Gene Therapy (2000) 7, 575–582

www.nature.com/gt

VIRAL TRANSFER TECHNOLOGY ***RESEARCH ARTICLE***

Gene transfer of virally encoded chemokine antagonists vMIP-II and MC148 prolongs cardiac allograft survival and inhibits donor-specific immunity

LA DeBruyne[1], K Li[1], DK Bishop[1,2] and JS Bromberg[1,2,3]

Departments of [1]Surgery, and [2]Microbiology and Immunology, University of Michigan Medical Center, Ann Arbor, MI; and [3]The Recanati-Miller Transplant Institute, Mount Sinai School of Medicine, New York, NY, USA

Introducing immunomodulatory molecules into allografts by gene transfer may avoid the side-effects of systemic immunosuppression. vMIP-II and MC148 are two recently identified chemokine homologues encoded by human herpes virus 8 and Molluscum contagiosum, respectively, that have antagonistic activities against multiple different CC and CXC chemokine receptors. We hypothesized that introduction of these molecules into cardiac allografts may block leukocyte infiltration into the grafts and prolong survival. Vascularized and nonvascularized cardiac allografts in mice were performed and plasmid DNA encoding vMIP-II, MC148 and/or vIL-10 was transferred into the allograft at the time of transplantation. Gene transfer of either vMIP-II or MC148 into cardiac allografts markedly prolonged graft survival. Combining gene transfer of either one of these chemokine antagonists with vIL-10 gene transfer, which has a mechanistically different immunosuppressive action, further enhanced graft survival. vMIP-II and MC148 gene transfer both resulted in a marked decrease of donor-specific cytotoxic T lymphocytes (CTL) infiltrating the grafts and inhibited alloantibody production. These results demonstrate that plasmid-mediated gene transfer of virally encoded chemokine antagonists vMIP-II and MC148 can block donor-specific lymphocyte immunity within cardiac allografts and prolong graft survival. This is a new mechanistic approach to analyze, treat, and prevent graft rejection. Delivery of these or related molecules by gene transfer or conventional pharmacologic means may represent a novel therapeutic modality for alloactivation. Gene Therapy (2000) **7,** 575–582.

Keywords: *gene therapy; transplantation; T cells; human herpes virus 8; Molluscum contagiosum*

Introduction

The recruitment and activation of recipient leukocytes in response to a transplanted organ constitute the initial steps of a complex immune response which ultimately culminates in rejection of the graft. Chemokines play a crucial role in initiating the rejection process, in that they are the primary molecules involved in leukocyte recruitment.[1] Chemokines consist of a large family of small secreted proteins that bind to G protein coupled membrane receptors, and are generally divided into two structural groups.[1–3] The CXC, or α chemokines, generally attract neutrophils whereas the CC, or β chemokines, are primarily chemoattractant for monocytes and lymphocytes.[1–3] CXC chemokines usually exhibit high affinity for a single receptor, whereas CC chemokines are more promiscuous and bind to and activate multiple receptors.[4] In addition, two new chemokines have been discovered that do not fall into the original CC and CXC classification. Lymphotaxin (C) has only one of the first two conserved cysteine residues,[5] and fractalkine (CXXXC) has three amino acids separating the first two cysteines.[6] Since chemokines play a crucial role in the physiologic process of leukocyte recruitment and activation, they have often been considered as potential targets for immunomodulation. However, antichemokine therapies have been hindered by the redundancy of the chemokine system. Multiple chemokines, many with overlapping activities, are usually induced at inflammatory sites and many different chemokine receptors can be expressed by a single type of cell.[1] Thus, it is difficult to inhibit inflammatory responses by antagonizing a single chemokine or its receptor. To overcome the redundancies of the chemokine system, molecules must be identified that have the capacity to bind to and antagonize multiple chemokine receptors.

Human herpes virus 8 (HHV8) is the infectious agent now believed to be responsible for Kaposi's sarcoma in both HIV and non-HIV infected individuals.[7–13] Unique among known human herpes viruses, HHV8 encodes a gene product, designated vMIP-II, which shows sequence similarity to the human chemokine family.[14,15] vMIP-II is most closely related to the CC chemokines, having 41.1% amino acid sequence homology to MIP-1α.[14,15] vMIP-II is unique in its ability to bind to both CC and CXC chemokine receptors.[16,17] Binding of vMIP-II to its receptors does not result in the normal, rapid mobilization of calcium from intracellular stores; instead it blocks calcium mobilization induced by endogenous chemokines.[16]

Correspondence: JS Bromberg, The Racanati/Miller Transplant Institute, Mount Sinai-NYU Health System, 1 Gustave L Levy Place, Box 1496, New York, NY 10029, USA
Received 20 July 1999; accepted 9 November 1999

Table 1 Gene transfer prolongs nonvascular cardiac allograft survival

Group	*Days*	*Mean ± s.e.m.*	*P value*
No treatment control	11, 13, 13, 15, 15	13.4 ± 0.8	—
vMIP-II	16, 21, 21, 23, 23	20.8 ± 1.3	0.001
MC148	24, 24, 26, 28	25.5 ± 1.0	<0.001
vMIP-II + MC148	19, 19, 21, 21, 24	20.8 ± 1.3	0.001
vIL-10	23, 23, 25, 25, 27	24.6 ± 0.8	<0.001
vMIP-II + vIL-10	35, 35, 35, 35, 37, 37	35.7 ± 0.4	<0.001
MC148 + vIL-10	26, 26, 28, 30	27.5 ± 1.0	<0.001

Thus, it appears that the virus has optimized vMIP-II, possibly as a viral defense mechanism, such that it is capable of binding and blocking multiple human chemokine receptors, in order to prevent the recruitment and activation of leukocytes.

Similar to HHV8, the 190 000 base pair genome of the pox virus *Molluscum contagiosum* also contains an open reading frame encoding a chemokine homologue.[18] *Molluscum contagiosum* encodes the gene product MC148, which is most closely related to human CC chemokines.[18] Similar to vMIP-II, MC148 is also capable of binding to multiple CC and CXC chemokine receptors, and inhibiting chemotaxis induced by endogenous chemokines.[19,20] In comparison with human CC chemokines, the amino terminal region is significantly shortened on MC148.[18] It is this amino terminal region of the protein that is believed to be involved in receptor activation,[21] and probably as a result of this deletion, MC148 is a chemokine antagonist. Interestingly, a remarkable feature of *Molluscum contagiosum* is that infectious skin lesions contain large numbers of viral particles yet lack an inflammatory cell infiltrate.[22] Based upon sequence similarity, it is believed that both vMIP-II and MC148 were probably acquired by these viruses through acts of molecular piracy on human chemokines. Since vMIP-II and MC148 are both potent chemokine antagonists capable of binding to multiple chemokine receptors, we hypothesized that introduction of these molecules into cardiac allografts may block leukocyte infiltration into the grafts and prolong survival.

Acute allograft rejection is normally controlled by administering immunosuppressive pharmacologic agents to transplant recipients. However, systemic immunosuppression often leads to opportunistic infections and drug-specific side-effects. Gene transfer has the potential to introduce immunoregulatory molecules directly into the graft, producing a localized immunosuppressive environment without systemic side-effects.[23–25] Thus, in the present study, we chose to utilize gene transfer technology to deliver more effectively vMIP-II and MC148 gene products into the graft *in vivo*, and identify immunologic mechanisms associated with enhanced graft survival.

Results

Chemokine gene transfer prolongs graft survival

Expression vectors encoding vMIP-II and MC148 were created by employing PCR cloning techniques. Utilizing the heterotopic, nonvascularized neonatal heart ear pinna model, plasmid DNA encoding either vMIP-II or MC148 was directly injected into C57BL/6 cardiac allografts placed into CBA/J recipients (Table 1). Administration of the vMIP-II plasmid significantly extended allograft survival to an average of 20.8 ± 1.3 days, compared with 13.4 ± 0.8 for controls. Similarly, injection of plasmid DNA encoding the virally derived MC148 molecule also significantly prolonged allograft survival (25.5 ± 1.0 days). Injecting grafts with a combination of vMIP-II and MC148 plasmid DNA did not further extend graft survival, implying that these two molecules function through related mechanisms. Previous studies in our laboratory demonstrated that gene transfer of another virally derived molecule, viral IL-10 (vIL-10), can also significantly extend graft survival.[24–26] Unlike vMIP-II and MC148, vIL-10 is not a chemokine antagonist. Instead, vIL-10 functions by inhibiting cytokine production and down-regulating important costimulatory molecules.[27–32] Thus, we hypothesized that combining either of the chemokine antagonists vMIP-II or MC148 with vIL-10 may further prolong graft survival by decreasing leukocyte infiltration into grafts, as well as inhibiting cytokine production and cell activation. Gene transfer of vMIP-II combined with vIL-10 had at least an additive effect, extending graft survival to 35.7 ± 0.4 days. The result of vIL-10 gene transfer combined with MC148 was no better than either molecule alone, prolonging graft survival to 27.5 ± 1.0 days.

The initial experiments utilized the nonvascularized neonatal heart ear pinna model. Although informative, this model system is not directly clinically applicable. To address the plausibility of utilizing vMIP-II and MC148 gene transfer in a more clinically relevant vascularized model and delineate the immune mechanisms responsible for extended survival, BALB/c cardiac allografts were perfused with plasmid DNA encoding vMIP-II or MC148 complexed with the lipid γAP DLRIE/DOPE, and graft survival determined following transplantation into C57BL/6 (Table 2). Both vMIP-II and MC148 gene transfer resulted in a modest, but statistically significant, increase in graft survival (Table 2). The small increase in survival times is likely due in part to the fact that while

Table 2 Gene transfer prolongs vascular cardiac allograft survival

Group	*Days*	*Mean ± s.e.m.*	*P value*
No treatment	7, 8, 8, 8, 8, 9, 9	8.1 ± 0.3	—
vMIP-II	9, 11, 11, 11	10.5 ± 0.5	0.001
MC148	10, 10, 11, 12	10.8 ± 0.5	0.001

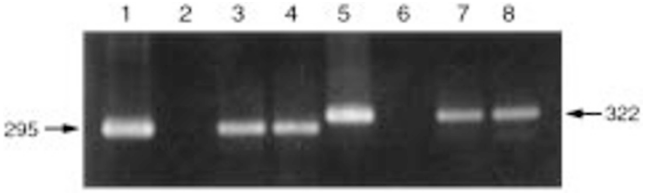

Figure 1 *vMIP-II and MC148 transgene transcription in cardiac allografts. RT-PCR was performed on DNase-treated RNA isolated from functioning grafts on days 7 and 11 after transplantation. vMIP-II primers produce a 295 base pair fragment, and MC148 primers produce a 322 base pair fragment. Lane 1, positive control for vMIP-II (PCR using pMP6A-vMIP-II as template); lane 2, negative control for vMIP-II (PCR without cDNA template); lane 3, RT-PCR on vMIP-II transduced allograft on day 7; lane 4, RT-PCR on vMIP-II transduced graft on day 11; lane 5, positive control for MC148 (PCR using pMP6A-MC148 as template); lane 6, negative control for MC148 (PCR without cDNA template); lane 7, RT-PCR on MC148 transduced allograft on day 7; lane 8, RT-PCR on MC148 transduced allograft on day 11.*

plasmid-mediated gene transfer is consistent in our model, the resulting transgene expression is low and transient.[24] Detection of gene products by ELISA or immunohistochemistry was not possible because antibodies specific for vMIP-II and MC148 do not exist. However, the results of RT-PCR analysis of graft tissues do show specific expression of gene transcripts (Figure 1). Although our previous studies with plasmid-mediated gene transfer in this model showed some transient and low level transcription of transferred genes in tissues outside the graft, the vast majority of transgene expression is intragraft.[26]

Chemokine antagonist gene transfer does not inhibit leukocyte accumulation within the graft

In order to determine the effects of vMIP-II or MC148 gene transfer on immune competence, animals were killed at 7 days after transplant, as this is the time the peak rejection response occurs in unmodified grafts. Graft infiltrating cells were isolated, and fluorescent flow cytometry was used to determine the composition of cells that had migrated into the grafts transduced with vMIP-II, MC148, or β-galactosidase, which was used as a control (Table 3). Somewhat unexpectedly, there were no significant differences in the total number of cells infiltrating vMIP-II or MC148 transfected grafts compared with β-galactosidase controls. In addition, there were similar percentages of macrophages and lymphocytes in all grafts evaluated, and there did not appear to be any significant changes in the ratio of CD4- to CD8-positive cells. Thus, transfer of chemokine inhibitor genes did not prevent homing to the allograft at the levels of gene expression achieved, but did enhance graft survival. The lack of effect on cell numbers may be due to the restricted and low expression of the peptides within the graft.

Table 3 Composition of graft infiltrating cells

Group	*CD3*	*CD4*	*CD8*	*MAC-1*
β-Galactosidase	31.5	13.8	16.4	65.2
vMIP-II	40.1	18.1	26.7	60.7
MC148	35.2	14.0	20.3	63.3

Data are expressed as the percent of the total cell number. Flow cytometry was performed on graft infiltrating cells harvested from two to three mice per group.

Intragraft, but not systemic, cell-mediated immunity is inhibited

Limiting dilution analysis was performed on graft infiltrating cells and splenocytes to determine donor alloantigen-specific precursor frequency for HTL and CTL. Results are representative of three separate experiments performed on groups of two to three animals at each time-point. At day 7 after transplant, there were no differences in the total number of donor-specific graft infiltrating or splenic HTL in vMIP-II- and MC148-transduced grafts, compared with β-galactosidase controls (Figure 2a). However, there was a large decrease in the number of donor-specific CTL infiltrating the chemokine antagonist-transduced grafts (Figure 2b). As previously seen in

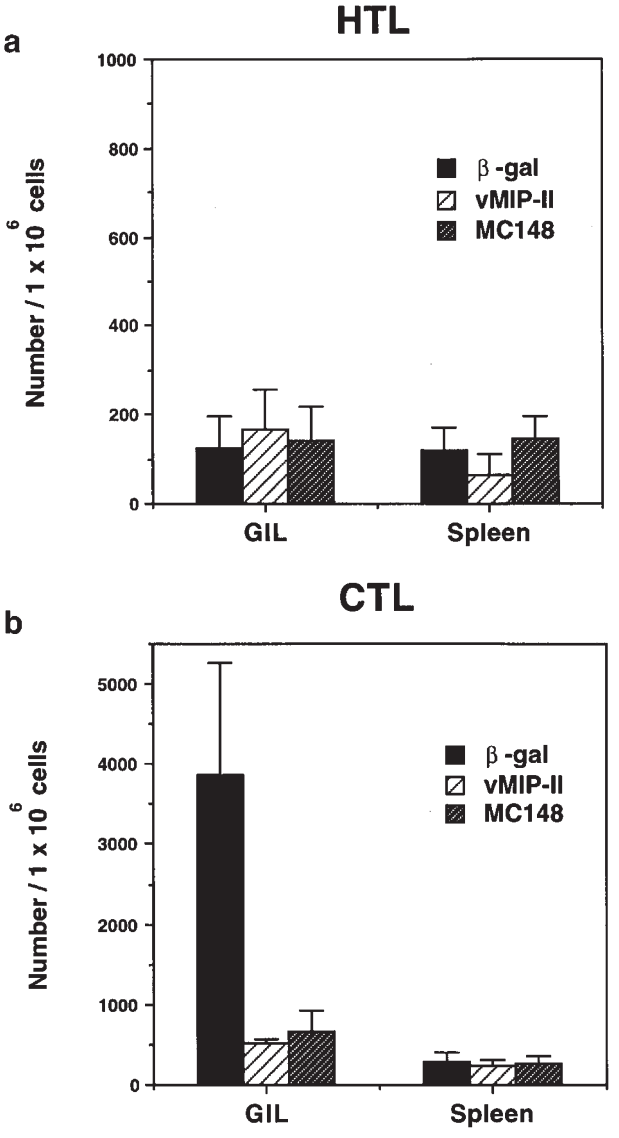

Figure 2 *Gene transfer inhibits graft infiltrating CTL but not HTL. Limiting dilution analysis of donor-specific graft infiltrating cells and splenic lymphocytes. Data are representative of three separate experiments performed on groups of two to three animals each. Results are presented as number per one million cells, including the 95% confidence interval.*

untreated allografts,[26] large numbers of donor-specific CTL infiltrated the β-galactosidase-transduced grafts (1/258 cells). In contrast, transducing grafts with either vMIP-II or MC148, inhibited the accumulation of donor-specific CTL within the grafts (1/1941 for vMIP-II and 1/1507 for MC148). This effect remained localized in that there were no significant differences in the number of donor-specific splenic CTL in animals receiving vMIP-II or MC148 grafts compared with β-galactosidase controls. This suggests that gene expression remained localized, as shown previously in our model.[24,25] Since graft infiltrating donor-specific CTL, but not splenic CTL, frequencies differed markedly between the experimental and control groups, while the total number of CD8-positive cells remained the same, it appears that the chemokine antagonists did not prevent homing to the graft, but rather maturation and/or proliferation of donor-specific CTL within the graft.

Humoral immunity is inhibited

At 7 days after transplantation, animals receiving grafts transduced with either vMIP-II or MC148 produced significantly less IgM alloantibody compared with β-galactosidase or no treatment control animals (Table 4). However, it appears that IgM alloantibody production was not eliminated, but rather delayed, since increased levels of IgM alloantibody were detected at later timepoints. None the less, little IgG1 and IgG2a alloantibody were detected at 11 days after transplantation, a time at which vMIP-II- and MC148-transduced grafts were eventually rejected. Differential production of Th1 and Th2 cytokines can be reflected in alloantibody isotype production. IgG1 is indicative of a Th2-type response, whereas IgG2a is indicative of a Th1-type response. In the vMIP-II- and MC148-treated animals that exhibited IgG production, there was consistently more IgG2a alloantibody produced than IgG1, suggesting a conventional Th1-type rejection response, similar to that seen in the β-galactosidase and untreated controls.

Since it was determined that vMIP-II and MC148 gene transfer partially inhibited components of both humoral and donor-specific cellular immune responses, we next questioned how the transduced grafts ceased functioning. Donor allogeneic cardiac grafts were harvested on days 7 and 11 after transplantation, fixed in formalin, sectioned, and stained with hematoxylin and eosin (Figure 3). Histologic analysis of vMIP-II- and MC148-transduced grafts at 7 days after transplant showed prominent mononuclear infiltrates, similar to those seen previously in unmodified control grafts (Figure 3a and c).[26,33] This day 7 histologic analysis correlated with the flow cytometric data (Table 3). In addition, the day 7 vMIP-II- and MC148-transduced hearts also had distinct areas of fibroblast-like infiltrate, as well as granulation tissue. Interestingly, when recipients are depleted of $CD8^+$ T cells this histologic picture of rejection is also observed.[33] Hence, the fibroblast containing infiltrate may be a reflection of the decrease in the number of donor-specific $CD8^+$ CTL. In contrast to the fibroblasts, the mononuclear cell infiltrate observed on day 7 after transplant was transient, and prolonged survival (day 11) of vMIP-II- and MC148-transduced grafts was associated with a lack of mononuclear infiltrate. Instead, large patches of dead myocytes and thrombosed arteries, typical of ischemic injury, were visible in day 11 post-transplant tissue samples (Figure 3b and d). Thus, the histologic data suggest that the eventual loss of graft function was the result of myocyte injury, possibly due to the eventual evolution of donor-specific cellular and humoral immune responses. None the less, this histology is markedly different from that of control unmanipulated rejecting allografts.[26,33]

Table 4 Gene transfer inhibits alloantibody production

Group	*Day*	*IgM*	*IgG1*	*IgG2a*
No treatment	7	41.2	8.5	7.2
βgal	7	46.8	5.9	8.1
vMIP-II	7	8.3	4.8	4.2
MC148	7	22.5	4.8	5.5
No treatment	10	23.9	21.6	31.3
vMIP-II	11	28.2	14.9	21.8
MC148	11	39.1	6.3	10.9
Isotype controls		4.1	4.4	4.4

Data are presented as mean channel fluorescence.

Discussion

This study addressed the feasibility of utilizing the virally derived chemokine antagonists vMIP-II and MC148 to transduce cardiac allografts and inhibit graft rejection, as well as defining the immunomodulatory mechanisms associated with enhanced graft survival. Direct injection of plasmids encoding either vMIP-II or MC148 into non-vascularized allografts significantly prolonged graft survival. Combining vMIP-II and MC148 did not further enhance survival, suggesting that these two molecules may be working by similar mechanisms, such as blocking the recruitment and/or activation of leukocytes. However, combining vIL-10, which functions by inhibiting cytokine production and down-regulating costimulatory molecules,[27–32] with vMIP-II markedly increased graft survival compared with either gene administered alone. In contrast, vIL-10 did not enhance MC148 immunosuppression, suggesting some differences between the two chemokine inhibitors. Alternatively, these findings may reflect different levels of protein expression. Although the vascularized grafts only exhibited modest prolongation of graft survival, our previous report[26] is the only other published study that evaluates plasmid gene transfer of immunomodulatory molecules to vascularized cardiac allografts, and those studies demonstrated that vIL-10 gene transfer can extend vascularized graft survival to a mean of 16.0 ± 2.3 days. As was the case with vIL-10, vMIP-II and MC148 gene transfer extended graft survival but this was not indefinite and tolerance was not achieved. This may be due in part to the fact that plasmid-mediated gene transfer is inefficient and the resulting transgene expression is low and transient.[24] Additional approaches will be required to improve non-viral and viral gene transfer and expression, such as optimization of nucleic acid delivery, transcellular transport, nuclear localizing signals and selective promoters. Furthermore, it is also unlikely that a single modality can achieve alloantigen-specific tolerance since there are multiple, separate arms of alloimmune responsiveness. Thus,

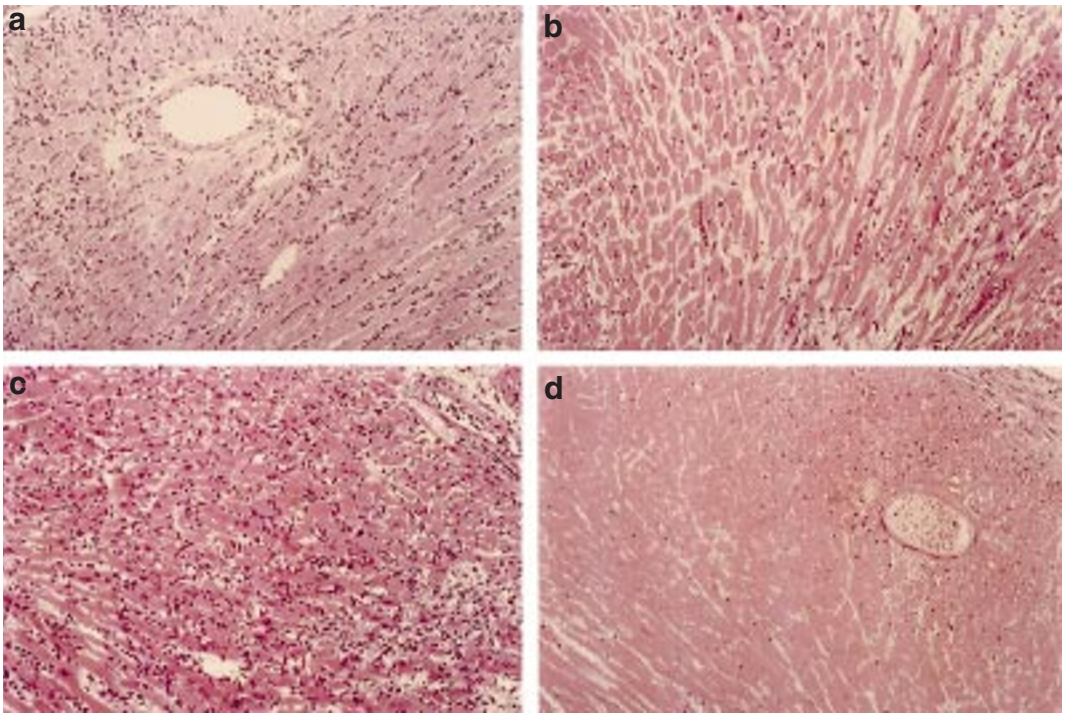

Figure 3 *Cardiac allografts were fixed in formalin, sectioned, and stained with hematoxylin and eosin for examination by light microscopy (× 200). (a) vMIP-II-transduced graft 7 days after transplantation; (b) vMIP-II-transduced graft 11 days following transplantation; (c) MC148-transduced graft 7 days after transplantation; (d) MC148-transduced graft 11 days following transplantation.*

chemokine antagonists may be more effective when combined with other agents.

Mechanistically, we were able to demonstrate that both vMIP-II and MC148 gene transfer inhibited humoral and cellular immune responses. Alloantibody production was decreased and delayed compared with β-galactosidase controls, even though the numbers of donor-specific IL-2 secreting HTL were unaffected. This could imply that the chemokine inhibitors act directly on B cells. In addition, even though flow cytometry indicated similar numbers of T cells infiltrating transduced grafts, vMIP-II and MC148 grafts had a significant decrease in the total number of donor-specific CTL residing in the grafts compared with controls. The lack of effect on donor-specific HTL implies a direct effect on CTL precursors. The data suggest that the primary effect of the peptides was to inhibit cellular maturation and/or activation rather than homing. In this regard, recent reports demonstrate that the chemokine fractalkine is highly expressed in heart tissue and that it has significant effects on $CD8^+$ but not $CD4^+$ T cells.[34] Although it is unknown if MC148 can interact with the fractalkine (CX_3CXR1) receptor, it has been reported that vMIP-II binds this receptor with high affinity.[6,34,35]

Several reports have now documented the ability of vMIP-II to act as a chemokine antagonist *in vitro*.[16,17,36] Kledal *et al*[16] found that vMIP-II binds to the human chemokine receptors CCR1, CCR2, CCR3, CCR5 and CXCR4, and antagonizes MIP-1α, MIP-1β and RANTES on human monocytes *in vitro*. Furthermore, evidence that vMIP-II acts as an antiinflammatory agent *in vivo* was recently reported by Chen *et al*,[36] utilizing a well established kidney inflammatory disease model in Wistar–Kyoto rats. Daily administration of nanomolar concentrations of vMIP-II effectively attenuated leukocyte infiltration into the kidneys and significantly reduced any ensuing renal injury.[36] In contrast, there have been no *in vivo* studies reported on the effects of MC148, and only two reports document the antagonistic effects of MC148 *in vitro*.[19,20] Krathwohl *et al*[19] initially reported that MC148 can block the *in vitro* chemotactic response of human monocytes to MIP-1α, and Damon *et al*[20] expanded this finding by demonstrating that MC148 was a potent chemotactic inhibitor of not only human monocytes, but of multiple leukocyte subsets responding to both CC and CXC chemokines. MC148 was found to functionally interact with human CCR1 and/or CCR5, CCR2, CCR8, CXCR1 and/or CXCR2 and CXCR4.[20] Our study is the first to demonstrate that MC148 is effective not only on human cells but also on rodent cells, and inhibits leukocyte function *in vivo*.

Initially, chemokines were identified as attractants of different types of leukocytes to sites of infection and inflammation.[1,2] However, chemokines are now known also to function as regulatory molecules in leukocyte maturation, trafficking and homing, and development of lymphoid tissues.[3] Furthermore, chemokines can function to costimulate lymphocyte proliferation, cytolysis, and cytokine production.[37–39] For example, β chemokines are capable of augmenting mouse and human CTL and NK cell, but not lymphokine-activated killer cell or antibody-dependent cell cytotoxicity-specific cytolytic responses.[39] In addition, various β chemokines can induce CTL and NK cells to degranulate and release granule-derived serine esterases.[37] The T cell costimulatory molecule B7–1 can be induced by various β chemokines.[37] Chemokines also enhance anti-CD3 mAb, antigen-specific, and alloantigen-specific T cell proliferation.[37,38] Chemokine treat-

ment of T cells *in vitro* increases the level of cell surface CD25 and soluble CD25 as well as augmenting IL-2 production.[38] In our studies we found no difference in the number of graft infiltrating $CD8^+$ T cells in vMIP-II and MC148 grafts compared with β-galactosidase controls, however, there was a significant decrease in the total number of donor-specific CTL. These data suggest that the virally encoded chemokine antagonists vMIP-II and MC148 inhibited the costimulatory functions of endogenous chemokines that are responsible for the maturation and/or activation of functional graft reactive CTLs. Since chemokines play such an essential role in the inflammatory process, they are good targets for the development of anti-inflammatory therapies. Yet, the redundancy of the chemokine system has made this approach problematic. Molecules such as vMIP-II and MC148, which have the ability to bind and antagonize multiple chemokine receptors, may prove to be highly effective. Because it is the amino terminal of chemokines that determines much of their specificity,[21] it may be possible to build chimeric molecules that incorporate the amino terminals of these chemokine blockers. The amino termini could then be grafted on to the β-sheet carboxyl scaffolding of molecules such as human IL-8 to 'humanize' the inhibitors and make reagents that are less likely to be immunogeneic *in vivo*. Our results demonstrate that plasmid-mediated gene transfer of the virally encoded chemokine antagonists vMIP-II and MC148 can block donor-specific CTL expansion in cardiac allografts, inhibit alloantibody production, and extend graft survival. Delivery of these or related molecules by gene transfer or conventional pharmacologic means may represent a novel therapeutic modality for the treatment of acute allograft rejection.

Materials and methods

Animals

Female CBA/J ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) mice 8 to 12 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Female C57BL/6 ($H\text{-}2^b$) mice 8 to 12 weeks of age and timed pregnant C57BL/6 ($H\text{-}2^b$) mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA).

Medium

The culture medium was DMEM supplemented with 1.6 mM L-glutamine, 0.27 mM L-asparagine, 10 mM Hepes buffer, 1.0 mM sodium pyruvate, 100 units/ml penicillin/streptomycin (all obtained from Gibco, Grand Island, NY, USA), 5×10^{-5} M 2-ME (Sigma Chemical, St Louis, MO, USA) and 10% heat inactivated fetal calf serum (Sigma Chemical).

Plasmids

PCR was used to amplify the vMIP-II gene from a Kaposi's sarcoma biopsy (a gift from Dr G Nabel, UMHS), and to clone the MC148 ORF from a vaccinia viral vector (a gift from Dr B Moss, NIH). The primers used to amplify the vMIP-II gene were 5′-ATTAGCTAGCATGGACACCAAGGGCATC-3′ (upstream) and 5′-ATTAGGATCCT CAGCGAGCAGTGACTGG-3′ (downstream), and the MC148 gene primers were 5′-ATTAGCTAGCATGG GGAGGGGCGGAGAC-3′ (upstream) and 5′-ATTAGGATCCTTACAGAGACTCGCACCC-3′ (downstream). The PCR cycle used for both vMIP-II and MC148 was 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min for 40 cycles. The MC148 and vMIP-II genes were then independently placed into the pMP6A expression plasmid.[40] The pMP6A expression plasmid utilizes the HCMVie promoter and an SV40 poly A tail. Sequencing of the clones was performed to confirm the correct gene product with no mutations. As a control, pMP6A-βgal was used.

Plasmid-mediated gene transfer to nonvascularized cardiac allografts

The heterotopic, nonvascularized cardiac transplantation model was used as previously described.[41,42] Briefly, donor neonatal C57BL/6 mice were killed, whole hearts removed, and placed in the subcutaneous position of the ear pinnae of CBA/J recipients. Twenty micrograms of plasmid DNA in 5 μl of phosphate-buffered saline was directly injected into the graft at the time of transplantation. Survival of cardiac allografts was followed with EKG monitoring (Polygraph 78 series with preamp filters; Grass Instruments, Quincy, MA, USA) every other day. Cessation of cardiac electrical activity was the determinant of rejection. There were at least four mice per group. Statistical comparison was performed using Students's *t* test, with $P < 0.05$ considered as significant.

Lipid-mediated gene transfer to vascularized cardiac allografts

Heterotopic cardiac transplantation was performed by anastomosing BALB/c donor hearts to the great vessels in the abdomen of C57BL/6 recipients as described by Corry *et al*.[43] For gene transfer, venae cavae of donor hearts were ligated *in situ* to retain the perfusate. The distal aortic arch was then ligated and held in position by the suture to facilitate perfusion of the coronary vasculature via the aortic root with a 1 cc syringe and a 30 gauge needle. The donor heart was first flushed with 400 μl of room temperature heparin (250 U/ml), then perfused with 250 μl of room temperature DNA–liposome complexes in lactated Ringer's solution.[26] DNA–liposome complexes were constituted by combining 375 μg plasmid DNA with 125 μg of the cationic lipid *N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-bis(dodecyloxy)-1-propaniminium bromide/dioleoyl phosphatidylethanolamine (γAP DLRIE/DOPE), (VICAL, San Diego, CA, USA). The DNA–liposome complexes were diluted in lactated Ringer's solution for a final volume of 250 μl, then incubated for 10 min at room temperature before being perfused into donor hearts. Following perfusion, donor grafts were immediately harvested and placed in iced Ringer's for approximately 1 h and then transplanted without further perfusion or wash out. In this model, the transplanted heart resumes contractions until acutely rejected, which occurs in this strain combination in 8–9 days.[26] Transplant function was monitored by daily abdominal palpation, and statistical comparison was performed with Student's *t* test. For histological analysis, cardiac allografts were fixed in formalin, sectioned at 5 μm, and stained with either hematoxylin and eosin or trichrome for examination by light microscopy.

RNA isolation, cDNA synthesis and PCR

Cardiac allografts were homogenized in 1 ml RNAzol B (Teltest, Friendswood, TX, USA), to isolate total RNA

which was then treated with DNase (Boehringer Mannheim, Indianapolis, IN, USA) and 1 μg used for random primer directed cDNA synthesis with reverse transcriptase (Invitrogen, Carlsbad, CA, USA). cDNA was amplified by PCR using the same primer sets and conditions that were used to clone the vMIP-II and MC148 genes. PCR products were run on 2.0% agarose gels and visualized by ethidium bromide staining. The vMIP-II primers yield a 295 base pair fragment, and the MC148 primers generate a 322 base pair fragment.

Recovery of heart infiltrating cells

Hearts were removed, minced and digested with 1 mg/ml collagenase A (Boehringer Mannheim) for 30 min at 37°C. Heart infiltrating cells were washed free of collagenase and viable leukocytes enumerated by trypan blue exclusion.

Flow cytometry

Graft infiltrating cells were stained with fluorescein-isothiocyanate (FITC)-conjugated anti-CD3, CD4, CD8, and MAC-1 (Pharmingen, San Diego, CA, USA). Phenotypic analysis was performed using a Becton Dickinson (San Jose, CA, USA) FACScan flow cytometer.

Limiting dilution analysis of helper T lymphocytes (HTL)

Dilutions of recipient cells were added as responder cells to microtiter wells along with gamma irradiated (5000 R) donor-type allogeneic splenocyte stimulator cells and incubated for 16 h.[44] CTLL-20 cells (an IL-2-dependent line) were then added directly to the microcultures, in order to detect IL-2 produced by the responder cells. The microcultures were incubated an additional 24 h, including a 16 h terminal pulse with ^{3}H-thymidine before harvesting by aspiration on to filter mats. ^{3}H-thymidine incorporation was determined on a scintillation counter. Individual microcultures were considered positive for IL-2 production if ^{3}H-thymidine incorporation exceeded the mean plus 3 standard deviations of ^{3}H-thymidine incorporation in microcultures lacking responder cells.

Limiting dilution analysis of cytolytic T lymphocytes (CTL)

Dilutions of recipient cells were added to microtiter wells along with gamma irradiated (5000 R) donor-type allogeneic splenic stimulator cells and incubated for 7 days.[45] To detect cytolytic activity, ^{51}Cr-labeled BALB/c splenocyte 3 day concanavalin A blasts were added as target cells to the microcultures. Following a 4 h incubation, microculture supernatants were assayed for release of ^{51}Cr in a gamma counter. Microcultures were considered cytolytic if observed chromium release exceeded the mean plus 3 standard deviations of the release observed in wells that contained targets and stimulators but no responder cells.

Limiting dilution analysis

Minimal estimates of HTL or CTL frequency were obtained according to the Poisson distribution equation as the slope of a line relating the number of responder cells per microwell (plotted on a linear x-axis) and the percentage of microwells that failed to produce IL-2 or develop cytolytic activity, respectively (plotted on a logarithmic y-axis). The slope of this regression line was determined using chi square minimization analysis, as described by Taswell.[46] This analysis yields the minimal frequency estimate, the 95% confidence interval of the frequency estimate, and a chi square estimate of probability.

Alloantibody isotype

P815 cells (H-2^{d}) were stained for FACS analysis using a 1:50 dilution of recipient serum as the primary antibody, followed by a 1:50 dilution of FITC-conjugated isotype-specific secondary antibodies. FITC-conjugated, affinity purified sheep anti-mouse IgM, IgG1 and IgG2a were obtained from The Binding Site (San Diego, CA, USA). Data are presented as the mean channel fluorescence at this dilution as determined on a Becton Dickinson FACScan.

Acknowledgements

We thank Dr John Magee for stimulating discussions and critical comments during the preparation of this manuscript. This work is supported in part by the National Institutes of Health T32 AI-07413 (LAD), RO1 AI-31946 (DKB), UM MAC P60-AR20557 (JSB); Edward Mallinckrodt, Jr Foundation (JSB); and the Baxter Extramural Grant Program (JSB).

References

1 Luster AD. Chemokines – chemotatic cytokines that mediate inflammation. *New Engl J Med* 1998; **338**: 436–445.
2 Baggiolini M, Dewald B, Moser B. Human chemokines: an update. *Annu Rev Immunol* 1997; **15**: 675–705.
3 Baggiolini M. Chemokines and leukocyte traffic. *Nature* 1998; **392**: 565–568.
4 Premack BA, Schall TJ. Chemokine receptors: gateways to inflammation and infection. *Nature Med* 1996; **2**: 1174–1178.
5 Kelner GS *et al*. Lymphotactin: a cytokine that represents a new class of chemokine. *Science* 1994; **266**: 1395–1399.
6 Bazan JF *et al*. A new class of membrane-bound chemokine with a CX_3C motif. *Nature* 1997; **385**: 640–644.
7 Zhong W, Wang H, Herndier B, Ganem D. Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. *Proc Natl Acad Sci USA* 1996; **93**: 6641–6646.
8 Weiss RA. Human herpesvirus 8 in lymphoma and Kaposi's sarcoma: now the virus can be propagated. *Nature Med* 1996; **2**: 277–278.
9 Boshoff C *et al*. Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. *Nature Med* 1995; **1**: 1274–1278.
10 Boshoff C *et al*. Kaposi's-sarcoma-associated herpesvirus in HIV negative Kaposi's sarcoma. *Lancet* 1995; **345**: 1043–1044.
11 Dupin N *et al*. Herpesvirus-like DNA sequences in patients with Mediterranean Kaposi's sarcoma. *Lancet* 1995; **345**: 761–762.
12 Moore PS, Chang Y. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and without HIV infection. *New Engl J Med* 1995; **332**: 1181–1185.
13 Chang Y *et al*. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994; **266**: 1865–1896.
14 Moore PS, Boshoff C, Weiss RA, Chang Y. Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science* 1996; **274**: 1739–1744.
15 Nicholas J *et al*. Kaposi's sarcoma-associated human herpesvirus-8 encodes homologues of macrophage inflammatory protein-1 and interleukin-6. *Nature Med* 1997; **3**: 287–292.
16 Kledal TN *et al*. A broad spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. *Science* 1997; **277**: 1656–1659.

17 Boshoff C *et al*. Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. *Science* 1997; **278**: 290–294.
18 Senkevich TG *et al*. Genome sequence of a human tumorigenic poxvirus: prediction of specific host response-evasion genes. *Science* 1996; **273**: 813–816.
19 Krathwohl MD *et al*. Functional characterization of the C-C chemokine-like molecules encoded by molluscum contagiosum virus types 1 and 2. *Proc Natl Acad Sci USA* 1997; **94**: 9875–9880.
20 Damon I, Murphy PM, Moss B. Broad spectrum chemokine antagonistic activity of a human poxvirus chemokine homolog. *Proc Natl Acad Sci USA* 1998; **95**: 6403–6407.
21 Clark-Lewis I *et al*. Structure-activity relationships of chemokines. *J Leuk Bio* 1995; **57**: 703–711.
22 Gottlieb SL, Myskowski PL. Molluscum contagiosum. *Int J Dermatol* 1994; **33**: 453–461.
23 DeBruyne LA, Magee JC, Buelow R, Bromberg JS. Gene transfer of immunomodulatory peptides correlates with heme oxygenase-1 induction and enhanced allograft survival. *Transplantation* (in press).
24 Qin L *et al*. Multiple vectors effectively achieve gene transfer in a murine cardiac transplantation model: immunosuppression with TGF-β1 or vIL-10. *Transplantation* 1995; **59**: 809–816.
25 Qin L *et al*. Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival. *J Immunol* 1996; **156**: 2316–2323.
26 DeBruyne LA *et al*. Lipid-mediated gene transfer of viral IL-10 prolongs vascularized cardiac allograft survival by inhibiting donor-specific cellular and humoral immune responses. *Gene Therapy* 1998; **5**: 1079–1087.
27 Moore KW *et al*. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein–Barr virus gene BCRFI. *Science* 1990; **248**: 1230–1234.
28 de Waal Malefyt R *et al*. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991; **174**: 1209–1220.
29 de Waal Malefyt R *et al*. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* 1991; **174**: 915–924.
30 Enk AH, Angeloni VL, Udey MC, Katz SI. Inhibition of Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance. *J Immunol* 1993; **151**: 2390–2398.
31 Ding L *et al*. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J Immunol* 1993; **151**: 1224–1234.
32 Willems F *et al*. Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. *Eur J Immunol* 1994; **24**: 1007–1009.
33 Chan SY *et al*. *In vivo* depletion of CD8+ T cells results in Th2 cytokine production and alternate mechanisms of allograft rejection. *Transplantation* 1995; **59**: 1155–1161.
34 Fong AM *et al*. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J Exp Med* 1998; **188**: 1413–1419.
35 Pan Y *et al*. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* 1997; **387**: 611–617.
36 Chen S *et al*. *In vivo* inhibition of CC and CX_3C chemokine-induced leukocyte infiltration and attenuation of glomerulonephritis in Wistar–Kyoto (WKY) rats by vMIP-II. *J Exp Med* 1998; **188**: 193–198.
37 Taub DD *et al*. β chemokines costimulate lymphocyte cytolysis, proliferation, and lymphokine production. *J Leuk Bio* 1996; **59**: 81–89.
38 Taub DD *et al*. Chemokines and T lymphocyte activation. I. β chemokines costimulate human T lymphocyte activation *in vitro*. *J Immmunol* 1996; **156**: 2095–2103.
39 Taub DD, Sayers T, Carter C, Ortaldo JR. α and β chemokines induce NK cell migration and enhance NK cell cytolytic activity via cellular degranulation. *J Immunol* 1995; **155**: 3877–3888.
40 Philip R *et al*. Efficient and sustained gene expression in primary T lymphocytes and primary and cultured tumor cells mediated by adeno-associated virus plasmid DNA complexed to cationic liposomes. *Mol Cell Bio* 1994; **14**: 2411–2418.
41 Fulmer R, Cramer A, Liebelt A. Transplantation of cardiac tissue into the mouse ear. *Am J Anat* 1963; **113**: 273–285.
42 Judd KP, Trentin JJ. Cardiac transplantation in mice: I. Factors influencing the take and survival of heterotopic grafts. *Transplantation* 1971; **11**: 298–302.
43 Corry RJ, Winn HS, Russell PS. Primarily vascularized allografts of hearts in mice. *Transplantation* 1973; **16**: 343–350.
44 Bishop DK, Orosz CG. Limiting dilution analysis for alloreactive, TCGF-secreting T cells: LDA methods that discriminate between unstimulated precursor T cells and *in vivo* alloactivated T cells. *Transplantation* 1989; **47**: 671–677.
45 Orosz CG, Horstemeyer B, Zinn NE, Bishop DK. Development and evaluation of an LDA technique that can discriminate *in vivo* alloactivated CTL from their naive CTL precursors. *Transplantation* 1989; **47**: 189–194.
46 Taswell C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J Immunol* 1981; **126**: 1614–1619.