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ORIGINAL ARTICLE

Downregulation of cytolytic activity of human effector cells by transgenic expression of human PD-ligand-1 on porcine target cells

Annegret Plege, Katja Borns, Lilli Beer, Wiebke Baars, Juergen Klempnauer and Reinhard Schwinzer

Transplant Laboratory, Department of General, Visceral and Transplantation Surgery, Hannover Medical School, Hannover, Germany

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Correspondence
Dr Reinhard Schwinzer, Transplantationslabor, Klinik für Allgemein-, Viszeral- und Transplantationschirurgie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30623 Hannover, Germany. Tel.: +49 511 532 4204; fax: +49 511 532 8326; e-mail: schwinzer.reinhard@mh-hannover.de

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Summary

Cellular rejection is a relevant hurdle for successful pig-to-primate xenotransplantion. We have shown previously that the induction of a human anti-pig T cell response (*in vitro* activation of $CD4^+$ T cells) can be suppressed by the overexpression of human negative costimulatory ligands (e.g. programmed death receptor ligand, PD-L1) on pig antigen presenting cells. Here, we asked whether PD-L1 mediated enhancement of negative signaling might also be efficient during the effector phase of human anti-pig cellular immune responses. The porcine B-cell line L23 was transfected with human PD-L1, and clones were selected stably expressing PD-L1 with low, medium, or high density. Mock-transfected L23 cells were effectively lysed by human cytotoxic effector cells (IL-2 activated $CD8^+$ T cells and $CD56^+$ cells). The lytic potential of the effectors decreased with increasing levels of PD-L1 and was reduced by about 50% in L23-PD-L^{high} targets. A proportion of activated $CD8^+$ effector cells underwent apoptosis when exposed to PD-L1 expressing L23 cells. These data suggest that the overexpression of PD-L1 on target cells may (a) trigger negative signals in effector cells that prevent the release of cytolytic molecules and/or (b) induce apoptosis in the attacking effector cells thereby protecting targets from destruction.

Introduction

Enormous progress has been made in recent years to overcome the barrier of hyperacute rejection after pig-to-primate xenotransplantation. For example, transgenic expression of human complement regulatory molecules in pigs has been shown to be an effective strategy to prevent hyperacute rejection in preclinical models of xenotransplantation [1]. Furthermore, alpha1,3-galactosyltransferase deficient pigs are now available and represent another approach to avoid hyperacute rejection mediated by preexisting antibodies [2]. Thus, for the further development of clinical xenotransplantation, immunological concepts are now required facilitating the control of human anti-pig cellular immune responses.

One approach for the downregulation of human anti-pig T cell responses by genetic modification of pigs may derive from the new concept of 'negative costimulation' [3]. It is well established that T cells need more than the signal through the antigen receptor ('signal 1') to get fully activated. Classical costimulatory molecules like the B7 family members CD80 or CD86 deliver a positive signal ('signal 2') to T cells which – in concert with 'signal 1' – leads to their complete activation. The B7 family of T cell costimulatory molecules has recently acquired several new members. Interestingly, some of them do not deliver a positive costimulatory 'signal 2' to T cells, but generate negative signals [4]. The programmed death 1 (PD-1) pathway has already been described in some detail.

The PD-1 receptor was initially found in cell lines undergoing programmed cell death and was thought to be involved in this process. Later, it was demonstrated that PD-1 is induced by T-cell and B-cell activation

and plays a role in the regulation of the activation process [5]. PD-1 contains a cytoplasmic immunoreceptor tyrosine-based switch motif [6]. Binding of programmed death receptor ligands (PD-L1 or PD-L2) to PD-1 leads to the recruitment of SHP-phosphatases, which downregulate T-cell receptor signaling accompanied by reduced T-cell proliferation and cytokine production [7].

The efficiency of PD-1/PD-ligand targeting to modulate immune responses to an allograft has been shown in various models. For example, prolongation of allograft survival could be obtained by the transfer of PD-L1.Ig gene to donor hearts in a rat transplantation model [8]. Furthermore, an enhancement of PD-1-mediated negative costimulation by the application of PD-L1.Ig prolonged graft survival in mouse models [9]. Recent data also show that the overexpression of PD-L1 on islet grafts in mice can partially prolong islet graft survival [10].

In pig-to-human xenotransplantation, there is the chance to create grafts exhibiting a strong negative costimulatory potential. We have previously shown that human $CD4^+$ T cells respond with reduced proliferation, a cytokine pattern which is dominated by IL-10 and an expansion of $CD4^+CD25^{high}Foxp3^+$ T_{reg} to stimulation with pig cells ectopically expressing human PD-ligands [11]. These findings suggested that an enhancement of negative costimulatory signals counteracts with the induction of a human-anti-pig T-cell response. Here, we investigated the effects of PD-1/PD-L1-mediated negative costimulation during the effector phase of the response. The data suggest that the augmentation of PD-1 signaling in human $CD8^+$ effector T cells and $CD56^+$ cells decreases their capacity to lyse pig target cells.

Materials and methods

Cells

The porcine peripheral blood B cell line L23 [12] was obtained from the European Collection of Cell Cultures (London, UK). L23-PD-L1 transfectants and L23-GFP controls were generated as described [11]. Clones of transfected L23-PD-L1 cells expressing PD-L1 in different intensities were generated by limiting dilution. Human peripheral blood mononuclear cells (PBMC) were isolated from leukotrap filters obtained from the Department of Transfusion Medicine (Hannover Medical School) by Ficoll gradient centrifugation. $CD8^+$ and $CD4^+$ T cell subsets were negatively isolated by MACS (Miltenyi Biotec; Bergisch Gladbach, Germany). Purity of $CD8^+$ T cells or $CD4^+$ T cells was usually ≥90%. To purify $CD56^+$ cells, PBMC were stained by monoclonal antibodies directed to CD56, and subsequently $CD56^+$ cells were isolated by electronic cell sorting (Cell Sorting Core Facility, Hannover Medical School).

Immunofluorescence staining and flow cytometry

The following mAb were used: anti-pig MHC class-I (74–11–10), anti-pig MHC class-II (MSA3) both provided by A. Saalmüller, Wien, Austria; anti-human CD8-PE (SK1; BD Biosciences; San Jose, CA, USA), anti-human CD56-APC (B159; BD Biosciences; San Jose, CA, USA), anti-human PD-L1-PE (MIH1; eBioscience; San Diego, CA, USA) and anti-human PD-1 (MIH4; BD Biosciences; San Jose, CA, USA). Binding of unlabeled primary reagents was visualized using FITC-conjugated goat anti-mouse IgG plus IgM (Dianova; Hamburg, Germany) or PE-conjugated rat anti-mouse IgG (BD Biosciences, San Jose, CA, USA). Apoptotic cells were stained by using Annexin-FITC (BD-Biosciences). Cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences) and data processed by using WINMDI software (free software).

Analysis of proliferation

A total of 1×10^5 $CD8^+$ T cells, human PBMC or porcine PBMC were stimulated with 1×10^3 L23 transfectants (irradiated with 30 Gray) in microtiter plates in a total volume of 200 µl culture medium (RPMI 1640 medium supplemented with 10% FCS, 50 U/ml penicillin, 4 mM L-glutamin, 50 µg/ml streptomycin, 1 mM sodium pyruvate and 0.05 mM β-mercaptoethanol). In some experiments, individual cells were coated with recombinant human PD-L1-Fc (R&D systems; Wiesbaden, Germany) at 15 µg/ml by incubating for 4 h at 37 °C. After 6 days, ^{3}H-TdR was added and cultures were harvested after an additional 16-h incubation period.

Analysis of apoptosis

A total of 4×10^6 human $CD8^+$ T cells were incubated with 5×10^5 irradiated L23-GFP transfectants for 6 days in a total volume of 10 ml culture medium. A sum of 2×10^6 $CD56^+$ cells were incubated with 2.5×10^5 irradiated L23-GFP transfectants in the presence of 50 U/ml IL-2 in a total volume of 5 ml culture medium for 6 days. Subsequently, $CD8^+$ T cells or $CD56^+$ cells were purified by Ficoll gradient centrifugation and cultured for additional 24 h without stimulus. Afterwards, 1×10^5 $CD8^+$ T cells or $CD56^+$ cells were co-cultured with 2×10^5 L23-GFP or L23-PD-L1 cells in microtiter plates in 200 µl culture medium for 4 h, and apoptotic cells were stained by anti-CD8 mAb or anti-CD56 mAb and Annexin-FITC. Frequencies of Annexin-positive cells were determined within CD8 positive and CD56 positive cells.

Cytotoxicity assays

The cytotoxic activity of human effector cells was assessed by ^{51}Cr-release assays. L23-GFP or L23-PD-L1 cells (3×10^6) were labeled with 100 μCi of sodium [^{51}Cr]-chromate (GE Healthcare, Buckinghamshire, Great Britain) and plated at 1×10^4 cells/well in microtiter plates as target cells. To generate CD56$^+$ and CD8$^+$ effector cells with cytotoxic potential purified, CD8$^+$ or CD56$^+$ cell subsets were cultured with 100 U/ml IL-2 for 4–5 days. Effector cells were added at the indicated effector:target ratios to the target cells in microtiter plates in a total volume of 200 μl. The plates were incubated for 4 h at 37 °C. After a second centrifugation, 25 μl of the culture supernatant was collected and assayed for radioactivity. The percentage of specific cell lysis mediated by effector cells was calculated as described previously [13].

Statistical analysis

Statistical analysis was performed by using the Student's *t*-test. Levels of significance are given as *P*-values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

Pig cells overexpressing human PD-L1 trigger diminished proliferative responses of human CD8$^+$ T cells

Proliferative responses of human CD4$^+$ T cells to porcine L23-PD-L1 transfectants have been described to be reduced compared with L23-GFP control cells. This suppression can be reversed by antibody-mediated blocking of PD-1/PD-L1 interactions [11]. To study whether downregulation of T-cell reactivity by PD-1/PD-L1-mediated negative signaling is particularly pronounced in the human CD4 subset or is a more general phenomenon, we analyzed the proliferative responses of the entire human PBMC population and isolated CD8$^+$ T and CD56$^+$ cells. Furthermore, porcine PBMC were used as control responders. Proliferation of human CD8$^+$ T cell and CD56$^+$ populations to L23-PD-L1 transfectants was markedly reduced compared with stimulation with L23-GFP control cells (Fig. 1). Neither L23-GFP nor L23-PD-L1 cells induced proliferation in CD56$^+$ cells (data not shown). When allogeneic porcine PBMC were used as responders, no difference in the stimulatory capacity of L23-PD-L1 and L23-GFP cells was observed (Fig. 1). Thus, it seems to be unlikely that transfection of L23 cells with PD-L1 led to a global drop of their stimulatory capacity, e.g., by downregulation of adhesion molecules or costimulatory molecules delivering positive signals. This is also supported by our previous finding that PD-L1 transfectants and control cells expressed similar amounts of MHC class-I and class-II molecules as well as CD80/86 and CD40 [11]. Furthermore, the observation that the responses of pig PBMC were not influenced by the L23 transfectants expressing the human PD-L1 transgene suggests that there is no functional compatibility between human PD-L1 and the porcine PD-1 receptor.

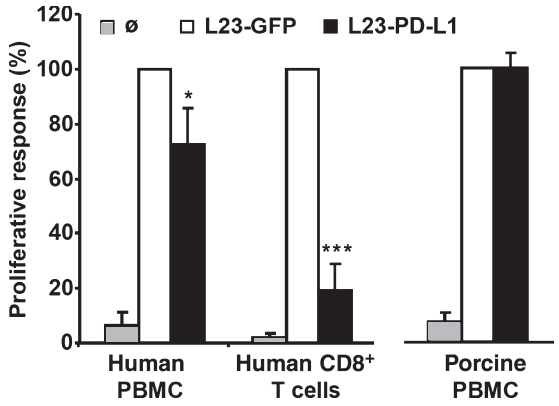

Figure 1 Suppression of human PBMC and CD8$^+$ T-cell proliferation after stimulation with L23-PD-L1 transfectants. A total of 1×10^5 human peripheral blood mononuclear cells (PBMC), purified human CD8$^+$ T cells and porcine PBMC were cultured with medium (gray bars), with 1×10^3 irradiated L23-GFP cells (white bars) or with L23-PD-L1 clones highly expressing PD-L1 (black bars). After 6 days, cells were pulsed with [^{3}H-TdR] and harvested after 16 h. Results are expressed as percentage of proliferative response ± SD of three independent experiments. *$P < 0.05$, ***$P < 0.005$.

To further confirm the role of PD-L1 in inhibiting CD8$^+$ T-cell proliferation, CD8$^+$ T cells were activated by L23-GFP cells in the presence or absence of recombinant human PD-L1-Fc. CD8$^+$ T cell proliferation was inhibited by about 40% by PD-L1-Fc (Table 1). Thus, PD-L1-Fc, similar to PD-L1 expressed on pig cells, has the capacity to downregulate proliferative responses of CD8$^+$ T cells (Table 1).

Table 1. PD-L1-Fc mediated inhibition of human CD8$^+$ T cell proliferation*.

	Stimulus		
Experiment no.	L23GFP	L23GFP plus PD-L1-Fc	L23-PD-L1
1	100†	54 ± 9	37 ± 7
2	100	68 ± 14	19 ± 3

*1×10^5 purified human CD8$^+$ T cells were stimulated with 1×10^3 L23-GFP cells or L23-PD-L1 clones highly expressing PD-L1 or L23-GFP cells and PD-L1-Fc. After 6 days, cells were pulsed with [3H-TdR] and proliferation was measured after 16 h.

†Proliferation of T cells stimulated by L23-GFP cells was set to 100%. Results are expressed as percent of proliferation ± SD of triplicate cultures.

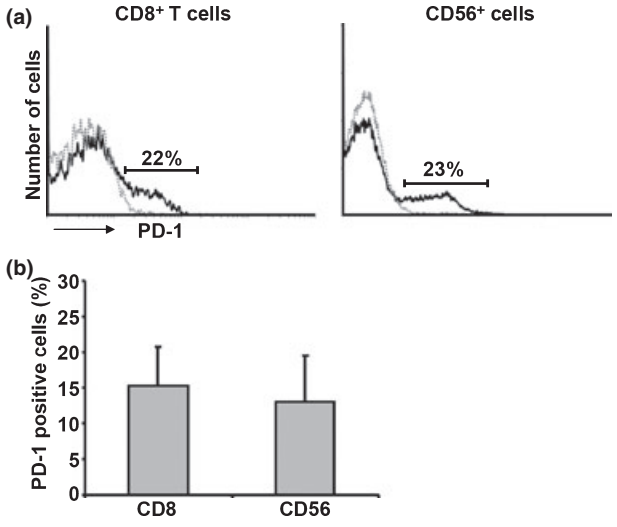

Figure 2 Flow cytometric analysis of PD-1 expression. Human peripheral blood lymphocytes were cultured for 5 days with 100 U IL-2/ml to generate effector cells. Cells were stained with anti-CD8-PE mAb or anti-CD56-APC mAb and with anti-PD-1 mAb that was detected by goat anti-mouse-FITC. Analysis of PD-1 expression was performed in gated $CD8^+$ T cells and $CD56^+$ cells. (a) Black lines represent fluorescence intensity obtained after staining of the cells with anti-PD-1 mAb and goat anti mouse-FITC, dashed lines were obtained by staining with an isotype control and goat anti-mouse-FITC. (b) PD-1 positive cells within $CD8^+$ and $CD56^+$ populations of five different blood donors (mean % ± SD).

PD-1 is expressed on human cytotoxic $CD8^+$ T cells and human $CD56^+$ cells

The data described above suggested that proliferation of human $CD8^+$ T cells can be suppressed by an enhancement of negative costimulatory signals. We next asked whether PD-L1-mediated negative signaling might also be efficient during the cytotoxic effector phase of an immune response. One prerequisite for PD-L1 to unfold its inhibitory capacity is the expression of an appropriate receptor (e.g. PD-1) on cytotoxic effector cells. Thus, we generated effector populations by the stimulation of human $CD8^+$ T cells and $CD56^+$ cells with high concentrations of IL-2 and analyzed PD-1 expression on these cells by flow cytometry. Cells expressing PD-1 on the cell surface could be detected in both, IL-2 activated $CD8^+$ T (15 ± 5%) and $CD56^+$ cell populations (13 ± 6%) (Fig. 2). The basic requirement to be susceptible to PD-L1-mediated negative signaling – namely expression of the PD-1 receptor – should therefore be fulfilled by both effector cell types.

PD-L1 protects pig cells from lysis mediated by human cytotoxic effector cells

IL-2-induced $CD8^+$ T cells exhibited a strong cytotoxic potential on L23-GFP targets (Fig. 3a). However, when

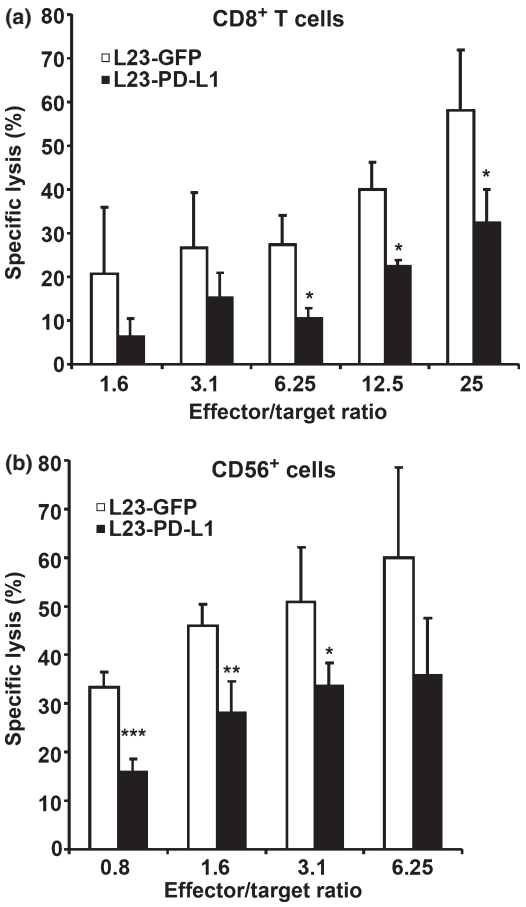

Figure 3 Influence of PD-L1 expression on $CD8^+$ T- and $CD56^+$-cell-mediated lysis of L23 cells. A total of 1×10^4 ^{51}Cr-labeled L23-GFP (white bars) or L23-PD-L1 cell clones highly expressing PD-L1 (black bars) were used as targets and cultured with increasing numbers of effector cells. The amount of radioactivity released into the supernatant was determined after 4 h and the percentage of specific lysis was calculated. (a) Cytotoxicity of $CD8^+$ T cells. Effector cells were generated by culturing purified $CD8^+$ T cells for 4–5 days in medium containing 100 U/ml IL-2. Presented data (mean ± SD) were obtained from three independent experiments. $*P < 0.05$. (b) Cytotoxicity of IL-2 activated $CD56^+$ cells. $CD56^+$ cells were purified by electronic cell sorting, cultured for 4–5 days in medium containing 100 U/ml IL-2 and used as effector cells. Presented data (mean ± SD) were obtained from three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

PD-L1 expressing target cells were used, specific lysis was considerably reduced. At an effector:target ratio of 25, lysis of L23-PD-L1 cells was usually reduced by about 50% compared with lysis of L23-GFP target cells. As expected, also IL-2-activated $CD56^+$ cells lysed porcine L23 cells very efficiently, even at low effector:target ratios (Fig. 3b). Again, lysis of L23-PD-L1 cells was reduced by about 50% compared with L23 control cells. It should be noted that complete protec-

tion of L23-PD-L1 cells was never observed, irrespective of whether activated $CD8^+$ T cells or $CD56^+$ cells were used as effectors. Thus, PD-L1 transfectants seem to be protected only partially from lysis by human effector cells.

The data obtained so far suggested that binding of the transgenic PD-L1 molecule to PD-1 on effector cells decreases their lytic potential. To confirm an essential role of PD-L1 in this scenario, we generated L23 clones expressing different levels of PD-L1 (Fig. 4a). These cells were used as targets for cytotoxic effector cells. As shown in Fig. 4b, L23-PD-$L1^{low}$ cells were only marginally protected from lysis. Intermediate protection was observed in L23-PD-$L1^{medium}$ targets, and L23-PD-$L1^{high}$ cells exhibited the most pronounced protective effect. The correlation of PD-L1 expression density with the inhibition of cytotoxicity strongly supports the assumption that PD-L1/PD-1 interactions are crucial for the observed protection of PD-L1 expressing targets and makes it unlikely that other reasons (e.g. differential expression of adhesion molecules by transfectants and control cells) are involved.

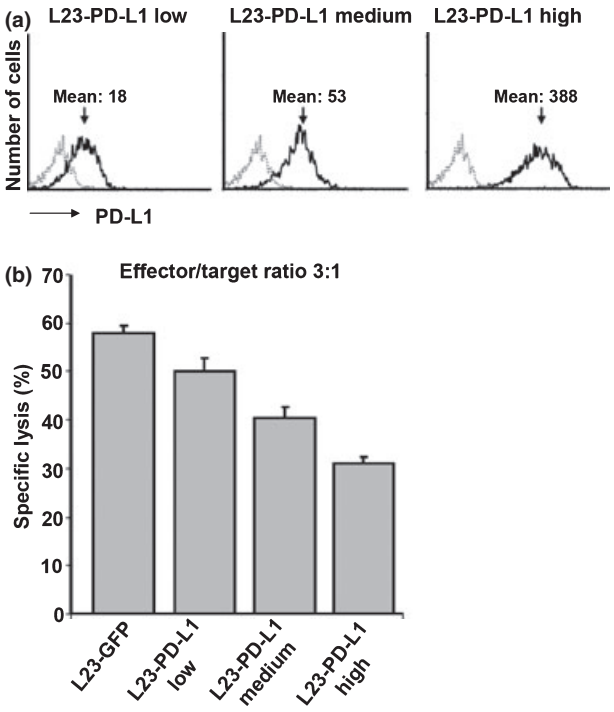

Figure 4 Dose-dependent effects of PD-L1 expression on $CD56^+$ cell cytotoxicity. (a) Expression of PD-L1 on different L23-PD-L1 clones. L23-PD-L1 clones were stained with mAbs directed to human PD-L1. Dashed lines represent staining of L23-GFP cells by the mAbs, black lines represent staining of L23-PD-L1 clones. (b) Lysis of L23-PD-L1 clones by $CD56^+$ cells. A total of 3×10^4 IL-2 activated $CD56^+$ cells were co-cultured with 1×10^4 ^{51}Cr-labeled L23-GFP or L23-PD-L1 clones. The amount of radioactivity released into the supernatant was determined after 4 h and the percentage of specific lysis was calculated. Presented data (mean ± SD) were obtained from two independent experiments.

PD-L1 expressing cells induce apoptosis in activated $CD8^+$ effector cells

Engagement of PD-L1 by PD-1 induces negative signals, but can also result in apoptosis of activated T cells in some experimental settings [14]. To assess whether activated $CD8^+$ T cells and $CD56^+$ undergo cell death when exposed to L23-PD-L1 transfectants, co-culture experiments were performed and the frequency of apoptotic $CD8^+$ T cells was determined by Annexin-FITC staining. Among activated $CD8^+$ T cells, only a small proportion of cells (1–2%) were stained by Annexin (Fig. 5a). This frequency increased when $CD8^+$ cells were co-cultured with L23-GFP cells and was further augmented by the presence of L23-PD-L1 cells. In a series of three experiments, 11 ± 3% Annexin-positive $CD8^+$ cells were found in co-cultures containing L23-GFP cells, but 21 ± 4% in L23-PD-L1 cultures (Fig. 5b). In contrast, among $CD56^+$ cells, neither in co-cultures containing L23-GFP nor in

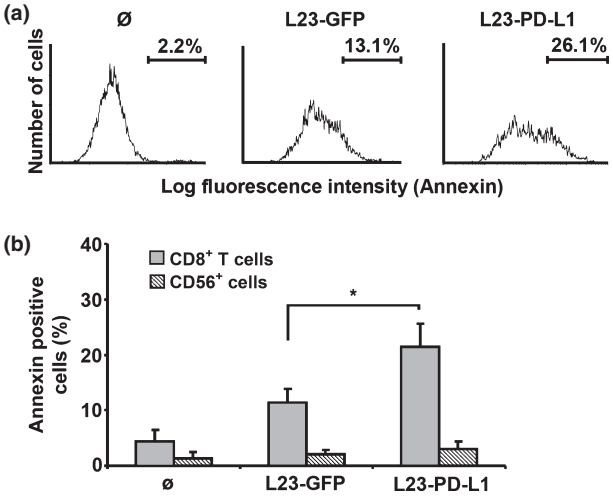

Figure 5 Induction of apoptosis in activated human $CD8^+$ T cells and $CD56^+$ cells by L23-PD-L1 cells. Purified $CD8^+$ T cells or $CD56^+$ cells were activated by stimulation for 6 days with irradiated L23-GFP cells. The cells were rested for 24 h and restimulated for 4 h with medium alone (Ø), L23-GFP, or L23-PD-L1 cell clones highly expressing PD-L1 (Effector:target ratio 1:2). Apoptotic $CD8^+$ T cells or $CD56^+$ cells were detected by Annexin-FITC binding. (a) A single experiment presenting Annexin-FITC staining of $CD8^+$ T cells is shown. Numbers represent the frequency (%) of Annexin-positive cells. (b) Frequencies of Annexin-positive cells (mean% ± SD) from three independent experiments with $CD8^+$ T cells (gray bars) are summarized. $^*P < 0.05$. Percentage of Annexin-positive cells (mean% ± SD) within $CD56^+$ cells (shaded bars) was determined from two independent experiments.

L23-PD-L1 cultures, enhanced frequencies of Annexin-positive cells were detected (Fig. 5b). Thus, in particular in $CD8^+$ T-cell effectors, PD-L-1 might protect target cells by inducing apoptosis in the attacking effector population in addition to switching off the effector potential of PD-1 expressing cells.

Discussion

Triggering of PD-1-mediated negative costimulation by the application of PD-L1.Ig fusion proteins has been described to prolong allograft survival in various mouse transplantation models [9]. Furthermore, we have recently shown that human $CD4^+$ T cells respond *in vitro* to PD-L1 expressing porcine transfectants with reduced proliferation, a cytokine pattern which is dominated by IL-10 and an expansion of $CD4^+CD25^{high}Foxp3^+$ T_{reg} [11]. Thus, an enhancement of negative costimulatory signals in T cells seems to be a promising concept to diminish T cell responses to allo and xenografts. Here, we addressed the question as to whether negative costimulatory signals might be capable of controlling cellular cytotoxicity during the effector phase of an anti-graft response. We found that PD-L1 expressing porcine target cells are partially protected from lysis by IL-2-activated $CD8^+$ T cells as well as $CD56^+$ cells.

Unseparated PBMC and purified $CD8^+$ T cells responded with reduced proliferation to stimulation by L23-PD-L1 cells (Fig. 1). PD-1 is not expressed on resting T cells but is upregulated during T-cell activation [7]. Stimulation of PD-1 by PD-ligand binding leads to phosphorylation on its two intracellular tyrosines and recruitment of phosphatases (SHP-1/SHP-2), which downregulate TcR signaling through direct dephosphorylation of signaling intermediates [15]. Thus, diminished proliferation of human T cells to stimulation with L23-PD-L1 cells (Fig. 1) in all likelihood results from dampening of TcR signals by PD-1-induced negative costimulation. On the cellular level, decreased production of IL-2 in $CD4^+$ T cells and $CD8^+$ T cells [11,16] might be key elements for the capacity of PD-L1 to counteract the induction of human anti-pig T-cell responses.

Activation of naïve/resting T cells requires signaling via the TcR (signal 1) as well as signals generated by triggering of positive costimulatory receptors like CD28 (signal 2). In contrast, in activated $CD8^+$ effector T cells, TcR-signaling alone is sufficient to deliver 'death signals' to target cells. Destruction of targets can be mediated by the release of cytotoxic granules or may involve CD95(fas)/CD95L-pathways to induce apoptosis. IL-2-activated $CD8^+$ T cells exhibited reduced cytotoxicity on PD-L1 expressing targets (Fig. 3a). Thus, PD-1-mediated negative signaling may not only interfere with TcR signals inducing cytokine gene transcription during the induction of immune responses, but may also dampen TcR signals required for the administration of effector molecules to target cells during the effector phase of a response.

Interactions of the PD-1 receptor with PD-ligands do not only result in the generation of negative costimulatory signals but can also induce apoptosis in some experimental settings [17]. Indeed, we found more Annexin-positive $CD8^+$ T cells in co-cultures containing PD-L1 transfectants than in control cultures suggesting that a portion of activated $CD8^+$ T cells undergoes apoptosis in response to PD-1 triggering (Fig. 5). Thus, it is likely that two different mechanisms are responsible for reduced proliferation of $CD8^+$ T cells to stimulation with L23-PD-L1 cells (Fig. 1). First, as discussed above, PD-1-mediated signaling may downregulate TcR signals that are required for the synthesis of growth promoting cytokines (e.g. IL-2), and second, PD-L1-induced apoptosis could decrease the number of cells responding to L23 xenoantigen. It is worth mentioning that we did not observe PD-L1-induced apoptosis in $CD4^+$ T cells [11] suggesting that the mechanisms leading to reduced proliferation of $CD4^+$ and $CD8^+$ T cells to stimulation by L23-PD-L1 cells may not be identical. The pronounced susceptibility of activated $CD8^+$ T cells to PD-L1-induced apoptosis may also contribute to protection of L23-PD-L1 cells against lysis by $CD8^+$ effectors. Thus, PD-1 expressing effector cells could achieve a death signal during first contact with PD-L1 expressing targets and die before they can kill further target cells. In this scenario, cell destruction will not proceed but comes to a standstill at low level.

Cytotoxicity of human $CD56^+$ cells was also reduced by PD-L1 expression on target cells (Fig. 3b). However, we did not detect more Annexin-positive cells in $CD56^+$ cells cocultured with L23-PD-L1 cells compared with control cultures (Fig. 5b), indicating that PD-L1 does not induce apoptosis in $CD56^+$ cells. The mechanisms underlying the suppressive effect of PD-L1 on NK cell cytotoxicity remain to be determined. PD-1 might recruit SHP phosphatases to its immunoreceptor tyrosine-based switch motif in NK cells as described for T cells, as other inhibitory receptors on NK cells act in similar manners [18]. Thus, it is conceivable that PD-1-mediated negative signals interfere with signals from activating NK receptors (e.g. NKG2D) thereby diminishing the release of effector molecules. The observation that the PD-1 receptor is upregulated on NK cells in patients with chronic hepatitis C infection [19] supports the assumption of a functional role of this molecule on NK cells.

It is well established that blockade of positive costimulatory signals (e.g., inhibition of CD80/86-CD28 interactions) can prolong allograft survival in mice [20]. However, this concept is limited by the fact that $CD8^+$ T

cells seem to be somehow resistant to costimulatory blockade, which can result in $CD8^+$ T cell-mediated graft rejection [21,22]. As discussed above, our own observations and those of others [9,16] suggest that $CD8^+$ T cells are particularly sensitive to PD-1-mediated negative costimulatory signals. Thus, enhancing these signals could be a strategy to suppress the activity of $CD8^+$ T cells, which are resistant to costimulatory blockade.

Concerning therapeutic application of the PD-ligand to inhibit human anti-pig cellular immune responses after xenotransplantation, it might be of interest to express the PD-L1 molecule on endothelial cells. In our model, we could show that PD-L1, expressed in high densities, has the capacity to downregulate human T cell responses against porcine antigen presenting B cells with a high stimulatory capacity. Compared with the L23 B-cell line, porcine endothelial cells have a much lower capacity to activate human T cells. Thus, human PD-L1 overexpression on porcine endothelial cells will probably result in similar or even more pronounced inhibitory effects on human T cells activation. This assumption is supported by the finding that mouse $CD8^+$ T cells exhibit reduced cytolytic activity to endothelial cells expressing PD-L1 [23,24].

In summary, we show that cytotoxicity of human $CD8^+$ T cells and $CD56^+$ cells can be suppressed *in vitro* by the overexpression of human PD-L1 on pig cells. Together with our previous observation that the induction of $CD4^+$ T cell proliferation is inhibited by an enhancement of negative signaling, this approach may interfere with both, the induction and the effector phase of human anti-pig cellular immune responses. It should be noted, however, that complete inhibition of cellular cytotoxicity was not achieved. Thus, for an effective control of cytotoxic responses genetically engineered overexpression of human PD-L1 in pigs might be combined with transgenic expression of human HLA-E to further prevent NK activity [25] and/or human TNF-related apoptosis-inducing ligand [26], which is expected to downregulate T-cell reactivity.

Authorship

AP: designed and performed research, analyzed data, wrote paper; KB: performed research; LB: performed research; WB: performed research; JK: contributed to data discussion; RS: designed research, analyzed data, wrote the paper.

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