ARGX-110, a highly potent antibody targeting CD70, eliminates tumors via both enhanced ADCC and immune checkpoint blockade

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Keywords: CD70, ARGX-110, POTELLIGENT®, immune checkpoint blockade

Overexpression of CD70 has been documented in a variety of solid and hematological tumors, where it is thought to play a role in tumor proliferation and evasion of immune surveillance. Here, we describe ARGX-110, a defucosylated IgG1 monoclonal antibody (mAb) that selectively targets and neutralizes CD70, the ligand of CD27.

ARGX-110 was generated by immunization of outbred llamas. The antibody was germlined to 95% human identity, and its anti-tumor efficacy was tested in several in vitro assays. ARGX-110 binds CD70 with picomolar affinity. In depletion studies, ARGX-110 lyzes tumor cells with greater efficacy than its fucosylated version. In addition, ARGX-110 demonstrates strong complement-dependent cytotoxicity and antibody-dependent cellular phagocytosis activity. ARGX-110 inhibits signaling of CD27, which results in blocking of the activation and proliferation of Tregs. In a Raji xenograft model, administration of the fucosylated version of ARGX-110 resulted in a prolonged survival at doses of 0.1 mg/kg and above. The pharmacokinetics of ARGX-110 was tested in cynomolgus monkeys; the calculated half-life is 12 days.

In conclusion, ARGX-110 is a potent blocking mAb with a dual mode of action against both CD70-bearing tumor cells and CD70-dependent Tregs. This antibody is now in a Phase 1 study in patients with advanced malignancies expressing CD70 (NCT01813539).

Introduction

CD70 belongs to the tumor necrosis factor (TNF) receptor ligand family and is transiently expressed on activated T and B cells and on mature dendritic cells.1-3 CD70 does not have an intracellular domain and it is the ligand for the CD27 receptor. Upon multimerization of the CD27 receptor by CD70, TNF-receptor associated factors (TRAF) adaptor proteins bind to its cytoplasmic domain, which results in activation of the NF-κB pathway leading to proliferation and survival.4 In some tumors, signaling via the CD70-CD27 interaction was induced in vitro. ARGX-110 binds CD70 with picomolar affinity. In depletion studies, ARGX-110 lyzes tumor cells with greater efficacy than its fucosylated version. In addition, ARGX-110 demonstrates strong complement-dependent cytotoxicity and antibody-dependent cellular phagocytosis activity. ARGX-110 inhibits signaling of CD27, which results in blocking of the activation and proliferation of Tregs. In a Raji xenograft model, administration of the fucosylated version of ARGX-110 resulted in a prolonged survival at doses of 0.1 mg/kg and above. The pharmacokinetics of ARGX-110 was tested in cynomolgus monkeys; the calculated half-life is 12 days.

In conclusion, ARGX-110 is a potent blocking mAb with a dual mode of action against both CD70-bearing tumor cells and CD70-dependent Tregs. This antibody is now in a Phase 1 study in patients with advanced malignancies expressing CD70 (NCT01813539).
to 95%) with the human germline equivalents. Although the human sequence homology for VL is somewhat lower than for VH, it is much better than for murine VL, where mainly the kappa isotype is used. Analysis of the folds of camelid derived V regions using their primary sequences with the publicly available software program (www.bioinf.org.uk/abs/chothia.html) predicted the presence of identical fold combinations as in human germlines, without exceptions. Information or sequences of germline lambda and kappa V regions of camelids is limited, but we were able to identify these for lama pacos and camelus ferus from publically accessible databases (manuscript in preparation). Both a high degree of human sequence homology and a binding site structure undistinguishable from human antibodies are relevant for therapeutic applications.

Here, we report the finding that active immunization of outbred llamas with the renal cell carcinoma cell line 786-O overexpressing CD70 yields a large diversity of conventional antibodies with high human sequence homology, binding to and blocking CD70. One anti-CD70 antibody was selected for clinical development. ARGX-110, is a germlined monoclonal antibody (mAb) that binds with picomolar affinity to human CD70. It has been modified using POTTLELIENT technology to induce enhanced antibody-dependent cell-mediated cytotoxicity (ADCC), making it a potent mAb for therapeutic use in cancer. In addition, we investigated the pharmacokinetics (PK) of this antibody in non-human primates.

### Results

**Generation and characterization of anti-CD70 monoclonal antibodies**

Four llamas were immunized with 786-O cells (renal cell carcinoma) and the antibody repertoire was cloned as a Fab phage display library. By selections on recombinant CD70, specific binding clones were obtained. Fabs present in the periplasmic extracts were screened by ELISA for blocking of the CD70-CD27 binding. Antagonistic hits were sequenced and grouped on the length and sequence of the HCDR3. We found 32 binders representing 12 VH-VL families. CD70 blocking Fab clones with high affinity for CD70 in Biacore and representing nine different VH-VL families were characterized into more detail. The potency in a CD70 blocking ELISA, off-rate for CD70 in Biacore, binding affinity for CD70 on 786-O cells and potency in ADCC assays (using four different PBMC donors).

| Name of clone | Fab | mAb |
|---------------|-----|-----|
| **Fab** | **IC50 blocking ELISA [ng/ml]** | **EC50 binding to 786-O cells [ng/ml]** | **Koff Biacore (s⁻¹) x E⁻⁴** | **IC50 blocking ELISA [ng/ml]** | **IC50 Raji cell based blocking assay [ng/ml]** | **EC50 binding to 786-O cells [ng/ml]** | **IC50 ADCC [fold 9D1]** |
| 5F4 | 95 | 121 | 20 | 0.8 | 93 | 31 | 370 | 5.7 |
| 5B2 | 91 | 392 | 20 | 12.4 | 83 | 19 | 272 | 1.8 |
| 9G2 | 95 | 234 | 56 | 1.8 | 38 | 45 | 164 | 1.9 |
| 4D2 | 91 | 62 | 7 | 3.3 | 69 | 40 | 607 | 8.9 |
| 9B2 | 95 | 383 | 37 | 2.8 | 71 | 217 | 348 | ND |
| 1C2 | 91 | 73 | 316 | 0.4 | 80 | ND | > 10000 | ND |
| 9E1 | 93 | 1436 | 92 | 1.9 | 1249 | 551 | 15000 | 25.4 |
| 7H8 | ND | 838 | 87 | 0.9 | 282 | 69 | 2762 | 14.2 |
| 9D1 | 91 | 123 | 6 | 4.8 | 44 | 29 | 115 | 1.0 |

The potency in a CD70 blocking ELISA, off-rate for CD70 in Biacore, binding affinity for CD70 on 786-O cells in FACS and human homology for these nine Fabs are summarized. The nine unique Fabs were converted into full-length human IgG1. The antibody were characterized for potency of blocking CD70 in ELISA and in a Raji cell-based assay, for binding affinity for CD70 on 786-O cells and potency in ADCC assays (using four different PBMC donors).
overlapped, as these Fabs all blocked the binding of anti-CD70 mAb 4D2. The epitope of 1C2, 9E1 and 7H8 are different as binding of the Fabs did not interfere with binding of mAb 4D2. Next, 786-O cells were saturated with the Fabs of 9E1, 1C2 and 7H8 and binding of biotinylated mAb 9E1 and 7H8 was tested. 7H8 mAb binding was blocked by 7H8 and 9E1 Fab only. 9E1 binding was blocked by all three Fabs (but not by a negative control Fab). This lead to the conclusion that our Fabs recognize at least three different epitopes, and that the epitope of 1C2 and 7H8 does not overlap with each other, but both epitopes overlap with 9E1 (Fig. 1). The nine unique Fabs were converted into full-length human IgG1. Remarkably, mAbs 1C2, 7H8 and 9E1 bind with lower affinity to 786-O cells, whereas they bound well as Fabs. These mAbs bind to a different and maybe less accessible epitope than the other mAbs. The antibody with the best potency of blocking CD70 in ELISA and in a Raji cell-based assay, with the highest binding affinity for CD70 on 786-O cells and best potency in ADCC assays (using four different PBMC donors) was 9D1 and was selected for VL shuffling and germlining (Table 1). The resulting clone, 41D12, was 95.2% identical to the framework region of the matching human VH3 and VL8 germline. This clone was produced transiently in HEK293 cells and stably transfected in CHOK1SVfut8KO cells. CHOK1SVfut8KO cells lack the FUT8 gene. FUT8 is the only gene coding for a 1,6-fucosyltransferase, which catalyzes the transfer of fucose from GDP-fucose to GlcNAc in a 1,6-linkage of complex oligosaccharides.17 41D12 produced in this cell line thus lacks fucose, and this version was called ARGX-110. The yield after purification of ARGX-110 was between 2 and 5 g/L.

Binding of ARGX-110 to CD70

Binding of ARGX-110 to CD70 expressed on different hematological cell lines was evaluated by FACS analysis. The results demonstrate that ARGX-110 binds to these cell lines with high affinity in the low nanomolar range (Fig. 2A). ARGX-110 does not bind to CD70 negative cell line SUP-T1 (T cell lymphoma cell line), demonstrating its specificity for CD70. Moreover, ARGX-110 does not bind to HEK293 cells, but does bind after transfection with CD70 (data not shown).

The binding affinity and binding kinetics of ARGX-110 were also analyzed by surface plasmon resonance analysis using a CD70-coated CM5 sensor chip. ARGX-110 had an equilibrium dissociation constant for binding to human CD70 of 17 pM. The “on” rate for binding was 2.8 × 10^5 M⁻¹s⁻¹ and the “off” rate 4.7 × 10⁻⁶ s⁻¹.

Specificity of ARGX-110 for CD70 was demonstrated by immunohistochemistry on cryosections of human tissues. Binding of ARGX-110 was not detected in any normal human tissues evaluated, whereas moderate (2+) staining was obtained on a positive control human RCC sample (data not shown). These data are consistent with the fact that CD70 is only transiently expressed on activated T and B cells, on mature dendritic cells and on tumor biopsies.

Binding of 41D12 and ARGX-110 to FcγRIIIa

Because de-fucosylation of mAbs increases binding to FcγRIIIa, binding of ARGX-110 was compared with the fucosylated 41D12. De-fucosylation of 41D12 increased binding in an ELISA to both human FcγRIIIa V158F (low affinity variant) and V158V (high affinity variant) 70-fold compared with the fucosylated mAb, 41D12. A 2.5-fold difference was observed for ARGX-110 in binding to the high and low affinity variants (EC₅₀ of 817 vs. 333 ng/ml, respectively).

Blocking of CD27 signaling

A bioassay was applied in which interleukin IL-8 production of HT1080-CD27 cells serves as a downstream readout of CD27 signaling.18 CD70+ cell lines, representing mantle cell lymphoma (Granta519 and Mino-1), multiple myeloma (U266) and Burkitt lymphoma (Raji), or recombinant CD70 (cross linked using Flag-specific mAb M2), were incubated with ARGX-110 and co-cultured with HT1080-CD27 cells. Effective inhibition of the CD70-CD27 interaction resulted in inhibition of IL-8 secretion. The results shown in Figure 2B demonstrate that ARGX-110 blocks CD70-CD27 signaling in these co-culture assays with picomolar potency. When HEK293 cells were applied, no IL-8 production was obtained, but when the cells were transfected with CD70, IL-8 was produced and this could be blocked with ARGX-110 dose dependently.

Effector functions: ADCC, CDC and ADCP, depletion of cell lines

ADCC was measured using a ⁵¹Cr-release assay using 786-O (renal cell carcinoma) as target cells. The results shown in Figure 3A demonstrate that de-fucosylation of 41D12 improved its IC₅₀ values by ~20-fold and increased the proportion of cells lysed by it under the chosen assay conditions.

To analyze ARGX-110-induced complement-dependent cytotoxicity (CDC) compared with that of 41D12, U266 cells (multiple myeloma) were mixed with the mAbs in the presence of human serum as source of complement. The number of viable and dead cells was determined by FACS. The results (Fig. 3B) show that de-fucosylation of 41D12 did not impair CDC activity.

To analyze antibody-dependent cellular phagocytosis (ADCP) activity 786-O cells were stained and incubated with the antibodies prior to the incubation with the monocyte-derived macrophages (MDM) effector cells. The MDM effector cells were stained and phagocytosis was analyzed by FACS. The
tumor, which promotes tumor growth. Blocking CD70 could thus potentially inhibit CD70-CD27-induced growth signals or impede Treg cell recruitment or activation within the tumor microenvironment.

Based on the observation from Van Oers and colleagues that Treg cells from healthy donors could be stimulated with CD70, we spiked PBMCs from a healthy donor with CD70-positive tumor cell lines, cells were spiked into freshly isolated peripheral blood mononuclear cells (PBMCs) from healthy donors and 41D12 or ARGX-110 was added at 5 μg/ml. After 2 d of incubation, lysis of the cell lines was measured by FACS analysis. The results demonstrate that ARGX-110 lysed the tumor cells more efficiently than 41D12 for most cell lines tested (Fig. 4A) and lysed 5–95% of the cells (Fig. 4A and B). Lysis is independent of the CD70 copy number, as the signal obtained in FACS for CD70 staining could be as low as 40 MFI (for SU-DHL-6 cells, corresponding to a copy number of 2,200) and still results in efficient lysis of 69% of the cells within 2 d of incubation (Fig. 4C).

Blocking of CD70-CD27 signaling has an effect on proliferation of Tregs

CD70 appears to play a role in evasion of immune surveillance by promoting proliferation and survival of Treg cells in the tumor, which promotes tumor growth. Blocking CD70 could thus potentially inhibit CD70-CD27-induced growth signals or impede Treg cell recruitment or activation within the tumor microenvironment.

Based on the observation from Van Oers and colleagues that Treg cells from healthy donors could be stimulated with CD70, we spiked PBMCs from a healthy donor with CD70-positive tumor cell lines [Raji (Burkitt lymphoma), SUDHL-6 (diffuse large B cell lymphoma) and U266 (multiple myeloma)] in the presence of soluble anti-CD3 mAb and ARGX-110. Samples were applied in duplicates. The copy number for CD70 is 2,200 for SU-DHL-6 cells, 46,000 for Raji cells and 150,000 for U266 cells. Consistent with its strong ADCC, ARGX-110 lysed most of the malignant CD70-expressing cells (Fig. 5A). Indeed, after 2 d of incubation, more than 60% of the cells were lysed with ARGX-110.

After two days of incubation the number of Tregs increased in the presence of CD70-positive cell lines to 9.5–10.0% compared with 6.7% in the absence of CD70-positive cell lines (P < 0.0001 ANOVA, P = 0.004–0.008 t test) (Fig. 5B). These data confirm reports in the literature showing that CD70-positive tumor cells give proliferation signals to CD27 presenting Tregs. ARGX-110 added to the wells abrogated the increase in Treg numbers (0.003–0.06 t test) (Fig. 5B). The supernatant was then analyzed for sCD27 levels. In all the wells where CD70-positive cell lines were added, sCD27 levels were increased: in the wells without cell lines, the sCD27 levels were 42 IU/ml, and in the presence of cell lines this level was at least doubled, which is consistent with active CD70-CD27 signaling that leads to release of sCD27 into the culture media (Fig. 5C). The lowest levels were obtained for the SU-DHL-6 cell line (85 IU/ml) which also has the lowest copy number of CD70 (2,200). The highest levels were obtained in the wells with Raji cells (257 IU/ml). The Raji cell line is not only CD70-positive, but also CD27-positive, which may explain these results. In the presence of ARGX-110, the sCD27 levels are lower than in the absence of ARGX-110 (Fig. 5C) except for the SU-DHL-6 cell line.

In vivo efficacy in a Raji xenograft model

Since it has been reported that the POTELLIGENT® technology with de-fucosylation of the mAbs does not show enhanced ADCC activity in mouse models, we were restricted to testing 41D12, the fucosylated version of ARGX-110, in the Raji Xenograft model. 41D12, was active in the Raji xenograft model with a plateau in survival prolongation observed at doses as low as 0.1 mg/kg (Fig. 6A). PK analysis revealed that at this dose, the extrapolated Cmax plasma level of 41D12 was 2.5 μg/ml. Serum concentrations of sCD27 were measured on the day of sacrifice. Figure 6B shows that tumor-bearing mice, which had to be euthanized during the course of the study (n = 28), had higher levels of sCD27 than mice that remained healthy until the end of the study (non-tumor bearing mice, n = 42). Indeed, after
ligation to CD70 and subsequent activation of the cell, CD27 is known to be cleaved off by metalloproteinases and the soluble form (sCD27) can be detected in blood. High sCD27 levels have been observed in blood of many types of cancer patients and high levels correlate with poor prognosis for the patient.

Pharmacokinetic study in cynomolgus monkeys

ARGX-110 was administered as a single dose in female cynomolgus monkeys (1, 3, or 10 mg/kg, three monkeys per dose). Analysis of ARGX-110 PK indicated a dose-proportionality of Cmax concentrations (50–151–504 μg/ml, respectively); area under curve (AUC: 444–1332–4442 d*μg/ml, respectively), and a terminal half-life (t ½) of ~12 d. The calculated clearance rate was 0.009 L/day for all dose levels.

Discussion

The modern era of cancer immunotherapy has been ushered in by therapeutic antibodies such as ipilimumab that block key immune checkpoints. In spite of noticeable efficacy improvements, the systemic toxicity remains clinically significant. Hence, there is a need to identify new relevant targets that are associated with a wide therapeutic index. Based on these parameters, CD70 appears to be an attractive target.

CD70 is a TNF receptor ligand that is normally transiently expressed on activated B and T cells and mature dendritic cells. Its role in the induction of a systemic immune response to viral infections is the subject of recent publications. Research on viral carcinogenicity suggests that dysregulation of normal CD70 physiology by pathogens such as the Epstein-Barr virus (EBV), results in sustained CD70 expression.

In the context of chronic expression, CD70 also appears to be involved in the recruitment of regulatory T cells to the tumor micro-environment, which may facilitate tumor evasion from immune surveillance. Hence, CD70 represents a potentially valid target in tumors that chronically express the antigen. Its limited expression in normal tissue suggests that a clinically useful therapeutic index might result from specific CD70 targeting with an antibody.

Claus et al. demonstrated that in mice bearing CD70-negative tumors, CD70 was expressed on TILs and that CD27 signaling increased both the frequency of intratumoral Tregs and the production of IL-2, a survival factor for Tregs, by a population of effector T cells. We, and others, have demonstrated by immunohistochemistry on human tumor biopsies that CD70 is chronically expressed on tumor and non-malignant tumor infiltrating cells (data not shown). Therefore, blocking CD70, and thus CD27 signaling, in humans is expected to stimulate the patient’s immune response against the tumor.

ARGX-110, a germlined mAb, binds to human CD70 and blocks CD27 signaling. In contrast to antibody-drug conjugates, which are designed to deliver a cytotoxic chemotherapy payload and require internalization for optimal function, ARGX-110 has been modified using POTELLIGENT® technology to augment the immune system activation that is induced by the Fc portion of the antibody. Hence ARGX-110 has a dual mechanism of action: (1) interruption of CD70-CD27 signaling, and (2) enhanced induction of ADCC directed against tumor cells expressing CD70. This has several important consequences: (1) ARGX-110 may deprive the tumor of an immune surveillance evasion mechanism by reducing the number of intratumoral Tregs while (2) inhibiting tumor proliferation and survival; and (3) its ADCC, CDC, and ADCP mechanisms may directly lyse CD70-expressing tumor cells.

Consistent with prior reports of CD70 expression, ARGX-110 has been shown to bind with high affinity to a large array of CD70-positive cell lines. Low nanomolar EC50 values were typical. In vitro experiments using different CD70-positive cell lines established that ARGX-110 inhibits, in a dose-dependent fashion, release of IL-8 with picomolar potency and blocks Treg...
proliferation. The ADCC activity of ARGX-110 was increased more than 20-fold compared with a fucosylated version of the antibody (41D12), with no impairment of CDC or ADCP, in both hematological and solid tumor cell lines. As a result, ARGX-110 was able to lyse cell lines independently from the CD70 copy number.

Finally, because the gold standard of anti-neoplastic therapy is prolongation of survival, the potency of the fucosylated version of ARGX-110 (41D12) was tested in an EBV-positive Burkitt lymphoma model. The survival plateau observed in the Kaplan-Meier curves at the 0.1 mg/kg levels and above suggests that saturation of the CD70 target may have occurred at doses that are easily achievable in the clinical setting. In addition, the demonstration of reduced sCD27 levels in mice that appear to derive a survival advantage from CD70 blockade suggests that this biomarker should be readily applicable in the clinic.

We believe that combining best-in-class CD70-blocking properties, which inhibit tumor cell proliferation and restore immunosurveillance by inhibiting CD27-mediated Treg activation, with optimal Fc-induced cell killing has the potential to result in clinical responses in CD70-dependent tumors.

**Materials and Methods**

**Cells**

The cell lines used in this study are: 786-O, Z-138, HH, MM1.s, MJ, Hey445 (ATCC), Karpas-299, L428, LP1, L540, L363, Raji, SUDHL6, SUDHL4, SUP-T1, JVM3, WSU-FSCLL, Granta519, Mino, U266, Sc-1, MHHPREB1, Daudi, Mec1, JVM2, ARH77, Jeko-1 (DSMZ) and Hut78 (CLS). Cells were used within 6 mo after receipt or resuscitation. Cells were propagated in culture conditions as recommended by the manufacturer.

**Generation and expression of 41D12 and ARGX-110**

Llamas were immunized with CD70 expressing 786-O cells (human renal cell carcinoma). PBLs were isolated for RNA extraction, RT-PCR and PCR-cloning of Fab in a phagemid as was described previously. Fab's with high affinity for CD70 in Biacore and with strong blocking potency in ELISA were
selected by panning from the immune libraries. Fabs were converted into mAbs (human IgG1 isotype) and produced by transient transfection in HEK293 cells and characterized. The antibody with the best potency of blocking CD70 in ELISA and in a Raji cell-based blocking assay, with the highest binding affinity for CD70 on 786-O cells and best potency in ADCC assays (using four different PBMC donors), was 9D1 and was selected for VL shuffling. The mAb with the best characteristics after VL shuffling of 9D1 was 27B3. The variable regions of 27B3 were germlined by genetic engineering to the framework regions of the closest human germline with the highest identity. Deviating amino acids in the framework regions were mutated by PCR using overlapping, degenerated oligonucleotide primers encoding both the human and llama residues as described for the germlining of a murine antibody, creating synthetic libraries with phage expressing germlined Fabs. Selection of germlined functional Fabs was performed using phage display and affinity selections (data not shown). The variable regions of 27B3 were germlined to 95.2% identity to the framework regions of the closest human germline, resulting in the 41D12 clone. The variable regions of 41D12 were fused in frame with the human lambda constant region and the human constant IgG1 region. 41D12 was transiently expressed in HEK293 cells, and then purified on Protein G followed by size-exclusion chromatography. 41D12 was also expressed by Lonza Biologics (Slough, United Kingdom) in stably transfected CHOK1SVfut8KO cells under the control of the GS (glutamine synthase) promoter to produce de-fucosylated 41D12 (ARGX-110). ARGX-110 was purified via protein A capture and ion exchange chromatography.

**Binding of Fabs and mAbs to CD70**

Binding of Fabs to CD70 on 786-O cells was determined by applying a dilution series of the Fabs (20 μg/ml-0.15 μg/ml) onto 786-O cells. Staining was done using anti-6x His tag PE (Abcam). Binding of mAbs to CD70 on 786-O cells was determined by

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**Figure 5.** Blocking of Treg activity by ARGX-110 demonstrated in an in vitro model. (A) PBMCs from a healthy donor were admixed with CD70-positive tumor cell lines (SUDHL6, Raji and U266) in the presence of soluble anti-CD3 mAb and ARGX-110. ARGX-110 lysed the cell lines during the experiment. (B) ARGX-110 blocks Treg proliferation. More Tregs are formed in the wells where the CD70-positive cell lines were added than in the wells without addition of cell lines and this can be blocked with ARGX-110. Results are shown as mean and SD of duplicates. (C) Proliferation of the Tregs is dependent on CD70 signaling and can be blocked with ARGX-110. After two days of incubation, ARGX-110 reduces sCD27 (as determined by ELISA) levels further witnessing of a role for CD70 in Treg proliferation.
using anti-hIgG1-FITC (The Binding Site) or anti-hIgG1-PE (eBioscience) antibody. Next, cell lines representing different histologies [U266 (multiple myeloma), Granta519 and Mino-1 (Mantle cell lymphoma) and Raji (Burkitt lymphoma)], were incubated with a dilution series of ARGX-110. Detection was done as for the other anti-CD70 mAbs. Fluorescence was measured using a flow cytometer (FACS CANTO, BD) and the mean fluorescence intensity of duplicate measurements was plotted. As a negative control, the CD70 negative [demonstrated with anti-CD70-PE (BD Bioscience)] T cell lymphoma cell line, SUP-T1, was tested.

The affinity of ARGX-110 for recombinant CD70 was determined using a Biacore3000. The purified recombinant FLAG-TNC-CD70 (20) was immobilized onto a CM5 biosensor chip (Biacore; BR-1000–12) using an amine coupling kit at 300 resonance units. A dilution series of 3–100 nM ARGX-110 was employed by serial 2-fold dilutions in HBS-EP buffer (Biacore; BR-1008–26). The running buffer was allowed to flow at a rate of 30 μl/min and the injections were performed using the KINJECT mode. For regeneration, 15 mM NaOH was injected.

To test the binding specificity of ARGX-110, the mAb was applied to cryosections of human tissues, and immunohistochemically detected using a biotinylated anti-human IgG secondary antibody (Jackson ImmunoResearch) in a pre-complexing method. Bound ARGX-110-secondary antibody complexes were visualized with a streptavidin-horseradish peroxidase (Labeling Solution) complex and diaminobenzidine (DAB) chromogen substrate. ARGX-110 binding was evaluated at concentrations of 20 and 50 μg/mL.

In all experiments, cryosections of human renal cell carcinoma, which is reported to express the target antigen (CD70), were used as positive control samples.

**Binding of Fab s and mAbs to human FcγRIIIa**

Maxisorb plates were coated with 100 ng/well of Neutravidin (Thermo Scientific) overnight at 4 °C. After blocking, 25 ng/well biotinylated human FcγRIIIa (CD16a) was added and allowed to bind. A dilution series of the mAbs was added and after washings, 50,000-fold diluted horseradish peroxidase (HRP) conjugated goat Fab specific anti-human IgG antibody (Sigma-Aldrich) was added. s(Hs)-TMB (SDT-reagents) was added after washings and the reaction was stopped with 1M H2SO4 and the optical density at 450 nm was read in a microplate reader (Tecan). The binding curves were plotted and the EC50 was calculated using GraphPad Prism.

**Epitope mapping of mAbs**

786-O cells were incubated for 30 min with 5 μg of the different Fabs at 4 °C, after which 1 μg of biotinylated anti-CD70 mAb 4D2, 7H8, or 9E1 was added. This mixture was incubated another 15 min at 4 °C before washing. Next, cells were incubated for 30 min with a mixture of streptavidin-PE (for detection of mAb binding) followed by several washing steps and analysis using FACS. Blockage of mAb binding indicates that the Fab and mAb share the same epitope.

**Blocking of binding of CD70 to CD27 in ELISA**

In this assay, Flag-TNC-CD7018 is captured by an anti-Flag mAb (Sigma) and binding of CD27-Fc (R&D Systems) is detected via a biotinylated anti-CD27 mAb (eBioscience) and strep-HRP (Jackson ImmuneResearch). Plates were read on a plate reader (Sunrise, Tecan) at 405 nm.

**Blocking of CD27 signaling in a cell based assay**

CD70-positive cell lines U266 (multiple myeloma), Granta519 and Mino-1 (Mantle cell lymphoma) and Raji (Burkitt lymphoma) or HEK293 cells with and without transfection with CD70 and cross linked Flag-TNC-CD70 (with anti-Flag mAb M2 (Sigma),18 were mixed in a 96-well tissue culture plate (50,000 cells per well) with ARGX-110 and incubated at room temperature for 1 h. HT1080-CD27 cells (human epithelial cells transfected with human CD27) were added (10,000 cells per well). Co-cultures were transferred to the incubator and grown overnight.18 Upon signaling of CD27, HT1080-CD27 cells produce IL-8. Supernatants were collected and analyzed for their IL-8 content by ELISA (BD Biosciences PharMingen). All experiments were performed in triplicate.

**Effector functions: ADCC, CDC and ADCP**

ADCC was measured using a 51Cr-release assay. Human PBMCs from a healthy donor were purified from heparinized whole blood by Ficoll separation. 786-O cells were loaded with

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**Figure 6.** In vivo efficacy study using Raji xenograft model. (A) Kaplan-Meier plot showing the antitumor effect of 41D12 in a disseminated Burkitt lymphoma xenograft model. SCID mice were injected intravenously with 10⁶ Raji B lymphoma cells, then treated with 41D12 or isotype control at the doses indicated. Mice were treated two times per week thereafter and received a total of five doses (n = 9 mice per group). (B) A plasma sample was obtained for each mouse at the time of sacrifice. sCD27 levels were determined by ELISA. Tumor bearing mice (n = 28) had higher levels of sCD27 than non-tumor bearing mice (n = 42). Statistics was done comparing groups treated with 41D12 vs. the isotype control.
μ were tested at final concentrations ranging from 0.01 μg/ml to 1 μg/ml. Chromium release was measured in the supernatant using a β counter. All experiments were done in triplicate.

To analyze CDC, U266 cells (multiple myeloma) were mixed with the mAbs at 0.001–10 μg/ml in the presence of 9% human serum and incubated for 2 h at 37 °C. Cells were sedimented and washed before propidium iodide was added. The number of viable and dead cells was determined by FACS. All experiments were done in triplicate.

To analyze the ADCP activity, human monocytes were purified from heparinized whole blood from a healthy donor with a monocyte enrichment cocktail (Stem Cell) and cultured with 500 U/ml GM-CSF (Milenyi) for 15 d. 786-O cells were stained with PKH26 (Sigma) and incubated with the antibodies for 30 min on ice prior to the incubation with the monocyte-derived macrophages effector cells (MDM) for 1 additional hour at 37 °C. The MDM effector cells were subsequently stained with CD11b FITC (EXBIO) and phagocytosis was determined by FACS by gating for PKH26/CD11b-positive cells. All experiments were done in triplicate.

**Depletion studies**

The lytic activity of ARGX-110 or 41D12 was tested against various hematological cell lines spiked into freshly isolated PBMCs from healthy donors. No serum was added. ARGX-110 or 41D12 were tested at 5 μg/ml. The samples were incubated for 2 d and lysis of the cell lines was analyzed by FACS using staining for specific target cell line markers. All experiments were performed in duplicate. The CD70 expression on these cell lines was determined by FACS as well. An anti-CD70-PE (BD Bioscience) was used for the detection and a mouse IgG3 PE (BD Bioscience) as the isotype control.

**Blocking of Treg proliferation and survival**

SU-DHL-6 (diffuse large B cell lymphoma), Raji (Burkitt lymphoma) and U266 (multiple myeloma) cell lines were spiked into freshly isolated PBMCs from healthy donors in the presence of soluble anti-CD3 mAb (Sanquin) and ARGX-110 or isotype control (human IgG1; Sigma) was added (5 μg/ml). The samples were incubated for 2 d and lysis of the cell lines was analyzed by FACS using staining for specific target cell line markers. All experiments were performed in duplicate. The CD70 expression on these cell lines was determined by FACS as well. An anti-CD70-PE (BD Bioscience) was used for the detection and a mouse IgG3 PE (BD Bioscience) as the isotype control.

**Pharmacokinetic study in cynomolgus monkeys**

The cross-species reactivity of ARGX-110 was established between human and cynomolgus monkeys via binding studies to recombinant CD70, as well as to primary B and T cells and cell lines from both species. All data were comparable to human binding (data not shown).

Captive-bred cynomolgus monkeys were ordered from Roberto C. Hartelust, 5001 CD Tilburg, The Netherlands. Prior to delivery the animals were kept in EC quarantine at the supplier’s facilities. Upon arrival at the test facility (LPT), the animals were subjected to a veterinary examination and allowed to acclimatise for a period of 4 wk before initiation of the study. ARGX-110 was administered as a single intravenous slow bolus (~1 min) of 1, 3, or 10 mg/kg in female cynomolgus monkeys (three monkeys per dose). Blood samples were collected at different time points and serum was prepared in order to assess the ARGX-110 concentration. Experiments were conducted according to German regulations.

**PK analysis of samples obtained from mouse or cynomolgus monkey studies**

A PK assay was developed in which anti-Flag (Sigma) was immobilized onto a 96-well microtiter plate. The plate was blocked and Flag-TNC-CD70 was added. Serum samples were diluted at least 100-fold and incubated for 2 h. Detection was done with a mouse anti-human IgG (Fc) HRP (Thermo scientific) for one hour and ABTS Substrate Solution. Plates were read on a plate reader (Sunrise, Tecan) at 405nm. ARGX-110 concentrations were determined on a standard curve obtained by plotting optical density vs. concentration using a four-parameter logistic curve-fitting program.

**Disclosure of Potential Conflict of Interest**

Silence K, Dreier T, Mosht M, Ulrichs P, Gabriels SME, Saunders M, Van Hauwermeiren T, Thibault A, and De Haard HJ are all employees of arGEN-X BVBA. Wajant H is consultant of arGEN-X. Brouckaert P and Huyghe L do not declare any conflict.

**Acknowledgments**

We would like to thank Prof Patrick Pauwels and David Lacey for reviewing the article carefully. We also thank Anna Hultberg for her input in the project and help in reviewing and submitting this article. This work was supported by Agentschap voor Innovatie door Wetenschappen en Technologie (IWT; grants IWT090297 and IWT100440).
Duggleby BC, Shaw TN, Jarvis LB, Kaur G, Gaston JS. CD27 expression discriminates between regulatory and non-regulatory cells after expansion of human peripheral blood CD4+CD25+ cells. Immunology 2007; 121:239-9; PMID:17425604; http://dx.doi.org/10.1111/j.1365-2567.2006.02550.x

Yang ZZ, Novak AJ, Ziemer SC, Witzig TE, Ansell SM. CD70+ non-Hodgkin lymphoma B cells induce Fasp3 expression and regulatory function in intratumoral CD4+CD25 T cells. Blood 2007; 110:2357-44; PMID:17605291; http://dx.doi.org/10.1182/blood-2007-03-082578

Jak M, Mouss R, Remmerswaal EB, Spijker J, Jaspers A, Yaguc A, Eldering E, Van Lieo RA, Van Oers MH. Enhanced formation and survival of CD4+CD25hi Foxp3+ T-cells in chronic lymphocytic leukemia. Leuk Lymphoma 2009; 50:788-801; PMID:19452318; http://dx.doi.org/10.1080/1042819090283677

Claus C, Riehler C, Schücht C, Matter MS, Hilmenyuk T, Ochsenbein AF. CD27 signaling increases the frequency of regulatory T cells and promotes tumor growth. Cancer Res 2012; 72:9664-76; PMID:22682472; http://dx.doi.org/10.1158/0008-5472.CAN-11-2791

Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012; 12:252-64; PMID:22437870; http://dx.doi.org/10.1038/nrc3239

Shinkawa T, Nakamura K, Yamane N, Shojo-Hosaka E, Kanda Y, Uchida K, Anazawa H, Saroh M, Yasamaki M, et al. The absence of fucose but not the presence ofgalactose or bisecting N-acetylgalosamime of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem 2003; 278:3466-73; PMID:12427744; http://dx.doi.org/10.1074/jbc.M210665200

Wysogol A, Müller N, Fick A, Munkel S, Grigoleit GU, Pfizenmaier K, Wajant H. Trimer stabilization, oligomerization, and antibody-mediated cell surface immobilization improve the activity of soluble trimers of CD27L, 4-1BBL, and glucocorticoid-induced TNF receptor ligand. J Immunol 2009; 185:1831-61; PMID:19596991; http://dx.doi.org/10.4049/jimmunol.0802597

Niwa R, Shojo-Hosaka E, Nakamura M, Shinkawa T, Uchida K, Nakamura K, Matsushita K, Ueda T, Hanai N, Shirata K. Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. Cancer Res 2004; 64:2127-33; PMID:15026353; http://dx.doi.org/10.1158/0008-5472.CAN-03-2068

Yano H, Ishida T, Imada K, Sakai T, Ishii T, Inagaki A, Iida S, Uchiyama T, Ueda R. Augmentation of antitumour activity of defucosylated chimeric anti-CCR4 monoclonal antibody in SCID mouse model of adult T-cell leukaemia/lymphoma using G-CSF. Br J Haematol 2008; 140:586-9; PMID:18205860; http://dx.doi.org/10.1111/j.1365-2457.2007.02494.x

Kara JO, Sahin B, Gunesarar R. Expression of soluble CD27 and interleukins-8 and -10 in B-cell chronic lymphocytic leukemia: correlation with disease stage and prognosis. Adv Ther 2007; 24:29-40; PMID:17526459; http://dx.doi.org/10.1007/s00269-004-0299-0

Lena SMA, Tessaar K, van Oers MHJ, van Lier RAW. Control of lymphocyte function through CD27-CD70 interactions. Semin Immunol 1998; 10:491-9; PMID:9826582; http://dx.doi.org/10.1006/smim.1998.0154

Weber JS, Dummer R, de Pril V, Lebhe C, Hodi FS; MDX010-20 Investigators. Patterns of onset and resolution of immune-related adverse events of special interest with ipilimumab: detailed safety analysis from a phase 3 trial in patients with advanced melanoma. Cancer 2013; 119:1675-82; PMID:23400564; http://dx.doi.org/10.1002/cncr.27969

Polak ME, Newell L, Zarabian VY, Pickard C, Healy E, Friedmann PS, Al-Shamkhi A, Ardern-Jones MR. CD70-CD27 interaction augments CD8+ T-cell activation by human epidermal Langerhans cells. J Invest Dermatol 2012; 132:1636-44; PMID:22377764; http://dx.doi.org/10.1038/jid.2012.26

Israel BF, Gulley M, Elmore S, Ferrini S, Feng WH, Kenney SC. Anti-CD70 antibodies: a potential treatment for EBV+ CD70-expressing lymphomas. Mol Cancer Ther 2005; 4:2037-44; PMID:16373719; http://dx.doi.org/10.1158/1535-7163.MCT-05-0253

Kenney S, Theodore E. Woodward Award: development of novel, EBV-targeted therapies for EBV-positive tumors. Trans Am Clin Climatol Assoc 2006; 117:55-73; discussion 73-4; PMID:18528464

McEarchern JA, Smith LM, McDonagh CF, Klauss K, Gordon RA, Morris-Tilden CA, Dunio S, Ryan M, Bouarlat TE, Cartter PJ, et al. Preclinical characterization of SGN-70, a humanized antibody directed against CD70. Clin Cancer Res 2008; 14:7763-72; PMID:19047165; http://dx.doi.org/10.1158/1078-0432.CCR-08-0493

Oflazoglu E, Stone JJ, Gordon K, Wood CG, Repasky EA, Grewal IS, Law CL, Gerber HP. Potent anticancer activity of the humanized anti-CD70 antibody h1F6 conjugated to the tubulin inhibitor auristatin via an uncleavable linker. Clin Cancer Res 2008; 14:6781-80; PMID:18809969; http://dx.doi.org/10.1158/1078-0432.CCR-08-0916

de Haard HJ, van Neer N, Reurs A, Hufton SE, Roovers RC, Henderikx P, de Bruïne AP, Arends JW, Klussman K, Gordon RA, Morris-Tilden CA, Dunio S, Ryan M, Boursalian TE, Carter PJ, et al. Preclinical characterization of SGN-70, a humanized antibody directed against CD70. Clin Cancer Res 2008; 14:7763-72; PMID:19047165; http://dx.doi.org/10.1158/1078-0432.CCR-08-0493

de Haard HJ, van Neer N, Reurs A, Hufton SE, Roovers RC, Henderikx P, de Bruïne AP, Arends JW, Hoogenboom HR. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J Biol Chem 1999; 274:18218-30; PMID:10373423; http://dx.doi.org/10.1074/jbc.274.26.18218

Baca M, Presta LG, O’Connor SJ, Wells JA. Antibody humanization using monoclonal phage display. J Biol Chem 1997; 272:10678-84; PMID:9099717; http://dx.doi.org/10.1074/jbc.272.10.10678