#4529: Neutralization of pro-apoptotic CD95L by Asunercept/APG101 does not impair anti-tumor immune responses

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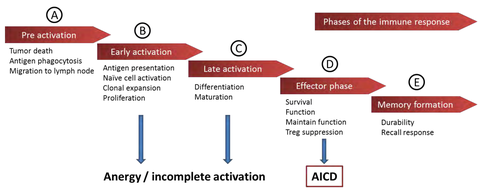
Abstract: The CD95 ligand (CD95L) is frequently overexpressed in cancers and tumor-associated endothelia, but also other immune cells like MDSCs and Tregs. Binding of CD95L expressed on tumors or regulatory immune cells to activated CD95-expressing effector cells triggers activation-induced apoptosis (AICD) or impairs their proliferation. In contrast, most tumors do also express CD95, but are intrinsically resistant to CD95-induced apoptosis. Thus, CD95L in the tumor microenvironment greatly contributes to the observed immunosuppression and escape from tumor surveillance by the immune system, making CD95 a potential immune checkpoint.

Here we examined the effects of APG101 on innate and adaptive immune cells and subsequent effects on tumor cell killing. Subtypes of *in vitro* differentiated macrophages generated with and without exposure to APG101 were functionally and phenotypically analyzed by ELISA and multi-color flow cytometry following various stimuli. APG101 did not alter differentiation patterns and response of M1- and M2-like macrophages *in vitro*. Direct co-culture of monocytes with tumor cells resulted in an M2/TAM-like phenotype which was not influenced by APG101, but re-programming to an M1-like state was achieved by addition of a CD40 agonist. Effects of APG101 on the proliferation and activation of CD8+ T cells in the presence of autologous CD4+ T(reg) cells and allogeneic APC was assessed by CFSE-dilution and multi-color flow cytometry, respectively. The proliferation rate of CD8 T cells in co-cultures with CD4 T(reg) cells in response to stimulation with APCs was increased in the presence of APG101. Real-time cell analysis was performed employing direct co-cultures of activated T cells and tumor cell lines. Tumor killing assays using direct co-cultures of *in vitro* activated T cells with and without APG101 demonstrate that tumor killing was not impaired by APG101.

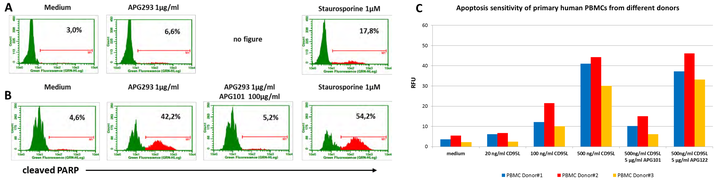
Conclusion: Asunercept (APG101) is a potent inhibitor of pro-apoptotic/anti-proliferative CD95/CD95L signaling in immune cells and protects activated immune cells from activation induced cell death (AICD). Our results suggest that APG101 does not impair CD8 T cell activation, but rather supports their proliferation by disrupting CD95/CD95L interaction with regulatory T cells. Importantly, the primary anti-tumor killing mechanisms is most likely CD95L-independent and remains unaffected by the presence of APG101. The inhibition of CD95 signaling as an immune checkpoint represents an attractive and novel concept for immunotherapeutic treatment of tumors and the combination of Asunercept/APG101 with co-stimulatory TNFR-SF agonists is currently being investigated.

Activation of immune cells requires multiple steps and is often countered by tumours



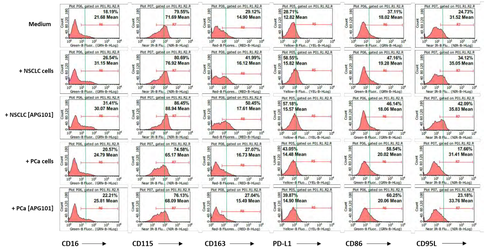
Elimination of excess effector cells is a normal process in immune homeostasis.
This process termed activation induced cell death (AICD) is mediated by the pro-apoptotic function of the CD95/CD95 ligand signaling pathway. Tumour cells frequently overexpress CD95 ligand and thereby prevent an efficient anti-tumour response of the immune system (= tumour counter-attack).

Asunercept inhibits acquired sensitivity of primary T cells and PBMCs to CD95L-induced killing



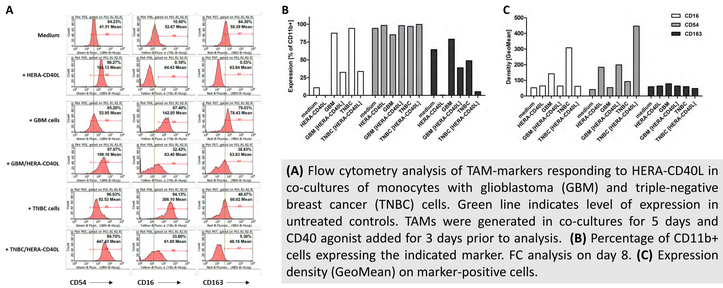
***In vitro* activation sensitizes primary T-lymphocytes to CD95L-induced apoptosis.**
Apoptosis in buffy coat derived naive T cells was either induced by soluble CD95L (APG293) or Staurosporine on day 0 **(A)** or following activation using PHA + IL-2 for three days prior to treatment **(B)**. CD95L induced apoptosis (i.e. appearance of cleaved PARP) is inhibited by Asunercept (APG101). **(C)** PBMCs from different donors activated with α–CD3 (TCR) and α–CD28 antibodies undergo apoptosis upon CD95 ligation. Caspase 3/7 activation is inhibited by Asunercept (APG101) but not by a CD95L-binding deficient APG101-mutant (APG122).

Primary monocytes acquire a TAM-like phenotype in direct co-cultures with tumor cells, which is not altered by Asunercept



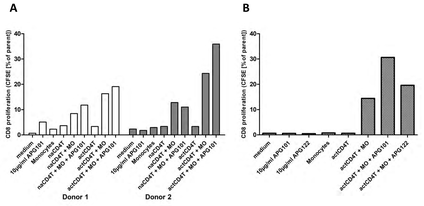
Flow cytometry analysis of tumor-associated macrophage (TAM) markers in co-cultures of monocytes with lung (NSCLC) and prostate cancer (PCa) cells. Green line indicates level of expression in untreated controls. TAMs were generated in direct co-cultures for 5 days without or with addition of APG101 to a final conc. of 10 µg/ml. Percentage of TAM-marker positive cells (gate: CD11b+) and expression density (GeoMean) on marker-positive cells is indicated in the figure. APG101 overall did not show an effect on TAM differentiation *in vitro*.

In vitro generated TAMs are repolarized by CD40 activation in co-cultures with tumor cells



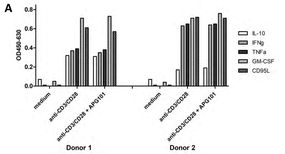
(A) Flow cytometry analysis of TAM-markers responding to HERA-CD40L in co-cultures of monocytes with glioblastoma (GBM) and triple-negative breast cancer (TNBC) cells. Green line indicates level of expression in untreated controls. TAMs were generated in co-cultures for 5 days and CD40 agonist added for 3 days prior to analysis. **(B)** Percentage of CD11b+ cells expressing the indicated marker. FC analysis on day 8. **(C)** Expression density (GeoMean) on marker-positive cells.

Asunercept increases proliferation of primary CD8+ T cells in response to allogeneic activation

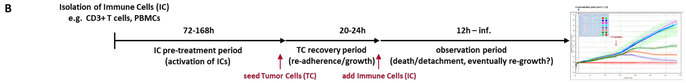


(A) Isolated CD8+ T cells of 2 healthy donors were labelled with CFSE and exposed to resting (naCD4T) or preactivated (actCD4T) autologous CD4+ T cells in the presence or absence of allogeneic monocytes and APG101 as indicated. Fowllowing incubation for 5 days, the dilution of CFSE on proliferating CD8+ T cells was analyzed by flow cytometry. **(B)** Repetition of experiment shown in (A) with cells from different donor. The specificity of the APG101 effect was confirmed using a CD95L-binding deficient mutant protein (APG122).

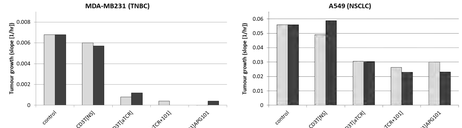
Asunercept preserves the anti-tumor response of T cells *in vitro:* Detection of cytokines by ELISA and direct killing of tumor cells analyzed by RTCA



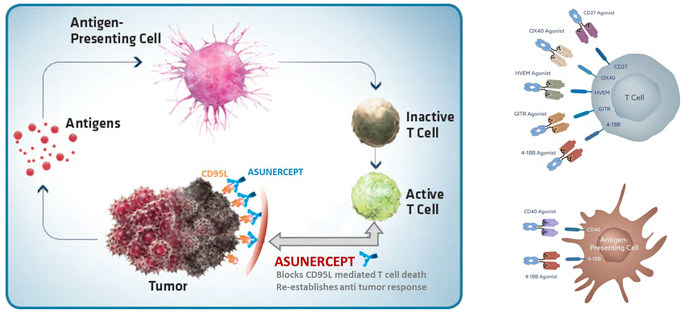
(A) Total CD8+ T cells were isolated from healthy donor buffy coats and cultured in medium containing 1 ng/ml IL-2. Cells were either left untreated [medium] or activation was performed by simultaneous stimulation of CD3 and CD28 using immobilized antibodies. APG101 was included at final conc. of 10 µg/ml during activation. Cytokine release into the supernatant of cultured cells is quantified by ELISA using commercially available kits from Peprotech and Bio-Techne.



(B) Total CD3+ T cells were isolated from healthy donor buffy coats and cultured in medium containing 1 ng/ml IL-2. Cells were either left untreated [NS] or activation was performed by simultaneous stimulation of CD3 and CD28 [aTCR] using immobilized antibodies. APG101 was included at final conc. of 10 µg/ml during activation phase only [TCR+101] and omitted for the RTCA kill-assay, or APG101 supplementation was carried forward during the assay [aTCR+101]APG101. Real time cell analysis (RTCA) of anti-tumor killing activity of the T cells was performed on A549 NSCLC cells and the TNBC cell line MDA-MB231. Tumor cells were seeded at 5000 cells/well in the RTCA instrument and grown for 22 hours. Following 5 days of activation, T cells were added at a effector:target ratio of 10:1. Reduction of TC growth is shown depicted as slope analysis of the growth curve (CI = 1/hr).



Asunercept – A Member of Apogenix Immuno-Oncology Pipeline



Asunercept (APG101) is a fully human fusion protein consisting of the extracellular domain of CD95 and the Fc-domain of human IgG1, which efficiently inhibits CD95/CD95L signaling. Clinical efficacy has been demonstrated in a controlled randomized phase 2 study in patients with recurrent glioblastoma and a phase 1 study in low-intermediate risk MDS.