Recombinant CD95-Fc (APG101) prevents graft-versus-host disease in mice without disabling anti-tumor cytotoxicity and T cell functions

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Abstract

Graft-versus-host disease (GVHD) induced by transplant-derived T cells represents a major complication after allogeneic bone marrow transplantation (BMT). These T cells, however, support engraftment, early T cell immunity and mediate the graft-versus-tumor (GVT) effect. Cytotoxic effector functions by transplanted T cells are predominantly mediated by the perforin/granzyme and the CD95/CD95L system. APG101, a novel recombinant human fusion protein consisting of the extracellular domain of CD95 and the Fc domain of an IgG1 antibody inhibited CD95L-induced apoptosis without interfering with T cell function in vitro and was therefore tested for its ability to prevent GVHD in murine BMT models across minor or major histocompatibility barriers. Starting APG101 treatment either one day before or six days after transplantation effectively reduced clinical GVHD and rescued survival between 60 – 100% if GVHD was CD95L mediated. APG101 did not interfere with the GVT effect, since P815 mastocytoma and most importantly primary Bcr-Abl-transformed B cell leukemias were completely eradicated by the alloantigen-specific T cells. Phenotype and homing of alloantigen-specific T cells or their perforin/granzyme-mediated cytotoxicity and proliferative capacity were not affected by APG101 treatment suggesting that APG101 therapy might be useful in GVHD prophylaxis without impairing T cell function and most importantly preserving GVT activity.
Introduction

Allogeneic bone marrow transplantation (BMT) is a potentially curative treatment modality for hematological malignancies, which involves the graft-versus-tumor or graft-versus-leukemia effect i.e. elimination of residual tumor cells by mature allogeneic T cells in the transplant. These T cells, however, are also responsible for the induction of graft-versus-host disease (GVHD), which is still a major complication after allogeneic BMT leading to significant morbidity and mortality despite routine immune suppression used in all protocols.\textsuperscript{1} T cell depletion of the graft significantly decreases the risk of GVHD, however it also delays engraftment, sensitizes the host for opportunistic infections and abrogates the GVT effect.\textsuperscript{2} Therefore, preventing GVHD while maintaining GVT activity and early T cell immunity is a major goal in allogeneic BMT.

GVHD represents a complex disease depending on alloantigen-specific T cell activation, proliferation, and subsequent induction of T cell effector functions, which are attempt to be inhibited by routinely given immunosuppressive therapy. This, however, causes general immunosuppression leading to an increased risk for opportunistic infections, cancer development and also severely suppresses the GVT effect.\textsuperscript{3} Acquiring effector functions is associated with versatile molecular and cellular changes in the T cell e.g. the expression of activation markers, homing molecules, cytokines and cytotoxic molecules which represent possible targets for new therapeutic approaches in GVHD-prevention and –treatment.\textsuperscript{4} Analysis of T cell subsets showed that memory T cells are unable to induce GVHD but efficiently eradicate tumor cells.\textsuperscript{5,6} We recently found that even in vitro-generated alloantigen-specific cytotoxic T cells (CTLs) mediate anti-tumor cytotoxicity in the absence of GVHD.\textsuperscript{7} Interfering with T cell trafficking or cytokine production offers other treatment strategies.\textsuperscript{8,9} The primary goal of most trials is the inhibition of host tissue destruction by attacking alloantigen-specific T cells. Destructive function of T cells is mediated either by death ligands CD95L, TRAIL, and TNF-\textalpha or the perforin/granzyme system. BMT-models using T cells
either deficient for CD95L or perforin or treatment with anti-TNF-α antibodies demonstrated that all three molecules are involved in GVHD induction although their contribution to GVHD pathology is different. While TNF-α and CD95L are indispensable for hepatic, intestinal and cutaneous GVHD, perforin plays an important role in the kinetics of GVHD pathophysiology.10-12 Efficient anti-tumor cytotoxicity, however, is predominantly dependent on TNF-α and perforin.11,13 The role of TRAIL in GVHD induction and GVT effect is controversially discussed.5,14,15 These studies indicate that interference with the CD95/CD95L pathway might be a successful treatment option to inhibit GVHD without abrogating the desired GVT effect after allogeneic BMT. APG101 is a human soluble fusion protein consisting of the extracellular domain of CD95 and the Fc portion of IgG1 thereby blocking the interaction of CD95L with its cognate receptor. APG101 has already been shown to be well tolerated up to high doses in a phase I first-in-man study16 and may therefore be applicable in GVHD prophylaxis. Since the CD95 system can mediate other cellular functions in addition to apoptosis such as migration, invasiveness and tumor promotion dependent on the specific cellular systems17,18, APG101 is also currently exploited in the treatment of gliomas.19 To test, whether the CD95L-mediated inhibition of tissue destruction by APG101 offers a new therapeutic approach to treat and/or prevent GVHD, we treated allogeneic BM-transplanted mice on a regular basis with APG101. Since CD95L is not only known for its function as an apoptosis inducer, but may also transmit signals into the CD95L-expressing cells, a process known as reverse signaling and predominantly affecting T cell proliferation20-22, we further analyzed the effect of APG101 on T cell function, anti-tumor cytotoxicity and homing.

In a parent into F1 MHC class I and II-mismatched BMT model and a BMT model differing in minor histocompatibility antigens (miHAg), we found that APG101 efficiently prevents clinical, CD95L-mediated GVHD. APG101 inhibited CD95-mediated apoptosis without disabling T cell functions since donor-derived T cells maintained their migratory ability,
proliferated after activation and most importantly exhibited efficient anti-tumor cytotoxicity with complete elimination of residual tumor cells. Thus, inhibiting CD95/CD95L interaction by APG101 might represent a clinically applicable strategy in allogeneic BMT to inhibit GVHD without disabling T cell functions indispensable for anti-tumor cytotoxicity and early T cell immunity.
Materials and Methods

Cell culture

All cell lines were grown in complete RPMI 1640 medium (GIBCO-BRL, United Kingdom) supplemented with 10% FCS (Lonza, Wuppertal, Germany), 2mM L-glutamine, 1mM sodium pyruvate at 37°C in a humidified atmosphere containing 7.5% CO2. CTLs were cultured in α-MEM (Lonza), 10% FCS (PAA, Heidelberg, Germany), 2mM L-glutamine, 1mM sodium-pyruvate, 0.05 mM 2-ME, 5% v/v of 0.5 M methyl-α-D-mannopyranoside and 5% v/v ConA supernatant.

Mice and BMT

Female C57BL/6 (B6; H-2^b, CD45.2), B6D2F1 (H-2^bd, CD45.2), DBA/2 (H-2^d), (Harlan-Winkelmann, Borchen, Germany), BALB/c (H-2^d) (Janvier, France), B10.D2/nSnJ (H-2^d) (Jackson Laboratory) and B6.SJL-Ptpcr^aPepc^b/BoyJ (B6.SJL; H-2^b, CD45.1) (breeding pairs from the Jackson Laboratory and bred at the University of Ulm) were used at an age of 6 - 10 weeks. On day -1 recipient mice were lethally irradiated with 13 Gy split into 2 doses 3 hours apart from a ^{137}Cs source and were reconstituted on day 0 with 5x10^6 BM cells via tail vein injection. If indicated, mature T cells were depleted from BM by incubation with hybridoma supernatant 30-H12 (anti-Thy-1.2) and subsequently lysed by Low-TOX-M rabbit complement (Cerdarlane, Ontario, Canada) (TCD BM = T cell-depleted BM). BM was co-injected with 2x10^7 spleen cells in B6D2F1 mice or with 3x10^7 spleen cells in BALB/c mice. APG101 treatment started either one day before BMT (day -1) or 6 or 13 days after BMT (day 6, day 13). APG101 was injected at a fixed dose of 1 mg/mouse twice a week i.p. in PBS. Weight loss, decreased activity, skin lesions, fur ruffling, and hunched posture were scored on a scale from 0 to 2 in the recipient mice every 2 days and the GVHD-score was determined by summation of the 5 criteria. Animals dying during the experiment remained included in the calculation until the end of the experiment with their final body weights and
GVHD-scores at day of death. Mice were sacrificed when more than 20% of their original weight was lost or if they became moribund. Survival was analyzed using Kaplan-Meier method. Tumor cells were injected i.v. either at the day of BMT (P815 (H-2\textsuperscript{d}), 2x10\textsuperscript{3}/mouse) or 7 days after BMT (GFP-Bcr-Abl-transformed B-ALL (H-2\textsuperscript{d}), 3x10\textsuperscript{2}/mouse\textsuperscript{24}). All animal studies were approved by the Regierungspräsidium, Tübingen, Germany.

**Histopathology and Immunohistochemistry**

Liver, intestin, and skin from the interscapular region were fixed in 4% formalin, paraffin-embedded, sectioned and stained with H&E. Slides were coded and examined by a pathologist who was blinded for the experimental history of the animals. Histopathology of GVHD was graded according to Kaplan et al.\textsuperscript{25}. To determine target cell apoptosis paraffin sections were stained at room temperature with a polyclonal cleaved caspase-3 (Asp175) antibody (Cell signaling, Danvers, MA), followed by anti-rabbit biotin (Jackson Immuno Research, West Grove, PA) and subsequently REAL\textsuperscript{TM} Detection System (Dako, Hamburg, Germany) according to the manufacturer’s protocol, nuclear counterlabeled with hemalaun and embedded in Aquatex medium (Merck, Darmstadt, Germany).

**Induction of apoptosis**

CD95-sensitivity was determined by incubating cells with 100 ng/ml CD95L and 1 µg/ml enhancer of ligands (Alexis Biochemicals, Lörrach, Germany). After 24 hours specific apoptosis was determined by 7-AAD staining on LSRII flow cytometer.

**Generation of alloantigen-specific T cells**

Spleen cells from B6 mice were weekly stimulated at 1:1 ratio with irradiated spleen cells from B6D2F1 mice as previously described.\textsuperscript{7} If indicated, APG101 at a concentration of 100 ng/ml was present during the whole stimulation time.
Chromium release assay

Chromium release assay was described previously. Assays were set up in triplicates and performed at day 6 after T cell stimulation. Cytotoxic effector mechanisms of CTLs were identified by 2h pre-incubation of CTLs with CMA (10 nM, Sigma-Aldrich) or APG101 (100 ng/ml). CMA and APG101 were constantly present during the whole assay. To determine cytotoxic activity of transplanted CD45.1+ T cells, spleen cells of transplanted mice were stained for CD45.1 expression before the assay and the total number of CD45.1+ T cells was calculated. Effector : target ratios were then adjusted for a ratio of CD45.1+ T cells : target cells.

CFSE-labeling

2x10^6 cells/ml were labeled with 100 µl 50 µM CFSE (Invitrogen, Darmstadt, Germany) for 10 min at 37°C. Cells were immediately washed and subsequently used for i.v. injection or in vitro assays.

Proliferation

2x10^5 CFSE-labeled cells were stimulated either with anti-CD3 and -CD28 antibodies (0.0125µg/ml, eBioscience, Frankfurt, Germany) or 2x10^5 irradiated allogeneic spleen cells and proliferation was measured on day 4 (after CD3/28 activation) or day 6 (after alloantigen or third party stimulation) on LSRII flow cytometer.

Flow cytometry

2x10^5 cells were stained with the following antibodies: CD3, CD4, CD25, NK1.1, TCRβ, CD122, CCR7, CD45.1, FoxP3, H-2K^d (eBioscience), H-2K^b, CD3, CD8, CD44, CD62L (BD Biosciences) and analyzed by flow cytometry on LSRII flow cytometer.
Isolation of CD45.1\(^+\) T cells

Spleen cells from transplanted mice were stained with CD45.2, CD11b, CD16/32 antibody (eBioscience) and supernatant from the hybridoma PK136 (anti-NK1.1) and were subsequently eliminated with goat-anti-mouse IgG and goat-anti-rat IgG magnetic beads (Invitrogen). Purity of the CD45.1\(^+\) T cell population was always > 87%.

Statistical analysis:

Differences between groups in survival studies were determined using logrank test. For all other data, a Student t test was used. Results were considered significant if p≤ 0.05.
Results

APG101 inhibits apoptosis but does not interfere with T cell function in vitro

APG101 is a recombinant human fusion protein binding to CD95L and thereby inhibiting apoptosis induction in CD95-expressing target cells. Although CD95L is best known for its role in apoptosis induction, it may also serve a function in reverse signaling, thereby modulating the immune function of CD95L-expressing cells. Therefore, we first tested the effect of APG101 on apoptosis inhibition and T cell function in vitro. B6-derived spleen cells were treated with recombinant CD95L and enhancer in the presence of increasing concentrations of APG101. After 24 hours CD95L-mediated apoptosis of CD4+ and CD8+ T cells was efficiently blocked with APG101 concentrations ranging from 5 - 1000 µg/ml. APG101 by itself did not induce apoptosis (Fig. 1A). Since CD95L can modulate T cell function as a reverse signaling co-stimulator, we tested the effect of APG101 on T cell proliferation and T cell cytotoxicity. CFSE-labeled B6-derived (H-2b) splenic CD4+ and CD8+ T cells activated for 6 days with irradiated allogeneic spleen cells from B6D2F1 mice (H-2^bd) efficiently proliferated independent of the presence or absence of APG101 (Fig. 1B). To exclude that APG101 interferes with the development of alloantigen-specific CTLs, B6-derived spleen cells were activated with irradiated B6D2F1 spleen cells in the presence or absence of APG101. After one week (1st stim.) the lytic capacity of induced CTLs was tested on H-2d-expressing target cells (P815). P815 cells were effectively lysed independent whether or not APG101 was added to the cultures while syngeneic H-2b-expressing cells (EL4) were not recognized. Cytotoxicity of H-2d-specific CTLs increased when CTLs (1st stim.) were subsequently re-stimulated by alloantigen-expressing cells (2nd and 3rd stim.) independent of the presence of APG101 (Fig. 1C). Taken together, these data clearly indicate that APG101 effectively prevents cell death induction but does not interfere with the proliferative or cytotoxic capacity of T cells in vitro.
APG101 treatment prevents clinical GVHD in a parent → F1 BMT model

In allogeneic BMT models where GVHD development depends on the presence of CD4+ T cells in the transplant recipient tissue destruction is predominantly mediated by CD95L-expressing allogeneic T cells attacking CD95 positive target cells.27 Since APG101 efficiently inhibited apoptosis induction without abrogating T cell proliferation or cytotoxicity in vitro, we studied whether APG101 might be a useful therapeutic agent in preventing GVHD after allogeneic BMT in a CD4+-dependent parent → F1 BMT model with a full mismatch for MHC class I and II molecules (B6 → B6D2F1). Lethally irradiated B6D2F1 mice were reconstituted with BM alone or together with spleen cells from B6 mice. APG101 treatment started one day before BMT (day -1) or six or 13 days after transplantation (day 6, day 13). Mice were treated twice a week with a fixed dose of 1 mg APG101/mouse i.p.. Induction of GVHD was determined by weight loss, a clinical scoring system23 and survival. Due to the toxic effect of irradiation GVHD scores increased and body weight decreased initially between day 4 and 10 after transplantation. After a short recovery phase mice reconstituted with BM and spleen cells exhibited severe weight loss, increased GVHD-scores and a mortality of 38%. Importantly, mice treated with APG101 prophylactically at day -1 and even therapeutically at day 6 showed only a modest weight loss and increase in GVHD-scores and a 100% survival comparable to mice transplanted with BM alone. However, starting treatment 13 days after transplantation APG101 did not prevent GVHD induction (Fig. 2 A, B, C). Mice injected with BM alone and treated with APG101 were indistinguishable from mice receiving BM and PBS. No differences in time to cellular reconstitution or the composition of repopulating cell subsets were observed (data not shown) indicating that APG101 does not influence donor chimerism. Although clinical GVHD was efficiently prevented by APG101 treatment starting at day -1 or 6, histological GVHD was not totally inhibited. Treatment start at day -1 or day 6 significantly inhibited GVHD of the intestine, while liver damage was only reduced in transplanted mice when APG101 treatment started one day before transplantation.
Histology of the ileum and colon of APG101-treated mice showed reduced mononuclear inflammatory infiltrates in the lamina propria, decreased numbers of intraepithelial lymphocytes, and reduced apoptosis of crypt epithelium depicted by active caspase-3 staining (Fig. 2E). In the liver reduced inflammation of the portal field and bile duct injury was observed in mice treated with APG101 starting day -1. To prove the specificity of APG101 and to exclude an off-target effect we tested the effect of APG101 in an allogeneic BMT model where GVHD induction is preferentially dependent on the perforin/granzyme system and CD8+ T cells in the transplant (B6 (H-2Kb) into B6.bm1 (H-2Kbm1)). B6.bm1 mice transplanted with BM and spleen cells and treated with APG101 on day -1 or day 6 developed clinical GVHD and showed no improved survival compared to the PBS-treated groups (Suppl. Fig. 1A, B). These data clearly show that CD95L-mediated GVHD can be specifically inhibited by APG101 treatment starting either one day before or six days after transplantation.

**APG101 treatment strongly attenuates clinical GVHD in a minor histocompatibility antigen mismatched BMT model**

The B6→B6D2F1 BMT model resembles the haploidentical BMT, but in humans transplantations with differences in only miHAgs are more frequently performed. Therefore, we tested the effect of APG101 in a miHAg-mismatched model (B10.D2→BALB/c). Lethally irradiated BALB/c mice reconstituted with B10.D2-derived TCD BM and spleen cells and treated with PBS developed severe GVHD with increased GVHD scores and a 100% mortality after 41 days. Treatment with APG101 starting one day before transplantation diminished GVHD severity and most importantly rescued mice from lethal GVHD with 60% (Fig. 3A, B). Mice transplanted with TCD BM alone and treated with PBS or APG101 survived and exhibited no signs of GVHD. Histological liver GVHD was significantly decreased by APG101 treatment, while skin GVHD was only slightly diminished and no
effect was observed in the intestine (Fig. 3C). These data show that in two clinically relevant BMT models APG101 attenuated clinical and histological GVHD and most importantly significantly improved the survival of transplanted animals.

**APG101 treatment does not influence engraftment, homing, phenotype or function of allogeneic T cells**

Since transplanted mature T cells support engraftment, promote early T cell immunity and mediate the GVT effect, we tested whether APG101 treatment in vivo interferes with reconstitution of the hematopoietic compartment, homing, phenotype and functionality of transplanted allogeneic T cells. Therefore, lethally irradiated B6D2F1 mice (H-2<sup>bxd</sup>, CD45.2<sup>+</sup>) were transplanted with B6-derived BM (H-2<sup>b</sup>, CD45.2<sup>+</sup>) and spleen cells from B6.SJL mice (H-2<sup>b</sup>, CD45.1<sup>+</sup>) and treated with APG101 or PBS starting on day -1. Using B6.SJL-derived spleen cells allows discrimination between transplanted mature allogeneic T cells and T cells developing out of the transplanted hematopoietic stem cells by the congeneric marker CD45.1. 14 days after transplantation mice were sacrificed and T cell numbers, phenotype, and function were analyzed. Independent of whether mice were treated with APG101 or PBS no differences in total spleen cell count or changes in the number of CD45.1<sup>+</sup> CD4<sup>+</sup>, CD8<sup>+</sup>, regulatory T cells, NK- or NK-T cells were detected (Fig. 4A). Transplanted CD4<sup>+</sup> and CD8<sup>+</sup> CD45.1<sup>+</sup> T cells developed into effector/memory T cells (CD44<sup>+</sup>, CD122<sup>+</sup>, CCR7<sup>-</sup>, CD62L<sup>-</sup>) (Fig. 4B). No differences in the expression of adhesion molecules CCR9, LPAM-1, LFA-1, and CXCR3 were observed in transplanted CD45.1<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup> T cells independent whether mice were treated with APG101 or PBS (Suppl. Fig.2A). Transplanted CD45.1<sup>+</sup> T cells maintained their proliferative capacity towards CD3/CD28 activation, alloantigen or third party stimulation (Fig. 4C) and produced Th1 and Th2 cytokines after allogeneic or PMA/Iono stimulation irrespective whether APG101 was present or not (Suppl. Fig. 2B). Most importantly, CD45.1<sup>+</sup> splenic T cells exhibited efficient cytotoxic capacity towards the
alloantigen-expressing tumor line P815, which was even slightly increased compared to
CD45.1+ T cells from PBS treated mice. Target cell lysis was mediated by the
perforin/granzyme system since addition of the vacuolar type H+-ATPase Concanamycin A
(CMA), which inhibits the perforin-dependent killing pathway28 abrogated specific
cytotoxicity while addition of APG101 showed no effect (Fig. 4D). APG101 treatment did not
interfere with the homing capacity of CD45.1+ T cells since 3, 7, 14 or 21 days after
transplantation the percentage of CD45.1+ T cells in spleen and liver (Fig. 4E) as well as
lymph nodes, thymus, bone marrow and lung (data not shown) was comparable between PBS-
and APG101-treated mice. All together, these data clearly show that despite the inhibitory
effects of APG101 on GVHD, alloantigen-specific T cell function required for tumor cell
rejection or early T cell immunity is not impaired by APG101.

GVT effect is maintained in APG101-treated mice after allogeneic BMT
Since APG101 did not abrogate effector functions of transplanted allogeneic T cells and the
CD95/CD95L system may be dispensable for tumor rejection11,13 we tested the anti-tumor
effect of alloantigen-specific T cells in APG101-treated mice. Lethally irradiated B6D2F1
mice were reconstituted with T cell-depleted BM (TCD BM) and spleen cells from B6 mice
and co-injected with P815 (H-2d) mastocytoma cells, which preferentially home to BM,
spleen, and liver. APG101 treatment was either started one day before BMT (day -1) or six
days after transplantation (day 6). H-2d-expressing P815 cells were detected by flow
cytometry and an increase in liver and spleen weights. Between day 17 and 20 after
transplantation all mice injected with P815 cells and TCD BM in the absence of
immunocompetent spleen cells died (Fig. 5A) due to tumor burden reflected by significantly
increased liver and spleen weights (Fig. 5B) and detection of H-2d single positive cells in liver
(Fig. 5C) and spleen (data not shown). APG101 had no direct effect on tumor growth since
survival times in APG101-treated and PBS-treated TCD BM-transplanted mice were similar.
Most importantly, mice receiving APG101 together with TCD BM and allogeneic spleen cells exhibited a survival of 95% independent whether treatment was started on day -1 or 6 (Fig. 5A). Death of one mouse in each APG101-treated group was not caused by tumor growth since no tumor cells could be detected in target organs by flow cytometry but increased GVHD-scores indicating GVHD development (data not shown). Analysis of all surviving animals at the end of the experiment showed no increase in organ weights and no detection of tumor cells by flow cytometry (Fig. 5B, C). Mice reconstituted with TCD BM plus spleen cells and treated with PBS were tumor-free but died until day 50 due to GVHD induction indicated by elevated GVHD scores (Suppl. Fig. 3A). As BMT is a curative therapy for leukemias, we tested whether APG101 treatment did also not affect the anti-tumor response towards Bcr-Abl-transformed primary B-ALL cells co-expressing GFP. B-ALL cells are highly aggressive since injection of 40 – 600 cells /mouse kills all animals between day 10 and 23 dependent on the individual experiment (data not shown). In order to prevent that tumor-mediated death is induced before the animals start to reconstitute their hematopoietic compartment we injected the tumor cells seven days after transplantation. Treatment of APG101 was started either 1 day before (day-1) or 6 days (day 6) after transplantation. Independent whether APG101 or PBS was injected, mice receiving B-ALL cells in the absence of transplanted immunocompetent spleen cells died around 13 days after tumor injection at day 20 or 21 (Fig. 5D) confirmed by high tumor loads of GFP+ cells in spleen and bone marrow (Fig. 5E). Most importantly, all animals receiving APG101 treatment and transplanted with BM and spleen cells remained 100 % tumor-free indicated by the absence of tumor cells in spleen and bone marrow (Fig. 5E). Survival was improved (71% day -1, 86% day 6) compared to PBS-treated mice (43%). Death of APG101-treated mice was not due to tumor growth but induced by GVHD. Mice receiving BM and spleen cells and treated with PBS were also all tumor-free, but developed GVHD indicated by elevated GVHD scores (Suppl. Fig. 3B). No interference of APG101 with the GVT effect was also detected in the
miHAg-mismatch BMT model. Lethally irradiated BALB/c mice reconstituted with TCD BM in the absence of immunocompetent cells, co-injected with Bcr-Abl+ B-ALL and treated with PBS or APG101 died of tumor development indicated by more than 75% of tumor cells in spleen, BM and liver. Co-transplantation of spleen cells totally prevented tumor development independent whether mice were treated with PBS or APG101 (Suppl. Fig. 4). All tumor experiments convincingly show that APG101 treatment does not abrogate the anti-tumor effect of transplanted allogeneic T cells. Taken together, our experiments show that the CD95/CD95L-pathway inhibitor APG101 efficiently prevents CD95L-mediated GVHD development in allogeneic BM-transplanted animals while GVT activity and T cell functions are preserved.
Discussion
The presence of allogeneic T cells in BM transplants ensures early T cell immunity and anti-tumor cytotoxicity but severely increases the risk of GVHD. Interfering with the CD95/CD95L pathway represents one possibility to dissect these two T cell effector functions. Treatment of allogeneic BM-transplanted animals with APG101, a human fusion protein, which prevents CD95L binding to its cognate receptor and therefore disrupts cell death induction in CD95-expressing target cells successfully prevented GVHD development in BMT models across major and minor histocompatibility barriers. Most importantly, however, transplanted allogeneic T cells remained functional since graft-versus-tumor activity and effector functions were preserved. To our knowledge, we show here for the first time, that a therapeutically applicable CD95/CD95L pathway inhibitor, which is already used for treating glioma patients in a clinical phase II study, can be applied successfully in GVHD prophylaxis without abrogating GVT activity and T cell immunity.

Treatment of allogeneic BM-transplanted animals with two weekly injections of APG101 efficiently prevented clinical GVHD and disease-induced morbidity in a major and a minor HAg-mismatched BMT model. APG101 treatment was successful when started one day before or six days after transplantation. Interestingly, T cell infiltration and GVHD-associated histological damages were not completely prevented. In line with this finding, clinical symptoms do not reliably correlate with the presence of gastrointestinal histopathology in human GVHD. This may indicate that the injected dose was not sufficient to completely eliminate all features of GVHD or alternatively, that mechanisms other than CD95-mediated apoptosis contribute to histological and/or clinical GVHD. APG101 treatment starting at day 13 after BMT was completely inefficient to prevent GVHD. This suggests either that target tissue destruction at day 13 is already advanced to such an extent that APG101 treatment cannot rescue organ function anymore or that GVHD at this time point is already driven by T cell- and CD95-independent mechanisms. In earlier studies prevention of CD95/CD95L-
induced GVHD was achieved to some extent with anti-CD95L antibodies or by using CD95L-deficient T cells as allogeneic effector cells.\textsuperscript{10,11,13,29} While anti-CD95L antibody treatment reduced morbidity to 50\% and was accompanied by a strongly extenuated weight loss, marked hepatic lymphocyte infiltration was still present in antibody-treated mice.\textsuperscript{29} In this study CD95L antibody injections were superior to human Fas-Fc treatment.\textsuperscript{29} In our experiments, however, APG101 treatment rescued 60 – 100\% of the mice from GVHD-induced cell death. Inhibition of GVHD was also reported after injections of a non-toxic anti-CD95 antibody mediating apoptosis of transplanted recipient-activated T cells\textsuperscript{30} suggesting that interference with the CD95 system in fact is effective in several models.

Defining effector mechanisms for GVHD induction, however, appears to be model system dependent. Contributions of the CD95/CD95L or the perforin/granzyme system to GVHD induction are partially defined by the type of MHC disparities between host and recipient. While the CD95/CD95L pathway plays a predominant role in CL II mismatches, the perforin/granzyme system contributes efficiently to GVHD induction in Cl I differences.\textsuperscript{27,31} APG101 efficiently prevents GVHD in major and minor histocompatibility mismatched models where GVHD development is dependent on the presence of CD4\(^{+}\) T cells in the transplant\textsuperscript{31-33} but is inefficient in the B6\(\rightarrow\)B6.bm1 model, in which GVHD induction is preferentially mediated by perforin/granzyme and CD8\(^{+}\) T cells\textsuperscript{27,34,35}. However, T cell deficiency in perforin and CD95L does not necessarily abrogate GVHD development\textsuperscript{36,37} indicating that other cytotoxic molecules such as TRAIL or TNF-\textit{\alpha} might also contribute to disease development\textsuperscript{10,11,15,37}.

To our knowledge none of the studies analyzed the effect of disrupting CD95/CD95L interaction on anti-tumor cytotoxicity, donor engraftment or T cell effector function. Here, we show that preventing CD95-mediated apoptosis by APG101 had no effect on the GVT activity of transplanted allogeneic T cells. Independent whether or not mice were treated with APG101, tumor cells were totally eradicated as soon as the transplant contained mature T
Not only the mouse mastocytoma cell line P815 was efficiently rejected but also primary Bcr-Abl transduced B cells, which are highly aggressive since injection of 40 tumor cells induced 100% mortality after 14 days. Differences in the effect of APG101 on allogeneic T cell effector functions, which induce host tissue destruction on the one hand and tumor cell eradication on the other hand, can most likely be explained by the requirement of specific effector molecules for cell death in certain cell types. Tumor cell destruction can be predominantly dependent on the presence of one cytotoxic mechanism but often CD95L and perforin-dependent killing can compensate for the absence of the other. Apparently, P815 and Bcr-Abl B-ALL tumor cell eradication does not predominantly require CD95L, since APG101 did not interfere with anti-tumor cytotoxicity, which was further supported by our in vitro data showing that allogeneic CTL-mediated tumor cell lysis cannot be blocked by APG101 but is totally abrogated by CMA. Although the contribution of TRAIL in GVHD and GVT effect is still under discussion, TRAIL seems to play only an inferior role in our BMT system.

Since the CD95/CD95L complex is now increasingly recognized as a system which does not only signal apoptosis but also mediates non-apoptotic cell type specific suppression or activation depending on the environment and co-stimulation, we investigated the effect of APG101 on donor engraftment and T cell function and homing. Allogeneic T cells from APG101 treated mice were phenotypically indistinguishable from T cells developing in PBS-injected animals and donor engraftment of CD4+ and CD8+ T, NK, and NK-T cells was similar. Even T-rep numbers were identical although these cells are susceptible to homeostatic control by CD95. Although CD95L activation is known to be a possible modulator of T cell expansion, re-isolated allogeneic T cells from APG101 treated mice showed no changes in proliferation towards alloantigen or third party antigens compared to T cells isolated from GVHD suffering mice. T cell expansion in APG101-treated mice was further confirmed by efficient T cell invasion of host tissues in vivo, which was reported to be impaired if CD95L-
deficient T cells were used as allogeneic effector cells.\textsuperscript{43,44} Taken together, these data indicate that APG101 predominantly prevents apoptosis of CD95-expressing target organs, which was confirmed by a decrease of active capsase-3-expressing cells in liver and intestine and the inefficiency of APG101 to prevent GVHD in a perforin/granzyme-dependent BMT model. Since APG101 prevents CD95L-mediated GVHD in murine allogeneic BMT models and does not inhibit the anti-tumor cytotoxicity of allogeneic T cells it might be a potential therapeutic reagent for prevention or even treatment of human GVHD after allogeneic BMT. However, while murine GVHD models are well defined by their dependence on either the CD95/CD95L or perforin/granzyme system\textsuperscript{27} the situation in humans appears to be more complex. Elevated CD95L expression was detected in patients with acute and chronic GVHD, however perforin and granzyme B serum levels were also increased indicating that both effector pathways contribute to human GVHD.\textsuperscript{45-48} Applying APG101 as a mono therapy in humans might therefore not be sufficient for GVHD inhibition and most likely requires the administration of other immunosuppressive drugs.

In summary, we showed that APG101 is an effective therapeutic reagent to prevent CD95L-mediated GVHD but preserves anti-tumor cytotoxicity and early T cell immunity. These data suggest that APG101 could be incorporated into protocols for GVHD prevention and treatment during BMT.
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Authorship Contributions

N.H. performed experiments, analyzed data and reviewed the manuscript. 
J.J.M. performed experiments, analyzed data, prepared the figures and reviewed the manuscript. 
F.L. performed histological analysis, analyzed data and reviewed the manuscript. 
M.W. performed experiments. 
M.K. provided APG101, designed research, and reviewed the manuscript. 
H.F. provided APG101 and reviewed the manuscript. 
K.-M.D. obtained funding, designed research, and reviewed the manuscript. 
G.S. designed research, wrote the paper, and obtained funding for the project.

Conflict of Interest Disclosures

N.H. received research funding from Apogenix GmbH. 
J.J.M. has no conflict of interest to declare. 
F.L. has no conflict of interest to declare. 
M.W. has no conflict of interest to declare. 
M.K. is employed by Apogenix GmbH. 
H.F. is employed by Apogenix GmbH and has ownership interests. 
K.-M.D. obtained research funding and has consultancy within the past two years. 
G.S. obtained research funding from Apogenix GmbH.
References


Figure legends

Fig. 1: APG101 inhibits CD95-mediated apoptosis but does not prevent T cell proliferation or cytotoxicity in vitro

A) Spleen cells from B6 mice were incubated with medium or recombinant CD95L plus enhancer in the presence or absence of increasing concentrations of APG101. After 24 h cells were stained for CD4+ and CD8+ expression and apoptosis was determined in both T cell populations by measuring 7-AAD positive cells. Data present the mean of three independent experiments with triplicates ± SD. B) CFSE-labeled B6-derived spleen cells were stimulated with medium or irradiated B6D2F1-derived allogeneic spleen cells in the presence or absence of increasing concentrations of APG101. After 6 days proliferation of CD4+ and CD8+ T cells was measured. Data represent the mean of triplicates ± SD of one representative experiment out of three experiments performed. C) H-2d-specific CTLs were established by co-incubation of B6-derived spleen cells with irradiated B6D2F1 spleen cells in the presence or absence of APG101 (100 ng/ml) (1st stim.). After each week living cells were isolated and re-stimulated with irradiated B6D2F1 spleen cells (2nd stim., 3rd stim.) in the presence or absence of APG101. Six days after each stimulation, H-2d-specific cytotoxicity of CTLs was tested on P815 (H-2d) or EL4 (H-2b) target cells in a chromium release assay. Data are representative for one experiment out of three done.

Fig. 2: APG101 prevents GVHD in a MHC class I and II-mismatched parent → F1 BMT model

A-E) Lethally irradiated B6D2F1 recipient mice were reconstituted with BM from B6 mice without or with B6-derived spleen cells (SC) and were treated with PBS or APG101 starting one day before transplantation (day -1) or with APG101 starting six (day 6) or 13 days (day 13) after transplantation. Weight reduction (A), GVHD-scores (B) and survival (C) were determined. C): BM+SC (PBS, day -1) vs. BM+SC (APG101, day-1), p<0.05*; vs. BM+SC
Surviving animals / total animals treated are indicated in C. On day 16 after transplantation paraffin sections of ileum, colon, and liver of 7 – 10 animals / treatment group were analyzed for histological signs of GVHD i.e. inflammation, active caspase-3 p<0.05*, p<0.001*** (D). On day 16 after transplantation active caspase-3 was stained at room temperature on colon sections of BM + spleen cells transplanted mice treated with PBS or APG101 on day -1 (Zeiss Axiphot microscope, Plan-Neofluar objective lens, 20x magnification, 0.50 numerical aperture, JVC-KY-F75U digital camera and DISKUS software, version 4.5) (E). Data represent the combination of two individual experiments.

**Fig. 3: APG101 significantly decreased GVHD-induced death in a minor histocompatibility-mismatched BMT model**

A-C) Lethally irradiated BALB/c recipient mice were reconstituted with TCD BM from B10.D2 mice with or without B10.D2-derived spleen cells (SC) and were treated with PBS or APG101 starting one day before transplantation (day -1). Survival (A) and GVHD-scores (B) were determined and surviving animals / total animals treated are indicated in A. TCD BM+SC (PBS) vs. TCD BM+SC (APG101), p<0.001*** Histological signs of GVHD in liver, skin, and intestine of 6-10 animals/ treatment group were either determined the day mice were killed due to their moribund state and for all surviving mice at the end of the experiment. Data are from one experiment performed.

**Fig. 4: APG101 treatment did not affect donor engraftment, T cell phenotype, function or homing in allogeneic BM-transplanted animals**

A-E) Lethally irradiated B6D2F1 recipient mice (H-2<sup>bd</sup>, CD45.2<sup>+</sup>) were reconstituted with BM from B6 mice (H-2<sup>b</sup>, CD45.2<sup>+</sup>) plus B6.SJL-derived spleen cells (H-2<sup>b</sup>, CD45.1<sup>+</sup>) and were treated with PBS or APG101 starting one day before transplantation. 14 days after
transplantation mice were sacrificed and spleen cells were analyzed for total cell count and the numbers of CD45.1+ T, NK, NK-T cells and T\textsubscript{reg} (A) and T cell phenotype (B) by flow cytometry. To measure the proliferative capacity and cytotoxicity of allogeneic T cells, CD45.1+ T cells were purified from spleens of PBS- and APG101-treated mice 14 days after transplantation. CFSE-labeled T cells were stimulated in vitro with anti-CD3 and CD28 antibodies, irradiated DBA/2 (H-2\textsuperscript{d}) spleen cells (alloantigen) or C3H (H-2\textsuperscript{k}) spleen cells (third party). Proliferation of CD3/CD28-activated CD4\textsuperscript{+} and CD8\textsuperscript{+} CD45.1\textsuperscript{+} T cells was measured after 4 days while allogeneic and third party proliferation was determined after 6 days (C). Cytotoxic specificity of splenic CD45.1\textsuperscript{+} T cells was analyzed on P815 and EL4 cells in chromium release assays. Addition of CMA (10 nM) or APG101 (100 ng/ml) during the assay indicates the cytotoxic effector mechanisms used (D). Different days after transplantation, spleen and liver of transplanted mice were analyzed for the percentage of infiltrating CD45.1\textsuperscript{+} T cells by flow cytometry (E). Data represent the mean value ± SD of 6 mice analyzed in (A), of triplicates from CD45.1\textsuperscript{+} T cells, which were re-isolated and pooled from spleens of 30 mice treated in (C), of triplicates from spleen cells, which were pooled from 30 mice treated and adjusted for an effector : target ration of CD45.1\textsuperscript{+} T cells : target cells as described in M+M in (D), and the mean values ± SD of three mice analyzed in (E). B is representative for one mouse out of 6 analyzed. All data are representative for one experiment out of at least two experiments performed. No statistical significant differences between APG101- or PBS-treated animals were detected in all experiments performed.

**Fig. 5: APG101 treatment does not prevent GVT activity**

A-E) Lethally irradiated B6D2F1 mice were transplanted with B6-derived TCD BM in the presence or absence of B6-derived spleen cells (SC) and treated with PBS starting one day before transplantation (day -1) or APG101 starting one day before (day -1) or six days after (day 6) transplantation. P815 mastocytoma cells were co-injected on day 0 (A-C) while
injection of GFP-expressing B-ALL cells was performed seven days after transplantation (D, E). P815-injected mice were analyzed for survival (A) and surviving animals / total animals treated are A. TCD BM+SC (PBS day -1) vs. TCD BM+SC (APG101 day -1) p<0.001***; vs. TCD BM+SC (APG101 day 6), p< 0.001***. Spleen and liver weights (B) were determined either the day mice were killed due to their moribund state or at the end of the experiment. (p<0.001***). Tumor manifestation was also determined by flow cytometry of liver lymphocytes. FACS analysis is shown for one representative mouse out of at least three mice analyzed at the end of the experiment (for mice transplanted with TCD BM plus SC and treated with APG101) or the day mice were killed due to their moribund state (for mice transplanted with TCD BM alone or TCD BM plus SC and treated with PBS) (C). B-ALL injected mice were analyzed for survival and surviving animals / total animals treated are indicated (D). TCD BM+SC (PBS day-1) vs. TCD BM+SC (APG101 day -1), p=0.3ns; vs. TCD BM+SC (APG101 day 6), p=0.1ns. Tumor manifestation by GFP-expression in bone marrow and spleen is shown by flow cytometry for one representative mouse out of at least three mice analyzed either at the end of the experiment (for mice transplanted with TCD BM plus SC and treated with APG101) or the day mice were killed due to their moribund state (for mice transplanted with TCD BM alone or TCD BM plus SC and treated with PBS) (E).
**Fig. 1**

**A**

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**B**

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**C**

- **- APG101**
  - ![Graph showing specific lysis vs effector:target ratio](image5)
  - ![Graph showing specific lysis vs effector:target ratio](image6)

- **+ APG101**
  - ![Graph showing specific lysis vs effector:target ratio](image7)
  - ![Graph showing specific lysis vs effector:target ratio](image8)

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Fig. 3

(A) Survival of mice within each group during the experiment. The survival rates are indicated by the black and red lines, with the corresponding group sizes shown in parentheses. The time axis is labeled in days (d).

(B) Graph showing the GvHD-score over time for each treatment group. The black, gray, pink, and blue lines represent different groups, and the time axis is labeled in days (d).

(C) Bar graphs showing the histological score for liver, skin, and ileum + colon for each treatment group. The scores are compared across different treatment groups, and the corresponding group sizes are shown in parentheses. The asterisks indicate statistical significance.
Recombinant CD95-Fc (APG101) prevents graft-versus-host disease in mice without disabling anti-tumor cytotoxicity and T cell functions

Natalie Hartmann, Joanna J. Messmann, Frank Leithäuser, Maxi Weiswange, Michael Kluge, Harald Fricke, Klaus-Michael Debatin and Gudrun Strauss

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