Direct and indirect anti-leukemic properties of activity-on-target interferons for the treatment of T-cell acute lymphoblastic leukemia
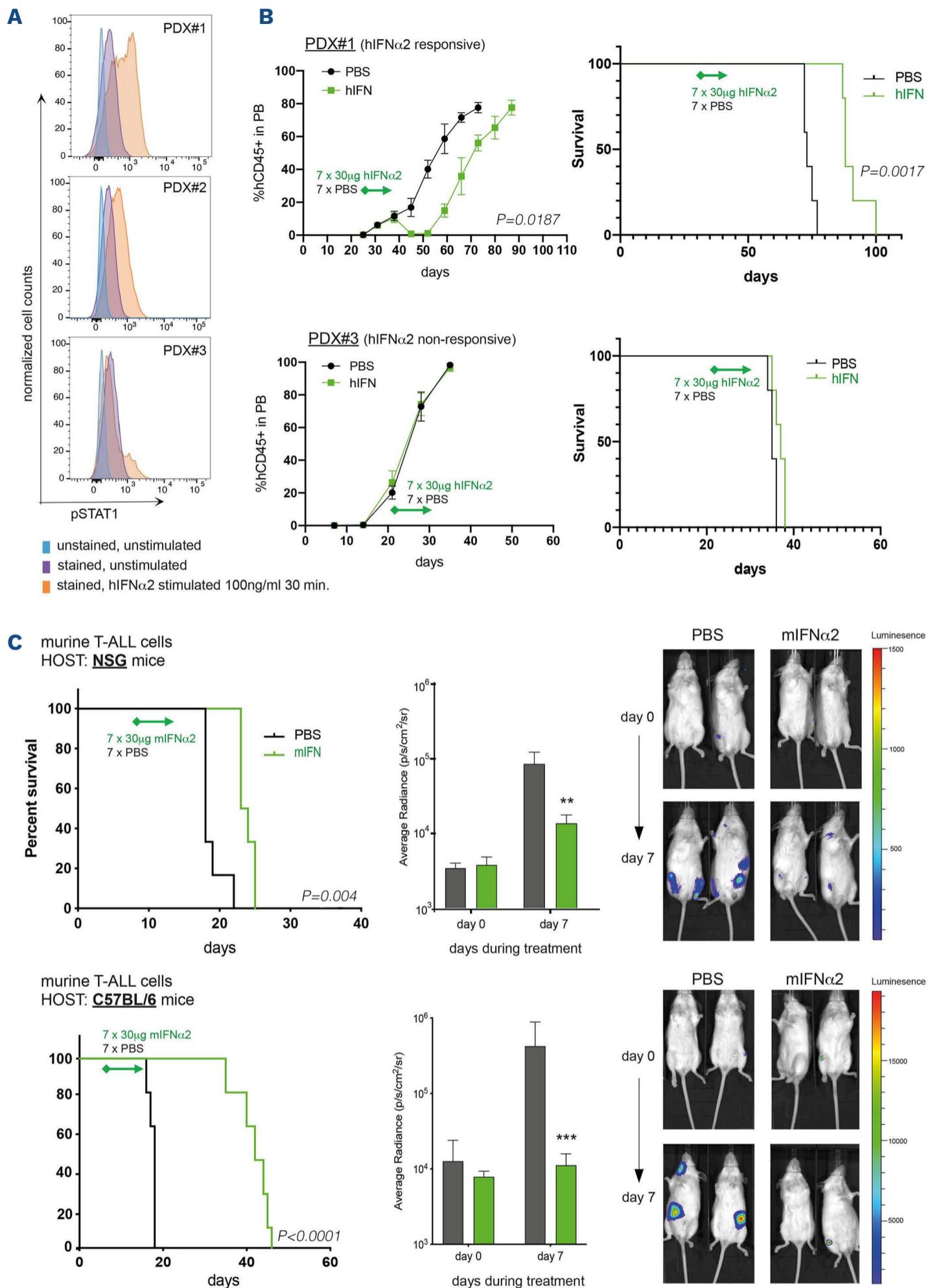
T-cell acute lymphoblastic leukemias (T-ALL) are rare aggressive hematologic tumors resulting from the malignant transformation of T-cell progenitors in the thymus.[1] T-ALL treatment currently consists of high-dose multi-agent chemotherapy, potentially followed by hematopoietic stem cell transplantation for high-risk patients.[2] Unfortunately, even with such a harsh and long treatment regime, the outcome of T-ALL patients with primary refractory or early relapsed leukemia remains poor. Novel therapeutic strategies to treat those high-risk patients remain an important unmet medical need.[3] Type-I interferons (IFN-I) have a long history in the treatment of cancer, including hematologic malignancies.[4] The pleiotropic anti-cancer effects induced by IFN-I result from a combination of: (i) direct cancer cell growth inhibition by inducing cell cycle arrest, apoptosis, and differentiation; and (ii) indirect effects by the activation of the immune system involving antigen presentation by dendritic cells and priming of cytotoxic CD8+ T cells.[5] As such, this cytokine, with both direct and indirect immunostimulatory anti-cancer effects, is unique in its kind. Nevertheless, IFN-I therapy experienced variable and unpredictable success in the clinic. Its clinical application is severely impeded by a complex pattern of adverse side-effects, due to the multifaceted pattern of activity of IFN-I.[6] Therefore, safe exploitation of the anti-cancer potential of IFN-I requires strategies to direct its activity to selected target cells only.

To document the direct anti-cancer effect of IFN-I on T-ALL, we stimulated nine human T-ALL cell lines with increasing concentrations of recombinant human interferon alpha-2 (hIFNα2) for 4 days and measured the effect on their *in vitro* growth. A variable anti-proliferative effect was observed, with no correlation between sensitivity to IFN-I and immunophenotype or molecular subtype (*Online Supplementary Figure S1*). Consistent with previous studies,[7,8] the anti-proliferative effect was associated with activation of the JAK/STAT1 pathway as documented by the increased levels of phosphorylated STAT1 (pSTAT1) determined by flow cytometry. We hypothesized that elevated pSTAT1 levels upon IFN-I stimulation could be used as a biomarker to stratify between IFN-I-sensitive and non-sensitive T-ALL patients. To validate our findings in primary T-ALL patients' material, we stimulated three patient-derived xenografts (PDX) samples with 100 ng/mL hIFNα2 for 30 min and quantified pSTAT1 levels (Figure 1A). As for the cell lines, a variable response to IFN-I was observed. Subsequently, we transplanted the IFN-I-responsive (PDX#1) and non-responsive (PDX#3) PDX samples

into immunodeficient NOD-*scid* IL2Rg[null] (NSG) mice. As soon as evidence of leukemia progression was observed (>5% human CD45+ [hCD45+] cells in peripheral blood), mice (5 mice/group) were treated for 7 consecutive days with intraperitoneal injections of 30 mg hIFNα2/mouse or vehicle (100 μL phosphate-buffered saline). A significant anti-leukemic effect was observed only for the IFN-I-responsive PDX#1, with a decrease in the percentage of hCD45+ cells in the peripheral blood at the end of the 7-day treatment regime and a prolonged leukemia-free survival of the mice (Figure 1B). Of note, IFNα2 has strict species specificity and human IFNα2 is unable to activate the mouse receptor complex,[7,9] indicating that the observed anti-leukemic effects can only be due to cell-intrinsic direct effects on the human leukemia cells.

To further document the indirect immune-mediated anti-leukemic properties of IFNα2, we generated an experimental T-ALL model that could be exploited to monitor leukemia progression both in an immunocompetent background as well as an immunodeficient background. To do this, we intercrossed the *Lck-cre*[tg/+]*;Pten*^{fl/fl} spontaneous murine T-ALL model[10] with a ROSA26-eGFP/luciferase reporter line[11] on a pure C57BL/6 background and derived eight primary murine T-ALL cell lines from diseased mice (*Online Supplementary Figure S1C*). As for the human T-ALL cell lines, a variable anti-proliferative effect with increased percentages of apoptotic cells was observed when murine T-ALL were treated for 3 days *in vitro* with increasing concentrations of recombinant mouse interferon alpha-2 (mIFNα2) (*Online Supplementary Figure S1D, E*). Subsequently, we transplanted the murine T-ALL cell lines in both immunodeficient NSG as well as immunocompetent syngeneic mice and treated them with intraperitoneal injections of 30 μg mIFNα2/mice or vehicle for 7 consecutive days (Figure 1C). As the injected T-ALL lymphoblasts express the eGFP-*Firefly* luciferase reporter from the *Rosa26* promoter, leukemic burden could be efficiently monitored using *in vivo* bioluminescence imaging.[11] mIFNα2 treatment resulted in a significant reduction of the leukemic burden, and prolonged survival of both immunodeficient and immunocompetent mice transplanted with murine T-ALL.

Notably, the therapeutic efficacy of mIFNα2 was consistently higher in the presence of an intact immune system (Figure 1C), suggesting an additive immune-mediated anti-leukemic effect. To uncouple these direct and putative indirect anti-leukemic properties of mIFNα2, we used our recently developed cell-type specific Activity-on-Target

Figure 1. Anti-leukemic effects of interferon alpha-2 on the progression of human and murine T-cell acute lymphoblastic leukemia *in vivo*. (A) Flow cytometric analysis of phospho-STAT1 (pSTAT1) levels in three established T-cell acute lymphoblastic leukemia (T-ALL) patient-derived xenografts (PDX) after 30 min. *ex vivo* stimulation with 100 ng recombinant human interferon alpha-2 (hIFNα2). (B) Direct anti-leukemic effects of recombinant hIFNα2 treatment (7 consecutive days with 30 μg/mouse) *versus* vehicle on type I interferon-responsive (PDX#1) and non-responsive (PDX#3) T-ALL patient-derived xenograft model transplanted in NSG mice (5 mice/group). Effect on leukemia progression was measured by flow cytometric quantification of the percentage of hCD45+ cells in peripheral blood (left) and Kaplan-Meier survival curves (right). (C) Anti-leukemic effects of recombinant murine interferon alpha-2 (mIFNα2) treatment (7 consecutive days; 30 μg/mouse) *versus* vehicle control (phosphate-buffered saline, PBS) on *in vivo* progression of murine T-ALL cell line transplanted in immunodeficient NSG animals or immunocompetent syngeneic C57BL/6 animals. Leukemia burden was quantified via *in vivo* bioluminescence imaging during treatment (right) and by Kaplan-Meier survival curves (left).

interferons (AcTaferons; AFN).[7,12-15] To obtain these AFN, we fused the hIFNa2[Q124R], a human IFNα2 mutation that enables binding to the murine interferon receptor complex with a 100-fold reduced affinity compared to wild-type (WT) mIFNα2, to VHH single domain antibodies targeting the murine CD8 (mCD8-AFN). We specifically chose the mCD8-AFN, as this CD8a epitope is not only present on 40% of T-ALL cases, but also on mouse classical dendritic cell type 1 (cDC1) which are particularly important for eliciting a CD8+ cytotoxic T lymphocyte response to kill cancerous cells upon IFNα2 stimulation.[12] Evaluating the therapeutic effects of the mCD8-AFN on both CD8+ *versus*
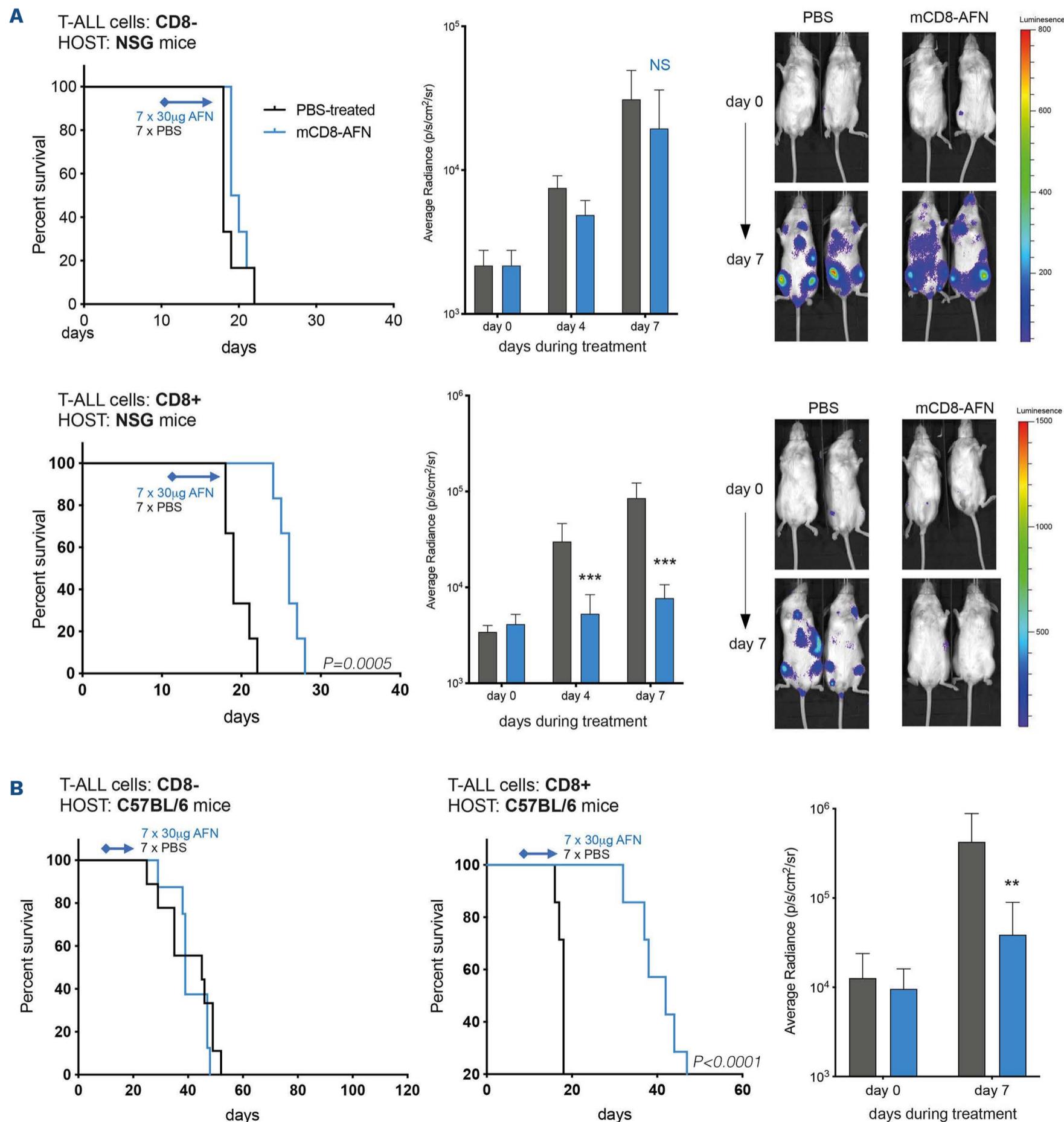
Figure 2. mCD8-AFN has direct but no indirect immune-mediated anti-leukemic effects on murine T-cell acute lymphoblastic leukemia. (A) Anti-leukemic effect of mCD8-AFN treatment (7 consecutive days; 30 μg/mouse) *versus* vehicle (phosphate-buffered saline, PBS) on *in vivo* progression of CD8– and CD8+ murine T-cell acute lymphoblastic leukemia (T-ALL) cell lines transplanted in immunodeficient NSG mice or (B) in syngeneic C57BL/6 mice. Leukemic burden was quantified via *in vivo* bioluminescence imaging during treatment regime (right) and Kaplan-Meier survival curves (left).
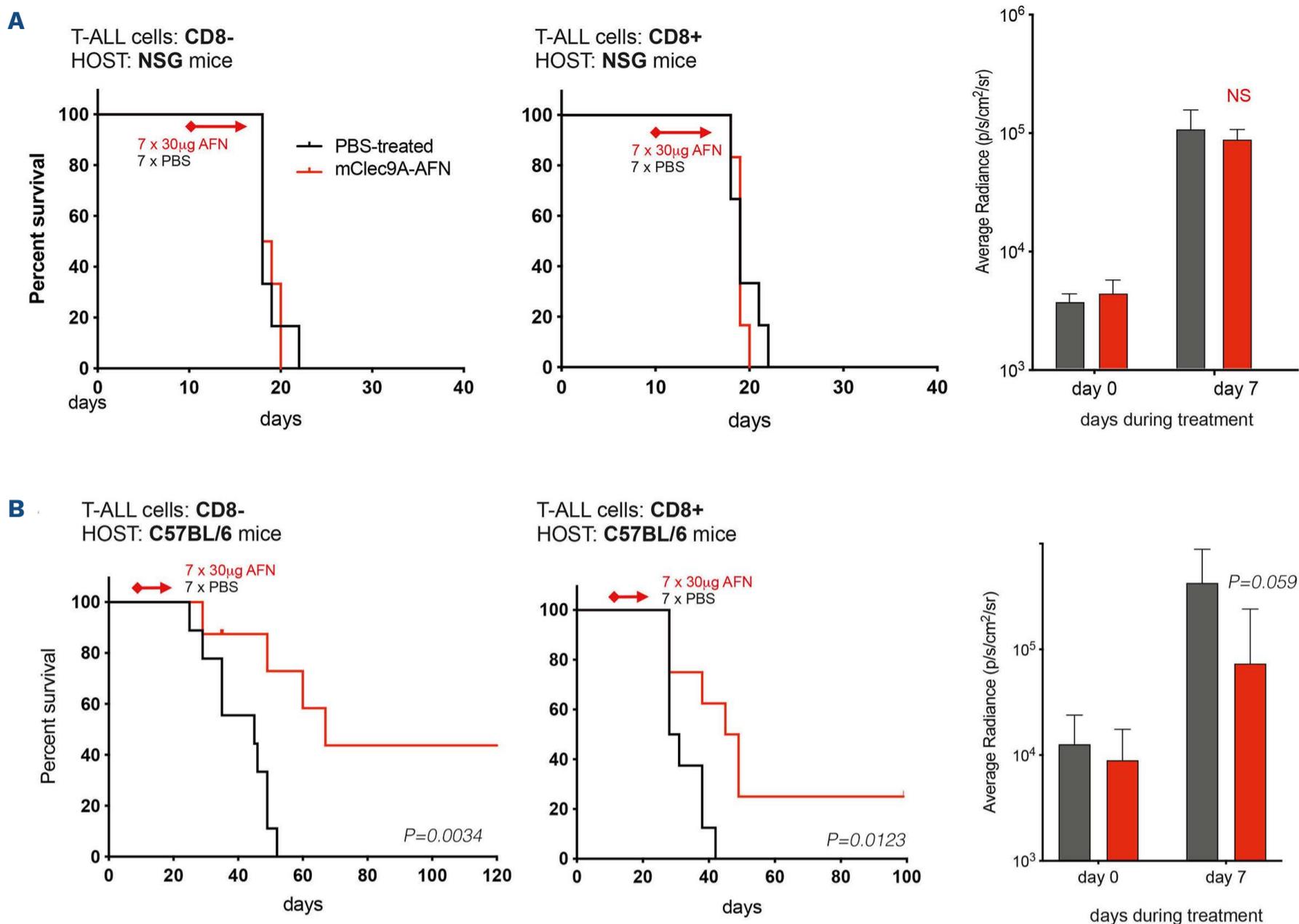
Figure 3. mClec9A-AFN has indirect but no direct anti-leukemic effects on murine T-cell acute lymphoblastic leukemia. (A) Anti-leukemic effect of mClec9A-AFN treatment (7 consecutive days; 30 mg/mouse) *versus* vehicle (phosphate-buffered saline, PBS) on *in vivo* progression of CD8⁻ and CD8⁺ murine T-cell acute lymphoblastic leukemia (T-ALL) cell lines transplanted in immunodeficient NSG mice or (B) in syngeneic C57BL/6 mice. Leukemic burden was quantified via *in vivo* bioluminescence imaging during treatment regime (right) and Kaplan-Meier survival curves (left).

CD8⁻ T-ALL in an immunocompetent *versus* immunodeficient background, allowed us to dissect the roles of the direct *versus* indirect anti-leukemic actions of IFNα2. To compare our results to those of a WT IFNα2 with a similar molecular weight as the AFN, we coupled this cytokine to a single-domain antibody targeting BclI10, an epitope that is absent in mice (mIFNα2-WT). As expected, the mCD8-AFN had only an anti-proliferative effect on the CD8⁺ T-ALL and not on the CD8⁻ T-ALL cell lines *in vitro* (*Online Supplementary Figure S2A-C*), confirming its cell-specificity. To evaluate the cell-specific direct anti-leukemic properties of mCD8-AFN on the growth of murine CD8⁺ T-ALL in an *in vivo* context, we transplanted both CD8⁺ and CD8⁻ T-ALL cell lines into immunodeficient NSG mice. As expected, the mCD8-AFN had a significant direct anti-leukemic effect only on the CD8⁺ T-ALL cell line, with a significant decrease of bioluminescence during the treatment and prolonged survival (Figure 2A). Of note, the direct anti-leukemic effect of the mCD8-AFN was more efficient than that of WT mIFNα2 (*Online Supplementary Figure S3*). As

AFN do not efficiently bind the ubiquitously expressed interferon receptor complex, they will not be cleared from the circulation before reaching their desired target population. This so-called 'sink-effect' can explain the improved direct anti-leukemic effect seen with the AFN.

As the CD8a epitope of the mCD8-AFN is also present on cDC1 cells, which have been shown to be superior in antigen cross-presentation, and on effector CD8⁺ T cells necessary to kill tumor cells, we hypothesized that the mCD8-AFN would also be able to induce an indirect anti-leukemic response. To test this, we repeated the experiment in an immunocompetent background. As an indirect immune-mediated effect would be independent of the immunophenotype of the T-ALL cells, we hypothesized that, in this setting, we would observe an anti-leukemic effect of the mCD8-AFN on both CD8⁻ and CD8⁺ T-ALL. However, no effect could be seen on the CD8⁻ T-ALL, only on the CD8⁺ T-ALL (Figure 2B), indicating that mCD8-AFN has only direct anti-leukemic T-ALL properties, and is not able to induce an indirect immune-mediated anti-leukemic response.

The inability of mCD8-AFN to elicit an immune-mediated anti-leukemic response could be either due to mature T-cell aplasia and leukemia progression in these mice, or due to the intrinsic nature of the AFN. We therefore tested the anti-leukemic properties of a second AFN, mClec9a-AFN, which we previously used to efficiently induce a cDC1-mediated anti-tumor response in the context of melanoma, breast carcinoma and lymphoma.[12] As Clec9A is not expressed in normal or malignant T cells, no direct anti-leukemic properties could be observed *in vitro* (*Online Supplementary Figure S2A-C*) or *in vivo* in immunodeficient NSG mice (Figure 3A). To evaluate the indirect immune-mediated anti-leukemic effects of mClec9A-AFN, we repeated the experiment in syngeneic C57BL/6 mice (7 mice/group). This time, a significant indirect effect on leukemic progression could be seen in the transplanted mice, irrespective of whether they were transplanted with CD8$^+$ or CD8$^-$ T-ALL (Figure 3B). These data demonstrate that mClec9A-AFN is able to induce a strong immune-mediated anti-tumor response. However, we currently cannot explain why we saw a difference between the ability of mCD8-AFN and mClec9A-AFN to induce an indirect immune-mediated anti-leukemic effect. In the mouse, the Clec9A-epitope is also present on CD8$^-$ immune cell subtypes, which may contribute to the immune response. Alternatively, binding of the CD8-AFN on cytotoxic CD8$^+$ T cells may (partially) hamper their cytotoxic functions, although the anti-CD8 single-domain antibody that was used for the design of the mCD8-AFN was demonstrated to be a non-neutralizing antibody.[15]

Finally, the adverse side-effects (body weight loss and hematologic deficiencies) observed in mice treated with mIFNα2-WT were significantly reduced in mice treated with the AcTaferons (*Online Supplementary Figure S2D, E*), as shown before.[7,12,13]

In summary, we used preclinical mouse models of T-ALL to show that immunocytokines with cell-specific activity can preserve the anti-leukemic properties of IFNα2 with a concomitant reduction of systemic toxic side-effects. As such, these AcTaferons can be used as a direct anti-leukemic agent in combination with classical chemotherapy, or as off-the-shelf targeted immunotherapy for T-cell malignancies.

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JT and AC are affiliated with Orionis Biosciences. JT holds equity interests and receives financial research support from Orionis Biosciences. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Contributions

SG, AC, PVV and JT conceived and designed the experiments and wrote the paper. SG, AJ, TP, ST and RDS performed experiments. SG and ST analyzed data. SG, TP and AA generated the mouse T-cell acute lymphoblastic leukemia cell lines. AC and JT produced the cytokines. SG, AC, WD, PVV and JT discussed results and critically reviewed the manuscript. SG and PVV directed and supervised the research. All authors approved the final manuscript.

Data-sharing statement

Original data can be made available in response to a reasonable, written request to the corresponding author.

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