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CUTTING EDGE

Cutting Edge: Immunosuppressant as Adjuvant for Tolerogenic Immunization

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Vaccination for autoimmune and alloimmune diseases has long been an attractive idea. Yet, there is no suitable adjuvant to forcefully steer the immune response toward tolerance. In this study we show that dexamethasone, a potent glucocorticoid immunosuppressant, can function as a tolerogenic adjuvant when applied together with peptide immunogen. BALB/c mice with pre-established delayed-type hypersensitivity to hen OVA were immunized with an OVA-derived, MHC II-restricted peptide (OVA323–339) in the presence of dexamethasone. The treatment caused long-term desensitization in treated animals to hen OVA via a dexamethasone-dependent tolerogenic mechanism that blocks maturation of dendritic cells and expands OVA323–339-specific CD4+CD25+Foxp3+ regulatory T cells in vivo. Similar treatment of NOD mice using dexamethasone and an insulin-derived, MHC II-restricted peptide (B9–23) prevented predisposed spontaneous diabetes. Remarkably, in both models, dexamethasone-augmented immunization induced long-term persistent, Ag-specific regulatory T cells responsive to recall Ags. These results reveal for the first time the potential usefulness of immunosuppressants as tolerogenic adjuvants. The Journal of Immunology, 2008, 180: 5172–5176.

Immunosuppressive drugs have long been used for treating allergy, autoimmune disease, and transplant rejection (1). Although effective, they only temporally dampen but do not permanently modify the immune responses toward pathogenic recall Ags and thus are not a cure. However, these drugs are generally anti-inflammatory, a condition that has been hypothesized to favor immune tolerance (2). To explore this drug-induced condition for the induction of Ag-specific tolerance, we devised a method of “suppressed immunization” where a glucocorticoid immunosuppressant, dexamethasone (DEX), is administered together with a peptide immunogen during the course of active immunization. In this report we show that DEX indeed induces tolerogenic responses to active immunization that are associated with preferential expansion of Ag-specific regulatory T cells (Treg) directly in vivo.

Materials and Methods

Mice and reagents

DO11.10 TCR-transgenic BALB/c mice were bred in the University of Illinois College of Medicine at Rockford (Rockford, IL) animal facility. Other mice were from The Jackson Laboratory. All animals were maintained in pathogen-free rooms and used in accordance with the institutional guidelines for animal care. The OVA323–339 (ISQAVHAAHAEINEAGR) and B9–23 (SHLVEALYLVCGER) peptides were from AnaSpec, DEX, hen OVA (grade VII), and hen lysozyme were from Sigma-Aldrich. IFA was from Pierce Biotechnology. All Abs were from eBioscience. The mouse CD4+ T cell negative selection kit was from Miltenyi Biotec. CFSE was from Invitrogen.

Ag sensitization

OVA in IFA was injected subcutaneously into BALB/c mice (100 µg/mouse) twice in a 2-wk interval. The mice were also similarly sensitized for hen lysozyme. Delayed-type hypersensitivity (DTH) to either sensitizing Ag was determined by rechallenge at a footpad (10 µg/injection) and measuring the net increase in footpad thickness (swelling) at 24 h.

Suppressed immunization

Mice were injected four times (on days 1, 4, 7, and 10) with DEX in the two hind footpads (8 µg/footpad). For the day-7 injection, OVA323–339 (1 µg/footpad) was coinjected with DEX. This regimen was given twice in a 2-wk interval.

Analysis of Treg in the blood

White cells from tail blood were blocked with anti-CD16/32 mAb, immunostained for CD4 and CD25, fixed (1% paraformaldehyde) and permeabilized (0.5% Tween 20), and intracellularly immunostained for Foxp3. CD4+CD25+Foxp3+ Treg were counted relative to total CD4+ cells by flow cytometry. To determine the Ag specificity of blood Treg, white cells were labeled with CFSE (Invitrogen) and stimulated in a 96-well plate with the immunizing peptide (10 µg/ml) or an irrelevant peptide as control in the presence of syngeneic accessory cells (2.5 × 104/well) and IL-2 (800 IU/ml). Three days later, cells were stained for CD4, CD25, and Foxp3 as described above and analyzed by CFSE dilution by flow cytometry, gating on Treg.

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Analysis of dendritic cells (DC) and T cells in draining lymph nodes (LN)

Total cellular content of popliteal LN was blocked with anti-CD16/32 mAb, immunostained with anti-CD11c, anti-CD83, and anti-CD86 mAbs (for DC analysis) or with anti-CD4, anti-CD25, and anti-Foxp3 mAbs (for T cell analysis), and analyzed by flow cytometry. To detect IL-10+ DC, LN cells were stained with anti-CD11c, fixed (1% paraformaldehyde), permeabilized (0.5% Tween 20), and intracellularly immunostained for IL-10.

In vivo proliferation assay

CFSE-labeled DO11.10 TCR-transgenic CD4+ T cells (2.5 \times 10^6/footpad) were coinjected with OVA323–339 (1 µg) into a footpad of BALB/c mice that had been pretreated with DEX on days 6–9. CFSE dilution was analyzed for CFSE dilution by flow cytometry, gating on CD4+ cells. On day 8, cells were stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry.

In vitro suppression assay

The assay was performed as previously described (3).

Treatment of NOD mice

Female NOD mice (6 wk of age) were injected four times (on days 1, 4, 7, and 10) with DEX in the two hind footpads (8 µg/footpad). For the day-7 injection, B-25–23 (1 µg/footpad) was coinjected with DEX. This regimen was given twice in a 2-wk interval. Glycercroat was checked weekly using Diastix strips (Bayer). Mice tested positive (>250 mg/dl) twice consecutively were deemed diabetic.

Analysis of Treg in the spleen

Mice were rechallenged (day 0) at a footpad with the immunizing peptide emulsified in IFA (50 µg/footpad). On day 5, splenic CD4+ T cells were isolated, labeled with CFSE, and stimulated in a 96-well plate with the immunizing peptide (10 µg/ml) or an irrelevant peptide (as control) in the presence of naive syngeneic accessory cells (2.5 \times 10^5/well) and IL-2 (8000 IU/ml). On day 8, cells were stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry. The observed cell division consisted of OVA323–339-specific Treg, because Treg count consisted of OVA323–339-specific Treg, because Treg compared with that of the non-treated mice (indicated as “Control”), and OVA323–339 Treg. White cells were labeled with CFSE, stimulated in culture with OVA323–339, and analyzed for CFSE dilution (cell division) by flow cytometry, gating on CD4+CD25+ cells (Treg). The observed cell division was Ag dependent because no CFSE dilution was seen in the absence of OVA323–339 (data not shown). Bar, mean and SD of two or three independent experiments, each using six mice per group (n = 6); *p < 0.05 between the indicated pair.

Results and Discussion

To test the idea of suppressed immunization, we first sensitized BALB/c mice for hen OVA. Using the resulting DTH to OVA as a model for recall response, we next tested suppressed immunization in these mice using an OVA-derived, MHC II-restricted peptide (OVA323–339) (4) as immunogen and DEX as adjuvant (test group). Some of the DTH-positive mice were control immunized with either or both the immunosuppressant and the peptide replaced by PBS (control groups).

Two weeks after the completion of the immunization, all groups were retested for DTH. Although the control groups remained fully sensitive to the recall Ag, the test group was markedly desensitized to OVA (Fig. 1A). The desensitization was OVA specific because the test group showed normal DTH to an irrelevant protein, hen lysozyme (data not shown). Importantly, the test group remained hyporesponsive to OVA when rechallenged 4–5 mo later (Fig. 1B), suggesting the establishment of long-term tolerance to the Ag. Consistent with such tolerance, a blood sample taken from the test group at 48 h following the Ag rechallenge showed an elevated count of CD4+CD25+Foxp3+ Treg compared with that of the non-treated control group (Fig. 1C). At least part of the increased Treg count consisted of OVA323–339-specific Treg, because they proliferated in culture in response to OVA323–339 (Fig. 1D) but not to an irrelevant peptide (data not shown). In comparison, no such Treg were detected in the blood of the non-treated control group (Fig. 1D).

Treg expansion is known to be linked to the function of immature DC (5, 6) and DEX was previously reported to prevent DC maturation in vitro (7, 8), suggesting that DEX might help expand Treg by increasing immature DC. We therefore analyzed the change in the ratio of mature to immature DC in the draining LN (popliteal) following footpad injection of DEX and OVA323–339. CD83 is a maturation marker for DC (9, 10). Using CD83 in conjunction with CD86, a costimulator up-regulated in DC upon maturation, we were able to identify immature and mature DC as CD11c++CD83−CD86low and CD11c++CD83+CD86high cells, respectively. Initial experiments in BALB/c mice showed that DEX blocked DC maturation in the draining LN (data not shown). However, it was difficult to detect corresponding changes in Ag-specific Treg, as they represented only a tiny fraction of total Treg in the LN.

Consequently, to allow simultaneous observation of DC and Ag-specific Treg, we switched to DO11.10 TCR-transgenic mice, where OVA323–339-specific CD4+ T cells are abundant (4). Injecting the mice with DEX alone increased immature DC count while decreasing mature DC count, indicating that DEX blocks DC maturation in vivo (Fig. 2A). Injecting the mice with OVA323–339 alone, however, stimulated DC maturation, as was
indicated by an increase in the mature DC count and a decrease in the immature DC count. Coinjection of both DEX and OVA323–339 led to an effect similar to that of DEX alone, indicating the dominance by DEX. Additional experiments showed that DEX augmented the differentiation of IL-10 (IL-10*) DCs, which may at least partly account for the efficacy of suppressed immunization.

We further tested suppressed immunization in the NOD autoimmune diabetes model (13) using DEX and the insulin-derived, MHC II-restricted peptide Ag B:9–23 (14, 15). Although nontreated mice developed the disease with a median incidence of 19 wk, mice treated with either DEX or B:9–23 showed a delayed median incidence of 24.5 and 26 wk, respectively (Fig. 3A). In contrast, most mice treated with both DEX and B:9–23 remained disease-free during the entire period of the experiment (35 wk), indicating that suppressed immunization is effective for delaying or preventing the progression of autoimmune diabetes. Moreover, ~40% of the animals (5 of 12 mice) in this group lived for 61 wk without diabetes and, upon rechallenge of these animals with B:9–23, Ag-specific Treg could be detected in the spleen, the primary site of memory T cells (Fig. 3B). These Treg showed greater sensitivity to restimulation with B:9–23, but not with control Ag, in comparison with control Treg (naive Treg isolated from nonimmunized, prediabetic mice). They also dominated the proliferative response to the recall Ag over splenic Teff (Foxp3-), suggesting that these Treg might be able to elicit adaptive response to recall stimulation.

To ascertain that suppressed immunization can preferentially prime Ag-specific Treg during the immunization phase and is therefore responsible for the generation of the “memory-like” Treg, we monitored the appearance of Ag-specific Treg and Teff in two different groups of prediabetic NOD mice that had just been treated with either DEX and B:9–23 (test) or PBS (control). At the completion of the treatment, peripheral blood
We have shown in this study that DEX can function as a tolerogenic adjuvant when applied in conjunction with a peptide immunogen. The particular effect of DEX on Treg expansion is consistent with prior findings by Chen et al. that DEX alone could alter the ratio of CD4^+CD25^+ Treg to CD4^+CD25^- conventional T cells in vivo in favor of the former (16) and that DEX could also amplify IL-2-dependent expansion of functional CD4^+CD25^-Foxp3^+ Treg in vivo (17). The novel finding from the present study is that when combined with antigenic immunization, the use of DEX can lead to preferential induction of Treg that are Ag-specific and long-term persistent. Furthermore, based on the mechanism revealed here, one may speculate that DEX, or glucocorticoids in general, may not be unique in its ability to serve as a tolerogenic adjuvant. Other small-molecule immunosuppressive drugs, such as vitamin D₃ and analogues, cyclosporine A, FK506, and rapamycin, have been shown to have similar pharmacological effects on DC maturation and function (18) and might therefore also be useful for tolerogenic immunization. Lastly, Van Overtvelt et al. recently reported their search for IL-10-inducing adjuvants and the observation that a combination of 1,25-dihydroxyvitamin D₃ and dexamethasone augmented the efficacy of sublingual immunotherapy in a murine asthma model in association with the induction of CD4^+CD25^-Foxp3^- Treg (19). Both their work and ours support the notion of tolerogenic adjuvants.

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