Increasing Tumor-Infiltrating T Cells through Inhibition of CXCL12 with NOX-A12 Synergizes with PD-1 Blockade

Dirk Zboralski, Kai Hoehlig, Dirk Eulberg, Anna Fromming, and Axel Vater

Abstract

Immune checkpoint inhibitors promote T cell–mediated killing of cancer cells; however, only a subset of patients benefit from the treatment. A possible reason for this limitation may be that the tumor microenvironment (TME) is immune privileged, which may exclude cytotoxic T cells from the vicinity of cancer cells. The chemokine CXCL12 is key to the TME-driven immune suppression. In this study, we investigated the potential of CXCL12 inhibition by use of the clinical-stage l-RNA-aptamer NOX-A12 (olaptesed pegol) to increase the number of tumor-infiltrating lymphocytes. We used heterotypic tumor–stroma spheroids that mimic a solid tumor with a CXCL12-abundant TME. NOX-A12 enhanced the infiltration of T and NK cells in a dose-dependent manner. NOX-A12 and PD-1 checkpoint inhibition synergistically activated T cells in the spheroids, indicating that the agents complement each other. The findings were validated in vivo in a syngeneic murine model of colorectal cancer in which the addition of NOX-A12 improved anti–PD-1 therapy. Taken together, our work shows that CXCL12 inhibition can break the immune-privileged status of the TME by paving the way for immune effector cells to enter into the tumor, thereby broadening the applicability of checkpoint inhibitors in cancer patients.

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Introduction

The presence of tumor-infiltrating lymphocytes (TIL) is predictive of patient survival for many tumor types (1). Insufficient infiltration of T and NK cells into tumors may be a limiting factor for efficacious cancer immunotherapy such as checkpoint inhibition (2). Despite considerable clinical benefit of checkpoint inhibition in various cancers, such as melanoma, non–small cell lung cancer, and renal cell cancer, only a minority of patients even in these indications actually benefit from this treatment. A proposed reason for tumor resistance to checkpoint inhibitors such as anti–PD-1 is the failure of cytotoxic T cells to physically reach the cancer cells in the tumor. This exclusion is due to the immune-privileged tumor microenvironment (TME), acting via soluble and cell-surface mediators (3). Interfering with the mechanisms that promote the exclusion of immune effector cells from the vicinity of cancer cells may thus support cancer immunotherapy combination treatments (4).

The chemokine CXCL12, also known as stromal cell–derived factor-1 (SDF-1), is expressed in the TME mainly by cancer-associated fibroblasts (5). In particular, CXCL12 has been shown to confer resistance to checkpoint inhibitors through T-cell exclusion in an autochthonous mouse model of pancreatic adenocarcinoma (6). In this model, high CXCL12 concentrations in cancer cell–dense areas were suggested to exclude T cells from these so-called cancer cell nests, thereby preventing the T cells from exerting antitumor activity. Blockade of the CXCL12/CXCR4 axis allowed the T cells and tumor cells to intermingle and enhanced the efficacy of anti–PD-1 immunotherapy (7).

NOX-A12 (olaptesed pegol) is an l-configured aptamer (Spiegelmer) (8) that binds CXCL12 with high affinity and specificity across various species, including human, mouse, and rat (9). NOX-A12 was previously shown to not only directly bind and inhibit, but also detach the cell-surface bound CXCL12, leading to abrogation of the CXCL12 gradient (10). Of note, NOX-A12 inhibits the interaction of CXCL12 with both its receptors, CXCR4 and CXCR7 (9, 11). Clinically, NOX-A12 has been shown to be efficacious on top of standard therapy in relapsed or refractory multiple myeloma and chronic lymphatic leukemia (12, 13). Here, we investigated the potential of CXCL12 inhibition by NOX-A12 to enhance the efficacy of anti–PD-1 therapy by modulating the cellular composition of the TME in vitro. Using tumor–stroma spheroids mimicking a solid tumor with a CXCL12-abundant TME, we found increased numbers of T and NK cells after treatment with NOX-A12. Furthermore, NOX-A12 in combination with PD-1 checkpoint inhibition synergistically enhanced T-cell activation in the spheroids. The synergy of NOX-A12 and anti–PD-1 was validated in a syngeneic murine model of colorectal cancer, resulting in reduced tumor growth in the combination-therapy group compared with the group that received PD-1 inhibition alone.

Materials and Methods

Spheroid generation

Murine stromal MS-5 cells (DSMZ) and various tumor cell lines, HT-29 and PSN-1 (both from ATCC), U251MG and H1299 (both from ECACC), were detached and filtered to exclude cell clumps. MS-5 cells (2.5 × 10^5) and 0.5 × 10^4 tumor cells were
Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by ficoll centrifugation and immediately frozen. thawed PBMCs were filtered and pan-T cells and NK cells were isolated using MACS (Miltenyi Biotec). One hour after addition of a serial dilution of NOX-A12, 5 × 10^6 immune cells were added per well for overnight incubation. Spheroids were washed three times with PBS to remove loosely attached immune cells. Spheroids were dissociated using Accumax (eBiosciences) for 90 minutes for immune cell quantification by flow cytometry (Guava easyCyte, Millipore).

**Immune cell quantification in the spheroids**

Immune cells were added to the spheroids 3 days after seeding. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by ficoll centrifugation and immediately frozen. Thawed PBMCs were filtered and pan-T cells and NK cells were isolated using MACS (Miltenyi Biotec). One hour after addition of a serial dilution of NOX-A12, 5 × 10^6 immune cells were added per well for overnight incubation. Spheroids were washed three times with PBS to remove loosely attached immune cells. Spheroids were dissociated using Accumax (eBiosciences) for 90 minutes for immune cell quantification by flow cytometry (Guava easyCyte, Millipore).

**Immunohistochemistry (IHC)**

Spheroids were washed with PBS three times and fixed with 10% (v/v) neutral buffered formalin for 2 hours. After paraffinization, 2-μm slides were cut for H&E or immunostaining. The following antibodies were used: panCK (DAKO, #M0821), CXCL12 (R&D Systems, #MAB350), CD3 (DAKO, #M0452). IHC experiments were performed by Provitro GmbH (Germany). T cells were counted by three blinded observers.

**3D PD-1-dependent T-cell activation reporter assay**

In order to investigate T-cell activation in the spheroids, a PD-1/CD154 blockade reporter bioassay (Promega) was adapted to the 3D format that used an alternative readout from traditional primary cell-based assays. Primary T cells were replaced with a Jurkat cell line stably expressing TCR, PD-1, and NAFAT-inducible luciferase. The target cells were CHO-K1 cells expressing human PD-L1 and an engineered cell surface protein designed to activate cognate TCRs in an antigen-independent manner. When cocultured, TCR activation of the Jurkat cells induced the NAFAT pathway, whereas PD-1 signaling decreased it. Upon addition of an anti–PD-1 or anti–PD-L1 agent, the Jurkat–TCR axis induced NAFAT-mediated luciferase expression that can be detected by addition of Bio-Glo reagent and quantified with a luminometer. Briefly, Jurkat PD-1−/− T cells (5 × 10^4/well) were preincubated with anti–PD-1 (clone PD1.3.1.3, Miltenyi Biotec) for 30 minutes and subsequently added to tumor–stroma spheroids (CHO PD-L1+ + MS-5: 0.5 × 10^6 + 2.5 × 10^5/well, respectively) in the presence or absence of NOX-A12. The next day, T-cell infiltration was quantified by flow cytometry as described above. T-cell activation was quantified after incubating the spheroids in 75 μL medium (DMEM + 5% FBS) with 50 μL BioGlo substrate (Promega) for 45 minutes. Relative luminescence of anti–PD-1 and/or NOX-A12–treated spheroids was normalized to untreated spheroids. Combination Index was calculated using CompuSyn software (CombioSyn, Inc.).

**CT-26 in vivo efficacy study**

Female BALB/c mice (6–7 weeks, Shanghai Lingchang Biotechnology Co. Ltd) were subcutaneously inoculated with 5 × 10^6 CT-26 colon cancer cells (Shanghai Institutes for Biological Sciences) at the right lower flank. Tumor growth was monitored three times per week by caliper measurement. Tumor volume was calculated using the formula: \( V = \frac{a \times b^2}{2} \) where \( a \) and \( b \) are the long and short diameters of the tumor, respectively. NOX-A12 (20 mg/kg s.c., every other day) treatment was initiated at day 5 when mean tumor volume (MTV) reached approximately 50 mm³. Anti–PD-1 (RMPI-1, BioXCell, 10 mg/kg i.p., twice weekly) treatment was initiated at day 7 when MTV in untreated mice reached approximately 100 mm³. Mice were sacrificed at day 20. All in vivo experiments were performed at Crown Bioscience Inc. in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care.

**Statistical analysis**

Differences of infiltrated T cells between groups in IHC were quantified by the Student t test. In FACS analysis of spheroids two-way ANOVA was used with the Dunnet test for multiple comparisons to compare NOX-A12–treated spheroids to nontreated ones. Tumor volumes were analyzed using a global Kruskal–Wallis test. In case of a statistically significant effect, this was followed by pairwise Wilcoxon rank sum tests (SPSS, version 18.0, IBM; and SAS 9.4, SAS Institute Inc.). All tests were two-sided. \( P < 0.05 \) was considered statistically significant. Statistical analyses were performed by SCO:SSiS Consulting.

**Results**

For mechanistic in vitro studies on the role of CXCL12 in lymphocyte tumor infiltration, heterotypic 3D spheroids consisting of CXCL12-secreting MS-5 stromal cells and the pancreatic cancer cell line PSN-1 were established. We found that NOX-A12 enhanced the infiltration of primary human T cells into PSN-1/MS-5 spheroids in a dose-dependent manner. The number of NK cells in the spheroids was also enhanced by CXCL12 inhibition. Likewise, B cells infiltrated the microtissue but to a lesser extent. Infiltration of monocytes was not observed (Fig. 1A). A closer look into the T-cell subsets by using isolated primary pan-T cells revealed a similar infiltration rate for CD8⁺ and CD4⁺ T cells, whereas Tregs migrated to a lesser degree into the spheroids after treatment with NOX-A12 (Fig. 1B). IHC analysis of paraffin-embedded sections showed a homogeneous distribution of tumor cells (PanCK staining) and stromal cells throughout the spheroids (Fig. 1C) CXCL12 was evenly expressed in the spheroid apart from the necrotic core which resembles a real tumor. IHC staining confirmed the finding of enhanced T-cell infiltration in the presence of NOX-A12 in the PSN-1/MS-5 spheroids as well as in the HT-29/MS-5 spheroids. The crescent-formed CD3⁺ T-cell localization of the latter spheroids was likely due to the fact that only the bottom of the spheroid had contact with the T cells and the cultures were not shaken overnight (Fig. 1D).

Next, we confirmed the enhanced lymphocyte infiltration upon CXCL12 inhibition in various other spheroid types using MS-5 stromal cells and tumor cell lines from four different origins (PSN-1 pancreatic adenocarcinoma cells, HT-29 colorectal carcinoma cells, H1299 non–small-cell lung cancer cells, and U251MG glioblastoma cells). In addition to T cells, the effect of CXCL12 inhibition on the migration of primary NK cells was tested in these experiments. We found a similar dose-dependent increase in spheroid T-cell infiltration in all four spheroid types. Approximately 30% of the T cells that were added to the assay infiltrated the spheroids at baseline. The addition of 10 nmol/L NOX-A12 led to a 2- to 3-fold increase in spheroid T-cell infiltration.
The baseline of NK-cell infiltration was lower (5–10%). However, when NOX-A12 was added to the spheroids, the NK-cell infiltration rate increased up to 8-fold (Fig. 2B).

To analyze whether enhanced T-cell infiltration would boost the efficacy of checkpoint inhibition, we used a bioluminescent PD-1/PD-L1 blockade reporter bioassay that we have adapted to

**Figure 1.**
Effect of NOX-A12 treatment on immune-cell infiltration into tumor-stroma spheroids. Spheroids consisting of CXCL12-expressing MS-5 stromal cells and PSN-1 pancreatic adenocarcinoma cells were generated. Peripheral blood mononuclear cells (A) or isolated primary human pan T cells (B-D) were added to the spheroids in the presence of various concentrations of NOX-A12. The next day, immune cell infiltration was quantified by flow cytometry (A, B). Alternatively, spheroids were paraffin-embedded for H&E or immunostaining (C, D). The tumor cell marker PanCK (pan-cytokeratin) revealed a homogeneous distribution of stromal and tumor cells in the spheroid which colocalized with the presence of CXCL12 (C). NOX-A12 increased the amount of CD3⁺ T cells (brown spots) in both tumor-stroma spheroid models (PSN-1 and HT-29). Bar graphs indicate the numbers of CD3⁺ T cells (mean ± SD) in sections of three different spheroids per group, counted by three individual analysts (D). Data are representative of three or more independent experiments.
the 3D format by generating spheroids consisting of MS-5 stromal cells with the CHOPD-L1 cancer cell line. JurkatPD-1/luc T cells were incubated with these spheroids in the presence or absence of NOX-A12. As expected, we found increased T-cell infiltration after CXCL12 inhibition by 10 nmol/L NOX-A12, whereas anti–PD-1 treatment did not influence T-cell trafficking (Fig. 3A). Macroscopically, enhanced T-cell migration into the spheroids was suggested by a reduced number of surrounding T cells (Fig. 3B). NOX-A12 treatment alone dose-dependently increased Jurkat T-cell activation, whereas the effect of treatment with anti–PD-

![Figure 2](image1.png)

**Figure 2.** NOX-A12 increases NK- and T-cell infiltration in various tumor–stroma spheroid models. Spheroids composed of MS-5 stromal cells and various cancer cell lines (PSN-1 pancreatic ductal adenocarcinoma, HT-29 colorectal carcinoma, H1299 non–small cell lung cancer, and U251MG glioblastoma) were treated with various concentrations of NOX-A12 and exposed to isolated primary human T cells (A) or NK cells (B) from healthy donors. After incubation overnight, infiltrated lymphocytes were quantified by flow cytometry. Results indicate immune cell infiltration relative to input cell counts representing the mean ± SD values of triplicates. Data are representative of three or more independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

![Figure 3](image2.png)

**Figure 3.** NOX-A12 synergizes with PD-1 checkpoint inhibition in vitro. A reporter-based PD-1/PD-L1 blockade bioassay was adapted to the 3D format. Spheroids consisting of MS-5 stromal cells and CHO PD-L1 cancer cells were incubated with NOX-A12 and exposed to JurkatPD-1/luc T cells in the presence or absence of anti–PD-1 antibody. The next day, T-cell infiltration was quantified by flow cytometry (A) and visualized by light microscopy (B). T-cell activation was normalized to untreated spheroids. The mean ± SD values of triplicates are shown. Data are representative of three independent experiments. Combination index below 1 indicating synergy was calculated using CompuSyn software. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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1 alone was rather weak. This is likely due to the fact that most of the T cells were located outside the anti–PD-1-treated spheroid whereas NOX-A12 facilitated the physical contact between effector and target. Consequently, the combination of anti–PD-1 with NOX-A12 synergistically increased T-cell activation (Fig. 3C), with a combination index of 0.001 (14).

The in vivo efficacy of NOX-A12/anti–PD-1 combination treatment was assessed in the murine syngeneic CT-26 colon cancer model. Mice treated with anti–PD-1 alone showed a nonsignificant reduction in tumor growth by day 20 \((P = 0.104)\) with only 2 out of 8 mice responding (Fig. 4A and B). NOX-A12 monotherapy did not significantly reduce tumor growth \((P = 0.564 \text{ vs. vehicle})\). However, when NOX-A12 treatment was combined with anti–PD-1 treatment, efficacy was enhanced with significantly reduced tumor volumes compared with either vehicle \((P = 0.002)\) or anti–PD-1 monotherapy \((P = 0.024)\). In fact, in this group, 5 of 8 mice responded to the treatment (Fig. 4A and B). The experiment was repeated at another independent contract research organization (Oncodesign, Dijon, FR) with similar results.

**Discussion**

The success of immune checkpoint inhibitors in a subset of cancer patients has raised interest in combining these therapies with other agents to broaden their applicability (4, 15). Checkpoint inhibitors are only able to unleash antitumor T-cell responses in an immunogenic TME. Therefore, turning an immunosuppressive tumor into an immunogenic tumor is necessary for successful cancer immunotherapy (2). Several combination therapies have been proposed that combine checkpoint inhibitors with other checkpoint inhibitors, inhibitors of suppressive metabolites, vaccines, targeted therapies, cytotoxics, and/or radiotherapy (16). Manipulation of T-cell trafficking into the tumor has rarely been addressed (3). Here, we provide evidence for the use of the CXCL12 inhibitor NOX-A12 to improve checkpoint inhibitor efficacy by enhancing T- and NK-cell migration into the tumor.

We used tumor–stroma spheroids in order to assess immune-cell infiltration. This approach allows a quantitative assessment of immune cell trafficking by flow cytometry. A similar spheroid model was used previously to evaluate NK-cell infiltration (17); however, we included CXCL12-expressing stromal cells in our...
CXCL12 Inhibition Synergizes with PD-1 Checkpoint Inhibition

In conclusion, we have shown in vitro and in an animal model that inhibition of CXCL12 by NOX-A12 can help to overcome the resistance to anti–PD-1 treatment. We anticipate that this concept can also be extended to inhibition of other checkpoints such as CTLA-4, TIM-3, and LAG-3, because NOX-A12 appears to break through the immune-privileged status of the TME, thereby paving the way for T-cell migration into the tumor. Considering NOX-A12 enhanced infiltration by not only T cells but also NK cells, which mediate antibody-dependent cellular cytotoxicity, a combination therapy that includes antibodies targeting immune checkpoints on cancer cells (e.g., PD-L1) seems reasonable. Furthermore, NOX-A12 has the potential to increase the efficacy of a variety of immuno-oncology approaches, including tumor vaccines, bispecific T-cell-engaging antibodies or modified immune cells, such as CAR-T and CAR-NK cells or TCR-modified T cells. A phase I/II clinical trial is currently ongoing to study the effects of combining NOX-A12 and anti–PD-1 treatment in patients with late-line colorectal cancer and pancreatic ductal adenocarcinoma (NCT03168139).

Disclosure of Potential Conflicts of Interest

D. Eulberg is VP Project Management at NOXXON Pharma AG. A. Vater has ownership interest (including patents) in NOXXON Pharma AG. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: D. Zboralski, K. Hoehlig, D. Eulberg, A. Fromming, A. Vater
Development of methodology: D. Zboralski
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Zboralski
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Zboralski, K. Hoehlig, A. Fromming
Writing, review, and/or revision of the manuscript: D. Zboralski, K. Hoehlig, D. Eulberg, A. Fromming, A. Vater
Other (interpretation of data in terms of hypothesis generation): A. Vater

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References


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