Poster 637

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

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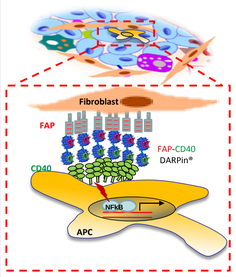
Summary

CD40 is a co-stimulatory molecule belonging to the tumor necrosis factor receptor superfamily which can activate both innate and adaptive immune system, making it an interesting target for tumor immunotherapy. Systemic activation of CD40 receptor, induced by administration of agonistic CD40 antibodies, has shown signs of activity in cancer patients, but dose-limiting toxicities have impaired the efficacy. New therapeutic approaches are therefore needed to increase the therapeutic index of CD40-targeting molecules and achieve better clinical outcomes. Here, we report a novel approach based on systemic administration of a tumor-localized CD40 agonistic DARPin® molecule targeting human CD40 and fibroblast activation protein (FAP) alpha, a tumor-associated antigen (TAA) abundantly expressed in many solid tumors, enabling CD40 pathway activation exclusively in the presence of TAA-expressing cells.

Using ribosome display technology, we generated a panel of bispecific CD40/FAP DARPin® molecules able to trigger specifically CD40 receptor and activate the NF-κB pathway in a reporter cell assay, in the presence of FAP-transfected Chinese hamster ovary (CHO) cells, but not in the presence of parental CHO cells. Selected bispecific DARPin® clones were then tested in more meaningful cell assays using human 1) primary B cells, 2) monocyte-derived dendritic cells and 3) monocyte-derived macrophages from whole blood, in the presence of FAP-positive or FAP-negative cells. In these immune cell populations, bispecific CD40/FAP DARPin® molecules confirmed a FAP-dependent activation of CD40 pathway inducing an upregulation of costimulatory molecules and proinflammatory cytokines, such as CD86 and interleukin (IL)-12, only in the presence of FAP-expressing cells. In order to properly address the *in vivo* activity, a surrogate mouse-specific CD40/FAP DARPin® molecule was also generated and tested in different *in vitro* assays showing a FAP-dependent activation and similar results as the human counterpart. *In vivo* pharmacokinetic and pharmacodynamic experiments, performed in tumor-free mice, showed a comparable half-life between mouse-specific CD40/FAP DARPin® molecule and the benchmark anti-mouse CD40 antibody (clone FGK45). However, DARPin® molecule, differently from the benchmark antibody FGK45, did not increase the serum level of IL-6, supporting a mode of action that is dependent on FAP-mediated crosslinking of CD40 receptor.

In conclusion, we have generated bispecific agonist CD40/FAP DARPin® molecules able to activate the CD40 pathway in cellular assays in a targeting-dependent manner, supporting the hypothesis that these DARPin® molecules could lead to a tumor-localized immune activation *in vivo*. *In vivo* experiments in mouse tumor models to test this hypothesis are ongoing. In *vivo* studies were approved by Veterinary Authorities of the Canton Zurich, approval number ZH102/16

Mode of Action



- DARPin® molecules are small engineered proteins, derived from natural ankyrin repeat proteins, able to bind to specific targets with high affinity
- 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

Figure 1. Mechanism of action.

Assay description

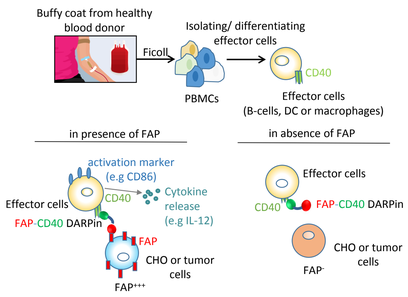


Figure 2. Illustration depicting the *in vitro* cellular assay used to characterize the activity of human CD40/FAP bispecific DARPin® molecule.
All graphs of this poster show representative data of at least two independent experiments. All graphs present means, and error bars represent standard deviations (SD).

Human DARPin®: B cell assay

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

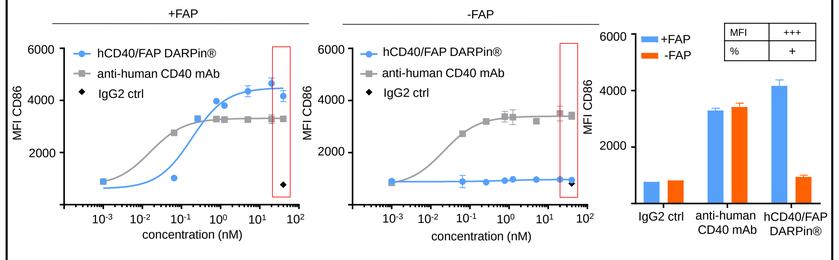
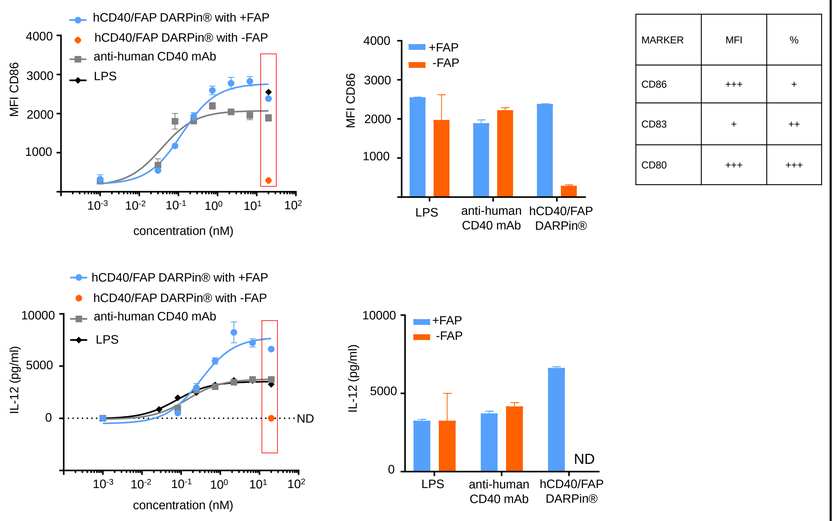


Figure 3. Functional characterization of human CD40/FAP bispecific DARPin® molecule in a B cell activation assay. Purified human CD19+ B cells were cocultured either with FAP-positive (+FAP) or FAP-negative (-FAP) CHO cells and treated with different concentration of reagents, as indicated in the titration 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, ++= 3<x ≤ 5 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 CD40/FAP 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 for 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

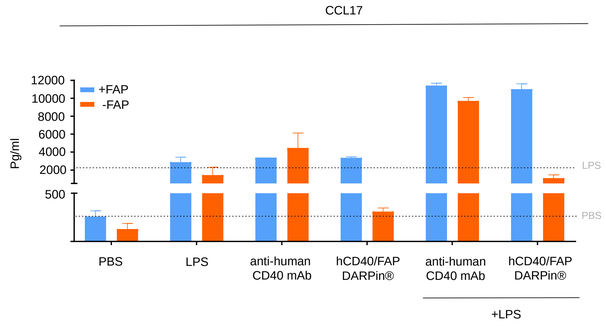
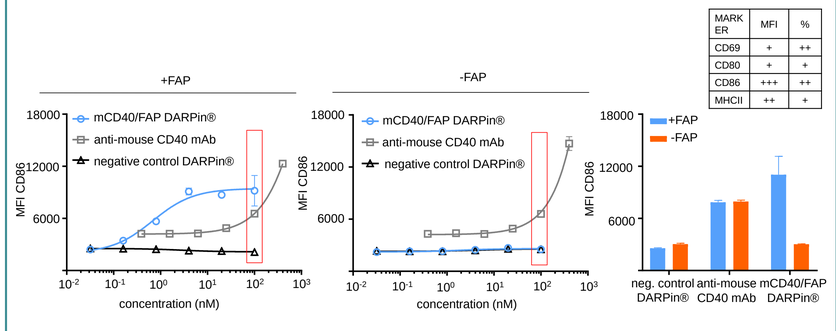


Figure 5. Functional characterization of human CD40/FAP bispecific DARPin® molecule in a macrophage activation assay. Human monocyte-derived CD68+ macrophages were cocultured with irradiated FAP-positive (+FAP) or FAP-negative (-FAP) CHO cells, as described in Fig.3 and CCL17 was measured in the supernatant by ELISA and cytokine array. Antibody (anti-human CD40 Ab) was used as +CTRL and clinical comparator. LPS was used as further +CTRL.

Mouse DARPin®: Dendritic cell assay

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

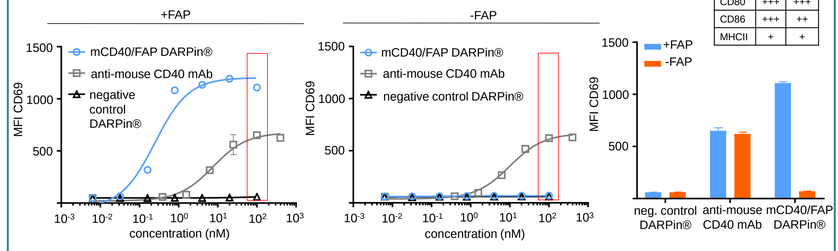


MARKER	MFI	%
CD69	+	++
CD80	+	+
CD86	+++	++
MHCII	++	+

Figure 6. Functional characterization of mouse CD40/FAP bispecific DARPin® molecule in a DC activation assay. Mouse bone marrow-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 CD40/FAP 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®: B cell assay

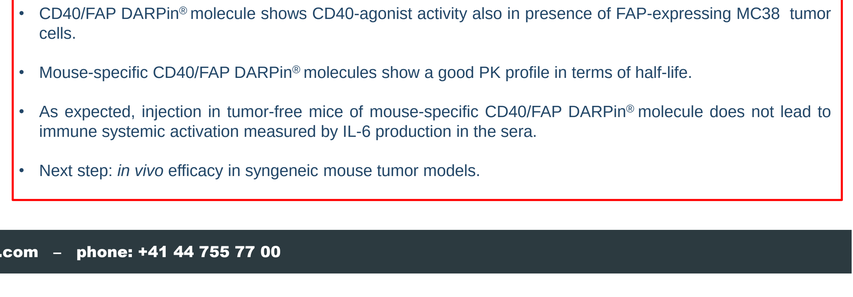
Mouse DARPin® molecule activates B cells in a FAP-specific manner



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

Figure 7. Functional characterization of CD40/FAP bispecific DARPin® molecules in a B cell activation assay. Purified splenic CD20+ B cells were cocultured with FAP-positive (+FAP) or FAP-negative (-FAP) CHO cells, as described in Fig.3 and CD69, CD80, CD86 and MHC-II expression on CD20+ 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 CD40/FAP DARPin® molecule. Table summarizes the expression of the other markers: + = 10 ≤ x < 20, ++ = 20 ≤ x ≤ 30 and +++ = x > 30. x = fold change.

Mouse DARPin® molecule activates B cells in presence of +FAP MC-38 tumor cells



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

Figure 8. Bispecific CD40/FAP DARPin® molecule activates B cells when FAP is expressed by tumor cells. Purified splenic CD20+ B cells were cocultured either with irradiated FAP-transfected MC38 (+FAP/MC38)- or wild type MC38 (-FAP/MC38)- tumor cells. After 48h incubation, CD20+ B cells were analyzed by FACS for the upregulation, in terms of MFI and % (shown only in the table), of CD69, CD80, CD86 and MHCII. Crosslinked antibody, clone FGK45, (anti-mouse CD40 Ab) was used as +CTRL. Negative control DARPin® is the human-specific CD40/FAP DARPin® molecule. Table summarizes the expression of the other markers: + = 10 ≤ x < 20, ++ = 20 ≤ x ≤ 30 and +++ = x > 30. x = fold change. FACS dot plot shows FAP expression on MC38 tumor cells.

In vivo

DARPin® molecule shows an half-life comparable to the benchmark CD40 Ab, FGK45

Differently from the FGK45, DARPin® molecule does not induce a systemic increase of IL-6

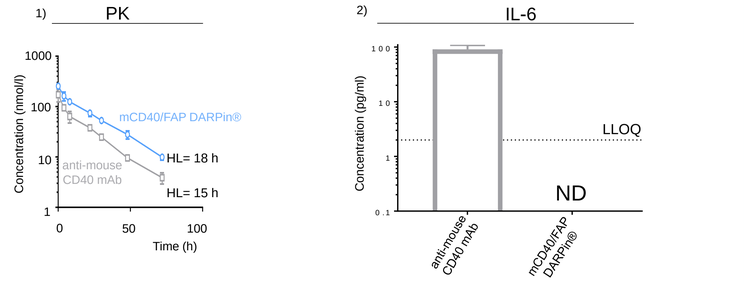


Figure 9. Pharmacokinetic and pharmacodynamic characterization of CD40/FAP bispecific DARPin® molecules. Tumor free mice were injected with 1 mg/kg of the indicated drugs and the sera concentrations of 1) the drug (left graph) at different time points and 2) IL-6 after 22 h (right graph) were measured by ELISA. Three mice per group were used. ND= Non-Detectable. LLOQ= Lower Limit of Quantification. HL= half life.

Conclusions

- Active and potent human-specific and mouse-specific CD40/FAP DARPin® molecules were generated and were able to activate different immune cell populations with a targeting (FAP)-dependent mechanism of action in *in vitro* cellular assays.
- CD40/FAP DARPin® molecule shows CD40-agonist activity also in presence of FAP-expressing MC38 tumor cells.
- Mouse-specific CD40/FAP DARPin® molecules show a good PK profile in terms of half-life.
- As expected, injection in tumor-free mice of mouse-specific CD40/FAP DARPin® molecule does not lead to immune systemic activation measured by IL-6 production in the sera.
- Next step: *in vivo* efficacy in syngeneic mouse tumor models.