transgenic T cells were labeled with fura 2-AM, and Ca²⁺ influx during cell-cell contacts with unirradiated or 200 J/m² UVB-irradiated DC were analyzed using a time lapse video microscopy system. DC-T cell contacts were sorted into three categories, depending on the resulting Ca²⁺ signal. A, Preapoptotic signals resulting in a breakdown of the membrane potential and massive Ca²⁺ influx; B, null events without any detectable Ca²⁺ changes; C, regular Ca²⁺ influx required for T cell activation. Statistical analysis of four independent experiments is depicted in the diagrams on the right. Bars represent the percentage of all events ± SEM for each category for untreated DC (□) or UVB-DC (■).
Indeed, toxin B-treated DC increased the number of TUNEL-positive T cells to 43%, similar to the effect of UVBR (46%), compared with the untreated control (29%) (Fig. 9C). Further, toxin B treatment of DC resulted in reduced T cell proliferation, similar to the effect observed with UVB-DC (data not shown). Taken together, these findings suggest that UVBR interferes with F-actin bundling in DC, critically affecting early T cell activation steps.

**Discussion**

In this study, we have shown that low dose UVBR inhibits the allostimulatory capacity of bone marrow-derived DC independent of B7/CD28 costimulation, because the exogenous addition of a stimulatory mAb for CD28 could not restore T cell proliferation. Previously, we demonstrated that the surface expression of MHC class II and costimulatory molecules, i.e., the B7 molecules CD80 and CD86, on DC is not affected by UVBR (15), in contrast to our findings with epidermal LC in that UVB-irradiated LC have a reduced expression of the B7-costimulatory molecules that can be overcome by exogenous CD28 costimulation (10). Whereas the Ag presentation of UVB-irradiated LC lacking a sufficient costimulatory signal leads to T cell tolerance (10, 12, 13, 19, 20), the reduced T cell response observed in coculture with bone marrow-derived UVB-DC is induced by a different mechanism.

In contrast to LC, UVB-DC dose-dependently induce T cell apoptosis after coculture, as determined by TUNEL, DNA laddering, and cell cycle analysis. The two major pathways involved in T cell apoptosis are AICD, an active process mainly mediated by the Fas-FasL and TNF-α-TNFRI pathways (23), and death by neglect, where the default apoptotic machinery is engaged in the absence of sufficient stimulatory factors, such as IL-2 (29). We have shown that both the Fas-FasL and TNF-α-TNFRI pathways are obviously not involved in this process. Therefore, AICD is unlikely to account for our observations. Conversely, UVBR of DC inhibited the up-regulation of the T cell activation markers CD69 and CD25 after their coculture, suggesting that UVBR interferes with early T cell activation. A very early event that occurs during DC-T cell interactions is an increase in the Ca$^{2+}$ concentration inside the T cell. This step is followed by the accumulation of MHC and costimulatory molecules to the contact point between the T cell and the APC. Recent studies using specific inhibitors of F-actin polymerization, like cytochalasin D and botulinum toxin, have shown that the active reorganization of the cytoskeleton on the DC surface is critically involved in T cell activation and proliferation (27, 28).

Interestingly, we found that UVBR of DC not only interferes with F-actin bundling but also leads to a massive Ca$^{2+}$ influx in ~10% of DC-T cell interactions. A role of sudden Ca$^{2+}$ liberation for the initiation of apoptosis has been inferred by several publications, e.g., by Macek et al., who proved calcium ionophores to cause apoptosis in a human leukemia T cell line (30, 31). We could show that specific inhibition of F-actin bundling in DC by toxin B leads to an increase of apoptosis in the cocultivated T cells, comparable with the induction of apoptosis by UVB-DC. Thus, inhibition of F-actin bundling of DC by UVBR might be a critical factor leaving the T cells more vulnerable to apoptosis.

However, there might be other contributing factors for UVB-induced T cell apoptosis, i.e., the secretion of immunosuppressive IL-12 p40 homodimers from DC after UVBR (32). Furthermore, injection of anti-IL-10 into UVB-irradiated mice reversed an UVB-induced APC defect, indicating that the production of IL-10 might be responsible for the impaired APC function (33). In vitro we could not confirm a regulatory role for IL-10 in cocultures of UVB-DC with naive, allogeneic, or syngeneic CD4$^+$ T cells (15). Also CTLA-4 is believed to be a negative regulator of T cell activation. CTLA-4 signaling blocks IL-2 production, CD69 and
CD25 expression, and cell cycle progression of activated T cells, eventually resulting in T cell apoptosis (34, 35). We considered the fact that CTLA-4 could be responsible for UVB-induced T cell apoptosis. However, FACS did not reveal an up-regulation of CTLA-4 on T cells cocultured with UVB-DC which makes CTLA-4 unlikely to play a role in UVB-induced T cell apoptosis (data not shown).

In conclusion, low dose UVB-DC inhibit T cell proliferation independent of B7/CD28 costimulation and induce apoptosis in CD4+ T cells by incomplete early T cell activation and not by AICD. These findings may be of interest for immunotherapeutic applications, e.g., in autoimmune diseases, allergy, and transplantation.

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