The role of the mineralocorticoid receptor in steroid-induced cytotoxicity in pediatric acute lymphoblastic leukemia

Glucocorticoids, e.g. prednisone and dexamethasone, were among the first classes of drugs used in the treatment of childhood acute lymphoblastic leukemia (ALL) and are still regarded as cornerstone drugs in ALL therapy.[1] Glucocorticoids can bind and activate the glucocorticoid receptor (GR, encoded by the *NR3C1* gene) and the mineralocorticoid receptor (MR, encoded by the *NR3C2* gene). The cytotoxic effect of glucocorticoids seems to be exerted mainly through the GR, and clinical as well as *in vitro* steroid resistance (a poor prognostic factor for the survival of patients with ALL) are related to *NR3C1* aberrations.[2,3] *NR3C2* mutations have been less frequently studied and the role of the MR in steroid cytotoxicity therefore remains unclear.

Synthetic glucocorticoids differ in their ability to activate the GR and MR. Prednisone has a high affinity for both receptors, whereas dexamethasone only has strong potency to activate the GR.[4] Hydrocortisone, i.e. the naturally occurring hormone cortisol, can bind both receptors, with a greater affinity for the MR.[4] Interestingly, hydrocortisone seems to potentiate the cytotoxic effect of both prednisolone and dexamethasone in steroid-sensitive ALL cells.[5] Moreover, hydrocortisone appeared to be as potent as dexamethasone or prednisolone in cytotoxicity assays.[5] Since hydrocortisone has far fewer side effects compared to dexamethasone or prednisone and could conceivably be used as an alternative therapy when dexamethasone toxicity is too high, it would be of interest to investigate the cytotoxic effect of hydrocortisone. The purpose of the present study was therefore to establish the role of the MR in steroid-induced cytotoxicity in patients with ALL and to evaluate the antileukemic activity of hydrocortisone.

To compare the differential cytotoxic effects of dexamethasone, prednisolone and hydrocortisone via the GR or the MR in leukemic cells, we generated bulk-transduced Reh cells with a doxycycline-inducible DDK-tagged *NR3C1* or *NR3C2* construct, respectively. Gateway multisite recombination (Invitrogen) was used for gateway cloning of lentiviral expression vectors as previously described.[2] The inducibility of the *NR3C1* or *NR3C2* constructs was assessed through flow cytometry following intracellular DDK staining (*Online Supplementary Figure S1A*). We selected two clones per cell line ($Reh^{NR3C1\text{-}A}$, $Reh^{NR3C1\text{-}B}$, $Reh^{NR3C2\text{-}A}$ and $Reh^{NR3C2\text{-}B}$), of which clones A were used primarily. Doxycycline exposure induced the expression of DDK-tagged NR3C1 or DDK-tagged NR3C2 proteins in Reh parental cells that lack a functional GR and MR (Figure 1A). Hydrocortisone and dexamethasone treatment further enhanced protein expression and correspondingly showed strong induction of BIM (Figure 1A).

Next, we studied the expression of *NR3C1* and *NR3C2* in both cell line models upon treatment with dexamethasone or hydrocortisone. For normalized expression levels, the expression of non-doxycycline-induced cells in the absence of steroid treatment was set at one. Doxycycline exposure induced *NR3C1* construct expression in the Reh^{NR3C1} cell line (Figure 1B, left panels). Dexamethasone and hydrocortisone enhanced endogenous *NR3C1* and *NR3C2* expression levels, signifying that *NR3C2* is a target gene of *NR3C1*. In Reh^{NR3C2}, doxycycline exposure induced expression of *NR3C2*, which was enhanced by hydrocortisone and to a lesser extent by dexamethasone (Figure 1B, right panels). In contrast to its effect on Reh^{NR3C1} cells, hydrocortisone treatment particularly induced *NR3C1* expression in Reh^{NR3C2} cells. Expression of known *NR3C1* transcriptional target genes *BIM*, *GILZ* and *FKBP5* was also strongly induced by hydrocortisone and dexamethasone in Reh^{NR3C1} cells, whereas strong transcriptional upregulation of these genes was predominantly achieved by hydrocortisone treatment in Reh^{NR3C2} cells (Figure 1C). Since Reh cells do not have a functional *NR3C1* allele, these results demonstrated that induction of GR-regulated target genes can also be achieved through activation of the MR, and especially through exposure to hydrocortisone.

The strong induction of BIM by hydrocortisone treatment in Reh^{NR3C2} cells (Figure 1A, C) is of interest since BIM mediates steroid-induced apoptosis of lymphoid cells.[7] The induction of BIM by hydrocortisone and to lesser extents by dexamethasone and prednisolone in Reh^{NR3C2} cells indicates that the MR may play a role in steroid-induced cytotoxicity. We examined the cytotoxic effects of steroid treatment in *NR3C1*- or *NR3C2*-expressing Reh cells using a methylthiazolyldiphenyl-tetrazolium bromide (MTT) cell viability assay. In the absence of doxycycline, Reh^{NR3C1} and Reh^{NR3C2} cells were completely refractory to dexamethasone, prednisolone or hydrocortisone treatment (Figure 2A, *Online Supplementary Figure S1B*). Doxycycline-induced *NR3C1* or *NR3C2* expression sensitized Reh cells to all three steroids. Hydrocortisone was the most potent steroid in both Reh^{NR3C1} and Reh^{NR3C2} cells. Furthermore, the cytotoxic effect induced by dexamethasone was comparable in cells expressing GR or MR. These results show that hydrocortisone can cause significant steroid-induced cell death of leukemic cells, through both MR and GR. Despite the (relative) lack of transcriptional upregulation of target genes, dexamethasone induces significant steroid-induced death of Reh^{NR3C2} cells.

To verify the role of the MR in steroid-induced cell death, we

treated Reh^{NR3C1} and Reh^{NR3C2} cells with RU28318, a specific MR antagonist.[8] RU28318 treatment in Reh^{NR3C2} cells completely inhibited the cytotoxic potential of the MR following steroid treatment, but not the GR potential in Reh^{NR3C1} cells (Figure 2B). To study the potential clinical relevance of these observations, we first determined the relative expression of *NR3C1* and *NR3C2* in 278 primary ALL patients' samples.[9] The relative expression of *NR3C1* was higher than that of *NR3C2* (Figure 3A) and patients with an *ETV6-RUNX1* fusion gene had the highest *NR3C2* expression, as described before.[5] As proof of concept, we treated one ALL patient-derived xenograft model and two primary patients' samples that

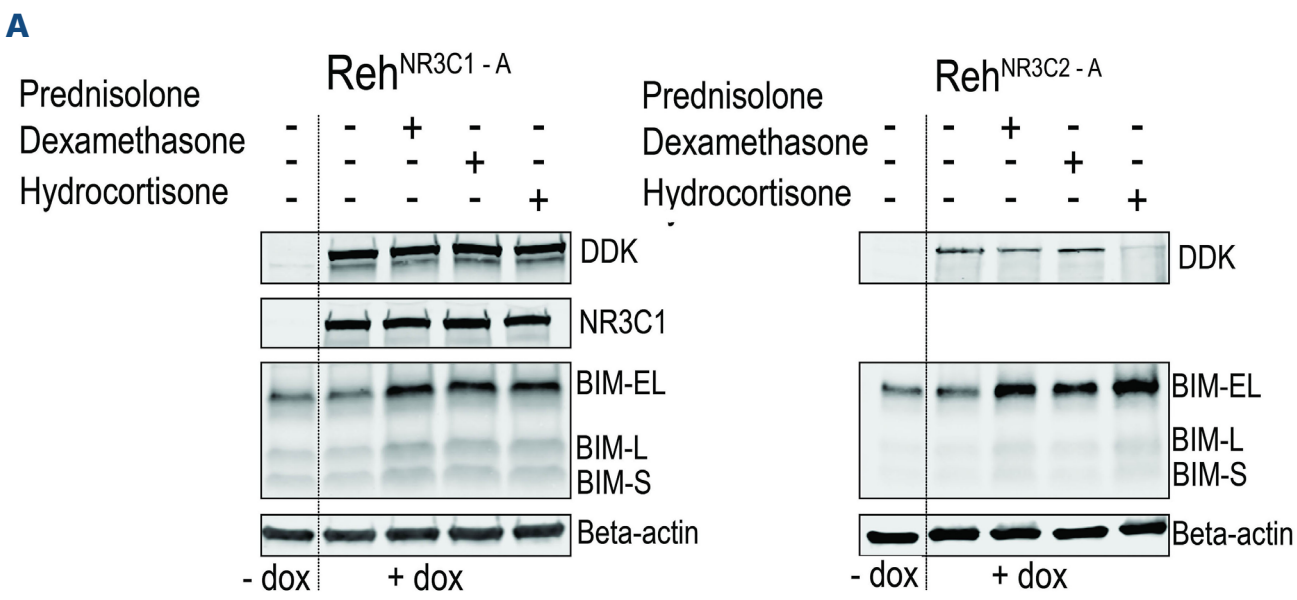

Figure 1. Hydrocortisone can induce expression of *NR3C1* and *NR3C2* via both the glucocorticoid receptor and the mineralocorticoid receptor. (A) Western blot analysis of DDK, NR3C1 and BIM in Reh cell lines that were transfected with either doxycycline-inducible DDK-tagged *NR3C1* or *NR3C2* constructs, after treatment with prednisone, dexamethasone or hydrocortisone, as indicated. (B) Transcriptional steroid response of Reh cell lines transfected with doxycycline-inducible *NR3C1* or *NR3C2* constructs. After doxycycline induction, cells were treated with 0.16 μM dexamethasone or 0.032 μM (Reh^{NR3C1}) or 0.0028 μM (Reh^{NR3C2}) hydrocortisone. Expression of *NR3C1* (upper panels) and *NR3C2* (lower panels) was measured in both cell lines, as was (C) the expression of *BIM, GILZ* and *FKBP5*, target genes of the glucocorticoid receptor and the mineralocorticoid receptor. Dox: doxycycline.

harbored the *ETV6-RUNX1* fusion gene with different concentrations of steroids in combination with RU28318. Viability was determined by amino staining and cytotoxicity was calculated as area under the curve (AUC) values. In contrast to Reh cells, we only observed a modest, not statistically significant decrease in steroid sensitivity after treatment with RU28318 (Figure 3B).

Furthermore, we studied the association between *NR3C1* and *NR3C2* expression and event-free survival in a subgroup of 131 ALL patients for whom outcome data were available (informed consent compliant with the biobanking procedure in the Princess Máxima Center [MEC-2016-739]). The levels of MR and GR expression were categorized as low or high, with the median as the cutoff value.[10] Event-free survival was estimated using the Kaplan-Meier methodology and the effect of prognostic factors on event-free survival was estimated with Cox proportional hazard regression models. In a univariable Cox regression, we did not find significant associations between either *NR3C1* expression (hazard ratio=0.96, 95% confidence interval: 0.40-2.30) or *NR3C2* expression (hazard ratio=0.57, 95% confidence interval: 0.24-1.33), and any of 25 observed events (*Online Supplementary Table S1*). Event-free survival was also not statistically significantly different between patients with high or low *NR3C1* and *NR3C2* expression (*Online Supplementary Figure S2A*). Together, these findings indicate that, even though MR made a pronounced contribution in our cell line models, the role of the MR in steroid-induced cytotoxicity in ALL patients appears to be limited.

In contrast to previous studies,[4,11,12] we found that hydrocortisone seemed to be more efficient in inducing cell death than was either prednisolone or dexamethasone in both Reh^{NR3C1} and Reh^{NR3C2} cell line models. This may be related to the high expression of *NR3C1* and *NR3C2* in our models compared to the expression in patient-derived xenografts or patients' samples (*Online Supplementary Figure S2B*). Nevertheless, the inter-patient variability in steroid sensitivity is high, with cytotoxicity values sometimes varying more than 1,000-fold among patients' samples.[11,12] It therefore cannot be excluded that certain patients may benefit from hydrocortisone treatment, especially when dexamethasone-induced side effects occur.

Patients with the *ETV6-RUNX1* fusion gene have high *NR3C1* and *NR3C2* expression (Figure 3A), as well as an excellent prognosis.[13] We speculated that the MR contributes to this superior survival, but our experiments only showed a minimal shift in cell toxicity curves after the addition of RU28318 to two primary ALL patients' samples and one patient-derived xenograft model with the *ETV6-RUNX1* fusion gene. An explanation for the difference between these samples and our experimental setting may be a lower expression or less transcriptional activity of *NR3C2* compared to *NR3C1* in

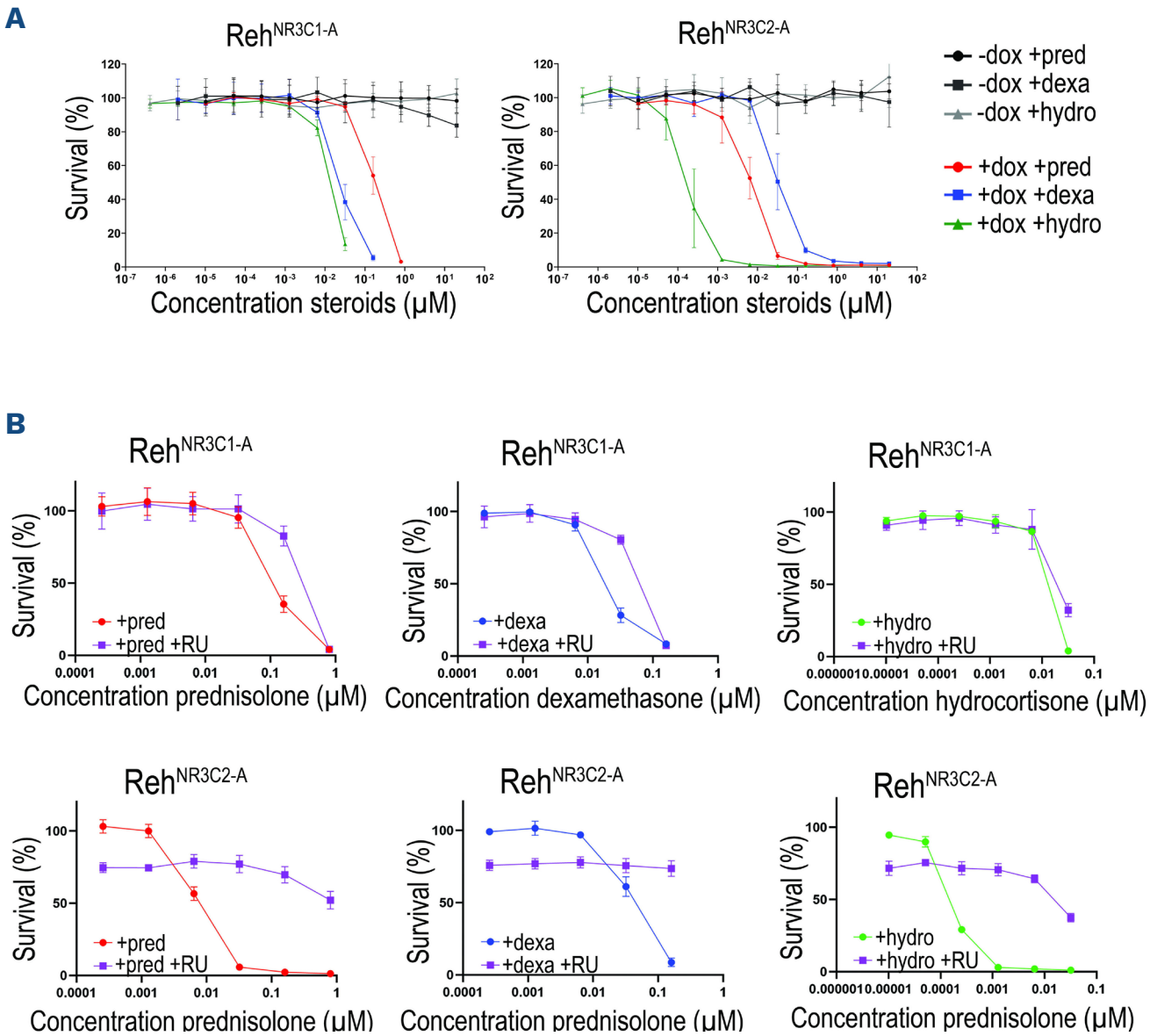

Figure 2. Hydrocortisone is the most potent steroid in *NR3C1*- and *NR3C2*-overexpressing cells. (A) Cell toxicity screening of Reh^{NR3C1} (left) and Reh^{NR3C2} (right) cells with (color) and without (gray-scales) doxycycline induction and after treatment with prednisolone, dexamethasone or hydrocortisone. Steroid sensitivity was determined with an MTT assay. Data represent biological triplicates, with standard deviations. (B) Cell toxicity screening of doxycycline-induced Reh^{NR3C1} (upper panels) and Reh^{NR3C2} (lower panels) with and without 4 µM RU28318 (mineralocorticoid receptor antagonist) treatment in combination with prednisolone, dexamethasone or hydrocortisone. RU28318 treatment in Reh^{NR3C2} cells reversed the acquired steroid sensitivity. Dox: doxycycline; pred: prednisolone; dexa: dexamethasone; hydro: hydrocortisone; RU: RU28318.

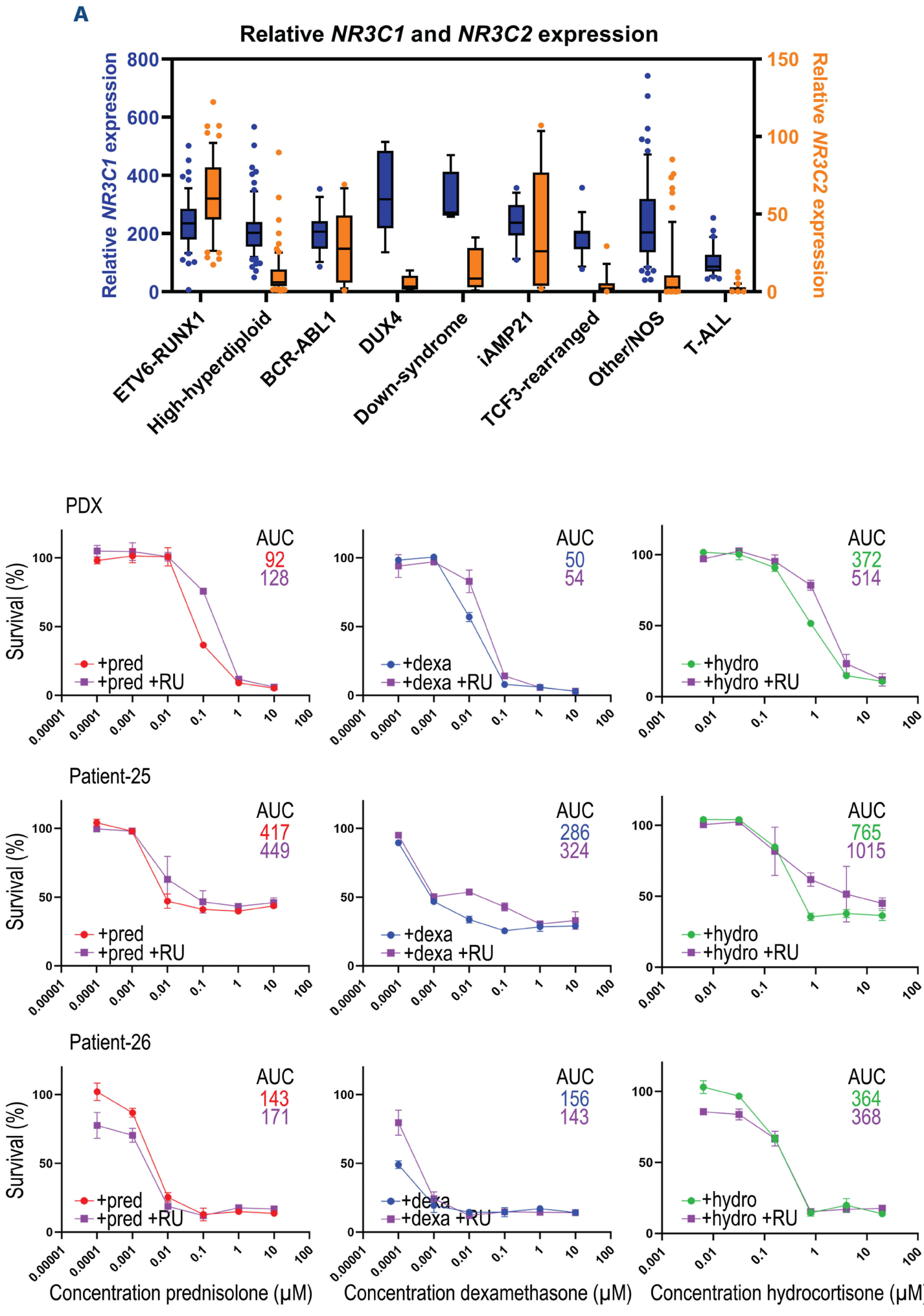

Figure 3. *NR3C2* expression in patients is relatively low. (A) Relative expression of *NR3C1* (blue) and *NR3C2* (orange) in 279 primary samples from patients with acute lymphoblastic leukemia, dissected according to genetic background. (B) Cell toxicity screening of two primary patients' samples and one patient-derived xenograft sample, all harboring the *ETV6-RUNX1* fusion gene. Toxicity screening was performed using amino staining and data represent technical duplicates with standard deviations. Samples were treated with prednisolone, dexamethasone or hydrocortisone, in the presence or absence of 4 μM RU28318 (a mineralocorticoid receptor antagonist). Area under the curve values were calculated using Prism software version 9.3.0 from GraphPad and statistically tested with a two-sided *t* test. iAMP21: intrachromosomal amplification of chromosome 21; NOS: not otherwise specified; T-ALL: T-cell acute lymphoblastic leukemia; PDX: patient-derived xenograft; AUC: area under the curve; pred: prednisolone; RU: RU28318; dexa: dexamethasone; hydro: hydrocortisone.

patients' leukemic cells (*Online Supplementary Figure S2B*) or the presence of other more dominant factors (genetic and/or cellular) in these patients. Due to the lack of a functional NR3C2 antibody, we were unable to test this at the protein level. Moreover, no genes have been identified to be specifically regulated by either *NR3C1* or *NR3C2*, preventing more specific transcriptional analyses.[4,14] The contribution of the MR in steroid-induced cytotoxicity in our patients does, therefore, remain unclear.

In our cohort of ALL patients (n=131), we did not find an association between *NR3C1* or *NR3C2* mRNA expression levels and event-free survival. This may be partially explained by the relatively short median follow-up of our cohort (median 26.1 months, 95% confidence interval: 23.8-28.4) and the relatively small sample size. It is however conceivable that other crucial processes play a more dominant role in relapse, such as chemotherapy-induced mutations.[15]

In conclusion, in experimental models of ALL, the MR (*NR3C2*) strongly induces steroid-induced cell death and hydrocortisone is a potent steroid to initiate this process. Although the cytotoxic contribution of the MR in leukemic patients' samples appears to be minimal, hydrocortisone may still be considered as a potential antileukemic agent, especially for those patients who suffer from severe dexamethasone-induced side effects.

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Disclosures

No conflicts of interest to disclose.

Contributions

AMvH and JCGvdZ designed the study, performed the experiments and wrote the manuscript. ELTvdA, MMvdHE and JM conceptualized the study and acquired funding. AMvH and MF performed statistical analyses. JBG and WS performed the experiments. RP, FNvL, MvdHE and ELTvdA provided critical input and wrote the manuscript. JPPM designed and supervised the study and wrote the manuscript. All authors read and approved the final version of the manuscript.

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Data-sharing statement

The data that support the findings of this study are available on request from the author for correspondence, MvdHE.

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