T Cells Against the Pathogenic and Protective Epitopes of Heat-shock Protein 65 Are Crossreactive and Display Functional Similarity: Novel Aspect of Regulation of Autoimmune Arthritis

MALARVIZHI DURAI, HONG RO KIM, KAMALESH BALA, and KAMAL D. MOUDGIL

ABSTRACT. ***Objective.*** In autoimmune situations, the outcome of immune response against a disease-related antigen is typically viewed in terms of the balance between the pathogenic versus the protective subsets of antigen-reactive T cells. Using the rat adjuvant arthritis (AA) model of human rheumatoid arthritis (RA), we examined the antigen specificity and the functional attributes of the T cell repertoire directed against defined pathogenic versus protective epitopes of heat-shock protein 65 (hsp65), and determined the immunologic basis of the AA-protective effect of subsets of T cells primed by the pathogenic determinant.

Methods. Lewis (RT.1^{l}) rats were pretreated subcutaneously with the pathogenic epitope 177–191 of mycobacterial hsp65 (B177) in adjuvant (incomplete Freund's adjuvant/complete Freund's adjuvant/CpG) and then immunized with heat-killed *M. tuberculosis* H37Ra for disease induction. The antigen specificity/crossreactivity of the T cells primed by B177 or the AA-protective determinant 465–479 of the homologous rat hsp65 (R465) was tested by using proliferation assay, cytokine ELISA, tolerance induction, and adoptive transfer.

Results. Pretreatment of Lewis rats with the arthritogenic determinant B177 using an immunogenic rather than a tolerogenic regimen affords protection against AA instead of initiation or aggravation of AA. This protective effect of B177 is mediated in part by activation of T cells that are crossreactive with R465.

Conclusion. Downmodulation of AA by a pathogenic foreign epitope involving T cells crossreactive with a distant, protective self-determinant represents a novel aspect of immune regulation, and suggests further exploration of the use of pathogenic epitopes for the treatment of autoimmune arthritis. (First Release Oct 15 2007; J Rheumatol 2007;34:2134–43)

Key Indexing Terms:
ANIMAL MODELS ARTHRITIS AUTOIMMUNITY
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The T cells specific for heat-shock protein 65 (hsp65) have been implicated in the pathogenesis of autoimmune arthritis in experimental rodent models[1-8] as well as in patients with rheumatoid arthritis (RA)[9-12]. Using the adjuvant-induced arthritis (AA) model in the Lewis rat, it has been shown that the mycobacterial hsp65 (Bhsp65) possesses not only an arthritogenic epitope[1,2,13,14], but also various disease-protecting T cell determinants[3,4,6,15,16]. The epitope region 180–188 of Bhsp65 is believed to harbor the pathogenic determinant for the Lewis rat. A T cell clone (A2b), which was generated from splenic T cells of an arthritic Lewis rat and shown to recognize epitope 180–188 of Bhsp65, could adoptively transfer arthritis to naive recipient Lewis rats[1,2]. The region 176–190/177–191 of Bhsp65, which contains the amino acid (aa) sequence of the arthritogenic epitope 180–188, is the dominant T cell epitope responded to by Lewis rats after AA induction or following immunization with Bhsp65[3,16]. Further, this longer epitope is also recognized by the arthritogenic clone A2b specific for the shorter epitope 180–188[17,18]. In regard to the disease-protecting epitopes of Bhsp65, we have reported that the T cells primed by Bhsp65 C-terminal determinants (BCTD) can adoptively transfer protection against AA to recipient Lewis rats[3,6].

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Additional AA-regulatory T cell epitopes within Bhsp65[4,16] as well as its mammalian self-homologs [e.g., human hsp60 and rat hsp65 (Rhsp65)] have been identified by others[19] and by us[5].

The evolution of an experimentally induced autoimmune disease is generally viewed in the context of the relative predominance of the T cell reactivity to the pathogenic versus the protective/regulatory antigenic determinants of the disease-associated antigen in a "see-saw" type of profile, such that the response to the pathogenic epitope is dominant in the acute phase of the disease, whereas the response to the protective epitope(s) is relatively higher in the recovery phase of the disease[20-22]. Moreover, the quantitative and/or qualitative changes in the T cell response to the pathogenic antigen/epitope might influence the disease outcome (susceptibility/acute phase vs resistance/recovery) following a potentially pathogenic antigenic challenge[23-25]. Although these concepts have contributed extensively both to the understanding of the pathogenesis of autoimmunity and to the development of new modalities for immunomodulation, there are gaps in the knowledge pertaining to the immune interactions between specific T cell subsets directed against disease-related antigenic determinants involved in autoimmunity.

We describe a novel aspect of the pathogenesis of autoimmunity involving a potentially arthritogenic epitope 177–191 within Bhsp65 (B177) that is crossreactive with a protective determinant 465–479 of Rhsp65 (R465) located at an entirely different position within the homologous self-hsp65. Further, B177 and R465 lack any significant aa identity. However, as we reported for R465[6], immunization of Lewis rats with B177 in an adjuvant to facilitate the priming and expansion of specific T cells leads to protection against AA instead of induction or exacerbation of AA. The B177-induced protection against AA is attributable in part to the priming of T cell subsets that are shared with R465. Moreover, tolerization of the R465-reactive repertoire prior to B177 immunization abrogates the AA-protective effect of B177. These results provide new insight into the regulation of autoimmunity by interaction between T cells reactive against non-overlapping but crossreactive epitopes of homologous disease-related antigens.

MATERIALS AND METHODS

Rats. Male inbred Lewis (LEW/SsNHsd; RT.1[l]) rats (5–8 wks old, weight 150–200 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA) and housed at the University of Maryland School of Medicine, Baltimore. All procedures were performed following the guidelines of the institutional animal care and use committee.

Antigens/mitogen/adjuvants. Heat-killed *Mycobacterium tuberculosis* $H_{37}Ra$ (Mtb) was obtained from Difco Laboratories (Detroit, MI, USA). Synthetic peptides of Bhsp65 and Rhsp65 (named with a prefix B/R and the first aa number) were procured from Macromolecular Resources and Global Peptide Services (both at Fort Collins, CO, USA). Our study is based on B177 as well as several of the C-terminal peptides of Bhsp65/Rhsp65 (BCTD/RCTD, respectively). (1) B177 contains within its sequence the shorter peptide 180–188 representing the arthritogenic epitope of Bhsp65. Moreover, B177 and B180 are crossreactive. The arthritogenic T cell clone A2b has been shown to recognize peptide 176–190[2,18,26], which is a variant of B177. (2) In our previous work, we have shown that BCTD as well as RCTD induce T cells that afford protection against AA[3,5,6]. Therefore, we selected the following 5 BCTD and 5 RCTD peptides to test in our study: Bhsp65 417–431 (e.g., B417), 441–455, 465–479, 513–527, and 521–535; and Rhsp65 418–432 (e.g., R418), 441–455, 465–479, 512–526, and 521–535. The aa sequences of the 2 homologous peptides studied in detail are as follows: B177, *ESN TFG LQL ELT EGM*; R177, *DGK TLN DEL EII EGM*; B465, *KVR NLP AGH GLN AQT*; and R465, *VEK ILQ SSS EVG YDA*. Hen eggwhite lysozyme (HEL), Keyhole limpet hemocyanin (KLH), concanavalin A (ConA), and incomplete Freund's adjuvant (IFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). CpG ODN (oligodeoxynucleotides, = ODN 1980; 5'-TCC ATG ACG TTC CTG ACG TT-3') containing the CpG motif[27] was obtained from Genosys (The Woodlands, TX, USA). For *in vivo* use, the appropriate ODN was solubilized in Tris-EDTA buffer (pH 7.0), filtered, and diluted appropriately in phosphate buffered saline (PBS).

Lymph node cell (LNC) proliferation assay. Lewis rats were immunized with a peptide (100 µg/rat) in IFA [IFA was used instead of complete Freund's adjuvant (CFA) to avoid any concurrent contribution of Bhsp65 of Mtb within CFA to the activation of T cells]. On day 9, the draining LNC were cultured in a 96-well plate ($2.5–5 \times 10^5$ cells/well) in HL-1 serum-free medium (BioWhittaker, Walkersville, MD, USA) as described[5,6]. The results of ^{3}(H)-thymidine incorporation were expressed as counts per minute (cpm) or stimulation index (SI) (ratio of cpm in the presence of antigen and cpm without antigen).

Measurement of cytokine secretion by antigen-primed LNC. Lewis rats were immunized subcutaneously (sc) with B177 or R465 (100 µg/rat each), each emulsified in IFA. After 8 days, the draining LNC were harvested and restimulated *in vitro* with the appropriate peptide (B177/R465) for 48 h at 37°C[5]. HEL was used as an irrelevant recall antigen. After 48 h, the culture supernatants were collected and tested for interferon-γ (IFN-γ), interleukin 10 (IL-10), and transforming growth factor-ß (TGF-ß) using ELISA kits (R&D Systems, Minneapolis, MN or Biosource International, Camarillo, CA, USA). The lower detection limit for IFN-γ, IL-10, and TGF-ß was 13.0, 5.0, and 31.2 pg/ml, respectively.

Induction and clinical assessment of AA. Lewis rats were immunized sc with heat-killed Mtb (2 mg/rat) at the base of the tail, and then observed regularly for clinical signs of arthritis like erythema and swelling. The severity of arthritis was graded on a scale from 0 to 4 as described[5,6]. The highest score for each paw was 4 and the maximum total score (sum of scores of all 4 paws) for each rat was 16.

Pretreatment of Lewis rats with peptide 177–191 of Bhsp65 (B177) to determine its influence on subsequent AA. (1) B177/CpG ODN injection: Rats were immunized sc at the base of the tail with B177 (50 or 150 µg/rat) in CpG ODN (50 µg/rat). The control rats received PBS in CpG ODN. (2) B177/IFA injection: Lewis rats were immunized sc at the lower back twice at 1 wk interval with B177 (100 µg/injection/rat) in IFA, whereas the control rats were challenged with PBS/IFA. All rats were observed for 4 weeks for any signs of arthritis. Thereafter, these rats were injected sc with Mtb and then graded regularly for severity of arthritis.

In vivo adoptive transfer of B177-primed LNC and its effect on subsequent AA. Lewis rats were immunized sc with 100 µg/rat of B177 in IFA, whereas control rats received KLH/IFA. On day 9, the draining LNC (3×10^6 cells/ml) were cultured for 48 h in complete RPMI-1640 in the presence of ConA (2.5 µg/ml)[5,6]. Thereafter, these cells were collected, washed, and transferred (5×10^7 cells/rat) intravenously (IV) into naive Lewis rats. On the same day, the recipient rats received Mtb sc. All rats were then observed regularly for signs of arthritis.

Tolerization of Lewis rats with peptide B177/R465 and its effect on peptide-specific T cell response and clinical AA. Lewis rats were given intraperi-

tonealy (ip) peptide B177 or R465 (200 µg peptide/rat each) in solution (PBS) on days –9, –7, and –5 for tolerance induction[28], followed by a subcutaneous injection on day 0 of B177/IFA or R465/IFA (100 µg peptide/rat each). Eight days after the last injection, the draining LNC of these rats were harvested and tested in a proliferation assay. For testing the disease-modulatory activity of R465, a group of Lewis rats were first tolerized with soluble R465 or KLH (control) and then immunized sc on day 0 with B177/IFA or HEL peptide 65–78/IFA (control). After 2 weeks, all rats were injected with Mtb and observed regularly for signs of arthritis.

RESULTS

Activation of T cells directed against the pathogenic determinant region 177–191 of Bhsp65 prior to Mtb injection leads to the downmodulation of AA rather than the induction or aggravation of AA. Lewis rats were immunized with B177 emulsified either in CpG ODN or in IFA to test the arthritogenicity of this peptide. A group of Lewis rats immunized with PBS/CpG ODN or PBS/IFA served as respective controls. [Immunization with B177 in adjuvant instead of in solution was aimed at the activation of epitope-specific T cells instead of tolerizing (inactivating) them.] Further, the choice of the 2 adjuvants (CpG ODN vs IFA) was based on the likely priming of T cells presumably under distinct cytokine environments, e.g., a primarily Th1 milieu following CpG ODN injection[29,30] versus a predominantly Th2 environment upon IFA injection[31], anticipating that the cytokine milieu in turn might influence the outcome of antigenic challenge[29,31]. All rats were then observed for 4 weeks for any signs of arthritis.

None of the above-mentioned immunization regimens was arthritogenic during the 4 weeks of observation period (data not shown). Thereafter, these rats were challenged with Mtb to determine the effect of prior activation of B177-reactive T cells on the course of subsequently induced AA. The results show that pretreatment of Lewis rats either with B177/CpG ODN (Figure 1A) or with B177/IFA (Figure 1B) before Mtb injection afforded protection against subsequent AA instead of aggravation of AA compared to controls. However, the severity and course of AA in rats pretreated with PBS/CpG or PBS/IFA were comparable to that of rats that had not received any pretreatment prior to disease induction (data not shown). These results document that regardless of the type of adjuvant (CpG ODN vs IFA) used, the activation of T cells directed against B177 leads to protection against AA instead of induction of AA.

Adoptive transfer of B177-primed T cells into naive Lewis rats before Mtb challenge affords protection against AA. (In the above experiment, the immunogenicity of B177/IFA was comparable to that of rats challenged with B177/CpG ODN, and both regimens led to protection against AA. Therefore, we used the commonly employed IFA over CpG ODN for the subsequent experiments.) A group of naive Lewis rats were injected IV with LNC primed *in vivo* by B177/IFA followed by subcutaneous challenge with Mtb on the same day. The control group was given KLH/IFA-primed LNC IV prior to Mtb challenge. Another group of rats not given any

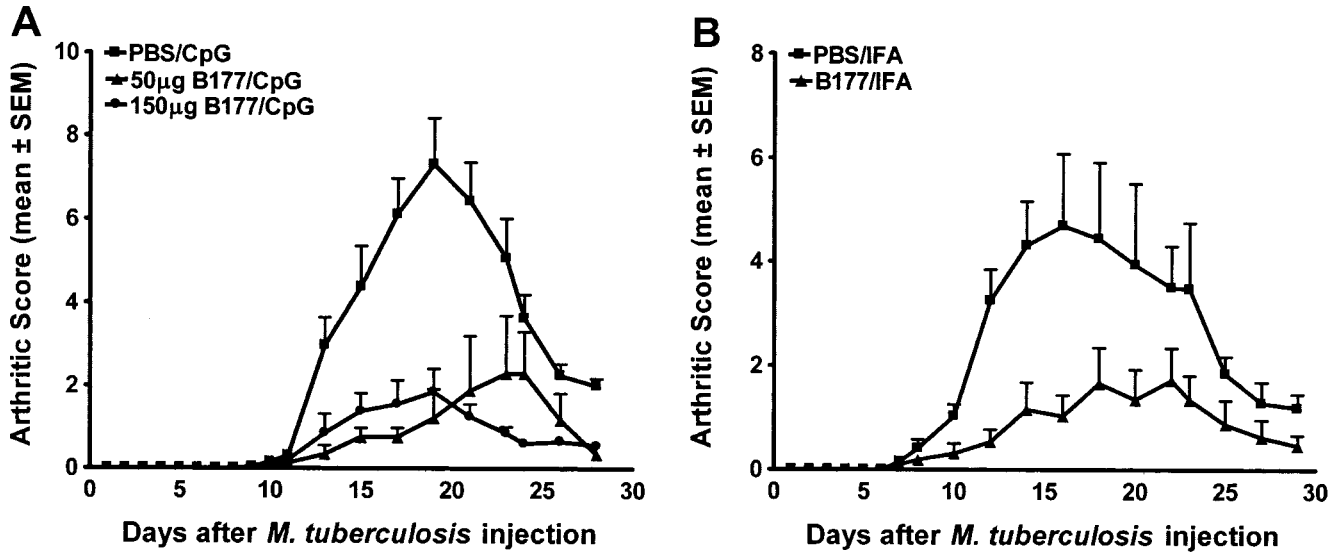

Figure 1. Immunization of Lewis rats with B177 leads to protection against AA. A. B177/CpG ODN injection: Lewis rats (n = 4–7) were immunized sc with B177 in CpG ODN (experimental group). The control group was injected with PBS in CpG ODN. Rats were observed for 4 weeks for any signs of arthritis. Then all rats were injected sc at the base of the tail with Mtb (for induction of AA) and graded regularly for signs of AA. The mean arthritic score of the experimental groups was significantly different from that of controls (PBS/CpG ODN) from day 13 through day 28 (p value between < 0.005 and < 0.05, Student t test). The difference between the indicated groups of rats was also significant ($p < 0.05$) by Wilcoxon rank-sum test). Similar results were obtained in repeat experiments. B. B177/IFA injection: Lewis rats were immunized at the lower back twice at 1-week interval with B177 (100 µg/rat, n = 6) in IFA. PBS/IFA-immunized rats (n = 6) served as controls. As in section A, rats were observed for 4 weeks for signs of arthritis. Then all rats were injected with Mtb and observed for arthritis as described above. The difference in the mean arthritic score of the experimental and the control groups was statistically significant from day 10 through day 28 (p value between < 0.005 and < 0.05, Student t test). The difference in the sum of the arthritic scores over the entire course of AA between the experimental and control groups was also significant ($p < 0.05$) by Wilcoxon rank-sum test). Similar results were obtained on repeat testing.

LNC, but immunized with Mtb, served as an additional control. Following Mtb immunization, all rats were observed and scored regularly for the signs of AA. The results (Figure 2) show that the adoptive transfer into Lewis rats of B177-primed, but not KLH-primed, LNC induced significant ($p < 0.05$) protection against subsequently induced AA, and thereby suggest the involvement of B177-primed T cells in regulation of AA. These findings further corroborate the above-mentioned results of protection against AA following active immunization with B177 (Figure 1).

Subsets of the T cells primed by B177 of Bhsp65 are cross-reactive with the disease-protective C-terminal epitope 465–479 of Rhsp65 (R465), and vice versa. We have reported that arthritic Lewis rats in recovery phase of AA raised T cell response to 5 each of the Bhsp65/Rhsp65 C-terminal determinants (BCTD/RCTD)[3], and that the adoptive transfer of the BCTD/RCTD-primed T cells into naive Lewis rats could afford protection against subsequent AA[5,6]. To define the relationship, if any, between the AA-protective T cell repertoires directed against B177 as well as the 5 C-terminal determinants each of Bhsp65/Rhsp65, we tested whether these 2 sets of determinants within hsp65 activate a shared subset of the T cell repertoire leading to a similar outcome, namely, protection against AA.

Lewis rats were challenged sc with B177 in IFA and the draining LNC of these rats were tested in a proliferation assay using individual peptides comprising BCTD/RCTD as recall antigens. B177 and ConA served as positive controls. The results show that the T cells primed *in vivo* by B177 could be recalled *in vitro* by Bhsp65 peptide 417–431 (B417) (Figure 3A), and Rhsp65 peptides 418–432 (R418) and 465–479 (R465) (Figure 3B). The level of recall response was the highest for R465, whereas there was no recall response to the corresponding Bhsp65 peptide 465–479, B465. The crossreactivity of B177-primed T cells was limited to C-terminal epitopes of hsp65 as there was no recall response to the self-counterpart of B177, namely peptide 177–191 of Rhsp65 (R177), and vice versa (data not shown). However, the control PBS/IFA-primed T cells did not show any recall response to the BCTD/RCTD peptides tested (data not shown).

Interestingly, the results of another set of experiments revealed that the T cells primed by R465 could be restimulated *in vitro* with B177, but not by the homologous B465 (Figure 3C). However, immunization of Lewis rats either with B417 or with R418 did not reveal any significant cross-reactivity with B177 (data not shown). Moreover, R465-primed T cells gave a positive recall response with 2 of

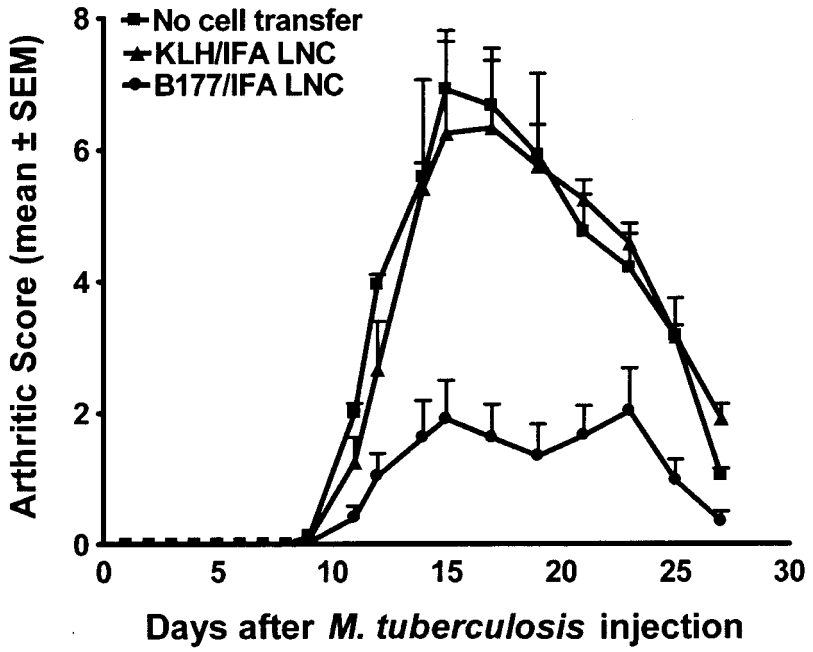

Figure 2. Modulation of AA in the Lewis rat by adoptive transfer of B177-primed LNC prior to Mtb injection. Rats were immunized with B177 (100 µg/rat) in IFA (experimental donor rats). Control donor rats were injected with KLH/IFA. After 8 days, the draining LNC of each group of rats were harvested separately, and then cultured with ConA for 48 h. After thorough washings, a total of 5×10^7 B177/IFA-primed LNC (●: experimental recipients; n = 4) or KLH/IFA-primed LNC (▲: control recipients; n = 3) were injected IV into naive Lewis rats. Another control group (■: no cell transfer; n = 3) did not receive any LNC. All groups of rats were then injected sc with Mtb on the same day and observed regularly for signs of AA. The difference in the mean arthritic score of the experimental and each of the control recipient groups was statistically significant from day 14 through day 27 (p value between < 0.005 and < 0.05, Student t test). The difference was also significant ($p < 0.05$) by Wilcoxon rank-sum test). Repeat experiments yielded similar results.

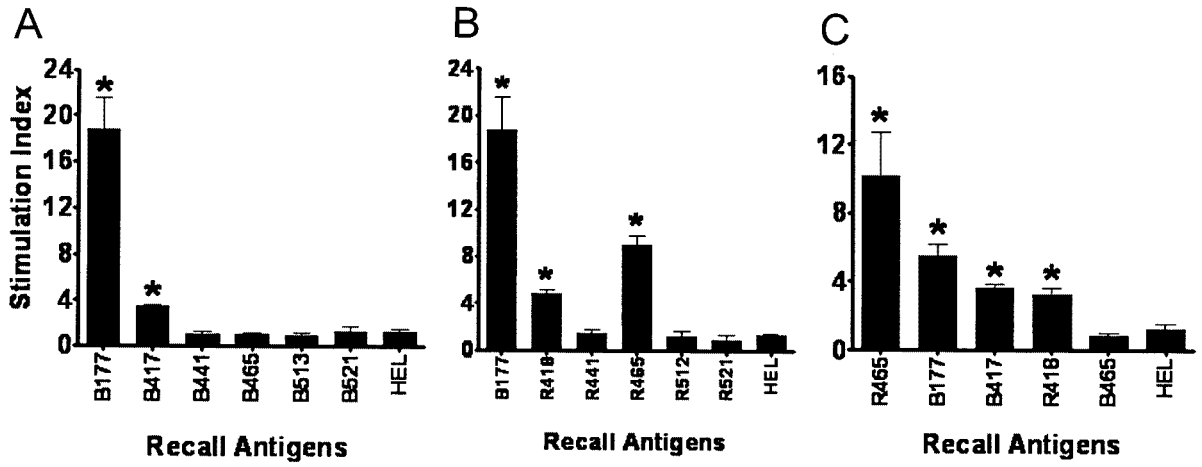

Figure 3. T cells primed *in vivo* by B177 can be activated *in vitro* with R465, and vice versa. Lewis rats (n = 4–5) were immunized sc at the tail base with B177 (A and B) or R465 (C) (each 100 µg/rat in IFA). On day 9, the draining LNC were harvested and tested in a proliferation assay using a panel of individual C-terminal peptides of Bhsp65 (A), C-terminal peptides of Rhsp65 (B), and C-terminal peptides of both Bhsp65 and Rhsp65 (C) as recall antigens[5,6]. Also included in the assay were HEL (negative control) and ConA (positive control). Results are expressed as a stimulation index (SI; mean ± SEM). A proliferative response with a SI value > 3 was considered to be positive in this and other experiments. In A and B, the SI for ConA was 370.5, and the cpm value for Medium (LNC in medium alone) was 1008. In C, the SI for ConA was 67.6, and the Medium background was 3991 (*$p < 0.05$, compared to response to HEL by Student t test).

BCTD/RCTD peptides tested, B417 and R418 (Figure 3C). This finding provides an explanation for the observed cross-reactivity of B177-primed T cells with B417 and R418. Together, these results show that the T cells directed against B177 and R465 (but not B417 and R418) display bidirectional crossreactivity. Therefore, we further examined in detail only the shared T cell repertoire directed against B177 and R465.

T cell repertoire shared by B177 and R465 can be tolerized by either B177 or R465 administered in solution. To further characterize the overlapping T cell repertoire directed against B177 and R465, we tested the effect of tolerance induction against one peptide on the subsequent immune response to the other, crossreactive peptide. Lewis rats were tolerized against B177 by ip injection of the soluble form of the peptide in PBS as described in Materials and Methods. On day 0, these rats were immunized sc either with B177/IFA or with R465/IFA to activate and expand specific T cells, and after 8 days, the draining LNC of these rats were tested in a proliferation assay. The results show that B177-tolerized rats (Figure 4B) had a significantly reduced T cell recall response not only to B177 but also to R465 compared to that of control rats (Figure 4A). Further, tolerization of Lewis rats with B177 could inactivate both the B177-specific as well as the B177-R465-crossreactive T cell subsets, leaving the R465-specific-B177-non-crossreactive T cell repertoire intact. These R465-specific T cells could be primed efficiently by R465/IFA (Figure 4B). Similar results were obtained using soluble R465 as the tolerogen in Lewis rats (Figure 4C).

Subsets of the T cells primed by B177 and R465 reveal a similar cytokine profile upon antigenic stimulation. To further examine the similarity in the functional characteristics of the T cells directed against B177 and R465, we tested their cytokine secretion profile. Lewis rats were immunized sc with B177/IFA. After 8 days, their draining LNC were harvested and restimulated *in vitro* with B177, and the culture supernatants collected were tested for IFN-γ, IL-10, and TGF-ß. A similar experiment was performed using R465/IFA as the immunogen. The results show that the T cells primed by B177 (Figure 5A) and R465 (Figure 5B) display similar cytokine profiles characterized by the secretion of IFN-γ and IL-10. In each case, the level of IFN-γ was relatively higher than that of IL-10 (Figure 5), whereas TGF-ß was not detectable (data not shown). Thus, both the cytokine profile (Figure 5) and the AA-protective attribute (Figures 1 and 2) of B177-reactive T cells are similar to that of R465-reactive T cells[6]. These results further reinforce the physiological significance of B177-R465 crossreactive T cell repertoire in the pathogenesis of AA.

Tolerization of the R465-reactive T cell repertoire compromises the AA-protective effect of B177 and enhances the severity of AA compared to control rats. On the basis of the above data showing that B177 and R465 can activate a shared T cell subset and are protective against AA, we reasoned that the AA-protective effect of B177 is mediated in part by the activation of B177-R465-crossreactive, shared T cell repertoire. To test this proposition, we examined the effect of tolerization of R465-reactive T cells on the protection induced against AA by B177/IFA. In addition, considering our results showing the AA-protective effect of immunization with R465/IFA[6], we also tested whether tolerization

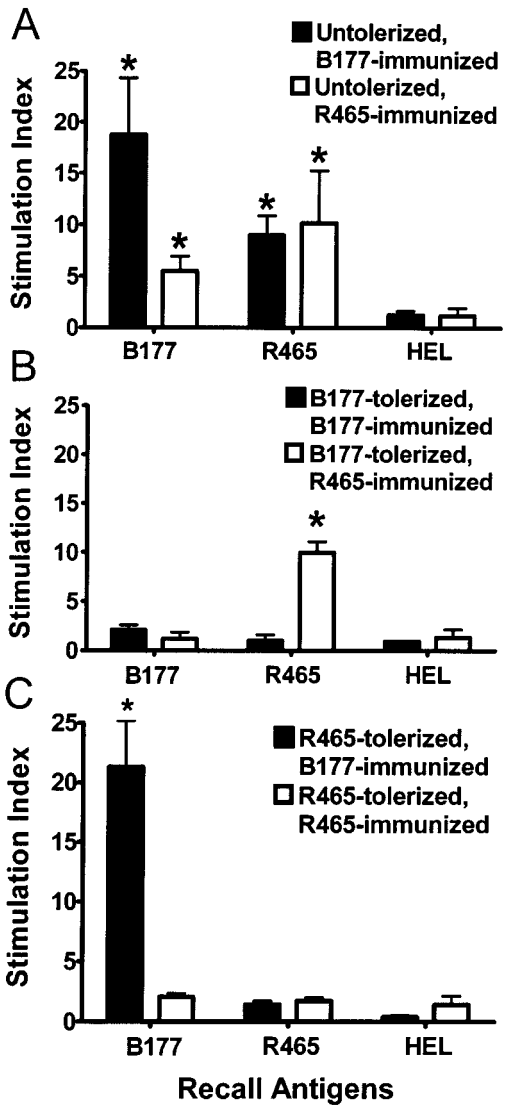

Figure 4. T cell repertoire shared between B177 and R465 can be tolerized by either B177 or R465. A group of Lewis rats (5–6 wks old) were either left untolerized (A), or tolerized by injecting ip B177 in PBS (B) or R465 in PBS (C) (each peptide 200 µg/rat) on day –9, –7, and –5. On day 0, each of these 3 groups of rats were immunized sc with either B177/IFA (black bars) or R465/IFA (white bars) separately (100 µg/rat each). After 8 days, the draining LNC were harvested and tested in a proliferation assay as described above. HEL and ConA were used as negative and positive controls, respectively. Results are given as SI (mean ± SD). SI values for ConA for the 2 subgroups of rats in each of the 3 groups were 370.5 and 67.6 (A), 13.8 and 17.9 (B), and 62.4 and 56.9 (C), respectively (*$p < 0.05$, compared to response to HEL in the respective group by Student t test).

of R465-reactive T cells had any effect on the course of subsequent AA, particularly on recovery from acute AA. A group of LEW rats were first tolerized with R465 or KLH in PBS and then immunized sc with B177/IFA. Rats that were tolerized with R465 prior to immunization with HEL peptide 65–78/IFA served as an additional control. Two weeks later, these rats were challenged with Mtb and then observed regularly for signs of arthritis. The results (Figure 6) show that Lewis rats tolerized with R465 prior to B177/IFA challenge displayed significantly less protection (i.e., de-protection or circumvention of protection) against AA compared to rats tolerized with a control antigen (KLH) before immunization with B177/IFA; the latter group of rats showed a profound suppression of the development of AA. Further, the severity of AA in the R465-tolerized "de-protected" rats was not significantly different from that of untolerized controls ($p > 0.05$). In addition, R465-tolerized but HEL p65–78 immunized rats displayed enhanced severity of AA, with a major effect on the recovery phase of the disease compared to untolerized rats ($p < 0.05$). Together, our results shown in Figure 6 demonstrate that (1) prior tolerization of R465-reactive T cells leads to a loss of protective effect of immunization with B177 on the course of subsequent AA, suggesting that the AA-protective effect of B177/IFA is attributable in part to the priming and expansion of T cells that are crossreactive with the AA-protective epitope, R465; and (2) tolerization of R465 reactive T cells prior to Mtb challenge leads to the aggravation of AA particularly in the recovery phase of the disease, further validating the AA-protective role of R465.

DISCUSSION

Evidence from a diverse set of experiments suggests that the T cells directed against hsp65 are involved in the pathogenesis of AA in the Lewis rat[1-6], and that the determinant region 180–188 (contained within B177) is believed to harbor the arthritogenic determinant within Bhsp65[1,2]. However, our results show that subsets of T cells directed against epitope B177 of Bhsp65 are protective against AA rather than being arthritogenic. We have shown that the epitope region R465 of Rhsp65 is naturally processed and presented from native self-hsp65[5], and that the pretreatment of naive Lewis rats with R465 affords significant protection against subsequent AA[6]. Interestingly, we now show that the subset of T cells directed against the determinant B177 of the foreign mycobacterial hsp65 (Bhsp65) are crossreactive with the AA-protective C-terminal epitope R465 within the self-hsp65, and vice versa. The significance of the crossreactivity between these 2 epitopes lacking any significant aa homology and located at entirely different molecular positions within the 2 homologous hsp65 proteins is further underscored by the observation that there is barely any crossreactivity in the corresponding regions pertaining to these 2 epitopes (B177 vs R177, or R465 vs B465). Moreover, the T cells primed with B177 or R465 also secrete similar cytokines (IFN-γ and IL-10) upon antigenic restimulation, further reinforcing the similarity in epitope recognition and functional attribute of these T cells. Generally, the T cell crossreactivity between foreign (e.g., microbial) and self (host tissue) antigens is considered to be one of the mechanisms that could lead to the initiation and

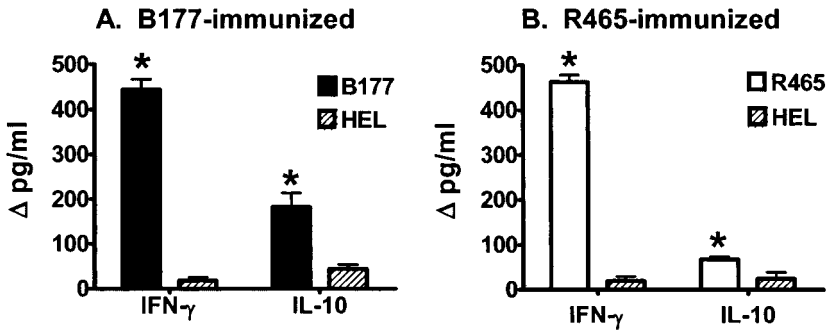

Figure 5. T cells directed against B177 and R465 show similar patterns of cytokine secretion. Lewis rats were immunized sc with B177 (A) or R465 (B) (100 µg/rat each), each emulsified in IFA. After 8 days, the draining LNC were harvested and restimulated *in vitro* with the appropriate peptide (B177/R465) for 48 h at 37°C. HEL was used as an irrelevant antigen control. Culture supernatants were tested for IFN-γ and IL-10 using ELISA kits. Results are expressed as Δ pg/ml (mean ± SD). In both A and B, the difference in the cytokine secretion of B177-/R465-restimulated LNC compared to HEL-recalled LNC was statistically significant (*$p < 0.05$, Student t test) for IFN-γ as well as IL-10. The ratio of IFN-γ to IL-10 was 2.4 (A) and 6.8 (B).

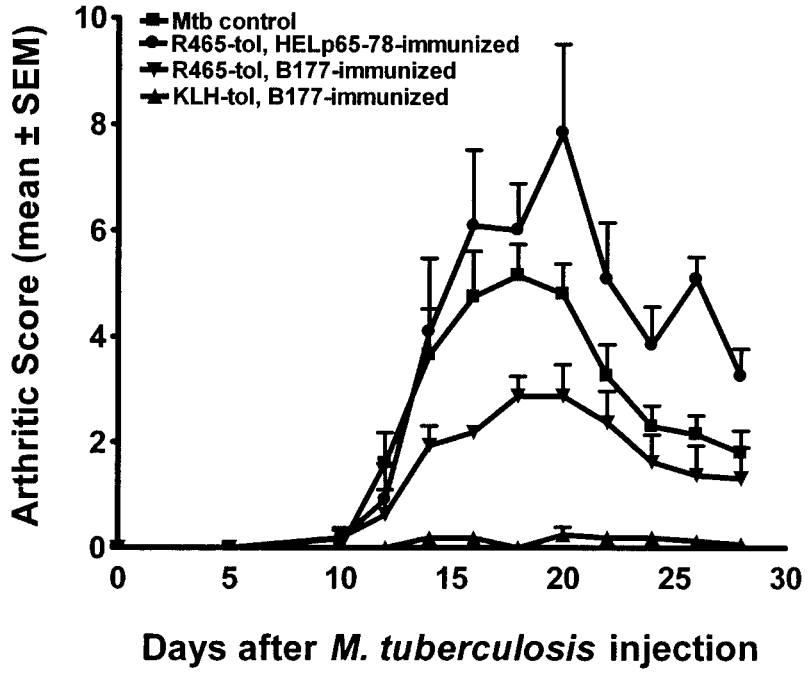

Figure 6. Tolerization of the T cell repertoire against R465 leads to a loss of AA-protective effect of B177. Lewis rats were injected ip with soluble R465 (▼: n = 4; experimental group) or KLH (▲: n = 4; control group) in PBS on days –9, –7 and –5 before immunization with B177/IFA sc at the base of the tail. Another group of rats (●: n = 3; control group) were tolerized with soluble R465 followed by sc injection of HEL peptide 65–78. A group of naive Lewis rats not tolerized or immunized with any peptide (■: n = 5; control group) served as an additional control. Two weeks after the last injection, all rats were injected with Mtb in oil sc and then followed regularly for the severity of AA. The difference in the mean arthritic scores of R465-tolerized experimental versus KLH-tolerized control rats (▼ vs ▲) was statistically significant from day 14 through day 28 (p value between < 0.0007 and < 0.04, Student t test). Similarly, the difference between R465-tolerized experimental versus R465-tolerized control groups (▼ vs ●) was significantly different from day 16 through day 28 ($p < 0.002$ to < 0.03, Student t test). This difference was also significant ($p < 0.05$) by Wilcoxon rank-sum test. However, the difference between R465-tolerized experimental versus naive group of rats (▼ vs ■) was not significant ($p > 0.05$). Also significant was the difference in the mean arthritic scores of R465-tolerized control versus naive control rats (● vs ■) ($p < 0.05$, Wilcoxon rank-sum test). Similar results were obtained on repeat experiments.

propagation of autoimmunity[26,32,33]. In contrast, our study shows a disease-protective aspect of the B177-R465-crossreactive shared T cell repertoire. This represents an example of functional degeneracy of immune recognition[34]. The contrasting pathogenic and protective roles of crossreactive T cell responses have also been invoked in the pathogenesis of diabetes mellitus[35]. In our study on AA, the observed crossreactivity between B177 and the distant C-terminal determinant 465 is different from that previously described by us[3] and others[16] for pairs of self-foreign determinants on the identical position within 2 homologous hsp65 proteins. Further, it is likely that a mechanism such as the network crossreactivity or immune crosstalk observed between 2 different heat-shock proteins might also apply to the epitopes under study here, B177 and R465. Immune crosstalk between hsp60 and hsp70/hsp90 was revealed by the induction of hsp60-specific T cell response following immunization with DNA plasmid expressing hsp70 or hsp90[36]. Moreover, self-vaccination with endogenous hsp induced following this DNA vaccination regimen was proposed as one of the mechanisms contributing to the generation of immune reactivity to hsp60. Our results of B177-induced protection against AA are supported by a similar observation in another model of autoimmunity, diabetes mellitus, where protection could be mediated by an hsp60 peptide that was recognized by pathogenic effector cells[37]. In addition, as for hsp60, B177 or R465 might also enhance the activity of CD4+CD25+ T cells leading to protection against AA[38].

The results pertaining to the *in vivo* priming with B177 (or R465) (Figure 3) and those of tolerance induction experiments (Figure 4) suggest that only a part of the epitope-directed T cell repertoire is crossreactive. Unlike well documented results in generating murine T cell clones, most investigators find it difficult to clone rat T cell lines and to maintain them in a functional state in culture, particularly after thawing them from frozen state[25] (Durai M, Moudgil KD, unpublished data). However, efforts to study the B177-R465-reactive protective T cell repertoire in AA at the T cell line and clonal level are under way in our laboratory.

Our results show that B177/R465, which induce protection against AA, activate T cells that secrete both IFN-γ and IL-10, with much higher level of IFN-γ compared to that of IL-10. We suggest the following possible mechanisms by which these T cells and their secreted cytokines might induce protection against AA. (1) Although counterintuitive, there is provisional evidence to suggest that IFN-γ might also contribute to protection against AA. For example, the injection of IFN-γ to Lewis rats immediately following the induction of AA afforded protection from the disease[39], and the administration of anti-IFN-γ antibody can exacerbate AA[40]. A protective effect of IFN-γ has also been observed in other Th1-mediated diseases[41-43]. The mechanism by which IFN-γ exerts its antiarthritic effect might involve one or more of the following: inhibition of proliferation of, and induction of apoptosis of, pathogenic T cells, influence on the migration/recruitment of T cells into the target organ, and alteration in the activity of antigen-presenting cells[44-46]. (2) IL-10 is known to be an immunosuppressive cytokine that can downregulate AA, which is considered to be a typical Th1-mediated disease. The protective role of IL-10 in autoimmunity is best defined through studies on Tr1 cells in inflammatory bowel disease[47,48]. IL-10 has also been shown to induce protection against type 1 diabetes and lupus[49,50]. (3) We have not observed significant production of TGF-ß by B177/R465-reactive T cells. However, we have not yet ruled out the possibility that a subset of these T cells might secrete TGF-ß in situ *in vivo*. The protective role of TGF-ß secreted by Th3 cells has been best elucidated in studies involving oral tolerance against defined disease-relevant antigens in experimental autoimmune encephalomyelitis (EAE)[51]. A protective effect against EAE was also documented by direct administration of TGF-ß[52]. Similarly, mucosal administration of antigen has been shown to induce protection against uveitis and arthritis via TGF-ß[53,54]. (4) CD4+Foxp3+ T regulatory cells (Treg) have been invoked in the regulation of a variety of autoimmune diseases, and these cells have been categorized as natural Treg versus induced/adaptive Treg[55,56]. Although cell to cell contact is believed to be the predominant mechanism of action of Treg, there is also evidence for the role of IL-10 and TGF-ß secreted by Treg[57]. We plan to examine the above possibilities in our studies in the future.

The finding that B177-reactive T cells can downmodulate the course of AA is supported by reports by other investigators showing that the T cell clones derived from the same T cell line (A2) generated from an arthritic Lewis rat could be categorized as being pathogenic (A2b) or protective/regulatory (A2c)[1,2]. The identical epitope specificity of both A2b and A2c clones for the region 180–188 of Bhsp65 suggests that the relative abundance of functionally disparate T cells may be important for the induction of AA versus recovery from the disease[1,39]. Considering that B177 contains within itself the shorter aa sequence 180–188 and also is crossreactive with it, the A2c clone probably portrays the characteristics of the B177-R465 crossreactive T cell subset. This functional resemblance is further supported by the observation that similar to the relatively higher level of IFN-γ compared to IL-10 secreted by the R465-reactive T cells (Figure 5), the A2c clone also secretes significant amounts of IFN-γ upon antigenic stimulation[39]. The results of our study provide support for, and contribute a new component to, the concept of "homuncular" or "immune network" regulation of autoimmunity[58]. These results advance our understanding of the pathogenesis of AA, and also provide insight into designing T cell epitope-based immunotherapeutic approaches for RA.

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