# Influence of Class II MHC-Specific Allopeptides via the Indirect Pathway of Allorecognition in Experimental Small Bowel and Kidney Transplantation

Susanne M. Lenhard, Martin Gasser, Cristophe Otto, Joana E. Kist-van Holthe, E. Navarro, Wolfgang Timmerman, Karin Ulrichs, Arnulf Thiede and Ana Maria Waaga

*Graft* 2002; 5; 182
DOI: 10.1177/1522162802005003016

# Influence of Class II MHC-Specific Allopeptides via the Indirect Pathway of Allorecognition in Experimental Small Bowel and Kidney Transplantation

*Susanne M. Lenhard,* Martin Gasser,* Christoph Otto, Joana E. Kist-van Holthe, E. Navarro, Wolfgang Timmerman, Karin Ulrichs, Arnulf Thiede, and Ana Maria Waaga*

Ana Maria Waaga, PhD
Brigham and Women's Hospital
Harvard Medical School
Renal Division
Laboratory of Immunogenetics
and Transplantation
75 Francis Street
Boston, Massachusetts, USA 02115
email: awaaga@rics.bwh.harvard.edu

*Co-first authors, both authors contributed equally to this work

## Introduction

T-cell recognition of alloantigens is the central and primary event initiating the rejection process, ultimately leading to the complete destruction of engrafted organs. It is generally accepted that there are 2 distinct pathways of allorecognition, each generating specific subsets of cells. T cells can either recognize intact allo-MHC (major histocompatibility complex) molecules on the surface of donor cells (via the direct pathway) or recognize them as processed allopeptides on the surface of self-antigen-presenting cells (APCs) (via the indirect pathway of allorecognition). There is increasing evidence that indirect allorecognition plays an important role in chronic allograft rejection. In this study, we investigated the functional role of class II MHC- specific allorecognition after both small bowel transplantation (SBTx) and kidney transplantation (KTx) in the rat.

## Materials and Methods

### *Class II MHC Peptides*

Eight overlapping peptides of 25 amino acids each, representing the full-length sequence of the β chain hypervariable domain of the donor strain RT1.B$^u$ (HLA-DQ like) and RT1.D$^u$ (HLA-DR like) were synthesized, as previously described.[1-5] Their immunogenicity was analyzed by testing for lymphocyte proliferation in vitro and DTH responses in vivo.[1,2,6] In this study, the immunogenic peptide RT1.D$^u$(20-44) was tested and the nonimmunogenic allopeptide RT1.B$^u$(20-44) was used as a control.

### *Experimental Model*

Lewis (LEW) (RT1$^l$) recipients of Wistar Furth (WF) (RT1$^u$) allografts (Harlan Sprague Dawley, Indianapolis, IN) were immunized subcutaneously on day –7 in the footpad (100 μg each) with the immunogenic peptide RT1.D$^u$(20-44) (group 1) or with the nonimmunogenic control peptide RT1.B$^u$(20-44) (group 2). Immunized animals were then transplanted with either small bowel or kidney (WF) allografts and were again injected with RT1.D$^u$(20-44) (group 1), or RT1.B$^u$(20-44) (group 2) (200 μg peptide in 2-ml phosphate-buffered saline on day 0 intraperitoneally). Small bowel transplants were performed using a standard technique as described earlier.[7] The kidneys were grafted heterotopically, with removal of the contralateral native kidney on the day of transplantation in animals from groups 1 and 2, or after 10 days in animals from groups 3 and 4. Recipients in groups 3 and 4, with small bowel or kidney grafts, were treated with cyclosporine (CsA; SBTx: 20 mg/kg from days 0 to 13, KTx: 5 mg/d0-4) and

Table 1 | **IMMUNOMODULATING TREATMENT PROTOCOL OF SMALL BOWEL TRANSPLANTATION (SBTx) AND KIDNEY TRANSPLANTATION (KTx) GROUPS**

| Group | Immunization Days –7 and 0 | Immunization Days +20 and 27 | CsA | Survival after SBTx Days, Mean ± SD | *N* | Survival after KTx Days, Mean ± SD | *N* |
|---|---|---|---|---|---|---|---|
| 1 | RT1.D$^u$(20-44) | — | — | 3.5 ± 0.6 | 4 | 4.0 ± 0.7 | 5 |
| 2 | RT1.B$^u$(20-44) | 1 | 1 | 4.0 ± 0.8 | 4 | 5.6 ± 0.9 | 5 |
| 3 | — | RT1.D$^u$(20-44) | + | 33.7 ± 4.1 | 6 | 37.0 ± 13.6 | 5 |
| 4 | — | RT1.B$^u$(20-44) | + | 69.7 ± 65.5 | 4 | 49.5 ± 11.2 | 6 |
| 5 | — | — | + | >250 | 6 | 51.0 ± 10.0 | 6 |
| 6 | — | — | — | 5.3 ± 0.5 | 6 | 7.5 ± 1.6 | 6 |

CsA = cyclosporin A; SD = standard deviation; *n* = number of animals per group.

challenged with RT1.D$^u$(20-44) (group 3) or RT1.B$^u$(20-44) (group 4) on days 20 and 27 after transplantation, or left without immunization (group 5), or without both CsA treatment and peptide immunization (group 6) (Table 1). The additional protocol of CsA in groups 3 and 4 was used to reliably treat the initial component of acute rejection, which requires a comparably higher dose of CsA after grafting a small bowel than after kidney transplantation. Animals were sacrificed during end-stage allograft failure.

### *Peptide Proliferation Assays*

Splenocytes and lymph node (LN) lymphocytes ($3 \times 10^5$) were cultured in RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) containing 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, $2 \times 10^{-5}$ M 2-mercaptoethanol, and 5 mM HEPES with 10 µg of RT1.D$^u$(20-44), or RT1.B$^u$(20-44) peptide in 96-well U-bottom plates (Costar, Cambridge, MA). All assays were set up in quadruplicate. Cells cultured in media, without the addition of peptide, served as negative controls. Cells incubated in media and 50 µg/ml of Concanavalin A (ConA, Sigma, Aldrich, St. Louis, MO) acted as positive controls. The plates were incubated for 96 h at 37 °C, with [$^3$H] thymidine (1 µCi/well) added for the final 6 hours of culture. The proliferation was assayed by measuring the incorporation of lymphocyte DNA. The data are expressed in counts per minute (CPM ± SEM).

### *Enzyme Linked-Immunosorbent Assay (ELISA) for Rat IFN-γ, IL-2, IL-4, IL-10*

The cultured supernatants from the proliferation assays were harvested after 48 h incubation at 37 °C with 5% $CO_2$ to determine IFN- γ, IL-2, IL-4, and IL-10 production. Culture supernatants were assayed by ELISA using BioSource Cytoscreen Rat ELISA Kits (BioSource, Camarillo, CA).

### *RNAse Protection Assay*

To analyze a whole panel of cytokines relevant for inflammatory events, RNase protection was performed using the Riboquant™ Multi-Probe RNase Protection assay system (BD Pharmingen, San Diego, CA). For this technique, a cocktail of RNA templates for cytokines, and control RNA radiolabelled with $^{32}$P and hybridized with the RNA extracted from the allografts was used. For each sample, RNA was isolated using Trizol. The RNA was then hybridized with a $^{32}$P-labelled cytokine template, HCK-1 (Pharmigen), and run on a polyacrylamide urea gel. Finally, the RNA was analyzed by a phosphoimager using Imagequant software, allowing accurate quantification of mRNA.

## Results

### *Animal Survival*

Unmodified animals in group 6 rejected their allografts after 5.3 ± 0.5 (SBTx, *n* = 6) and 7.5 ± 1.6 days (KTx, *n* = 6). The mean survival of SB control animals (group 5) was > 250 days (4/5 without signs of chronic rejection, with secondary transplanted WF heart and skin being specifically accepted) versus 51.0 ± 10.0 days for kidney transplant recipients (group 5, *n* = 6). Animals immunized with the nonimmunogenic RT1.B$^u$ (20-44) peptide, and treated with CsA (group 4), rejected their grafts after 69.7 ± 65.5 (1/6 survived > 250 days, SBTx) and 49.5 ± 11.2 (KTx, *n* = 4)

days. In contrast, group 3 animals, immunized with RT1.D$^u$(20-44) peptide and treated with CsA, rejected their grafts in an accelerated fashion (day 33.7 ± 4.1 SBTx, $n$ = 6 or 37.0 ± 13.6 KTx, $n$ = 5). The animals that were immunized with RT1.D$^u$ (20-44), and left without additional CsA (group 1), rejected their allografts after 3.5 ± 0.6 (SBTx, $n$ = 4) and 4.0 ± 0.7 (KTx, $n$ = 5) days versus 4.0 ± 0.8 (SBTx, $n$ = 4) and 5.6 ± 0.9 (KTx, $n$ = 5) days for animals immunized with the nonimmunogenic RT1.B$^u$(20-44) peptide (Table 1). These data confirm the specificity of the observed effect of a donor class II MHC peptide priming in accelerating small bowel and kidney allograft rejection.

### *Peptide Proliferation Assays*

Animals bearing kidney allografts and immunized with RT1.B$^u$(20-44) (group 4) demonstrated an identical low reactivity to the specific RT1.D$^u$ (20-44) and RT1.B$^u$(20-44) peptide (splenocytes: 2001 ± 276 versus 2084 ± 415 CPM ± SEM, LN: 2763 ± 788 versus 2507 ± 343 CPM ± SEM). In contrast, splenocytes and LN lymphocytes from group 3 animals, immunized with the RT1.D$^u$ (20-44) peptide, showed a significant reactivity to the specific peptide RT1.D$^u$(20-44), compared with the nonimmunogenic peptide RT1.B$^u$(20-44) (splenocytes: 14776 ± 2599 versus 721 ± 239, LN: 15521 ± 1377 versus 853 ± 381 CPM ± SEM). Proliferation of splenocytes and LN lymphocytes, in absence of peptide, was 661 ± 125 versus 759 ± 137 CPM ± SEM (group 4) and 741 ± 231 versus 822 ± 174 CPM ± SEM (group 3), respectively. The proliferative responses of splenocytes and LN lymphocytes, incubated with ConA, were 38375 ± 3527 versus 37083 ± 2236 (group 4) and 38008 ± 4123 versus 37016 ± 5438 CPM ± SEM (group 3), respectively.

### *ELISA for Rat IFN-γ, IL-2, IL-4, IL-10*

Cytokine analysis by ELISA of the culture supernatants obtained from the proliferation assays was performed. Splenocytes from the animals immunized with RT1.B$^u$(20-44) (group 4) produced IFN-γ, in response to the specific peptide RT1.D$^u$ (20-44), or to the nonimmunogenic peptide RT1.B$^u$(20-44) (184 ± 11 versus 59 ± 7 pg/ml), IL-2 (125 ± 7 versus 60 ± 6 pg/ml), IL-4 (102 ± 19 pg/ml versus 42 ± 9), and IL-10 (193 ± 7 versus 85 ± 4 pg/ml). In contrast, splenocytes and LN lymphocytes from animals immunized with the RT1.D$^u$(20-44) peptide (group 3) produced higher amonts of IFN-γ response to the specific peptide RT1.D$^u$(20-44), or to the nonimmunogenic peptide RT1.B$^u$(20-44) (401 ± 87 versus 84 ± 15 pg/ml), IL-2 (337 ± 13 versus 115 ± 7 pg/ml), but lesser Il-4 (34 ± 12 versus 28 ± 2 pg/ml) and IL-10 (80 ± 10 versus 56 ± 6). These findings indicate that immunization with peptide RT1.D$^u$(20-44) mediates the relevant inflammatory cytokines involved in the acceleration of the allograft rejection.

### *RNAse Protection Assay*

Acutely and accelerated rejected allografts from animals primed with the immunogenic RT1.D$^u$ (20-44) peptide showed IL-2, –4, –6, IFN-γ TNF-β (groups 1 and 3), and TNF-α gene expression in group 1 kidneys. In contrast to RT1.B$^u$(20-44) immunized animals, those immunization with RT1.D$^u$(20-44) expressed high levels of IL-6, and less predominant, but significantly different IFN-γ (both cytokines $P < 0.0001$) in small bowel and kidney allografts (groups 1 and 3), whereas nonimmunogenic RT1.B$^u$(20-44) peptide-immunized animals demonstrated higher levels of IL-4 (groups 2 and 4) ($P < 0.0001$) than those immunized with RT1.D$^u$(20-44). These results confirm the production of the cytokines measured by ELISA and clearly demonstrate a Th1 cytokine profile in kidneys from RT1.D$^u$(20-44) immunized animals, which is related to the severity of the allograft rejection.

## Discussion

These data demonstrate for the 1st time that T cells primed in vivo via the indirect pathway with donor class II MHC-specific peptides can mediate a specific immune response after both kidney and small bowel transplantation. These results are in accordance with recently published data obtained by others using the same peptides in a heart transplant model.[8] This indicates a uniform immune response in the recipient, independent of the type of transplanted organ, after priming with donor class II peptides via the indirect pathway of allorecognition.

It has been suggested that early acute allograft rejection is predominantly mediated by the direct

pathway of allorecognition, as organ allografts contain a significant number of donor-derived passenger cells, with the APCs presenting a high density of intact allo-MHC molecules. This seems to be important for the small bowel, because this organ contains a higher number of passenger cells than organs such as the heart or the kidney. However, there is increasing evidence that indirect allorecognition also has a significant role in mediating allograft rejection. Several reports demonstrate that T cells, primed by the indirect pathway, are present during acute allograft rejection in experimental transplant models, as well as in humans.[2,4,6,9-11] It has been suggested that transplant recipients are primed to donor class II MHC peptides via the indirect pathway during the course of acute or subacute rejection episodes.[11] Over the long term, this T cell–mediated process persists and may be responsible for the induction of chronic allograft rejection.[12,13] In fact, the precursor frequency of donor allopeptide–specific T cells increased significantly under these circumstances. Therefore, this may have implications in terms of novel therapeutic strategies—and may be particularly important after small bowel transplantation since this organ undergoes acute rejection episodes more frequently during the early phase after transplantation. Due to the large amount of passenger cells within the bowel, this organ contains an immense source of alloantigen (MHC class I and II) that can be shed from these passenger cells as intact antigens and, in a soluble form, trigger cellular processes via the indirect pathway. Although acute rejection, via the direct pathway, may be treated successfully in this case with interventional immunosuppressive therapy, indirect primed T cells may subsequently induce and/or perpetuate chronic cellular processes.

In our previous study with T cell lines and clones, generated from long-term surviving LEW recipients of WF kidney allografts and from acutely rejecting recipients against the immunodominant donor MHC peptide RT1.D$^{u}$(20-44), we demonstrated that the cytokine pattern, produced by the donor-specific peptide-primed T cells, is associated with the presence or absence of allograft disfuntion. Stable graft function is associated with a Th2 pattern of cytokines, IL-4 and IL-10, whereas allograft dysfuntion is associated with Th1 cytokine production, IFN-γ and IL-2.[14] In this study, we found a correlation between acute rejection and the expression of a predominantly Th1 cytokine profile, and between long-term survival and the expression of IL-4 and IL-10 in supernatants of splenocytes obtained from small bowel transplant recipients. The current findings in peptide-primed recipients could relate to the direct effects of the Th1 cytokine IFN-γ, or arise as a result of the induction of alloantibodies and their binding to the graft, or both. These data were confirmed by the cytokine gene expression of TNF-α/β, IFN-γ, IL-2, in acutely rejected allografts. Moreover, animals immunized with RT1.D$^{u}$(20-44) rejected their allografts consistently in an accelerated manner, which was reflected by gene expression of IL-4/IFN-γ in the SB and renal allografts. Animals immunized with the immunogenic RT1.D$^{u}$(20-44) peptide expressed higher levels of IFN-γ than IL-4, compared with animals immunized with the nonimmunogenic RT1.B$^{u}$(20-44) peptide, which expressed more IL-4 than IFN-γ. These results, coupled with our recent data on allopeptide-specific T cell clones, clearly demonstrate that indirectly primed Th1 cells specifically target the allograft (DTH), resulting in severe cellular rejection. Moreover, the immunization of transplanted animals with the immunogenic peptide RT1.D$^{u}$(20-44) at a critical time point after transplantation (days 20 and 27) resulted in acute rejection, which occurred in an accelerated manner when compared with nonimmunized controls. However, animals that were immunized at this late time point with the nonimmunogenic peptide RT1.B$^{u}$(20-44) rejected their renal allografts acutely like the controls, or had late acute rejection after small bowel transplantation. This indicates an immunodulatory role for MHC-derived peptides acting at several different stages in T cell activation and proliferation, with clear dominance of the immunogenic peptides. Thus, priming through the indirect pathway can accelerate or induce allograft rejection. This study indicates the importance of the indirect pathway and may have implications for the development of new therapeutic strategies using such peptides in combination with additional immunosuppressants to achieve allospecific tolerance.

## REFERENCES


1. Sayegh MH, Khoury SJ, Hancock WW, , Weiner HL, Carpenter CB. Induction of immunity and oral tolerance with polymorphic class II major histocompatibility complex allopeptides in the rat. **Proc Natl Acad Sci U S A 1992;89(16):7762-6**.
2. Gallon L, Watschinger B, Murphy B, Akalin E, Sayegh MH, Carpenter CB. The indirect pathway of allorecognition: the occurrence of self-restricted T cell recognition of allo-MHC peptides early in acute renal allograft rejection and its inhibition by conventional immunosuppression. **Transplantation 1995;59(4):612-16**.
3. Chen W, Murphy B, Waaga AM, et al. Mechanisms of indirect allorecognition in graft rejection: class II MHC allopeptide-specific T cell clones transfer delayed-type hypersensitivity responses in vivo. **Transplantation 1996;62(6):705-10**.
4. Vella JP, Vos L, Carpenter CB, Sayegh MH. Role of indirect allorecognition in experimental late acute rejection. **Transplantation 1997;64(12):1823-28**.
5. Waaga AM, Chandraker A, Spadafora-Ferreira M, et al. Mechanisms of indirect allorecognition: characterization of MHC class II allopeptide-specific T helper cell clones from animals undergoing acute allograft rejection. **Transplantation 1998;65(7):876-83**.
6. Watschinger B, Gallon L, Carpenter CB, Sayegh MH. Mechanisms of allo-recognition: recognition by in vivo-primed T cells of specific major histocompatibility complex polymorphisms presented as peptides by responder antigen-presenting cells. **Transplantation 1994;57(4):572-6**.
7. Deltz E, Thiede A. Microsurgical technique of small-intestine transplantation. In: Thiede A, Deltz E, Engemann R, Hamelmann H, editors. Microsurgical model in rats for transplantation research.**Berlin: Springer-Verlag; 1985. p. 51-5**.
8. Vella JP, Magee C, Vos L, et al. Cellular and humoral mechanisms of vascularized allograft rejection induced by indirect recognition of donor MHC allopeptides. **Transplantation 1999;67(12):1523-32**.
9. Benichou G, Takizawa AP, Olson AC, McMillan M, Sercar EE. Donor major histocompatibility complex (MHC) peptides are presented by recipient MHC molecules during graft rejection. **J Exp Med 1992;175:305-8**.
10. Shirwan H, Leamer M, Wang HK, Makowka L, Cramer DV. Peptides derived from alpha-helices of allogeneic class I major histocompatibility complex antigens are potent inducers of $CD4^+$ and $CD8^+$ T cell and B cell responses after cardiac allograft rejection. **Transplantation 1995;59(3):401-10**.
11. Liu Z, Colovai AI, Tugulea S, et al. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. **J Clin Invest 1996;98(5):1150-7**.
12. Vella JP, Spadafora FM, Murphy B, et al. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. **Transplantation 1997;64(6):795-800**.
13. Ciubotariu R, Liu Z, Colovai AI, et al. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. **J Clin Invest 1998;101(2):398-405**.
14. Waaga AM, Gasser M, Kist-van Holthe JE, Najafian N, Muller A, Vella JP, et al. Regulatory functions of self-restricted MHC class II allopeptide-specific Th2 clones in vivo. **J Clin Invest 2001;107(7):909-916.**

# THE NEUROSCIENTIST

**Editor**
**Stephen G. Waxman**
*Yale University School of Medicine,*
*New Haven, Connecticut*

SAGE SCIENCE PRESS

## New at Sage Science Press!

Edited by Stephen G. Waxman of Yale University School of Medicine with help from a renowned international editorial board that includes Nobel Prize winners and chairs of top university neuroscience, neurology, neurosurgery, and psychiatry departments from around the world, **The Neuroscientist** reviews and evaluates the noteworthy advances and key trends in molecular, cellular, developmental, behavioral/systems, and cognitive neuroscience in a unique, disease-relevant format.

Aimed at basic neuroscientists, neurologists, neurosurgeons, and psychiatrists in research, academic, and clinical settings, **The Neuroscientist** reviews and updates the most important new and emerging basic and clinical neuroscience research. It presents original and innovative topics such as:


- Neural transplantation, stem cells, and neurogenesis
- Development and aging of the brain
- Trophic and growth factor; guidance molecules
- Channels, receptors, and transmitters
- Neural plasticity and regeneration
- Applications of neurobiology to therapy of diseases of the brain and spinal cord
- Functional imaging and cognitive neuroscience
- Molecular and cellular mechanisms of disease
- Neural substrates of behavior and behavioral disorders
- Computational neuroscience
- Genetics of neurologic and psychiatric diseases

***Volume 7 (2001), 6 issues***
***Bimonthly: February, April, June, August, October, December***
***Subscription rates: Individual $125 / Institution $300***
***Prices subject to change without notice.***
***ISSN: 1073-8584***

**SUBSCRIBE TODAY!**
**Phone: 800-818-7243 Fax: 800-583-2665**
**International: 805-499-9774 Fax: 805-499-0871**
*E-mail: order@sagepub.com*

**www.sagesciencepress.com**

F398001