

An Arthritis-Suppressive and Treg Cell-Inducing CD4+ T Cell Epitope Is Functional in the Context of HLA-Restricted T Cell Responses

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Objective. We previously showed that mycobacterial Hsp70-derived peptide B29 induced B29-specific Treg cells that suppressed experimental arthritis in mice via cross-recognition of their mammalian Hsp70 homologs. The aim of the current study was to characterize B29 binding and specific CD4+ T cell responses in the context of human major histocompatibility complex (MHC) molecules.

Methods. Competitive binding assays were performed to examine binding of peptide B29 and its mammalian homologs to HLA molecules. The effect of B29 immunization in HLA-DQ8-transgenic mice with proteoglycan-induced arthritis was assessed, followed by ex vivo restimulation with B29 to examine the T cell response. Human peripheral blood mononuclear cells were used to investigate the presence of B29-specific T cells with immunoregulatory potential.

Results. The binding affinity of the B29 peptide was high to moderate for multiple HLA-DR and HLA-

DQ molecules, including those highly associated with rheumatoid arthritis. This binding was considered to be functional, because B29 immunization resulted in the suppression of arthritis and T cell responses in HLA-DQ8-transgenic mice. In humans, we demonstrated the presence and expansion of B29-specific CD4+ T cells, which were cross-reactive with the mammalian homologs. Using HLA-DR4+ tetramers specific for B29 or the mammalian homolog mB29b, we showed expansion of cross-reactive T cells, especially the human FoxP3+ CD4+CD25+ T cell population, after in vitro stimulation with B29.

Conclusion. These results demonstrated a conserved fine specificity and functionality of B29-induced Treg cell responses in the context of the human MHC. Based on these findings, a path for translation of the experimental findings for B29 into a clinical immunomodulatory therapeutic approach is within reach.

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease that is characterized by synovial inflammation of the peripheral joints, resulting in pain and stiffness and often irreversible joint damage, deformity, and disability. Conventional therapy for patients with RA is based on general immune suppression, which reduces both pathogenic and physiologic immune responses. Although the majority of RA patients benefit from such therapy, a drawback is that life-long treatment is necessary, because any amelioration of symptoms will usually disappear when the treatment is discontinued. Ideally, long-term restoration of tolerance after a short period of therapy should be realized, and therefore, re-education instead of temporal suppression of the immune system is required. Treg cells may have therapeutic potential for reestablishment of tolerance. Ideally, Treg

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Drs. van der Zee and van Eden hold a patent for peptide B29.

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cells should be retained at the site of inflammation (i.e., the joints) and exert their activity locally. The fact that Treg cells need to be activated via their T cell receptor in order to maintain their suppressive potential suggests that their cognate antigen should be abundantly present and presented in the context of major histocompatibility complex (MHC) class II molecules in inflamed joints.

A major group of proteins that is highly expressed at sites of inflammation consists of the immunodominant heat-shock proteins (HSPs), which are up-regulated under conditions of stress and are presented by MHC class II molecules to a large extent (1). HSPs are well conserved between species, which has led to extensive sequence similarities between microbial and mammalian homologs. Their abundant presence as self antigens, together with their abundant presence in the gut microbiota, may have evoked the highly conserved HSP sequences to be targeted by Treg cells. This may well explain the down-modulation of disease by HSPs observed in many experimental models of autoimmunity (2–6).

To determine the presence of HSP-specific Treg cells in mice and to investigate whether such cells can have therapeutic value in arthritis, we recently performed adoptive transfer studies. CD4+CD25+ Treg cells or CD4+CD25– Teff cells were isolated from donor mice that had been immunized with mycobacterial Hsp70 peptide B29 and transferred to healthy acceptor mice. Induction of arthritis in the acceptor mice was significantly diminished when the donor cells were Treg cells from B29-immunized mice but not when the cells were derived from mice immunized with a control peptide (7). Apparently, B29 immunization of the donor mice resulted in expansion of B29-specific Treg cells. The reduction in arthritis severity was also observed when the Treg cells were given to acceptor mice in which severe arthritis had already developed (7). This demonstrates that B29-specific T cells are present in mice, and importantly, that therapeutic use of B29 is possible under specific conditions.

To examine whether therapeutic application of B29 is also possible in patients with RA, we investigated the presence and function of such HSP-specific T cells with immunoregulatory potential in the context of human MHC molecules. We observed that both mycobacterial peptide B29 and its mammalian homologs could bind with high to moderate affinity to multiple HLA–DR and HLA–DQ molecules, including RA-associated HLA–DR4 and HLA–DQ8. We used an HLA–DQ8–transgenic mouse model to further investigate the functionality of B29 peptide binding to MHC class II molecules, because these transgenic mice develop arthritis in response to immunization with human proteoglycan (PG) (8). More-

over, we performed *in vitro* priming and restimulation of human peripheral blood mononuclear cells (PBMCs) with peptide B29 and its mammalian homologs to evaluate the presence of B29-specific CD4+ T cells and their cross-reactivity with the mammalian B29 homologs. Finally, we investigated the presence of *in vitro*–expanded human Treg cells cross-reactive with B29 peptide.

MATERIALS AND METHODS

Mice. Three-month-old female HLA–DQ8–transgenic BALB/c mice were used for PG-induced arthritis (PGIA) experiments (8). The mice were maintained under standard conditions at the animal facility, and all experiments were approved by the Institutional Animal Care and Use Committee at Rush University Medical Center.

Peptides, tetramers, and antibodies. Mycobacterial peptide B29 (Hsp70 peptide 141–155; VLRVINEPTAAALAY) and the mammalian homologs mB29a (VLRVINEPTAAALAY), mB29b (VLRINEPTAAAIAY), and mB29c (VMRIVNEPTAAAIAY) were obtained from GenScript and used for HLA binding affinity assays, cell cultures, and *in vivo* immunization and restimulation. Ovalbumin peptide (pOVA) 323–339 (GenScript) was used as control for the immunization and restimulation experiments. Adenoviral peptide 475–489 (A5; Ansynth Service B.V.) was used as control for the cell culture experiments. DRB1*04:01-B29 (VLRVINEPTAAALAY), DRB1*04:01-mB29b (VLRINEPTAAAIAY), and DRB1*04:01-CLIP (PVSKM RMATPLLMQA) complexes were provided as allophycocyanin (APC)–conjugated tetramers by the National Institutes of Health Tetramer Core Facility (contract HHSN272201300006C).

All of the antibodies used for coculture flow cytometry analysis were purchased from Miltenyi Biotec, unless stated otherwise. Cells were surface-labeled with the monoclonal antibodies VioBlue-conjugated CD3 (clone BW264/56), fluorescein isothiocyanate (FITC)–conjugated CD4 (clone SK3; BD Biosciences), and PerCP-conjugated HLA–DR (clone AC122). After fixation and permeabilization using Cytotfix/Cytoperm and Perm/Wash reagents (BD Biosciences), the cells were labeled intracellularly with the antibodies APC-conjugated CD40L (clone 5C8) and phycoerythrin (PE)–conjugated interferon- γ (IFN γ) (clone 45-15).

All antibodies used for tetramer flow cytometric analysis were purchased from eBioscience, unless stated otherwise. Cells were surface-labeled with the monoclonal antibodies eFluor 450–conjugated CD4 (clone SK3) and FITC-conjugated CD25 (clone M-A251; BD Biosciences). After fixation and permeabilization using Cytotfix/Cytoperm and Perm/Wash reagents (BD Biosciences), the cells were labeled intracellularly with a PE-labeled antibody specific for FoxP3 (clone 236A/E7).

The samples used for flow cytometry coculture assays were measured using a BD LSR II analyzer (BD Biosciences), and those used for flow cytometry tetramer assays were measured using a BD FACSCanto II system (BD Biosciences). FlowJo software (Miltenyi Biotec) was used for the analyses.

Immunization, assessment of arthritis, and *ex vivo* restimulation. Mice received intranasal treatment with peptide B29 (100 μ g) in 10 μ l of phosphate buffered saline (PBS) on days –7, –5, and –3 before the first PG immunization. The dose of B29 peptide was determined based on previous

experiments using the same protocol described herein. Control mice received 10 μ l of PBS, alone or with pOVA (100 μ g).

Arthritis was induced on days 0, 21, and 42 by 3 intraperitoneal injections of 100 μ g PG protein and 1.5 mg adjuvant dimethyldioctadecylammonium bromide (Sigma) in a total volume of 300 μ l. Arthritis scores were determined using a visual scoring system based on swelling and redness of the paws, as previously described (9), starting 10 days before the third injection with PG.

On day 54 after the first PG immunization, splenocytes were obtained and used in a lymphocyte stimulation test. The cells were isolated and cultured with PG (50 μ g/ml) alone or with a combination of PG and peptide B29 (20 μ g/ml) or a combination of PG and pOVA (20 μ g/ml). Concanavalin A (2.5 μ g/ml) (Sigma) was used as a positive control in the proliferation assays. T cell proliferation was determined on day 5 by the incorporation of 3 H-thymidine (0.25 μ Ci/well), which was added during the last 18–20 hours of culture.

Binding affinity assay. HLA–DR molecules were immunopurified from homologous Epstein-Barr virus (EBV)–transformed cell lines using the monomorphic monoclonal antibody L243, as described previously (10). The binding of B29 peptides to HLA–DR molecules was assessed by competitive enzyme-linked immunosorbent assay (ELISA), as previously reported (10). Briefly, HLA–DR molecules were diluted with an appropriate biotinylated marker peptide and serial dilutions of competitor peptides. Samples (100 μ l/well) were incubated in 96-well polypropylene plates (Nunc) at 37°C for 24–72 hours. After pH neutralization, samples were applied to 96-well MaxiSorp ELISA plates (Nunc) that had been previously coated with 10 μ g/ml monoclonal antibody L243 (ATCC). Bound biotinylated peptides were detected by streptavidin–alkaline phosphatase conjugate (GE Healthcare) and 4-methylumbelliferyl phosphate as substrate (Sigma). Fluorescence (excitation at 365 nm, emission at 450 nm) was measured on a Gemini SpectraMax Fluorimeter (Molecular Devices). Nonbiotinylated marker peptides were used as reference peptides.

HLA–DQ molecules were immunopurified from EBV-transformed B cells homozygous for DQ2 or DQ8 using the SPV-L3 monoclonal pan DQ antibody, and binding of the B29 peptides to these HLA molecules was determined as previously described (11). Briefly, 96-well FluoroNunc plates (Nunc) were coated with 20 μ g/ml monoclonal antibody SPV-L3, and subsequently HLA–DQ molecules were added. Test peptides were serially diluted, and a fixed concentration of biotinylated marker peptide was added. Samples (100 μ l/well) were added to the SPV-L3/HLA–DQ-coated plates and incubated for 48 hours at 37°C. After incubation, streptavidin–europium in assay buffer (Wallac) and enhancement buffer (Wallac) were added to detect bound biotinylated peptides. Fluorescence was measured on a Wallac time-resolved fluorometer (model 1234).

Data were first expressed as the concentration of test peptide that prevented binding of 50% of the labeled marker peptide (50% inhibition concentration [IC₅₀]) and then as the relative affinity (ratio of the test peptide IC₅₀ and that of the nonbiotinylated marker peptide).

Cell isolation and coculture assay. Detection of antigen-specific T cell responses was based on a previously described assay (12). Briefly, PBMCs were acquired by Ficoll-Paque density-gradient centrifugation from buffy coats derived

from 14 healthy Sanquin Blood Supply donors of different ages. Ten of these donors were determined to have the HLA–DRB1*04:01 (by DNA typing), and 6 of them were determined to also have the HLA–DQ8 serotype. The HLA type of the remaining 4 donors was unknown. A portion of the PBMCs was used to isolate CD14+ monocytes by positive selection, using magnetic beads according to the instructions of the manufacturer (Miltenyi Biotec); the remaining cells were cryopreserved at –80°C until further processing was performed. The CD14+ cells were cultured in serum-free X-Vivo 15 medium (Lonza) for 7 days with 100 ng/ml granulocyte–macrophage colony-stimulating factor and 25 ng/ml interleukin-4 (IL-4) (both from Miltenyi Biotec) in order to differentiate them into monocyte-derived dendritic cells (DCs). For monocyte-derived DC maturation, 25 ng/ml tumor necrosis factor α (TNF α) (Miltenyi Biotec) was added during the last culture day, and the monocyte-derived DCs were simultaneously loaded with peptide. Matured monocyte-derived DCs were defined as CD11c+HLA–DR+CD83+CD86+ cells.

PBMCs were thawed for CD4+ T cell purification by negative selection, using a CD4+ T Cell Isolation Kit II (Miltenyi Biotec) according to the instructions of the manufacturer. The cells were cultured in the presence of the peptide-pulsed monocyte-derived DCs at a ratio of 50:1 for 14 days at 37°C in an atmosphere of 5% CO₂ in serum-free X-Vivo 15 medium (Lonza). Thereafter, the cells were harvested and restimulated for 7 hours with newly differentiated monocyte-derived DCs loaded with either specific or irrelevant peptide (at a DC:T cell ratio of 1:4 to 1:16). During the last 5 hours of restimulation, 1 μ g/ml Brefeldin A (Sigma) was added to the cultures. Cells were subsequently analyzed for T cell reactivity, using flow cytometry.

Cell enrichment, expansion, and tetramer staining. PBMCs from a DRB1*04:01 buffy coat (Sanquin Blood Supply) were incubated with CD4 and CD25 antibodies at 4°C for 30 minutes. CD4+CD25– and total CD4– cells were

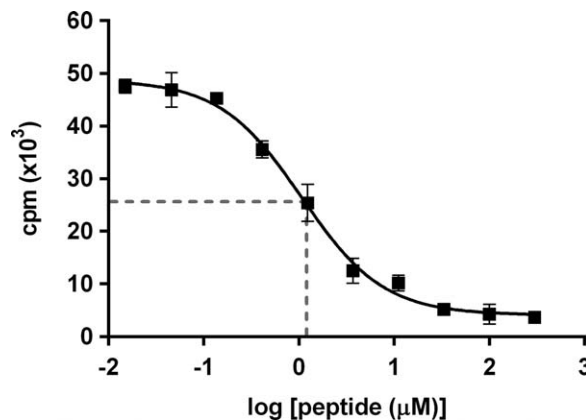


Figure 1. Representative dose-response curve of B29 peptide binding to HLA–DQ2 molecules. HLA molecules were immunopurified, and binding of mycobacterial peptide B29 and its mammalian homologs was assessed by competitive binding assay. The peptide concentration that prevented binding of 50% of the biotinylated marker peptide (50% inhibition concentration [IC₅₀]) was measured. The point at which the broken lines meet indicates an IC₅₀ value of 1.07 μ M. Values are the mean \pm SEM.

Table 1. Binding of mycobacterial peptide B29 and its mammalian homologs mB29a, mB29b, and mB29c to HLA-DR and HLA-DQ molecules

	Binding affinity*			
	B29	mB29a	mB29b	mB29c
HLA-DR1	5	35	5	2
HLA-DR3	>1,000	35	190	1
HLA-DR4	0.01	0.2	0.03	0.2
HLA-DR7	24	153	3	18
HLA-DR11	2	137	2	23
HLA-DR13	>5,000	750	>5,000	Not available
HLA-DR15	0.04	1	0.1	0.03
HLA-DQ2	2	10	2	31
HLA-DQ8	2	2	0.6	Not available

* High affinity = 0.01–20; moderate affinity = 21–100; weak affinity = 101–1,000; no binding = >1,000.

sorted using a Cytopeia Influx sorter (BD Biosciences). The sorted CD4⁺CD25[−] cells (with <1% FoxP3⁺ expression) were expanded in serum-free X-Vivo 15 medium (Lonza) supplemented with 10% normal human serum (Sanquin), with irradiated (3,000 rad) autologous CD4[−] cells as antigen-presenting cells (at a 1:2 ratio) and with 0.5 μ g/ml influenza hemagglutinin (HA) peptide 307–309 (Ansynth Service B.V.) or 0.5 μ g/ml peptide B29. On day 7, 200 IU/ml IL-2 (aldesleukin; Novartis) was added to the cells to stimulate antigen-specific T cell expansion. After 14 days of culture, cells were washed and incubated with B29 or mB29b tetramers (5 μ g/ml) at 37°C for 90 minutes. The human class II-associated invariant-chain peptide (CLIP) tetramer was used as a negative control to rule out nonspecific staining. Samples were subsequently stained for flow cytometric analysis.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 6 software. Comparisons between 2 groups were performed using a 2-sample 2-tailed *t*-test. Comparisons between 3 or more groups were performed by one-way analysis of variance followed by Bonferroni post hoc test. *P* values less than 0.05 were considered significant.

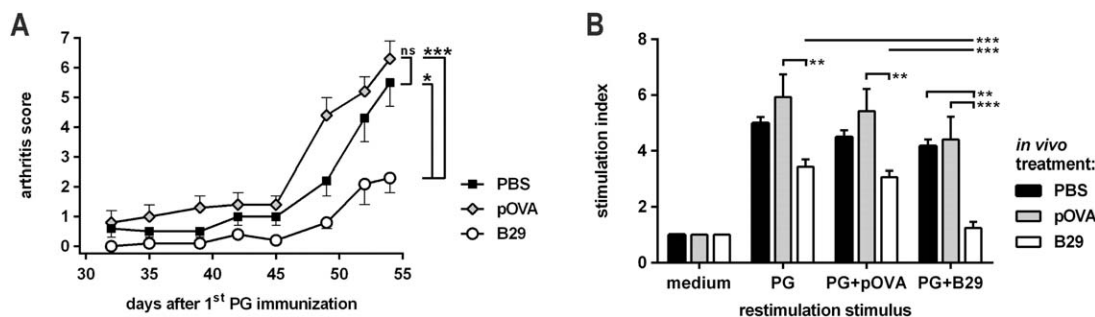


Figure 2. Intranasal treatment with peptide B29 suppresses the development of arthritis and proteoglycan (PG)-specific proliferative responses in mice. **A**, Arthritis scores in HLA-DQ8-transgenic mice that were treated intranasally with peptide B29, ovalbumin peptide (pOVA), or phosphate buffered saline (PBS) prior to the initiation of arthritis by immunization with PG. **B**, Proliferative response in splenocytes from intranasally treated mice (as described in A) after restimulation with PG as shown in MHC peptide elution studies (7), alone or in combination with peptide B29 or pOVA. T cell proliferation was determined on day 5 by the incorporation of ³H-thymidine. Values are the mean \pm SEM of 2 independent experiments (*n* = 5–8 mice per group). * = *P* < 0.05; ** = *P* < 0.01; *** = *P* < 0.001 by one-way analysis of variance followed by the Bonferroni post hoc test. NS = not significant.

RESULTS

Binding capacity of B29 peptides in the context of human MHC class II molecules. Based on the literature, it is known that Hsp70-derived peptides are relatively abundant in MHC class II molecules from mice and humans, as shown in MHC peptide elution studies (7). To evaluate the binding capacity of the B29 peptide and its mammalian homologs to human MHC class II molecules, we performed HLA binding assays with the 7 most common HLA-DR molecules in the Caucasian population and 2 HLA-DQ molecules. Analysis of the IC₅₀ (Figure 1) and relative affinity (Table 1) showed that the B29 peptides exhibited broad binding specificity. Except for HLA-DR3 and HLA-DR13, the B29 peptides bound to most HLA-DR molecules with high to moderate affinity. The differences in binding affinity indicate specific binding between the B29 peptides and the individual HLA molecules. Interestingly, all of the homologous peptides showed high affinity for the RA-associated HLA-DR4 and HLA-DQ8 molecules.

Effect of intranasal treatment with peptide B29 on arthritis severity. We next sought to test the immunosuppressive activity of the B29 peptide in the context of human MHC class II *in vivo*. For this, we investigated the *in vivo* effect of B29 in HLA-DQ8-transgenic mice, because these mice have been shown to develop arthritis in response to immunization with human PG (8). HLA-DQ8-transgenic mice were treated intranasally with peptide B29 or with PBS or pOVA as control. Subsequently, arthritis was induced with human PG, and disease development was monitored using a visual scoring system. Prophylactic intranasal treatment with B29

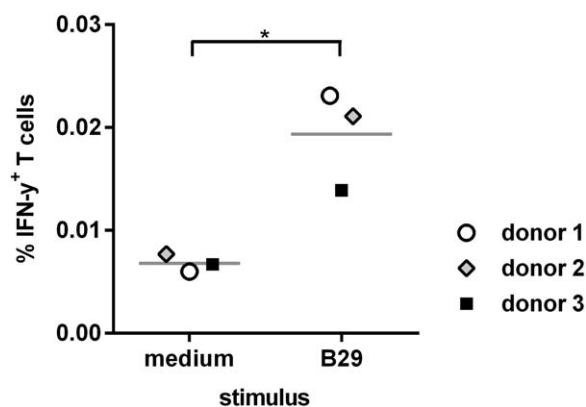


Figure 3. Presence of B29-specific T cells in peripheral blood mononuclear cells from healthy blood donors. Hsp70 peptide-loaded (B29) or unloaded (medium) monocyte-derived dendritic cells were cocultured with autologous CD4+ T cells for 24 hours. The percentage of interferon- γ (IFN γ)⁺ cells within the CD3+CD4+ T cell population was determined by flow cytometric analysis. Each data point represents a single donor; horizontal lines show the mean. Each experiment was performed in duplicate. * = $P < 0.05$ by 2-sample 2-tailed *t*-test.

significantly reduced arthritis development compared with treatment with PBS or pOVA (Figure 2A).

After 54 days, splenocytes from the pretreated mice were restimulated *ex vivo* with PG alone or in combination with B29 or pOVA. Pretreatment with peptide B29 reduced all PG responses; this response was further reduced when B29 was present during restimulation (Figure 2B) but only when the mice had been pretreated with B29. These results indicate that binding of peptide B29 to HLA-DQ8 is functional and induces immune regulatory cells *in vivo*, which also become active during *ex vivo* restimulation in the presence of peptide B29.

Presence of B29-specific T cells in healthy blood donors. Subsequently, we determined whether B29-specific T cells are present in healthy DR4+DQ8+ blood donors. CD4+ T cells from 3 healthy blood donors were stimulated for 24 hours with mature B29-loaded or unloaded monocyte-derived DCs. Although the frequency was very low, IFN γ + cells were observed within the CD3+CD4+ T cell population of all 3 donors whose T cells were stimulated with unloaded monocyte-derived DCs. However, increased numbers of IFN γ + cells were present when the cells had been stimulated with B29-loaded monocyte-derived DCs (Figure 3).

Because we did not know whether B29-specific T cells would be present as naive or Teff cells, we used a sensitive assay that allows *in vitro* priming and restimulation of naive human T cells (12). To prevent the detection of bystanders (nonspecific T cells) instead of

antigen-specific T cells, we focused on IFN γ +CD40L+ cells (12). As expected, CD4+ T cells that had been cultured for 14 days in the presence of unloaded autologous monocyte-derived DCs did not show any peptide-specific response above the background level upon restimulation (Figure 4A). Priming of CD4+ T cells with B29-loaded monocyte-derived DCs resulted in an average 3.1-fold increase in IFN γ +CD40L+ cells in responding donors (HLA-DR4+ donors with a fold increase of >2) after restimulation with B29 compared with restimulation with medium only (Figure 4A). However, the percentage of IFN γ +CD40L+ cells after restimulation with unloaded monocyte-derived DCs had already increased as a consequence of prior activation with B29-loaded monocyte-derived DCs (Figure 4A). This resulted in underestimation of the increase in IFN γ +CD40L+ cells after B29 restimulation compared with restimulation with unloaded monocyte-derived DCs. When we compared priming with B29 and priming with medium only, the increase in B29-specific CD4+ T cells (measured as the percentage of IFN γ +CD40L+ cells) observed after B29 restimulation was up to 104-fold (average 28-fold).

In most HLA-DR4+ donors, the B29-stimulated CD4+ T cells not only responded to monocyte-derived DCs loaded with B29 but also responded after being loaded with the mammalian homologs, especially mB29b (Figures 4B and C), indicating that cross-reactive B29-specific T cells are present in human PBMCs and can be expanded with B29 peptide.

The presence of HLA-DRB1*04:01 (HLA-DR4) is associated with an increased susceptibility to RA (13,14). Furthermore, the presence of HLA-DQB1*03:02 (HLA-DQ8) can contribute to disease severity in HLA-DR4+ patients with RA (15,16). Therefore, we specifically analyzed the response to peptide B29 in healthy HLA-DR4+ donors; 10 of the 14 donors we used for *in vitro* priming and restimulation assays were determined to be HLA-DR4+, and 6 of these HLA-DR4+ donors were also determined to be HLA-DQ8+. Among all of the donors tested, 71% showed a B29-specific response upon restimulation (>2-fold increase in IFN γ +CD40L+ T cells). B29-specific T cells were detected in 50% of donors with an unknown HLA type, in 80% of donors positive for HLA-DR4, and in 83% of donors positive for both HLA-DR4 and HLA-DQ8 (Figure 4D). We can therefore conclude that B29-specific T cells are present in the majority of healthy blood donors, including donors with an RA-susceptible HLA type.

Induction and expansion of cross-reactive FoxP3+CD25+ T cells by peptide B29. We previously showed that in mice, B29 immunization resulted in the induction of FoxP3+CD4+CD25+ Treg cells that sup-

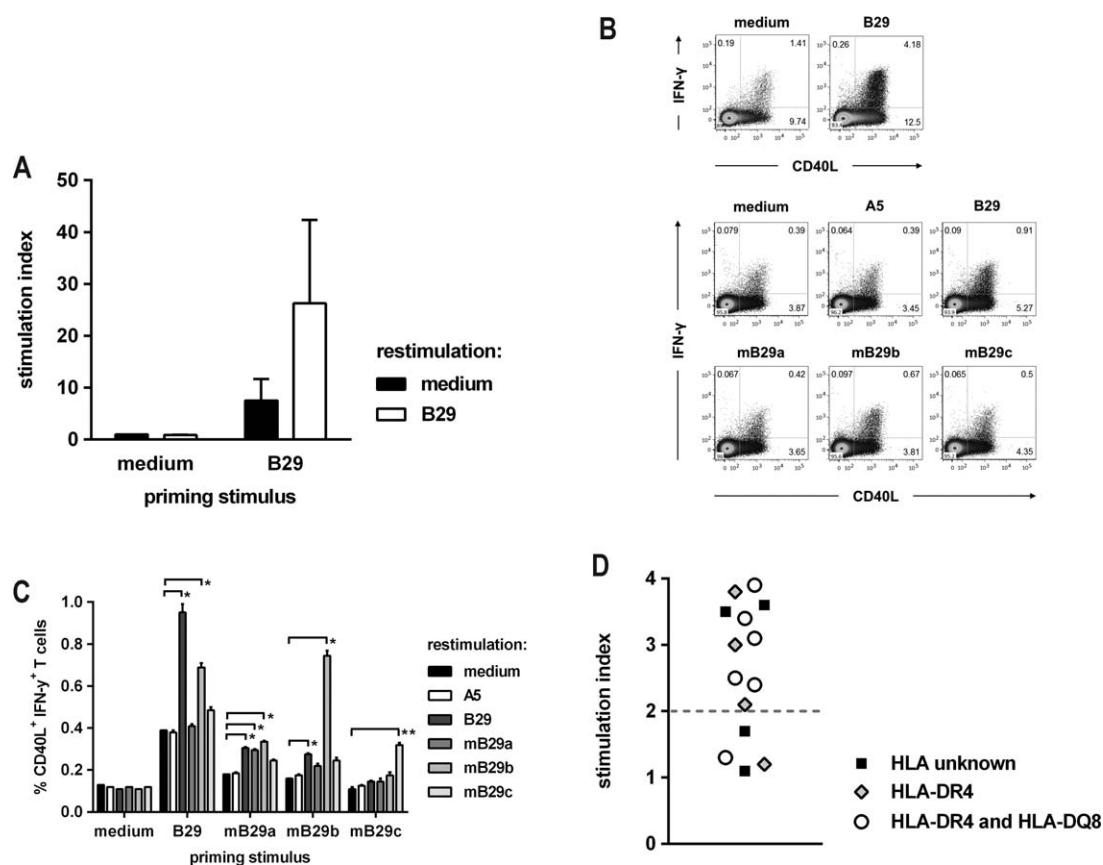


Figure 4. Priming with B29 peptides induces B29-specific and cross-reactive T cell responses. Isolated CD4⁺ T cells were primed with peptide B29-loaded monocyte-derived dendritic cells (DCs) only (**B** and **D**) or with unloaded (medium) monocyte-derived DCs (**A** and **C**) for 14 days. T cell responses were measured as the percentage of IFN γ +CD40L⁺ cells within the CD3+CD4⁺ T cell population after a subsequent restimulation. **A**, Response of CD4⁺ T cells obtained from 5 HLA-DR4⁺ blood donors after priming with medium or B29, expressed as the stimulation index (the percentage of IFN γ +CD40L⁺ cells after priming and restimulation with medium or B29 divided by the percentage of IFN γ +CD40L⁺ cells after priming and restimulation with unloaded monocyte-derived DCs). Values are the mean \pm SEM. **B**, Flow cytometric staining for CD40L and IFN γ within the CD3+CD4⁺ T cell population after priming with B29 or its mammalian homologs (n = 2 different donors). **C**, Cross-reactivity of CD4⁺ T cells after priming with B29 or its mammalian homologs. Values are the mean \pm SEM and are representative of 6 donors. * = $P < 0.05$; ** = $P < 0.01$ by 2-sample 2-tailed *t*-test. **D**, Percentage of IFN γ +CD40L⁺ cells within the CD3+CD4⁺ T cell population of 14 healthy HLA-DR4⁺ donors after priming with B29, expressed as the stimulation index (the percentage of IFN γ +CD40L⁺ cells after restimulation with B29-loaded monocyte-derived DCs divided by the percentage of IFN γ +CD40L⁺ cells after restimulation with unloaded monocyte-derived DCs). Donors with an increase of >2-fold (indicated by the broken line) were considered to have B29-specific T cells.

pressed PG-induced arthritis (7). To analyze whether B29 can also induce Treg cells in humans susceptible to RA, we expanded CD4⁺CD25⁻ T cells from a healthy HLA-DR4⁺ donor with B29 peptide or HA peptide as control. After 14 days, expanded cells were stained with tetramers specific for B29, mB29b, or human CLIP. After 14 days of culture, CD4⁺ T cells showed enhanced expression of CD25 (~16%) and FoxP3 (~12%). However, culturing with peptide B29 specifically expanded B29 tetramer-positive and mB29b tetramer-positive cells, in both the total CD4⁺ T cell population (Figure 5A) and the FoxP3+CD4⁺CD25⁺ T cell population (Figure 5B). The HA control peptide did not induce B29 tetramer-positive or mB29b tetramer-positive T cells. These

data suggest that peptide B29 activates and expands B29-specific Treg cells, which are cross-reactive with the human homologs.

DISCUSSION

Faulty immune regulation appears to be the fundamental mechanism of autoimmune diseases such as RA. In the past, the pathogenesis of inflammation was thought to be initiated by the inadvertent emergence of an unwanted clone of self-reactive Teff cells. However, we have seen that inflammation and autoimmune diseases are associated with the long-term activation or repeated reactivation of self-reactive lymphocytes.

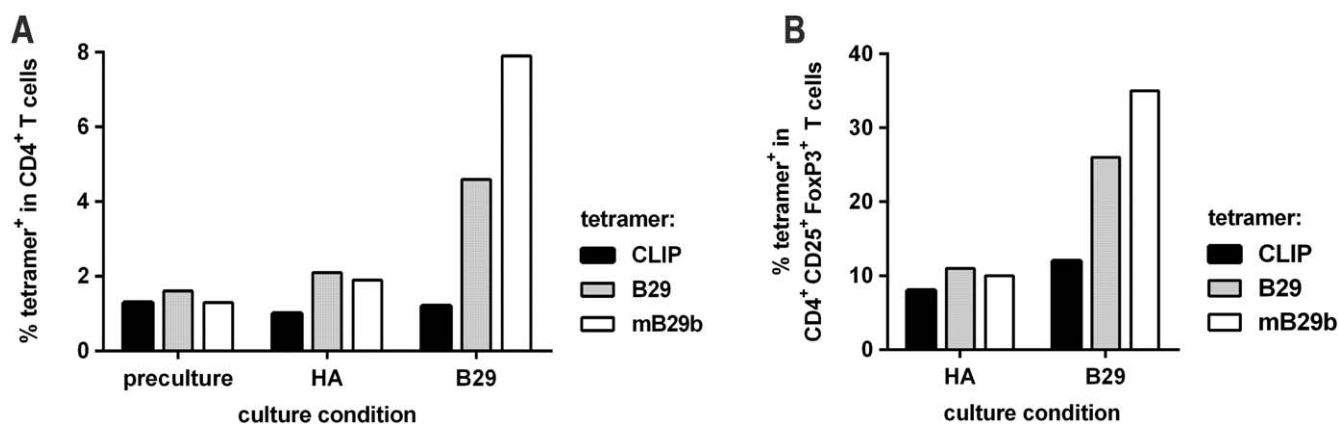


Figure 5. Induction of cross-reactive HLA-DR4+ Treg cells by B29 peptide. CD4+CD25- T cells from a healthy HLA-DR4+ donor were cultured with B29 peptide or HLA-DR4-restricted influenza control peptide (hemagglutinin [HA]) for 14 days. Cells were stained with tetramers specific for B29, mammalian homolog mB29b, or human class II-associated invariant-chain peptide (CLIP) to analyze expansion of antigen-specific T cells. **A**, Percentage of tetramer-positive cells within the CD4+ T cell population before and after culture. **B**, Percentage of tetramer-positive cells within the FoxP3+CD4+CD25+ T cell population after culture with B29 or HA peptide.

Inflammatory disease results from failure of the immune system to regulate its own potentially dangerous cells. Thus, the rational goal of intervention in diseases characterized by unregulated inflammatory activation is to reinstate physiologic regulation (17).

Antigen-specific T cell regulation appears to be the most physiologic form of control of inflammation. The therapeutic use of this form of regulation will depend on the definition of a target antigen that is abundantly present, selectively at sites of inflammation. Therefore, HSPs that become highly expressed under inflammatory stress conditions are a possible source of such antigens.

HSPs can induce the immune system to regulate the inflammatory response. Previous studies have shown the regulatory potential of HSPs and HSP-derived peptides. In particular, stress-inducible Hsp70 has been demonstrated to effectively target regulation in inflamed tissue (5,18,19). Peptide B29 is a major epitope in stress-inducible Hsp70 family members (amino acids 141–155 in mycobacterial Hsp70). Both in humans and in mice, B29 was shown to be a major contributor to the MHC class II ligandome (7,20). Because its presence is ubiquitous, B29 can be an attractive target for T cell regulation. Immunization of BALB/c mice with B29 induced a lymphocyte activation gene 3-positive, FoxP3+CD25+ Treg cell population that suppressed PG-induced arthritis upon transfer (7). Only 4,000 of such Treg cells were required to fully suppress arthritis. Antibody-mediated *in vivo* depletion of these Treg cells abrogated disease suppression, confirming the anti-inflammatory activity of B29-induced Treg cells. The transferred cells were observed to exhibit a stable phe-

notype *in vivo* for >50 days. In addition, nasal administration of B29 suppressed disease and was shown to induce B29-specific Treg cells. Therefore, B29 has the unique quality to induce highly effective Treg cells with specificity for Hsp70, which is a very abundantly expressed and inflammation-associated molecule.

Recently, we evaluated binding of B29 and its homologs to human MHC class II, by using *in silico* prediction methods and reviewing published MHC class II elution studies (7). Interestingly, a previous study had already demonstrated the presence of a human homolog of peptide B29 in the elution profile obtained from a stressed HLA-DR4+ human B cell line (21). Other elution profiles showed the same peptides as well as many other Hsp70-derived peptides (20). Based on these observations, a promiscuous binding profile was predicted (for review, see ref. 7). In the present study, we further substantiated this notion by performing direct binding assays (Table 1). B29 proved to be a good-to-strong binder of HLA-DR1, HLA-DR4, HLA-DR7, HLA-DR11, HLA-DR15, HLA-DQ2, and HLA-DQ8. Based on the relative frequencies of these HLA molecules in Caucasian populations, we conclude that >80% of humans can present B29 to their T cells. Given the high frequency of HLA-DR4 and HLA-DQ8 in RA patients, the B29-specific T cell frequency could even be higher in these patients.

Subsequently, we tested B29 in a HLA-DQ8-transgenic mouse model of PG-induced arthritis, in which murine MHC class II is completely lacking (8). Intranasal administration of B29 prior to arthritis induction dramatically suppressed disease development. In addition, splenocytes from these immunized mice were

restimulated with PG, and in vivo immunization with B29 reduced all ex vivo PG responses. This response was further reduced when B29 was also present during restimulation. This indicates that B29, when presented in vivo in the context of human MHC, induced disease-suppressive regulation that was further activated during ex vivo B29 restimulation.

An in vitro priming and restimulation assay (12) was performed to investigate whether human CD4+ T cells could be primed with B29-loaded monocyte-derived DCs and to analyze the resulting T cell phenotype by staining for intracellular CD40L and IFN γ . As already expected based on the HLA binding data, B29 triggered CD4+ T cell responses in humans with a high frequency, because this peptide induced B29-specific T cell responses in 10 of 14 healthy individuals. Among these healthy individuals, 8 of 10 HLA-DR4+ donors responded to B29. With the use of B29-specific and mB29b-specific tetramers, we showed that B29 also induced cross-reactive Treg cells in humans (in vitro), which is consistent with our previous findings in mice (7).

Based on various observations made using mouse models of preclinical arthritis, we originally formulated the hypothesis that conserved sequences present in mycobacterial Hsp60 and Hsp70 were capable of inducing a T cell response, which was protective due to its self-HSP cross-reactive qualities. In the case of B29, we have seen close homologies of this microbial epitope with both constitutive and inducible mammalian Hsp70 family members (mB29a present in Hsp9, mB29b present in HspA1A and HspA8, and mB29c present in Hsp5 [7]). Consequently, in the mouse model of PG-induced arthritis, CD4+ T cells primed with B29 were shown to cross-react with these mammalian homologs. In addition, protection against arthritis was seen not only after immunization with B29 itself but also after immunization with the mammalian homologs mB29a and mB29b (7). For B29 to become protective against RA in humans, the same cross-reactivities must be present in the human T cell responses. As shown in Figure 4, in vitro priming with B29 did lead to an enhanced secondary response to mammalian homolog mB29b. Because mB29b is present both in a constitutive Hsp70 family member (HspA8) and in one of the major inducible Hsp70 family members (HspA1A), the 2-amino acid difference between B29 and mB29b apparently does not negatively impact the cross-reactive nature of the peptides at the T cell level in both mice and humans.

Taken together, these data indicate a conserved specificity and functionality of B29-induced Treg cell responses in the context of the human MHC. Based on these findings, we conclude that a translational path for

the clinical development of peptide B29 may be a real possibility in the near future. The antiinflammatory activities of B29-induced Treg cells, as shown in models of preclinical arthritis, indicate that similar antiinflammatory activities can be anticipated when the peptide is used in human RA and possibly other chronic inflammatory conditions.

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All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Broere had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Clinical Images: Prevention of hand osteoarthritis by hemiparesis

The patient, a 93-year-old woman, was hospitalized in the rheumatology unit for acute low back pain associated with an L4 spinal fracture. She had a 60-year history of right hemiparesis secondary to a stroke. Physical examination revealed right hemiparesis with dystonia. Muscle strength for the right upper extremity was $>3/5$, and she still used her right hand for daily activity. No deficit was found on the left side of the body. The fingers of the right hand had no deformity or joint swelling. In contrast, the left hand exhibited typical changes of osteoarthritis (OA), with both Heberden's and Bouchard's nodes in association with irregular deformation (A). Radiographic examination (B) confirmed the diagnosis of OA, with osteophytic lipping, sclerosis, and joint space narrowing of several distal and proximal joints. Asymmetric OA in a patient with hemiparesis has been described previously (1). Reported underlying mechanisms (2) are mechanical, with less use of the hand on the hemiparetic side, and neurogenic, with the possible implication of inflammatory neuropeptides (like adipokines in OA).

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