Poster 3251

Fibroblast activation protein (FAP)-selective delivery of CD40 agonistic DARPin® molecule for tumor-localized immune activation

Nicolo' Rigamonti[1*], Anja Schlegel[1], Sophie Barsin[1], Jonas Schwestermann[1], Susanne Mangold[1], Yvonne Kaufmann[1], Christof Zitt[1], Niina Veitonmäki[1], Victor Levitsky[1], Pamela A.Trail[1] and Clara Metz[1]

[1]Molecular Partners AG, Schlieren-Zurich, Switzerland.
*Corresponding Author: nicolo.rigamonti@molecularpartners.com

Summary

CD40 is a co-stimulatory molecule belonging to the tumor necrosis factor receptor superfamily which can activate both the innate and adaptive immune system, making it an interesting target for tumor immunotherapy. Agonistic anti-CD40 monoclonal antibodies (mAbs) have shown signs of activity in cancer patients, but have been limited by systemic toxicities. New approaches are therefore needed to increase the therapeutic index of CD40-targeting molecules and achieve better clinical outcomes. Here, we report an alternative approach designed to activate CD40 within the tumor microenvironment (TME), and not systemically, in order to increase efficacy and reduce systemic toxicity. This novel approach is based on a bispecific DARPin® molecule, designed to target both CD40 and fibroblast activation protein (FAP) alpha, to induce immune activation only when clustered by binding to FAP-expressing cells in the TME.

The bispecific FAPxCD40 DARPin® molecule induced a phenotypic and functional activation of primary human 1) B cells, 2) dendritic cells and 3) macrophages, resulting in upregulation of co-stimulatory molecules (CD80, CD86, CD69 and MHCII) and secretion of proinflammatory cytokines (IL-12, TNFα and IL-6) and chemokines (CCL17 and CXCL10), only in the presence of FAP-positive, but not with FAP-negative cells, confirming a mechanism of action strictly dependent on FAP-mediated crosslinking.

A surrogate mouse-specific FAPxCD40 DARPin® molecule (mFAPxCD40) was generated and tested in similar *in vitro* assays and showed FAP-dependent activation of CD40 and comparable results as the human construct.

In vivo experiments, performed in tumor-free mice, showed a comparable half-life between mFAPxCD40 and an anti-mouse CD40 Ab (clone FGK45). However, mFAPxCD40, in contrast to the FGK45 Ab, did not increase the serum level of IL-6, supporting a mode of action that is dependent on FAP-mediated crosslinking of CD40 receptors *in vivo*. A transgenic murine tumor model expressing FAP was generated to test the *in vivo* activity of mFAPxCD40 in tumor bearing mice. These experiments are currently ongoing.

In conclusion, we have generated bispecific agonist FAPxCD40 DARPin® molecules able to activate the CD40 pathway with a targeting (FAP)-dependent mechanism of action.

Mode of Action

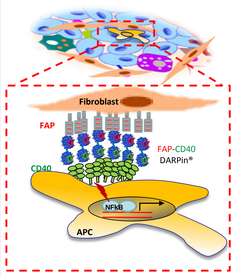


Figure 1. Mechanism of action.

- DARPin® molecules are small, engineered proteins, derived from natural ankyrin repeat proteins, able to bind to specific targets
- Half-life extension using serum albumin-binding DARPin® domain
- Individual DARPin® molecules can be linked together genetically in order to create multi-specific drug molecules
- CD40 receptor-mediated signaling requires a high level of oligomerization of the CD40 receptor
- FAP is abundantly expressed in the stroma of many solid tumors, in contrast to healthy human tissues
- Targeting-dependent mechanism of action: tumor-confined antigen presenting cell (APC) activation by binding to FAP-expressing cells

Assay description

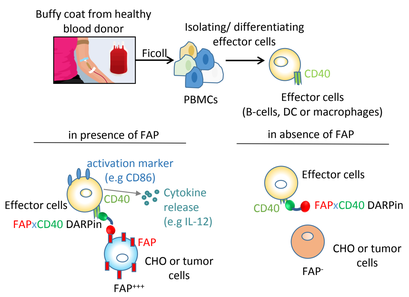


Figure 2. Illustration depicting the *in vitro* cellular assay used to characterize the activity of human FAPxCD40 bispecific DARPin® molecule.
All graphs of this poster show representative data of at least two independent experiments. All graphs present means, and error bars represent standard deviations (SD).

Human DARPin®: B cell assay

DARPin® molecule activates B cells in a FAP-specific manner

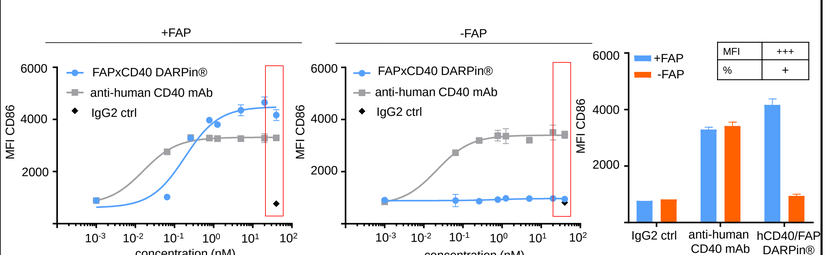
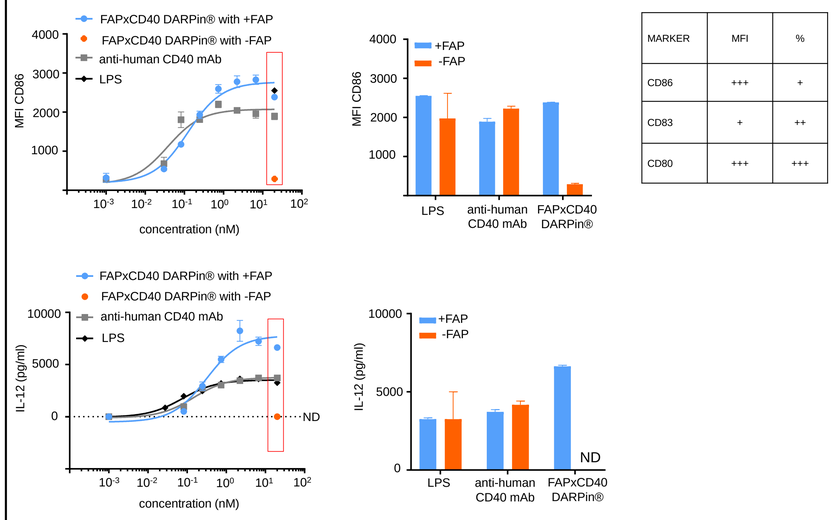


Figure 3. Functional characterization of human FAPxCD40 bispecific DARPin® molecule in a B cell activation assay. Purified human CD19+ B cells were cocultured either with FAP-positive (+FAP) or FAP-negative (-FAP) CHO cells and treated with different concentration of reagents, as indicated in the titration graphs. After 24 h incubation, CD20+ B cells were analyzed by FACS for the upregulation of CD86. Mean fluorescence intensity (MFI) and percentage (%, shown only in the table) were measured. Antibody (anti-human CD40 Ab) was used as positive control (+CTRL) and clinical comparator. Column graph highlights the values obtained for the highest dose of the titration graphs (red square). += 2<x≤3 and +++= 5<x ≤ 10. x= fold change.

Human DARPin®: Dendritic cell assay

DARPin® molecule activates dendritic cells (DC) in a FAP-specific manner



MARKER	MFI	%
CD86	+++	+
CD83	+	++
CD80	+++	+++

Figure 4. Functional characterization of human FAPxCD40 bispecific DARPin® molecule in a DC activation assay. Human monocyte-derived DC were cocultured with irradiated FAP-positive (+FAP) or FAP-negative (-FAP) CHO cells as described in Fig.3, and CD86, CD80 and CD83 expression on CD14- cells was analyzed by FACS as MFI and % (shown only in table). IL-12 production was measured by ELISA in the supernatant. Column graphs highlight the values obtained from the highest dose of the titration graphs (red square). Antibody (anti-human CD40 Ab) was used as +CTRL and clinical comparator. Lipopolysaccharide (LPS) was used as further +CTRL. Table summarizes the expression of the other markers: += 2<x≤3, ++= 3<x≤5 and +++= 5<x≤10. x= fold change. ND= non-detectable.

Human DARPin®: Macrophage assay

DARPin® molecule activates macrophages in a FAP-specific manner

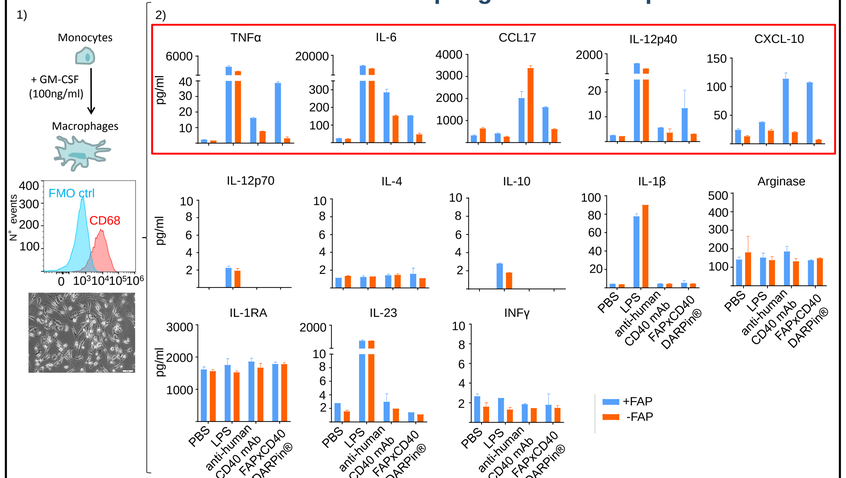
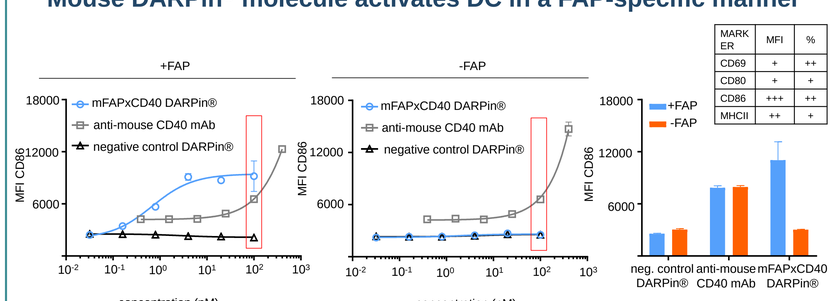


Figure 5. Functional characterization of human FAPxCD40 bispecific DARPin® molecule in a macrophage activation assay. Human monocyte-derived CD68+ macrophages were cocultured with irradiated FAP-positive (blue) or FAP-negative (orange) CHO cells, as described in Fig.3 and 13 cytokines were measured in the supernatant by cytokine array. Antibody (anti-human CD40 Ab) was used as +CTRL and clinical comparator. LPS was used as further +CTRL. Macrophage differentiation was monitored by CD68 expression by FACS (histogram graph) and light transmitted microscopy.

Mouse DARPin®: Dendritic cell assay

Mouse DARPin® molecule activates DC in a FAP-specific manner



MARKER	MFI	%
CD69	+	++
CD80	+	+
CD86	+++	++
MHCII	++	+

Figure 6. Functional characterization of mouse FAPxCD40 bispecific DARPin® molecule in a DC activation assay. Mouse bone marrow (BM)-derived DC were cocultured with irradiated FAP-positive (+FAP) or FAP-negative (-FAP) CHO cells, as described in Fig.3 and CD69, CD80, CD86 and MHC-II expression on CD45+CD11c+ double positive cells was analyzed by FACS as MFI and % (shown only in table). Column graph highlights the values obtained for the highest dose of the titration graphs (red square). Crosslinked antibody, clone FGK45, (anti-mouse CD40 Ab) was used as +CTRL. Negative control DARPin® is the human-specific FAPxCD40 DARPin® molecule. Table summarizes the expression of the other markers: + = 1.5 ≤ x < 2, ++ = 2 ≤ x ≤ 3 and +++ = x > 3. x = fold change.

Mouse DARPin®: Macrophage assay

DARPin® molecule skews M2-like macrophages towards M1-like macrophages

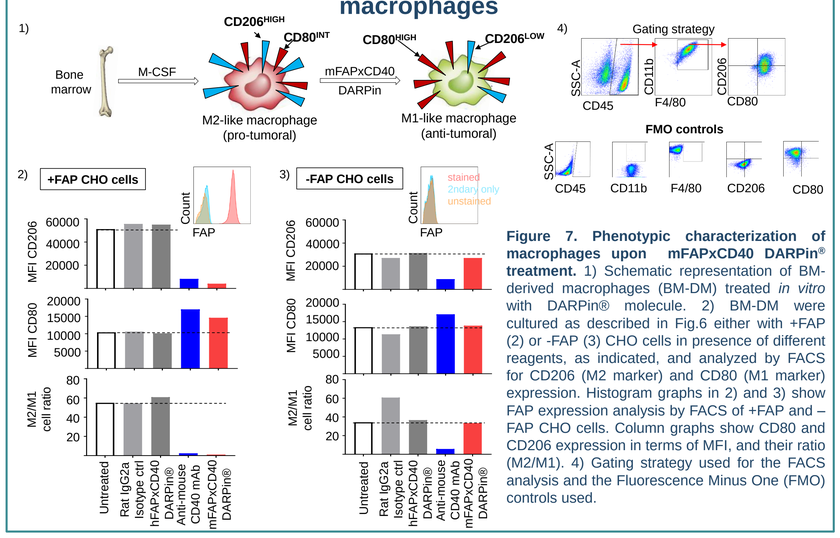


Figure 7. Phenotypic characterization of macrophages upon mFAPxCD40 DARPin® treatment. 1) Schematic representation of BM-derived macrophages (BM-DM) treated *in vitro* with DARPin® molecule. 2) BM-DM were cultured as described in Fig.6 either with +FAP (2) or -FAP (3) CHO cells in presence of different reagents, as indicated, and analyzed by FACS for CD206 (M2 marker) and CD80 (M1 marker) expression. Histogram graphs in 2) and 3) show FAP expression analysis by FACS of +FAP and –FAP CHO cells. Column graphs show CD80 and CD206 expression in terms of MFI, and their ratio (M2/M1). 4) Gating strategy used for the FACS analysis and the Fluorescence Minus One (FMO) controls used.

In vivo

DARPin® molecule is active also with +FAP MC38 tumor cells

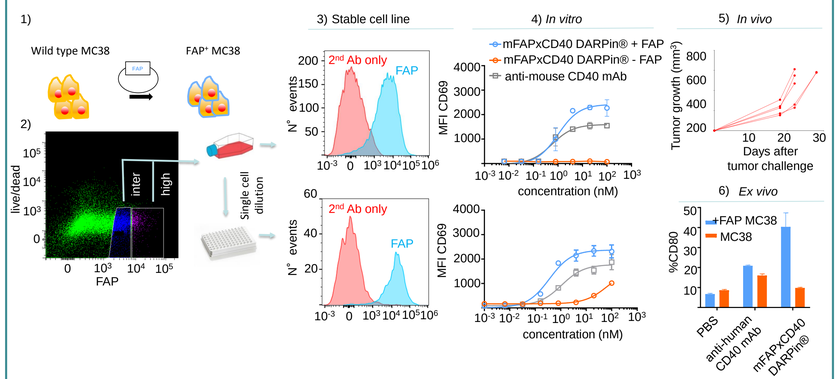


Figure 8. Generation of a +FAP *in vivo* tumor model. 1) Representation of the FAP transfection on MC38 cells. 2) FACS sorting of intermediate (inter) and high FAP-expressing transfected MC38 cells. 3) FAP analysis by FACS of the stable cell lines generated from sorted FAP-positive transfected MC38 cells, these cell lines are termed as +FAP MC38. 4) B cell activation assay (as described in Fig. 3) using +FAP MC38, showing that the amount of FAP provided by +FAP MC38 is enough to activate B cells. 5) In vivo tumor growth in immunocompetent mice of +FAP MC38. 6) B cell activation assay using +FAP MC38 harvested from tumor-bearing mice (blue) or wild type MC38 cells (orange), showing that FAP+MC38 maintains FAP expression *in vivo* that is enough to activate B cells *in vitro* in presence of mFAPxCD40 DARPin molecule.

1) DARPin® molecule shows systemic half-life comparable to the anti-CD40 Ab, FGK45; 2) In contrast to FGK45, the DARPin® molecule does not induce a systemic increase of IL-6.

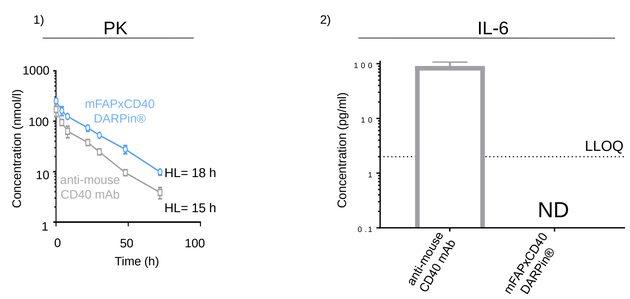


Figure 9. Pharmacokinetic and pharmacodynamic characterization of mFAPxCD40 bispecific DARPin® molecule. Tumor free mice were injected with 1 mg/kg of the indicated drugs and the sera concentrations of 1) the drug at different time points and 2) IL-6 after 22 h were measured by ELISA. Three mice per group were used. ND= Non-Detectable. LLOQ= Lower Limit of Quantification. HL= half life;

Conclusions

- The FAPxCD40 DARPin® molecule activates CD40 on B cells, macrophages and dendritic cells in a FAP-dependent manner. FAP-restricted activation provides the potential to localize CD40 mediated activation to the tumor within a subsequent improvement in efficacy and reduction in systemic toxicity;
- FAPxCD40 DARPin® molecule reduces M2-like (pro-tumoral) and increases M1-like (anti-tumoral) macrophages;
- FAPxCD40 DARPin® molecule shows CD40-agonist activity also in presence of FAP-expressing MC38 tumor cells;
- As expected, injection in tumor-free mice of mouse-specific FAPxCD40 DARPin® molecule does not lead to immune systemic activation measured by IL-6 production in the sera.