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ARTICLE

Allogeneic lymphocyte-licensed DCs expand T cells with improved antitumor activity and resistance to oxidative stress and immunosuppressive factors

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Adoptive T-cell therapy of cancer is a treatment strategy where T cells are isolated, activated, in some cases engineered, and expanded *ex vivo* before being reinfused to the patient. The most commonly used T-cell expansion methods are either anti-CD3/CD28 antibody beads or the "rapid expansion protocol" (REP), which utilizes OKT-3, interleukin (IL)-2, and irradiated allogeneic feeder cells. However, REP-expanded or bead-expanded T cells are sensitive to the harsh tumor microenvironment and often short-lived after reinfusion. Here, we demonstrate that when irradiated and preactivated allosensitized allogeneic lymphocytes (ASALs) are used as helper cells to license OKT3-armed allogeneic mature dendritic cells (DCs), together they expand target T cells of high quality. The ASAL/DC combination yields an enriched Th1-polarizing cytokine environment (interferon (IFN)-γ, IL-12, IL-2) and optimal costimulatory signals for T-cell stimulation. When genetically engineered antitumor T cells were expanded by this coculture system, they showed better survival and cytotoxic efficacy under oxidative stress and immunosuppressive environment, as well as superior proliferative response during tumor cell killing compared to the REP protocol. Our result suggests a robust *ex vivo* method to expand T cells with improved quality for adoptive cancer immunotherapy.

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INTRODUCTION

Adoptive T-cell therapy is a treatment strategy where tumor-infiltrating lymphocytes or genetically engineered T cells are isolated, activated, and expanded *ex vivo* before being reinfused into cancer patients.[1] Interleukin (IL)-2 and an agonistic stimulator of CD3, such as the OKT-3 antibody, are crucial factors in most T-cell expansion protocols. By immobilizing anti-CD3 and anti-CD28 antibodies on beads to simultaneously deliver signal-1 and costimulatory signal-2, T-cell proliferation can be increased without provoking anergy or early apoptosis.[2] However, while $CD4^+$ T cells respond strongly to anti-CD3/CD28 antibody beads, $CD8^+$ T cells proliferate less well. Given the importance of $CD8^+$ T cells in the antitumor response, this is a concern.[3] Another commonly used approach for T-cell expansion is the "rapid expansion protocol" (REP) where T cells are expanded with IL-2, OKT-3, and irradiated allogeneic peripheral blood mononuclear cells (PBMCs) as feeder cells, including accessory cells expressing Fc-γ I receptor (FcγRI).[3,4] The Fc-portion of immunoglobulin (Ig)G2a-subclass mouse antibodies, including the OKT-3 antibody,[5] attach to FcγRI on human feeder cells. An anti-CD3 antibody bund to FcγRI induces a more optimal proliferation/differentiation signal to $CD8^+$ T cell than anti-CD3/CD28 immobilized on a solid surface.[6] This reflects the dual benefit of anti-CD3-T-cell receptor (TCR) crosslinking and the costimulation provided by cell-cell interaction between T cells and $FcγRI^+$ accessory cells.[3] The REP approach has been used extensively for expansion of T-cell clones and lines for clinical adoptive transfer studies.[1,7,8]

Several factors need to be considered to obtain substantial tumor regression in the clinical setting. The reinfused T cells must proliferate and sustain upon tumor cell-recognition/killing within an immunosuppressive tumor microenvironment. However, human $CD8^+$ cytolytic T lymphocytes (CTLs) obtained *ex vivo* using current protocols are often suboptimal in triggering substantial tumor regression *in vivo* in otherwise unmanipulated cancer patients.[9] Considerable evidence suggests that one of the mechanisms limiting their efficacy is the failure of these CTLs to persist *in vivo*.[10]

Mature dendritic cells (mDCs) provide strong costimulatory signals to T cells,[11] and deliver a third signal which is a polarization signal toward Th1, Th2, Th9, or Th17 immunity. The polarization is strongly associated with differential abilities to induce CTL-mediated tumor cell killing where Th1 polarization is desired.[12] It is generally accepted that DCs have to be helped by $CD4^+$ T cells, a process known as licensing or conditioning, in order to induce long-lived memory $CD8^+$ T cells.[13,14] The interaction between CD40L (CD154) on $CD4^+$ T cells and CD40 on DCs is instrumental for this licensing process.[15] $CD4^+$ T-cell help enhances the expression of costimulatory molecules on DCs and induces secretion of bioactive IL-12,[16] a signal-3-cytokine, which is central for induction of Th1 immunity. Moreover, $CD4^+$ helper T cells may secrete factors like IL-2

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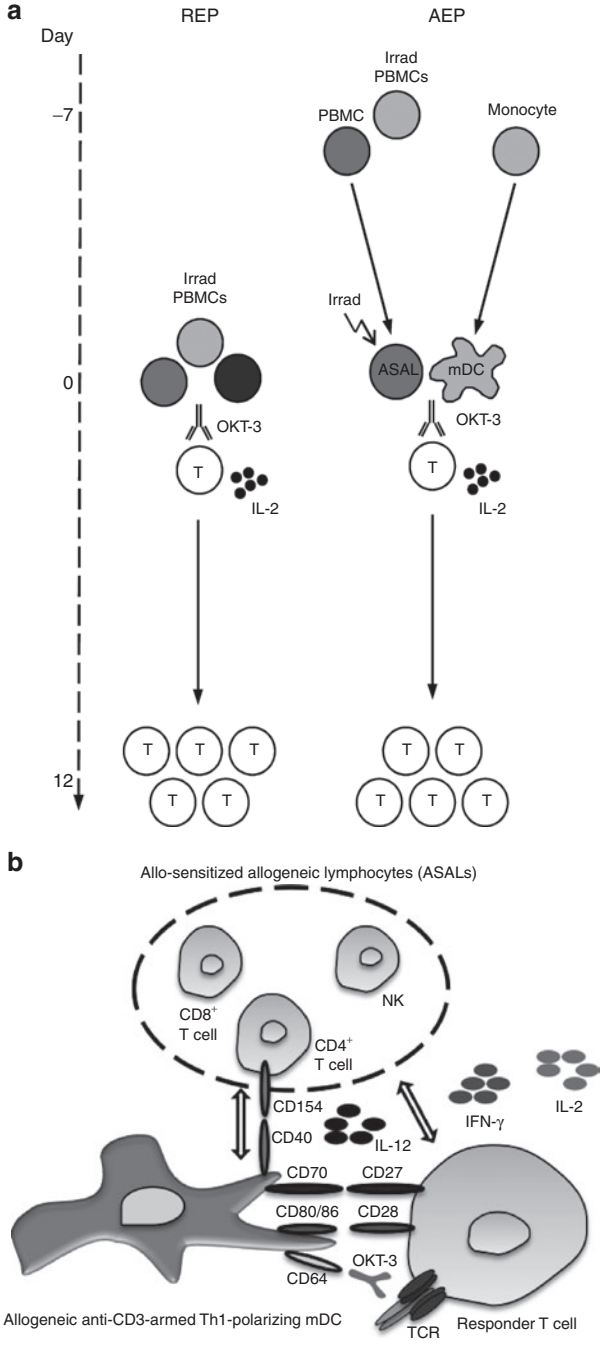

Figure 1 Schematic illustration of the rapid expansion protocol (REP) and allosensitized allogeneic lymphocytes (ASAL) expansion protocol (AEP) T-cell expansion protocols and proposed mechanisms of action for the AEP protocol. (**a**) REP: T cells were expanded for 12 days by stimulation with irradiated feeder cells (peripheral blood mononuclear cells (PBMCs) from three donors), OKT-3 and IL-2. ASAL expansion protocol (AEP): Prior to expansion: Monocytes from one donor were differentiated into immature dendritic cells (DCs) with IL-4 and granulocyte-macrophage colony-stimulating factor for 6 days and then matured for 24 hours with IFN-γ, poly(I:C) and R848. ASALs were generated over 7 days by coculturing PBMCs from one donor with irradiated PBMCs from a second donor (autologous to the DCs) at a ratio of 1:1. The ASALs and mDCs can be frozen and stored or used immediately. Expansion phase: T cells, allogeneic to both the ASAL and mDCs, were expanded for 12 days by coculture with irradiated ASAL, mDCs, OKT-3, and IL-2. When comparing the two protocols, the same amount of T cells from the same donors were expanded and analyzed in parallel. (**b**) A proposed illustration why the AEP protocol leads to activation of responder T cells, which are insensitive to immunosuppressive and oxidative stress. ASALs provide "help signals", which lead to upregulation of CD70, CD80, CD64, and CD40 on allogeneic mDCs. The interaction between ASALSs and mDC also leads to secretion of IL-2 and IFN-γ. Furthermore, CD40L expression on helper cells in the ASAL population can interact with CD40 on mDCs and thereby induce IL-12 secretion and Th1 polarization. CD64 expression on the mDCS ensures proper Fc binding of the OKT-3 antibody. The combination of allogeneic anti-CD3-armed Th1-polarizing mDCs and ASALs leads to T cells, which are insensitive to immunosuppressive and oxidative stress.

and interferon (IFN)-γ that directly affect CD8+ T-cell survival, cell cycle progression, and differentiation to either effector or memory cells.[17,18] Recent data further indicate that activated CD8+ T cells and NK cells, usually regarded as classical effector cells, also may play a "helper" role in DC-mediated activation of CD8+ T cells.[19,20]

A potential explanation for the suboptimal efficiency *in vivo* of T cells expanded with the current protocols could be that anti-CD3/CD28 beads and allogeneic PBMCs are unable to fully replace lymphocyte-licensed DCs for optimal activation of CTLs. In this study, we therefore established a novel T-cell expansion protocol based on (i) allogeneic anti-CD3-armed mDCs providing signal-1, signal-2 and a Th1-polarizing signal-3 to the T cell and (ii) irradiated allosensitized allogeneic lymphocytes (ASALs), comprising a heterogeneous population of preactivated CD4+ T cells, CD8+ T cells, and NK cells potentially acting as helper cells in DC-licensing and direct lymphokine-dependent communication with cocultured cytolytic T cells. We defined this protocol as the "ASAL expansion protocol" (AEP). Notably, the AEP protocol was found to promote an efficient expansion of genetically engineered T cells with improved resistance to oxidative stress and immunosuppressive cytokines, as compared to T cells expanded by the commonly used REP protocol.

RESULTS

The AEP protocol efficiently expands CD8+ T cells with higher frequency of costimulatory receptor expression, lower frequency of exhaustion markers, and better survival *in vitro* than the REP protocol

The REP and AEP protocols are illustrated in Figure 1a. For the REP protocol, irradiated allogeneic PBMCs from three different donors are used as feeder cells. For the AEP protocol, the ASALs, mDCs, and T cells for expansion are allogeneic with respect to each other. Irradiated PBMCs are used to stimulate allogeneic PBMCs for 7 days to become ASALs. These irradiated PBMCs are from the same donor as the mDCs, meaning that the ASALs will reexperience the allogeneic major histocompatibility complex class I and class II molecules on mDCs when they are mixed for T-cell expansion. ASALs and mDCs can be prepared in advance over 7 days and used either directly or kept frozen until T-cell expansion is initiated. The ASALs are irradiated before they are added to the DCs and T cells.

T-cell expansion efficiency was compared side by side for anti-CD3/CD28 beads, the REP protocol and the AEP protocol starting with the same amount of peripheral blood lymphocytes (1×10^5) from the same donor. A ratio of 1 T cell per 400 irradiated PBMCs was used for the REP protocol. The AEP protocol yielded as efficient T-cell (CD3+) expansion over 12 days as the bead and REP protocols when a ratio of 1:1:4 (T-cell:mDC:ASAL) was used (Figure 2a). Therefore, this ratio was kept in all subsequent AEP experiments. Next, omitting each of the three major components from the AEP protocol (OKT-3, ASALs, mDCs) revealed that all three components are needed for optimal T-cell expansion (Figure 2b). Notably, T cells (CD3+) expanded from the AEP protocol had a significantly higher ($P = 0.03$) frequency of CD8+ T cells ($83 \pm 3\%$) compared to the REP

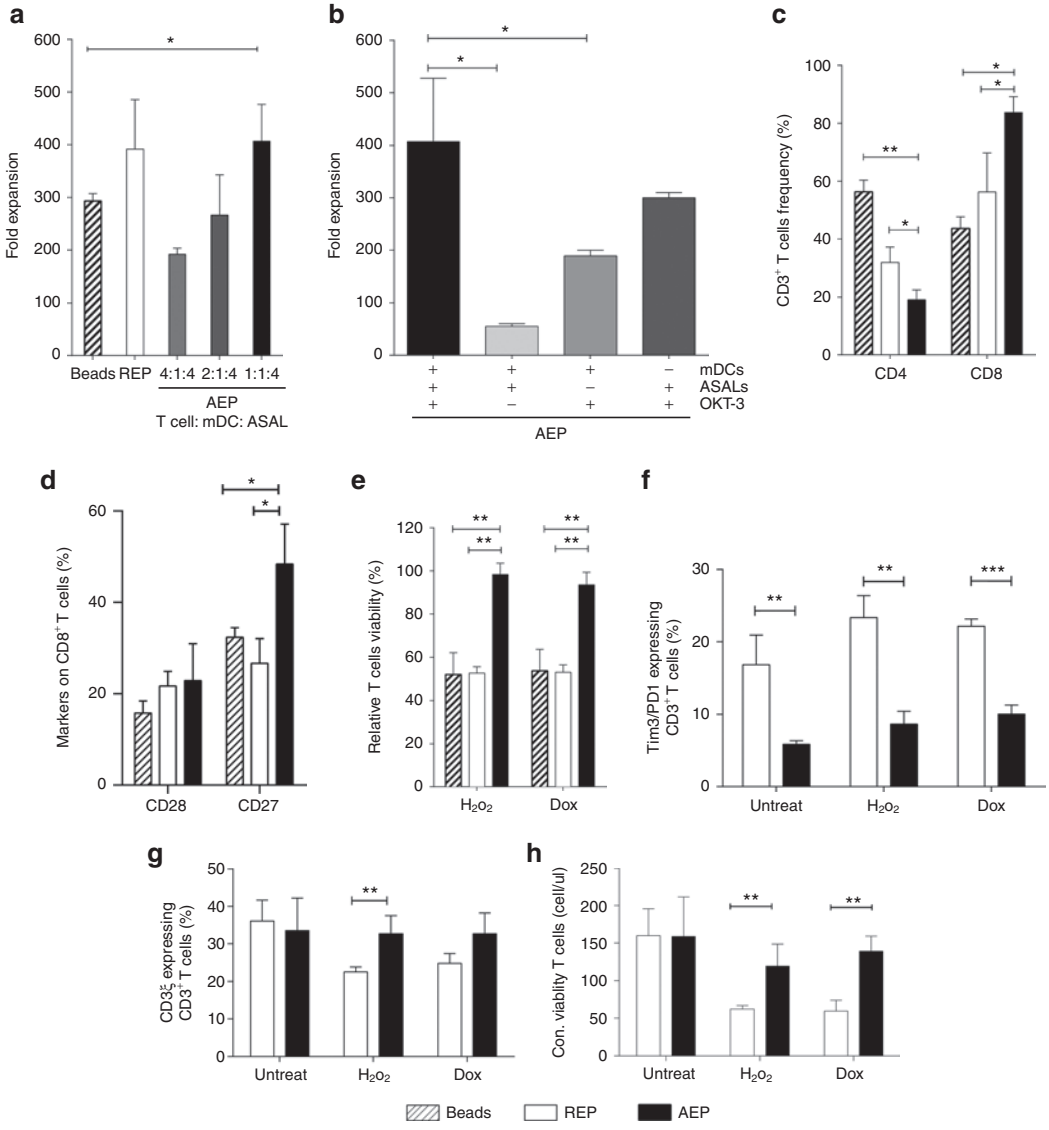

Figure 2 Comparison of T cells expanded by the rapid expansion protocol (REP) and allosensitized allogeneic lymphocytes (ASAL) expansion protocol (AEP) in terms of expansion capacity, phenotype, and viability after exposure to oxidative and apoptotic stress. (**a**) Peripheral blood lymphocytes (PBLs) (1×10^5) were expanded for 12 days by either the anti-CD3/CD28 bead protocol, the REP protocol, or the AEP protocol and then counted. Fold expansion of T cells is presented. For the REP protocol, irradiated PBMCs (mix from three donors) were used. For the AEP protocol, various T-cell:mDC:ASAL ratios were investigated. Since the 1:1:4 ratio expanded T cells as efficiently as the REP protocol, this ratio was kept for all subsequent experiments. (**b**) Fold expansion of T cells using the AEP protocol with various components omitted. (**c**) Frequency of $CD4^+$ T cells and $CD8^+$ T cells among $CD3^+$ T cells after expansion. (**d**) Phenotypic marker expression on $CD8^+$ T cells after expansion. (**e–h**) Expanded T cells were exposed to either 25 µmol/l H_2O_2 or 0.1 µmol/l doxorubicin for 24 hours. Various flow cytometry analyses were then used to evaluate the expanded T cells. Values were normalized against expanded and otherwise untreated T cells from the same expansion protocol. (**e**) Annexin-V and PI staining was used to assess T-cell viability after stress exposure. (**f**) Expression of the exhaustion/dysfunction markers Tim3 and PD1 after stress exposure. (**g**) Expression of CD3 zeta-chain after stress exposure. (**h**) Absolute T-cell numbers after stress exposure. (**a–h**) All experiments were performed at least three times with three new and different donors each time. Error bars represent SD, and statistical significance is depicted by symbols (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

protocol (56 ± 8%) and beads (43 ± 7%) (Figure 2c). As reported previously,[3] our data verified that anti-CD3/CD28 beads primarily expand the $CD4^+$ T-cell population (Figure 2c).

Rosenberg and colleagues reported that highly persistent T-cell clonotypes *in vivo* express relatively higher levels of the costimulatory receptors CD28 and CD27.[21] It has also been reported that concurrent upregulation of Tim3 and PD1 on tumor antigen-specific $CD8^+$ T cells corresponds to T-cell dysfunction (exhaustion) in melanoma patients.[22] Therefore, we examined the phenotype and quality of AEP-expanded T cells in comparison with bead-expanded and REP-expended T cells. The AEP protocol generated a significantly higher ($P = 0.02$) frequency of CD27-expressing $CD8^+$ T cells (48 ± 5%) compared to the REP protocol (27 ± 2%) and the bead protocol (32 ± 2%), while the frequency of CD28 expression remained at the same level for the three protocols (Figure 2d). Other phenotypic markers were also examined including CD25, CCR7, CTLA-4, and CD45RO/CD62L. For none of these markers did we observe any difference in expression between the various protocols (data not shown). We noted that the expanded T cells preferentially had an effector phenotype, which is not surprising given the extensive *in vitro* activation.

In order to mimic the oxidative stress and proapoptotic factors that infused T cells will meet in the tumor environment, the expanded T cells were exposed to H_2O_2 or doxorubicin for 24 hours. The AEP protocol yielded T cells ($CD3^+$) with significantly improved

survival (Figure 2e), significantly lower expression of Tim3 and PD1 (Figure 2f), which together is regarded as a sign of exhaustion, and a tendency toward higher CD3 zeta chain expression (Figure 2g) compared to the REP protocol. The absolute numbers of viable T cells after exposure to H_2O_2 and doxorubicin were also significantly higher for AEP-expanded T cells (Figure 2h). Control experiments verified that a T-cell:mDC:ASAL ratio of 1:1:4 is optimal to expand T cells with high resistance to oxidative and apoptotic stress and that all components of the AEP protocol are needed (Supplementary Figure S1a–c).

Culturing DCs with ASALs leads to enhanced expression of costimulatory molecules on DCs and high secretion of IFN-γ, IL-2, and IL-12

Licensing of DCs by helper cells is supposed to enhance the expression of costimulatory molecules on DCs and to induce secretion of bioactive IL-12.[16] Moreover, helper cells may secrete factors like IL-2 and IFN-γ that directly affect $CD8^+$ T-cell survival, cell cycle progression, and differentiation to either effector or memory T cells.[17,18] The frequency of mature DCs expressing CD80 and CD70 significantly increased ($P = 0.04$ and $P = 0.006$, respectively) after coculture with ASALs, while the already high frequency of CD86 on mature DCs did not further increase (Figure 3a). There was also a tendency that the frequency of mature DCs expressing FcγRI (CD64) and CD40 were increased (Figure 3a). Representative flow cytometry histograms for CD70 and CD40 expression on DCs are shown as Supplementary Figure S2a,b. Coculture of mDCs and ASALs for 24 hours induced a substantial release of IFN-γ, IL-2, and IL-12p70 (Figure 3b). Depletion experiments of $CD4^+$, $CD8^+$, and $CD56^+$ cells from the ASAL population showed that both T cells and NK cells are needed as helper cells in this process. Depletion of either $CD4^+$ or $CD56^+$ cells led to a significant drop in CD70 expression on the mature DCs (Supplementary Figure S2c). For T-cell expansion, it was observed that depletion of $CD4^+$, $CD8^+$, or $CD56^+$ cells from the ASAL population translated to a reduction in T-cell expansion (Supplementary Figure S2d), a shift in phenotype with relatively less expanded $CD8^+$ T cells (Supplementary Figure S2e) and reduced T-cell viability after exposure to oxidative stress in the form of H_2O_2 (Supplementary Figure S2f).

Various signaling pathways are involved in the beneficial effects obtained by the AEP protocol

Stimulation of the costimulatory receptor CD27 by its ligand CD70 has proven important for the generation of primary and memory $CD8^+$ T-cell responses in various models of antigenic challenge (see Supplementary Materials and Methods).[23] The interaction between CD40L on $CD4^+$ T cells and CD40 on DCs plays a pivotal role in the DC licensing process and induction of IL-12 production and thereby in priming and clonal expansion of antigen-specific CTLs.[24] We therefore performed blocking experiments with an antagonistic anti-CD70 antibody, an antagonistic anti-CD40 antibody and a neutralizing IL-12p70 antibody. As shown in Figure 4, the addition of anti-CD40 or neutralizing IL-12p70 antibody efficiently reduced the expression of CD70 on mature DCs (Figure 4a). The neutralizing IL12p70 antibody also reduced the expression of CD40 on mature DCs (Figure 4b). Both the anti-CD70 and anti-CD40 antibody efficiently blocked secretion of IL-12 (Figure 4c). Furthermore, the anti-CD70 antibody significantly reduced IFN-γ and IL-2 secretion (Supplementary Figure S3).

We next looked at the phenotype of expanded T cells when CD70/CD70 or CD40/CD40L signaling or IL-12 secretion was altered in the AEP protocol. The frequency of CD27 expression on $CD8^+$ T cells dropped from 40±2% to 23±2%, 30±2%, and 20±3%, respectively, when CD70, CD40, or IL-12 was blocked (Figure 4d), indicating that all three pathways are important for high CD27 expression on the expanded T cells. We also detected CD40L expression on the expanded $CD8^+$ T cells and the frequency of CD40L expression decreased by the addition of the anti-CD40 antibody (Figure 4e).

Next, we wanted to examine whether blockage of those pathways during T-cell expansion had an effect on the T-cell quality. This was analyzed by exposing the expanded T cells to either oxidative or apoptotic stress. The viability of T cells expanded in the presence of the antagonistic anti-CD40 antibody or the neutralizing IL-12p70 antibody decreased significantly in response to oxidative stress (H_2O_2) and apoptotic stress (doxorubicin), indicating a significant contribution of T-cell quality to these signals (Figure 4f). There was also a trend that the viability of T cells expanded in the presence of the antagonistic anti-CD70 antibody decreased by ~20% after exposure to H_2O_2 and ~15% after exposure to doxorubicin compared to T cells expanded in the absence of anti-CD70 antibody (Figure 4f).

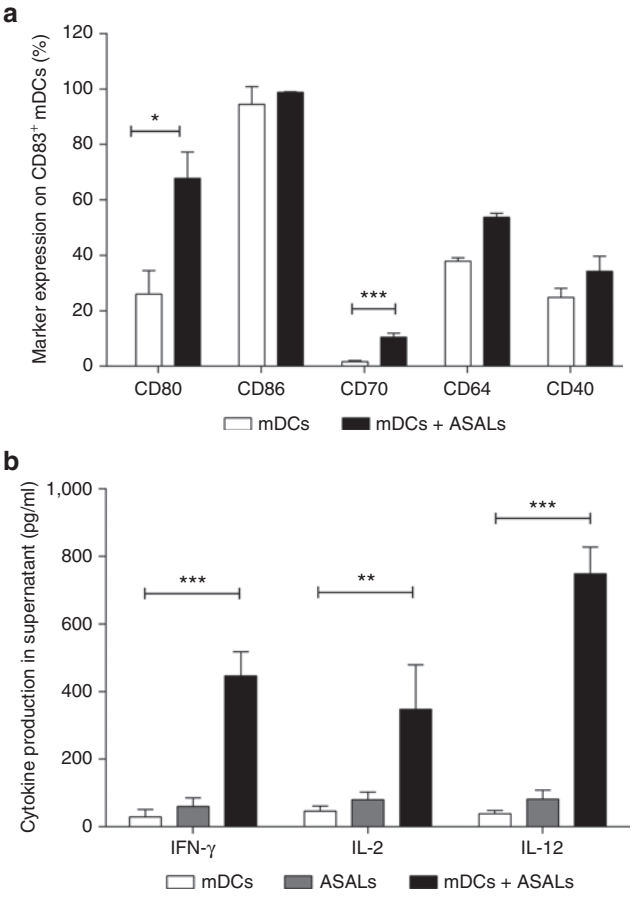

Figure 3 Stimulatory molecule expression and cytokine release are increased on mature dendritic cells (DCs) after coculture with allogeneic allosensitized allogeneic lymphocytes (ASALs). (**a**) Mature DCs ($CD83^+$) either cultured alone or cultured with ASALs for 48 hours were analyzed for CD80, CD86, CD70, and CD64 expression by flow cytometry. (**b**) Enzyme-linked immunosorbent assay (ELISA) was used to analyze IFN-γ, IL-2, and IL-12p70 secretion from mDCs cultured alone, ASALs cultured alone, and a coculture of mDCs and ASALs after 48 hours. (**a, b**) The experiments were performed at least three times with three new and different donors each time. Error bars represent SD, and statistical significance was depicted by symbols where there was a significant difference (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

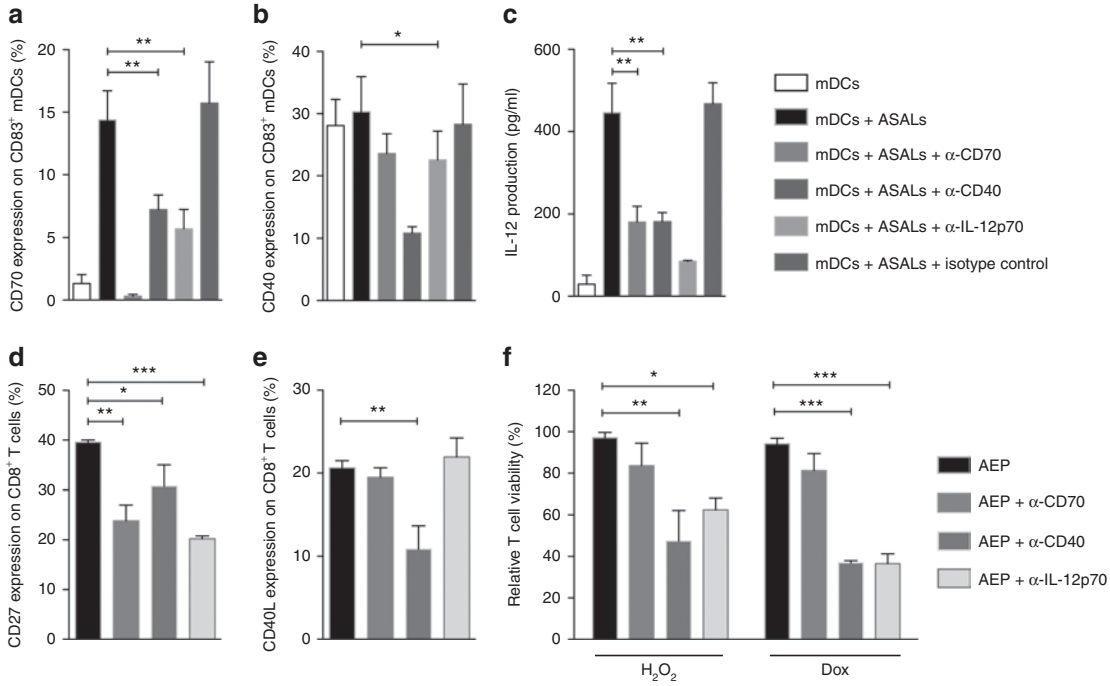

Figure 4 Blockage of CD70/CD27 ligation, CD40L/CD40 ligation, and IL-12 secretion each partly inhibits the function of allosensitized allogeneic lymphocytes (ASAL) expansion protocol (AEP)–expanded T cells. An antagonistic anti-CD70 antibody, an antagonistic anti-CD40 antibody and a neutralizing IL-12p70 antibody were used to block different signaling pathways in the AEP protocol. mDCs were cultured alone or together with ASALs or together with ASALs and blocking antibodies or an isotype-matched control antibody. (**a–c**) CD70 expression and CD40 expression on mDCs, as assessed by flow cytometry, and IL-12p70 secretion, as assessed by ELISA, with or without addition of blocking antibodies. (**d–f**) T cells were expanded by the AEP protocol with the addition of blocking antibodies or an isotype control antibody. (**d, e)** CD27 expression and CD40L expression, as assessed by flow cytometry, on $CD8^+$ T cells expanded by the AEP protocol with or without addition of blocking antibodies during the T-cell expansion phase. (**f**) Viability of T cells expanded by the AEP protocols, with or without addition of blocking antibodies, when exposed to either 25 µmol/l H_2O_2 or 0.1 µmol/l doxorubicin for 24 hours. Viability was then analyzed by flow cytometry using Annexin-V/PI staining and relative viability was normalized against nontreated T cells. (**a–f**) The experiments were performed at least three times with three new and different donors each time. Error bars represent SD, and statistical significance is depicted by symbols where there was a significant difference (*$P < 0.05$, **$P < 0.01$).

AEP-expanded genetically engineered T cells are resistant to oxidative and apoptotic stress

Given the recent impressive responses in cancer patients for adoptive T-cell therapy using genetically engineered T cells,[25,26] it was of interest to investigate the performance of such T cells using the AEP protocol. We therefore engineered T cells to express a chimeric antigen receptor (CAR) against the disialoganglioside GD2, which is overexpressed on neuroblastoma cells and a relevant target for T-cell therapy of neuroblastoma.[27] $CD8^+$ T cells isolated from peripheral blood were transduced with a lentiviral vector encoding the GD2-CAR (Figure 5a), in order to generate GD2-specific T cells. The engineered T cells were then used for comparison of the AEP and REP protocols. Fold expansion was similar between the two protocols (Figure 5b). Specific cytotoxicity against GD2-expressing neuroblastoma target cells (IMR-32) was also similar, but with a tendency that AEP-expanded T cells performed somewhat better (Figure 5c). Importantly, the ability to resist oxidative (H_2O_2) and apoptotic (doxorubicin) stress was significantly better for AEP-expanded T cells (Figure 5d).

Genetically engineered T cells expanded by the AEP protocol possess high cytotoxic capacity and proliferation in an environment with oxidative stress and immunosuppressive cytokines

The immunosuppressive microenvironment in tumors is partly due to regulatory T cells, myeloid suppressor cells, and stromal cells and their release of suppressive cytokines and reactive oxygen spices, which can hamper the effect of transferred T cells.[28] Therefore, the AEP-expanded GD2-CAR T cells were evaluated for resistance by exposing them to H_2O_2 and subsequently adding the immunosuppressive cytokines IL-10 and tumor growth factor (TGF)-β and mixing the T cells with IMR-32 target cells at a 1:1 ratio. Expression of the degranulation marker CD107a was examined by flow cytometry and normalized to untreated T cells for both protocols. The relative frequency of CD107a-expressing GD2-CAR T cells after exposure to H_2O_2 was found to be significantly higher ($P = 0.0006$) in the AEP protocol (91 ± 4%) than in the REP protocol (45 ± 1%; Figure 5e). There was a tendency ($P = 0.053$) for this also after exposure to H_2O_2 and immunosuppressive cytokines (Figure 5e). Furthermore, the T-cell production of IFN-γ after exposure to H_2O_2 or H_2O_2 and immunosuppressive cytokines was significantly higher ($P = 0.008$) in the AEP protocol (88 ± 1%) than in the REP protocol (49 ± 3%; Figure 5f). The proliferation of AEP-expanded GD2-CAR T cells was superior to proliferation of REP-expanded GD2-CAR T cells after restimulation with specific target cells (IMR-32; Figure 5g, top panel). By adding suppressive cytokines and oxidative stress, the proliferation ability was reduced somewhat especially if the expanded T cells were first pretreated with H_2O_2 for 1 day and the cultured with IL-10 and TGF-β for 4 days (Figure 5g, bottom panel).

The efficacy of expanded GD2-CAR T cells to control tumor growth *in vivo* was examined using SK-N-FI neuroblastoma cells stably transduced to express GD2. Tumor cells were injected subcutaneously in nude mice and the mice were treated three times by intravenous administration of GD2-CAR T cells. Mice treated with GD2-CAR T cells exhibited suppression of tumor growth

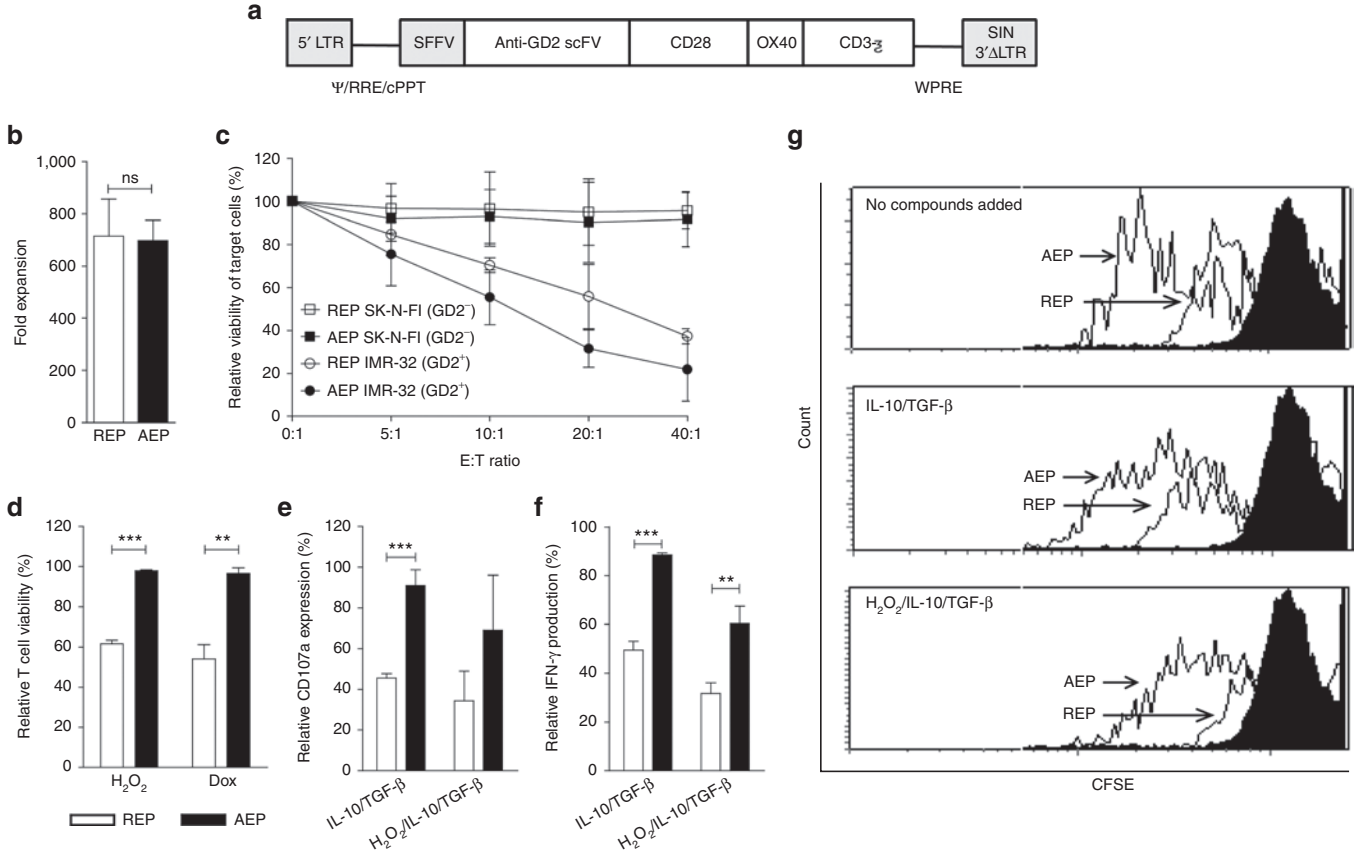

Figure 5 GD2-CAR-engineered T cells, expanded by the allosensitized allogeneic lymphocytes (ASAL) expansion protocol (AEP), are more resistant to immunosuppressive cytokines and oxidative and apoptotic stress than GD2-CAR T cells expanded by the rapid expansion protocol (REP). (**a**) An illustration of the GD2-CAR-encoding lentiviral vector used to transduce T cells. (**b**) $CD8^+$ T cells were transduced, isolated by magnetic beads, and expanded by the REP or AEP protocols. The starting number of GD2-CAR T cells was 1×10^5 for both protocols and a ratio of 1:1:4 (T-cell:mDC:ASAL) was used in the AEP protocol. Fold expansion of GD2-CAR T cells is presented. (**c**) The cytotoxic ability of GD2-CAR T cells was analyzed by coculture with luciferase-expressing $GD2^+$ (IMR-32) or $GD2^-$ (SK-N-FI) neuroblastoma target cells at different E:T ratios. Viability of target cells were measured by luminescence and related to signals from target cells alone. (**d**) GD2-CAR T cells expanded by the REP and AEP protocols were exposed to either 25 µmol/l H_2O_2 or 0.1 µmol/l doxorubicin for 24 hours. Viability was analyzed by flow cytometry using Annexin-V and PI staining and relative viability was normalized against nontreated GD2-CAR T cells. (**e–f**) The REP- and AEP-expanded GD2-CAR T cells were treated with IL-10/TGF-β for 4 hours or with H_2O_2 for 24 hours, followed by 4 hours treatment with IL-10/TGF-β and then cocultured with $GD2^+$ target cells (IMR-32) in the presence of IL-10/TGF-β for 24 hours. (**e**) Flow cytometry was used to analyze CD107a expression on GD2-CAR T cells ($CD3^+$) after exposure to IL-10/TGF-β or H_2O_2/IL-10/TGF-β and normalized against nontreated GD2-CAR T cells. (**f**) ELISA was used to analyze IFN-γ production from GD2-CAR T cells after exposure to IL-10/TGF-β or H_2O_2/IL-10/TGF-β and normalized against nontreated GD2-CAR T cells. (**g**) GD2-CAR T cells were treated with H_2O_2 for 24 hours, followed by 4 hours labeling with CFSE in the presence of IL-10/TGF-β and then cocultured with $GD2^+$ target cells (IMR-32) in the presence of IL-10/TGF-β for 4 days before proliferation analysis by flow cytometry. (**a–g**) The experiments were performed three times with three new and different donors each time. Error bars represent SD, and statistical significance is depicted by symbols (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

(Supplementary Figure S4a) and prolonged survival (Supplementary Figure S4b) compared to mice treated with phosphate-buffered saline. There was a tendency that AEP-expanded T cells delayed tumor growth more sufficiently than REP-expanded T cells (Supplementary Figure S4a) and there was a small but statistically significant difference in survival of mice treated with AEP-expanded T cells compared to REP-expanded T cells (Supplementary Figure S4b).

We also engineered T cells with a TCR directed against an HLA-A2-restricted epitope of the TARP antigen,[29] which is uniquely expressed by normal prostate epithelial cells and by prostate and breast adenocarcinomas and therefore a relevant target for T-cell therapy of prostate cancer and breast cancer. The same set of experiments were performed for TARP-TCR engineered T cells with very similar results, again showing significantly better performance for AEP-expanded genetically engineered T cells than REP-expanded genetically engineered T cells (Supplementary Figure S5a–f).

DISCUSSION

At present, very little is known about how T cells generated for adoptive T-cell transfer respond to antigenic restimulation *in vivo*. However, in a recent study,[30] using the classical REP protocol, expanded $CD8^+$ MART1-specific tumor-infiltrating lymphocytes were found to be hyporesponsive to restimulation *in vitro*. Such hyporesponsiveness obviously contrasts to the efficient responsiveness seen in $CD8^+$ memory T cells, which are generated after viral and certain bacterial infections. Our working hypothesis is that the suboptimal survival and efficiency of T cells expanded *ex vivo* with CD3/CD28 beads or by the REP protocol is due to the inability to fully replace properly activated mature DCs and lack of help from $CD4^+$ T cells, and potentially also help from other "helper" lymphocytes such as activated $CD8^+$ T cells and NK cells.[13,19,31] Our novel AEP T-cell expansion protocol is based on (i) allogeneic anti-CD3-armed Th1-polarizing mDCs and (ii) ASALs, comprising a heterogeneous population of lymphocytes, including $CD4^+$ T cells, $CD8^+$ T cells, and

NK cells that have been preactivated against irradiated allogeneic PBMCs that are autologous to the DCs.

A proposed image of function of the AEP protocol is presented in Figure 1b, including surface molecules and cytokines addressed in the paper. ASAL helper cells can stimulate allogeneic mDCs through CD40L-CD40 ligation, which triggers the release of IL-12 from the DCs, a polarization signal toward a Th1-type immune response and induction of CTLs. We found CD70 expression both on the mDC (Figure 3a) and the $CD4^+$ ASAL cells (data not shown). This is also a sign of Th1 response as it has been reported that CD70 is selectively expressed on Th1 but not on Th2 cells and is required for Th1-type immune responses.[32] It has also been reported that CD70 expression on DCs plays a key role in CD40-dependent $CD8^+$ T-cell responses.[33,34] The results from the experiment with depletion of $CD4^+$ T cells from the ASAL population strengthen the notion that they are needed for efficient generation of $CD8^+$ T cells with high viability. It should be noted that depletion of NK cells and to some extent also $CD8^+$ T cells from the ASAL population led to similar results, pointing at important helper roles also for these cells. T cells expanded by the AEP protocol induced a superior expansion of $CD8^+$ T cells with a higher frequency of CD27 expression (the receptor for CD70), as compared to T cells expanded by the REP protocol. The increased frequency of CD27-expressing $CD8^+$ T cells is most likely of relevance since long-term persistence of infused T cells has been found to correlate with their expression of CD27.[35] We also investigated the expression of other T-cell markers such as CD45RO/CD62L, CCR7, CTLA-4, and CD25 but did not detect any significant differences between T cells expanded by the REP or AEP protocols. After 12 days of expansion *in vitro* most T cells exhibited an effector-like phenotype. It has been shown that T cells loose their initial memory phenotype when expanded *in vitro* but can regain their phenotype when the T cells are transferred *in vivo*.[36] It is therefore possible that the memory phenotype for AEP- and REP-expanded T cells would differ after *in vivo* exposure.

In order to get some insight in potential mechanisms behind the advantages of the AEP protocol, we further investigated the contribution of the CD70/CD27 and CD40L/CD40 signaling pathways and the IL-12 cytokine. We found that blocking of CD70 or CD40 or neutralization of IL-12 during AEP-expansion reduced the frequency of CD27-expressing $CD8^+$ T cells and reduced T-cell viability after exposure to oxidative stress and apoptotic factors. CD70/CD27 ligation has costimulatory properties, which is crucial in the generation of antigen-specific $CD8^+$ T cells[37] and it is involved in naive T-cell expansion into a Th1-type immune response.[32] Addition of anti-CD70 antibody to the ASAL/mDC cocultures significantly reduced the release of the lymphocyte-derived cytokines IL-2 and IFN-γ (Supplementary Figure S3). Addition of an antagonistic anti-CD70 antibody also decreased the production of IL-12p70, which probably leads to further reduced levels of IFN-γ.[38]

Adoptive T-cell therapy using T cells genetically engineered to express a CAR against CD19 has recently shown great success in patients with advanced B-cell chronic lymphocytic leukemia and B-cell acute lymphocytic leukemia, with CAR T cells proliferating extremely well upon adoptive transfer.[25,26] In the case of B-cell leukemia, the malignant cells are derived from antigen-presenting cells, which may provide costimulation to CAR T cells. This will not be the case for CAR T cells targeting non–antigen-presenting cells tumors, including all solid tumors, where the *ex vivo* T-cell stimulation and expansion protocol may be the tipping point between success and failure. Therefore, it would be of great interests to investigate the performance of genetically engineered T cells targeting solid tumor using the AEP-protocol. The cytotoxic ability of AEP-expanded engineered T cells was at least as good as REP-expanded T cells in a nonsuppressive *in vitro* environment. When the cytotoxic capacity against relevant tumor cells was analyzed after a period of oxidative stress and presence of the immunosuppressive cytokines IL-10 and TGF-β, the AEP-expanded T cells showed significantly higher signs of cytotoxic degranulation (CD107a expression) and produced significantly more IFN-γ, as compared to REP-expanded T cells.

The increased resistance to oxidative stress and apoptotic factors is of importance since high oxidative stress levels characterize the tumor microenvironment and this can seriously suppress antigen-specific T-cell responses.[39,40] Malmberg *et al*.[40] reported on inhibition of T-cell activity within 4 hours after H_2O_2 administration and Otsuji *et al*.[39] reported that oxidative stress caused by macrophages induces abnormality of the TCR complex with reduced CD3ζ signaling. Our data show that AEP-expanded T cells partly overcome damage from oxidative stress that indicates that the AEP protocol could be useful for *ex vivo* expansion of T cells for adoptive T-cell therapy.

The immunosuppressive tumor microenvironment is a challenge for adoptive T-cell transfer therapy protocols. IL-10 is normally produced in the tumor microenvironment by DC, macrophages, or natural Tregs and it inhibits the activity of Th1 cells, NK cells, and effector macrophages, all of which are required for optimal pathogen or tumor clearance. TGF-β, which is produced by tumor fibroblasts and other cells in the microenvironment, also interferes with the generation and proliferation of tumor-specific CTLs, induces promotion of angiogenesis and tumor stroma formation and leads to the production of other immunosuppressive cytokines.[41] It is therefore encouraging that improved proliferative response was observed for our AEP-expanded engineered T cells after interaction with cognate antigens on tumor cells, even in models including oxidative stress (H_2O_2) and immunosuppression by cytokines (IL-10 and TGF-β). Our data from the mouse model of solid tumor indicate that AEP-expanded T cells perform more optimal *in vivo* than REP-expanded T cells, when it comes to delay of tumor growth and prolongation of survival of mice. The rather modest improvement observed *in vivo* may be attributed to a suboptimal tumor model when using human T-cell transfer to nude mice.

The new AEP protocol is more time consuming than the established REP protocol. However, irradiated ASALs and mDCs can be prepared in advance over 7 days and kept frozen until T-cell expansion is initiated. Large amount of clinically approved helper cells for T-cell expansion can therefore be prepared in advance. We have verified that T cells are as efficiently expanded using frozen ASALs and mDCs as using fresh cells. The costs for expansion of clinical cells using the AEP protocol have not been addressed in this paper. It is of course an important factor to keep in mind. The time consumption and costs associated with the AEP protocol must be weighted against the possible benefits of transferring T cells that appear to be insensitive to oxidative stress and immunosuppression. Taken together, our data indicate that the AEP protocol could be used to generate genetically engineered CAR or TCR T cells from peripheral blood and it is likely that they will perform better in cancer patients than T cells expanded with current protocols.

MATERIALS AND METHODS

Isolation of PBMCs and monocytes and generation of ASALs and mDCs

Buffy coats were obtained freshly from the blood bank at the Uppsala University Hospital, Uppsala, Sweden. PBMCs from different donors were isolated by Ficoll-Paque Premium separation (GE Healthcare Life Science, Uppsala, Sweden). Culture medium was prepared as follows: RPMI-1640 supplemented with 10% heat-inactivated human AB serum (our own production), 2 mmol/l L-glutamine, 10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 IU penicillin/ml and 100 µg streptomycin/ml, and 20 µmol/l

β-mercaptoethanol. All components and culture media were from Invitrogen (Carlsbad, CA). All cells were cultured in a humidified incubator with a 5% CO_2 atmosphere at 37 °C.

Generation of ASALs. ASALs were generated over 7 days by coculturing PBMCs from one donor with irradiated (50 Gy) PBMCs from a second donor at a ratio of 1:1 in T-25 flasks (Corning, New York, NY) leaning at ~30 degrees angel in the incubator.

Generation of DCs. PBMCs were incubated in T-75 flasks (Corning) for 2 hours and nonadherent cells (mostly peripheral blood lymphocytes) were saved. Adherent cells (mostly monocytes) were washed three times with phosphate-buffered saline, differentiated over 6 days into immature DCs with granulocyte-macrophage colony-stimulating factor (Gentaur, Brussels, Belgium, 50 ng/ml) and IL-4 (Gentaur, 25 ng/ml). Medium was changed every 2 days. Immature DCs were then maturated for 24 hours in culture medium with IFN-γ (Shenandoah Biotechnology, Warwick, PA, 1,000 IU/ml), polyinosinic:polycytidylic acid (poly(I:C); Sigma-Aldrich, St Louis, MO, 20 μg/ml) and R848 (InvivoGen, San Diego, CA, 2.5 μg/ml).

Allogenicity. ASALs and DCs are allogeneic toward each other. The irradiated PBMCs used for ASAL production are from the same donor as the monocytes used for generation of DCs.

T-cell expansion by the anti-CD3/CD28 beads, REP, and AEP protocols

Peripheral blood lymphocytes were obtained from fresh PBMCs by adhering the monocytic fraction of cells to the plastic surface to T-75 flasks (Corning) for 90 minutes. The nonadherent fractions of peripheral blood lymphocytes were then used as responder cells.

Bead expansion. Responder cells (1×10^5) were expanded for 12 days by mixing them at a bead-to-cell 1:1 ratio with Dynabeads Human T-Activator CD3/CD28 (Invitrogen) according to the manufacturer's instruction, in culture medium supplemented with IL-2 (Proleukin; Novartis, Basel, Switzerland, 50 IU/ml). Fresh culture medium with IL-2 was changed every 2–3 days and cells were maintained at a concentration below 1×10^6 cells/ml.

REP. Responder cells (1×10^5) were expanded for 12 days by stimulation with 4×10^7 irradiated (50 Gy) allogeneic PBMCs (mix from three donors), OKT-3 (BioLegend, San Diego, CA, 50 ng/ml), and IL-2 (100 IU/ml) in a T-25 flask (Corning). Fresh culture medium with IL-2 was changed every 2–3 days, and cells were maintained at a concentration below 1×10^6 cells/ml.

AEP. Responder cells (1×10^5) were expanded for 12 days by coculture with irradiated ASALs (50 Gy) (1×10^5 to 4×10^5) and allogeneic mDCs (2.5×10^4 to 1×10^5) with OKT-3 (50 ng/ml) and IL-2 (100 IU/ml) in a T-25 flask (Corning). Fresh medium with IL-2 was changed every 2–3 days and cells were maintained at a concentration below 1×10^6 cells/ml.

T-cell viability and proliferation assays

T cells expanded by using the REP and AEP protocols were exposed to 25 μmol/l hydrogen peroxide (H_2O_2, Sigma-Aldrich) or 0.1 μmol/l doxorubicin (Meda, Solna, Sweden) for 24 hours. The Annexin-V apoptotic assay (BD Biosciences, San Jose, CA) was then used to evaluate T-cell viability by flow cytometry analysis on BD FACS Canto II (BD Biosciences). The CellTrace CFSE cell proliferation kit (Invitrogen) was used to evaluate T-cell proliferation after restimulation with target tumor cells and analysis by flow cytometry. Absolute numbers of viable T cells were determined by flow cytometry with Count Bright absolute counting beads (Invitrogen) according to the manufacturer's instructions.

Cytokines release assays, T-cell and DC phenotype analyses

Mature DCs were cocultured with ASALs at a 1:4 ratio in 96-well round bottom plates in 200 μl of culture media. Enzyme-linked immunosorbent assay kits (Mabtech, Nacka Strand, Sweden) were used to measure IFN-γ, IL-2, and IL-12p70 in the supernatants. Phenotypic analysis of T cells was performed using the following fluorophore-labeled mouse monoclonal antibodies: anti-human CD3, anti-human CD4, anti-human CD8, anti-human CD28, anti-human CD27, anti-human CD107a, anti-human Tim3, anti-human PD1, anti-human CD3 zeta chain, and matched mouse IgG isotype controls (all from BD Biosciences). Phenotypic analysis of DCs was performed using the following fluorophore-labeled mouse monoclonal antibodies: anti-human CD80, anti-human CD86, anti-human CD70, and anti-human CD64 expression and matched mouse IgG isotype controls (all from BD Biosciences). These data were on analysis BD FACS Canto II (BD Biosciences).

Blockade of the CD70/CD27 signaling CD40/CD40L signaling and IL-12 cytokine signaling pathway

Mature DCs were incubated with an antagonistic anti-CD70 antibody (Abcam, Cambridge, UK, 10 μg/ml), antagonistic anti-CD40 antibody (Nordic Biosite, Taby, Sweden, 40 ng/ml), neutralizing IL12p70 antibody (Nordic Biosite, 20 ng/ml) and a matched isotype control antibody (Nordic Biosite) for 4 hours and then cocultured with ASALs. Enzyme-linked immunosorbent assay was used to analyze cytokines secretion as described above. T cells were expanded according to the AEP protocol described above in the presence or absence of blocking antibodies. CD27 expression and the relative viability after exposure to H_2O_2 and doxorubicin and cytokines secretion was measured as described above.

Generation of GD2-CAR T cells and TARP-TCR T cells by lentiviral transduction

The sequence encoding the anti-GD2 CAR (scFv derived from 14G2a) was kindly obtained from Dr Eric Yvon and Dr Malcolm Brenner, Baylor College of Medicine, Houston, TX. It was subcloned into a third-generation self-inactivating lentiviral vector under transcriptional control of the spleen-focus forming virus promoter. The generation of lentivirus particles encoding the anti-GD2 CAR and HLA-A2-restricted TARP-TCR has been described previously.[29] PBMCs (5×10^6 cells) were activated for 3 days in culture medium with OKT-3 (50 ng/ml) and IL-2 (100 IU/ml). Thereafter, $CD8^+$ T cells were isolated (Miltenyi Biotec, Auburn, CA). The T cells were resuspended in 20 μl concentrated virus together with 5 μl of Sequa-brene (1 mg/ml; Sigma-Aldrich), and IL-2 (100 IU), followed by 4 hours of incubation. The T cells were transduced again the day after in the same manner and cultured in 5 ml culture medium supplied with IL-2 (20 IU/ml) for 7 days before being analyzed for CAR and TCR expression by flow cytometry. GD2-CAR-transduced T cells were stained with the high-performance liquid chromatography–purified antibody 1A7 (anti-idiotype antibody against 14G2a, kindly provided by Dr Eric Yvon) and a secondary phycoerythrin (PE)-conjugated anti-mouse IgG1 antibody (BD Biosciences). TCR transduced T cells were stained with a PE-conjugated $TARP(P5L)_{4-13}$/HLA-A2-dextramer (Immudex, Copenhagen, Denmark). Anti-phycoerythrin Microbeads (Miltenyi Biotec) were then used to isolate GD2-specific and TARP-TCR-specific T cells. The purity after sorting was more than 80%.

Cell lines used for assays of genetically engineered and expanded T cells

The IMR-32 ($GD2^+$) neuroblastoma cell line (kindly provided by F. Hedborg, Uppsala University) was used as a target cell line for GD2-CAR T cells and the SK-N-FI ($GD2^-$) neuroblastoma cell line, (kindly provided by F. Hedborg) were used as irrelevant target control. The HLA-A2 positive mel526 melanoma cell line with stable expression of the prostate tumor-associated antigen TARP (T cell receptor gamma chain alternate reading frame protein) was used as a target cell line for TARP-TCR T cells, while the same cell line stably expressing prostate stem cell antigen (PSCA) was used as an irrelevant target control.[29] All cell lines were also engineered to express firefly luciferase. The stable luciferase-expressing cell lines were used for T-cell cytotoxicity assay while the parental cell lines were used for T-cell cytokines release and T-cell proliferation assays.

Cytotoxic assay for genetically engineered and expanded T cells

T cells were cultured in low-dose IL-2 (20 IU/ml) for 3 days after expansion in order to reduce their hyperreactivity. They were then mixed with luciferase-tagged target cells at effector to target (E:T) cell ratios ranging from 5:1–40:1 in 96-well plates, total volume was 100 μl per well, followed by 72 hours of coculture. The luciferase activity (from alive target cells) was analyzed using Bright-Glo kit (Promega, Madison, WI) and measured with a luminometer (Wallac VICTOR2; PerkinElmer, Turku, Finland). The relative cell viability was calculated as relative light unit and normalized against the luciferase activity from target cells without T cells. Background activity from wells only containing medium was set to 0% viability. Triplicate samples were used.

Assays for cytokine release and degranulation of expanded T cells

T cells were cultured in low-dose IL-2 (20 IU/ml) for 3 days after expansion in order to reduce their hyperreactivity. They were then treated with H_2O_2 for 24 hours, washed twice, incubated with IL-10 (Nordic Biosite, 2.5 ng/ml) and TGF-β (Nordic Biosite, 10 ng/ml) for 4 hours and then mixed with target cells at an effector:target cell (E:T) ratio of 1:1. The cells were then cultured for 24 hours in the presence of IL-10 (2.5 ng/ml) and TGF-β (10 ng/ml). Enzyme-linked immunosorbent assay was used to measure IFN-γ secreted and flow cytometry was used to measure the degranulation marker CD107a on T cells.

Assay for proliferation of expanded T cells

T cells were cultured in low-dose IL-2 (20 IU/ml) for 3 days after expansion in order to reduce their hyperreactivity. They were then were treated with H_2O_2 for 24 hours, washed twice, stained with CellTrace CFSE cell proliferation kit (Invitrogen) for 4 hours in the presence of IL-10 (2.5 ng/ml) and TGF-β (10 ng/ml) and then mixed with target cells at an effector:target cell (E:T) ratio of 1:1. The cells were cultured for 4 days in the presence of IL-10 (2.5 ng/ml) and TGF-β (10 ng/ml) before the content of CFSE was analyzed by flow cytometry.

Statistical analysis

The data are reported as means and SD. Statistical analysis was performed by GraphPad prism software version 6.01 (La Jolla, CA). Statistical analyses in all figures were performed using student's *t*-test. Values with $P < 0.05$ were considered to be statistically significant.

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CONFLICT OF INTEREST

A.K.-P. and A.W. are founders of Immunicum AB, a company in the field of allogeneic dendritic cell–based immunotherapeutic of cancer. This does not alter the authors' adherence to all The Molecular Therapy Methods and Clinical Development policies on sharing data and materials. The other authors have no conflicting financial interests.

REFERENCES

1 Rosenberg, SA and Dudley, ME (2004). Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. *Proc Natl Acad Sci USA* **101** (suppl. 2): 14639–14645.
2 Levine, BL, Bernstein, WB, Connors, M, Craighead, N, Lindsten, T, Thompson, CB *et al*. (1997). Effects of CD28 costimulation on long-term proliferation of CD4+ T cells in the absence of exogenous feeder cells. *J Immunol* **159**: 5921–5930.
3 Li, Y and Kurlander, RJ (2010). Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: differing impact on CD8 T cell phenotype and responsiveness to restimulation. *J Transl Med* **8**: 104.
4 Klapper, JA, Thomasian, AA, Smith, DM, Gorgas, GC, Wunderlich, JR, Smith, FO *et al*. (2009). Single-pass, closed-system rapid expansion of lymphocyte cultures for adoptive cell therapy. *J Immunol Methods* **345**: 90–99.
5 Ceuppens, JL, Bloemmen, FJ and Van Wauwe, JP (1985). T cell unresponsiveness to the mitogenic activity of OKT3 antibody results from a deficiency of monocyte Fc gamma receptors for murine IgG2a and inability to cross-link the T3-Ti complex. *J Immunol* **135**: 3882–3886.
6 Yang, S, Dudley, ME, Rosenberg, SA and Morgan, RA (2010). A simplified method for the clinical-scale generation of central memory-like CD8+ T cells after transduction with lentiviral vectors encoding antitumor antigen T-cell receptors. *J Immunother* **33**: 648–658.
7 Kobayashi, H, Tanaka, Y, Yagi, J, Minato, N and Tanabe, K (2011). Phase I/II study of adoptive transfer of γδ T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma. *Cancer Immunol Immunother* **60**: 1075–1084.
8 Wang, QJ, Hanada, K, Feldman, SA, Zhao, Y, Inozume, T and Yang, JC (2011). Development of a genetically-modified novel T-cell receptor for adoptive cell transfer against renal cell carcinoma. *J Immunol Methods* **366**: 43–51.
9 Gattinoni, L, Klebanoff, CA, Palmer, DC, Wrzesinski, C, Kerstann, K, Yu, Z *et al*. (2005). Acquisition of full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest* **115**: 1616–1626.
10 Berger, C, Turtle, CJ, Jensen, MC and Riddell, SR (2009). Adoptive transfer of virus-specific and tumor-specific T cell immunity. *Curr Opin Immunol* **21**: 224–232.
11 Banchereau, J and Steinman, RM (1998). Dendritic cells and the control of immunity. *Nature* **392**: 245–252.
12 Kaliński, P, Hilkens, CM, Wierenga, EA and Kapsenberg, ML (1999). T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* **20**: 561–567.
13 Janssen, EM, Lemmens, EE, Wolfe, T, Christen, U, von Herrath, MG and Schoenberger, SP (2003). CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* **421**: 852–856.
14 Shedlock, DJ and Shen, H (2003). Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* **300**: 337–339.
15 Ridge, JP, Di Rosa, F and Matzinger, P (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* **393**: 474–478.
16 Cella, M, Scheidegger, D, Palmer-Lehmann, K, Lane, P, Lanzavecchia, A and Alber, G (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med* **184**: 747–752.
17 Lu, Z, Yuan, L, Zhou, X, Sotomayor, E, Levitsky, HI and Pardoll, DM (2000). CD40-independent pathways of T cell help for priming of CD8(+) cytotoxic T lymphocytes. *J Exp Med* **191**: 541–550.
18 Williams, MA, Tyznik, AJ and Bevan, MJ (2006). Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* **441**: 890–893.
19 Kalinski, P, Nakamura, Y, Watchmaker, P, Giermasz, A, Muthuswamy, R and Mailliard, RB (2006). Helper roles of NK and CD8+ T cells in the induction of tumor immunity. Polarized dendritic cells as cancer vaccines. *Immunol Res* **36**: 137–146.
20 Fernandez, NC, Flament, C, Crépineau, F, Angevin, E, Vivier, E and Zitvogel, L (2002). Dendritic cells (DC) promote natural killer (NK) cell functions: dynamics of the human DC/NK cell cross talk. *Eur Cytokine Netw* **13**: 17–27.
21 Huang, J, Khong, HT, Dudley, ME, El-Gamil, M, Li, YF, Rosenberg, SA *et al*. (2005). Survival, persistence, and progressive differentiation of adoptively transferred tumor-reactive T cells associated with tumor regression. *J Immunother* **28**: 258–267.
22 Fourcade, J, Sun, Z, Benallaoua, M, Guillaume, P, Luescher, IF, Sander, C *et al*. (2010). Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med* **207**: 2175–2186.
23 Feau, S, Garcia, Z, Arens, R, Yagita, H, Borst, J and Schoenberger, SP (2012). The $CD4^+$ T-cell help signal is transmitted from APC to $CD8^+$ T-cells via CD27-CD70 interactions. *Nat Commun* **3**: 948.
24 Koch, F, Stanzl, U, Jennewein, P, Janke, K, Heufler, C, Kämpgen, E *et al*. (1996). High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J Exp Med* **184**: 741–746.
25 Porter, DL, Levine, BL, Kalos, M, Bagg, A and June, CH (2011). Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* **365**: 725–733.
26 Grupp, SA, Kalos, M, Barrett, D, Aplenc, R, Porter, DL, Rheingold, SR *et al*. (2013). Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* **368**: 1509–1518.
27 Seeger, RC (2011). Immunology and immunotherapy of neuroblastoma. *Semin Cancer Biol* **21**: 229–237.
28 Yu, P and Fu, YX (2006). Tumor-infiltrating T lymphocytes: friends or foes? *Lab Invest* **86**: 231–245.
29 Hillerdal, V, Nilsson, B, Carlsson, B, Eriksson, F and Essand, M (2012). T cells engineered with a T cell receptor against the prostate antigen TARP specifically kill HLA-A2+ prostate and breast cancer cells. *Proc Natl Acad Sci USA* **109**: 15877–15881.
30 Li, Y, Liu, S, Hernandez, J, Vence, L, Hwu, P and Radvanyi, L (2010). MART-1-specific melanoma tumor-infiltrating lymphocytes maintaining CD28 expression have improved survival and expansion capability following antigenic restimulation *in vitro*. *J Immunol* **184**: 452–465.
31 Bevan, MJ (2004). Helping the CD8(+) T-cell response. *Nat Rev Immunol* **4**: 595–602.
32 Kawamura, T, Ogawa, Y, Shimozato, O, Ando, T, Nakao, A, Kobata, T *et al*. (2011). CD70 is selectively expressed on Th1 but not on Th2 cells and is required for Th1-type immune responses. *J Invest Dermatol* **131**: 1252–1261.
33 Taraban, VY, Rowley, TF and Al-Shamkhani, A (2004). Cutting edge: a critical role for CD70 in CD8 T cell priming by CD40-licensed APCs. *J Immunol* **173**: 6542–6546.
34 Taraban, VY, Rowley, TF, Tough, DF and Al-Shamkhani, A (2006). Requirement for CD70 in CD4+ Th cell-dependent and innate receptor-mediated CD8+ T cell priming. *J Immunol* **177**: 2969–2975.
35 Robbins, PF, Dudley, ME, Wunderlich, J, El-Gamil, M, Li, YF, Zhou, J *et al*. (2004). Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J Immunol* **173**: 7125–7130.

36 Berger, C, Jensen, MC, Lansdorp, PM, Gough, M, Elliott, C and Riddell, SR (2008). Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest* **118**: 294–305.
37 Hendriks, J, Xiao, Y and Borst, J (2003). CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J Exp Med* **198**: 1369–1380.
38 Trinchieri, G and Gerosa, F (1996). Immunoregulation by interleukin-12. *J Leukoc Biol* **59**: 505–511.
39 Otsuji, M, Kimura, Y, Aoe, T, Okamoto, Y and Saito, T (1996). Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 zeta chain of T-cell receptor complex and antigen-specific T-cell responses. *Proc Natl Acad Sci USA* **93**: 13119–13124.
40 Malmberg, KJ, Arulampalam, V, Ichihara, F, Petersson, M, Seki, K, Andersson, T *et al*. (2001). Inhibition of activated/memory (CD45RO(+)) T cells by oxidative stress associated with block of NF-kappaB activation. *J Immunol* **167**: 2595–2601.
41 de Visser, KE and Kast, WM (1999). Effects of TGF-beta on the immune system: implications for cancer immunotherapy. *Leukemia* **13**: 1188–1199.

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