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MHC class I manipulation on cell surfaces by gene transfer of anti-MHC class I intrabodies—a tool for decreased immunogenicity of allogeneic tissue and cell transplants

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Abstract

Intrabodies (IB) are suitable tools to down-regulate the expression of cell surface molecules in general. In this work, the appearance of major histocompatibility (MHC) class I molecules on the cell surface could be prevented by the expression of intracellularly localized anti-MHC class I antibodies. The expression of MHC antigens presenting intracellularly synthetised peptides on the cell surface is the predominant reason for immunologic detection and rejection of allogeneic cell and tissue transplants. Allogeneic keratinocyte sheets might be a suitable tool for skin grafting. Within this study primary rat keratinocytes have been transfected with anti-MHC I-IB. Strong IB-expressing cells showed a MHC I "knockout" phenotype. The cells did not exhibit any significant alterations compared to non-transfected cells: the cell growth and the expression of other surface molecules were unaltered. Merely an enhanced intracellular accumulation of MHC I molecules could be detected. Notably, IB-expressing keratinocytes displayed a reduced susceptibility to allogeneic cytotoxic T cells in vitro compared to unmodified cells with a normal level of MHC I surface expression. These MHC I-deficient keratinocytes might be utilized in tissue-engineered allogeneic non-immunogeneic skin transplants. The principle of MHC class I manipulation in general can be used for other allogeneic cell and tissue-engineered transplants as well.

Keywords: Intrabodies; Antibodies intracellular; Major histocompatibility antigen class I; Tissue engineering; Transfection; Fusionprotein; Bicistronic expression

1. Introduction

During the last decade, advances in cell culture have opened a new avenue for transplantation: the generation of artificial tissues from single cell suspension by tissue engineering [1–3]. To date, the starting material for tissue engineering is of autologous origin. However, the use of autologous material in transplantation in emergency settings involves multiple limitations, as there are availability and time of generation. To compensate this shortage of autologous material, the use of allogeneic tissue might be a solution. Yet, the immunogenicity of allogeneic transplants mediated by the expression of MHC I molecule/peptide complexes remains to be a problem. The ex vivo generation of an artificial tissue starting from a small number of cells allows the in vitro manipulation of the antigenic properties of these cells, e.g., the elimination of genes that mediate rejection of grafts or introduction of genes that would protect the graft from destruction by the recipient's immune system. This approach might lead to the generation of allogeneic tissue that requires little or no immunosuppression to prevent its rejection. One potential means to lower the immunogenicity of artificial tissues could be the down-regulation of donor MHC class I molecules on the cell surface.

The expression of the MHC class I molecule comprises three different genes that include a high number of

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alleles with co-dominant expression [4]. Because of this complex genetic structure of the MHC I, standard gene therapeutic techniques to target the gene sequences of the MHC proteins are not applicable for this approach. Thus in the past alternative methods were employed to achieve MHC I deficiency: such as the deletion of the MHC I associated protein, β_2 microglobulin (β_2m) and the deletion or inhibition of the intracellular TAP (transporter associated with antigen processing). The absence of the monomeric β_2 microglobulin leads to the instability of the MHC I complex and to diminished expression of MHC class I on the cell surface [5]. Also, the decreased availability of peptides following TAP blockade leads to a reduced number of MHC I molecules on the cell surface. Cells lacking the functional peptide transporter are characterized by a reduced MHC I surface expression and a lowered ability of MHC I-dependent antigen presentation [6]. Based on these observations β_2m and TAP knockout-transplants were generated and tested in vivo [7–11]. $TAP^{-/-}$ and $\beta_2m^{-/-}$ cells still exhibit small amounts of MHC I molecules on their cell surface—about 10% of the constitutive expression [7,9]. Consequently, the heavy chain of the class I molecules is transported to the cell surface without β_2m association, or alternative pathways of peptide loading exist, like the generation of peptides in the endoplasmic reticulum (ER) resulting from the leader sequences of exported proteins [12].

In the past, several approaches using MHC I down-regulated grafts have been employed in transplantation. In this setting, either $\beta_2m^{-/-}$ or $TAP^{-/-}$ organs have been used. Based on the stringency of the transplantation model prolonged up to indefinite survival has been achieved [13–17]. Skin transplants originating from $TAP^{-/-}/\beta_2m^{-/-}$ mice are rejected in 13 days, e.g., only 5–6 days delayed than grafts from normal donors [10,11,18]. Although the in vitro generated skin sheets do not contain professional antigen-presenting cells (APC) and are less immunogeneic than full skin, the allogeneic MHC class I expressing keratinocytes are able to induce an alloimmune response via infiltrating recipient APC that pick up the donor MHC class I molecules and present them to recipient's T cells. Thus, the rejection of allogeneic keratinocyte sheets is only marginally protracted in comparison to full skin containing donor APC [19,20]. In all these findings, one has to acknowledge that MHC class I expression was not fully abrogated since the deletion of TAP and/or β_2m does not lead to complete loss of the complex on the cell surface.

A novel promising approach for inhibiting the expression of cell surface molecules is the use of IB [21,22]. IB are antibody molecules that are retained in the ER by terminal expression of a common carboxy-terminal retention amino acid sequence (SE)KDEL [23–27]. IB are commonly used as single chain (sFv) or Fab fragments. Depending on the antibody molecule one or the other form is more stable. Generally the smaller form, the single chain molecule, is used which consists only of the variable regions of the heavy and the light chains of the antibody. To avoid the dissociation of heavy and light chains, they are connected by a linker peptide which commonly consists of the sequence $(Gly_4Ser)_3$ [28]. To date, several IB targeting cell surface molecules have been employed successfully in experimental settings. Their application led to the down-regulation of several cell surface molecules, e.g., the T cell molecule CD2 [29], the α chain of the IL-2 receptor on several T cell lines including HTLV-1-transformed cells [30,31], the EGF receptor [32], and the VLA-2 antigen [33]. Very recently, we described the potential of an anti-HLA I-IB for different human cell types [34]. The generation of transplantable skin sheets for replacement of skin defects is one of the most advanced applications of tissue-engineered material today [35,36]. Keratinocytes can be cultured in vitro for many passages [37,38]. The application of autologous cultured epidermis has become a standard method for the treatment of burns and chronic wounds [39–41]. Besides the generation of pure epidermal skin sheets more complex composed skin equivalents have been generated by tissue engineering [42–47]. The fact that tissue-engineered epidermal skin can be essentially created from a single keratinocyte makes it possible to engineer a graft with every cell expressing the transgene.

Here, we describe the method of generation of rat keratinocytes with a MHC I "knockout" phenotype by the application of an anti-MHC I (RT1.A)-IB. This manipulation of the surface MHC I expression on a primary cell type could serve as a proof of principle for the generation of non-immunogeneic cells for transplantation purposes.

2. Methods

2.1. Expression plasmids

For construction of the anti-rat MHC I-IB (rat-IB)-containing expression vectors, the hybridoma cell line OX-18 (ECACC, No. 84112011, Oxford, UK) was used as starting material. Total mRNA was prepared from OX-18 cells and single-stranded cDNA was synthesized. For the generation of the rat-IB bicistronic constructs, heavy and light chains of the OX-18 antibody gene were amplified using the primers V_H forward (A: 5′-tac-tag-gcg-cgc-cat-ggm-ttg-ggt-gtg-gam-ctt-gct-att-cct-g-3′; annealing to V_H leader sequences) plus J_H reverse (B: 5′-aat-tat-gag-ctc-aga-tcc-gcc-gcc-acc-gct-ccc-acc-acc-tcc-gga-gcc-acc-gcc-acc-tga-gga-gac-ggt-gac-cga-ggt-3′) and V_κ forward (C: 5′-ggt-ggc-tcc-gga-ggt-ggt-ggg-agc-ggt-ggc-ggc-gga-tct-gag-ctc-gtg-ctg-acc-caa-act-cca-ctc-3′) plus C_κ-KDEL reverse (D: 5′-ttg-act-taa-tta-att-att-aca-gct-cgt-cct-ttt-cgc-tta-cag-ttg-gtg-cag-cat-c-3′), respectively.

Primers B and C contain partly complementary pieces of the interchain linker (ICL) $(Gly_4Ser)_3$ [28], and were assembled by overlap-extension PCR [48]. The construct was then cloned into a multicloning site (MCS) modified version of pIRES-EGFP (Clontech, Palo Alto, USA) using restriction enzymes *Asc*I and *Pac*I (primer sequences contained restriction sites). For the pZeo-rat-IB-GFP constructs, heavy and light chains of the OX-18 antibody gene were amplified using the primers V_H forward (A) plus J_H reverse (B) and V_κ forward (C) plus C_κ reverse (E: 5′-tag-taa-tcg-att-aca-gtt-ggt-gca-gca-tc-3′), respectively, and assembled by overlap-extension PCR. The GFP gene was amplified from pEGFP-N2 using the primer pair GFP forward (F: 5′-tac-taa-tcg-atg-aat-tcg-cgg-ccg-cta-tgg-tga-gca-agg-gcg-ag-3′) and GFP-KDEL reverse (G: 5′-tag-tag-ggc-cct-taa-tta-att-att-aca-gct-cgt-cct-ttt-cgc-tct-tgt-aca-gct-cgt-cca-tgc-cga-gag-tga-t). The latter contains the *Cla*I restriction site for cloning the IB gene in-frame in front of the GFP gene. Both genes were then cloned into a MCS modified version of pcDNA3.1-Zeo (Invitrogen, Leek, The Netherlands). The anti-human MHC I-IB (hu-IB) has been described previously [34]. It was sub-cloned into the plasmids pIRES-rat-IB and pZeo-rat-IB-GFP by re-amplification of the hu-IB using the primers H + I (H: 5′-tac-taa-tcg-atg-gcg-cgc-cga-tat-gga-aca-tct-gtg-gtt-c-3′, I: 5′-ttc-tga-gat-gag-ttt-ttg-ttc-att-att-aat-taa-agc-ggc-cgc-tac-agt-tgg-tgc-agc-atc-3′) and J + K (J: 5′-tac-tag-gcg-cgc-cat-gga-aca-tct-gtg-gtt-c-3′, K: 5′-tag-taa-tcg-att-aca-gtt-ggt-gca-gca-tc-3′), respectively, replacing the rat-IB gene. All plasmids were propagated in *Escherichia coli* strain XL-1 Blue (Stratagene, La Jolla).

In summary, the anti-MHC I-IB (rat-IB and hu-IB) were cloned in two different expression vectors containing the green fluorescent protein (GFP) for detection of IB-expressing cells (Fig. 1). The first construct contained the IB in form of an IB-GFP fusionprotein (Fig. 1A), in the second construct the IB was cloned into a bicistronic vector with GFP in the second cassette (Fig. 1B).

2.2. Cell isolation and culture

2.2.1. Isolation and culture of rat keratinocytes

Skin from the ears of Dark Agouti (DA) rats (M&B A/S, Ry, Denmark) was cut into small pieces and digested with 0.25% (w/v) trypsin, 0.1% (w/v) EDTA overnight in a glass petri dish at 4 °C. The following day the dermis was separated from the epidermis by forceps and discarded. The remaining epidermal tissue was pipetted in the trypsin/EDTA solution for 5 min and subsequently passed through a 100 μm nylon mesh. Trypsination was stopped with an equivalent volume of fetal calf serum (FCS; Biochrom KG, Berlin, Germany) and the cells were spun down for 5 min at 250*g*. The cells were then resuspended in keratinocyte culture medium (3 parts DMEM 1 part HAM's F12 (both Biochrom KG, Berlin, Germany)) supplemented with 1 mM sodium pyruvate (Life Technologies GmbH, Karlsruhe, Germany), 2 mM L-glutamine (Biochrom KG, Berlin, Germany), 100 U/ml penicillin (Life Technologies GmbH, Karlsruhe, Germany), 100 μg/ml streptomycin (Life Technologies GmbH, Karlsruhe, Germany), 5 μg/ml insulin (Sigma, Deisenhofen, Germany), 0.4 μg/ml hydrocortisone (Sigma, Deisenhofen, Germany), 10 ng/ml murine epidermal growth factor (Sigma, Deisenhofen, Germany), 1 nM Choleratoxin (Sigma, Deisenhofen, Germany), 10% FCS and seeded together with NIH-3T3 feeder cells (DSM ACC 59, DSMZ, Braunschweig, Germany) in culture flasks at a density of 1×10^5 cells/cm^2. The strongly adherent keratinocytes were separated from the feeder cells by subsequent trypsination with 0.05% trypsin, 0.02% (w/v) EDTA for removal of the feeder cells in the first step (about 3 min) and further 15 min at 37 °C to detach the rat keratinocytes. The cells could be passaged several times.

2.2.2. Cell culture of epithelial cell lines

The human embryonic kidney cell line 293 (DSM ACC 305, DSMZ, Braunschweig, Germany) and rat epithelial cell line NRK-52E (DSM ACC 199, DSMZ, Braunschweig, Germany) were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and passaged every 3–4 days.

2.3. Transfection protocols

2.3.1. Transfection of primary rat keratinocytes

Primary rat keratinocytes were grown in 6-well plates to about 50% confluence and transfected using the DMRIE-C reagent (Life Technologies, Karlsruhe, Ger-

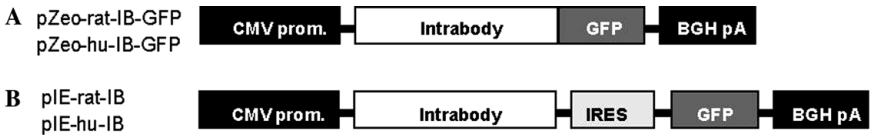

Fig. 1. Expression vectors containing anti-MHC I-IB. Anti-rat MHC I (RT1.A)-IB were cloned either as GFP-fusionproteins (A) or in a GFP-containing bicistronic vector (B). Control constructs were human IB-containing expression vectors. GFP fusionprotein-containing vectors were designated pZeo-rat-IB and pZeo-hu-IB- bicistronic vectors were termed pIE-rat-IB and pIE-hu-IB, respectively. All constructs are driven by the CMV enhancer/promoter and contained the bovine growth hormone poly adenylation signal (BGH pA) for mRNA stabilization. The IB configuration is I_GV_H leader—V_H– ICL–V_K plus GFP (A) or minus GFP (B).

many). Briefly, 2 μg of supercoiled DNA and 5 μl DMRIE-C reagent were diluted in 500 μl OPTI-MEM (Life Technologies, Karlsruhe, Germany) separately and combined thereafter. Complex formation was carried out at room temperature for 30 min. Cells were incubated with the 1 ml transfection mix for 5 h and the mix was replaced with keratinocyte growth medium thereafter. For transient expression, transgene expression was analyzed 48 h post-transfection.

2.3.2. Transfection of epithelial cell lines

Human 293 cells were transfected using the calcium phosphate method as described elsewhere [49]. Briefly, transfection was carried out for 12 h with 15 μg DNA in 10 cm tissue culture dishes containing cells grown to 50% confluence.

2.3.3. Selection of stable IB-expressing cell clones

To isolate stable IB-expressing cell clones, the cells were cultured the following 48 h after transfection either in medium with 200 μg/ml Zeocin™ (Invitrogen, Leek, The Netherlands) for rat keratinocytes or 400 μg/ml G418 (Life Technologies GmbH, Karlsruhe, Germany) for 293 cells. Thereafter, the adherent cells were passaged into 15 cm culture dishes and cultured together with non-transfected cells in selection medium. After 10 days of culture in selection medium, several cell clones were visible by microscopic investigation. In case of the transfection with GFP containing constructs, the cell clones additionally could be identified by the green fluorescence. For the isolation of single cell clones, the adherent cell clusters were washed once with PBS and single clones were picked by small sheets of trypsin/EDTA moisted filter paper pieces. After an incubation time of 10–15 min, the small paper pieces were transferred with a forceps into 24-well plates containing selection medium. The cells adhered in the 24 wells and proliferated further until confluency. The filter pieces could be removed after 3–4 days of culture. After reaching confluency, the cells were expanded into larger culture dishes.

2.4. Flow cytometry analysis

2.4.1. Detection of surface molecule expression

Surface expression of MHC I was detected by indirect immunofluorescence using antibodies against rat RT1.A (MHC I), RT1.B (MHC II), rat ICAM, human HLA-A,-B,-C (all obtained from BD Biosciences, Heidelberg, Germany) and isotype controls (Dianova, Hamburg, Germany) in combination with a PE-conjugated $F(ab')_2$ Fragment Donkey anti-mouse-IgG (H + L) secondary antibody (Dianova). Analysis of the immunophenotype was performed using a FACSort flow cytometer (BD Biosciences, Heidelberg, Germany) and the CellQuest software.

2.4.2. Detection of intracellular MHC I expression

Intracellular staining of the cells was conducted using the commercially available Cytofix/Cytoperm staining kit from PharMingen (BDBiosciences, Heidelberg, Germany). To avoid the staining of the cell surface MHC I molecules, the rat keratinocytes were treated with papain to remove all surface MHC I molecules. The cells were incubated for 30 min at 37 °C with freshly prepared papain solution (5 mg/ml papain; Sigma, Deisenhofen, Germany), 20 mM Cystein (Sigma, Deisenhofen, Germany), 1 mM EDTA (Sigma, Deisenhofen, Germany), and 80 mM Na_2HPO_4 (Merck, Darmstadt, Germany). After papain treatment, the cells were washed two times with culture medium and further used for the intracellular staining procedure. Briefly, the cells were washed once in PBS, resuspended in 200 μl of Cytofix/Cytoperm-solution, and incubated for 20 min at 4 °C. After one washing step with 1 ml of Perm-Wash solution, the cells were incubated with 0.5 μg/50 μl anti-MHC I antibody (F16-4-4-11, ECACC No 89062109, Oxford, UK) for 30 min at 4 °C. After a washing step with 1 ml Perm-Wash solution, the cells were further incubated with the secondary PE-conjugated antibody (see Section 2.4.1) for 30 min at 4 °C. After a final washing step with 1 ml Perm-Wash, the cells were resuspended in 200 μl PBS and analyzed by flow cytometry.

For the extra-/intracellular combination staining of 293 cells, surface MHC I molecules were first saturated with a 0.5 μg/50 μl FITC-conjugated anti-HLA-A,-B,-C antibody (BD Biosciences, Heidelberg, Germany). The staining of intracellular human MHC I molecules thereafter was carried out with a lower concentration (0.2 μg/50 μl) of the same PE-conjugated anti-HLA-A,-B,-C antibody (BD Biosciences, Heidelberg, Germany) using the Cytoperm/Cytofix-kit as described above. Analysis of the immunophenotype was performed by flow cytometry.

2.5. Functional assays for the verification of MHC I manipulation

2.5.1. Generation of effector T cell populations

Cytotoxic T lymphocytes (CTL) specific for the MHC phenotype of keratinocytes from the rat strain Dark Agouti (DA) were generated by a mixed lymphocyte culture of spleen cells which originated from the rat strain LEWIS (responder) with DA spleen cells (stimulator) for 5 days. Irradiated (30 Grey) DA stimulator cells were incubated with responder cells (LEWIS) at a ratio of 1:5 in 75 cm^2 upright standing tissue culture flasks with 1×10^8 stimulator cells in 40 ml RPMI culture medium (Biochrom KG, Berlin, Germany) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM β-mercaptoethanol (Sigma, Deisenhofen, Germany), 1 mM N^6-monomethyl-L-arginine-monoacetate (L-NMMA) (QBIOGENE-Alexis GmbH, Grün-

berg, Germany), 10% fetal calf serum, and 2% autologous LEWIS rat serum per flask.

2.5.2. Cytotoxicity assays

For determination of the immunogeneic properties of IB-expressing cells, europium release assays were performed [50,51] using DA rat keratinocytes as target cells. To assess the non-specific lytic activity of the CTL, the rat epithelial tumor cell line NRK-52E was utilized as third party. 1×10^4 target cells were incubated with CTL effector cells at different effector: target ratios in V-bottomed 96-well plates in 200 μl total volume. All labeling steps were carried out at 4 °C. Target cells for the CTL assays were loaded with europium by washing the cells once with washing buffer 1 (WB1: 50 mM Hepes, 93 mM $NaCl_2$, 5 mM KCl, and 2 mM $MgCl_2$, pH 7.4) and incubating 10^7 cells in 1.5 ml cold labeling buffer (for rat keratinocytes: 800 μM $EuCl_3$, 4 mM DTPA, and 500 μM dextransulfate MW 500.000 in WB1; for NRK-52E cells: 600 μM $EuCl_3$, 3 mM DTPA, and 500 μM dextransulfate MW 500.000 in WB1) for 15 min with occasional shaking. Subsequently, 63 μl of 100 mM $CaCl_2$ was added to the labeling mix and the cells were incubated for additional 5 min at 4 °C. The cells were then washed with cold wash buffer 2 (WB2: 2 mM $CaCl_2$, 10 mM glucose in WB1) and three times with culture medium (RPMI, 10% FCS) whereas the temperature was increased to room temperature. The target cells were then incubated with the effector cells at the indicated ratios for 2 h. After this incubation time, 20 μl of the supernatant from each well was collected and added to 200 μl enhancement solution (Wallac, Turku, Finland). The released europium molecules were quantified by time-resolved fluorometry on a Spectrafluor fluorimeter (Tecan, Crailsheim, Germany). The specific europium release was calculated as follows: ((experimental release − spontaneous release)/(maximum release − spontaneous release)) × 100%. The maximum release was obtained by lysis of the cells with a 0.9% Triton X-100 (final concentration in culture medium) solution.

3. Results

3.1. Expression of rat-IB in primary rat keratinocytes

Primary rat keratinocytes were transfected with constructs either encoding for the rat-IB as an IB-GFP fusionprotein or as a bicistronic vector with GFP. Forty-eight hours post-transfection the MHC I surface expression was analyzed (Fig. 2). As a control an anti-human MHC I-IB (hu-IB) was used which does not recognize the rat MHC class I molecule.

The anti-RT1.A antibody clone F16-4-4-11, which recognizes a different epitope than the rat-IB OX-18, was used as staining antibody for rat MHC I surface molecule detection to ensure the detection of escaped class I molecules on the cell surface that were masked by the rat-IB OX18. Rat-IB-expressing rat keratinocytes showed a clear inversed correlation between IB expression (indicated by the green fluorescence) and MHC I expression (red fluorescence). Cells with very strong IB expression displayed a MHC I "knockout" phenotype matching the cell surface staining of the isotype control (mean < 10 U). In contrast to rat-IB-transfected keratinocytes (Figs. 2A and C), control IB-transfected cells did not show a decreased MHC I surface expression (Figs. 2B and D). A certain basal expression of the IB was necessary to give a visible effect of down-regulation of the MHC I surface expression (at a green fluorescence of > 200 U). Furthermore, the IB-GFP fusionprotein (Fig. 2A) was much more effective in down-regulating MHC class I expression than the IB alone in the bicistronic vector (Fig. 2C). Reasons for this finding could include enhanced stability or better retention of the fusionprotein.

3.2. Expression of other cell surface markers on rat-IB-expressing keratinocytes

Morphologically, IB-transfected rat keratinocytes did not differ from unmodified cells. They merely exhibited a slightly bigger size and a stronger granulation pattern probably due to the accumulation of trapped rat MHC I molecules and intracellularly expressed IB (Fig. 3). They also showed the same growth kinetics in vitro as non-transfected keratinocytes. To ensure that the rat-IB expression in rat keratinocytes does not have any impact on other cell surface molecules, inducible and constitutively expressed molecules were analyzed. Fig. 4 shows the analysis of the surface molecules RT1.B (rat MHC class II) and ICAM-1 (CD54). RT1.B is induced by interferon γ (IFN-γ) stimulation on non-transfected and IB-transfected rat keratinocytes to a comparable extent.

Similarly, a strong ICAM-1 expression was observed in non-transfected, control IB-transfected, and rat-IB-transfected rat keratinocytes. ICAM-1 expression was only weakly enhanceable by IFN-γ in all cells analyzed. Thus, IB-transfected as well as non-transfected rat keratinocytes can be activated by IFN-γ and do not differ in the analyzed cell surface antigen expression.

3.3. Fate of IB-bound MHC I molecules

Flow cytometry analysis showed that IB-expressing cells displayed an enhanced number of intracellularly localized class I molecules (Fig. 5). Rat-IB-transfected rat keratinocytes (Fig. 5A) showed accumulation of the IB trapped MHC I molecules inside the cell. The number of intracellularly accumulated class I molecules correlated with the degree of MHC I surface reduction as shown for human 293 cells (Fig. 5B): intermediate MHC

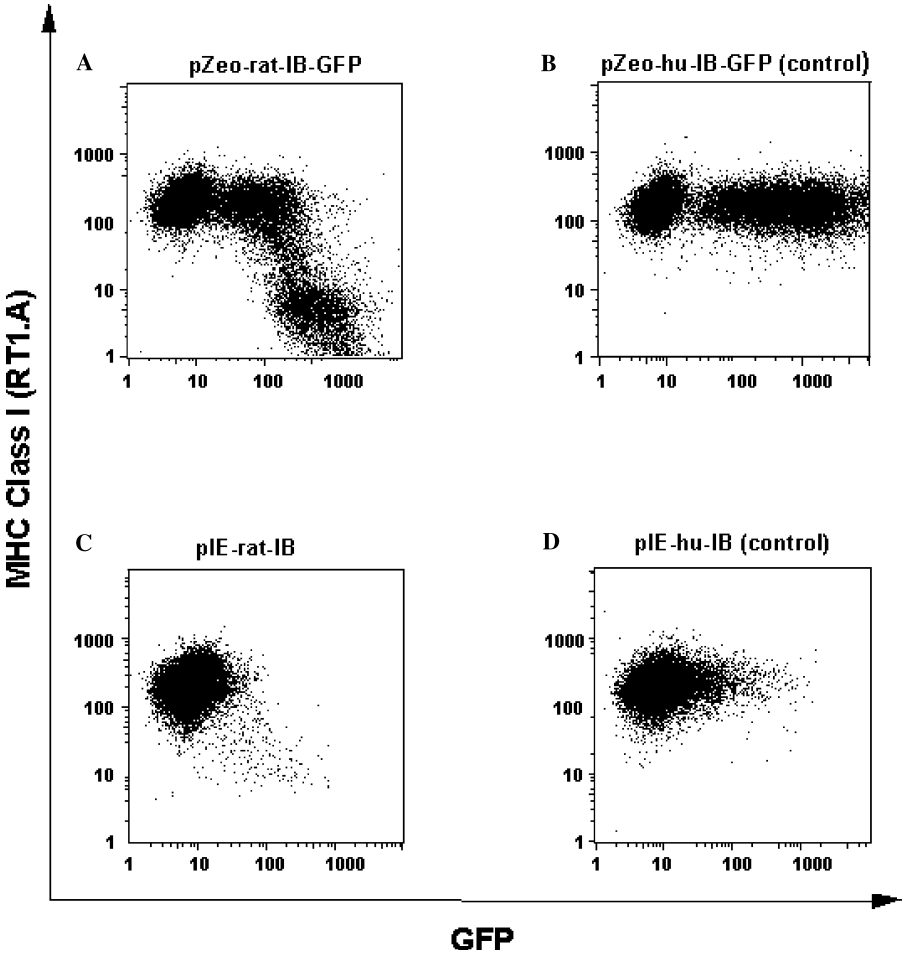

Fig. 2. Strong reduction of MHC class I (RT1.A) cell surface expression on rat keratinocytes by rat-IB (A and C) but not by control hu-IB (B and D). Primary rat keratinocytes were transfected with the designated constructs and analyzed by flow cytometry 48 h post-transfection. Constructs used: IB-GFP fusionproteins cloned into pcDNA3.1-Zeo (A, B) and IB cloned into the GFP-containing bicistronic vector pIRES-EGFP (C, D).

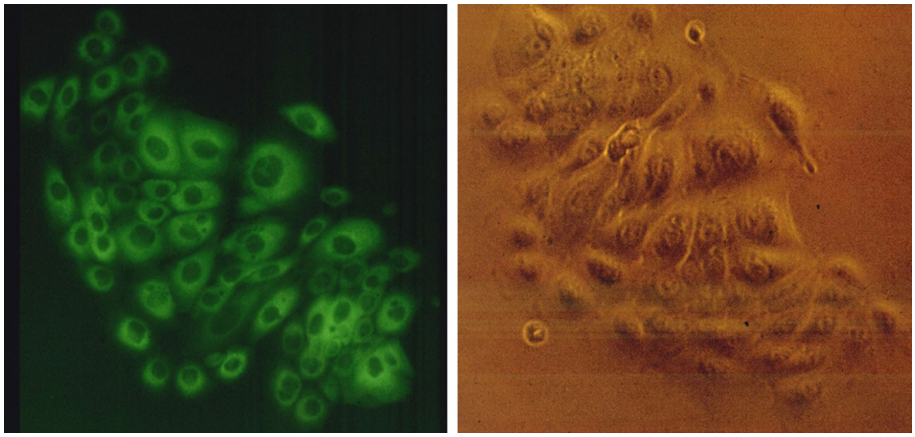

Fig. 3. Strong intracellular expression of rat-IB-GFP in primary rat keratinocytes. Stable IB-expressing cell clones were established by selection with Zeocin™. Isolated clones show a strong GFP staining (left) and no changes in the typical morphology of keratinocytes, shown here in the light microscopic picture (right). Original magnification, 400×.

I expressing cells showed moderate staining for intracellular class I molecules (IB-transfected clone 1) whereas strongly MHC I down-regulated cells displayed large numbers of MHC I molecules inside the cell (IB-transfected clone 2).

3.4. Susceptibility of anti-MHC I-IB-expressing rat keratinocytes to alloreactive cytotoxic T cells

To test the immunogenicity of IB-expressing cells, we generated clones from transfected keratinocytes with

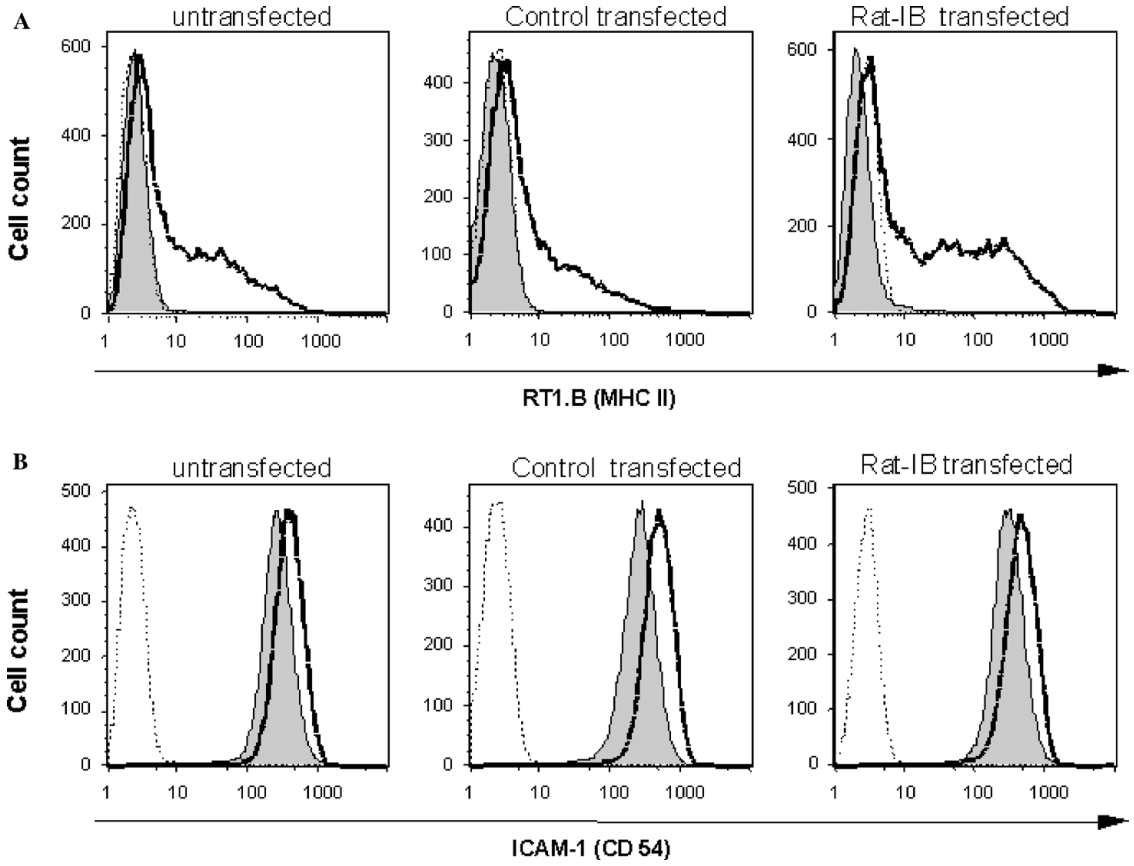

Fig. 4. No changes in expression of other cell surface molecules by rat IB-expressing rat keratinocytes. Stably rat IB-expressing rat keratinocytes (right) were analyzed for their cell surface expression of MHC II (RT1.B) (A) or ICAM-1 (B) with (bold line) or without IFN-γ treatment (single line, filled) and compared to control IB-expressing (middle) or non-transfected cells (left). Cells were treated with IFN-γ at 10 ng/ml for 48 h. Isotype controls are shown in dashed lines.

extremely low MHC I expression. Rat-IB-expressing but not control hu-IB (anti-human MHC I)-expressing keratinocytes derived from DA rats ($RT1.A^{av1}$) are almost completely resistant to the donor-specific killing by DA-alloreactive cytotoxic T cells of LEWIS rats ($RT1.A^{l}$) that were primed against the DA haplotype by mixed lymphocyte culture (Fig. 6). This result shows the functional capacity of the anti-MHC I-IB in lowering the immunogenicity of the selected cells.

4. Concluding remarks

The MHC class I antigens are the major target of alloreactivity to cells and tissues. We could successfully abolish the immunogenicity of rat keratinocytes by trapping their MHC I molecules inside the cell utilizing anti-MHC I-IB and thereby preventing their expression on the cell surface. By applying this approach, a truly MHC I "knockout" phenotype has been generated in contrast to transplants originating from $\beta_2m^{-/-}$ or $TAP^{-/-}$ donors which do have residual MHC I molecules on their cell surface. As primed alloreactive T cells are very efficient in killing of appropriate target cells and require only very few MHC class I molecules (<50/cell) for triggering the cytotoxic cascade, the absence of killing demonstrates the efficiency of our approach. It is argued that the residual MHC class I expression might be the reason for the rejection of allogeneic β_2m-deficient skin transplants by wild type mice [10].

Importantly, IB-expressing cells displayed unaltered patterns of other cell surface molecules, their response to IFN-γ, and their growth kinetics in culture. The retained MHC class I molecules accumulated in the IB-expressing cells but their final fate is uncertain. When the number of accumulated molecules inside the cell reaches a toxic level, they will probably be degraded. The number of class I molecules that is tolerated by the cell will presumably vary between cell types. Yet, the large number of class I molecules inside the cell might have a negative feedback on the regulation of the MHC I protein synthesis.

A problem in lowering MHC I surface expression could be the detection of these cells by natural killer (NK) cells which recognize the "missing self" by being not inactivated by the specific killer inhibitory receptors. NK cells recognize and lyse syngeneic lymphoblasts and

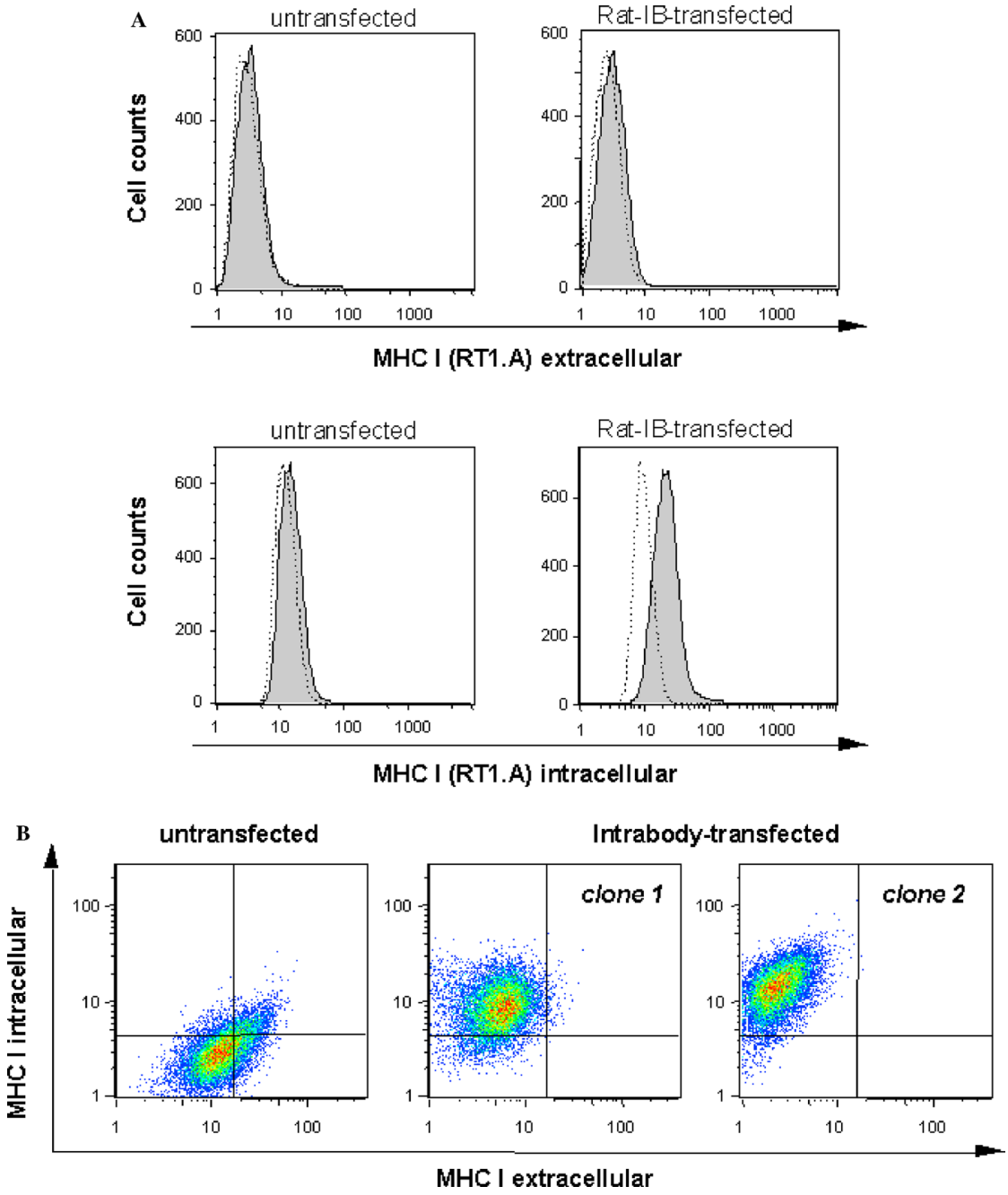

Fig. 5. Class I molecules accumulate intracellularly in IB-transfected cells. (A) Prior to intracellular staining of MHC I (RT1.A) molecules, rat keratinocytes were treated with papain to cleave off all molecules on the cell surface (upper histograms). Intracellular staining shows accumulation of RT1.A molecules in rat IB-transfected keratinocytes as opposed to very little intracellular RT1.A staining in non-transfected cells (isotype controls = dashed lines). (B) Stably hu IB-transfected and non-transfected human 293 cells were sequentially extra- and intracellularly stained with anti-MHC I antibodies. In the first step, cell surface MHC I molecules were saturated using a FITC-coupled anti-MHC I antibody. Subsequently the cells were stained intracellularly with the same but PE-coupled antibody. Cells with a very strong MHC I cell surface reduction show the strongest intracellular accumulation of class I molecules (IB-transfected clone 2).

bone marrow cells from $\beta_2m^{-/-}$ or $TAP^{-/-}$ donors [52–54]. However, allogeneic skin grafts from β_2m-deficient mice were not recognized by NK cells of irradiated (and thereby T cell depleted) recipients [18]. These findings might implicate that NK cells are not able to injure MHC-deficient skin tissue.

MHC I-deficient allogeneic keratinocytes might be used in skin transplantation as readily available material: they could be grafted alone as an epidermal allograft or they could be used in combination with other bio-engineered synthetic products. The manipulation of the MHC I surface expression by gene transfer of

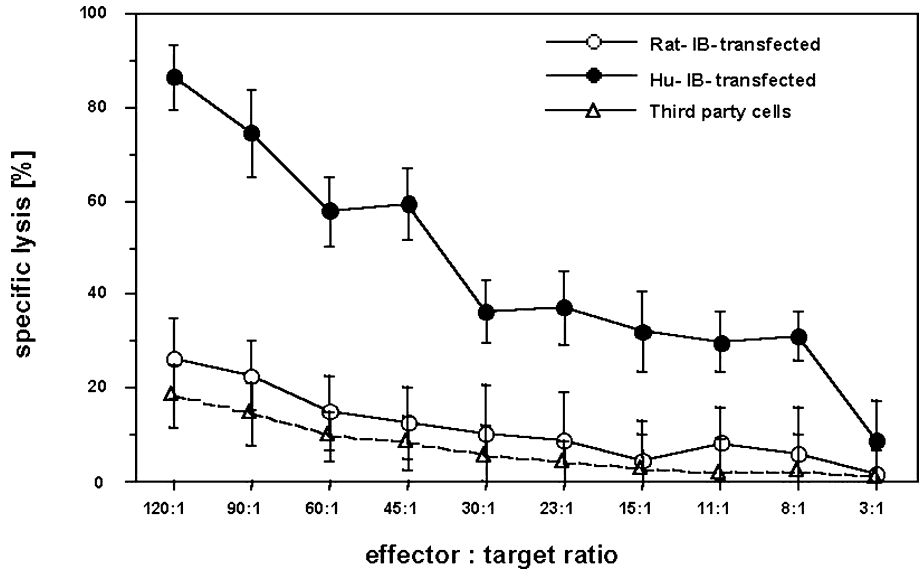

Fig. 6. CTL assay with rat keratinocytes from DA rats as target cells. 1×10^4 target cells were incubated with the indicated numbers of effector cells. As effector cells allogeneic (LEWIS rat origin) spleen cells, that were pre-stimulated with spleen cells of DA origin, were used. Target cells were rat IB-transfected rat keratinocytes (clear circles), control hu IB-transfected rat keratinocytes (filled circles) or third party cells (cell line NRK 52E) of a different RT1.A haplotype that is not recognized by the T cells (clear triangles).

anti-MHC I-IB could be further applied to other tissue-engineered transplants.

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