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RESEARCH ARTICLE

Intrabody-mediated phenotypic knockout of major histocompatibility complex class I expression in human and monkey cell lines and in primary human keratinocytes

AM Mhashilkar[1], C Doebis[3], M Seifert[3], A Busch[3], C Zani[1], J Soo Hoo[1], M Nagy[4], T Ritter[3], H-D Volk[3] and WA Marasco[1,2]

[1]*Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, MA, USA;* [2]*Department of Medicine, Harvard Medical School, Boston, MA, USA;* [3]*Institute of Medical Immunology, Medical School (Charité), Berlin, Germany; and* [4]*Institute for Forensic Medicine, Medical School (Charité), Berlin, Germany*

Cultured keratinocyte allografts from unrelated donors can be readily grown as sheets in large-scale cell culture and have been used as an immediate skin cover for severely burned patients. Despite the absence of passenger leukocytes and the unlimited amount of material that can be obtained for permanent skin coverage, the allografts are susceptible to rejection. Since MHC class I (MHCI) antigens serve as targets for allograft rejection, we investigated whether 'phenotypic knockout' of human MHCI could be achieved through expression of an ER-directed anti-human MHCI single-chain intrabody (sFvhMHCI) that is directed against a monomorphic, conformational epitope, expressed across species lines, on the MHCI heavy chain. Co-immunoprecipitation of both MHCI heavy chain and β2-microglobulin occurred in transfected monkey COS-1 cells, while Jurkat T cells stably expressing the ER-directed sFvhMHCI intrabody showed that complete phenotypic knockout of MHCI cell surface expression could be achieved. Infection of several human cell lines of divergent tissue sources and different HLA haplotypes resulted in marked down-regulation of MHCI expression, even under conditions where inflammatory cytokines (eg γ-IFN) which up-regulate MHCI expression were used. Finally, when adenovirus encoding the anti-human MHCI intrabody was used to transduce primary human keratinocytes, a marked reduction of surface MHCI expression was observed. These in vitro studies set the groundwork for in vivo studies to determine if intrabody-mediated knockout of MHCI can impair alloantigen expression and prolong the survival of keratinocyte allografts.
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Keywords: *gene therapy; MHC-I; transplantation; MHC class I; tolerance; intracellular antibody*

Introduction

During the past several decades organ transplantation has been developed to a well-established therapeutic option for replacement of irreversible injured parenchymal organs. Improvements in tissue engineering have opened up new therapeutic opportunities in the field of transplantation. In contrast to organ transplantation, permanent immunosuppression is not an option for those patients receiving tissue-engineered materials therefore autologous materials are mostly used. However, there is a medical need for using allogeneic material as well. In comparison to allogeneic organ grafts, *in vitro* generated tissues have several advantages: (1) they are generated from single cell suspensions. This readily allows gene therapeutic or pharmacologic *ex vivo* manipulations for decreasing immunogenicity and resistance to inflammatory attacks by the immune system; and (2) they do not contain professional antigen-presenting cells that are the main stimulators of alloreactivity in the host. Manipulating cells to decrease their immunogenicity during tissue generation *in vitro* would open new therapeutic applications for those grafts of different sources (keratinocytes, endothelial cells etc).

Skin is the largest organ of the body and it is the primary interface between the body and the environment. The spectrum of insults to which skin is susceptible includes disorders caused by chemical and microbial agents, thermal and electromagnetic radiation, and mechanical trauma.[1] Rapid and effective coverage of burn wounds is an important determinant of survival after major thermal injury. However, many patients lack enough donor sites to completely cover the burn wound with autologous split-thickness skin grafts. This limitation has lead to a search for alternative sources of skin coverings.

Correspondence: WA Marasco, Dana-Farber Cancer Institute, JFB 824, 44 Binney Street, Boston, MA 02115, USA and H-D Volk, Institute of Medical Immunology, Medical School (Charité), Schumannstr. 20/21, D-10117 Berlin, Germany
The first two authors contributed equally to this work
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Since human epidermal keratinocytes were first cultured and serially passaged from a single cell suspension,[2] sheets of keratinocyte autograft have been used for skin cover.[3] Unfortunately for emergency cases, autografts cannot be used because of the lengthy delay entailed in culturing autografts, generally a minimum of 10 days to generate confluent sheets and 3 to 4 weeks if large areas requiring secondary cultures are needed. Allogeneic keratinocyte grafts from unrelated donors have been used as a readily available alternative for immediate cover of severely burned patients.[4–6] Keratinocyte allografts are devoid of passenger leukocytes and could potentially provide immediate, unlimited amounts of material for permanent wound coverage, if they were not susceptible to rejection. However, although early reports of successful keratinocyte allograft transplantation[4,7] were supported by the finding that recipient blood group antigen expression was absent on epidermal cells at the allografted site and by the lack of stimulation of the mixed epidermal cell lymphocyte reaction between donor keratinocytes and recipient lymphocytes,[8] more recent investigations have failed to provide convincing evidence that the surviving grafted keratinocytes are of donor origin even though frank allograft rejection is not observed.[9–13]

It is now well established that allografts may be rejected even in the absence of direct allopresentation (lack of professional donor antigen-presenting cells) via the indirect allopresentation pathway mediated by host antigen-presenting cells that infiltrate the graft within 2 weeks following transplantation and pick up donor alloantigens (especially MHC class I molecules (MHCI)), and present them to host T cells in the draining lymph nodes. These controversial results have highlighted the difficulties in establishing whether keratinocyte allografts can be used for more than a temporary skin covering.

MHCI molecules, composed of a polymorphic 45-kDa heavy chain, a noncovalently associated 12-kDa light chain, termed β2-microglobulin and a bound peptide of 8 to 10 amino acids generally, though not exclusively derived from an endogenously synthesized protein, are expressed on the surface of almost every cell of the body. Surface expression of these molecules on keratinocytes can be up-regulated by cytokines.[14–16] The heavy chain possesses three external domains designated α1, α2 and α3, a transmembrane domain, and a cytoplasmic tail. X-ray crystallographic studies of a human MHCI molecule indicate that the polymorphic α1 and α2 domains are structurally similar and are paired, as are the monomorphic α3 domains and β2-microglobulin.[17,18] During biosynthesis, the heavy chain associates with β2-microglobulin in the ER and is accompanied by changes in the conformation of the heavy chain.[19,20] Assembly of the heavy chain, β2-microglobulin, and peptide is required for normal MHC class I surface expression,[21] and the bound peptide contributes to the antigenic determinants recognized by the TCR.[22,23]

Because MHCI antigens serve as targets for allograft rejection,[24,25] the present study was undertaken to determine if an ER-directed anti-human MHCI single-chain intrabody that is directed against a monomorphic, conformational epitope on the MHCI heavy chain[26,27] could block transport of MHCI molecules from the ER to the cell surface of cells. We present data demonstrating that an ER-directed sFvhMHCI intrabody is able to effectively block MHCI cell surface expression on both monkey and human cell lines with different HLA-A,B,C haplotypes. Furthermore, when an adenovirus encoding the anti-human MHCI intrabody was used to transduce primary human keratinocytes, a marked reduction of surface MHCI expression was observed. These *in vitro* studies set the groundwork for *in vivo* studies to determine if intrabody-mediated knockout of MHCI can prolong the survival of keratinocyte allografts.

Results

Cloning and characterization of anti-hMHCI-5 and anti-hMCHI-8 single-chain antibodies

The hybridoma cell line BB7.7 that was used for this project originally was prepared by fusing spleen cells of BALB/c mice immunized with papain-solubilized purified HLA-A2,B7 with the NS-1 myeloma line.[26] The BB7.7 hybridoma produces a cytotoxic MAb (IgG_{2b}) that reacts against a combinatorial determinant of HLA-A,B,C and β-2 microglobulin. However, based on competition studies with free β_2-microglobulin and papain-solubilized HLA-A2 antigens, it is likely that MAb BB7.7 recognizes a monomorphic determinant on the heavy chain that is conformationally dependent on the presence of β_2-microglobulin.[27] BB7.7 MAbs also react with lymphocytes from apes, old and New World monkeys, the slow loris (a prosimian) and cows.[27,28]

Fingerprinting experiments with *Bst*NI digestion of sFv fragments cloned into pHEN demonstrated two different patterns. DNA sequence analysis typified by clones 5 and 8 showed identical VH genes, but different Vκ genes, a finding that is not uncommon when using mRNA from hybridoma cell lines as the starting material.[29–31] The nucleotide and deduced amino acid sequences of sFvhMHCI-5 and sFvhMHCI-8 are shown in Table 1a and b. The VBASE database was used to make V gene family and germline gene assignment[32] for the rearranged VH, VK5 and VK8 genes. The rearranged VH gene is a member of the VH5 gene family, most closely matches the DP-7/21-2 germline VH gene, and is rearranged with the JH4d germline gene, however, alignment for the D segment was not possible. The rearranged VK5 gene is a member of the VK1 subgroup, most closely matches the DPK1/018 germline VK gene, and is rearranged with the JK2 germline gene. Interestingly, the rearranged VK8 gene is also a member of the VK1 subgroup, most closely matches the same DPK1/018 germline VK gene, but is rearranged with the JK4 germline gene.

Co-immunoprecipitation of MHCI heavy chains and β2-microglobulin by anti-MHCI sFv intrabodies expressed in African green monkey-derived COS-1 cells

The sFvhMHCI-5 and sFvhMHCI-8 genes were further modified to allow ER-directed expression in eukaryotic cells. For these studies, the human VH F105 leader sequence was added to direct the sFv into the lumen of the ER, where the MHCI epitope would be available. A carboxy-terminal ER-retention signal SEKDEL was included in some constructs to produce the ER-retained intrabodies sFvhMHCI-5K and sFvhMCHI-8K.[33–35]

Table 1a Nucleotide and deduced amino acid sequence of murine sFvhMHC-5

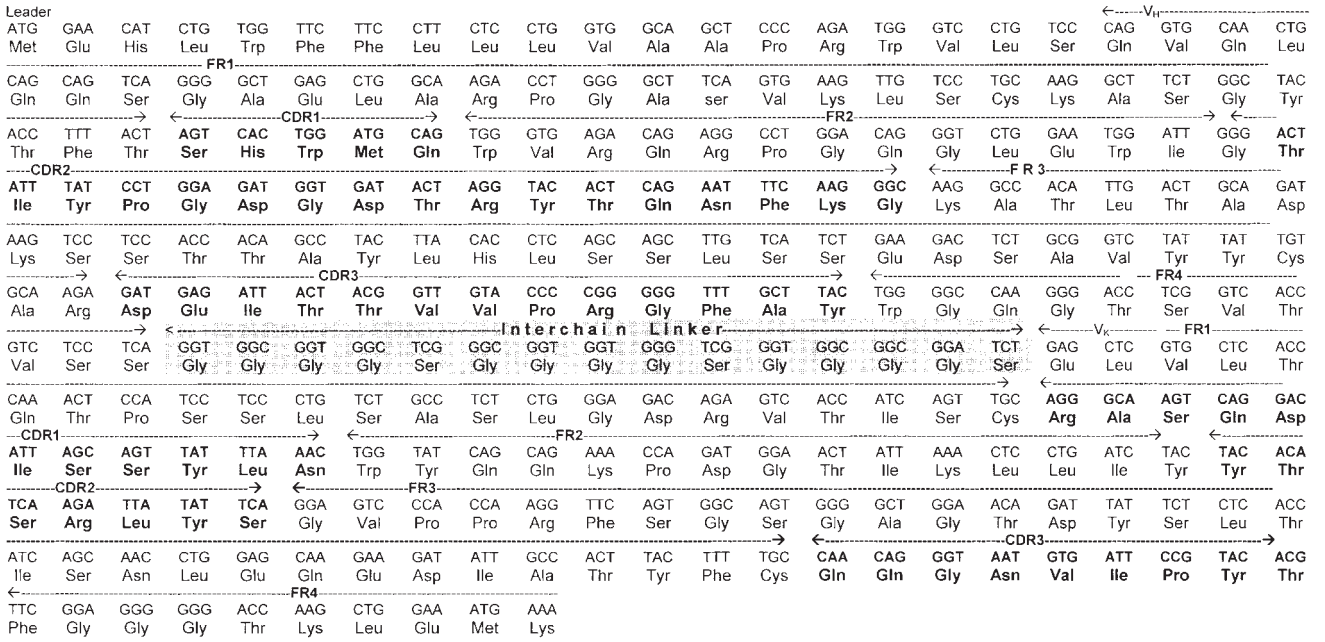

Table 1b Nucleotide and deduced amino acid sequence of murine sFvhMHC-8

COS-1 cells, a fibroblast-like cell line derived from the kidney of an African green monkey, were transiently transfected with the sFvhMHCI-5K and sFvhMHCI-8K intrabody expression plasmids and then analyzed for sFv expression using radioimmunoprecipitation. As shown in Figure 1, immunoprecipitation with anti-mouse IgG identified a distinct circa 30 kDa band of expected molecular weight for the sFv in both sFvhMHCI-5K- and sFvhMHCI-8K-transfected cells. In addition, two specific bands corresponding to approximately 50 and 23 kDa

proteins are also co-immunoprecipitated which correspond to MHCI heavy chains and β_2-microglobulin, respectively. Thus, these experiments demonstrate that both sFvhMCHI-5 and sFvhMHCI-8 have comparable binding activity for a conformationally sensitive epitope displayed by the MHCI/β_2-microglobulin complex expressed in these monkey cells.

Anti-MHCI sFv intrabody-mediated phenotypic 'knockout' of MHCI in the human CD4+ Jurkat T cell line

Human CD4+ Jurkat T cells were initially used to assess the effects of the sFvhMHCI intrabodies on MHCI expression, since the HLA haplotype of these cells is known (Table 2) and use of this cell line would allow us to determine if phenotypic 'knockout' of multiple alleles expressed from different HLA loci could be achieved. The sFvhMHCI-5K and sFvhMHCI-8K intrabody genes in both pRc/CMV and pCMV4 expression plasmids were transfected into human CD4+ Jurkat T cells and stable subclones were established after G418 selection. Figure 2 shows the results of FACS analysis of surface MHCI staining of six randomly chosen subclones expressing each construct. As can be seen, when compared with Jurkat cells stably transfected with either the empty pRc/CMV (clones V1–V4) or pCMV4 (clones V1–V4) vectors, all subclones expressing the sFvhMHCI-5K or sFvhMHCI-8K intrabodies showed a decrease in MHCI expression and several clones showed nearly complete phenotypic 'knockout' of MHCI (pRc/CMV, clone 5K6; pCMV4, clones 5K4 and 8K2).

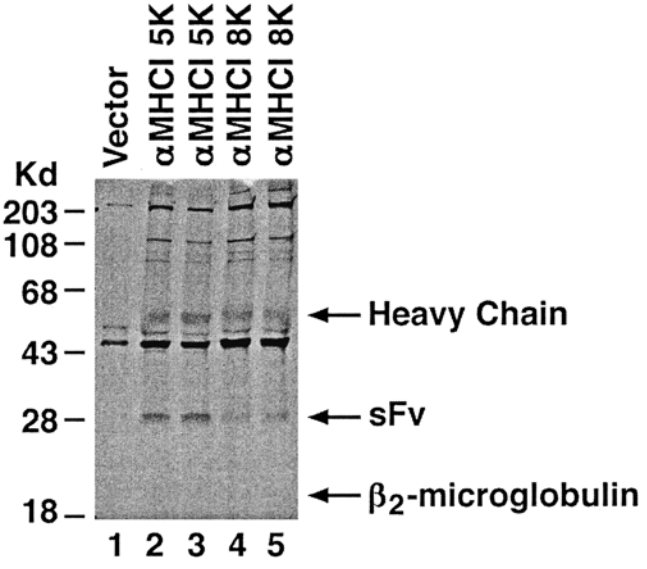

Figure 1 *Transient expression of sFvhMHCI intrabodies in COS-1 cells. Radio-immunoprecipitation of transiently transfected and metabolically radiolabeled cells were carried out using anti-mouse IgG (whole molecule, Sigma) bound Protein A Sepharose. The samples were run on a 12.5% SDS-PAGE denaturing gel. Lane 1, pRc/CMV vector control; lanes 2 and 3, two different plasmid preparations of pRc/CMV-sFvhMHCI-5K; Lanes 4 and 5, two different plasmid preparation of pRc/CMV-sFvhMHCI-8K.*

Table 2 HLA haplotype of human cell lines and primary keratinocytes

Cell line	*HLA-A*	*HLA-B*	*HLA-C*
Jurkat	A9, A25	B7, B41	ND
A549[a]	A30	B18, B44	C12, C15, C17[b]
HeLa[a]	A02, A68	B15	C12, C17
HaCaT[a]	A31	B40 (4001), B51	C03, C15 (1502)
Primary keratinocytes			
VH29[a]	A01, A30	B27, B35	C02, C04
VH34[a]	A24(9)	B08, B2702	C02,C07
VH38[a]	A24(9), A30	B35, B44 (12)	C04

[a]HLA assignments are obtained by HLA-DNA typing. Nucleotide sequence polymorphism at the HLA class I loci were analyzed by sequence specific PCR (SSP-typing).
[b]Not clearly detectable.
ND, not determined.

Radioimmunoprecipitation studies were next performed to determine the level of intrabody expression in the stable clones that showed loss of MHCI surface expression. As shown in Figure 3, lanes 2–5, comparable levels of the circa 30 kDa sFvhMCHI-5K and sFvhMCHI-8K proteins were seen in phenotypic 'knockout'-selected stable subclones of Jurkat cells, regardless of which expression vector was used for stable transfections.

To determine if the phenotypic 'knockout' of surface MHCI expression was specific in the Jurkat subclones expressing sFvhMHCI-5K or sFvhMHCI-8K intrabodies, FACS staining of a number of structurally unrelated cell surface molecules was examined. As shown in Figure 4, when compared with stably transfected Jurkat cells expressing the pRc/CMV vector (top row), the two sFvhMHCI-5K and -8K expressing subclones that were examined showed similar levels of MHCII, CD3, CD3, CD4 expression. As expected, CD8 expression was undetectable in the CD4+ Jurkat T cell lines. β2-microglobulin expression was reduced, but not absent in the two intrabody-expressing clones suggesting that β2-microglobulin transport to the cell surface was only partially disrupted in these cells that displayed nearly undetectable levels of surface MHCI. Thus, these studies demonstrate that expression of sFvhMHCI intrabodies can lead to phenotypic 'knockout' of MHCI heavy chains representing different haplotypes encoded at different HLA loci.

MHCI down-regulation on human epithelial cell lines by adenovirus encoding sFvhMHCI-8K intrabody

Several human cell lines of divergent tissue sources and representing different HLA haplotypes (Table 2) were transduced with recombinant adenovirus encoding sFvhMHCI-8K (Ad-8ksFv) and the expression of MHCI heavy chains on the cell surface was determined after 48 h by FACS analysis. Figure 5 shows the clear down-regulation of the MHCI heavy chains on all human cell lines tested, which is in contrast to cells transduced with a control vector (Ad-β-gal). In particular there was a strong shift of the whole cell population to a lower mean value of fluorescence staining by the cell lines A549 and HeLa (Figure 5a and b). When the human keratinocyte cell line

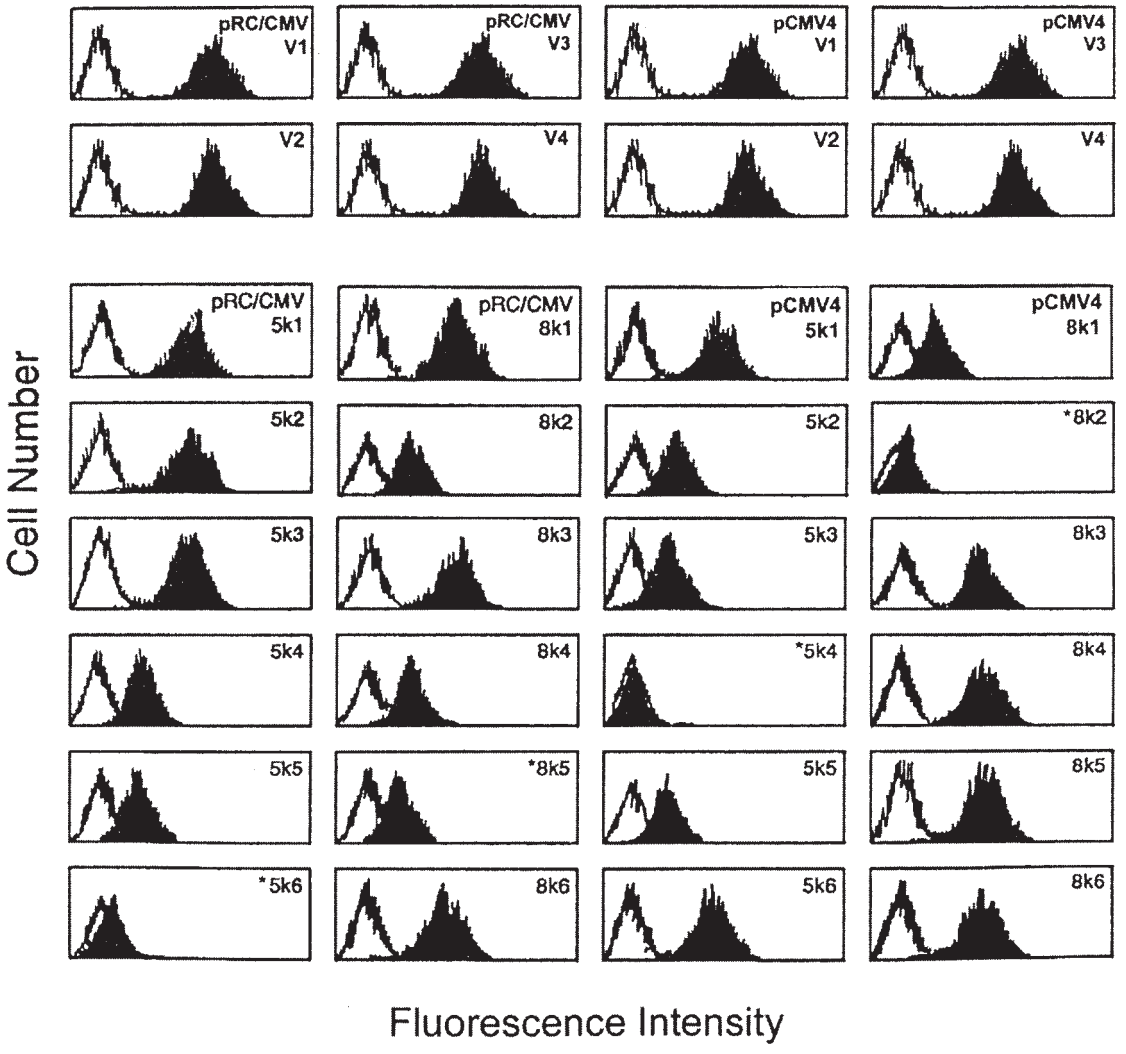

Figure 2 *FACS analysis of MHCI surface staining in stably transfected CD4+ Jurkat subclones expressing sFvhMHCI-5K or sFvhMHCI-8K. Jurkat cells expressing empty vectors, sFvhMHCI-5K or sFvhMHCI-8K intrabodies were incubated first with HB94 hybridoma supernatant, followed by a FITC-labeled anti-mouse IgG (Sigma). These cells were monitored for MHCI cell surface expression. Column 1, pRc/CMV-vector clones V1 and V2 or sFvhMHCI-5K subclones 1–6; column 2, replicate pRc/CMV-vector clones V3 and V4 or pRc/CMV-sFvhMHCI-8K subclones 1–6; column 3, pCMV4-vector clones V1 and V2 or pCMV4-sFvMHCI-5K subclones 1–6; column 4, replicate pCMV4-vector clones V3 and V4 or pCMV4-sFvhMHCI-8K subclones 1–6. (∗) denotes subclones described in Figures 3 and 4.*

HaCaT was transduced with Ad-8ksFv, a clear shift of the main population of cells to lower staining intensities and a marginal shift of a small subpopulation of cells was observed (Figure 5c). The level of the MHCI heavy chain expression after transduction of these three cell lines with the control construct Ad-β-gal was similar to the levels of MHCI expressed on uninfected (non-transduced) cells (data not shown). Table 3 shows the mean fluorescence intensities (MFI) for the MHCI heavy chain staining of all three tested cell lines comparing controls with the anti-MHC I intrabody gene (Ad-8ksFv). Control infection alone induced no changes in the MHCI expression, only Ad-8ksFv down-regulated this molecule on the cell surface, indicating specific binding of 8ksFv to MHCI. These corroborating results demonstrate that the level of sFvhMHCI-8K intrabody expression from Ad-8ksFv is sufficient to result in phenotypic 'knockout' of diverse MHCI heavy chains representing different HLA haplotypes.

Modulation of MHCI expression on human primary keratinocytes

The next investigations examined the capacity of Ad-8ksFv to diminish the level of MHCI heavy chain expression on primary human keratinocytes isolated from normal human skin and representing different HLA haplotypes (Table 2). MHCI heavy chain expression was analyzed 48 h after adenovirus infection of primary keratinocytes from three different donors. As shown in Figure 6, the transduction of human primary keratinocytes with Ad-8ksFv resulted in a dramatic shift of the mean fluorescence intensity to lower values (Figure 6), although the extent of down-regulation for all donors was different. In addition, soluble MHCI heavy chain was barely detect-

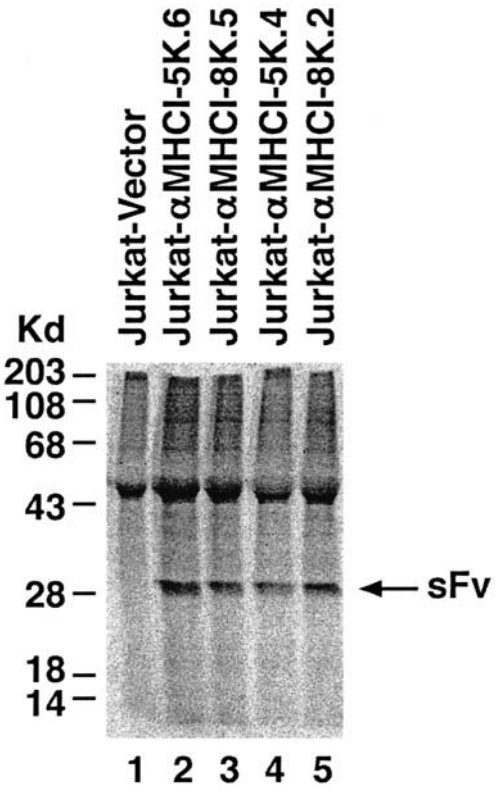

Figure 3 *Stable expression of sFvhMHCI intrabodies in CD4+ Jurkat T cell. To analyze for sFvhMHCI intrabody expression, radio-immunoprecipitation of selected subclone cell lysates using anti-mouse IgG (whole molecule, Sigma) bound Protein A Sepharose was performed. The samples were run on a 12.5% SDS-PAGE denaturing gel. Lane 1, stable Jurkat-pRc/CMV vector cells; lane 2, Jurkat-pRc/CMVsFvhMHCI-5K cells, clone 6; lane 3, Jurkat-pRc/CMV-sFvhMHCI-8K cells, clone 5; lane 4, Jurkat-pCMV4-sFvhMCHI-5K cells, clone 4; lane 5, Jurkat-pCMV4-sFvhMHCI-8K cells, clone 2.*

Table 3 Down-regulation of MHCI heavy chain expression of human cell lines transduced with adenovirus encoding sFvhMHCI-8K intrabody

Cell line	*MFI of MHC I expression*[a]	
	Ad-control-transduced	*Ad-8ksFv*
A549	8.42	2.91
HeLa	23.36	4.37
HaCaT	74.90	16.45

[a] Results are presented as MFI (geometric mean) of MHC I staining. A representative experiment for each cell line is shown ($n = 3$).

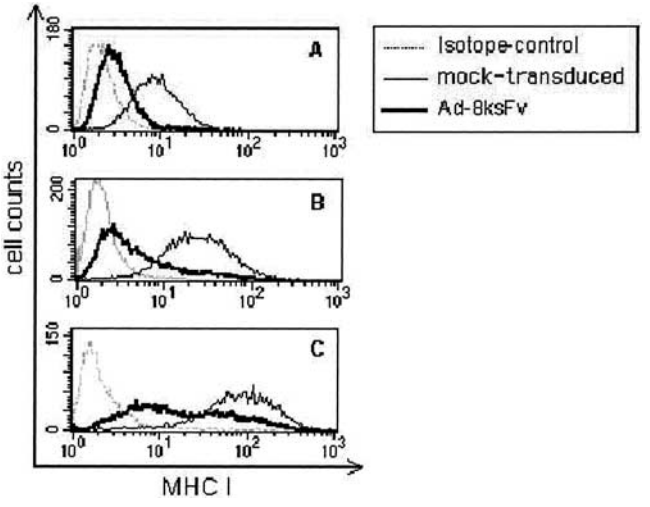

Figure 5 *A comparison of MHCI expression on the cell surface of three different cell lines after adenoviral infection. The cell lines A549 (a); HeLa (b); and HaCaT (c) were cultured and grown to confluence and then infected with Ad-8ksFv (bold line) and Ad-control (Ag-β-Gal) (thin line), respectively. Thereafter, cells were harvested and incubated with the FITC-labeled anti-MHC class I antibody. Fluorescence intensity was determined by flow cytometry. An isotype control for background staining is shown (dotted gray line). A representative result out of three independently performed experiments for each cell line is presented.*

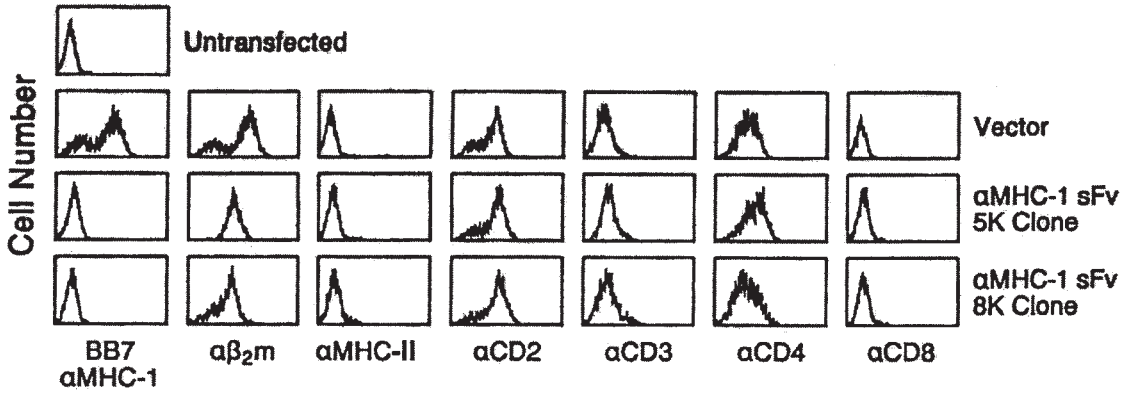

Figure 4 *FACS analysis of stable Jurkat subclones expressing pRc/CMV empty vector (top row), pRc/CMV-sFvhMHCI-5K, clone 6 (middle row) or pCMV4-sFvhMHCI-8K, clone 2 (bottom row). Cell surface expression levels of MHCI, MHCII, β_2-microglobulin, CD2, CD3, CD4 and CD8 were analyzed.*

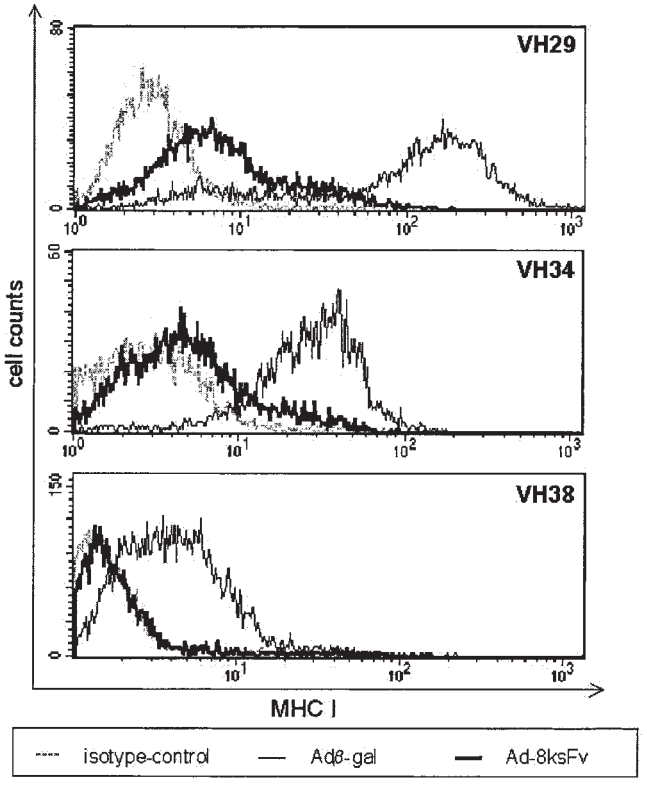

Figure 6 *Diminished MHCI expression on Ad-8ksFv infected human primary keratinocytes. After cultivation and infection with Ad-8ksFv (bold line) and Ad-control (Ad-hAAT) (thin line), respectively, MHCI expression on the cell surface was analyzed by FACS. One sample incubated with a FITC-labeled isotype-matched control IgG antibody for unspecific binding is shown (dotted gray line). Fluorescence histograms of three different donors of keratinocytes are presented (VH29, VH34 and VH38).*

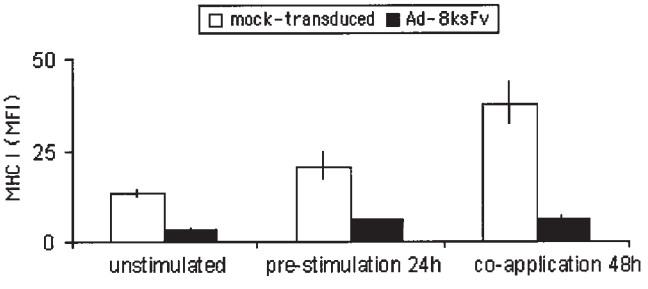

Figure 7 *Down-regulation of MHCI by Ad-8ksFv under inflammatory conditions. The cell line A549 was cultured to confluence and infected with Ad-8ksFv (black column) and an Ad-control (Ad-hAAT, white column), respectively. Cells were pre-stimulated with IFN-γ (24h) or IFN-γ-co-applicated (simultaneous to transduction for 48 h). MHCI expression after Ad-8ksFv infection are presented as geometric means ± s.e.m. (n = 3) and refer to the expression after infection with the control vector in each independently performed experiment.*

able in the supernatant of Ad-8ksFv treated cells (data not shown). The infection with the control Ad-vector (Ad-hAAT) did not influence the MHCI expression on the cell surface of keratinocytes (data not shown), indicating the specificity of the down-regulation by intracellular production of the sFvhMHCI-8K intrabody. Thus, these experiments demonstrate that Ad-8ksFv infection of human primary keratinocytes can result in the dramatic down-regulation of MHCI heavy chain expression.

Influence of IFN-γ on the MHC class I down-regulation by Ad-8ksFv

To analyze the capacity of the sFvhMHCI-8K intrabody to retain MHCI heavy chain molecules intracellularly under inflammatory conditions when MHCI is up-regulated, the cell line A549 was stimulated at different timepoints (prestimulation for 24 h and coapplication for 48 h) with recombinant human IFN-γ. The cells were then analyzed by FACS 48 h later to detect the level of MHCI heavy chain expression. As shown in Figure 7, stimulation with IFN-γ led to elevated levels of MHCI expression. Importantly, even under inflammatory conditions Ad-8ksFv is able to down-regulate MHCI expression quite efficiently (Figure 7, middle and right bar graphs), whereas in contrast increased MHCI expression could not be downmodulated by control vector Ad-hAAT. These results demonstrate the capacity of Ad-8ksFv to effectively down-regulate MHCI both under normal and inflammatory conditions.

Discussion

The results presented in this manuscript demonstrate that expression of an ER retained anti-hMHCI sFv intrabody can result in phenotypic 'knockout' of human MHCI heavy chains representing different HLA haplotypes. In addition, soluble MHC I was hardly detectable in supernatants of 'knockout' keratinocytes. These results extend previous studies which demonstrate that ER-directed intrabodies can lead to phenotypic knockout of a variety of cell surface molecules including IL-2Rα,[36] α4 integrin,[37] αV integrin,[38] c-erbB-2,[39,40] and EGFR,[41] as well as inhibition of surface Galα1,3Gal carbohydrate expression through inactivation of golgi associated α-1,3, galactosyltransferase.[42,43] Inhibition of maturation and surface expression of viral glycoproteins has also been demonstrated.[33,44] Moreover, despite the extraordinary polymorphism of this diverse class of cell surface molecules both within and between multiple loci, phenotypic knockout of MHCI is made possible because of a monomorphic, combinatorial epitope that is expressed by these molecules. This mechanism of phenotypic knockout is due to direct retention of the MHCI heavy chains through binding of an ER-retained intrabody and not to an indirect mechanism as occurs in the case of β2-microglobulin or transporter associated with antigen presentation (TAP) deletion mutants which result in incomplete folding and accelerated decay of heavy chain.[45,46]

The BB7.7 MAb that was used for the construction of the anti-hMHCI intrabody has some interesting properties worth mentioning.[27–29,47–53] First, BB7.7 MAb recognizes an epitope which is a monomorphic, combinatorial determinant on the MHCI heavy chain that is confor-

mationally dependent on the presence of β2-microglobulin. Second, BB7.7 MAb shows extensive cross-reactions in other species and these reactions are also monomorphic, ie the reactions with every individual within a species, whether positive or negative, is the same. Third, BB7.7 MAb reacts with a combinatorial determinant in apes and most monkeys[28] and has been reported to co-immunoprecipitate HLA-A,-B homologues with β2-microglobulin in Owl monkeys,[47] a finding similarly seen in COS-1 cells with the anti-hMHCI intrabody immunoprecipitates (Figure 1). Fourth, BB7.7 MAb inhibits dissociation of papain-solubilized HLA heavy chain and β2-microglobulin at 37°C and also increases the rate of association of HLA heavy chain and β2-microglobulin.[49,50] Finally, BB7.7 MAb does not bind to human HLA-A,-B,-C heavy chains associated with mouse β2-microglobulin.[26,27] The latter finding implies that the unique combinatorial epitope that is recognized by BB7.7 MAb requires a precise interaction with a β2-microglobulin that is highly conserved in primary amino acid sequence, a requirement that is not fulfilled by mouse β2-microglobulin. Indeed, it is well known that a number of heavy chain-associated monomorphic epitopes are modified upon assembly with structural variants of β2-microglobulin, a further indication of the profound influence of β2-microglobulin on the folding of heavy chains.[54,55] In addition, although the exact region of MHCI heavy chain that is recognized by BB7.7 MAb is not known, it is proposed to reside in the α3 domain which is monomorphic, although not invariant,[56,57] and has extensive contact with β2-microglobulin.[45] Other Mabs that are directed against monomorphic, combinatorial epitopes on MHCI also map to the α2 and/or α3 domains.[58–61]

Since MHC class I antigens serve as the dominant targets for skin allograft rejection, it is reasonable to hypothesize that impaired antigen processing of donor MHC class I via the indirect pathway, as well as decreased CTL susceptibility via the direct pathway of allorecognition may permit the long-term survival of keratinocyte allografts. As a result of several recent breakthroughs in genetic engineering, MHC-deficient mice have been generated by disrupting components of class I and class II antigens. These studies have reaffirmed that MHC class I plays a dominant role in skin graft rejection.[62,63] Soluble MHC class I shed by the donor keratinocytes are taken up by recipient's APC and presented to CD4 T cells. The CD4 T cells primed via the indirect pathway of allorecognition mediate inflammatory effects (DTH) as well as activate CD8 CTL that recognize donor MHC class I via the direct pathway.

Several investigators have reported that full-thickness skin grafts deficient in class I antigens, class II antigens, or both retain significant immunogenicity and are readily rejected by immunocompetent hosts.[25,62,64–67] However, β2-microglobulin and TAP single/double knockout mice still express residual amounts of the MHCI heavy chain that reach the cell surface and are sufficient to trigger a CTL response.[68–70] These data suggest that an almost complete MHC class I knockout phenotype is necessary to prevent CTL triggering and action. Here we show that such phenotype is demonstrable by the intrabody technology.

Interestingly, MHC-deficient cultured keratinocyte sheets were considerably less immunogenic than full-thickness MHC-deficient skin *in vivo* and when assayed *in vitro* failed to induce a CTL response to alloantigen.[67] This may reflect the intrinsically decreased amount of alloantigen within these grafts since cultured keratinocytes do not contain antigen-presenting cells. Hultmann *et al*[67] demonstrated that MHC II-deficient sheets were less immunogenic than MHC I-deficient ones. However, this could be explained by the residual expression of MHC I heavy chain on cells of the β2-microglobulin knockout mice that were used in this study. Therefore the application of an intrabody to achieve a near complete MHC I heavy chain knockout phenotype could be more effective in preventing an alloimmune response and might be complemented by a MHC II deficiency of grafted cells.

Our data show that the MHC class I down-regulation on keratinocytes by intrabodies could not be overcome by inflammatory stimuli like IFN-γ (see Figure 7) or TNF-α (data not shown). This is a very important observation as inflammatory stimuli are common after skin/sheet transplantation *in vivo*. One problem with the reduction of surface MHC I on keratinocytes might be a susceptibility to NK cell-mediated killing as MHC class I binding triggers the specific inhibitory receptors (KIRs). It has been demonstrated by other groups that NK cells recognize and lyse syngeneic lymphoblasts and bone marrow cells from β2-microglobulin −/− or TAP −/− donors.[71–73] In contrast, data from β2-microglobulin-deficient mice showed that skin grafts from those deficient mice were not recognized by NK cells.[64] The same may be true for the human immune system however, this still must be elucidated *in vivo*. Obviously, various tissues show distinct susceptibility to NK-mediated killing.

Currently, the application of cultured keratinocyte allografts has limited but potential efficacy in burn wound management and other disorders of the skin arising from trauma, necrotizing infection, venous ulcers and excisional surgery. Despite their cost and technical limitations, keratinocyte allografts can function as temporary skin substitutes and permit more aggressive wound excision and coverage. Thus, MHC-deficient keratinocyte allografts, achieved by intrabody-mediated phenotypic knockout of MHCI may provide a technological advance toward their use as permanent skin substitutes. Likewise, the use of bilayered living skin equivalent, made of type I bovine collagen and cultured allogeneic cells (keratinocytes and fibroblasts) isolated from human neonatal foreskin, although already showing clinical benefit for the treatment of several skin disorders, may also benefit from supplying MHC-deficient keratinocytes and fibroblasts to this form of tissue therapy.[74–76] However, these keratinocyte allografts and/or living skin equivalents will first need to be tested *in vivo* to determine if the MHC-deficient cells have significantly decreased immunogenicity compared with their untreated counterparts.[77]

In addition to the studies described above, further manipulation of the major histocompatibility complex via the combined disruption of both class I and class II antigens could potentially permit the indefinite survival of keratinocyte allografts in patients with significant thermal injury, as class II knockout keratinocytes have been shown to be even less immunogenic than class I knockout keratinocytes, possibly as a result of the role of MHC class II antigens in the afferent arm of antigen processing,

in amplifying T-helper activity and ultimately in activating allospecific CTLs.[67] Indeed, this intrabody approach can be tailored to systematically evaluate different components of the antigen presentation pathways to find optimal target(s) to prevent keratinocyte allograft rejection and the rejection of other tissue engineered grafts.

Materials and methods

Cell cultures

The human $CD4^+$ Jurkat T-lymphocyte cell line was cultured in RPMI-1640 medium supplemented with 10% fetal serum, glutamine (2 mM), penicillin-streptomycin (100 μg/ml) at 37°C and 5% CO_2. The African green monkey fibroblast-like kidney cell line, COS-1 (ATCC-CRL-1650), was grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and antibiotics. The human keratinocyte cell line HaCaT,[78] the human lung epithelial cell line A549 and the human epithelial cell line HeLa were cultured in DMEM supplemented with 5–10% FCS. Human epidermal cell suspensions were obtained from normal donors undergoing foreskin surgery and cultured in serum-free medium (KBM; BioWhittaker, Heidelberg, Germany) with full supplements according to the manufacturer's instruction. All cells were cultured at 37°C in a 5% CO_2 humidified atmosphere. The concentration of neomycin used for selection of stable Jurkat cell lines was 800 μg/ml.

Construction of single-chain antibody against human major histocompatibility complex class I molecules (hMHCI)

The hybridoma cell line BB7.7 was obtained from American Type Tissue Collection (ATCC HB94 hybridoma cells). The HB94 cells were used to isolate mRNA and produce single-stranded cDNA. Forward murine VH primer, 5′-cc-ctc-tag-aca-tat-gtg-aat-tcc-acc-atg-gcc-cag-gt(c/g)-(a/c)a(a/g)-ctg-cag-(c/g)ag-tc(a/t)-gg-3′ and reverse JH primer, 5′-tg(a/c)-gga-gac-ggt-gac-c(a/g)(a/t)-ggt-ccc-t-3′ were used to amplify VH fragment and forward murine Vκ primer, 5′-gag-ctc-gtg-ctc-ac(c/a)-ca(g/a)-t/a)ct-cca-3′ and reverse murine Cκ primer, 5′-att-tgc-ggc-cgc-tac-agt-tgg-tgc-agc-atc-3′ were used to amplify the Vκ fragment. The interchain linker $(Gly_4Ser)_3$ was PCR-amplified from sFvtat3[79] using the forward JH primer 5′-ggg-acc-tcg-gtc-acc-gtc-tcc-tca-3′ and reverse Vκ primer 5′-tgg-aga-ctg-ggt-gag-cac-gag-ctc-aga-tcc-3′. The sFv was assembled using V_H, interchain linker and Vκ fragments by overlap-extension PCR.[80] The anti-hMHCI sFv was cloned into the phagemid vector pHEN1 following *Nco*I/*Not*I digestion.[81] Positive clones were further screened by *Bst*NI fingerprinting after reamplification of the inserts using primers 7452 and 11816.[82]

Construction of endoplasmic reticulum (ER)-directed anti-hMCHI sFv intrabodies

The HB94 sFvs were used to construct ER-directed and ER-retained intrabodies. First, to direct the intrabody into the lumen of the ER, the human F105 VH leader sequence was PCR amplified from F105 Fab-protamine[83,84] as template. The forward primer 5′-tt-taa-gct-tgg-cgc-gcc-acc-atg-gaa-cat-ctg-tgg-3′ (VH71-4) and the reverse primer 5′-ctg-ctg-cag-ttg-cac-ctg-gga-cag-gac-cca-tct-3′ was used to PCR amplify the F105 VH leader sequence. This fragment, which contains a 15 nucleotide overlap with HB94 VH FR1, was used together with HB94 sFv, the 5′ leader primer (VH71-4) and either of the 3′ primers 5′-t-cga-ctt-aat-taa-tta-tta-tac-agt-tgg-tgc-agc-atc-3′ or 5′-t-tga-ctt-aat-taa-tta-tta-cag-ctc-gtc-ctt-ttc-gct-tac-agt-tgg-tgc-agc-atc-3′ for overlap extension assembly of ER-directed and ER-retained HB94 sFv, respectively. The HB94 sFv fragments were cloned into modified pRc/CMV vector (Invitrogen, Carlsbad, CA, USA) after *Asc*I/*Pac*I digestion.[85] A 1.8 kb DNA fragment obtained by *Sca*I/*Hin*dIII digestion of the pCMV4 plasmid, which contains the 5′-untranslated region of the Alfalfa Mosaic Virus 4 after the CMV promoter and acts as a translation enhancer in this position (gift from David Russell, University of Texas Southwestern Medical Center, Dallas, TX, USA), was exchanged for a 1.3 kb *Sca*I/*Hin*dIII digested CMV promoter containing DNA fragment in pRc/CMV and used in some experiments in an effort to enhance intrabody expression.

Adenoviral vectors

Recombinant adenovirus encoding sFvhMHCI-8K (Ad-8ksFv), E. coli β-galactosidase (Ad-β-gal) and human alpha-1-antitrypsin (Ad-hAAT) were used for gene transfer studies. Briefly, for Ad-8ksFv construction, sFvhMHCI-8K was subcloned into pACCMV, which contains 1.3 map units of sequence from the left end of the adenovirus (Ad5) genome, the CMV early promoter, the pUC19 polylinker, SV40 poly (A) signal sequences, and finally, map units 9 to 17 of the Ad5 genome.[86] The recombinant plasmid was co-transfected into the 911 cell line[87] together with the large adenoviral plasmid pJM17[88] using the Ca-phosphate method.[89] Adenoviral genomes formed by homologous recombination between the pJM17 vector and the pACCMV vector contained the sFv cDNA, were replication-defective and were efficiently packaged to form infectious virus. Individual adenovirus plaques were picked and propagated in 60-mm plates of 911 cells. Viral DNA was extracted from the supernatant of single plaques and analyzed for homologous recombination by PCR with appropriate primers (5 -primertactaggatccggcgcgccatggaacatctgtggttcttc; 3 -primeragcgaaaaggacgagctgtaataataattaattaaaagctttacta). Plaques that were positive for the transgene were further propagated. For the propagation and purification of recombinant adenovirus, 911 cells were grown in DMEM supplemented with 10% FCS in 15-cm plates and infected at a multiplicity of 5–10. After 36–48 h when the cytopathic effect was complete, cells were harvested, virus was released by several freeze–thaw cycles and recombinant adenovirus was purified over two rounds of CsCl-gradient.[86] Banded virus was recovered and desalted over Sephadex G25 column (Pharmacia, Freiburg, Germany) and stored in virus preservation buffer at –80°C after the addition of 10% glycerol. To titer the final preparation, an aliquot of the virus was serially diluted and assayed for ability to form plaques on 911 cell monolayers.[88] The capacity of forming plaques from different virus preparations was 1×10^{11} plaque forming units (pfu/ml). The generation of the recombinant adenoviruses encoding *E.*

coli β-galactosidase[86] or human alpha-1-antitrypsine (gift of Dr Mark Kay)[90] has been described elsewhere.

Transfection of mammalian cells

For transient transfection, COS-1 cells were plated at a density of 10^7 cells/100-mm petri plate 24 h before transfection. DEAE-Dextran method of transfection was used.[79] In short, 10 μg of supercoiled plasmid DNA (sFvhMHC-1 in pRc/CMV or pCMV4 vector) was diluted with 1.8 ml of PBS and 100 μl of DEAE-Dextran (10 mg/ml stock made in water) was added to the mixture. The adherent cells were washed 2× with PBS before transfection. DNA-DEAE-Dextran mixture was layered on the cells and the plates were incubated at 37°C for 30 min. The cells were reacted with chloroquine (80 μM, final concentration) in 5 ml of serum-free DMEM media and allowed to incubate for another 2.5 h at 37°C. The medium was aspirated and replaced by 5 ml of fresh serum-free DMEM with 5% DMSO. After 2.5 min of further incubation, the medium was drained and the cells were washed twice with PBS and 7 ml of fresh DMEM media containing 10% fetal calf serum was added and incubated until the cells were processed for metabolic labeling or exposed to neomycin (48–60 h after transfection) for selection for stable cell lines.

Transfection of non-adherent Jurkat T-lymphocytic cell lines was also performed using either a DEAE-Dextran or electroporation method. In short, cells were washed three times with PBS and suspended in 0.8 ml of serum-free RPMI media to which 10 μg of plasmid DNA and 12.5 μl of DEAE-Dextran (10 mg/ml) was added. The DNA-DEAE-Dextran cell mixture was incubated for 30 min at 37°C. The cells were then washed twice with serum-free RPMI and then plated with 10% fetal calf serum in RPMI for 48–60 h. For electroporation, a BioRad Gene Pulser was used with the same number of cells and pulsing them with 10 μg plasmid DNA at settings of 250 V, capacitance of 960 μF for 18–24 s. The transfected cells were then placed in RPMI growth media, and 48–60 h after transfection, cells were either analyzed for protein expression or exposed to neomycin selection.

Adenovirus infections

For infection with recombinant adenovirus, cells were seeded at 5×10^5 cells per culture dish (60 × 15 mm style) and grown to 90–95% confluence. Subsequently 1×10^6 cells were infected with recombinant adenovirus at a multiplicity of infection (MOI 100–250) for 30 min at room temperature. Then medium was added and cells were further incubated for 48 h at 37°C and 5% CO_2. To increase the infection efficiency of A549, HaCaT and primary human keratinocytes, polybrene (hexadimethrine bromide; Sigma, Deisenhofen, Germany) was added to the infection solution at a concentration of 8 μg/ml (A549) and 4 μg/ml (HaCaT and human keratinocytes), respectively.[91] Non-infected cells were used as negative controls. After 48 h in culture, cells were prepared for FACS analysis.

Immunoprecipitation of sFvhMHCI intrabodies

For immunoprecipitation, 10^7 transiently transfected or stably transfected cell lines were exposed to cysteine-free RPMI media (for 2 h) and then metabolically labeled with 100–150 uCi of ^{36}S-cysteine. Cells were washed three times with PBS and lysed with RIPA$^+$ lysate buffer. Soluble proteins from the cell lysate were immunoprecipitated with rabbit-anti-mouse IgG (whole molecule, Sigma)-tagged protein A Sepharose beads. Proteins were resolved on 12.5% SDS-PAGE and visualized by autoradiography.[92]

Flow cytometry

Fluorescence-activated cell sorter (FACS) analysis was used to analyze cell surface expression of MHCI molecules. Transiently transfected COS-1 cells and stably transfected Jurkat cells were washed three times with PBS (with 1% fetal calf serum), and incubated with mouse anti-human MHCI (BioSource, Camarillo, CA, USA) or HB94 hybridoma cells supernatant (1:50 dilution) for 2 h at 4°C, following which the cells were washed three times with PBS and the incubated with FITC-conjugated rabbit anti-mouse IgG (1:500 dilution, Sigma) for 2 h at 4°C. Cells were then washed three times with PBS and resuspended in 0.4 ml of PBS with 4% formaldehyde. FACS analysis of MHCII, β2-microblobulin, CD2, CD3, CD4 and CD8 was performed similarly using mouse anti-human MHCII (Ancell, Bayport, MN, USA), mouse anti-β2 microglobulin (BioSource), mouse anti-human CD2, CD3, CD4 and CD8 (Becton Dickinson, Lincoln Park, NJ, USA). The cells were then analyzed by flow cytometry in the Core-Facility of Dana-Farber Cancer Institute. Adenovirus-infected cells were harvested and washed twice with FACS-buffer (PBS, 2% FCS, 0.1% sodium azide), then cells (1×10^5) were incubated for 30 min with the FITC-labeled anti-human MHC class I IgG_1 mab (Pharmingen, San Diego, CA, USA) or with a FITC-labeled isotype matched control IgG_1 mab (Immunotech, Marseille, France). After washing cells were fixed with 1% paraformaldehyde and analyzed thereafter. Fluorescence intensity was determined by using FACSort (Becton Dickinson). Data were analyzed with CellQuest software (Becton Dickinson).

Stimulation assay with IFN-γ

A549 cells were stimulated with recombinant human IFN-γ (Genzyme, Cambridge, MA, USA) at a concentration of 20 ng/ml. IFN-γ was added to the medium either 24 h before or simultaneously to transduction with the recombinant adenovirus. Forty-eight hours later, cells were harvested and analyzed for MHCI expression on the cell surface by FACS analysis.

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