Unraveling Glucocorticoid Resistance In *MLL*-rearranged Infant Acute Lymphoblastic Leukemia

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Unraveling Glucocorticoid Resistance In *MLL*-rearranged Infant Acute Lymphoblastic Leukemia

Het ontrafelen van glucocorticoïd-ongevoeligheidsmechanismen
bij zuigelingen met acute lymfatische leukemie met een *MLL*-herschikking

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"De betekenis van ons leven ligt in het verschil
dat we maken in de levens van anderen"

-Nelson Mandela (1918-2013)-

CONTENTS

CHAPTER 1

General Introduction

Leukemia

In the Netherlands, approximately 650 children aged between 0 and 18 years are diagnosed with cancer every year, including ~120 patients suffering from leukemia. Leukemia (Greek for *leukos* - white, and *haima* for blood) is a type of cancer characterized by an abnormal increase of immature (non-functional) white blood cells in the bone marrow. As a result, the production of all healthy, functional blood cells is impaired, leading to anemia (loss of functional red blood cells), infections (loss of functional white blood cells) and (internal) bleeding (loss of functional platelets), and eventually to leukemic infiltration of other tissues such as liver, spleen, skin and in some instances even in the central nervous system. Depending on the rate of disease progression, leukemia is classified into "acute" (rapidly developing) or "chronic" (slowly developing). Acute leukemias are usually characterized by uncontrolled proliferation of highly immature (leukemic) white blood cells, whereas chronic leukemias more often involve the malignant transformation of more differentiated white blood cells. Leukemia can further be classified into lymphoid (B-cell or T-cell leukemias) or non-lymphoid (myeloid) types of leukemia, depending on the type of white blood cell that was subjected to leukemic transformation.

Childhood Acute Lymphoblastic Leukemia (ALL)

The most prevalent type of leukemia among children is acute lymphoblastic leukemia (ALL), affecting ~80% of all children diagnosed with leukemia (**Figure 1A**), with a peak incidence at the age of 2-5 years (Pui *et al.* 2008).

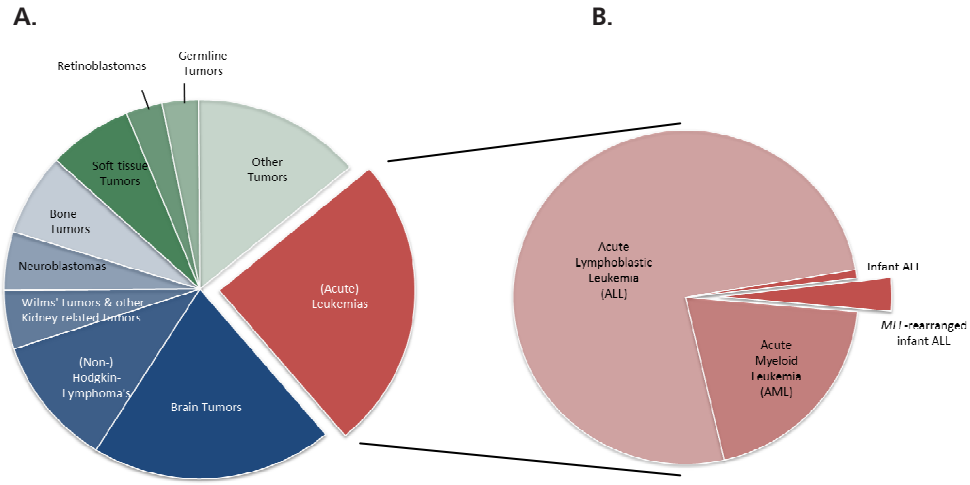

Figure 1. Distribution of childhood cancer in the Netherlands.
A. The most common type of cancer in children (0-18 year of age) represents acute leukemia (~25% of all cases), particularly leukemias that affect the lymphoblastic lineage (*i.e.* acute lymphoblastic leukemia: ALL). **B.** Within the subgroup of childhood ALL, only a small subset ALL is diagnosed in infants (<1 year of age; ~4% of all cases). In turn, infant ALL is characterized by an exceedingly high incidence of translocations of the *Mixed Lineage Leukemia* (*MLL*) gene), which are found in ~80% of all infant ALL cases (data derived from "Stichting Kinderoncologie Nederland (SKION)).

Over the last 50 years, the cure rate for childhood ALL has increased tremendously due to the availability of more adequate chemotherapeutics and improved treatment strategies. As a result, event-free survival (EFS) rates for children with ALL have increased from 5-10% in the early 1960s to an impressive 80-90% at present (Pui *et al.* 1996, Frankel *et al.* 1997, Reaman *et al.* 1999, Dördelmann *et al.* 1999, Chessells *et al.* 2002, Pieters *et al.* 2007, Tomizawa *et al.* 2007). This spectacular improvement in clinical outcome still represents one of the most successful achievements in pediatric oncology. Unfortunately, these improved treatment regimes did not appear beneficial to all patients with ALL. Perhaps the most striking example of a subgroup of ALL patients to whom the improved therapies were only marginally advantageous is infants (*i.e.* children less than 1 year of age). ALL in infants is relatively rare (**Figure 1B**), and with EFS rates of approximately 50%, the prognosis for infant ALL patients still remains poor (Pieters *et al.* 2007).

Infant ALL is characterized by a high incidence of balanced chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene, occurring in ~80% of all cases. As a result of these translocation events, the N-terminal part of the *MLL* gene (on chromosome 11q23) becomes fused to the C-terminal part of one of its many translocation partner genes derived from other chromosomes. To date, 79 different *MLL* translocations partner genes have been identified (Meyer *et al.* 2013). Among *MLL*-rearranged infant ALL patients, the most recurrent types of *MLL* translocations are t(4;11), t(11;19), and t(9;11), leading to the formation, transcription, and translation of the chimeric proteins MLL-AF4, MLL-ENL, and MLL-AF9, respectively (Gu *et al.* 1992, Tkachuk *et al.* 1992, Iida *et al.* 1993, Huret *et al.* 2001, Meyer *et al.* 2005, Jansen *et al.* 2007). The presence of leukemia-specific *MLL*-gene rearrangements has been shown to be a strong and independent adverse prognostic factor (Pieters *et al.* 2007), defining infant ALL patients at high risk of therapy failure. The EFS rates for *MLL*-rearranged infant ALL patients currently are at best 30-40%, whereas infant ALL patients who do not carry *MLL* translocations (*i.e.* wild-type *MLL* infant ALL) fare significantly better, with EFS rates of 75-90% (Pui *et al.* 1996, Heerema *et al.* 1999, Reaman *et al.* 1999, Pieters *et al.* 2007, Tomizawa *et al.* 2007) (**Figure 2**).

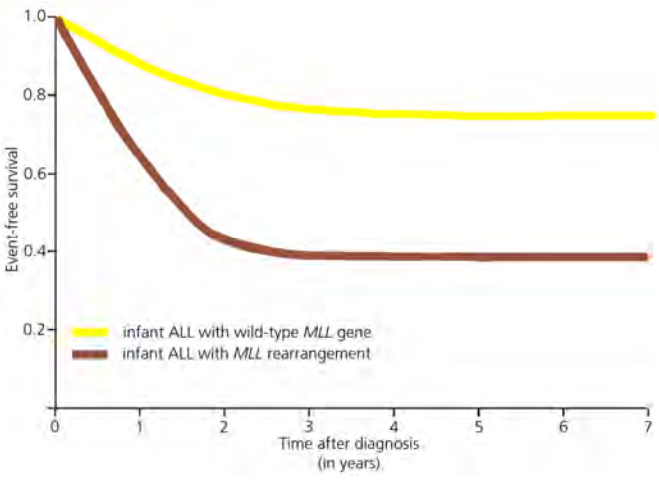

Figure 2. Event-free survival rates in infant ALL

Event-free survival of infant ALL patient samples divided into wild-type *MLL* infant ALL (~75%; yellow) or *MLL*-rearranged infant ALL (~30-40%; purple) (Kaplan-Meier curve is adapted from Pieters *et al.* 2007)

Cellular drug resistance

A likely contributing factor to the poor prognosis of *MLL*-rearranged infant ALL is cellular drug resistance, as *MLL*-rearranged ALL cells are notoriously resistant to multiple chemotherapeutics currently used in the treatment of ALL. This is especially true for glucocorticoids (GCs) such as prednisone and dexamethasone (see box 1).

Box 1 | Glucocorticoids (GCs; *e.g.* prednisone) in *MLL*-rearranged infant ALL

Prednisone and dexamethasone are synthetic glucocorticoid drugs that bind and activate the glucocorticoid receptor (GR). The activated GR-GC complex is dissociated from its chaperone proteins and either binds to activating protein (AP-1) or nuclear factor-kB (NFkB) and subsequently represses their activity; or the GR-GC complex dimerizes and binds to GC responsive elements (GREs) and thereby activating gene expression (*i.e.* cell proliferation and survival pathways, NFkB signaling and glucose metabolism) (Inaba *et al.* 2010). Prednisone and dexamethasone exert potent anti-cancer activity, and represent important drugs in the treatment of acute lymphoblastic leukemia (ALL), (Non-) Hodgkin lymphoma, and multiple myeloma. In fact, glucocorticoids represented one of the first classes of drugs used in the treatment of ALL, and currently it is unthinkable to treat ALL patients on regimes lacking glucocorticoids. Moreover, in ALL both the *in vitro* and *in vivo* resistance to glucocorticoids has become an important predictor of an adverse clinical outcome. From that perspective, the characteristically poor prognosis for infant patients diagnosed with *MLL*-rearranged ALL may to great extent be associated to a notoriously poor response to glucocorticoids.

For instance, *in vitro* cytotoxicity data showed that infant ALL cells are ~500-fold more resistant to prednisolone (*i.e.* the active metabolite of prednisone) as compared with ALL cells from children older than 1 year of age (Pieters *et al.* 1998). Moreover, about one-third of all *MLL*-rearranged infant ALL cases show a poor *in vivo* response to a 7-day window of prednisone mono-therapy, whereas for ALL patients who do not carry translocations of the *MLL* gene <10% responds poorly to prednisone. Yet, despite the GC-resistant character of *MLL*-rearranged infant ALL, GC including induction chemotherapy courses induce morphological complete remission in ~95% of the cases. Therefore, GCs should not be excluded from the current infant ALL treatment protocols. Unfortunately, the majority of these patients experience relapses within the first two years of diagnosis, while still on therapy. As both *in vitro* and *in vivo* prednisone responses have been identified as prognostic markers in childhood ALL (Kaspers *et al.* 1998, Dördelmann *et al.* 1999, Den Boer *et al.* 2003), overcoming GC resistance may well decrease the risk of disease relapse and lead to an improvement in clinical outcome. For this, however, a firm understanding of the biological mechanisms underlying GC resistance is still needed. Unfortunately, still little is known about GC resistance mechanisms in *MLL*-rearranged ALL (see box 2).

Box 2 | No involvement of the glucocorticoid receptor (GR) in GC resistance mechanisms

In an inactive state, the cytosolic glucocorticoid receptor (GR) forms a multi-protein complex involving numerous proteins, including p23, heat-shock proteins (HSP90 and HSP70), immunophilins and Src kinases. Upon glucocorticoid (GC) binding to the GR, HSP90 is acetylated resulting in GR release from the complex and GR homodimerization, and subsequent GR activation. Eventually, activated GR homodimers translocate to the nucleus, where they bind to glucocorticoid responsive elements (GRE) sequences within the promoter regions of associated target genes. Binding to GREs activates or inhibits either transcription, transactivation, or transrepression (Ray *et al.* 1994, Hayashi *et al.* 2004, Pratt *et al.* 2006, Buckingham *et al.* 2006). Thus aberrant expression of either the GR or its co-chaperone proteins might induce glucocorticoid resistance in pediatric ALL. However, GC resistance did not appear to correlate with high-level GR mRNA expression, neither with aberrant expression of its co-chaperone proteins (Tissing *et al.* 2005a/b). Additionally, genetic variations of the GR were also not the origin for glucocorticoid resistance, whereas no differences in mutations were found when comparing glucocorticoid-resistant to glucocorticoid-sensitive pediatric ALL samples (Tissing *et al.* 2005c).

Recently, Holleman *et al.* defined gene signatures associated with drug resistance, including a prednisolone-resistance signature in primary childhood ALL (Holleman *et al.* 2004). Yet, given the tremendous differences in transcriptome activity between *MLL*-rearranged infant ALL and pediatric (non-infant) ALL cells (Stam *et al.* 2010a), this prednisolone-resistance signature does not fully apply to *MLL*-rearranged ALL patients.

Therefore we set out to define a gene expression signature associated with *in vitro* prednisolone resistance specifically for *MLL*-rearranged infant ALL cells, using our recently published gene expression profiling data (Stam *et al.* 2010b). Comparing the prednisolone-resistance gene expression signatures associated with pediatric (non-infant) ALL with those defined for *MLL*-rearranged infant ALL, indeed demonstrated highly distinctive gene lists with only a few overlapping genes associated with prednisolone resistance in both types of leukemia (Stam *et al.* 2010b). Hence, the mechanisms underlying prednisolone resistance in pediatric ALL are likely to be different from those in *MLL*-rearranged infant ALL. Despite several reported attempts to elucidate the mechanisms of prednisolone resistance in ALL (see: Box 2), the molecular basis of this phenomenon still remained largely unknown. In this thesis, we used the specific prednisolone-resistance gene signature to explore the involvement of several of these genes in the *in vitro* prednisolone resistance, and to unravel possible mechanisms of resistance in *MLL*-rearranged ALL.

OUTLINE OF THE THESIS

Chapter 2 of this thesis describes a study investigating the correlation of *in vitro* drug sensitivity with clinical outcome and known prognostic parameters in a relatively large cohort of primary *MLL*-rearranged infant ALL cases. We recently identified high-level expression of anti-apoptotic *MCL1* to be associated with prednisolone resistance in *MLL*-rearranged ALL. Therefore, in **Chapter 3** we explored the value of potent MCL1 inhibitors in inducing prednisolone sensitivity in *MLL*-rearranged infant ALL. In **Chapter 4** we investigated the role of two S100 family members, *i.e. S100A8* and *S100A9*, which are both highly expressed in prednisolone-resistant primary *MLL*-rearranged infant ALL cells. S100A8 and S100A9 dimerize together to form a protein complex that highly efficiently bind and absorb free-cytosolic Ca^{2+}. Interestingly, cytosolic Ca^{2+} plays a pivotal role in GC-induced apoptosis in lymphoid cells. Upon exposure to GCs, Ca^{2+} is released from the endoplasmic reticulum (ER). Continuous increases in cytosolic Ca^{2+} subsequently leads to increased uptake of Ca^{2+} in the mitochondria, which eventually triggers the apoptotic program inevitably resulting in programmed cell death. In **Chapter 5** we studied the role of an additional calcium-binding protein Annexin A2 (encoded by the gene *ANXA2*) related to prednisolone resistance. *ANXA2* appeared to be highly expressed in prednisolone-resistant *MLL*-rearranged infant ALL patient samples, but not in prednisolone-sensitive samples. In **Chapter 6** we applied Connectivity Map analysis to search for agents potentially capable of reversing the prednisolone-resistance gene expression signature, and induce prednisolone sensitivity. The Connectivity Map represents a large collection of gene expression profiling data obtained from cultured human cancer cells treated with a variety of small molecule drugs, which via pattern-matching algorithms allows the discovery of functional connections between drugs and gene patterns through the transitory feature of common gene expression changes. In **Chapter 7** we focused on the frequency of *RAS* mutations in *MLL*-rearranged infant ALL. Finally, in **Chapter 8** and **Chapter 9**, we discuss, review and summarize all these novel findings in terms of GC resistance in *MLL*-rearranged infant ALL. **Chapter 10** covers a layman's summary in Dutch.

REFERENCES

Buckingham JC. Glucocorticoids: exemplars of multi-tasking. *Britisch Journal of Pharmacology*. 2006. 147(S1):S258-268.

Chessells JM, Harrison CJ, Watson SL, Vora AJ, Richards SM. Treatment of infants with lymphoblastic leukaemia: results of the UK Infant Protocols 1987–1999. *British Journal of Haematology*. 2002. 117:306–314.

Dördelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, *et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*. 1999. 94(4):1209-1217.

Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, *et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *Journal of Clinical Oncology*. 2003. 21(17):3262-3268.

Frankel LS, Ochs J, Shuster JJ, Dubowy R, Bowman WP, Hockenberry-Eaton M, Borowitz M, Carroll AJ, Steuber CP, Pullen DJ. Therapeutic trial for infant acute lymphoblastic leukemia: the Pediatric Oncology Group experience (POG 8493). *Journal of Pediatric Hematology/oncology*. 1997. 19:35–42.

Gu Y, Nakamura T, Alder H, Prasad R, Canaani O, Cimini G, Croce CM, Canaani E. The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to Drosophila trithorax, to the AF-4 gene. *Cell*. 1992. 71(4):701-708.

Hayashi R, Wada H, Ito K, Adcock IM. Effects of glucocorticoids on gene transcription. European Journal of Pharmacology. 2004. 500(1-3):51-62.

Heerema NA, Sather HN, Ge J, Arthur DC, Hilden JM, Trigg ME, Reaman GH. Cytogenetic studies of infant acute lymphoblastic leukemia: poor prognosis of infants with t(4;11) - a report of the Children's Cancer Group. *Leukemia*. 1999. 13(5):679-86.

Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJ, Kazemier KM, Pei D, Cheng C, Pui CH, Relling MV, Janka-Schaub GE, Pieters R, Evans WE. Gene expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *New England Journal of Medicin*. 2004. 351(6):533-542.

Huret JL, Dessen P, Bernheim A. An atlas of chromosomes in hematological malignancies. Example: 11q23 and MLL partners. *Leukemia*. 2001. 15(6):987-989.

Iida S, Seto M, Yamamoto K, Komatsu H, Tojo A, Asano S, Kamada N, Ariyoshi Y, Takahashi T, Ueda R. MLLT3 gene on 9p22 involved in t(9;11) leukemia encodes a serine/proline rich protein homologous to MLLT1 on 19p13. *Oncogene*. 1993. 8(11):3085-3092.

Inaba H, Pui CH. Glucocorticoid use in acute lymphoblastic leukaemia. *Lancet Oncology*. 2010. 11(11):1096-1106.

Jansen MW, Corral L, van der Velden VH, Panzer-Grümayer R, Schrappe M, Schrauder A, Marschalek R, Meyer C, den Boer ML, Hop WJ, Valsecchi MG, Basso G, Biondi A, Pieters R, van Dongen JJ. Immunobiological diversity in infant acute lymphoblastic leukemia is related to the occurrence and type of MLL gene rearrangement. Leukemia. 2007. 21(4):633-641.

Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood*. 1998. 92(1):259-266.

Meyer C, Schneider B, Reichel M, Angermueller S, Strehl S, Schnittger S, Schoch C, Jansen MW, van Dongen JJ, Pieters R, Haas OA, Dingermann T, Klingebiel T, Marschalek R. Diagnostic tool for the identification of MLL rearrangements including unknown partner genes. Proceedings of the National Academy of Sciences. 2005. 102(2):449-454.

Meyer C, Hofmann J, Burmeister T, Gröger D, Park TS, Emerenciano M, Pombo de Oliveira M, Renneville A, Villarese P, Macintyre E, Cavé H, Clappier E, Mass-Malo K, Zuna J, Trka J, De Braekeleer E, De Braekeleer M, Oh SH, Tsaur G, Fechina L, van der Velden VH, van Dongen JJ, Delabesse E, Binato R, Silva ML,Kustanovich A, Aleinikova O, Harris MH, Lund-Aho T, Juvonen V, Heidenreich O, Vormoor J, Choi

WW, Jarosova M, Kolenova A, Bueno C, Menendez P, Wehner S, Eckert C, Talmant P, Tondeur S, Lippert E, Launay E, Henry C, Ballerini P, Lapillone H, Callanan MB, Cayuela JM, Herbaux C, Cazzaniga G, Kakadiya PM,Bohlander S, Ahlmann M, Choi JR, Gameiro P, Lee DS, Krauter J, Cornillet-Lefebvre P, Te Kronnie G, Schäfer BW, Kubetzko S, Alonso CN, Zur Stadt U, Sutton R, Venn NC, Izraeli S, Trakhtenbrot L, Madsen HO, Archer P, Hancock J, Cerveira N, Teixeira MR, Lo Nigro L, Möricke A, Stanulla M, Schrappe M, Sedék L,Szczepański T, Zwaan CM, Coenen EA, van den Heuvel-Eibrink MM, Strehl S, Dworzak M, Panzer-Grümayer R, Dingermann T, Klingebiel T, Marschalek R. The *MLL* recombinome of acute leukemias in 2013. *Leukemia*. 2013. Apr 30.

Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, van Wering ER, Veerman AJ. Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia – implications for treatment of infants. *Leukemia*. 1998. 12(9):1344-1348.

Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, Hovi L, LeBlanc T, Szczepanski T, Ferster A, Janka G, Rubnitz J, Silverman L, Stary J, Campbell M, Li CK, Mann G, Suppiah R, Biondi A, Vora A, Valsecchi MG. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007. 370(9583):240-250.

Pratt WB, Morishima Y, Murphy M, Harrell M. Chaperoning of glucocorticoid receptors. *Handbook of Experimental Pharmacology*. 2006. 172:111-138.

Pui CH, Ribeiro RC, Campana D, Raimondi SC, Hancock ML, Behm FG, Sandlund JT, Rivera GK, Evans WE, Crist WM, Krance R. Prognostic factors in the acute lymphoid and myeloid leukemias of infants. *Leukemia*. 1996. 10(6):952-956.

Pui CH, Robinson LL, Look AT. Acute lymphoblastic leukaemia. *Lancet*. 2008. 371(9617):1030-1043.

Ray A, Prefontaine KE. Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *PNAS*. 1994. 91(2):752-756.

Reaman GH, Sposto R, Sensel MG, Lange BJ, Feusner JH, Heerema NA, Leonard M, Holmes EJ, Sather HN, Pendergrass TW, Johnstone HS, O'Brien RT, Steinherz PG, Zeltzer PM, Gaynon PS, Trigg ME, Uckun FM. Treatment outcome and prognostic factors for infants with acute lymphoblastic leukemia treated on two consecutive trials of the Children's Cancer Group. *Journal of Clinical Oncology*. 1999. 17(2):445-55.

Riehm H, Reiter A, Schrappe M, Berthold F, Dopfer R, Gerein V, Ludwig R, Ritter J, Stollmann B, Henze G. Corticosteroid-dependent reduction of leukocyte count in blood as a prognostic factor in acute lymphoblastic leukemia in childhood (therapy study ALL-BFM 83). *Klinische Pädiatrie*. 1987. 199(3):151-160.

Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, de Lorenzo P, Valsecchi MG, Pieters R. Gene expression profiling-based dissection of *MLL* translocated and *MLL* germline acute lymphoblastic leukemia in infants. *Blood*. 2010a. 115(14):2835-2844.

Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, de Lorenzo P, Sallan SE, Brady HJ, Armstrong SA, Pieters R. Association of high-level MCL1 expression with *in vitro* and *in vivo* prednisone resistance in *MLL*-rearranged infant acute lymphoblastic leukemia. *Blood*. 2010b. 115(5):1018-1025.

Tissing WJ, Lauten M, Meijrink JP, den Boer ML, Koper JW, Sonneveld P, Pieters R. Expression of the glucocorticoid receptor and its isoform in relation to glucocorticoid resistance in childhood acute lymphocytic leukemia. *Haematologica*. 2005a. 90(9):1279-1281.

Tissing WJ, Meijerink JP, den Boer ML, Brinkhof B, Pieters R. mRNA expression levels of (co)chaperone molecules of the glucocorticoid receptor are not involved in glucocorticoid resistance in pediatric ALL. *Leukemia*. 2005b. 19(5):727-733.

Tissing WJ, Meijerink JP, den Boer ML, Brinkhof B, van Rossum EF, van Wering ER, Koper JW, Sonneveld P, Pieters R. Genetic variations in the glucocorticoid receptor gene are not related to glucocorticoid resistance in childhood acute lymphoblastic leukemia. *Clinical Cancer Research*. 2005c. 11(16):6050-6056.

Tkachuk DC, Kohler S, Cleary ML. Involvement of a homolog of Drosophila trithorax by 11q23 chromosomal translocations in acute leukemias. *Cell*. 1992. 71(4):691-700.

Tomizawa D, Koh K, Sato T, Kinukawa N, Morimoto A, Isoyama K, Kosaka Y, Oda T, Oda M, Hayashi Y, Eguchi M, Horibe K, Nakahata T, Mizutani S, Ishii E. Outcome of risk-based therapy for infant acute lymphoblastic leukemia with or without an MLL gene rearrangement, with emphasis on late effects: a final report of two consecutive studies, MLL96 and MLL98, of the Japan Infant Leukemia Study Group. Leukemia. 2007. 21(11):2258-2263.

CHAPTER 2

Relation between *in vitro* drug responses and prognostic markers in *MLL*-rearranged infant acute lymphoblastic leukemia

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Submitted.

ABSTRACT

Despite the current successful treatment results in pediatric acute lymphoblastic leukemia (ALL), the prognosis for *MLL*-rearranged infant ALL patients remains poor, and novel strategies are needed to identifiy and predict patients at high risk of therapy failure. We explored to what extent *in vitro* drug resistance is associated with clinical outcome and established prognostic markers, such as age <6 months, *MLL* rearrangement and white blood cell count (WBC) at diagnosis.

We found that *in vitro* response to none of the drugs tested by itself is capable of predicting clinical outcome, although a strong trend between *in vitro* glucocorticoid (*i.e.* prednisolone and dexamethasone) resistance and a poor outcome was observed. Also, *in vitro* resistance to glucocorticoids was significantly associated with age <6 months and high WBC. However, here we showed that the combined *in vitro* response to prednisolone, vincristine and L-asparaginase (PVA) was the most important factor predicting an adverse outcome in *MLL*-rearranged infant ALL. PVA-resistant *MLL*-rearranged infant ALL patients are at extremely high risk of therapy failure. PVA-resistant *MLL*-rearranged infant ALL patients showed cross-resistance towards dexamethasone but not to other drugs.

Taken together, we conclude that *in vitro* PVA-sensitivity testing in *MLL*-rearranged infant ALL may be a useful tool to stratify patients at extreme high risk of therapy failure, and that this approach allows identification of patients most urgently requiring more effective novel treatment options.

INTRODUCTION

Over the last decades, the prognosis for childhood acute lymphoblastic leukemia (ALL) in general has improved tremendously. Yet, the clinical outcome for infants (< 1 year of age) diagnosed with ALL remained dismal (1-5). Infant ALL is characterized by a high incidence of balanced chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene located on chromosome 11, which are detected in ~80% of the cases (6-8). As a result of such translocations, the N-terminal region of *MLL* fuses to the C-terminal region of one of its many translocation partner genes. Among infant ALL patients, the most recurrent *MLL* translocations are t(4;11), t(11;19) and t(9;11), which generate the chimeric fusion proteins MLL-AF4, MLL-ENL and MLL-AF9, respectively. The presence of leukemia-specific *MLL* translocations on its own represents a strong predictor of an unfavorable clinical outcome (2,3,5,9,10). Additional parameters predicting a poor prognosis include age at diagnosis (<6 months of age and white blood cell count at disease presentation (WBC) (5). Moreover, infant ALL cells are notoriously resistant to glucocorticoids and L-asparaginase (11,12). The *in vitro* responses of prednisolone, vincristine and L-asparaginase combined (known as the PVA score), appeared highly predictive for clinical outcome in childhood ALL (13,14). To date it remains largely unclear whether *in vitro* drug resistance patterns obtained in primary infant ALL cells correlate with prognosis or to what extent cellular resistance is associated with abovementioned prognostic factors. Therefore we generated *in vitro* cytotoxicity profiles in a relatively large cohort of infant ALL patients enrolled in the INTERFANT treatment protocols, and investigated the relation between *in vitro* drug responses, clinical outcome, and known prognostic factors, in order to attain an evidence-based improvement towards personalized medicine.

MATERIALS AND METHODS

Patient samples

In the present study we used a total of 67 samples obtained from infant ALL patients (<1 year of age) enrolled in either the INTERFANT-99 or the INTERFANT-06 study, for whom a complete overview of clinical parameters and follow up data was available, as well as sufficient material for *in vitro* cytotoxicity testing. This cohort of infant ALL patients consists of *MLL*-rearranged infant ALL cases (*n*=55) as well as patients carrying germline (or wild-type) *MLL* genes (*i.e.* no translocation (*n*=12). The *MLL*-rearranged infant ALL patient group comprises patients carrying t(4;11) (*n*=28), t(11;19) (*n*=18) translocations, as well as patients with other 11q23 rearrangements, as determined by fluorescence *in situ* hybridization (FISH) and RT-PCR analyses. Bone marrow aspirates or peripheral blood was collected at the Erasmus MC-Sophia Children's Hospital, and within 24 hours leukemic cells were isolated by density gradation centrifugation (Lymphoprep; density 1.077 g/ml, Nycomed Pharma, Oslo, Norway). Samples were enriched for leukemic blasts by removal of contaminating normal cells using monoclonal antibodies linked to magnetic beads as described by Kaspers *et al.* (15). Consequently, all samples used for *in vitro* drug testing contained >90% leukemic cells, as determined morphologically on May-Grunwald Giemsa (Merck, Darmstadt, Germany) stained cytospins.

In vitro drug response and *in vivo* prednisone response

For all samples, *in vitro* drug cytotoxicity was assessed by MTT assays as described previously (16). Briefly, leukemic cells were incubated for 4 days in a humidified incubator at 37°C and 5% CO_2, both in the absence and presence of a range of drug concentrations. Then, 10 µl of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) was added to the cells, following an additional 6-hour incubation under the same conditions, during which the yellow MTT is reduced to blue formazan crystals by viable cells only. Next, the blue formazan crystals were dissolved using HCL-isopropyl alcohol, allowing spectrophotometrical measurement of the optical density. Culture medium without cells or drugs served as blanks (*i.e.* 0% survival), and leukemic cells cultured in the absence of drugs (*i.e.* controls) were adjusted to 100% survival and used to calculate the LC_{50}-value. Results were considered evaluable only if the mean control OD, after correction for the background (as determined by the blanks), at day 4 exceeded 0.05 arbitrary units. The following drugs and concentration ranges (6 dosages per drug) were used: Prednisolone, 0.08-250 ug/mL (PRED; Bufa, Uitgeest, The Netherlands); Vincristine, 0.05-50 ug/mL (VCR; Pharmacy Erasmus Medical Center, Rotterdam, The Netherlands); L-Asparaginase, 0.003-10 IU/mL (L-ASP; Nycomed BV, Hoofddorp, The Netherlands); Daunorubicin, 0.002-2.0 ug/mL (DNR; Sanofi Aventis, Gouda, The Netherlands); Cytarabine, 0.04-2.5 ug/mL (Ara-C; Hospira Benelux BVBA, Brussel, Belgium) and Dexamethasone, 0.0002-6.0 ug/mL (DEX; Pharmacie Erasmus Medical Center, Rotterdam, The Netherlands). The PVA score was based on the *in vitro* drug responses (*i.e.* LC_{50}-values) of Prednisolone, Vincristine and L-Asparaginase, in which sensitivity towards each drug individually was scored as 1, intermediate responses as 2, and resistance was scored as 3. Following, cut-off LC_{50}-values were applied: prednisolone sensitivity: <0.1 µg/mL and resistance: >150 µg/mL; vincristine sensitivity: <0.3906 µg/mL and

resistance: >1.7578 ug/mL; L-asparaginase sensitivity: <0.033 IU/mL and resistance: >0.912 IU/mL. Hence, the PVA scores vary between 3 (sensitive to all three drugs) and 9 (resistant to all three drugs). Identical LC_{50} cut-off values for these 3 drugs were previously shown to be of high clinical relevance in childhood ALL (14).

The *in vivo* response to prednisone was determined after one intrathecal dose of methotrexate and a 7-day window of prednisone mono-therapy (before the initiation of combination chemotherapy). Patients were defined as prednisone poor responders (PPRs) when >1000 leukemic cells/μL remained present in the peripheral blood (17). When the amount of leukemic cells dropped below 1000/μL patients were defined as prednisone good responders (PGRs).

Statistical analysis

Differences in the distribution of variables between patient groups were analyzed using the Mann-Whitney *U*-test or the Kruskall-Wallis test. Cross–resistance patterns were studied by correlating the LC_{50} values for different drugs using the Kruskall-Wallis test and the Spearman's Rho-test was performed to correlate LC_{50} values from different drugs. The probability of event-free survival (EFS) was calculated using the Kaplan-Meier method and the Log-rank (Mantel-Cox) test was performed to analyze differences in outcome between patient groups. Furthermore, a correlation between *in vitro* prednisolone response and age at diagnosis was analyzed using the Spearman's Rho-test. The EFS rate is defined as time from diagnosis to death in induction, failure to achieve complete remission after induction, disease relapse, the emergence of secondary malignancies, or death in complete remission. Patients who did not achieve complete remission were assigned an event at time-point zero in the EFS analyses. All analyses were two-tailed and differences with *p*-value <0.05 were considered statistically significant.

RESULTS

Prognostic relevance of *in vitro* PVA scoring in infant ALL

The combined *in vitro* responses to prednisolone, vincristine and L-asparaginase (*i.e.* PVA score) has been shown to be predictive for clinical outcome in pediatric ALL (*i.e.* children >1 year of age) (13,14,17). We analyzed whether this was also valid for infants with ALL (<1 year of age). Patients were divided into a sensitive (PVA scores of 3-4), an intermediate (PVA scores of 5-6), or a resistant (PVA scores of 7-9) group. PVA-resistant infant ALL cases (including both patients with and without *MLL* translocations) tended to have a worse outcome when compared to patients with sensitive and intermediate PVA scores (**Figure 1A**); although this did not reach statistical significance ($p=0.0686$). As the presence of *MLL* translocations represents a strong and independent prognostic factor (5), we also analyzed the influence of the PVA score on clinical outcome excluding patients with wild-type *MLL* genes. PVA-resistant *MLL*-rearranged infant ALL patients showed significantly (Log-rank; $p<0.05$) worse event-free survival rates compared with both PVA-sensitive and PVA-intermediate *MLL*-rearranged infant ALL cases (**Figure 1B**). Strikingly, the event-free survival chances at 5 year for PVA-resistant *MLL*-rearranged infant ALL patients is only ~10%. Furthermore, we also analyzed whether *in vitro* sensitivity towards single chemotherapeutics (prednisolone, vincristine, L-asparaginase, daunorubicin, cytarabine or dexamethasone) is predictive for event-free survival. *MLL*-rearranged infant ALL patients *in vitro* resistant to prednisolone ($p=0.1756$), L-asparaginase ($p=0.2970$) or daunorubicin ($p=0.1396$) have a non-significant worse outcome when compared to their sensitive counterpart; no remarkable differences in outcome were observed for dexamethasone, vincristine or cytarabine (data not shown).

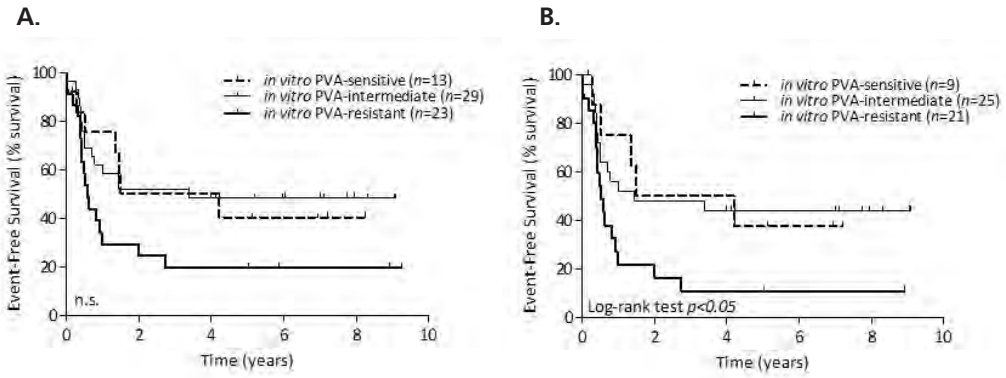
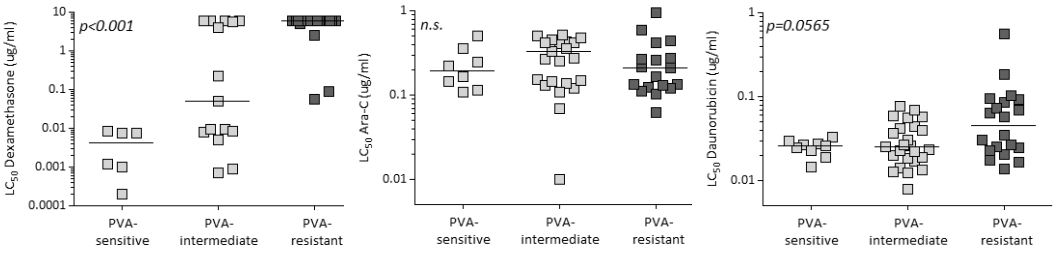


Figure 1. Prognostic relevance of prednisolone, vincristine and L-asparaginase (PVA) in infant acute lymphoblastic leukemia.

Event-free survival analysis of **A.** total infant ALL patients (with and without *MLL*-rearranged infant ALL) and **B.** *MLL*-rearranged infant ALL cases only. Infant ALL patient samples were divided based on their *in vitro* response to prednisolone, vincristine and L-asparaginase (PVA): PVA-sensitive (score 3 or 4), PVA-intermediate (score 5 or 6) and PVA-resistant (score 7-9). Differences in outcome were statistically analyzed using the Log-rank test.

Next, we analyzed whether PVA resistance showed cross-resistance to other drugs. In *MLL*-rearranged infant ALL patients, PVA resistance was correlated to dexamethasone resistance, but not to resistance to daunorubicin or Ara-C (**Figure 2A**). Interestingly, when analyzing cross-resistance between prednisolone, vincristine and L-asparaginase, only a correlation between prednisolone and vincristine emerged (Spearman's Rho-test=0.5520, *p<0.005*) (**Figure 2B**); there was no correlation between prednisolone and L-asparaginase (Spearman's Rho-test=0.044, *p=0.7494*) nor between vincristine and L-asparaginase (Spearman's Rho-test=0.247, *p=0.0688*). Furthermore, *MLL*-rearranged infant ALL patient samples which were *in vitro* sensitive to prednisolone or vincristine only displayed increased *in vitro* sensitivity to the glucocorticoid dexamethasone (Spearman´s Rho-test prednisolone-dexamethasone=0.8951, *p<0.0001*; Spearman´s Rho-test vincristine-dexamethasone=0.6611, *p<0.0001*) and the antracycline daunorubicin (Spearman´s Rho-test prednisolone-daunorubicin=0.4036, *p=0.0025*; Spearman´s Rho-test vincristine-daunorubicin=0.3681, *p=0.0061*) (**Figure 2C**). No cross-resistance or cross-sensitivity was observed for either L-asparaginase nor Ara-C with other drugs (data not shown).

A.



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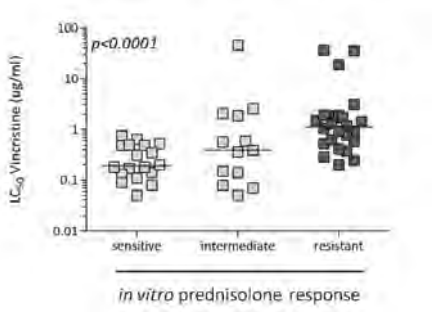

Figure 2. *In vitro* cross-resistance of PVA to other induction-therapeutic drugs

A. LC_{50} values of dexamethasone, daunorubicin or cytarabine (Ara-C) of *MLL*-rearranged patient samples divided into *in vitro* sensitive, intermediate or resistant to PVA. *P*-values were determined by the Kruskall Wallis-test. **B.** LC_{50} values of vincristine of *MLL*-rearranged patient samples divided into *in vitro* sensitive, intermediate or resistant to prednisolone. The indicated Spearman´s Rho index was determined by comparing LC_{50} values of prednisolone with LC_{50} values of vincristine.

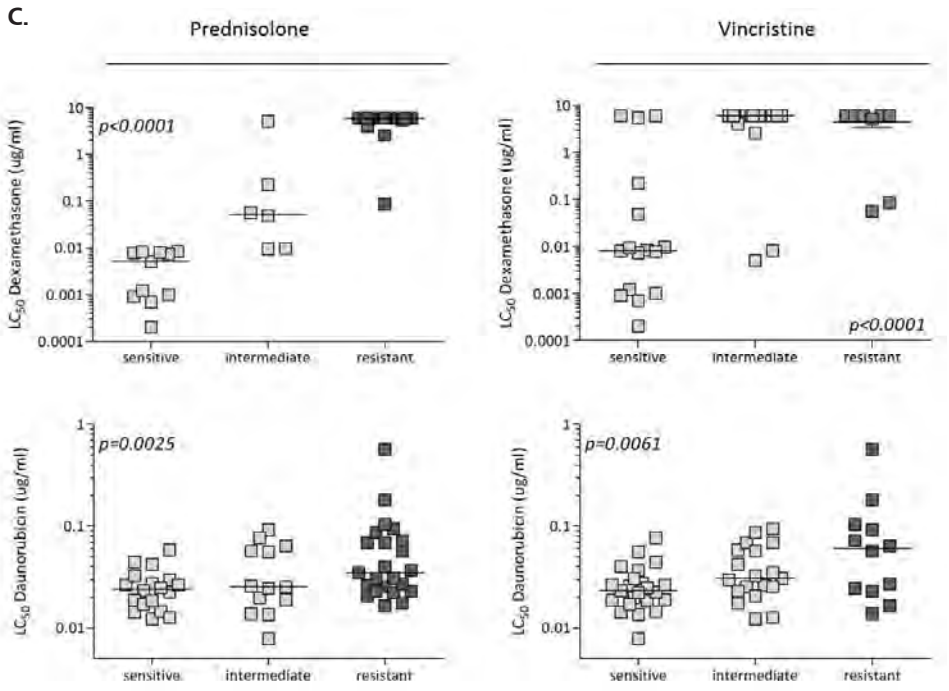

Figure 2. *In vitro* cross-resistance of PVA to other induction-therapeutic drugs
C. LC_{50} values of dexamethasone, daunorubicin or cytarabine (Ara-C) of *MLL*-rearranged patient samples divided into *in vitro* sensitive, intermediate or resistant to either prednisolone or vincristine. Indicated *p*-value was determined by Kruskall Wallis test. Correlation index was determined by Spearman's Rho-test, in which LC_{50} values of both indicated drugs were correlated to each other.

Both the *in vitro* prednisolone response, as well as the *in vivo* prednisone response have been identified as prognostic makers in pediatric ALL (17) and infant ALL (2,5). Here, we investigated to what degree the clinical prednisone response correlates with *in vitro* prednisolone response. As shown in **Figure 3**, prednisone good responders (PGR) are more frequently sensitive to prednisolone *in vitro* as compared with prednisone poor responders (PPR). Similar results are obtained when analyzing infant ALL in general (**Figure 3A**), or when analyzing only *MLL*-rearranged infant ALL cases (**Figure 3B**). Although the observed differences are statistically significant ($p<0.005$) and the median LC_{50} values of the patient groups show that PPRs are >1000-fold more resistant to prednisolone *in vitro*, a discrepancy was found in 8/39 of the PGRs that appeared to be *in vitro* resistant to prednisolone, whereas 3/21 of the PPRs appeared to be sensitive to prednisolone *in vitro*. Interestingly, *in vitro* prednisolone response was only non-significantly predictive for outcome within *MLL*-rearranged infant ALL patients that were PGR (**Figure 3C** and **3D**).

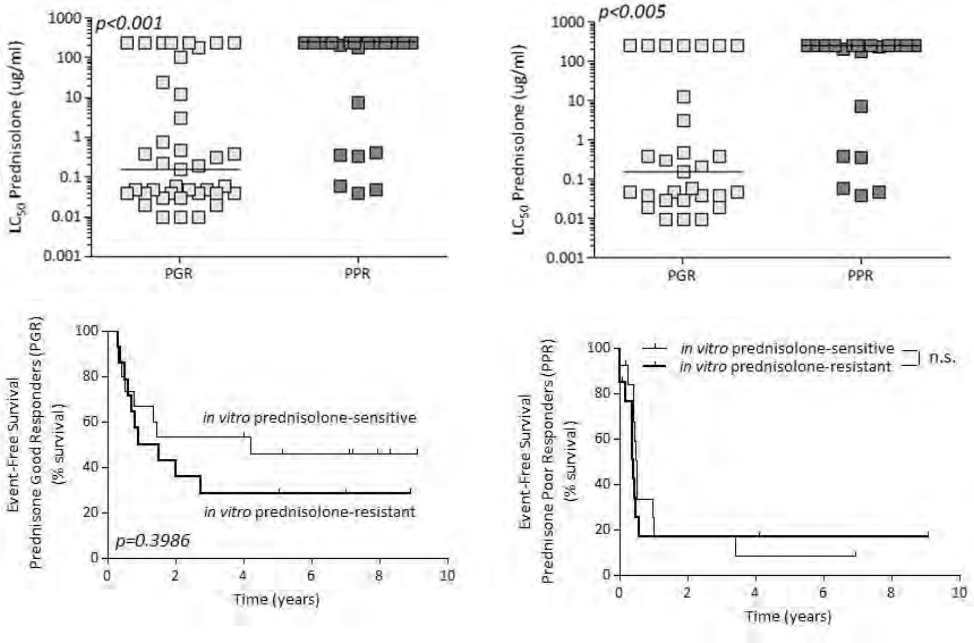

Figure 3. Correlation of *in vivo* and *in vitro* prednisolone resistance
LC_{50} values of prednisolone of **A.** infant ALL or **B.** *MLL*-rearranged infant ALL that are *in vivo* prednisone good responders (PGR, *n*=39) or *in vivo* prednisone poor responders (PPR, *n*=29). *In vivo* prednisone response is based on the white blood cell count after one-week of prednisone monotherapy. PPRs demonstrate to be *in vitro* prednisolone resistant whereas PGRs are *in vitro* sensitive to prednisolone. *P*-values were determined by the Mann–Whitney *U*-test.
Event-free survival analysis of prednisolone-sensitive and prednisolone-resistant within **C.** PGRs and **D.** PPRs. The median *in vitro* prednisolone response was used as a cut-off. *In vitro* prednisolone response is non-significantly predictive in PGRs, and not in PPRs.

In vitro drug responses and *MLL* translocations

MLL-rearranged infant ALL patients have an inferior outcome when compared with infant ALL patients who do not carry translocations of the *MLL* gene (5). We compared *in vitro* drug responses between *MLL*-rearranged and wild-type *MLL* infant ALL samples. *MLL*-rearranged infant ALL appeared to be more resistant to vincristine, and a trend was found for increased resistance towards glucocorticoids (*i.e.* prednisolone and dexamethasone) and cytarabine (Ara-C) (**Figure 4**). Analysis by type of *MLL* rearrangement showed that t(4;11)-positive infant ALL samples were significantly more resistant to prednisolone, dexamethasone and vincristine (**Figure 4**).

Strikingly, t(11;19)-positive infant ALL samples displays a remarkable sensitivity towards the glucocorticoids prednisolone and dexamethasone, with median LC_{50} values comparable to that of wild-type *MLL* infant ALL samples.

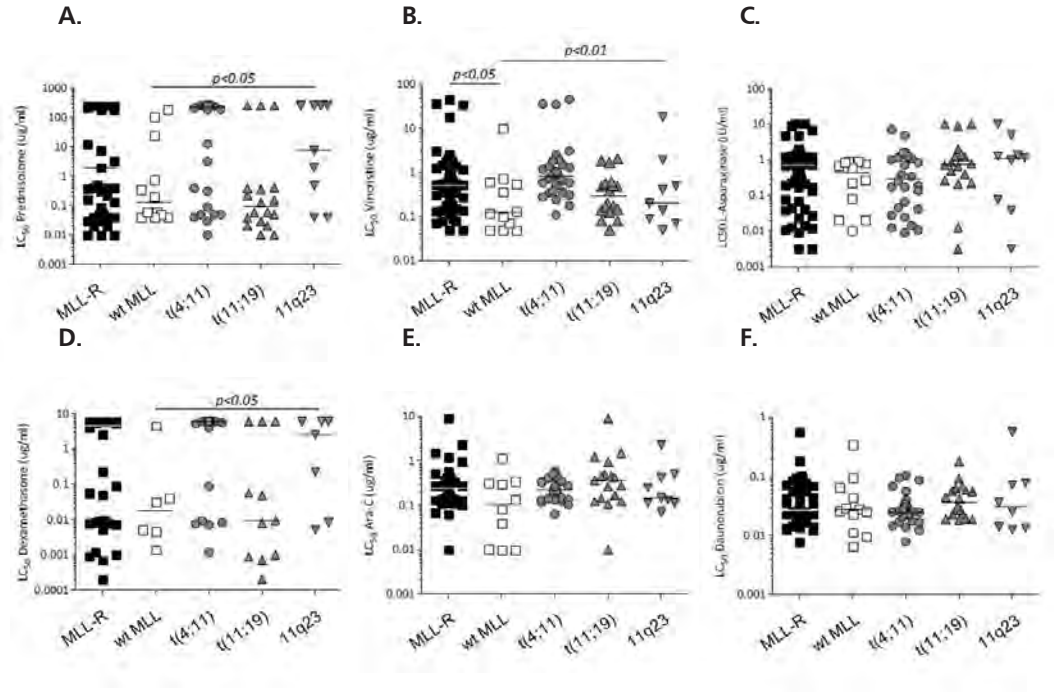

Figure 4. *In vitro* drug response and the type of *MLL* rearrangement
LC_{50} values of **A.** prednisolone, **B.** vincristine, **C.** L-asparaginase, **D.** dexamethasone, **E.** cytarabine (Ara-C) and **F.** daunorubicin in infant ALL patient samples with (*MLL*-R; in black) or without an *MLL* rearrangement (wild-type *MLL*, denoted as wt *MLL*; in white). *P*-values were determined by the Mann–Whitney *U*-test, when comparing *MLL*-R and wt *MLL* patient samples. *In vitro* drug response was also depicted in *MLL-AF4*-rearranged ("t(4;11)"), *MLL-ENL*-rearranged ("t(11:19)") and other *MLL*-rearranged ("11q23") infant ALL (in grey). *P*-values were determined by the Kruskall-Wallis test, when comparing LC_{50} values of the groups "wt MLL", "t(4;11)", "t(11;19)" and "11q23".

Relation between *in vitro* drug response and prognostic factors in *MLL*-rearranged infant ALL

Among *MLL*-rearranged infant ALL patients, predictors of an adverse outcome identified in the INTERFANT-99 study (currently representing the most comprehensive analyses available on the largest cohort of uniformly treated infant ALL patients), include young age, high WBCs and poor prednisone response (5). Central nervous system (CNS) involvement and a highly immature (CD10-negative) pro-B cell immunophenotype have been identified as factors adversely influencing outcome (3, 18), but did not significantly contribute to prognosis in the INTERFANT-99 study (5). Infant ALL patients <6 months of age have a worse outcome than infant ALL patients >6 month of age (2-5) and therefore we analyzed *in vitro* drug sensitivity in *MLL*-rearranged infant ALL samples in both age groups. Differences in *in vitro* drug response were only observed for the glucocorticoids prednisolone and dexamethasone and for the anthracycline daunorubicin (**Figure 5**), although the differences in LC_{50} values of the latter drug for both age groups were not substantial.

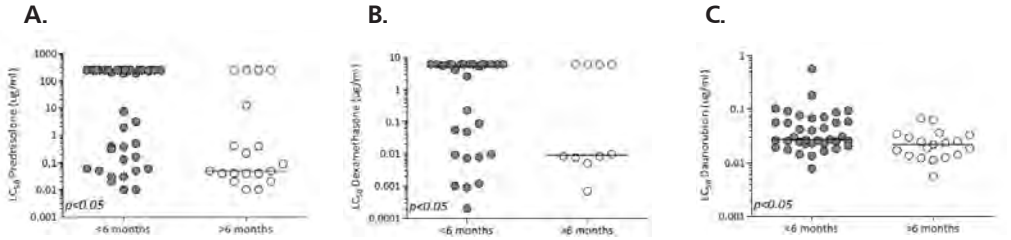

Figure 5. Relationship between age and *in vitro* drug resistance in *MLL*-rearranged infant ALL.
In vitro drug response of *MLL*-rearranged infant ALL aged <6 months were compared with their counterpart, *MLL*-rearranged infant ALL with aged >6 months. All LC_{50} values are depicted in the figures for **A.** prednisolone, **B.** dexamethasone and **C.** daunorubicin. The median is shown as a horizontal bar. *P*-values were determined by the Mann–Whitney *U* test.

MLL-rearranged infant patients with high WBCs (>300*10^6 cells/mL), had a worse outcome when compared to patients with low (<100*10^6 cells/mL) or intermediate WBC numbers (100-300*10^6 cells/mL) (3,5). Here we show that samples from *MLL*-rearranged patients with high WBC numbers tend to be more resistant to prednisolone (**Figure 6A**; Kruskall-Wallis Test *p=0.1446*) and are *in vitro* more resistant to L-asparaginase when compared to samples from patients with low WBC numbers (**Figure 6B**; Kruskall-Wallis Test *p<0.05*).

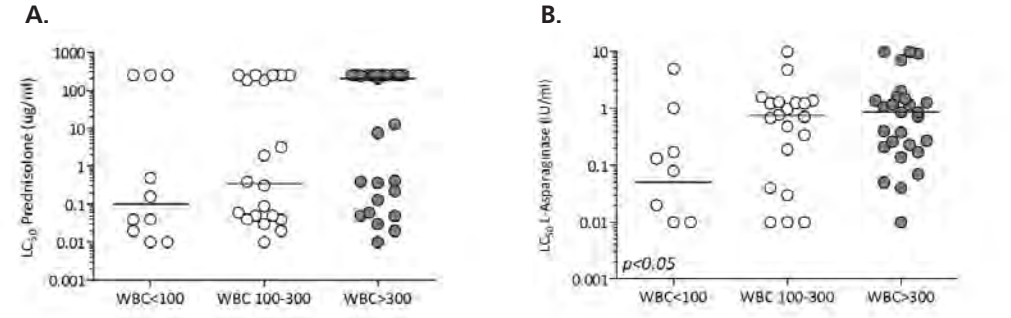

Figure 6. Correlation of white blood cell counts (WBC) and *in vitro* drug response in *MLL*-rearranged infant ALL
In vitro drug response of *MLL*-rearranged infant ALL with WBCs <100*10^6 cells/mL were compared with their *MLL*-rearranged infant ALL with WBCs 100-300*10^6 or >300*10^6 cells/mL. All LC_{50} values are depicted in the figures for **A.** prednisolone and **B.** L-asparaginase. The median is shown as a horizontal bar. *P*-values were determined by the Kruskall-Wallis test.

DISCUSSION

In vitro cytotoxicity testing (MTT assays) has been very informative in childhood acute lymphoblastic leukemia (ALL), providing an indication of therapy effectiveness before the initiation of actual treatment. Prospective analyses showed that patients who are *in vitro* sensitive to several drugs have a superior prognosis over patients displaying *in vitro* resistance (13,14). Here we provide clear evidence of a marked association between clinical parameters (*e.g.* white blood count, age at diagnosis, *in vivo* prednisone response) and *in vitro* cytotoxicity for a variety of drugs used in the treatment of *MLL*-rearranged infant ALL patients.

We show that the *in vitro* sensitivity to none of the drugs tested individually is capable of predicting clinical outcome. However, *MLL*-rearranged infant ALL patients displaying combined *in vitro* resistance to prednisolone, vincristine and L-asparaginase (*i.e.* PVA score), had inferior event-free survival rates. This is in concordance with studies in pediatric ALL (13,14). Moreover, the *in vitro* sensitivity to prednisolone appeared to correlate with the clinical response to prednisone. Yet, the correlation between the *in vivo* prednisone response and the *in vitro* prednisolone response was not absolute. Interestingly, both *in vitro* prednisolone resistance as well as *in vivo* prednisone resistance have been identified as predictors of an adverse prognosis in pediatric ALL (14,17). For infant ALL patients, the *in vitro* prednisolone is marginally indicative for clinical outcome, but not significantly (data not shown). Additionally, within the PGR patient group, *in vitro* prednisolone sensitivity showed to be predictive for a better prognosis in childhood ALL (17) and in *MLL*-rearranged infant ALL. Hence, the strongest indicator for an adverse outcome in infant ALL patients in terms of drug resistance is the combined *in vitro* resistance to prednisolone, vincristine, and L-asparaginase (PVA), especially for *MLL*-rearranged infant ALL cases.

Furthermore, infant ALL patients samples carrying high risk features such as a t(4;11) translocation, age at presentation is <6 months, or a WBC >100*10^6 cells/mL, demonstrated to be *in vitro* resistant to the glucocorticoids prednisolone and dexamethasone. In contrast, infant ALL patients carrying the translocation t(11;19) appeared to be more sensitive to these glucocorticoids. Although, t(4;11)- and t(11;19)-infant ALL patients, as well as patients carrying other types of *MLL* translocations (designated 11q23-rearranged) show a comparable poor clinical outcome (5), no similarity was observed based in terms of *in vitro* drug response profiles. Interestingly, Ramakers-van Woerden *et al.* observed that infants ALL (<1 year of age) were *in vitro* more resistant to the glucocorticoids prednisolone and dexamethasone when compared to pediatric ALL (>1 year of age) (12). Intriguingly, here we investigated age at presentation within only *MLL*-rearranged infant ALL patients; and demonstrated that *MLL*-rearranged infant ALL patients <6 months of age were *in vitro* more resistant to the glucocorticoids prednisolone and dexamethasone, when compared to *MLL*-rearranged infant ALL patients >6 months of age.

In summary, we show that for *MLL*-rearranged infant ALL patients the *in vitro* drug cytotoxicity profile can predict clinical outcome, but only by combining the responses of prednisolone, vincristine, and L-asparaginase (PVA). Moreover, established prognostic markers such as high WBC and young age at diagnosis are associated with resistance to prednisolone *in vitro*. We

therefore conclude that *in vitro* PVA testing identifies *MLL*-rearranged infant ALL patients at very high risk of therapy failure, and that PVA-scoring may be used to stratify these patients more accurately if confirmed in an independent patient cohort. Clearly, PVA-resistant *MLL*-rearranged infant ALL cases form a patient group that needs alternative, more effective therapeutic strategies.

REFERENCES

1. Frankel LS, Ochs J, Shuster JJ, Dubowy R, Bowman WP, Hockenberry-Eaton M, Borowitz M, Carroll AJ, Steuber CP, Pullen DJ. Therapeutic trial for infant acute lymphoblastic leukemia: the Pediatric Oncology Group experience (POG 8493). *Journal of Pediatric Hematology/oncology*. 1997. 19:35–42.

2. Dördelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, *et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*. 1999 Aug;94(4):1209-1217.

3. Reaman GH, Sposto R, Sensel MG, Lange BJ, Feusner JH, Heerema NA, Leonard M, Holmes EJ, Sather HN, Pendergrass TW, Johnstone HS, O'Brien RT, Steinherz PG, Zeltzer PM, Gaynon PS, Trigg ME, Uckun FM. Treatment outcome and prognostic factors for infants with acute lymphoblastic leukemia treated on two consecutive trials of the Children's Cancer Group. *Journal of Clinical Oncology*. 1999. 17:445–455.

4. Chessells JM, Harrison CJ, Watson SL, Vora AJ, Richards SM. Treatment of infants with lymphoblastic leukaemia: results of the UK Infant Protocols 1987–1999. *British Journal of Haematology*. 2002. 117:306–314.

5. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, Hovi L, LeBlanc T, Szczepanski T, Ferster A, Janka G, Rubnitz J, Silverman L, Stary J, Campbell M, Li CK, Mann G, Suppiah R, Biondi A, Vora A, Valsecchi MG. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007. 370(9583):240-250.

6. Pui CH, Kane JR, Crist WM. Biology and treatment of infant leukemias. *Leukemia*. 1995. 9(5):762-9.

7. Felix CA, Lange BJ.Leukemia in infants. *Oncologist*. 1999. 4(3):225-40.

8. Biondi A, Cimino G, Pieters R, Pui CH. Biological and therapeutic aspects of infant leukemia. *Blood*. 2000. 96: 24–33.

9. Pui CH, Behm FG, Downing JR, Hancock ML, Shurtleff SA, Ribeiro RC, Head DR, Mahmoud HH, Sandlund JT, Furman WL, Roberts M, Crist WM, Raimondi SC. 11q23/MLL rearrangement confers a poor prognosis in infants with acute lymphoblastic leukemia. *Journal of Clinical Oncology*. 1994. 12(5):909-15.

10. Heerema NA, Sather HN, Ge J, Arthur DC, Hilden JM, Trigg ME, Reaman GH. Cytogenetic studies of infant acute lymphoblastic leukemia: poor prognosis of infants with t(4;11) – a report of the Children's Cancer Group. *Leukemia*. 1999. 13(5):679-686.

11. Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, van Wering ER, Veerman AJ.Relation between age, immunophenotype and *in vitro* drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants. *Leukemia*. 1998. 12(9):1344-8.

12. Ramakers-van Woerden NL, Beverloo HB, Veerman AJ, Camitta BM, Loonen AH, van Wering ER, Slater RM, Harbott J, den Boer ML, Ludwig WD, Haas OA, Janka-Schaub GE, Pieters R. *In vitro* drug-resistance profile in infant acute lymphoblastic leukemia in relation to age, MLL rearrangements and immunophenotype. *Leukemia*. 2004. 3:521-9.

13. Kaspers GJ, Veerman AJ, Pieters R, Van Zantwijk CH, Smets LA, Van Wering ER, Van Der Does-Van Den Berg A. *In vitro* cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. *Blood*. 1997. 7:2723-9.

14. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, Graubner U, Haas RJ, Jorch N, Spaar HJ, Kaspers GJ, Kamps WA, Van Der Does-Van Den Berg A, Van Wering ER, Veerman AJ, Janka-

Schaub GE. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *Jouranl of Clinical Oncology*. 2003. 17:3262-3268

15. Kaspers GJ, Veerman AJ, Pieters R, Broekema GJ, Huismans DR, Kazemier KM, Loonen AH, Rottier MA, van Zantwijk CH, Hahlen K, Van Wering ER. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. *British Journal of Cancer*. 1994. 6:1047-1052.

16. Pieters R, Loonen AH, Huismans DR, Broekema GJ, Dirven MW, Heyenbrok MW, Hählen K, Veerman AJ. *In vitro* drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. *Blood*. 1990. 11:2327-2336.

17 Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood*. 1998. 92:259-266.

18. Stam RW, den Boer ML, Pieters R. Toward targeted therapy for infant acute lymphoblastic leukaemia. *British Journal of Haematology*. 2006. 132(5):539-551.

CHAPTER 3

Glucocorticoid sensitization in *MLL*-rearranged infant ALL cells by pan-BCL-2 family member inhibitors gossypol and AT-101

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ABSTRACT

Resistance to glucocorticoids (GCs) remains a major problem for successful treatment infants with acute lymphoblastic leukemia (ALL) carrying *MLL* translocations. Despite intensive research, the mechanism underlying GC resistance remains poorly understood.

Recent studies suggested an important role for the pro-survival BCL-2 family member MCL1 in GC resistance in *MLL*-rearranged ALL. We exposed GC-resistant *MLL*-rearranged cells to potent MCL1-inhibiting agents, including gossypol, AT-101, rapamycin, SU9516 and obatoclax (GX15-070). Only gossypol and its synthetic analogue AT-101 induced GC sensitivity in *MLL*-rearranged ALL cells. Remarkably, the GC-sensitizing effects of gossypol and AT-101 appeared not to be mediated by down-regulation of MCL1 or other anti-apoptotic BCL-2 family members, but rather involved up-regulation of multiple pro-apoptotic BCL-2 family members. Interestingly, incubation of resistant *MLL*-rearranged cells with gossypol or AT-101 in the presence of prednisolone increases the expression of only BIM and BID more rapidly.

In conclusion, gossypol and AT-101 induce GC sensitivity in *MLL*-rearranged ALL cells, most likely mediated by the activation of BID and BIM without the necessity to down-regulate anti-apoptotic BCL-2 family members like MCL1. Hence, co-administration of either gossypol or AT-101 during GC treatment of GC-resistant *MLL*-rearranged ALL patients may overcome GC resistance and improve prognosis in this high-risk childhood leukemia.

INTRODUCTION

The prognosis for childhood acute lymphoblastic leukemia (ALL) has been progressively improved over the last decades. Unfortunately, a comparable therapeutic efficiency in infant ALL patients (<1 year of age) with translocations of the *Mixed Lineage Leukemia* (*MLL*) gene (occurring in ~80 % of the cases) is still lacking. Nowadays, infants with ALL in general reach event-free survival (EFS) rates of ~50%, while *MLL*-rearranged infant ALL cases in particular only reach EFS rates of 30-40% (1). As *MLL*-rearranged infant ALL patients typically relapse within two years from diagnosis (while still on treatment), the primary obstacle for obtaining satisfactory treatment results seems to be overcoming cellular drug resistance, especially to glucocorticoids (GCs) like prednisone and dexamethasone. Interestingly, *MLL*-rearranged infant ALL patients are highly resistant to prednisone. Thus, in order to improve the prognosis of *MLL*-rearranged infant ALL patients, it is important to unravel the mechanism underlying glucocorticoid resistance.

The activation of pro-apoptotic and inhibition of anti-apoptotic proteins, have been extensively investigated in drug-induced cell death, and deregulated expression of BCL-2 family members have been shown to correlate with GC resistance in ALL (2-11). Recently, Holleman *et al.* generated a gene expression signature related to the *in vitro* GC response in pediatric ALL patients (12), in which 42 probe sets were found to be differentially expressed between GC-resistant and GC-sensitive samples. Yet, underlining the genetic differences between pediatric ALL and *MLL*-rearranged infant ALL, this pediatric ALL GC-resistance signature appeared not able to discriminate between GC-resistant and GC-sensitive *MLL*-rearranged infant ALL cases (13). Nonetheless, a limited number of genes appeared to be commonly over-expressed in GC-resistant pediatric ALL and GC-resistant *MLL*-rearranged infant ALL, like for instance the anti-apoptotic BCL-2 family member MCL1 (12). Subsequently, shRNA-mediated knock-down experiments established a role for MCL1 in GC-resistance in both pediatric ALL (6) and *MLL*-rearranged infant ALL (13). Moreover it was shown that small molecule inhibitors like rapamycin and obatoclax repress MCL1 expression and sensitize ALL cells to GCs (6,7).

Apart from rapamycin and obatoclax, a number of other small molecule inhibitors have been shown to inhibit MCL1. Gossypol, which is derived from the cotton plant *Gossypium*, and AT-101, a R-enantiomer of gossypol (apogossypol), both modulate MCL1 expression and concomitantly induce apoptosis in leukemic cells (14-16). Furthermore, AT-101 has been shown to induce drug sensitivity by inhibition of stromal cell-mediated MCL1 protein expression in chronic lymphoblastic leukemia (CLL) cells (15). Also, the CDK2 inhibitor SU9516 was shown to repress MCL1 expression, and induce apoptosis in human leukemic cells (17). Interestingly, most of these agents have already been evaluated in clinical phase I or II leukemia trials (reviewed in Quinn *et al.* 2011, clinical trials.gov #NCT00286780, clinical trial.gov #NCT00275431) (18).

However, most of these MCL1-inhibiting compounds have not been tested in *MLL*-rearranged ALL cells. Therefore, we here studied the ability of rapamycin, obatoclax, SU9516, gossypol and AT-101 to induce leukemic cell death and/or *in vitro* GC-sensitization in *MLL*-rearranged ALL cells. We show that rapamycin, obatoclax, and SU9516 were not able to induce GC sensitivity

in *MLL*-rearranged ALL cells, whereas gossypol and AT-101 did. Remarkably however, the GC-sensitizing effects of these potent MCL1 inhibitors were not mediated by down-regulation of MCL1 itself, but rather by activation of the pro-apoptotic BCL-2 family members.

METHODS

Patient samples

Diagnostic bone marrow or peripheral blood samples from infants (*i.e.* children <1 year of age) with *MLL*-rearranged ALL were collected at the Sophia Children's Hospital (Rotterdam, the Netherlands) as part of the international collaborative INTERFANT treatment protocol (1). Approval for these studies was obtained from the Erasmus MC Institutional Review Board. Informed consent was obtained in accordance with the Declaration of Helsinki. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation, and contaminating non-leukemic cells removed using immunomagnetic beads as described before by Kaspers *et al.* (19). All samples used in this study contained more than 90% of leukemic blasts.

Microarray and bioinformatics analyses

Gene expression profiles (Affymetrix HGU133plus2.0 GeneChips) were generated for primary infant ALL samples with or without a *MLL* rearrangement and pediatric ALL. The gene expression data has been deposited in the NCBI Gene Expression Omnibus under the GEO Series accession number GSE19475 (20) and GSE13551 (21). For the analysis of differentially expressed genes between infant ALL patients with or with a MLL rearrangment and pediatric ALL, raw microarray data (Affymetrix HU133plus2.0 GeneChips) were collectively normalized using variance-stabilizing normalization (VSN) (22), and differential gene expression was statistically evaluated using linear models for microarray analyses (LIMMA) (23,24).

Gene set enrichment analysis (GSEA) (25) was used to evaluate enrichment of genes encoding the apoptotic pathway family members in prednisolone-resistant *MLL*-rearranged infant ALL samples.

Cell line culture and treatment with modulator agents of the apoptotic pathway

The *MLL-AF4*-rearranged cell line SEMK2 and the *TEL-AML*-rearranged cell line REH (both *in vitro* resistant to prednisolone) were maintained as suspension cultures in RPMI growth medium containing Glutamax (Invitrogen, Life Technologies, Breda, The Netherlands), 10% fetal calf serum (FCS), and 1% penicillin/streptomycin/fungizone (PSF; Invitrogen, Life Technologies, Breda, the Netherlands). Cells were grown in a humidified tissue culture incubator at 37^{o}C in 5% CO_2.

Both cell lines were incubated with prednisolone (the active metabolite of prednisone; Bufa, Uitgeest, The Netherlands) in the absence or presence of potent MCL1 inhibitors or pan-BCL-2 family member inhibitors, rapamycin, gossypol, synthetic analogue of gossypol (AT-101), and SU9516 (all derived from Tocris, Minneapolis, MN, USA), and Obatoclax (GX15-070; SelleckChem, Houston, TX, USA).

In vitro prednisolone cytotoxicity testing

The *in vitro* prednisolone response in the absence or presence of potent MCL1 inhibitors or pan-BCL-2 family member inhibitors, was determined using MTT assays as described before (26). Briefly, leukemic cells were exposed to increasing concentrations of prednisolone (0.0076-250 ug/mL) for 4 days. Subsequently, cells were incubated with the yellow tetrazole MTT, for 6 hrs at 37^{o}C, 5% CO_2. In viable cells only, the yellow-colored MTT is reduced to blue-colored formazan crystals, which were subsequently solubilzed with acidified isopropanol before measuring absorption using a spectrophotometer (420 nm). The obtained OD values correlate with the amount of viable cells (27).

Western blot analysis

Whole cellular protein extracts (25 ug) were electrophoretically resolved on pre-casted SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked and subsequently probed with antibodies directed against MCL1 (Sigma-Aldrich, St. Louis, MO, USA), phospho-MCL1, phospho-BCL-2, BCL-XL, BID and PARP (Cell Signaling, Danvers, MA, USA), BCL-2 and BAX (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-Bcl-X (Pierce Biotechnology, Rockford, IL, USA), BIM, BAD, NOXA and PUMA (Abcam, Cambridge, UK). Membranes were counterstained with IRDye® 680/800 conjugated secondary antibodies and were scanned by an Odyssey imaging system (LICOR biotechnology, Lincoln, NE, USA). All membranes were subsequently re-probed with mouse monoclonal anti-β-actin antibodies (Sigma-Aldrich, St. Louis, MO, USA) to assure equal protein loading between lanes.

Statistical analysis

Differences in *in vitro* responses were performed using the (one-tailed) Bonferroni's Multiple Comparison Test and were considered statistically significant at *P* values *<0.05*. Differences in gene expression between patients groups were statistically evaluated using the Kruskall-Wallis Test. All analyses were 2-tailed, and differences were considered statistically significant at *P* values *<0.05* (indicated in experiments with asterisks).

RESULTS

MCL1-inhibiting agents reduce cell viability and induce prednisolone sensitivity in ALL cells

Specific down-regulation of MCL1, either by pharmacological inhibitors or shRNA-mediated knock-down, has been shown to lead to *in vitro* GC sensitivity in ALL cell lines (6,7,13). To test the potency of MCL1-inhibiting compounds in ALL cells (with or without *MLL* translocations), we tested a variety of potential agents, including the pan-BCL-2 family member inhibitors rapamycin, gossypol, AT-101 (synthetic gossypol analogue: apogossypol), SU9516, and obatoclax (GX15-070). The ALL cell lines SEMK2 (*MLL*-rearranged) and REH (*TEL-AML*-rearranged) were incubated in the presence of a range of concentrations of these inhibitors. The *MLL*-rearranged cell line SEMK2 was responsive to all inhibitors, except for rapamycin. Even at high concentrations, rapamycin was not able to reduce cell viability of SEMK2 cells (LC_{50} value: >100 nM; **Figure 1A**). Interestingly, this is in sharp contrast with the *TEL-AML*-rearranged cell line REH, which was highly sensitive to rapamycin (LC_{50} value: 12.8 nM), but hardly responded to gossypol (**Figure 1B**). Both REH and SEMK2 cells were sensitive towards SU9516 and obatoclax, however the SEMK2 cell line showed a higher sensitivity. Both cell lines were also responsive to AT-101, but did not reach a LC_{50} value at the concentrations tested (**Figure 1**).

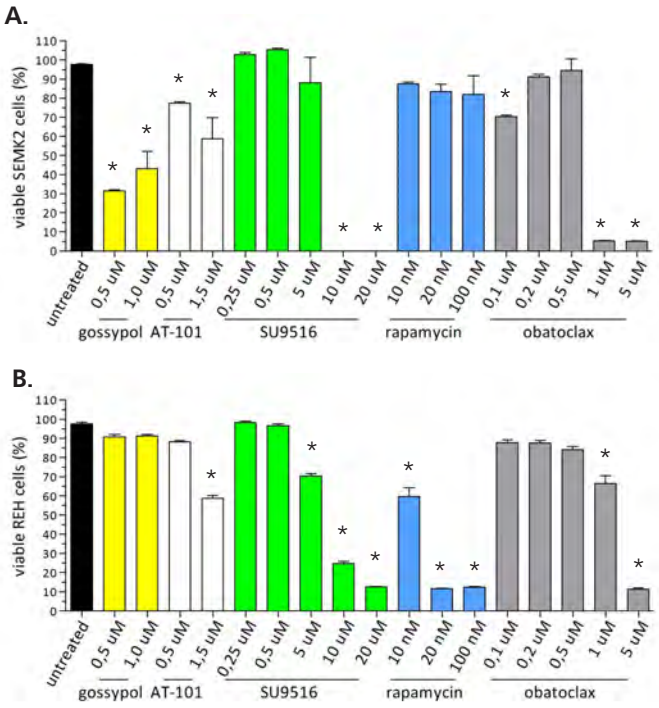


Figure 1. Cell viability of *MLL*-rearranged or *TEL-AML*-rearranged cells after treatment with potent MCL1 inhibiting compounds. Percentage of viable cells of depicted leukemia cell lines exposed to increasing concentrations of gossypol, AT-101 (apogossypol), SU9516, rapamycin and obatoclax (GX15-070), as determined by 4-day MTT assays**.** The *in vitro* drug responses are shown for the cell lines **A.** SEMK2 (*MLL*$^+$ ALL) and **B.** REH (*TEL-AML*$^+$ ALL). Experiments were performed in duplicate. * Indicate significant differences at *p*-values of *<0.05* (when compared to untreated cells).

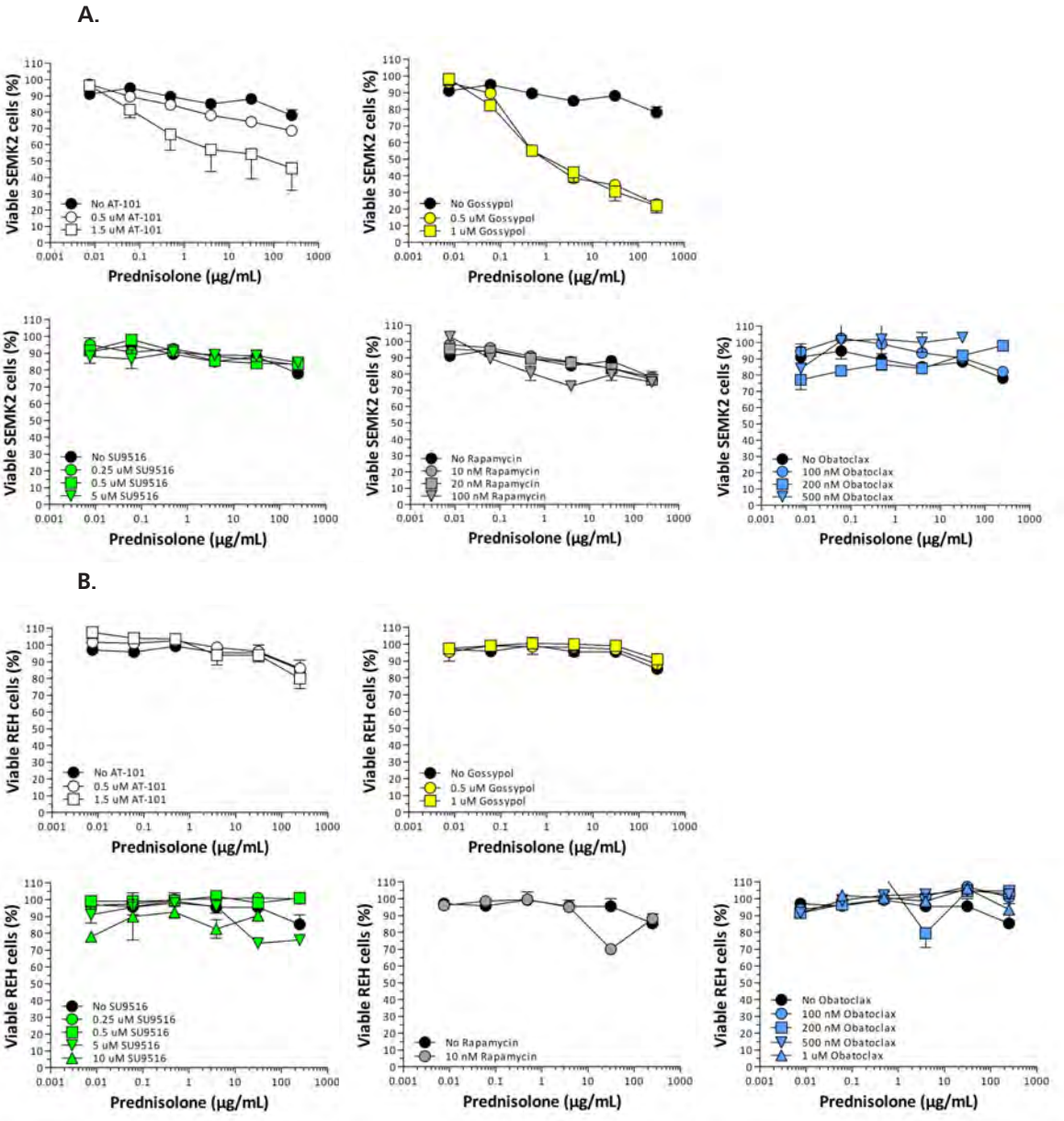


Figure 2. Potent MCL1 inhibiting compounds sensitize prednisolone-resistant *MLL*-rearranged SEMK2 cells, and not *TEL-AML*-rearranged REH cells

4-day *in vitro* cytotoxicity testing (MTT assay) of **A.** the prednisolone-resistant *MLL*-rearranged cell line SEMK2 or **B.** the prednisolone-resistant *TEL-AML*-rearranged precursor B-ALL cell line REH in the absence or presence of prednisolone (concentration range 0.0076-250 µg/ml), showing *in vitro* prednisolone sensitization of the potent MCL1 inhibiting agents gossypol and AT-101, in only SEMK2 cells. * Indicate significant differences at *p*-values of *<0.05*. All cytotoxic dose-response curves were corrected for the inhibitor-specific cell death and only show normalized effects of prednisolone.

In order to investigate GC-sensitizing effects of the MCL1-inhibiting compounds we assessed prednisolone cytotoxicity in the absence and presence of these inhibitors. Both, SEMK2 and REH are well-established cell lines that are highly resistant to prednisolone *in vitro*. We here demonstrate that gossypol and AT-101, but not rapamycin, SU9516 or obatoclax, induced prednisolone sensitivity in the *MLL*-rearranged SEMK2 cells. Even at very high concentrations, SU9516 (5 μM), rapamycin (100 nM), or obatoclax (500 nM) were not able to modulate the *in vitro* prednisolone response (**Figure 2A**). The sensitizing effects of gossypol and AT-101 appeared to be restricted to *MLL*-rearranged ALL cells, as neither gossypol nor AT-101 induced prednisolone sensitivity in the non-*MLL*-rearranged REH cells. However, to strengthen this phenomenom, more cell lines should be tested (**Figure 2B**). Interestingly, the sensitizing effects of gossypol and AT-101 were only restricted to glucocorticoids (*i.e.* prednisolone and dexamethasone), whereas no sensitization was observed for other drugs, such as L-asparaginase and daunorubicin (**Figure 3**).

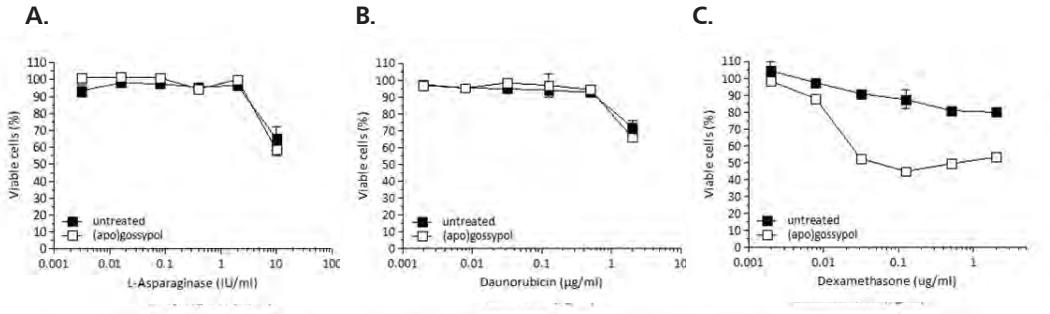


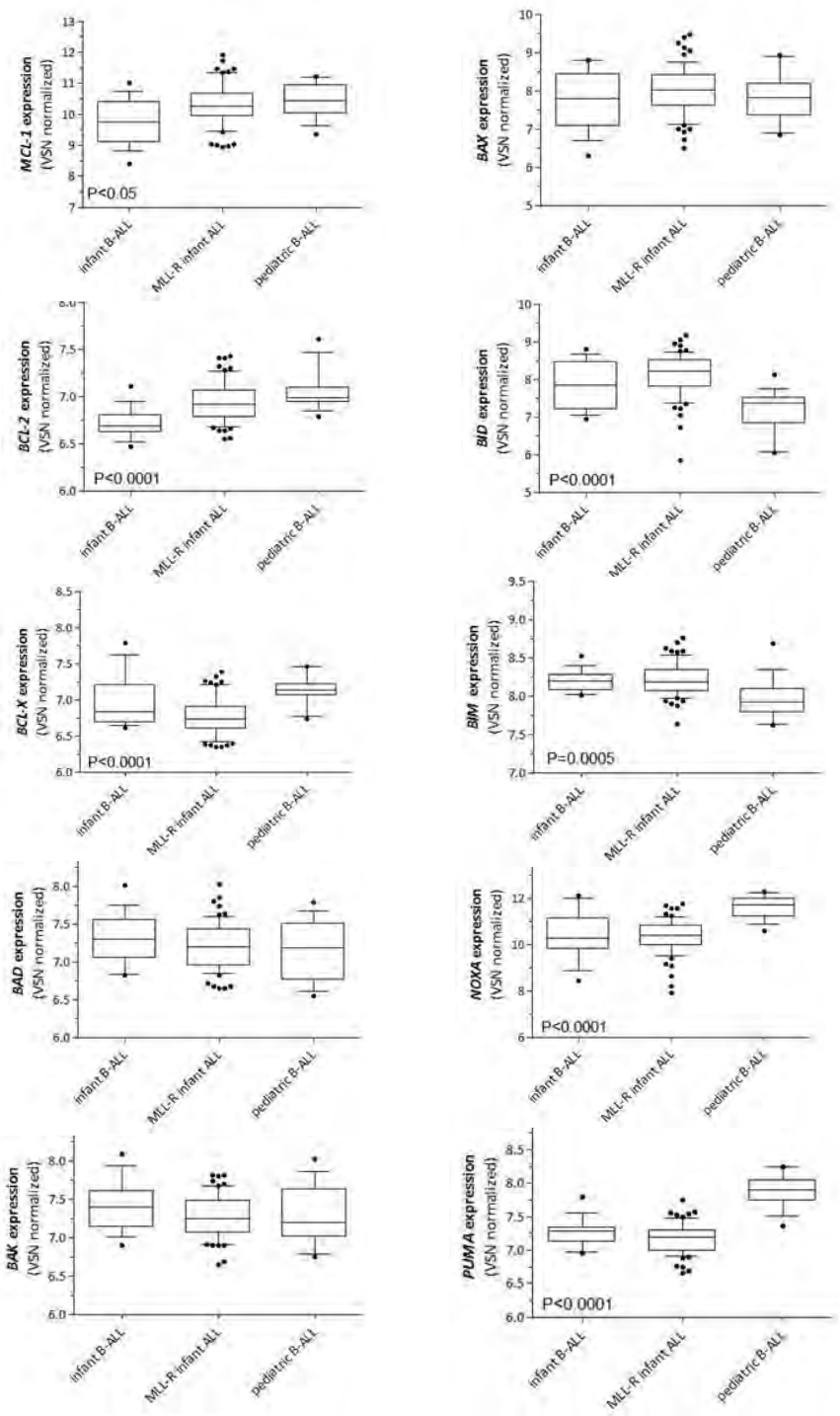
Figure 3. *In vitro* cytotoxicity testing of (apo)gossypol in prednisolone-resistant *MLL*-rearranged cells.
In vitro cytotoxicity as determined by 4-day MTT assays in the glucocorticoid-resistant *MLL*-rearranged ALL SEMK2 cell line. Cytotoxic dose-response curves are shown for **A.** L-asparaginase, **B.** daunorubicin and **C.** dexamethasone. All cytotoxic dose-response curves were corrected for the drug-specific cell death and only show normalized effects of the drug tested.

Expression of pro-apoptotic and anti-apoptotic BCL-2 family members

We recently showed that *MCL1* is one of the few genes related to GC resistance in both pediatric ALL and *MLL*-rearranged infant ALL patients (13). Moreover, Robinson *et al.* showed that not only *MCL1*, but also *BCL-2* is abundantly expressed in *MLL*-rearranged leukemias (28). As the MCL1-inhibiting agents used in this study have been shown to not only repress MCL1 expression, but also influence other pro-survival and pro-apoptotic BCL-2 family members (29-32), we explored the expression patterns of these genes. For this, we used our recently published gene expression profiling data (13) to compare the levels of expression of important pro-survival and anti-apoptotic BCL-2 family members (including *MCL1, BCL-2, BCL-XL, BAD, BAK, BAX, BID, BIM, PUMA* and *NOXA*) between primary *MLL*-rearranged infant ALL, wild-type *MLL* infant ALL and pediatric B-cell precursor ALL. As shown in **Figure 4A**, both *MCL1* and *BCL-2* are indeed abundantly expressed in *MLL*-rearranged infant ALL and pediatric B-cell precursor ALL. Furthermore, *BCL-X* expression was significantly lower in *MLL*-rearranged infant ALL, compared to pediatric B-cell precursor ALL and wild-type *MLL* infant ALL patients. Furthermore,

compared to pediatric BCP-ALL, both *MLL*-rearranged infant ALL as well as wild-type *MLL* infant ALL patients express higher levels of pro-apoptotic *BID* and *BIM* and lower levels of pro-apoptotic *NOXA* and *PUMA* expression (**Figure 4A**).

A.



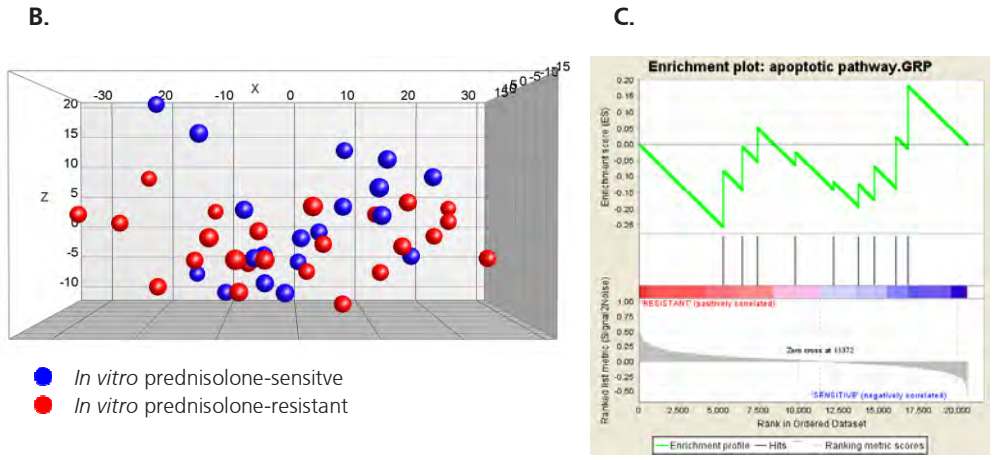


Figure 4. Gene expression analysis of apoptotic regulators in *MLL*-rearranged infant ALL
A. VSN-normalized expression of *MCL1, BCL-2, BCL-X, BAD, BAK* (left panel), *BAX, BID, BIM, NOXA, PUMA* (right panel) in infant B-ALL with either wild-type *MLL* or *MLL* translocations, and pediatric B-cell precursor ALL. **B.** Principle Component Analysis (PCA) of apoptotic gene signature (23 probe sets of Affymetrix HU133plus2 GeneChips, corresponding to 10 genes involved in apoptosis: *BAD, BAK, BAX, BID, BIM, NOXA, PUMA, BCL-2, BCL-X, MCL1*), visualizing the degree of separation between prednisolone-resistant (red; *n*=24) and prednisolone-sensitive (blue; *n*=19) patient samples. **C.** Gene expression profiling data (Affymetrix HU133plus2 GeneChips) associated with *in vitro* prednisolone resistance in primary *MLL*-rearranged infant ALL samples was used for Gene Set Enrichment Analysis (GSEA) interrogating enrichment of the probesets used in Figure 4A, involving the apoptotic pathway. As shown in the enrichment plot, genes related to apoptosis are not involved in GC resistance or GC sensitivity mechanisms.

Next, we investigated whether variations in expression in these pro-survival and anti-apoptotic BCL-2 family members are related to the response to GCs. As shown by principal component analysis (PCA; **Figure 4B**) prednisolone-resistant and prednisolone-sensitive *MLL*-rearranged infant ALL samples could not be separated based on the expression of the BCL-2 family members. In line with this, neither prednisolone-resistant nor prednisolone-sensitive patients showed any sign of gene-set enrichment for these genes (**Figure 4C**). This suggests that GC resistance is not dependent on the gene expression of either pro-survival or pro-apoptotic genes, but rather involves the activation of these apoptotic family members at protein level.

Effects of MCL1-inhibiting agents on the expression of pro-apoptotic and anti-apoptotic proteins

Finally we set out to explore the expression of the BCL-2 family members at the protein level, and test the ability of these inhibitors to modulate these proteins. For this, we focused on agents capable of inducing GC sensitivity in the *MLL*-rearranged ALL cell line SEMK2 (*i.e.* gossypol and AT-101), and used rapamycin, to which SEMK2 cells appeared not to be responsive, as a control.

SEMK2 cells were incubated in the presence of prednisolone alone, or in combination with gossypol, AT-101, or rapamycin (co-treated with prednisolone), followed by western blot analysis of multiple BCL-2 family members. Prednisolone-treated SEMK2 cells did not show any alteration in protein expression of any of the BCL-2 family members (data not shown). Gossypol

and AT-101 did not reduce the levels of MCL1, nor the expression of other anti-apoptotic BCL-2 family member proteins such as BCL-2 and BCL-XL, but rather increased expression of the pro-apoptotic proteins BAD, BAX, BID, BIM and NOXA (**Figure 5A** and **5B**; ≥72 hrs). Interestingly, for BIM and BID, we observed augmentation in protein levels when co-incubated with prednisolone at an even earlier time point (<72 hrs, **Figure 5C**). In contrast, although rapamycin did not induce GC sensitivity (Figure 2), this compound induced significantly up-regulation of pro-apoptotic BAK and BAX, as well as down-regulation of anti-apoptotic MCL1 (**Figure 5A** and **5B**), suggesting that modulation of BAK, BAX and MCL1 are not sufficient to induce GC sensitization in *MLL*-rearranged ALL cells.

Recently, it has been demonstrated that gossypol induces apoptosis in the acute promyelocytic leukemia cell line HL60, by inhibiting phosphorylation of the pro-survival protein BCL-2 (33). Moreover, phosphorylation of MCL1 initiates MCL1 degradation and primes cells for apoptosis initiation (34-36). Since MCL1 expression did not change in response to gossypol or AT-101 treatment, we queried the role of post-translational modifications in inducing apoptosis, and investigated whether gossypol, AT-101 or rapamycin altered the phosphorylation status of the pro-survival proteins MCL1, BCL-2 and BCL-X (*i.e.* BCL-XL and BCL-XS) in prednisolone-resistant *MLL*-rearranged SEMK2 cells. Interestingly MCL1 only became significantly phosphorylated by rapamycin or AT-101 (**Figure 5D** and **5E**). Yet, down-regulation of MCL1 was only observed in response to rapamycin, but not in response to AT-101. Moreover, rapamycin induced severe reduction of phosphorylated BCL-X long isoform (BCL-XL; 96 hrs), but at the same time increased phosphorylation of the BCL-X short isoform (BCL-Xs). Phosphorylation of BCL-Xs was also induced by gossypol and AT-101, albeit to a lesser extent. (**Figure 5D** and **5E**). Taken together, it appears that gossypol and AT-101 induce GC sensitivity in *MLL*-rearranged ALL cells by up-regulation of multiple pro-apoptotic BCL-2 family members, whereas down-regulation of anti-apoptotic members without the up-regulation of pro-apoptotic BCL-2 family proteins (*e.g.* by rapamycin) does not seem sufficient to induce GC sensitivity.

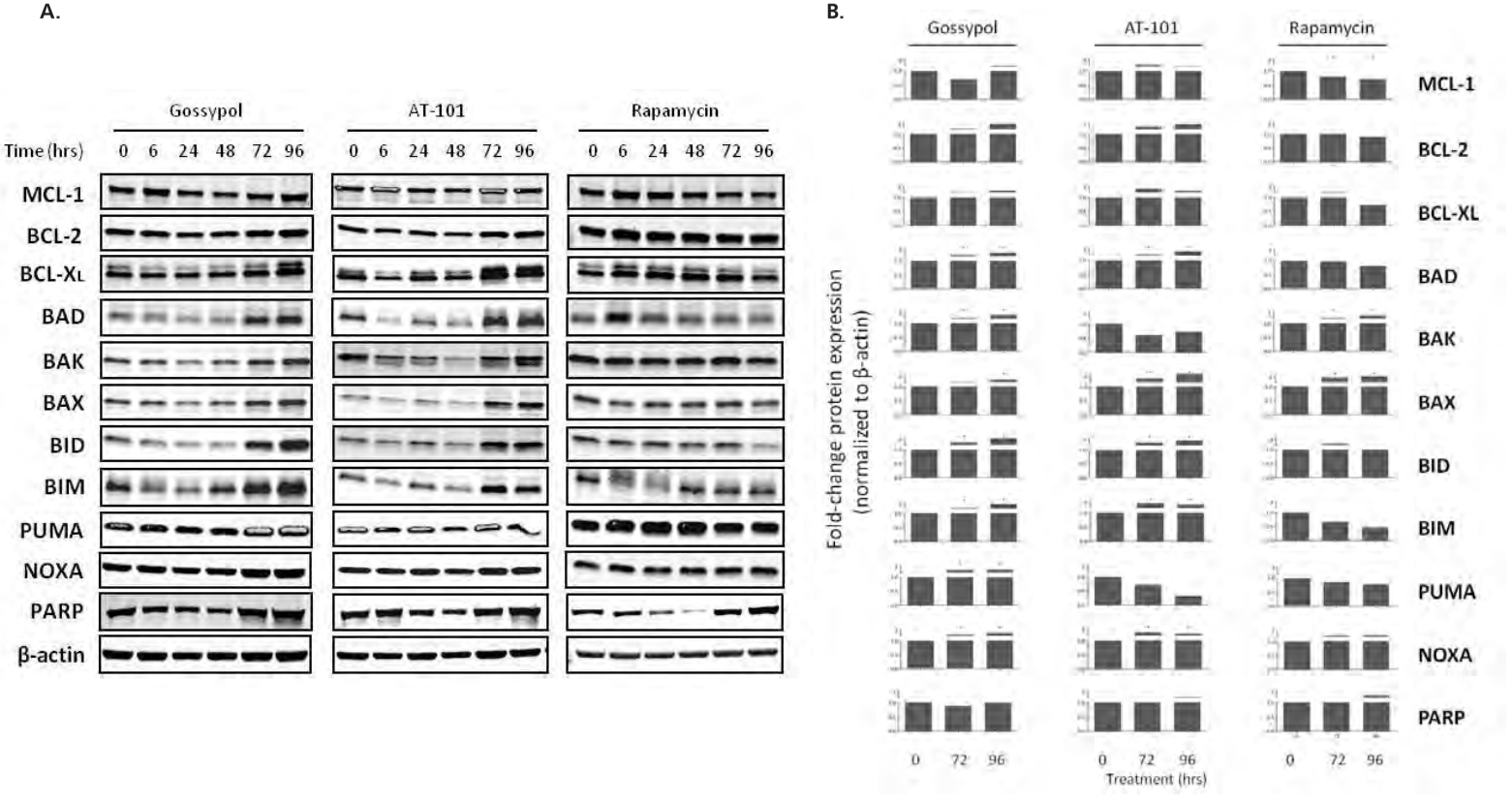


Figure 5. Repression of pro-survival or activation of pro-apoptotic proteins after treatment with prednisolone-sensitizing agents
Western blot analysis of BCL-2 family member proteins involved in apoptosis (MCL-1, BCL-2, BCL-X_L, BID, BIM, BAD, BAK, BAX, PUMA, NOXA, PARP). **A.** Showing western blot analysis of SEMK2 cells treated with prednisolone (125 μg/ml); no modulation of the aforementioned proteins is observed after cells were treated with prednisolone. **B.** Relative expression of the proteins analyzed in figure A, normalized to β-actin expression after treatment with the GC-sensitizing agents gossypol (1 μM; left panel), AT-101 (1.5 μM; middle panel) or rapamycin (10 nM; right panel). Rapamycin was used as a reference drug. * Indicate expected protein modulation (either repression or activation) after treatment with indicated drugs.

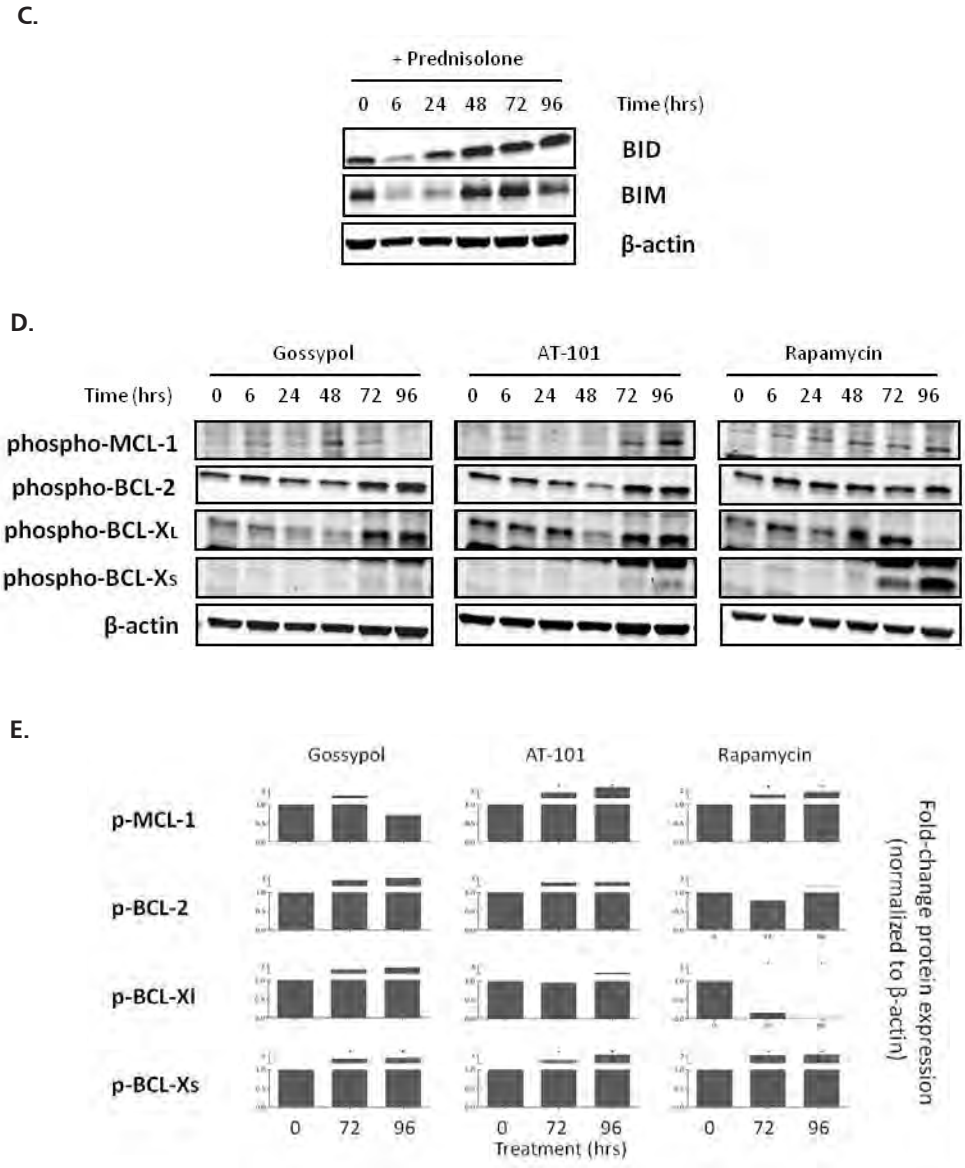


Figure 5. Repression of pro-survival or activation of pro-apoptotic proteins after treatment with prednisolone-sensitizing agents

C. Western blot analysis of BID and BIM protein expression in SEMK2 cells after treatement with gossypol (1 μM) in the presence of prednisolone (125 μg/ml). The on-set of both proteins was even earlier when treated in combination with prednisolone. **D.** Phosphorylated of either pro-apoptotic or anti-apoptotic proteins after incubation with the GC-sensitizing agents gossypol (1 μM), AT-101 (1.5 μM) or rapamycin (10 nM). **E.** Relative expression of the phosphorylated proteins analyzed in figure D, normalized to β-actin expression after treatment with the GC-sensitizing agents gossypol (1 μM; left panel), AT-101 (1.5 μM; middle panel) or rapamycin (10 nM; right panel). Rapamycin was used as a reference drug. * Indicate expected protein modulation (either repression or activation) after treatment with indicated drugs.

DISCUSSION

Infant acute lymphoblastic leukemia (ALL) patients carrying rearrangements of the *Mixed Lineage Leukemia* (*MLL*) gene are characterized by a poor treatment outcome and cellular drug resistance to chemotherapeutics, such as glucocorticoids (GCs). Both *in vivo* and *in vitro* GC resistance contribute to a poor prognosis in infant as well as in childhood ALL (1, 37-41). One of the major mechanisms involved in GC resistance in ALL involves impaired induction of the apoptotic pathway. This impairment includes a deregulated expression of BCL-2 family members, being either pro-apoptotic (4, 9, 10, 11) or pro-survival (3, 5-8, 12, 13, 42). Interestingly, *MCL1* is highly up-regulated in pediatric and infant ALL patient samples resistant to glucocorticoids *in vitro* (13). In this study, we searched for potent MCL1-inhibiting compounds that were able to induce leukemic cell death and/or GC sensitization in *MLL*-rearranged ALL cells. Additionally, we determined the alterations in pro-survival or pro-apoptotic BCL-2 family member activation after exposing *MLL*-rearranged ALL cells to the selected inhibitors.

Recently, drug-based *in silico* screening for small molecule compounds revealed that rapamycin induces glucocorticoid sensitivity in otherwise glucocorticoid-resistant cells, via inhibition of MCL1 (6). Other studies indicated that inhibition of MCL1 by the small molecule GX15-070 (obatoclax) induced apoptotic cell death in GC-resistant ALL cells (7, 8). Still, the concentrations of obatoclax used to induce apoptosis in GC-resistant ALL cells (8) as well as higher concentrations proved inadequate to induce GC sensitivity in *MLL*-rearranged ALL cells in the present study. Although rapamycin and obatoclax are both potent MCL1 inhibitors, these compounds failed to either repress MCL1 expression or to induce prednisolone sensitivity in *MLL*-rearranged ALL cells. In addition, previous results from our laboratory showed that shRNA-mediated knock-down of MCL1 did result in GC sensitivity in *MLL*-rearranged ALL cells, however these effects were very moderate (13), suggesting that other proteins involved in inducing GC sensitivity play a more prominent role.

Interestingly, the present study shows that the potent MCL1-inhibiting compounds gossypol and its synthetic analogue AT-101, induced GC sensitivity specifically in *MLL*-rearranged ALL cells mainly by modulating the pro-apoptotic pathway, while anti-apoptotic BCL-2 family members largely remained unaffected, and appeared surprisingly independent of MCL1 down-regulation. Gossypol has been shown to markedly reduce the expression of the pro-survival proteins BCL-2, BCL-X or MCL1 in human cancer cells (31, 33, 43), which might block the interaction of the pro-survival proteins to BAK and BAD, leading to activation of the pro-apoptotic proteins NOXA and PUMA (31). However, gossypol-induced prednisolone sensitization in *MLL*-rearranged ALL cells was not associated with down-regulation of any of the anti-apoptotic proteins BCL-2, BCL-X or MCL1 in the present study. Moreover, gossypol has been shown to inhibit the phosphorylation of BCL-2 in the acute promyelocytic leukemia cell line HL60. Phosphorylation of BCL-2 is associated with a poor survival in AML and down-regulation of BCL-2 phosphorylation leads to sensitivity towards chemotherapeutics (33, 44). Since phosphorylation of BCL-2 is necessary to exert its anti-apoptotic effect phosphorylation of BCL-2 might be involved in maintaining GC resistance (33). Yet, we did not observe decreased

levels of phosphorylated BCL-2 in *MLL*-rearranged ALL cells exposed to the GC-sensitivity inducing compounds gossypol and AT-101. Instead, we found gossypol and AT-101 to up-regulate multiple pro-apoptotic BCL-2 family members, including BAD, BAK, BAX, BID, BIM, PUMA and NOXA, suggesting that the prominent induction of a pro-apoptotic phenotype represents an alternative mechanism to mediate GC sensitization, independent of the modulation of the anti-apoptotic BCL-2 family proteins. In line with this, rapamycin was able to suppress MCL1 protein expression in *MLL*-rearranged ALL cells, while only two pro-apoptotic BCL-2 family members, *i.e.* BAK and BAX, were significantly up-regulated and no GC sensitization was observed. A previous gene expression study demonstrating up-regulation of *BIM* upon GC treatment in lymphoma cells, thymocytes and leukemia cells (45), which is also in concordance with our findings. The role of BIM in GC resistance was recently confirmed by Zhao *et al.*, demonstrating GC-induced up-regulation of BIM protein expression in GC-sensitive, but not in GC-resistant cells (10). In addition, Erlacher *et al.* found that the absence of BIM protected thymocytes against GC-induced apoptosis (46). Taken together, these observations collectively suggest a prominent role of BIM in GC resistance. Interestingly, both gossypol and AT-101 appeared able to significantly up-regulate the BIM expression in *MLL*-rearranged ALL cells and induce GC sensitivity, whereas rapamycin failed to do so. These findings thus suggest that the GC-sensitizing effects of gossypol and AT-101 in *MLL*-rearranged ALL cells are more dependent on the up-regulation of the pro-apoptotic proteins instead of the down-regulation of anti-apoptotic proteins such as MCL1. Emphasizing this hypothesis, we found that, like rapamycin, SU9516 and obatoclax induce complete down-regulation of MCL1 protein expression (**Figure 6**), but did not lead to GC sensitization. Instead, both SU9516 and obatoclax effectively induced leukemic cell death, and therefore may become useful agents in the treatment of *MLL*-rearranged ALL.

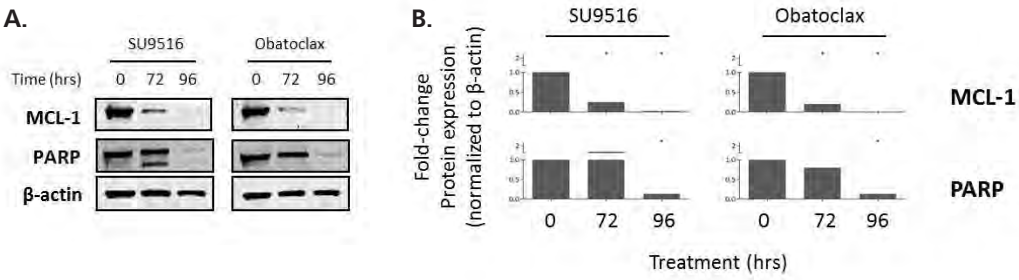


Figure 6. Downregulation of MCL1 in *MLL*-rearranged cells.
The prednisolone-resistant *MLL*-rearranged SEMK2 cell line was incubated with either SU9516 (10 uM) or Obatoclax (1 uM). **A.** Western blot analysis of MCL1 and PARP protein. **B.** Relative expression of MCL1 or total PARP normalized to B-actin expression. Both figures demonstrate a reduction of MCL1 and total PARP expression when either incubated with SU9516 or Obatoclax.

In conclusion, among the spectrum of known MCL1-inhibiting compounds tested in the present study, only gossypol and AT-101 were able to induce GC sensitivity in *MLL*-rearranged ALL cells, via the up-regulation of multiple pro-apoptotic BCL-2 family members. BID and BIM have a prominent role in mediating GC resistance in *MLL*-rearranged ALL cells. Hence, co-

administration of gossypol or AT-101 during GC treatment in resistant *MLL*-rearranged ALL patients may represent an attractive strategy to overcome GC resistance and improve prognosis for this high-risk type of leukemia.

REFERENCES

1. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, *et al.* A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007. 370(9583):240-250.

2. Haarman EG, Kaspers GJ, Pieters R, van Zantwijk CH, Broekema GJ, Hählen K, *et al.* BCL-2 expression in childhood leukemia versus spontaneous apoptosis, drug induced apoptosis, and in vitro drug resistance. Advances in Experimental Medicine and Biology. 1999. 457:325-333.

3. Baghdassarian N, Bertrand Y, French P, Duhaut P, Bryon PA, French M. Role of BCL-2 and cell cycle regulatory proteins for corticosensitivity assessment in childhood acute lymphoblastic leukaemia. *British Journal of Haematology*. 2000. 109(1):109-116.

4. Abrams MT, Robertson NM, Yoon K, Wickstrom E. Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM mRNA with small interfering RNA and short hairpin RNA. *Journal of Biological Chemistry*. 2004. 279(53):55809-55817.

5. Pituch-Noworolska A, Hajto B, Balwierz W, Klus K. Induction of apoptosis and bcl-2 expression in acute lymphoblastic leukaemia and non-Hodgkin's lymphoma in children. *Haematologica*. 2001. 31(3):191-207.

6. Wei G, Twomey D, Lamb J, Schlis K, Agarwal J, Stam RW, *et al.* Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. *Cancer Cell*. 2006. 10(4):331-342.

7. Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, Niggli FK, *et al.* Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. *Journal of Clinical Investigation*. 2010. 120(4):1310-1323.

8. Heidari N, Hicks MA, Harada H. GX15-070 (obatoclax) overcomes glucocorticoid resistance in acute lymphoblastic leukemia through induction of apoptosis and autophagy. *Cell Death and Disease*. 2010. 16;1:e76.

9. Jiang N, Koh GS, Lim JY, Kham SK, Ariffin H, Chew FT, *et al.* BIM is a prognostic biomarker for early prednisolone response in pediatric acute lymphoblastic leukemia. *Experimental Hematology*. 2011. 39(3):321-329.

10. Zhao YN, Guo X, Ma ZG, Gu L, Ge J, Li Q. Pro-apoptotic protein BIM in apoptosis of glucocorticoid-sensitive and -resistant acute lymphoblastic leukemia CEM cells. *Medical Oncology*. 2011. 28(4):1609-1617.

11. Ploner C, Rainer J, Niederegger H, Eduardoff M, Villunger A, Geley S *et al.* The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. *Leukemia*. 2008. 22(2):370-377.

12. Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJ, Kazemier KM, *et al.* Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *New England Journal of Medicin*e. 2004. 351(6):533-542.

13. Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, *et al.* Association of high-level MCL-1 expression with *in vitro* and *in vivo* prednisone resistance in *MLL*-rearranged infant acute lymphoblastic leukemia. *Blood*. 2010. 115(5):1018-1025.

14. Balakrishnan K, Wierda WG, Keating MJ, Gandhi V. Gossypol, a BH3 mimetic, induces apoptosis in chronic lymphocytic leukemia cells. *Blood*. 2008. 112(5):1971-1980.

15. Balakrishnan K, Burger JA, Wierda WG, Gandhi V. AT-101 induces apoptosis in CLL B cells and overcomes stromal cell-mediated MCL1 induction and drug resistance. *Blood*. 2009. 113(1):149-153.

16. Billard C. Design of novel BH3 mimetics for the treatment of chronic lymphocytic leukemia. *Leukemia*. 2012. 26(9):2032-2038.

17. Gao N, Kramer L, Rahmani M, Dent P, Grant S. The three-substituted indolinone cyclin-dependent kinase 2 inhibitor 3-[1-(3H-imidazol-4-yl)-meth-(Z)-ylidene]-5-methoxy-1,3-dihydro-indol-2-one (SU9516) kills human leukemia cells via down-regulation of MCL1 through a transcriptional mechanism. *Molecular Pharmacology*. 2006. 70(2):645-655.

18. Quinn BA, Dash R, Azab B, Sarkar S, Das SK, Kumar S, *et al.* Targeting MCL1 for the therapy of cancer. *Expert Opinion on Investigational Drugs*. 2011. 20(10):1397-1411.

19. Kaspers GJ, Veerman AJ, Pieters R, Broekema GJ, Huismans DR, Kazemier KM, *et al.* Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. *British Journal of Cancer*. 1994. 70(6):1047-1052.

20. Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, *et al.* Gene expression profiling-based dissection of MLL translocated and MLL germline acute lymphoblastic leukemia in infants. *Blood*. 2010 Apr 8;115(14):2835-2844.

21. Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines J, Peters S, Van Zutven L, Beverloo HB, Van der Spek PJ, Escherich G, Horstmann MA, Janka-Schaub GE, Kamps WA, Evans WE, Pieters R. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. *Lancet Oncology*. 2009. 10(2):125-134.

22. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002;18 Suppl 1:S96-104

23. Smyth GK. Limma: linear models for microarray data. In: Gentleman R, Dudoit S, Irizarry R, Huber W, eds. Bioinformatics and Computational Biology Solutions Using R and Bioconductor. (New York, NY: Springer; 2005:397-420).

24. Wettenhall JM, Smyth GK. limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics*. 2004 Dec 12;20(18):3705-3706.

25. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceeding of the National Academy of Sciences*. USA. 2005.102(43):15545-15550.

26. Spijkers-Hagelstein JAP, Mimoso Pinhanços S, Schneider P, Pieters R, Stam RW. SRC kinase-induced phosphorylation of annexin a2 mediates glucocorticoid resistance in *MLL*-rearranged infant acute lymphoblastic leukemia. *Leukemia*. 2013. 27(5):1063-1071.

27. Pieters R, Loonen AH, Huismans SR, Broekema GJ, Dirven MW, Heyenbrok MW, *et al. In vitro* drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions *Blood*. 1990. 76(11):2327-2336.

28. Robinson BW, Behling KC, Gupta M, Zhang AY, Moore JS, Bantly AD, *et al.* Abundant anti-apoptotic BCL-2 is a molecular target in leukaemias with t(4;11) translocation. *British Journal of Haematology*. 2008. 141(6):827-839.

29. Masood A, Chitta K, Paulus A, Khan AN, Sher T, Ersing N, *et al.* Downregulation of BCL2 by AT-101 enhances the antileukaemic effect of lenalidomide both by an immune dependant and independent manner. *British Journal of Haematology*. 2011. 157:59–66.

30. Li ZM, Jiang WQ, Zhu ZY, Zhu XF, Zhou JM, Liu ZC, *et al.* Synergistic cytotoxicity of Bcl-xL inhibitor, gossypol and chemotherapeutic agents in non-Hodgkin's lymphoma cells. *Cancer Biology and Therapy*. 2008. 7(1):51-60.

31. Meng Y, Tang W, Dai Y, Wu X, Liu M, Ji Q, *et al.* Natural BH3 mimetic (-)-gossypol chemosensitizes human prostate cancer via Bcl-xL inhibition accompanied by increase of Puma and Noxa. *Molecular Cancer Therapy*. 2008. 7(7):2192-202.

32. Kang MH, Reynolds CP. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clinical Cancer Research*. 2009.15(4):1126-1132.

33. Huang LH, Hu JQ, Tao WQ, Li YH, Li GM, Xie PY, *et al.* Gossypol inhibits phosphorylation of Bcl-2 in human leukemia HL-60 cells. *European Journal of Pharmacology*. 2010. 645(1-3):9-13.

34. Ding, Q, He X, Hsu JM, Xia W, Chen CT, Li LY, *et al.* Degradation of MCL1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. *Molecular Cell Biology*. 2007. (11):4006–4017.

35. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL1. *Molecular Cell*. 2006. 1(6):749-760.

36. Thomas LW, Lam C, Edwards SW. MCL1; the molecular regulation of protein function. *FEBS Letters*. 2010. 584(14):2981-2989.

37. Greaves MF. Infant leukaemia biology, aetiology and treatment. *Leukemia*. 1996 Feb;10(2):372-377.

38. Kaspers GJ, Pieters R, van Zantwijk CH, van Wering ER, van der Does-van den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood*. 1998. 92(1):259-266.

39. Dördelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, *et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*. 1999. 94(4):1209-1217.

40. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, *et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *Journal of Clinical Oncology*. 2003. 17:3262-3268.

41. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med*. 2004 Apr;350(15):1535-1548.

42. Ruvolo PP, Deng X, May WS. Phosphorylation of Bcl2 and regulation of apoptosis. *Leukemia*. 2001. 15(4):515-522.

43. Jarvis WD, Turner AJ, Povirk LF, Traylor RS, Grant S. Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. *Cancer Research*. 1994. 54(7):1707-1714.

44. Kurinna S, Konopleva M, Palla SL, Chen W, Kornblau S, Contractor R, *et al.* Bcl2 phosphorylation and active PKC alpha are associated with poor survival in AML. *Leukemia*. 2006. 20(7):1316-1319.

45. Wang Z, Malone MH, He H, McColl KS, Distelhorst CW.Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. *Journal of Biological Chemistry*. 2003. 278(26):23861-23867.

46. Erlacher M, Michalak EM, Kelly PN, Labi V, Niederegger H, Coultas L, *et al.* BH3-only proteins Puma and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells *in vivo*. *Blood*. 2005. 106(13):4131-4138

CHAPTER 4

Elevated *S100A8/S100A9* expression causes glucocorticoid resistance in *MLL*-rearranged infant acute lymphoblastic leukemia

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ABSTRACT

MLL-rearranged Acute Lymphoblastic Leukemia (ALL) in infants is characterized by a poor clinical outcome and resistance to glucocorticoids (*e.g.* prednisone and dexamethasone). As both the response to prednisolone *in vitro* and prednisone *in vivo* are predictive for clinical outcome, understanding and overcoming glucocorticoid resistance remains an essential step towards improving prognosis.

Prednisolone-induced apoptosis depends on glucocorticoid-evoked Ca^{2+} fluxes from the endoplasmic reticulum towards the mitochondria. Here we demonstrate that in *MLL*-rearranged infant ALL, over-expression of *S100A8* and *S100A9* is associated with failure to induce free-cytosolic Ca^{2+} and prednisolone resistance. Furthermore, we demonstrate that enforced expression of *S100A8/S100A9* in prednisolone-sensitive *MLL*-rearranged ALL cells, rapidly leads to prednisolone resistance as a result of S100A8/S100A9-mediated suppression of prednisolone-induced free-cytosolic Ca^{2+} levels. In addition, the Src kinase inhibitor PP2 markedly sensitized *MLL*-rearranged ALL cells otherwise resistant to prednisolone, via down-regulation of S100A8 and S100A9, which allowed prednisolone-induced Ca^{2+} fluxes to reach the mitochondria and trigger apoptosis. Based on this novel mechanism of prednisolone resistance, we propose that developing more specific S100A8/S100A9 inhibitors may well be beneficial for prednisolone-resistant *MLL*-rearranged infant ALL patients.

INTRODUCTION

Since the early 1960s, treatment results for childhood Acute Lymphoblastic Leukemia (ALL) began to improve steadily and continued to progress ever since. Consequently, the survival chances for childhood ALL in general nowadays exceed 85% (1). Unfortunately, this tremendous step forward has not been equally beneficial for all patients. This is especially true for infants (<1 year of age) with ALL carrying leukemia-specific chromosomal translocations involving the *MLL* gene, which occur in ~80% of the infant ALL cases (2-3). Depending on the treatment protocol, survival chances for *MLL*-rearranged infant ALL patients are at best ~40% (3). Considerably contributing to this poor outcome is cellular resistance to multiple chemotherapeutic drugs, in particular to glucocorticoids like prednisone and dexamethasone, which form the cornerstone of childhood ALL treatment regimes. Prednisolone (the biologically active metabolite of prednisone) dosages needed to eliminate 50% of infant ALL cells *in vitro* typically are ~500-fold higher than the dosages required to eradicate similar amounts of precursor B-ALL cells from children older than one year of age (*i.e.* non-infants) (4). Moreover, approximately 30% of infants with *MLL*-rearranged ALL show a poor prednisone response *in vivo*, compared to only ~10% of non-infant pediatric precursor B-ALL cases (5). As the *in vitro* prednisolone response and the *in vivo* prednisone response represent important prognostic factors (6-8), the poor prognosis for *MLL*-rearranged infant ALL seems to a large extent associated with glucocorticoid resistance. Therefore, the mechanism(s) underlying poor glucocorticoid responses should be unraveled in order to adapt treatment strategies and improve prognosis.

We recently demonstrated that high-level expression of the anti-apoptotic BCL-2 family member *MCL1* is associated with prednisolone resistance (9). However, modulation of MCL1 in prednisolone-resistant *MLL*-rearranged cell line models only resulted in moderate sensitization (9-10), implying that other and more relevant resistance mechanisms are involved. In our earlier efforts to define a prednisolone-resistance-associated gene expression profile, the obtained gene signature was compromised by a relatively low accuracy, presumably due to small numbers of patient samples (9). Recently, however, we published high-resolution gene expression profiling data on a larger cohort of infant ALL samples, which even allowed specification of differential gene expression between distinct sub-types of infant ALL (11). Studying these profiles we observed that *MLL*-rearranged infant ALL frequently displays high-level expression of genes encoding members of the S100 protein family. For example, *S100A10* and *S100A4* appeared in the top50 of over-expressed genes in *MLL*-rearranged infant ALL when compared to non-infant pediatric precursor B-ALL (11). Moreover, a recent paper by Qazi *et al.* demonstrated that, in their gene expression profiling analyses comparing ALL in infants and non-infants, over-expression of both *S100A8* and *S100A9* was highly characteristic for infant ALL cells (12). Intrigued by these observations we set out to explore the meaning of this phenomenon, and found that the expression of S100 family members often is discriminative between prednisolone resistant and sensitive *MLL*-rearranged infant ALL samples. Among other S100 family members, this appeared true for both *S100A8* and *S100A9*. Given that S100A8 and S100A9 form functional heterotetramer protein complexes (13-15), we here specifically

focused on this particular protein complex in relation to prednisolone-resistance. Both S100A8 and S100A9 contain calcium(Ca^{2+})-binding EF-hand motifs (16), which provides the S100A8/S100A9 protein complex the capacity to bind, and therefore buffer, free-cytosolic Ca^{2+} (17). Interestingly, over 30 years ago, Kaiser and Edelmann demonstrated that glucocorticoid-induced lymphocytolysis is dependent on free-cytosolic Ca^{2+} (18) released from the endoplasmic reticulum (ER) upon glucocorticoid exposure. From this, we hypothesized that over-representation of the S100A8/S100A9 complex in the cytosol may establish glucocorticoid resistance in *MLL*-rearranged ALL by trapping free-cytosolic Ca^{2+}, preventing it to reach the mitochondria, and thereby forestalling apoptosis.

MATERIALS AND METHODS

Patients sample collection and processing

Bone marrow and/or peripheral blood samples from untreated infants (*i.e.* children <1 year of age) diagnosed with *MLL*-rearranged ALL, were collected at Sophia Children's Hospital (Rotterdam, the Netherlands) as part of the international collaborative INTERFANT-99treatment protocol (3). Mononuclear cells were isolated and contaminating non-leukemic cells removed using immunomagnetic beads as described before (19). As a result, all leukemic samples used in this study contained more than 90% of leukemic blasts.

In vitro prednisolone and *in vivo* prednisone response

In vitro prednisolone sensitivity was assessed by a 4-day MTT assays as described elsewhere (20). Patient samples were characterized as *in vitro* sensitive or resistant to prednisolone based on the LC_{50} value (*i.e.* the concentration of prednisolone lethal to 50% of the leukemic cells). Samples were defined as prednisolone-sensitive at LC_{50} values <0.1 µg/mL prednisolone, prednisolone-intermediate at LC_{50} values 0.1-150 µg/mL, and prednisolone-resistant at LC_{50} values >150 µg/mL prednisolone.

The *in vivo* response to prednisolone was determined after a 7-day window of prednisone monotherapy (before the initiation of combination chemotherapy). Patients were defined as prednisone poor responders (PPRs) when >1000 leukemic blasts/µL remained present in the peripheral blood. When the amount of leukemic blasts dropped below 1000/µL patients were defined as prednisone good responders (PGRs).

RNA extraction and cDNA synthesis

Total RNA was extracted from a minimum of 5 $x10^6$ leukemic cells using TRIzol reagent (Invitrogen, Life Technologies, Breda, the Netherlands) according to the manufacturer's guidelines. The quality of the extracted RNA was assessed on 1.5% agarose gels, and the RNA integrity using RNA 6000 Nano Assay LabChips on the Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, USA). Further cDNA synthesis was performed as described previously (21).

Microarray and bioinformatics analyses

Gene expression profiles (Affymetrix HGU133plus2.0 GeneChips) were generated for primary *MLL*-rearranged infant ALL samples to produce an *in vitro* prednisolone resistance signature. The gene expression data has been deposited in the NCBI Gene Expression Omnibus under the GEO Series accession number GSE32962. For the analysis of differentially expressed genes between prednisolone sensitive and resistant patients, raw microarray data (Affymetrix HU133plus2.0 GeneChips) were collectively normalized using variance-stabilizing normalization (VSN) (22), and differential gene expression was statistically evaluated using linear models for microarray analyses (LIMMA) (23-24). A detailed description of the processing of RNA samples and the generation of the gene expression profiles are previously described elsewhere (11).

Gene set enrichment analysis (GSEA) (25) was used to evaluate enrichment of genes encoding S100 protein family members in prednisolone-resistant *MLL*-rearranged infant ALL samples. **Table 1** lists the gene enrichment scores for all S100 family members.

Quantitative real time PCR analysis

S100A8 and *S100A9* mRNA expression was quantified by quantitative RT-PCR analysis (26) using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). Oligonucleotide primers used for PCR amplification were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO) and purchased from Eurogentec (Seraing, Belgium). Primer sequences were as follows: sense strand: 5'-GTGGGCATCATGTTGAC-3' and antisense strand: 5'-TGCACCATCAGTGTTGATA-3' (for *S100A8*); sense strand: 5'-TCGGCTTTGACAGAGTG-3' and anti-sense strand: 5'-GGTCCTCCATGATGTGTT-3' (for *S100A9*); sense strand: 5'-GTCGGAGTCAACGGATT-3' and anti-sense strand: 5'-AAGCTTCCCGTTCTCAG-3' (for *GAPDH*; used as a reference gene).

Cell culturing and flow cytometry

The leukemia cell lines RS4;11, BEL-1, SEM (acute lymphoblastic leukemia (ALL) cell lines) and MV4-11 (acute myeloid leukemia (AML) cell line) all carry the *MLL* translocation t(4;11) generating the *MLL-AF4* fusion protein, and were maintained in RPMI 1640 with glutamax (Invitrogen, Life Technologies, Breda, the Netherlands) supplemented with 10% (v/v) FCS and 1% penicillin/streptomycin/fungizone (PSF; Invitrogen, Life Technologies, Breda, the Netherlands) at 37^{o}C in humidified air-containing 5% CO_2. RS4;11 and BEL-1 represent *in vitro* prednisolone-sensitive cell lines, whereas SEM and MV4-11 are *in vitro* prednisolone-resistant. Depending on the experiments, cell lines were cultured in the presence of prednisolone (25 µg/ml; Bufa, Uitgeest, The Netherlands), EGTA (50 µM; Sigma, St Louis MO, USA), BAPTA-AM (2 µM; Sigma, St Louis MO, USA) or PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo (27)pyrimidine; 10 µM; Sigma, St Louis MO, USA) even prior to *in vitro* cytotoxicity testing. Free-cytosolic Ca^{2+} was stained using Calcium Green-1 (Invitrogen, Life Technologies, Breda, the Netherlands) and measured on a flow cytometer (Facs Calibur, Becton Dickinson, USA). For this, leukemic cells were washed twice in phosphate-buffered saline (PBS) and incubated with 2 µM of Calcium Green-1 for 1 hour at RT in the dark. Prior to detection by flow cytometry,

incubated cells were washed twice in PBS to exclude aspecific binding Calcium Green-1. Similarly, mitochondrial depolarization was detected using JC-1, a cationic dye that displays potential-dependent accumulation in the mitochondria, indicated by a fluorescence emission shift from green to red.

Western blotting

Western blot analysis was performed as described previously (28). Briefly, cellular proteins were resolved on the polyacrylamide gels and transferred to nitrocellulose membrane. Membranes were probed using mouse monoclonal antibodies against human S100A8 and rabbit polyclonal antibodies against human S100A9 (Santa Cruz, CA, USA). Anti-β-actin mouse monoclonal antibodies (Sigma-Aldrich, St. Louis, MO, USA) were used to confirm equal loading in all lanes.

Transfection of leukemia cell lines with S100A8 and S100A9 expressing constructs

Full-length cDNA of human S100A8 and S100A9 were cloned into mammalian expression vectors: *S100A8* into pLPCX containing a puromycin selection marker (Clontech, Mountain View, USA) and *S100A9* into pLNCX2 containing a neomycin selection marker (Clontech, Mountain View, USA). The primer sequences used to amplify the entire coding regions of these genes were as follows: sense strand: 5'-TGTCAGCTGTCTTTCAGAAG-3' and anti-sense strand: 5'-TGGGCCCAGTAACTCA-3' for *S100A8*; sense strand: 5'-CTCGGCTTTGACAGAGTG-3' and anti-sense strand: 5'-CTGGCCTCCTGATTAGTG-3' for *S100A9*. *S100A8* was cloned into pLPCX using the restriction enzymes *HindIII* and *NotI.* The same enzymes were used to insert *S100A9* into pLNCX2. Next, prednisolone-sensitive RS4;11 cells were transfected with *S100A8* pLPCX or *S100A9* pLNCX2 constructs by square-pulse electroporation (at 600V, for 2 msec). Cells transfected with the constructs were cultured under selection of neomycin (1 mg/ml; Gibco BRL, Life Technologies) and puromycin (10 μg/ml; Sigma, St Louis MO, USA) in order to obtain a pure population of transfected cells.

Statistical analysis

Differences in *S100* gene expression between patients groups or cell lines were statistically evaluated using the Mann Whitney *U*-test. The student *t*-test or the one-way ANOVA test was used to analyze differences in mean cytotoxicity responses and to determine differences in mitochondrial membrane depolarization. All analyses were 2-tailed, and differences were considered statistically significant at *P* values <0.05 (indicated in experiments with asterisks). Spearman's Rho test was used to assess the correlation between VSN-normalized expression of *S100A9* and VSN-normalized expression of *S100A8* or *MCL1*. Log-rank test was performed to analyze differences in outcome between patient groups expressing high and low levels of *S100A8* and *S100A9*.

RESULTS

High-level *S100A8/S100A9* expression in prednisolone-resistant *MLL*-rearranged infant ALL

In order to gain more insight into the mechanisms that underlie prednisolone resistance in *MLL*-rearranged infant ALL, we continuously search for genes that are differentially expressed in prednisolone-resistant and prednisolone-sensitive patient samples. For this, *in vitro* response to prednisolone is assessed by 4-day cytotoxicity (MTT) assays, and the LC_{50} values (*i.e.* the concentration of prednisolone required to eliminate 50% of the leukemic cells) are used to determine prednisolone sensitivity. Patient samples are deemed sensitive at LC_{50} values of <0.1 μg/mL of prednisolone, and resistant at LC_{50} values of >150 μg/mL. Next, high-resolution gene expression profiling data (Affymetrix HU133plus2.0 microarrays) is used to evaluate differential gene expression between prednisolone resistant and sensitive primary *MLL*-rearranged infant ALL samples. Remarkably, applying this approach we often encountered differentially expressed genes encoding members of the S100 protein family. Although these genes do not represent the most significant genes discriminating between prednisolone resistant and sensitive patients, Gene Set Enrichment Analysis (GSEA) revealed pronounced enrichment of high-level expression of multiple S100 family members in prednisolone-resistant *MLL*-rearranged infant ALL samples (**Figure 1A** and **Table 1**).

Table 1. Probe sets of S100 genes used for Gene Set Enriched Analysis (GSEA).

Probe set	Gene Symbol	Gene title	Core Enrichment
200660_at	**S100A11**	S100 calcium binding protein A11 (calgizzarin)	Yes
204268_at	**S100A2**	S100 calcium binding protein A2	Yes
204351_at	**S100P**	S100 calcium binding protein P	Yes
202917_s_at	**S100A8**	S100 calcium binding protein A8 (calgranulin A)	Yes
203535_at	**S100A9**	S100 calcium binding protein A9 (calgranulin B)	Yes
205863_at	**S100A12**	S100 calcium binding protein A12 (calgranulin C)	Yes
217728_at	**S100A6**	S100 calcium binding protein A6 (calcyclin)	Yes
207763_at	**S100A5**	S100 calcium binding protein A5	Yes
200872_at	**S100A10**	S100 calcium binding protein A10 (annexin II ligand)	Yes
203186_s_at	**S100A4**	S100 calcium binding protein A4	No
205334_at	**S100A1**	S100 calcium binding protein A1	No
202598_at	**S100A13**	S100 calcium binding protein A13	No
209686_at	**S100B**	S100 calcium binding protein, beta (neural)	No
218677_at	**S100A14**	S100 calcium binding protein A14	No
1554876_a_at	**S100Z**	S100 calcium binding protein, zeta	No
206027_at	**S100A3**	S100 calcium binding protein A3	No
205916_at	**S100A7**	S100 calcium binding protein A7 (psoriasin 1)	No

Therefore we studied VSN-normalized gene expression (Affymetrix HU133plus2 microarray data) for all probe sets corresponding to *S100* genes in infant and non-infant pediatric B-ALL samples. In general, all genes encoding evaluated members of the S100 protein family appeared to be consistently expressed at higher levels in infant ALL cells as compared to pediatric precursor B-ALL cells from children older than 1 year of age, with the exception of *S100A13* (**Figure 1B**). Moreover, comparing *MLL*-rearranged infant ALL samples, either resistant or sensitive to prednisolone, demonstrated that *S100* expression often is higher in prednisolone-resistant samples (**Figure 1B**). Although the strongest enrichment in prednisolone-resistant *MLL*-rearranged infant ALL samples was observed for *S100A11*, *S100A2*, and *S100P* (**Table 1**), the expression levels of these particular genes was rather low when compared to, for example, *S100A8* and *S100A9* (**Figure 1B**). In addition, studying the differences in expression of S100 encoding genes between prednisolone resistant and sensitive *MLL*-rearranged leukemia cell lines (**Figure 1C**), showed that the largest fold-change was observed for *S100A8* (**Figure 1D**). Given that the expression of these two S100 family members appeared extremely high in *MLL*-rearranged infant ALL patient samples, and show pronounced differences in expression between prednisolone resistant and sensitive cases (**Figure 1B**), and displayed the highest enrichment after *S100A11*, *S100A2*, and *S100P* (**Table 1**), we decided to further focus on *S100A8* and *S100A9*. Further supporting our choice to here focus on these to particular members of the S100 protein family is the fact that, at the protein level, S100A8 and S100A9 preferably function cooperatively in heterotetramer complexes (13-15).

To validate our gene expression data, quantitative RT-PCR analysis was performed using primer sets specifically amplifying either *S100A8* or *S100A9*. This confirmed that prednisolone-resistant patients expressed >100-fold higher levels of both *S100A8* and *S100A9* compared to prednisolone-sensitive patients. Moreover, patients displaying intermediate *in vitro* sensitivity to prednisolone (LC_{50} values of >0.1 and <150 μg/mL) exhibit intermediate *S100A8* and *S100A9* expression levels (**Figure 2A** and **2B**). Of note is that the *S100A8* and *S100A9* expression levels, as determined by quantitative RT-PCR analysis, appeared to be exceedingly high. Whereas the majority of genes is expressed at relative levels of ~0.5 to 5% of the housekeeping gene *GAPDH*, in prednisolone-resistant *MLL*-rearranged infant ALL cells the expression levels of both *S100A8* and *S100A9* appeared near comparable to that of *GAPDH*.

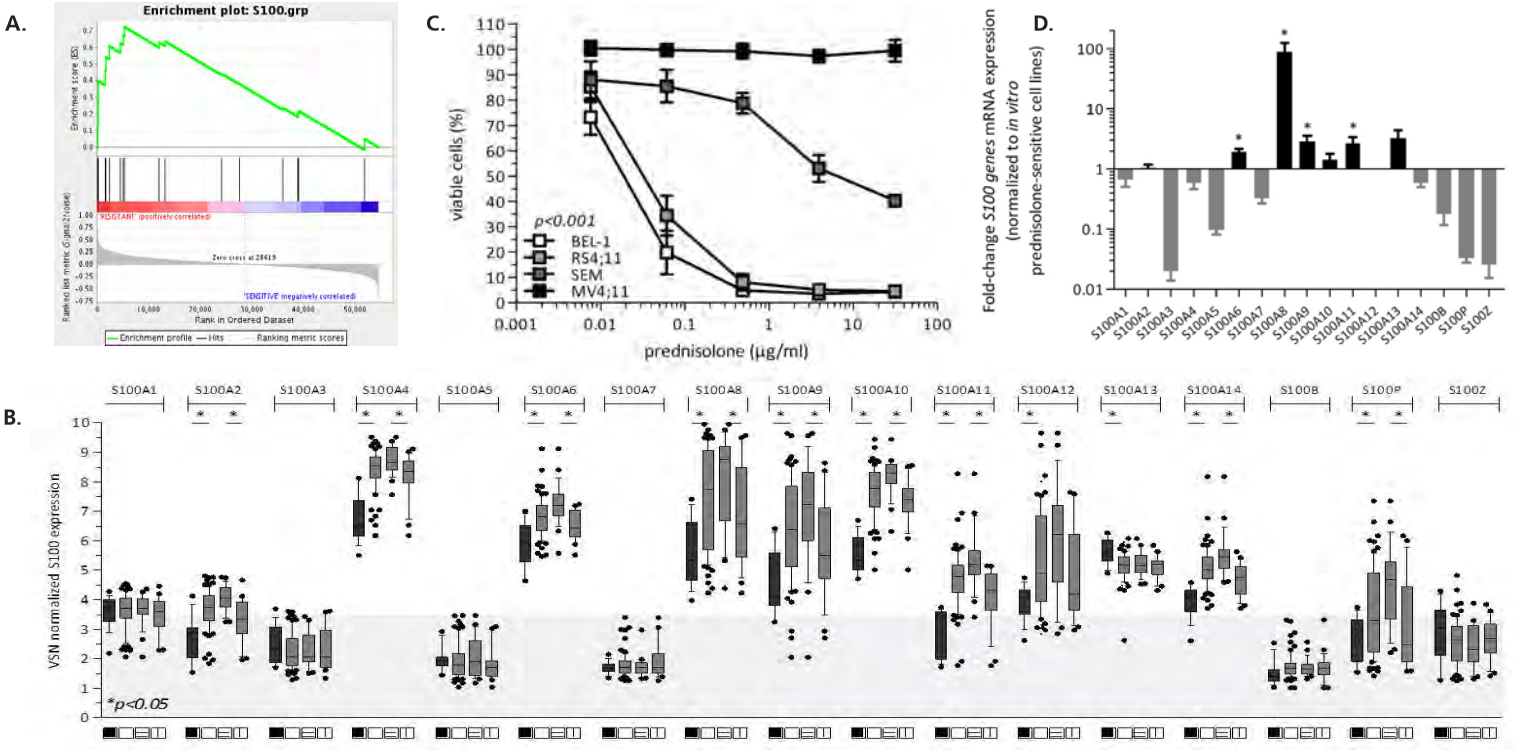

Figure 1. Enrichment and distribution of the expression of the *S100* gene family in prednisolone-resistant *MLL*-rearranged infant ALL samples. **A.** Gene expression profiling data (Affymetrix HU133plus2 GeneChips) associated with *in vitro* prednisolone resistance in primary *MLL*-rearranged infant ALL samples was used for Gene Set Enrichment Analysis (GSEA) interrogating enrichment of the entire *S100* gene family. As shown in the enrichment plot, high-level *S100* expression appears highly associated with resistance to prednisolone ($p<0.05$). **B.** VSN normalized gene expression of all members of the S100 gene family is depicted for childhood (>1 year of age) precursor B-ALL (black boxes), infant (<1 year of age) ALL including both *MLL*-rearranged and wild-type *MLL* cases (white boxes), prednisolone-resistant *MLL*-rearranged infant ALL (horizontally striped boxes), and prednisolone-sensitive *MLL*-rearranged infant ALL (vertically striped boxes). VSN expression values of <3.5 imply very low to no expression and coincide with the background noise (grey border). **C.** *In vitro* prednisolone responses in the *MLL*-rearranged leukemia cell lines BEL-1, RS4;11 (prednisolone-sensitive), and SEM and MV4-11 (prednisolone-resistant). **D.** Shows the fold-change in mRNA expression of *S100* genes in *MLL*-rearranged *in vitro* prednisolone-sensitive cell lines (RS4;11 and BEL-1) compared to *in vitro* prednisolone-resistant cell lines (SEM and MV4-11). Fold-change expression of *S100* genes in the resistant cell lines was normalized to the expression in of the same genes in the sensitive cell lines. S100 genes that are up-regulated in resistant cell lines are depicted in black, and down-regulated genes are depicted in grey**.** Differences in *S100* expression were statistically analyzed using the Mann Whitney *U*-test; * indicate significant differences with p-values of *<0.05*.

As both the *in vitro* prednisolone response, as well as the *in vivo* prednisone response, have been shown to be predictive for clinical outcome (6-8), we next asked whether the observed correlation between high-level *S100A8/S100A9* expression and resistance to prednisolone also holds true for the *in vivo* prednisone response. As part of the INTERFANT-99 treatment protocol (3), infant ALL patients receive prednisone monotherapy before initiation of combination chemotherapy. After 7 days, the drop in leukemic blasts in the peripheral blood reflects the *in vivo* prednisone response, and is used to ascribe patients to either the standard-risk (PGRs: prednisone good responders) or the high-risk (PPRs: prednisone poor responders) arm of the treatment protocol. To evaluate how the *S100A8/S100A9* expression correlates with the *in vivo* prednisone response, we plotted the distribution of *S100A8* and *S100A9* expression between PGRs and PPRs. As shown in **Figure 2C**, patients showing a poor *in vivo* prednisone response (PPR) are clearly expressing higher levels of both *S100A8* and *S100A9*. However, statistical analysis showed that these differences did not reach significance, presumably due to a few patients in the PPR-group expressing low *S100A8/S100A9* levels. Together this suggests that in general the correlation between high-level *S100A8* and *S100A9* expression and both the *in vitro* prednisolone and *in vivo* prednisone response exists, but also implies that in some patients other (presumably pharmacodynamical) mechanisms play a role.

High-level *S100A8/S100A9* expression in *MLL*-rearranged infant ALL is associated with a poor prognosis

Expression of several S100 family members, including *S100A8* and *S100A9*, have been shown to correlate with an adverse prognosis in invasive ductal carcinomas of the breast (29) and in *de novo* pediatric AML patients (30). Likewise, low *S100A8* and *S100A9* expression has been shown to correlate with a good prognosis in childhood AML patients carrying *IDH1/2* mutations (31). Therefore we asked whether high-level *S100A8* and *S100A9* expression is also associated with a poor prognosis in *MLL*-rearranged infant ALL. For this, we divided patient samples into two groups either expressing high or low levels of *S100A8* and *S100A9* based on our quantitative RT-PCR data, using the median *S100A8/S100A9* expression levels as cut-off values. Interestingly, the event-free survival varied significantly between both subgroups ($P<0.05$), with *MLL*-rearranged ALL patients expressing high-level *S100A8* and *S100A9* expression being at extreme high risk of disease relapse or other complications during treatment, whereas patients expressing low levels of S100A8 and S100A9 seem to fare markedly better (**Figure 2D**).

Multiple studies identified the *in vivo* prednisone response as a prognostic marker for *MLL*-rearranged infant ALL (6). However, despite clear trends towards a correlation between high-level *S100A8/S100A9* expression and the *in vivo* prednisone response (**Figure 2C**), these differences did not reach significance. Therefore we also determined clinical outcome between patients showing a good or a poor prednisone response *in vivo* (**Figure 2D**). Remarkably, although PPRs showed a worse outcome than PGRs, the level of *S100A8* and *S100A9* expression appeared to be a much stronger predictor of prognosis in these patients.

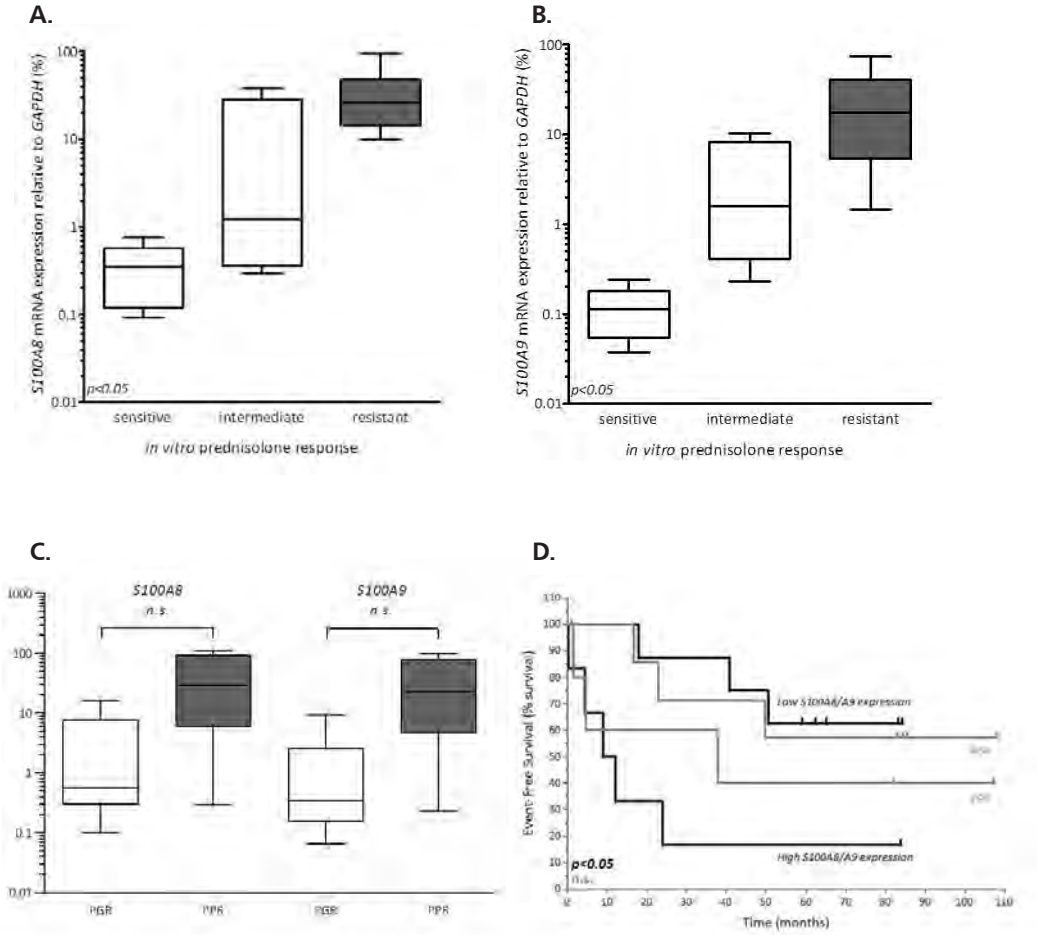

Figure 2. High-level *S100A8/S100A9* expression is correlated with *in vitro* prednisolone resistance and a poor prognosis in *MLL*-rearranged ALL.

Relative mRNA expression of **A.** *S100A8* and **B.** *S100A9* as determined by quantitative RT-PCR analysis in an independent cohort of *MLL*-rearranged infant ALL patient samples either *in vitro* sensitive (*n*=5) or resistant (*n*=5) to prednisolone, and in patients displaying intermediate responses (*n*=5). **C.** Relative *S100A8* and *S100A9* mRNA expression in *MLL*-rearranged infant ALL patients based on the *in vivo* prednisone response. PGR: prednisone good responders (*n*=9), PPR: prednisone poor responders (*n*=4).

D. Event-free survival for *MLL*-rearranged infant ALL patients expressing high (*n*= 6) or low (*n*= 8) levels of *S100A8/S100A9*. The median expression value in the entire cohort was used as the cut-off value in order to divide the two patient groups (in black). Event-free survival for *MLL*-rearranged infant ALL patients, displaying *in vivo* prednisone good response (PGR; *n*=9) or prednisone poor response (PPR; *n*=4) (in grey). Differences in outcome were statistically analyzed using the Log-rank test.

Prednisolone sensitivity is dependent on prednisolone-induced Ca^{2+} release

Already in 1977, Kaiser and Edelmann showed that glucocorticoid-induced lymphocytolysis requires cytosolic Ca^{2+} (18) which is released from the endoplasmic reticulum (ER) upon glucocorticoid exposure (32-34). Given the Ca^{2+}-binding properties of the S100A8/S100A9 protein complex (26-28) and the association of high-level *S100A8/S100A9* expression with prednisolone resistance, we set out to explore the importance of Ca^{2+} release in prednisolone-induced apoptosis in *MLL*-rearranged ALL cells. For this the levels of free-cytosolic Ca^{2+} were determined in prednisolone-sensitive and prednisolone-resistant *MLL*-rearranged leukemia cell line models using flow cytometry. These analyses showed that in *MLL*-rearranged leukemia cell lines that are sensitive to prednisolone *in vitro*, pronounced levels of prednisolone-induced free-cytosolic Ca^{2+} are detectable (**Figure 3A**). In contrast, in prednisolone-resistant *MLL*-rearranged leukemia cell lines, the levels of free-cytosolic Ca^{2+} were hardly elevated upon prednisolone exposure (**Figure 3A**).

To confirm that prednisolone-induced apoptosis depends on free-cytosolic Ca^{2+}, sensitive cells were exposed to prednisolone either in the absence or presence of the Ca^{2+} chelators EGTA (50 μM) or BAPTA-AM (2 μM), that have been shown to effectively scavenge free-cytosolic Ca^{2+}. Both EGTA and BAPTA-AM fully inhibited prednisolone-induced Ca^{2+} release, within 4 hours of exposure (**Figure 3B**). As a consequence, both EGTA and BAPTA-AM to some extent induced resistance to prednisolone in otherwise sensitive *MLL*-rearranged ALL cells, albeit the effects for both co-incubations appear moderate in 2-day cytotoxicity assays (**Figure 3C-D**). Moreover the effects of BAPTA-AM and EGTA induced inhibition of free-cytosolic Ca^{2+} on prednisolone sensitivity seems to vary between both cell lines tested. A plausible explanation for this variability as well as for the moderate effects on prednisolone cytotoxicity is the fact that optimal inhibition of free-cytosolic Ca^{2+} by BAPTA-AM and EGTA takes place at 4 hours of exposure, but after that rapidly diminishes. As *in vitro* prednisolone sensitivity is here determined in 2-day MTT assays, the effects of BAPTA-AM and EGTA largely depend on Ca^{2+} inhibition during the first 4 hours of the cytotoxicity assay. Nevertheless, these results clearly demonstrate that short-term inhibition of free-cytosolic Ca^{2+} certainly affected the *in vitro* prednisolone response in otherwise highly sensitive cells.

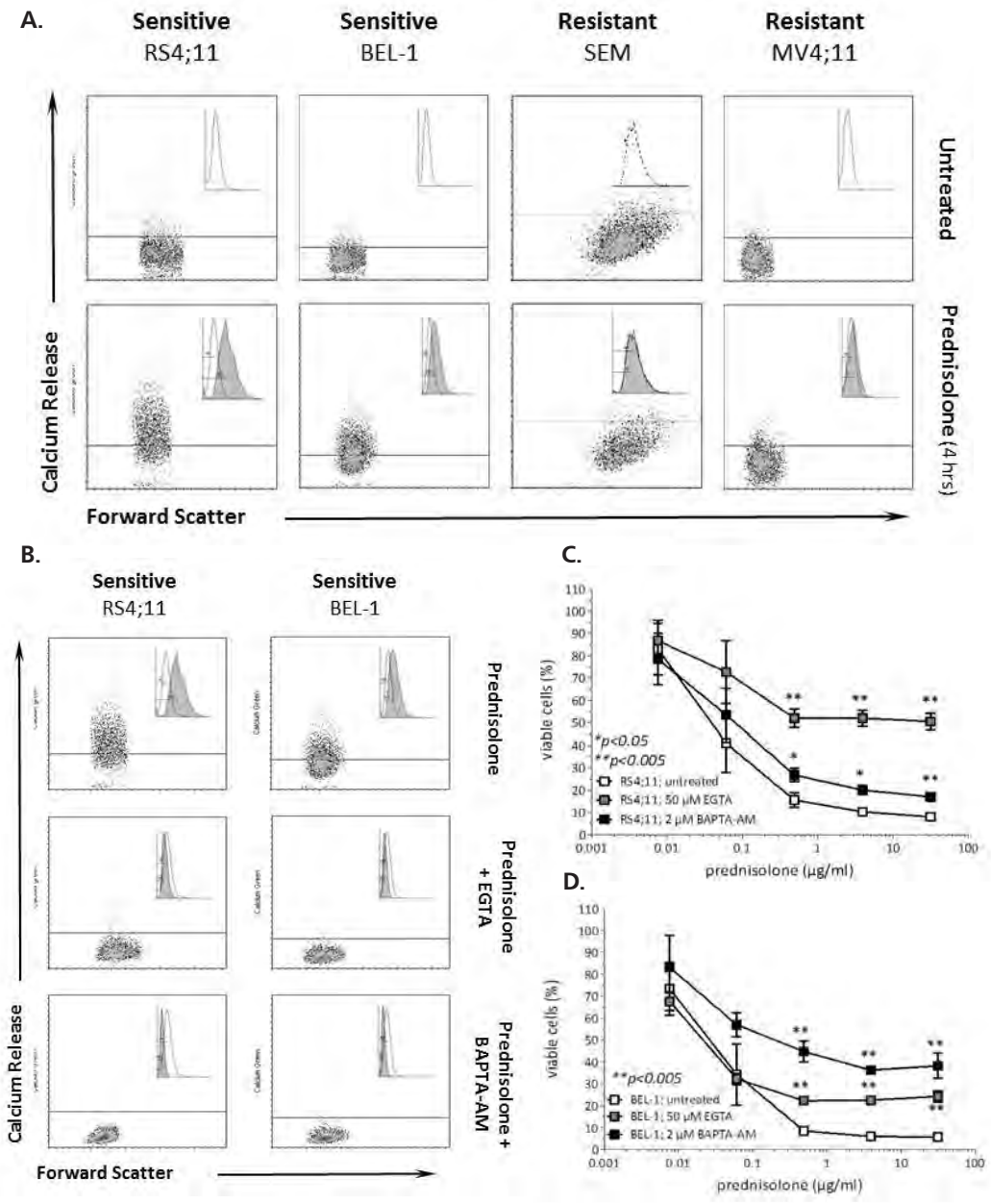

Figure 3. Cytosolic Ca^{2+} depletion confers prednisolone resistance in *MLL*-rearranged ALL cells.
A. Qualitative measurement of free-cytosolic Ca^{2+} in prednisolone-sensitive (BEL1 and RS4;11) and prednisolone-resistant (SEM and MV4-11) *MLL*-rearranged ALL cells before and after prednisolone (25 µg/ml) exposure for 4 hours. **B.** Analyses of prednisolone-induced free-cytosolic Ca^{2+} in the prednisolone-sensitive cell lines RS4;11 and BEL-1 in the absence or presence of the Ca^{2+} chelators EGTA (50 µM) or BAPTA-AM (2 µM), after 4 hrs incubation with prednisolone (25 µg/ml). *In vitro* prednisolone response for **C.** RS4;11 and **D.** BEL-1 in the absence or presence of either EGTA (50 µM) or BAPTA-AM (2 µM), as determined by 2-day MTT assays. * Indicate significant differences at *p*-values of *<0.05*, and ** *p<0.005*.

High-level *S100A8/S100A9* expression inhibits free-cytosolic Ca^{2+} and induces prednisolone resistance

With the above confirmation that prednisolone-induced apoptosis requires free-cytosolic Ca^{2+}, we hypothesized that the observed over-representation of both *S100A8* and *S100A9* obstruct prednisolone-induced apoptosis by binding and thereby preventing cytosolic Ca^{2+} released from the ER. To test this, prednisolone-sensitive *MLL*-rearranged ALL cells were transduced with eukaryotic expression vectors encoding human *S100A8* and/or *S100A9* and free-cytosolic Ca^{2+} levels measured upon prednisolone exposure. The transfection experiments resulted in pronounced up-regulation of both genes as determined by quantitative RT-PCR analysis (*$P<0.05$) (**Figure 4A**). Prednisolone-induced Ca^{2+} release was partially inhibited in cells transduced with *S100A8* or *S100A9* alone, whereas no free-cytosolic $Ca^{2+}$was detected in cells over-expressing both *S100A8* and *S100A9* (**Figure 4B**). In line with this, prednisolone-sensitive *MLL*-rearranged ALL cells transduced with either *S100A8* or *S100A9* separately became somewhat more resistant to prednisolone resulting in increases of leukemic cell survival of ~30-40% (**Figure 4C**). However, when transduced with both *S100A8* and *S100A9* simultaneously, the prednisolone-sensitive *MLL*-rearranged ALL cells became highly resistant to prednisolone (*$P<0.05$) (**Figure 4C**).

Mitochondrial depolarization is inhibited by S100A8 and S100A9

Free-cytosolic Ca^{2+} rapidly migrates towards the mitochondria and successively leads to the depolarization of the mitochondrial membrane, cytochrome c release from the mitochondria, the formation of the apoptosome, and the initiation of apoptosis (35-38). As proposed in the present study, high-level expression of S100A8 and S100A9 prevent cytosolic Ca^{2+} to reach to the mitochondria. Thus, transfection of prednisolone-sensitive *MLL*-rearranged ALL cells with both S100A8 and S100A9 should lead to an unaffected mitochondrial membrane potential when these cells are exposed to prednisolone. Therefore, the prednisolone-sensitive *MLL*-rearranged ALL cell line RS4;11 was transduced with S100A8 and/or S100A9 and incubated with prednisolone (25 µg/ml). Subsequently the mitochondrial potential was measured using flow cytometry. Mitochondrial depolarization was indeed observed in prednisolone-sensitive cells transduced with empty vectors (*$P<0.05$), whereas the mitochondrial membrane potential remained unaffected in cells over-expressing S100A8 and/or S100A9 after prednisolone exposure (**Figure 4D**).

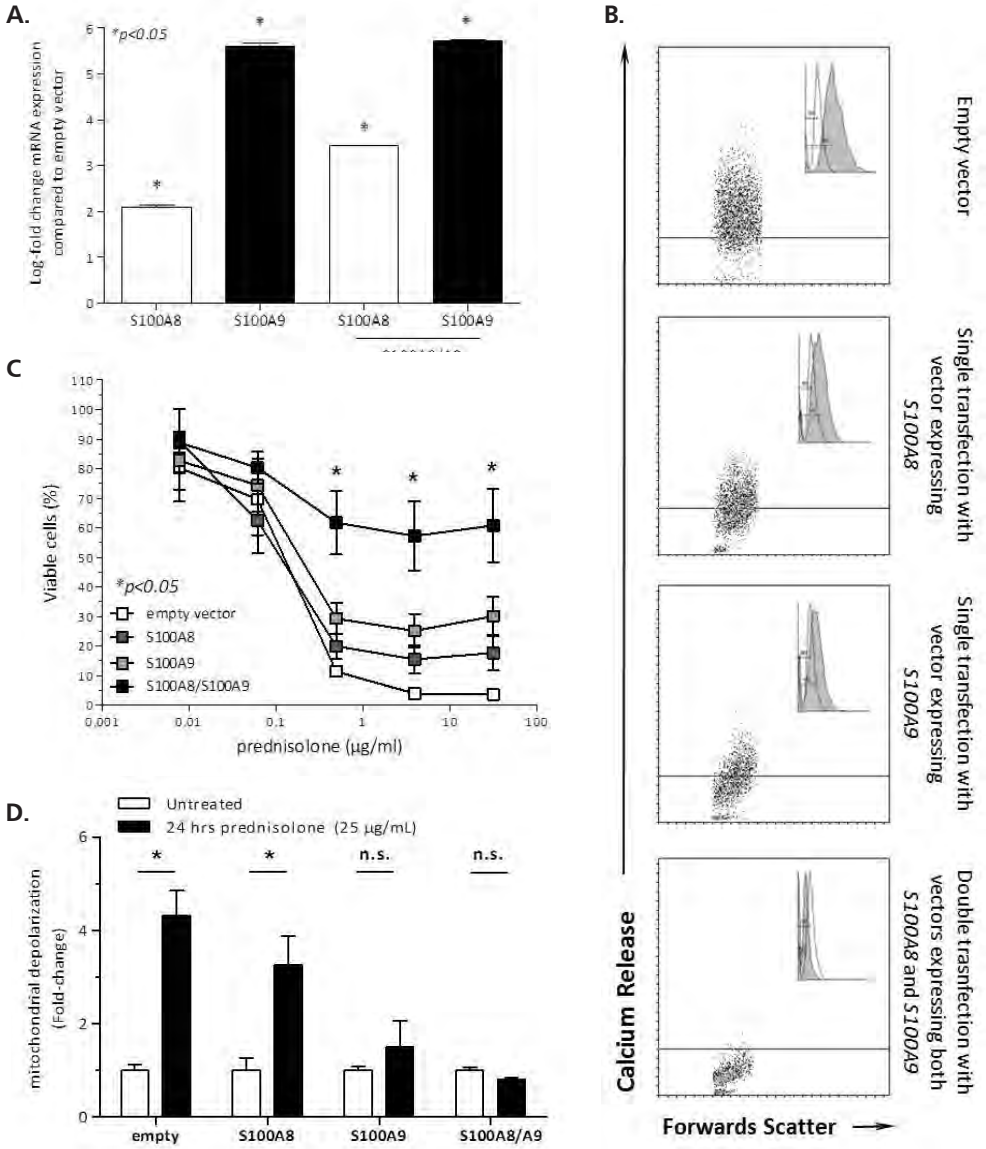

Figure 4. S100A8/S100A9 over-expression depletes free-cytosolic Ca^{2+} and induces prednisolone resistance in *MLL*-rearranged ALL cells.

A. Quantitative RT-PCR analysis showing the mRNA expression of *S100A8* or *S100A9* in the prednisolone-sensitive cell line RS4;11 transduced with eukaryotic expression vectors encoding human S100A8 and/or S100A9. **B.** Measurement of prednisolone-induced free-cytosolic Ca^{2+} in prednisolone-sensitive RS4;11 cells after transduction with vectors enforcing S100A8 and/or S100A9 expression or with empty vectors (controls). Inserts represent the shift in free-cytosolic Ca^{2+} compared to untransduced cells. **C.** *In vitro* prednisolone response of prednisolone-sensitive RS4;11 cells in the absence and presence of enforced S100A8 and/or S100A9 expression. **D.** Mitochondrial depolarization analysis in RS4;11 cells before and after transfection with eukaryotic expression vectors encoding S100A8 and/or S100A9, when challenged with prednisolone (25 µg/ml) for 24 hrs.

Inhibition of S100A8 and S100A8 by PP2 induces prednisolone sensitivity in prednisolone-resistant *MLL*-rearranged ALL cells

Next we asked whether inhibition of the S100A8/S100A9 protein complex leads to sensitization to prednisolone in resistant *MLL*-rearranged ALL cells expressing high levels of *S100A8* and *S100A9*. In a recent paper, Ryckman *et al.* showed that the Src kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo-3,4-d-zpyrimidine) was able to inhibit S100A8/S100A9 release from neutrophils by approximately 50% (39). Based on these findings, we investigated the potential of PP2 to sensitize prednisolone-resistant *MLL*-rearranged ALL cells. Co-incubation of 10 μM of PP2 with prednisolone in prednisolone-resistant *MLL*-rearranged ALL cell line SEM, indeed showed sensitization to prednisolone (**Figure 5A**), whereas no additional sensitization for prednisolone occurred in *in vitro* prednisolone-sensitive cell line RS4;11 (**Figure 5B**). Importantly, PP2 induced prednisolone sensitivity in *MLL*-rearranged ALL cells was accompanied by detectable levels of cytosolic Ca^{2+} in response to prednisolone exposure (**Figure 5C**), and down-regulation of both S100A8 and S100A9 at the protein level (**Figure 5D and 5E**). Although the levels of free-cytosolic Ca^{2+} were apparently not increased at 10 μM, of PP2, it must be taken into consideration that the detection of free-cytosolic Ca^{2+} is performed after 4 hours of exposure, whereas *in vitro* prednisolone cytotoxicity was assessed in 4-day MTT assays. Measurements of free-cytosolic Ca^{2+} at increasing concentrations of PP2, show that PP2 exposure indeed results in increased levels of free-cytosolic Ca^{2+} (already after 4 hours of exposure) when cells are challenged with prednisolone.

Interestingly, the sensitizing effects of PP2 were only observed for the glucocorticoids prednisolone and dexamethasone, but not for other therapeutics used in the treatment of childhood ALL such as L-asparaginase, vincristine, daunorubicin, cytarabine (Ara-C) or cladribine (2CdA) (**Figure 6**).

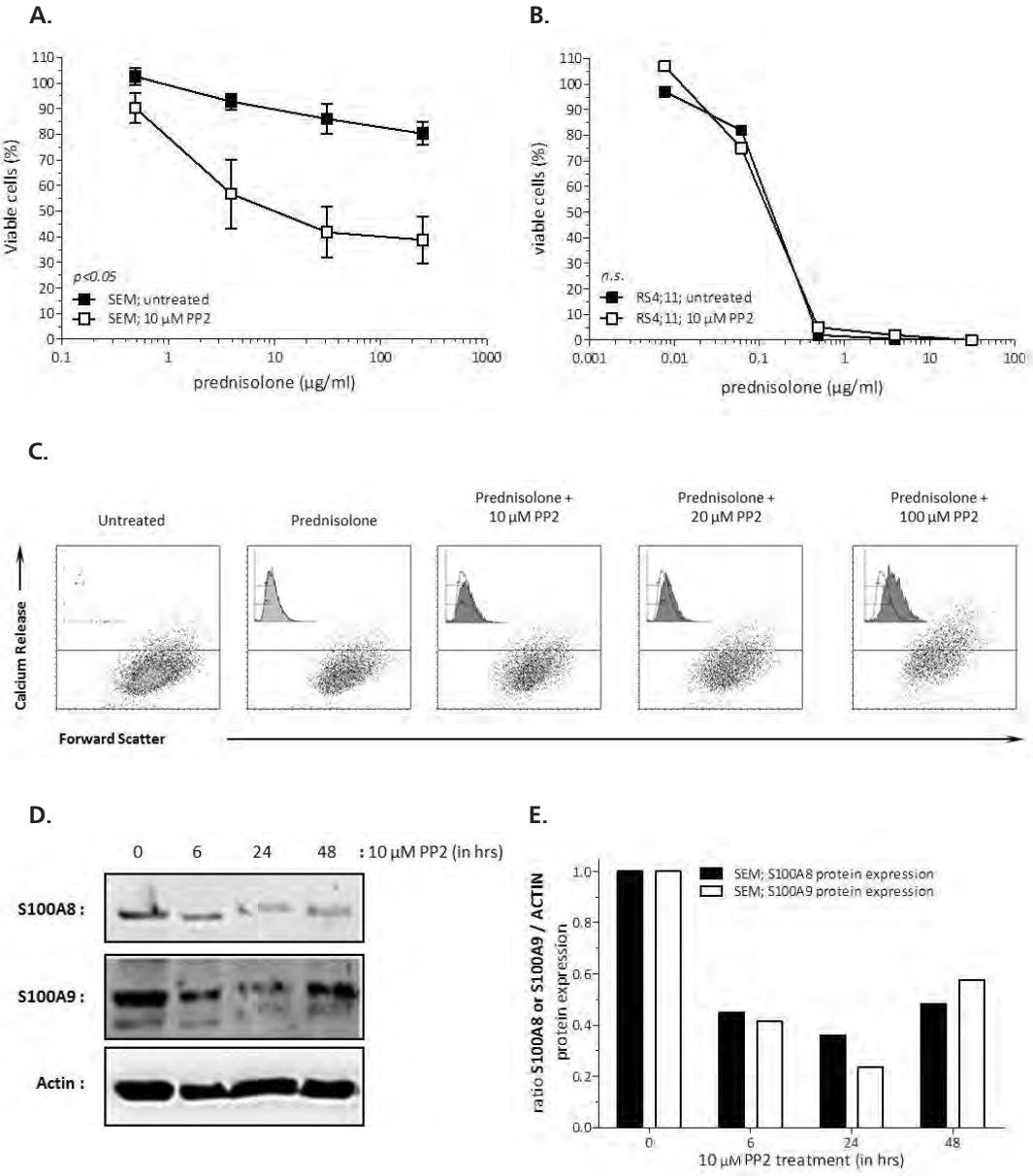

Figure 5. Inhibition of S100A8/S100A9 sensitizes prednisolone-resistant *MLL*-rearranged ALL cells. **A.** *In vitro* prednisolone response for the prednisolone-resistant *MLL*-rearranged ALL cell line SEM, and **B.** the prednisolone-sensitive *MLL*-rearranged ALL cell line RS4;11 in the absence or presence of the Src kinase inhibitor PP2 (10 µM). **C.** Measurement of prednisolone-induced free-cytosolic Ca^{2+} in SEM cells in the absence or presence of the Src kinase inhibitor PP2, after 4 hours of exposure. The inserts represents the shift in Ca^{2+} release compared SEM cells exposed to prednisolone alone. **D.** Shows Western blot analysis of the protein expression of S100A8 and S100A9 in SEM cells upon exposure to PP2 (10 µM) at indicated time points. **E.** Depicts the calculated ratios of S100A8 or S100A9 protein expression normalized against actin expression in these samples in the absence or presence of 10 µM of PP2.

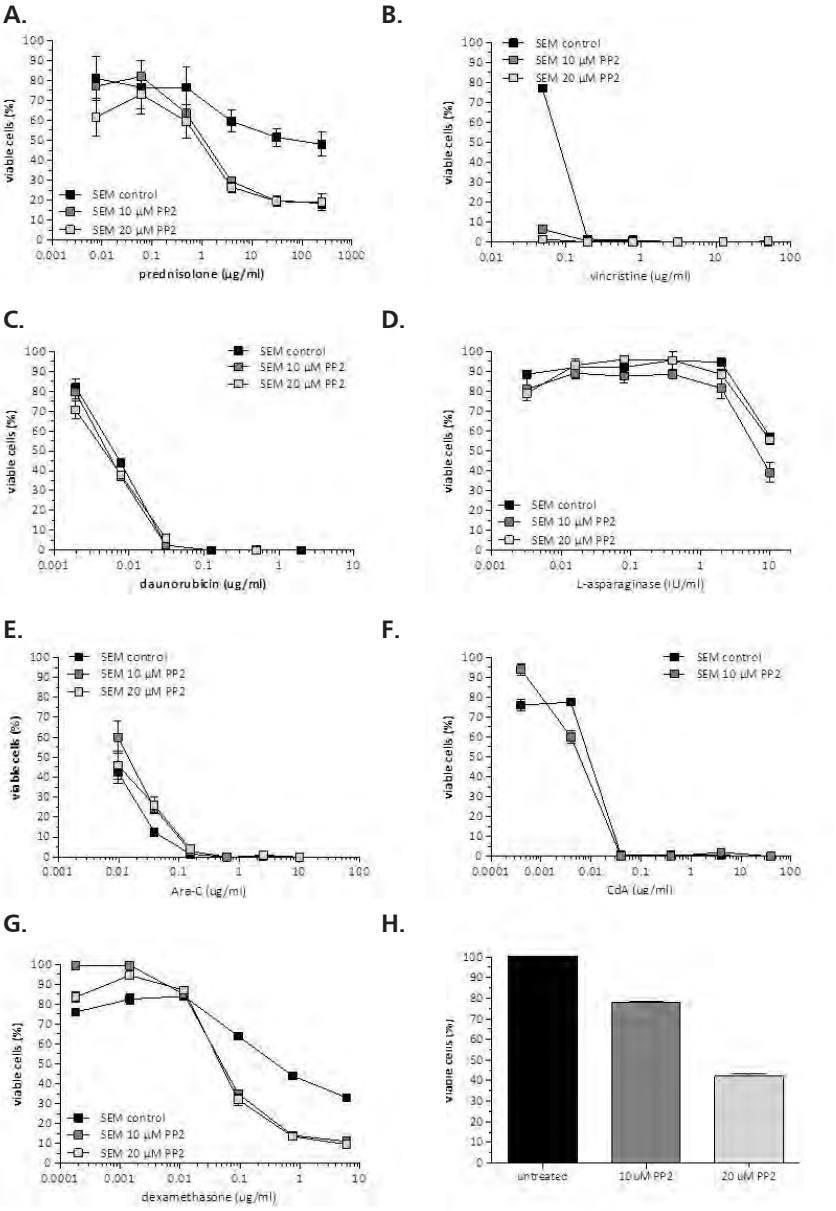

Figure 6. *In vitro* cytotoxicity testing in the *MLL*-rearranged ALL cell line SEM.
In vitro cytotoxicity as determined by 4-day MTT assays in the glucocorticoid-resistant *MLL*-rearranged ALL cell line SEM in the absence or presence of 10 or 20 µM of the Src kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d]pyrimidine). Cytotoxic dose-response curves are shown for **A.** prednisolone, **B.** vincristine, **C.** daunorubicine, **D.** L-asparaginase, **E.** cytarabine (Ara-C), **F.** cladribine (2CdA), **G.** dexamethasone. Sensitizing effects of PP2 were only observed for the glucocorticoids *i.e.* prednisolone and dexamethasone, whereas no effects were detectable for the other types of cytotoxic drugs used for the treatment of ALL. **H.** shows the percentage of viable SEM cells after 4 days of culturing in the presence of 10 or 20 µM PP2 alone. All cytotoxic dose-response curves were corrected for PP2 specific cell death and only show normalized effects of the drugs tested.

DISCUSSION

Cellular resistance to the glucocorticoids prednisone and dexamethasone is a major obstacle in reaching successful treatment results for *MLL*-rearranged infant ALL. Glucocorticoids induce apoptosis by releasing Ca^{2+} from the endoplasmic reticulum (ER; Ca^{2+} storage), which was first demonstrated by Kaiser and Edelmann who showed that glucocorticoid-induced lymphocytolysis is correlated with a higher Ca^{2+} influx (18). These findings were later confirmed by McConkey *et al.* who showed that prednisolone-induced apoptosis in thymocytes also depends on the release of free Ca^{2+} into the cytosol (40). Subsequently, free-cytosolic Ca^{2+} migrates towards the mitochondria. Mitochondrial Ca^{2+} uptake results in opening of the permeability transition pore (PTP) and in depolarization of the mitochondrial membrane (36-37). In turn, disruption of the mitochondrial membrane induces apoptosis by the release of cytochrome c and apoptosis-inducing factor (AIF), resulting in activation of the downstream caspase cascade (35, 38). Thus, hindered migration of prednisolone-induced cytosolic Ca^{2+} towards the mitochondria, for example by Ca^{2+}-binding proteins may well cause prednisolone resistance. Here we show that in prednisolone-resistant *MLL*-rearranged infant ALL, high-level expression of *S100A8* and *S100A9* is associated with resistance to glucocorticoid-induced apoptosis. Given the Ca^{2+}-binding capacity of the S100A8/A9 protein complex, it may be tempting to assume that these complexes simply act as Ca^{2+} chelators, binding all or most of the Ca^{2+} ions released into the cytosol. Although the here observed *S100A8* and *S100A9* mRNA levels in primary prednisolone-resistant *MLL*-rearranged infant ALL samples appeared extremely high and comparable to the level of expression of the housekeeping gene *GAPDH*, it remains uncertain and perhaps unlikely that the Ca^{2+}-binding capacity of S100A8/A9 is sufficient to absorb all Ca^{2+} released into the cytosol. Additional or alternative mechanisms may therefore not be excluded. For instance, S100A8/S100A9 may exert direct inhibitory effects on the release mechanisms of cellular Ca^{2+} stores, effectively blocking Ca^{2+} release at its source without the necessity of binding high concentrations of free-cytosolic Ca^{2+}. Since in the present study we measured the levels of free-cytosolic Ca^{2+} in the presence or absence of high-level S100A8/A9 expression, these experiments do not reveal whether cytosolic Ca^{2+} release is blocked before reaching the cytosol.

Since the dismal prognosis of *MLL*-rearranged infant ALL seems tightly linked to cellular resistance to glucocorticoids, unraveling the underlying resistance mechanisms represents an important step toward improved prognosis. Recently, we showed that *MLL*-rearranged infant ALL cells that are *in vitro* resistant to prednisolone express high levels of the anti-apoptotic BCL2 family member *MCL1* (9). Although MCL1 knock-down experiments in prednisolone-resistant *MLL*-rearranged leukemia cells demonstrated a clear but modest induction of prednisolone sensitivity in these cells (9), it remained speculative whether elevated *MCL1* expression embodied the most important mechanism of resistance. Interestingly, Minagawa *et al.* (41) showed how MCL1 obstructs apoptosis by inhibiting mitochondrial Ca^{2+} levels. As such, MCL1 is able to delay Ca^{2+}-induced apoptosis, however, when repetitive Ca^{2+} signals reach certain

thresholds, MCL1 is not able to prevent apoptosis (42). From the perspective of Ca^{2+}-dependence in glucocorticoid-induced apoptosis, high-level MCL1 expression represents a rather downstream line of defense. As shown in the present study, elevated levels of S100A8/S100A9 appear to already inhibit prednisolone-induced Ca^{2+} levels in the cytosol, providing yet another and more upstream defense mechanism against glucocorticoid-induced cell death. If so, high-level *MCL1* expression only becomes relevant in case *S100A8/S100A9* expression is low. Interestingly, we found a strong correlation between *S100A8* and *S100A9* expression, but a correlation between *S100A8/S100A9* and *MCL1* expression appeared completely absent (**Figure 7A**). Supposedly, high-level *MCL1* only becomes protective against prednisolone-induced apoptosis when the levels of *S100A8/S100A9* are not high enough to suppress sufficient amounts of free Ca^{2+} released by the ER in response to glucocorticoid exposure. Emphasizing this, our observation that *MLL*-rearranged infant ALL cells expressing high-levels of *S100A8* and *S100A9* are *in vitro* resistant to prednisolone is regardless of the level *MCL1* expression (**Figure 7B**). Only when the level of *S100A8/S100A9* expression is low, *MCL1* expression levels become discriminative for the *in vitro* prednisolone response (**Figure 7B**). Together these findings explain that, as shown before, high-level *MCL1* expression is to some extent associated with prednisolone resistance (9) but that *MCL1* knock-down or inhibition may not be sufficient to completely reverse the prednisolone-resistant phenotype in *MLL*-rearranged ALL. In contrast to *MLL*-rearranged infant ALL, high-level *S100A8/S100A9* expression appeared largely absent in other types of childhood ALL (Figure 1B), suggesting that the inhibition of MCL1 in prednisolone resistant non-infant pediatric precursor B-ALL cells may be far more effective (9-10).

Furthermore we show that high-level expression of *S100A8* and *S100A9* is not only associated with *in vitro* prednisolone resistance, but also correlates (although not significantly) with poor *in vivo* prednisone responses.

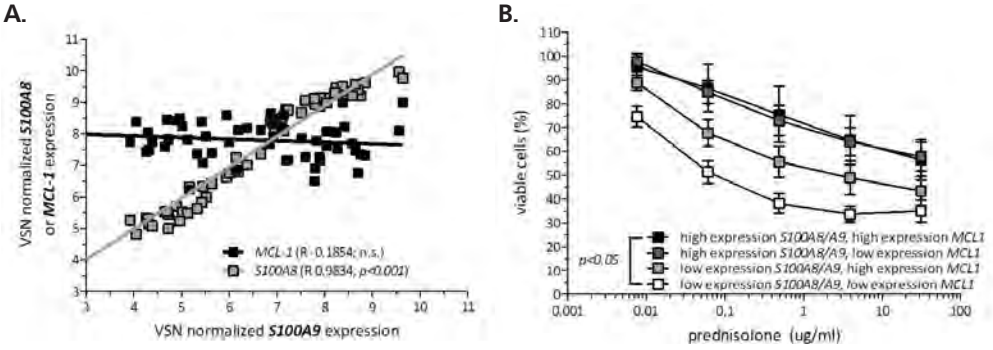

Figure 7. Correlation of *MCL1* to *S100A8/S100A9* in *MLL*-rearranged infant ALL

A. Correlation between VSN-normalized expression (Affymetrix HU133plus2 microarray data) of *S100A9* (x-axes) to *S100A8* or *MCL1* (y-axes) in primary *MLL*-rearranged infant ALL samples (*n*=57). **B.** *In vitro* cytotoxicity for prednisolone as determined by 4-day MTT assays in *MLL*-rearranged infant ALL, expressing high or low levels of *S100A8/S100A9* in combination with either high or low levels of *MCL1*. For each gene the median expression level in the entire patient cohort was used as the cut-off value to distinguish between high or low level expression.

Interestingly, the level of *S100A8/A9* expression appeared a stronger predictor of a poor clinical outcome than the *in vivo* prednisone response. This may either indicate that *S100A8/A9* expression more accurately discriminates between favourable or poor responses to glucocorticoid-based therapies, or implies that that the S100A8/S100A9 protein complex exerts additional functions contributing to the aggressive nature of *MLL*-rearranged ALL. In line with this, low expression of *S100A8* and *S100A9* was recently reported to represent a predictor of a good prognosis in *IDH1/2*-mutated childhood AML patients (31). Similarly, Nicolas *et al.* demonstrated that high-level expression *S100A8* represents poor prognostic marker in *de novo* pediatric AML (30). In concordance, our data shows that high-level *S100A8/S100A9* expression not only correlates with glucocorticoid resistance, but in itself seems to accurately predict an inferior outcome in *MLL*-rearranged infant ALL. As high-level *S100A8/S100A9* expression has been associated with tumor development, invasion, and metastasis in several types of human cancers (43), a role in MLL fusion-driven leukemogenesis might be plausible, at least in a subset of *MLL*-rearranged infant ALL patients.

In any case, the present study demonstrates that the S100A8/S100A9 protein complex represents an attractive target for therapeutic intervention in order to improve the response to glucocorticoids and with that improve the prognosis for *MLL*-rearranged infant ALL. As shown, the Src kinase inhibitor PP2 to some extent targets and down-regulates the S100A8/A9 complex, and sensitizes otherwise resistant *MLL*-rearranged ALL cells to prednisolone. Obviously, PP2 does not represent a highly specific S100A8/S100A9 inhibitor, as the compound was designed to target a broad spectrum of Src kinase family members (44). Additional influences of Src kinase inhibition on prednisolone resistance can therefore not be excluded. Also, S100A8 and S100A9 may not be the only S100 family members targeted by this inhibitor, as PP2 was recently shown to also inhibit S100A4 expression in colon carcinomas (45). From this perspective, the development of more specific S100A8/S100A9 inhibitors that are able to reverse the prednisolone-resistant phenotype in *MLL*-rearranged infant ALL may be desirable. For example, small molecules that prevent the formation of the S100A8/S100A9 heterodimeric complex, or that block the Ca^{2+}-binding sites in these proteins, could potentially diminish its suppressive actions on prednisolone-induced Ca^{2+} release, and allow apoptosis. The development of such compounds represents a potentially attractive challenge providing new therapeutic options for *MLL*-rearranged ALL, and possibly for other human malignancies as well.

REFERENCES

1. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med.* 2004 Apr;350(15):1535-1548.

2. Greaves MF. Infant leukaemia biology, aetiology and treatment. *Leukemia*. 1996 Feb;10(2):372-377.

3. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, *et al.* A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007 Jul;370(9583):240-250.

4. Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, *et al.* Relation between age, immunophenotype and *in vitro* drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants. *Leukemia*. 1998 Sep;12(9):1344-1348.

5. Riehm H, Reiter A, Schrappe M, Berthold F, Dopfer R, Gerein V, *et al.* Corticosteroid-dependent reduction of leukocyte count in blood as a prognostic factor in acute lymphoblastic leukemia in childhood (therapy study ALL-BFM 83). *Klin Padiatr.* 1987 May-Jun;199(3):151-160.

6. Dördelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, *et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*. 1999 Aug;94(4):1209-1217.

7. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, *et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *J Clin Oncol*. 2003 Sep;21(17):3262-3268.

8. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood*. 1998 Jul;92(1):259-266.

9. Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, *et al.* Association of high-level MCL1 expression with *in vitro* and *in vivo* prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia. *Blood*. 2010 Feb;115(5):1018-1025.

10. Wei G, Twomey D, Lamb J, Schlis K, Agarwal J, Stam RW, *et al.* Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. *Cancer Cell*. 2006 Oct;10(4):331-342.

11. Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, *et al.* Gene expression profiling-based dissection of MLL translocated and MLL germline acute lymphoblastic leukemia in infants. *Blood*. 2010 Apr;115(14):2835-2844.

12. Qazi S, Uckun FM. Gene expression profiles of infant acute lymphoblastic leukaemia and its prognostically distinct subsets. *Br J Haematol*. 2010 Jun;149(6):865-873.

13. Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N. Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J Biol Chem*. 1991 Apr;266(12):7706-7713.

14. Strupat K, Rogniaux H, Van Dorsselaer A, Roth J, Vogl T. Calcium-induced noncovalently linked tetramers of MRP8 and MRP14 are confirmed by electrospray ionization-mass analysis. *J Am Soc Mass Spectrom*. 2000 Sep;11(9):780-788.

15. Teigelkamp S, Bhardwaj RS, Roth J, Meinardus-Hager G, Karas M, Sorg C. Calcium-dependent complex assembly of the myeloic differentiation proteins MRP-8 and MRP-14. *J Biol Chem.* 1991 Jul;266(20):13462-13467.

16. Kawasaki H, Nakayama S, Kretsinger RH. Classification and evolution of EF-hand proteins. *Biometals*. 1998 Dec;11(4):277-295.

17. Carafoli E. Calcium signaling: a tale for all seasons. *Proc Natl Acad Sci U S A*. 2002 Feb;99(3):1115-1122.

18. Kaiser N, Edelman IS. Calcium dependence of glucocorticoid-induced lymphocytolysis. *Proc Natl Acad Sci U S A.* 1977 Feb;74(2):638-642.

19. Kaspers GJ, Veerman AJ, Pieters R, Broekema GJ, Huismans DR, Kazemier KM, *et al.* Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. *Br J Cancer.* 1994 Dec;70(6):1047-1052.

20. Pieters R, Loonen AH, Huismans DR, Broekema GJ, Dirven MW, Heyenbrok MW, *et al. In vitro* drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. *Blood.* 1990 Dec;76(11):2327-2336.

21. Stam RW, Schneider P, de Lorenzo P, Valsecchi MG, den Boer ML, Pieters R. Prognostic significance of high-level FLT3 expression in MLL-rearranged infant acute lymphoblastic leukemia. *Blood.* 2007 Oct;110(7):2774-2775.

22. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002;18 Suppl 1:S96-104.

23. Smyth GK. Limma: linear models for microarray data. Edited by: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, eds. Bioinformatics and Computational Biology Solutions Using R and Bioconductor. New York: *Springer*; 2005:397-420.

24. Wettenhall JM, Smyth GK. limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics*. 2004 Dec;20(18):3705-3706.

25. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005 Oct;102(43):15545-15550.

26. Stam RW, den Boer ML, Meijerink JP, Ebus ME, Peters GJ, Noordhuis P, *et al.* Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. *Blood.* 2003 Feb;101(4):1270-1276.

27. Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU, *et al.* H3K79 methylation profiles define murine and human MLL-AF4 leukemias. *Cancer Cell.* 2008 Nov;14(5):355-368.

28. Stam RW, den Boer ML, Passier MM, Janka-Schaub GE, Sallan SE, Armstrong SA, *et al.* Silencing of the tumor suppressor gene FHIT is highly characteristic for MLL gene rearranged infant acute lymphoblastic leukemia. *Leukemia*. 2006 Feb;20(2):264-271.

29. Arai K, Takano S, Teratani T, Ito Y, Yamada T, Nozawa R. S100A8 and S100A9 overexpression is associated with poor pathological parameters in invasive ductal carcinoma of the breast. *Curr Cancer Drug Targets.* 2008 Jun;8(4):243-252.

30. Nicolas E, Ramus C, Berthier S, Arlotto M, Bouamrani A, Lefebvre C, *et al.* Expression of S100A8 in leukemic cells predicts poor survival in de novo AML patients. *Leukemia*. 2011 Jan;25(1):57-65.

31. Damm F, Thol F, Hollink I, Zimmermann M, Reinhardt K, van den Heuvel-Eibrink MM, *et al.* Prevalence and prognostic value of IDH1 and IDH2 mutations in childhood AML: a study of the AML-BFM and DCOG study groups. *Leukemia*. 2011 Nov;25(11):1704-1710.

32. Bootman MD, Collins TJ, Peppiatt CM, Prothero LS, MacKenzie L, De Smet P, *et al.* Calcium signalling--an overview. *Semin Cell Dev Biol.* 2001 Feb;12(1):3-10.

33. Distelhorst CW, Dubyak G. Role of calcium in glucocorticosteroid-induced apoptosis of thymocytes and lymphoma cells: resurrection of old theories by new findings. *Blood*. 1998 Feb;91(3):731-734.

34. Rizzuto R, Pinton P, Ferrari D, Chami M, Szabadkai G, Magalhaes PJ, *et al.* Calcium and apoptosis: facts and hypotheses. *Oncogene*. 2003 Nov;22(53):8619-8627.

35. Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, *et al.* Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med.* 1996 Oct;184(4):1331-1341.

36. Duchen MR. Mitochondria and calcium: from cell signalling to cell death. *J Physiol.* 2000 Nov;529 Pt 1:57-68.

37. Smaili SS, Hsu YT, Youle RJ, Russell JT. Mitochondria in Ca2+ signaling and apoptosis. *J Bioenerg Biomembr.* 2000 Feb;32(1):35-46.

38. Holleman A, den Boer ML, Kazemier KM, Janka-Schaub GE, Pieters R. Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lymphoblastic leukemia. *Blood.* 2003 Dec;102(13):4541-4546.

39. Ryckman C, Gilbert C, de Medicis R, Lussier A, Vandal K, Tessier PA. Monosodium urate monohydrate crystals induce the release of the proinflammatory protein S100A8/A9 from neutrophils. *J Leukoc Biol*. 2004 Aug;76(2):433-440.

40. McConkey DJ, Nicotera P, Hartzell P, Bellomo G, Wyllie AH, Orrenius S. Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca2+ concentration. *Arch Biochem Biophys.* 1989 Feb;269(1):365-370.

41. Minagawa N, Kruglov EA, Dranoff JA, Robert ME, Gores GJ, Nathanson MH. The anti-apoptotic protein MCL1 inhibits mitochondrial Ca2+ signals. *J Biol Chem.* 2005 Sep;280(39):33637-33644.

42. Vrana JA, Bieszczad CK, Cleaveland ES, Ma Y, Park JP, Mohandas TK, *et al.* An MCL1-overexpressing Burkitt lymphoma subline exhibits enhanced survival on exposure to serum deprivation, topoisomerase inhibitors, or staurosporine but remains sensitive to 1-beta-D-arabinofuranosylcytosine. *Cancer Res*. 2002 Feb;62(3):892-900.

43. Srikrishna G. S100A8 and S100A9: New Insights into Their Roles in Malignancy. *J Innate Immun*. 2011 Sep 6. [Epub ahead of print].

44. Kim LC, Song, L. and Haura, E.B. Src kinases as therapeutic targets for cancer. *Nat Rev Clin Oncol.* 2009 Oct;6(10):587-595.

45. Chen M, Sastry SK, O'Connor KL. Src kinase pathway is involved in NFAT5-mediated S100A4 induction by hyperosmotic stress in colon cancer cells. *Am J Physiol Cell Physiol.* 2011 May;300(5):C1155-1163.

CHAPTER 5

Src kinase-induced phosphorylation of Annexin A2 mediates glucocorticoid resistance in *MLL*-rearranged infant acute lymphoblastic leukemia

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ABSTRACT

MLL-rearranged infant ALL (<1 year of age) are frequently resistant to glucocorticoids, like prednisone and dexamethasone. As poor glucocorticoid responses are strongly associated with therapy failure, overcoming glucocorticoid resistance may be a crucial step towards improving prognosis. Unfortunately, the mechanisms underlying glucocorticoid resistance in *MLL*-rearranged ALL largely remain obscure.

We here defined a gene signature that accurately discriminates between prednisolone-resistant and prednisolone-sensitive *MLL*-rearranged infant ALL patient samples, demonstrating that, among other genes, high-level *ANXA2* is associated with prednisolone resistance in this type of leukemia. Further investigation demonstrated that the underlying factor of this association was the presence of Src kinase-induced phosphorylation (activation) of annexin A2, a process requiring the adaptor protein p11 (encoded by human *S100A10*). shRNA-mediated knock-down of either *ANXA2, FYN, LCK*, or *S100A10*, all led to inhibition of annexin A2 phosphorylation and resulted in marked sensitization to prednisolone. Likewise, exposure of prednisolone-resistant *MLL*-rearranged ALL cells to different Src kinase inhibitors exerting high specificity towards FYN and/or LCK had similar effects.

In conclusion, we here present a novel mechanism of prednisolone resistance in *MLL*-rearranged leukemias, and propose that inhibition of annexin A2 phosphorylation embodies a therapeutic strategy for overcoming resistance to glucocorticoids in this highly aggressive type of leukemia.

INTRODUCTION

Glucocorticoids (*e.g.* prednisolone and dexamethasone) are successfully used in the treatment of childhood Acute Lymphoblastic Leukemia (ALL), and represent extremely important agents in current combination chemotherapy regimes. Although most pediatric ALL patients respond well to glucocorticoid-based therapies, some subsets of patients appeared highly resistant to these drugs. A characteristic group of ALL patients highly resistant to glucocorticoids, and as such notoriously difficult to treat, involve infants (<1 year of age) carrying leukemia-specific chromosomal translocations of the *Mixed Lineage Leukemia* (*MLL)* gene. *MLL*-rearranged infant ALL cells are often characterized by cellular resistance to glucocorticoids (1-2), and approximately one-third of all infant ALL cases show a poor *in vivo* response to a 7-day window of prednisone mono-therapy (3). In contrast, among wild-type *MLL* childhood ALL patients, a poor *in vivo* prednisone response is only observed in ~10% of the cases. Currently facing survival chances of at best ~50% (4), this particular group of patients would surely benefit from glucocorticoid-sensitizing treatment strategies. As both the *in vitro* and *in vivo* glucocorticoid responses have been identified as strong prognostic factors (3, 5-6), glucocorticoid resistance remains a major obstacle in reaching optimal treatment results. Unfortunately, the mechanisms underlying glucocorticoid resistance remain largely obscure, which significantly hampers the development of such sensitizing therapies.

In search of insights into glucocorticoid resistance mechanism, we recently compared gene expression profiles from *MLL*-rearranged infant ALL patients either *in vitro* resistant or sensitive to prednisolone (the biologically active metabolite of prednisone). This study demonstrated that the gene expression signatures associated with glucocorticoid resistance in *MLL*-rearranged infant ALL significantly deviate from glucocorticoid-resistance signatures defined in non-infant pediatric precursor B-ALL patients (7). Moreover, our recently published glucocorticoid-resistance signature in *MLL*-rearranged infant ALL was to some extent compromised by a relatively low accuracy, presumably due to the limited number of patient samples (7). Therefore, we here set out to produce a stronger glucocorticoid-resistance profile for *MLL*-rearranged infant ALL samples, which more accurately discriminates between patients either resistant or sensitive to prednisolone *in vitro*. To validate our improved glucocorticoid-resistance profile, we validated one of the genes (*i.e. ANXA2*) prominently over-expressed in prednisolone-resistant *MLL*-rearranged infant ALL patients. *ANXA2*, encoding annexin A2, appeared to be represented in the glucocorticoid-resistance signature by three different probe sets. Annexin A2, a member of the annexin family of calcium-dependent phospholipid-binding proteins, plays important roles in various types of human cancer, including hematopoietic malignancies. For instance, high-level annexin A2 expression has been observed in multiple myeloma cells, in which siRNA-mediated down-regulation leads to impaired proliferation and invasion potential, as well as to increased induction of apoptosis (8). In human breast cancer cells, annexin A2 was markedly up-regulated when cells acquired multi-drug resistance (9). In human prostate cancer, annexin A2 expression is usually absent, but upon loss of androgen dependence annexin A2 is rapidly over-expressed. Inhibiting annexin A2 expression resulted in significant decreases in human prostate tumour growth in mouse models (10). Finally, annexin A2 phosphorylation (activation)

at tyrosine 23 is associated with tumour development and disease progression in pancreatic ductal adenocarcinoma. Interestingly, knock-down of annexin A2, mutations in tyrosine 23, or treatment with anti-ANXA2 antibodies inhibited metastasis and prolonged survival in mouse pancreatic ductal adenocarcinoma models (11).

Based on the above, we studied the role of high-level *ANXA2* expression in *MLL*-rearranged ALL. Our results show that elevated expression of *ANXA2* is associated with increased levels of phosphorylated (activated) annexin A2 protein expression. Annexin A2 phosphorylation is mediated by Src kinases, a process that requires the presence of the adaptor protein p11 (encoded by the *S100A10* gene) (12-14). We here demonstrate that exposing glucocorticoid-resistant *MLL*-rearranged ALL cells to a variety of known Src kinase inhibitors results in glucocorticoid sensitization. Likewise, shRNA-mediated knock-down of annexin A2, the Src kinases FYN and LCK, or the annexin A2-adaptor protein p11, all induce prednisolone sensitivity in otherwise prednisolone-resistant *MLL*-rearranged cells.

MATERIALS AND METHODS

Patient samples and cell line cultures

Bone marrow and/or peripheral blood samples from untreated infants (*i.e.* children <1 year of age) diagnosed with *MLL*-rearranged ALL, were collected at Sophia Children's Hospital (Rotterdam, the Netherlands) as part of the international collaborative INTERFANT treatment protocol (4). Approval for these studies was obtained from the Erasmus MC Institutional Review Board. Informed consent was obtained according to the Declaration of Helsinki. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation, and contaminating non-leukemic cells removed using immunomagnetic beads as described before (15). All samples used in this study contained more than 90% of leukemic blasts.

The *MLL*-rearranged ALL cell lines SEM, KOPN8, RS4;11, and BEL-1 were maintained as suspension cultures in RPMI 1640 supplemented with glutamax (Invitrogen, Life Technologies, Breda, the Netherlands), 10% (v/v) fetal calf serum (FCS), and 1% penicillin/streptomycin/fungizone (PSF; Invitrogen, Life Technologies, Breda, the Netherlands) at 37^{o}C in humidified air containing 5% CO_2. SEM, BEL-1, and RS4;11 all carry translocation t(4;11) generating MLL-AF4 fusion proteins. KOPN8 is a t(11;19)-positive ALL line expressing the MLL-ENL fusion. Both SEM and KOPN8 are resistant to prednisolone *in vitro*, whereas RS4;11 and BEL-1 are highly sensitive.

In vitro prednisolone and *in vivo* prednisone response

The *in vitro* prednisolone response was assessed by 4-day MTT assays as described elsewhere (16). Patient samples were characterized as prednisolone sensitive or resistant based on the LC_{50} value (*i.e.* the concentration of prednisolone lethal to 50% of the leukemic cells). Samples were typified as prednisolone-sensitive at LC_{50} values <0.1 µg/mL and prednisolone-resistant at LC_{50} values >150 µg/mL.

The *in vivo* response to prednisolone was determined after a 7-day window of prednisone monotherapy (before the initiation of combination chemotherapy). Patients were defined as

prednisone poor responder (PPR) when >1000 leukemic blasts/μL remained present in the peripheral blood at day 7. When the amount of leukemic blasts dropped below 1000/μL patients were defined as prednisone good responder (PGR).

Microarray data analysis

A gene expression signature associated with prednisolone-resistance in *MLL*-rearranged infant ALL was produced using a selection of recently generated expression profiles (Affymetrix HU133plus2.0 GeneChips), deposited in the NCBI Gene Expression Omnibus under the GEO Series accession number GSE32962 (17). Differential gene expression between prednisolone-resistant and prednisolone-sensitive patient samples were statistically evaluated using linear models for microarray analyses (LIMMA). Differences in gene expression were considered significant at P values <0.05 (adjusted for multiple testing by the False-discovery rate (FDR) according to the step-up procedure of Benjamini and Hochberg (18)). Heatmaps were generated in GenePattern (19) and graphical representations of principal component analysis (PCA) were produced using the GeneMath XT 1.6.1. software (Applied Maths).

***In vitro* assessment of the prednisolone response and sensitization**

In vitro prednisolone cytotoxicity was determined by 4-day MTT assays as described before (16). Briefly, leukemic cells were cultured in 96-well plates in the presence of increasing concentrations of prednisolone (Bufa, Uitgeest, The Netherlands) in duplicate. Controls were cultured in the absence of prednisolone, and wells containing culture medium only were used as blanks. After 4-day incubation periods at 37°C in humidified air containing 5% CO_2, 10 μL MTT (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added and plates were incubated for an additional 6 hours during which the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals by viable cells only. Next, formazan crystals were dissolved and the optical density, which is linearly related to the number of viable cells, was measured on a spectrophotometer. Assay results were deemed successful when a minimum of 70% leukemic cells remained present in the control wells after 4 days of culturing.

Prednisolone-sensitizing effects were determined by performing conventional MTT assays, either in the absence or presence of indicated Src kinase inhibitors. In all instances, observed prednisolone-sensitization was normalized against the effects of the co-administered agent by culturing control cells (not exposed to prednisolone) in the presence of these agents at similar concentrations. The Src kinase inhibitors used include: Lavendustin A, Piceatannol, PP1 (1-(1,1-Dimethylethyl)-1-(4-methylphenyl)-1*H*-pyrazolo[3,4pyrimidin-4-amine), PP2 (3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine), SKI-606 (bosutinib), Herbamycin A ((15*R*)-17-demethoxy-15-methoxy-11-*O*-methyl-geldanamycin), MNS (3,4-Methylenedioxy-β-nitrostyrene), Dasatinib, Imatinib (Glivec), SU6656, AZD0530. PP3 (1-Phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine), an inactive molecule structurally related to PP2 was used as a negative control. All drugs were obtained from Tocris Bioscience (Missouri, USA).

Quantification of *ANXA2* mRNA expression levels using quantitative RT-PCR

Total RNA was extracted from a minimum of $5x10^6$ leukemic cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified on a spectrophotometer. Next, RNA was reverse transcribed and the obtained cDNA was used to quantify *ANXA2* mRNA expression relative to the housekeeping gene *B2M* using quantitative RT-PCR analysis. For this, PCR products were amplified using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) according to the manufacturer's recommendations, using SYBR Green as a fluorophore to detect transcripts on an ABI Prism 7900 sequence detection system (Applied Biosystems). Oligonucleotide primers used for PCR amplification were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO) and purchased from Eurogentec (Seraing, Belgium). Primer sequences for *ANXA2* were as follows: forward primer 5'-GTGGGCATCATGTTGAC-3' and reverse primer 5'-TGCACCATCAGTGTTGATA-3'. *B2M* primer sequences were: forward primer 5'-GGAGCAATCAGACTTGTCTT-3' and reverse primer 5'-ATGCGGCATCTTCAAA-3'. For each sample, assessment of the *ANXA2* mRNA expression levels was carried out in duplicate.

Assessment of protein expression by immunoblot analysis

Annexin A2, FYN, LCK, and p11 (S100A10) protein expression were determined by Western Blot analysis, essentially as described before(20). Briefly, total cellular protein lysates were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleichler & Schuell, Dassel, Germany). Membranes were probed with rabbit polyclonal anti-annexin A2 (Abcam, Cambridge, UK), mouse monoclonal anti-phospho-annexin A2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-FYN (Epitomics Inc., Burlingame, CA, USA), goat polyclonal anti-phospho-FYN (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-LCK (Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-phospho-LCK (Abnova, Taipei City, Taiwan), or mouse monoclonal anti-S100A10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Next, membranes were counterstained with IRDye® 680/800 labelled secondary antibodies, and the target proteins were visualized using the Odyssey imaging system (LICOR biotechnology, Lincoln, NE, USA). All membranes were re-probed with mouse monoclonal anti-β-actin antibodies (Sigma-Aldrich, St. Louis, MO, USA) to assure equal protein loading between lanes.

shRNA-mediated RNA interference

In order to knock-down *ANXA2*, *FYN*, *LCK,* or *S100A10* expression by means of RNA interference, SEM cells were virally infected by shRNA-expressing pLKO.1 vectors obtained from Sigma (St. Louis, MO, USA). shRNA target sequences were 5'-CGG GAT GCT TTG AAC ATT GAA-3' for *ANXA2*, 5'-CTT ACC GAT CTG TCT GTC AAA-3' for *FYN,* 5'-GCA CAC ATC AGG AGT TCA ATA-3' for *LCK*, and 5'-CCA TGA TGT TTA CAT TTC ACA-3' for *S100A10*. A similar vector expressing a non-human targeting shRNA was used as a none-silencing control. Virus particles were produced by transient transfection of 293T cells with a mixture of psPAX-2, pMD2G-VSVG and pLKO.1 (3:1:4). Medium was refreshed after 24 hours, and virus-containing

medium was harvested at 48 hours following transfection. Upon filtration through 0.45 μm cellulose acetate filters, and the obtained virus stock was used to infect SEM cells. To produce pure populations of shRNA expressing SEM cells, infected cells were selected with puromycin (1 μg/ml), which is lethal to uninfected cells. Next, annexin A2, FYN, LCK and p11 (S100A10) protein expression was evaluated by immunoblotting to confirm successful knock-down. In addition, the *in vitro* prednisolone sensitivity in these cells was determined by 4-day MTT assays performed in triplicate as described above.

BrdU cell proliferation assay and cell viability.

Cell proliferation was measured using the FITC BrdU Flow Kit (BD Biosciences, San Diego, CA, USA) following the manufacturer's instruction manual. For this, a total of $1x10^6$ SEM cells were incubated with 10 μM BrdU for 45 minutes. Next, cells were fixated and treated with DNase to expose BrdU epitopes, and subsequently incubated with FITC-conjugated anti-BrdU antibodies. In parallel, total DNA was stained with 7-AAD. This staining combination permits flow cytometric evaluation of actively synthesizing DNA (BrdU incorporation) in terms of cell cycle phases (G0/1, S, or G2/M) defined by 7-AAD intensities. These analyses were performed using a FACSCalibur flow cytometer (Becton Dickinson, USA). In addition, trypan blue exclusion was used to microscopically monitor cell expansion of viable cells. For this, the percentage of viable cells at a given time point was derived from the number of viable cells per mL divided by the total amount of cells per mL present in the samples.

Free-cytosolic calcium measurement

Free-cytosolic calcium was stained using Calcium Orange-1 (Invitrogen, Life Technologies, Breda, The Netherlands) and quantified on a flow cytometer (Facs Calibur, Becton Dickinson, USA). For this, leukemic cells were washed twice in phosphate-buffered saline (PBS) and incubated with 2 μM of Calcium Orange-1 for 1 hour at RT in the dark. Prior to detection by flow cytometry, incubated cells were washed twice in PBS to exclude aspecific binding Calcium Orange-1.

Statistical analysis

Statistical significance in differential *ANXA2* expression was determined using the Mann-Whitney *U*-test. The Kruskall-Wallis test was performed to evaluate differences in *in vitro* prednisolone response between samples expressing high and low levels of either *ANXA2* or *S100A10*. Spearman's Rho test was used to assess the correlation between *ANXA2* and *S100A10* expression. The student *t*-test was used to analyze differences in mean cytotoxicity responses. All analyses were 2-tailed, and differences were considered statistically significant at *P* values <0.05. Event-free survival (EFS) curves were generated using the Kaplan-Meier method and analyzed by Log-rank (Mantel-Cox) tests. Multivariate analysis was performed using a Cox regression model based on the EFS, and the Wald Backward Test was used for the joint analysis of the *in vitro* prednisolone response, *ANXA2* and *S100A10* expression.

RESULTS

Establishing a prednisolone-resistance expression signature for *MLL*-rearranged infant ALL

We here generated a gene expression signature associated with *in vitro* prednisolone resistance, by comparing gene expression profiles (GEPs; Affymetrix HU133plus2.0 GeneChips) obtained from prednisolone-resistant patients (LC_{50} values >150 µg/mL; *n*=24) with profiles from prednisolone-sensitive patients (LC_{50} values <0.1 µg/mL; *n*=19). LIMMA (Linear Models for Microarray data) analyses revealed differential expression (FDR <0.05) of 74 probe sets, corresponding to 66 genes (**Figure 1A**). The level of separation between resistant and sensitive patients was explored using principle component analysis (PCA). This approach revealed a separation, but the presented gene signature was not able to completely isolate both patient groups: some degree of overlap remained present (**Figure 1B**). In order to evaluate the relevance of this *in vitro* prednisolone response signature in relation to *in vivo* prednisone responses, prednisolone LC_{50} values obtained *in vitro* were compared with *in vivo* responses established after 7 days of prednisone mono-therapy. As shown in **Figure 1C**, prednisone poor responders (PPRs) are significantly more resistant to prednisolone *in vitro* as compared to prednisone good responders (PGRs) *in vivo* (Mann Whitney *U*-test *p=0.005*) .

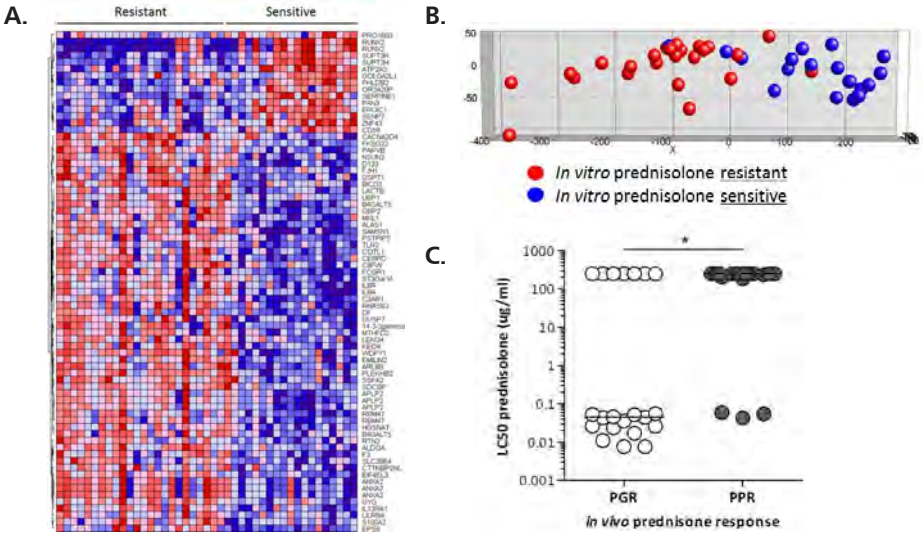


Figure 1. Establishing a prednisolone-resistance gene expression signature in *MLL*-rearranged infant ALL

A. Heatmap showing 74 probe sets (HU133plus2.0 Affymetrix GeneChips) corresponding to 66 genes differentially expressed (FDR <0.05) between prednisolone-resistant (*n*=24) and prednisolone-sensitive (*n*=19) *MLL*-rearranged infant ALL samples. Columns represent individual patient samples, and the rows represent single probe sets corresponding to the genes listed on the right side of the heatmap. Normalized gene expression is depicted in red (high expression) or blue (low expression). **B.** Shows the Principle Component Analysis (PCA) corresponding to prednisolone-resistance signature (74 probe sets), visualizing the degree of separation between prednisolone-resistant (red) and prednisolone-sensitive (blue) patient samples. **C.** Distribution of the *in vitro* prednisolone response depicted as LC_{50} values (concentrations of prednisolone lethal to 50% of the leukemic cells) between *in vivo* prednisone good responders (PGRs) and prednisone poor responders (PPRs). * Indicate significant differences with *p*-values of *<0.05*.

Relation between annexin A2 expression, phosphorylation, and resistance to prednisolone in *MLL*-rearranged infant ALL

One of the genes highly expressed in our *MLL*-rearranged infant ALL prednisolone-resistance signature represented by three different probe sets (*i.e.* 213503_x_at, 210427_x_at, and 201590_x_at) is *ANXA2*, encoding human annexin A2 (**Figure 1A**). For all probe sets significant higher expression was found in prednisolone-resistant *MLL*-rearranged infant ALL patient samples ($p<0.001$; **Figure 2A**). Moreover, *ANXA2* expression also appeared significantly higher in *MLL*-rearranged infant ALL samples when compared to infant ALL patients without *MLL* translocations or non-infant pediatric (>1 year of age) ALL cases ($p<0.05$; **Figure 2B**). Moreover, among non-infant pediatric precursor BCP-ALL or T-cell ALL patients, no differences in *ANXA2* expression was observed between prednisolone-sensitive and prednisolon-resistant cases (**Figure 2C** and **2D**).

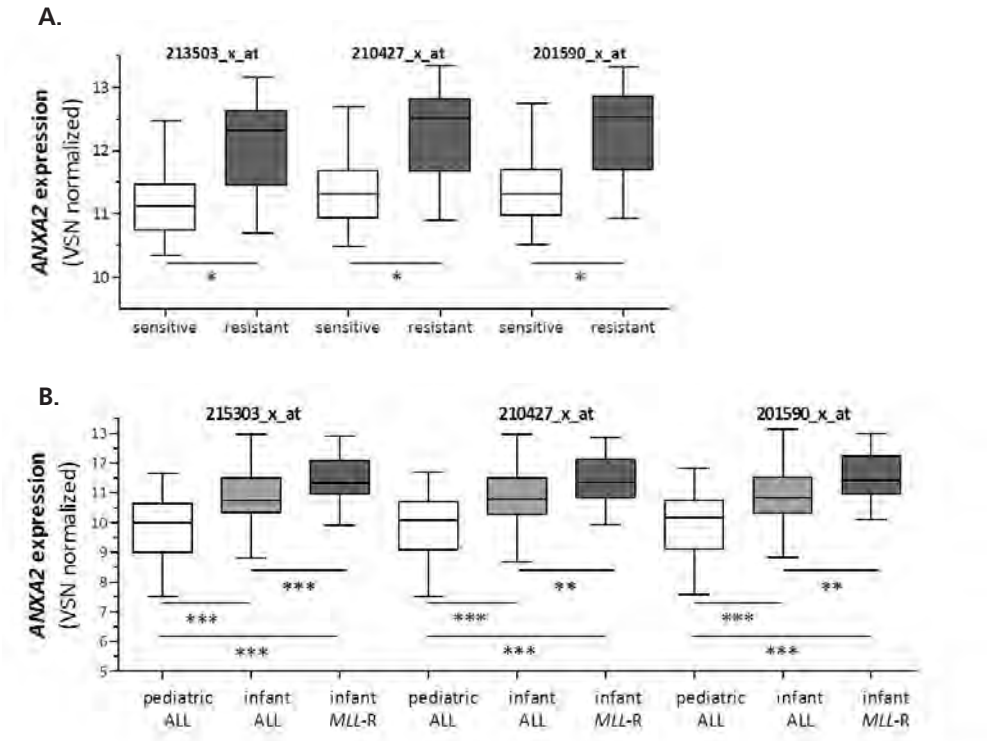


Figure 2. Distribution of annexin A2 expression in infant and pediatric ALL

VSN-normalized gene expression of *ANXA2* of three different probe sets (213503_x_at, 210427_x_at and 201590_x_at) in **A.** *MLL*-rearranged infant ALL patient samples either *in vitro* resistant or sensitive to prednisolone; **B**. *MLL*-rearranged infant ALL patient samples display high-level expression of *ANXA2* mRNA when compared to either infant ALL (without *MLL* translocations) or pediatric ALL (>1 year of age); * Indicate significant differences with *p*-values of *<0.005, **p<0.001 and ***p<0.0001*

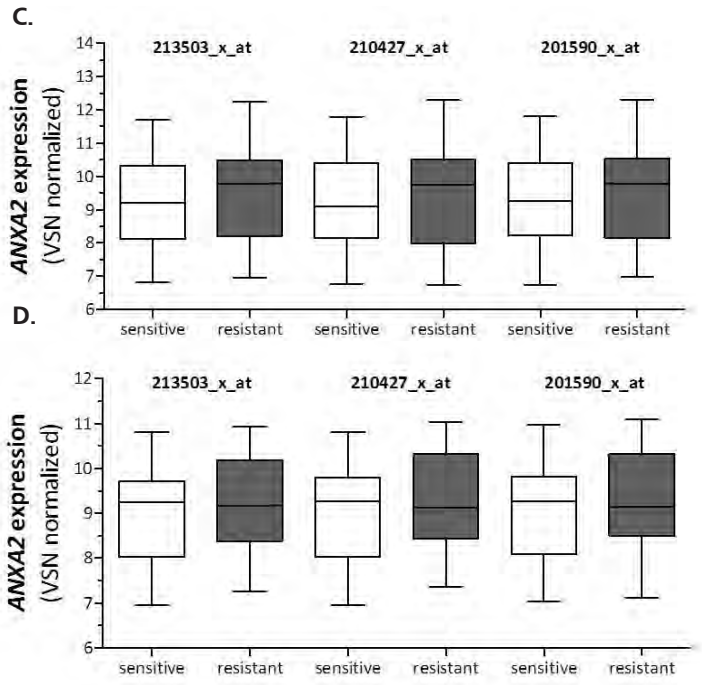


Figure 2. Distribution of annexin A2 expression in infant and pediatric ALL
VSN-normalized gene expression of *ANXA2* of three different probe sets (213503_x_at, 210427_x_at and 201590_x_at) in **C.** pediatric precursor BCP-ALL or **D**. pediatric T-cell ALL patient samples either *in vitro* resistant or sensitive to prednisolone. In both patient groups, no significant differences of *ANXA2* mRNA expression was observed when comparing prednisolone-resistant with prednisolone-sensitive patients.

Using quantitative RT-PCR analysis, we validated high-level *ANXA2* mRNA expression in prednisolone-sensitive (RS4;11 and BEL-1) and prednisolone-resistant (SEM and KOPN8) *MLL*-rearranged ALL cells lines. The prednisolone response curves for these cell lines are presented in **Figure 3A**. As shown in **Figure 3B**, prednisolone-resistant cell lines significantly expressed higher levels of *ANXA2* mRNA as compared to the prednisolone-sensitive cell lines ($p<0.05$). Next, we analyzed whether high-level expression of *ANXA2* mRNA also translates into increased levels of annexin A2 protein expression, and elevated annexin A2 phosphorylation (activation). As depicted in **Figure 3C**, prednisolone-resistant *MLL*-rearranged ALL cell lines clearly showed increased annexin A2 protein expression, as well as marked annexin A2 phosphorylation (especially in SEM cells). Annexin A2 phosphorylation was largely absent in the prednisolone-sensitive *MLL*-rearranged ALL cell lines (**Figure 3C**). Similar results were obtained for primary *MLL*-rearranged infant ALL patient samples. Prednisolone-resistant patient samples (n=5) significantly higher levels of *ANXA2* mRNA as compared to prednisolone-sensitive samples (n=5) ($p<0.01$; **Figure 3D**). Annexin A2 protein levels only appeared marginally higher in prednisolone-resistant samples, yet these samples evidently displayed increased levels of annexin A2 phosphorylation (**Figure 3E**).

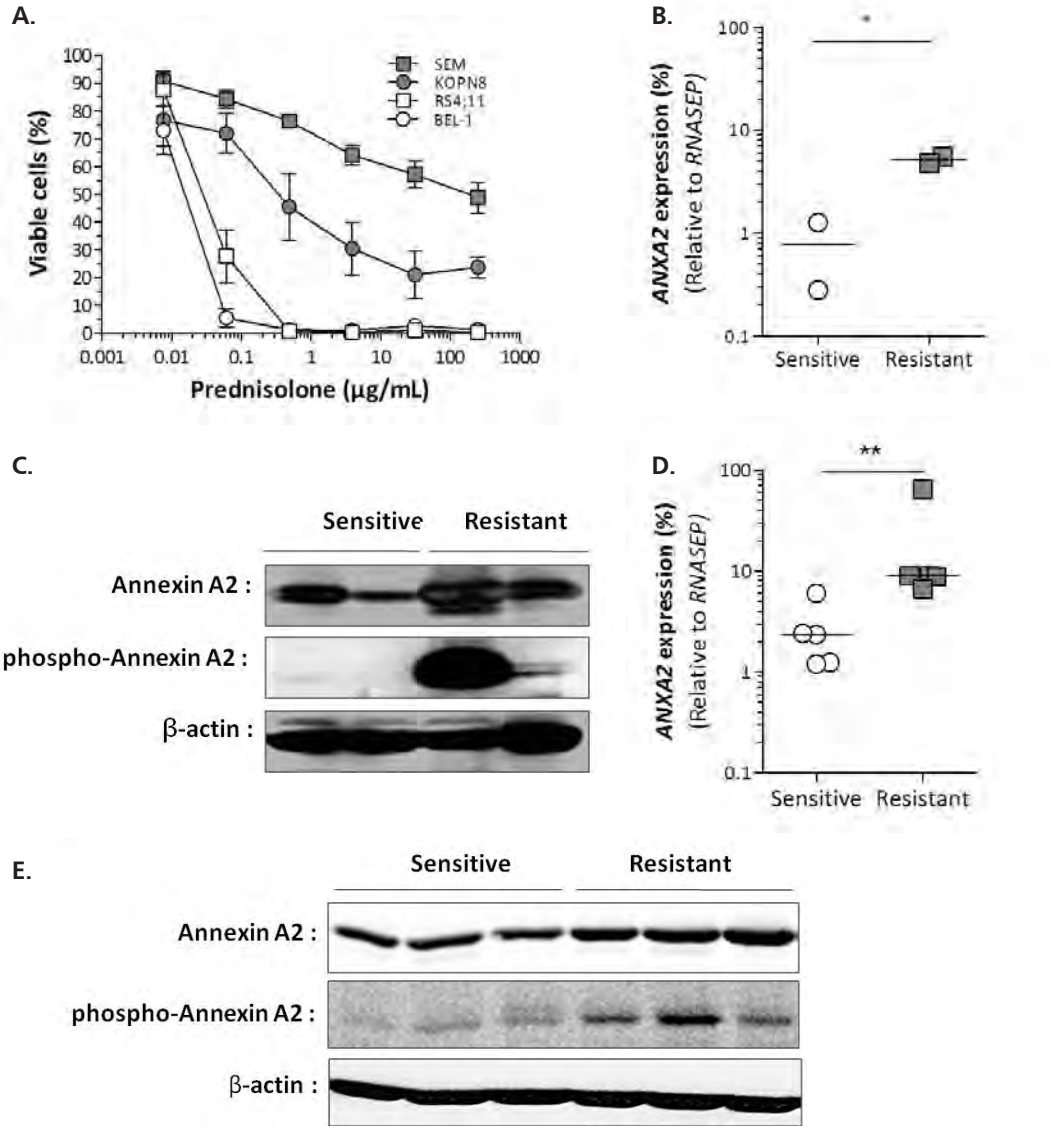
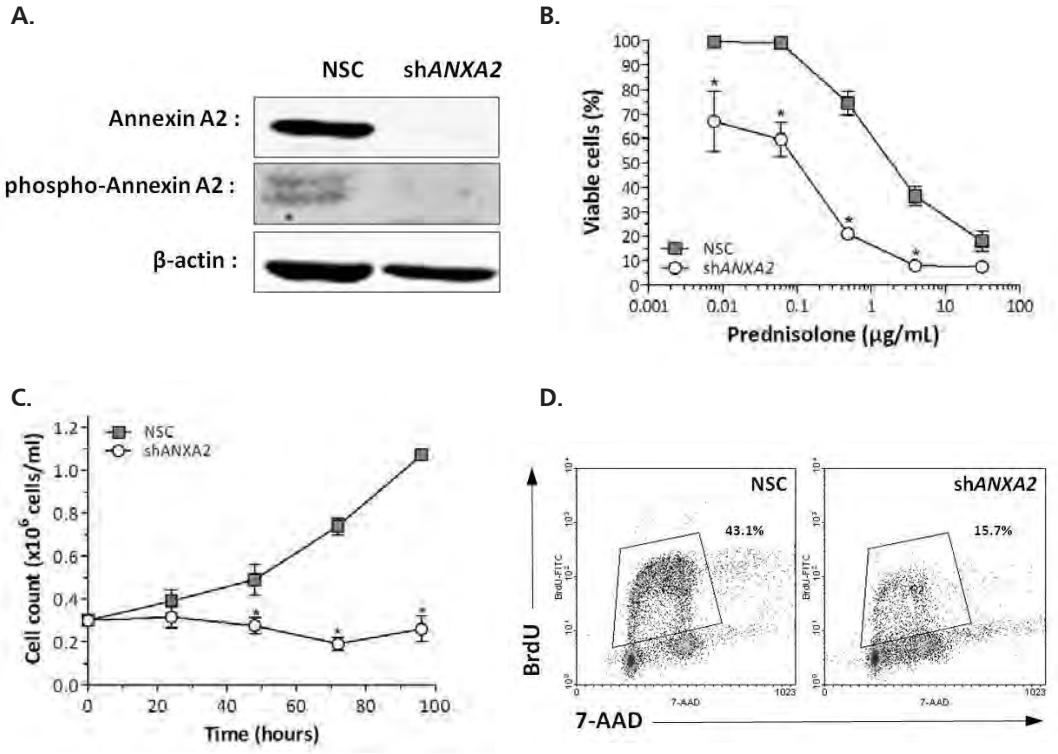


Figure 3. High-level *ANXA2* expression confers resistance to prednisolone in *MLL*-rearranged infant ALL.

A. *In vitro* prednisolone response curves for the *MLL*-rearranged ALL cell line models either sensitive (RS4;11 and BEL-1) or resistant (SEM and KOPN8) to prednisolone as determined by 4-day MTT assays. In these cell lines **B.** *ANXA2* mRNA levels were determined by quantitative RT-PCR analysis, and **C.** total and phosphorylated annexin A2 as well as annexin A2 phosphorylation was determined by Western Blot analysis. **D.** Similarly, *ANXA2* mRNA levels were determined in primary *MLL*-rearranged infant ALL patient samples either sensitive (n=5) or resistant to prednisolone (n=5) *in vitro*. **E.** Shows annexin A2 and phospho-annexin A2 protein levels in prednisolone-resistant (n=3) and prednisolone-sensitive (n=3) *MLL*-rearranged infant ALL patient samples as determined by Western Blot analysis. * Indicate significant differences with p-values of <0.05, ** indicate significant differences with p-values of <0.01.

shRNA-mediated annexin A2 knock-down blocks proliferation and induces prednisolone sensitivity

To further investigate the relation of high-level *ANXA2* expression, annexin A2 phosphorylation, and prednisolone resistance, we performed *ANXA2* knock-down experiments in a *MLL*-rearranged ALL cell line model. For this, prednisolone resistant SEM cells were transduced with lentiviral constructs stably expressing shRNAs against human *ANXA2* mRNA, or with vectors expressing non-targeting shRNAs as a non-silencing control (NSC). Western blot analysis confirmed successful knock-down of the target protein (**Figure 4A**). Compared to the NSC, SEM cells challenged with anti-*ANXA2* shRNAs appeared to have almost complete loss of both total and phosphorylated annexin A2 expression (**Figure 4A**). As anticipated, down-regulation of annexin A2 sensitized resistant SEM cells ~20-fold to prednisolone (**Figure 4B**). Intriguingly, loss of annexin A2 expression strongly inhibited leukemic cell proliferation (**Figure 4C**). A BrdU-based proliferation assay showed a near 30% S-phase reduction in SEM cells in which annexin A2 was suppressed (**Figure 4D**). The notion that annexin A2 down-regulation leads to impaired proliferation is completely in line with earlier observations (8-9, 11).



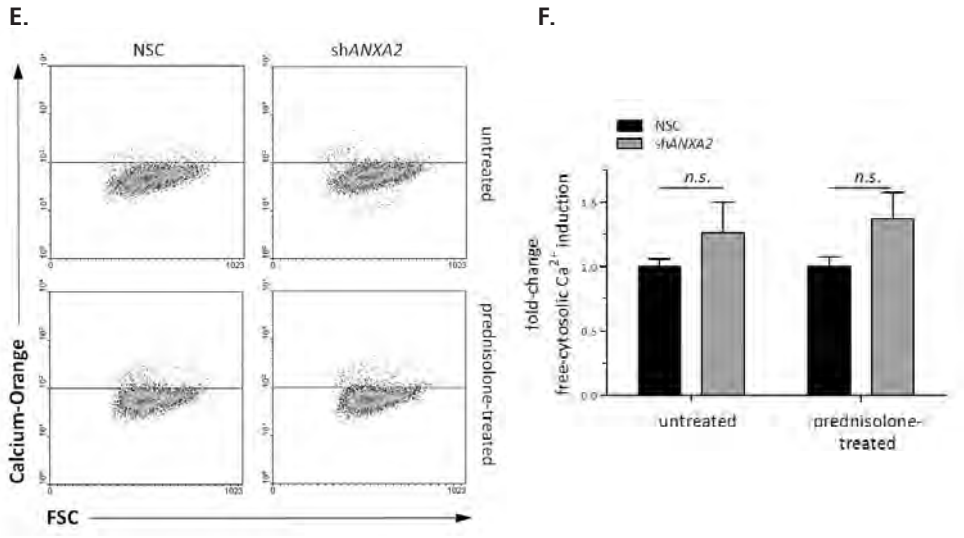
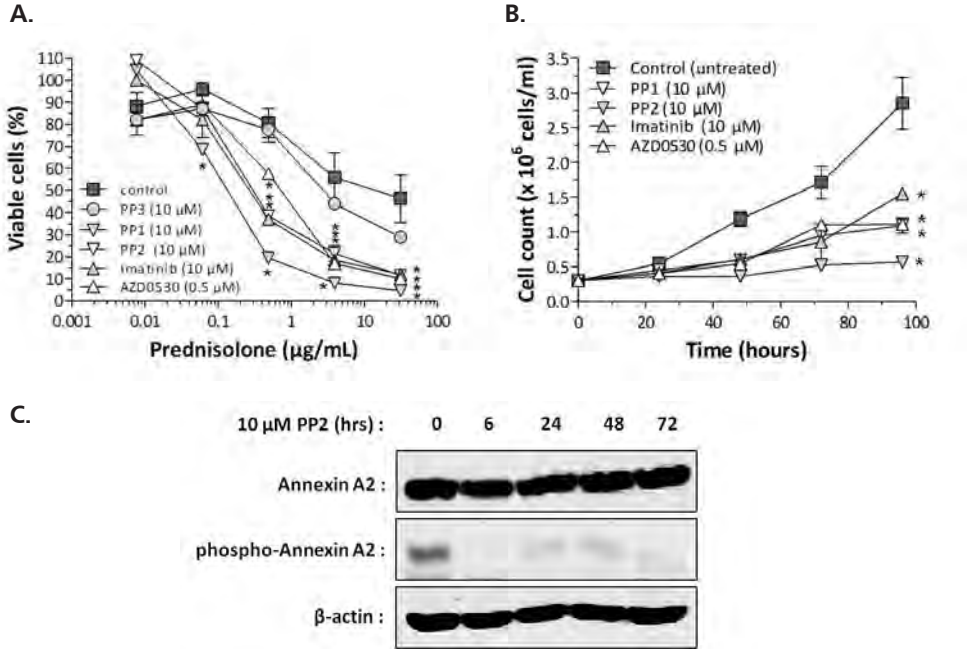


Figure 4. Knock-down of annexin A2 inhibits cell proliferation and induces prednisolone sensitivity in *MLL*-rearranged ALL cells, without affecting cytosolic Ca^{2+}
A. Western Blot analysis showing protein expression of total and phosphorylated annexin A2 in the *MLL*-rearranged ALL cell line SEM challenged with shRNAs directed against *ANXA2* mRNA or non-silencing control (NSC) shRNAs. **B.** *In vitro* prednisolone response (as determined by 4-day MTT assays) for SEM cells upon annexin A2 knock-down, compared to cells transduced with NSC shRNAs. **C.** Shows viable cell counts at indicated time points in SEM cells displaying knocked-down annexin A2, or NSC cells. **D.** The same cells were subjected to BrdU/7-AAD staining and subsequently analyzed by flow cytometry, in order to more accurately confirm differences in cell expansion. The selected regions (skewed squares) represent cells residing in the S-phase of the cell cycle. All experiments were performed in triplicate. * Indicate significant differences with *p*-values of *<0.05*.**E.** Measurement of free-cytosolic Ca^{2+} in SEM cells transduced with either NSC or shRNAs directed against *ANXA2*, in the presence or absence of prednisolone (50 µg/ml; 4 hours). **F.** Depicts the calculated ratios of the prednisolone-induced free-cytosolic Ca^{2+} in NSC-transduced or sh*ANXA2*-transduced SEM cells, with or without prednisolone (50 µg/ml; 4 hours). This figure demonstrates no prednisolone-induced Ca^{2+} release into the cytosol, suggesting that the prednisolone resistance mechanism caused by annexin A2 is independent of the calcium released from the endoplasmic reticulum by prednisolone.

Recently, we demonstrated that high-level expression of the calcium-binding protein complex S100A8/A9 blocks glucocorticoid-induced Ca^{2+} release form the endoplasmic reticulum into the cytosol, and with that inhibits prednisolone-induced apoptosis (17). To determine whether the calcium-binding protein annexin A2 maintains prednisolone resistance via a similar mechanism, we challenged sh*ANXA2*-transduced SEM cells, as well as NSC-transduced cells, to prednisolone (50 µg/ml) and determined the levels of free-cytosolic Ca^{2+}. Interestingly, knock-down of annexin A2 did not lead to increased levels of free-cytosolic Ca^{2+} upon exposure to prednisolone (**Figure 4E-F**). Thus, in contrast to high-level S100A8/A9, phosphorylated annexin A2 mediates resistance to prednisolone independent of prednisolone-induced free-cytosolic Ca^{2+}.

Src kinase inhibition blocks annexin A2 phosphorylation and induces prednisolone sensitivity

Although high-level *ANXA2* expression is associated with prednisolone resistance in *MLL*-rearranged ALL, the actual factor conferring resistance may in fact be the presence of phosphorylated annexin A2. Phosphorylation of annexin A2 is mediated by Src kinases, a process that requires the inducible adaptor protein p11 (encoded by the *S100A10* gene) (12-14). Thus theoretically, Src kinase inhibition should block annexin A2 phosphorylation and by that induce prednisolone sensitivity. As the Src kinase family comprises multiple members (including SRC, LCK, HCK, FYN, YES, FGR, BLK, LYN, and FRK) with varying substrate specificities, we tested the effects of a broad spectrum of known Src kinase inhibitors on the *in vitro* prednisolone response in resistant SEM cells. The used inhibitors included PP1, PP2, AZD0530, dasatinib, imatinib, herbamycin A, lavendustin A, piceatannol, SU6656, and SKI-606, exerting varying specificity towards different members of the Src kinase family (21-25). As shown in **Figure 5A**, only a few of the inhibitors, *i.e.* PP1, PP2, imatinib and AZD0530 significantly sensitized SEM cell to prednisolone, while the other agents had little or no effect. As expected, PP3, which is structurally comparable to PP2 but represents an inactive agent (commonly used as a negative control for PP2), did not induce prednisolone sensitivity in contrast to PP2 (**Figure 5A**). Interestingly, like we observed in our annexin A2 knock-down experiments, all Src kinase inhibitors that were able to induce prednisolone sensitivity also inhibited cell proliferation (**Figure 5B**). Although we recently reported that the Src kinase inhibitor PP2 induces prednisolone sensitization by inhibition of S100A8 and S100A9 (17), we here also investigated whether PP2 influences annexin A2 expression. To test this, we incubated SEM cells with PP2 for indicated time points and determined total and phosphorylated annexin A2 protein expression.



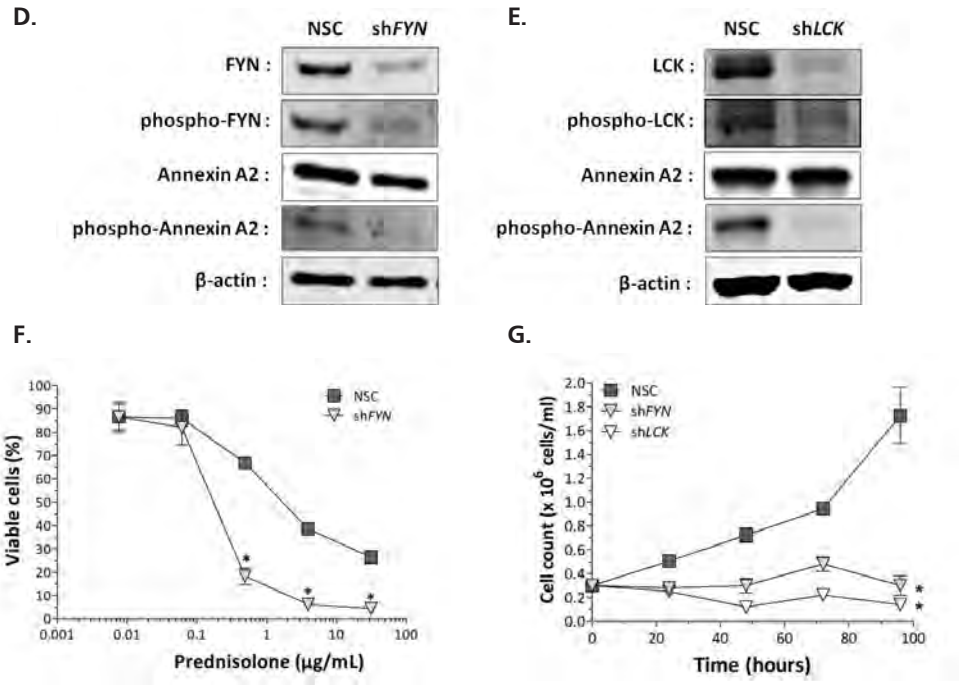


Figure 5. Src kinase inhibition induces prednisolone sensitivity in otherwise resistant *MLL*-rearranged ALL cells.
A. *In vitro* prednisolone responses (4-day MTT assays) in the *MLL*-rearranged ALL cell line model SEM in the absence or presence of the Src kinase inhibitors PP1 (10 µM), PP2 (10 µM), PP3 (inactive molecule; 10 µM), imatinib (10 µM), or AZD0530 (0.5 µM). In all instances, observed prednisolone-sensitization was normalized against Src kinase inhibitor-specific cytotoxicity. **B.** Shows viable cell counts of SEM cells in the absence or presence of indicated Src kinase inhibitors, at indicated time points. **C.** Shows Western Blot analysis of total and phosphorylated annexin A2 in SEM cells upon exposure to PP2 (10 µM) at indicated time points. **D.** Western blot analysis detecting FYN, LCK, total annexin A2, and phosphorylated annexin A2 protein expression in SEM cells challenged with shRNAs directed against *FYN* or *LCK*. Control cells were transduced with non-silencing control (NSC) shRNAs. **E.** *In vitro* prednisolone response (as determined by 4-day MTT assays) for SEM cells upon FYN knock-down, compared to cells transduced with NSC shRNAs. **F.** Shows viable cell counts at indicated time points in SEM cells displaying knocked-down FYN or LCK, or in SEM cells transduced with NSC shRNAs. All experiments were performed in triplicate. * Indicate significant differences with *p*-values of *<0.05*.

Interestingly, in addition to inhibiting S100A8/S100A9 (17), PP2 clearly inhibited annexin A2 phosphorylation (**Figure 5C**). Apart from the fact that these results demonstrate that several Src kinase inhibitors are indeed capable of inducing prednisolone sensitivity in *MLL*-rearranged ALL, these data may also point out which of the Src kinases play prominent roles in annexin A2 phosphorylation. As the prednisolone-sensitizing Src inhibitors PP1 and PP2 effectively inhibit both FYN and LCK (21-22), these may well represent the key kinases that phosphorylate annexin A2. To further investigate whether these Src kinases are indeed important in prednisolone resistance in *MLL*-rearranged ALL, we separately knocked-down FYN and LCK expression using shRNA-mediated RNA interference in prednisolone-resistant SEM cells.

Western blot analyses showed that compared to the NSC, anti-*FYN* and anti-*LCK* shRNAs markedly down-regulated FYN and LCK protein expression respectively, and inhibited annexin A2 phosphorylation (**Figure 5D**). As a result of FYN and LCK suppression, total annexin A2 expression appeared largely unaffected, whereas phosphorylated annexin A2 was almost completely down-regulated (**Figure 5D**). Subsequently, we asked whether FYN and LCK down-regulation would also sensitize *MLL*-rearranged ALL cells to prednisolone. Unfortunately, no reliable assay results could be obtained for sh*LCK*, as leukemic cell survival in SEM cells in which sh*LCK*-transduced cells was poor, and too few cells survived four days of culturing required to determine the *in vitro* prednisolone response. However, knock-down of FYN clearly led to prednisolone sensitization (**Figure 5E**). Again, knock-down of FYN and LCK was accompanied by impaired leukemic cell proliferation (**Figure 5F**), supposedly due to the loss of phosphorylated annexin A2.

Relevance of p11 (*S100A10*) in Src kinase induced annexin A2 phosphorylation and prednisolone resistance in *MLL*-rearranged infant ALL

As mentioned above, Src kinase induced phosphorylation of annexin A2 requires the presence of the adapter protein p11 (encoded by *S100A10*) (13). At the same time, p11 requires partnering with annexin A2 to become stable and protected from proteosome-dependent degradation (13). Interestingly, in our recently published *MLL*-rearranged infant ALL gene expression profiling study, we found *S100A10* to be highly expressed in all *MLL*-rearranged infant ALL patients (26). In fact, *S100A10* expression appeared among the top 50 of most significant genes discriminating between *MLL*-rearranged infant ALL and pediatric precursor B-ALL (26). Although these data only reflects mRNA expression, which does not necessarily predict the levels of p11 protein expression, we attempted to verify the importance of *S100A10* expression regarding prednisolone resistance. First, we determined the relation between VSN-normalized *ANXA2* and *S100A10* expression (HU133plus2.0 Affymetrix microarray data), and found a positive correlation (Spearman's Rho-test=0.682, $p<0.0001$) (**Figure 6A**). Next, to test the importance of *S100A10* regarding prednisolone resistance, we divided our *MLL*-rearranged infant ALL patient samples into four groups based on the levels *ANXA2* and *S100A10* expression as follows: I. samples expressing low *ANXA2* and low *S100A10*, II. samples expressing high *ANXA2* but low *S100A10*, III. low *ANXA2* but high *S100A10* expression, and IV. samples highly expressing both *ANXA2* and *S100A10*. In this, "low" and "high" expression should be interpreted cautiously, as both *ANXA2* and *S100A10* are expressed at relatively high levels throughout the patient cohort. Therefore the median expression values were used as cut-off values to classify patients expressing "high" (*i.e.* very high) or "low" (moderately high) expression levels. Next, we compared *in vitro* prednisolone sensitivity among these four groups (**Figure 6B**). Interestingly, high-level expression of *S100A10* appeared a stronger predictor for prednisolone resistance than high *ANXA2* expression (**Figure 6B**). *MLL*-rearranged infant ALL samples over-expressing *S100A10* at the mRNA level were significantly ($p<0.001$) more resistant to prednisolone *in vitro*, regardless of their *ANXA2* expression level. In addition, expression of *S100A10* appeared more predictive for clinical outcome than *ANXA2* expression. *MLL*-

rearranged infant ALL patient with high-level expression of *S100A10* experienced a worse outcome than patients expressing low-levels of *S100A10* (Log-rank test *p<0.005*). Similar analysis for *ANXA2* expression revealed that the correlation with clinical outcome and the level of *ANXA2* expression was far less evident (Log-rank test *p=0.1278;* **Figure 6C**). Moreover, multivariate Cox regression analysis demonstrated that *S100A10* expression is more important in conferring prednisolone resistance then *ANXA2* expression (Wald test *p=0.038*).

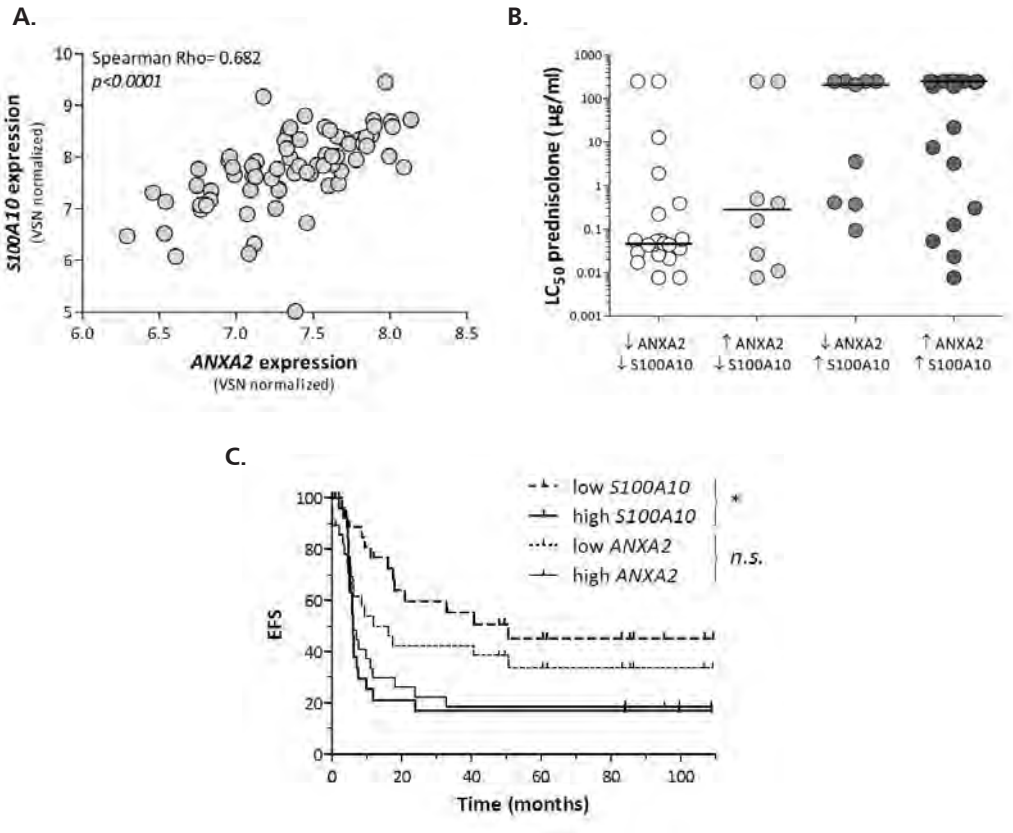


Figure 6. Correlation of *S100A10* with *ANXA2* in *MLL*-rearranged infant ALL.
A. Correlation between VSN-normalized expression (Affymetrix HU133plus2 microarray data) of *ANXA2* (average of probe sets 213503_x_at, 210427_x_at, and 201590_x_at) and *S100A10* (200872_at) in primary *MLL*-rearranged infant ALL samples (*n*=72). (Spearman Rho's correlation: 0.682; *p<0.0001*). **B.** Distribution of the *in vitro* prednisolone response (LC_{50} values: concentrations of prednisolone lethal to 50% of the leukemic cells) in primary *MLL*-rearranged infant ALL samples expressing high levels of *ANXA2* and/or *S100A10*. **C.** Event-free survival (EFS) for *MLL*-rearranged infant ALL patients expressing either high (solid lines) or low (dashed lines) levels of *ANXA2* (thin lines) or *S100A10* (thick lines). The median expression value in the entire cohort was used as the cut-off value. Differences in outcome were statistically analyzed using the Log-rank test. *Indicates differences with *p*-values of *<0.005*.

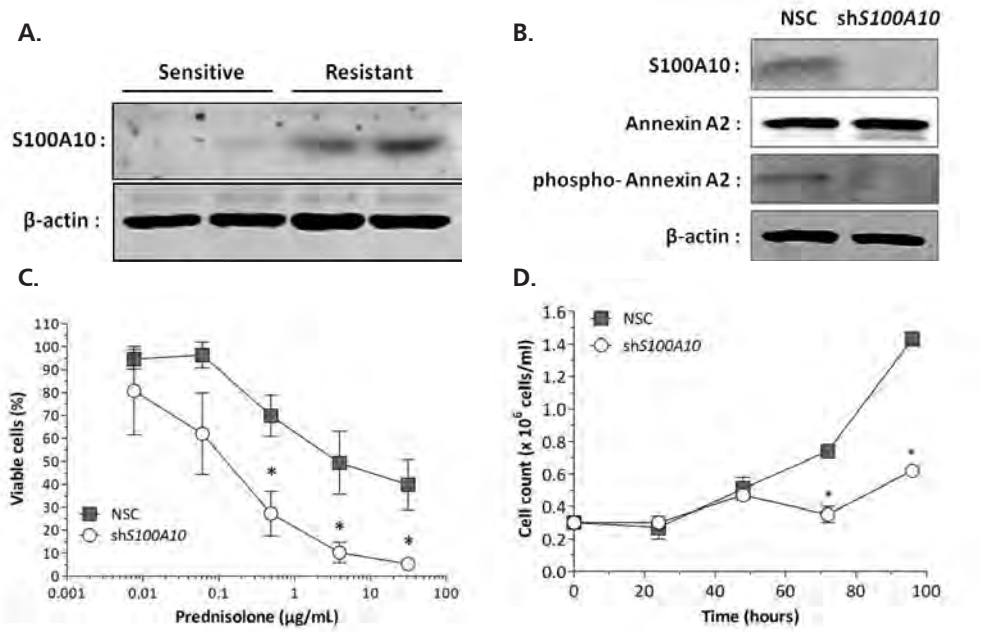


Figure 7. Relevance of p11 (*S100A10*) in Annexin A2 phosphorylation and prednisolone resistance in *MLL*-rearranged ALL cells.
A. Western blot analysis was used to determine p11 (*S100A10*) expression in *in vitro* prednisolone-sensitive (RS4;11 and BEL-1) and prednisolone-resistant (SEM and KOPN8) cell lines. **B.** Western Blot analysis showing p11, total annexin A2, and phosphorylated annexin A2 in SEM cells challenged with shRNA-mediated knock-down of *S100A10*, or non-silencing control (NSC) shRNAs. **C.** *In vitro* prednisolone response (as determined by 4-day MTT assays) in SEM cells with and without suppressed p11 (*S100A10*) expression. **D.** Viable cell counts at indicated time points in SEM displaying knocked-down p11 (*S100A10*), or cells transduced with NSC shRNAs. Experiments were performed in duplicate. *Indicate significant differences with *p*-values of *<0.05*.

Moreover, prednisolone-resistant *MLL*-rearranged cells also displayed higher p11 protein expression when compared to cells sensitive to prednisolone (**Figure 7A**). Thus, although high-level *ANXA2* expression, annexin A2 phosphorylation, and the presence of (active) Src kinases (like FYN and LCK) to catalyze tyrosine phosphorylation of annexin A2, are associated with prednisolone resistance in *MLL*-rearranged ALL, this association is largely dependent on *S100A10* expression. Apparently, sufficient amounts of p11 protein (encoded by *S100A10*) are necessary for successful phosphorylation of annexin A2 by FYN and LCK. Thus, in case of insufficient availability of p11 protein, FYN and LCK mediated phosphorylation of annexin A2 is hindered and should lead to prednisolone sensitivity. To test this hypothesis, we knocked-down *S100A10* expression by shRNA-mediated RNA interference in prednisolone resistant SEM cells. Western blot analyses showed that this led to complete down-regulation of p11 protein (**Figure 7B**). Moreover, while total annexin A2 expression remained unaltered, annexin A2 phosphorylation was indeed suppressed (**Figure 7B**). As expected, this sensitized otherwise resistant SEM cells to prednisolone (**Figure 7C**), which again was accompanied by a reduction in leukemic cell proliferation (**Figure 7D**).

DISCUSSION

MLL-rearranged infant leukemia is characterized by poor *in vitro* and *in vivo* glucocorticoid responses, which are both of prognostic value (3, 5-6). Here we demonstrate that, among other genes, *ANXA2* (encoding human annexin A2) is highly expressed in prednisolone-resistant *MLL*-rearranged infant ALL samples. Elevated *ANXA2* expression appeared to be specific for *in vitro* prednisolone resistance in *MLL*-rearranged infant ALL, since *ANXA2* expression could not explain prednisolone resistance in non-infant BCP-ALL nor in T-cell ALL. Whether *ANXA2* expression is also associated with prednisolone resistance in older children or adults remains to be tested. Furthermore, we show that suppression of annexin A2 expression indeed sensitizes otherwise resistant *MLL*-rearranged ALL cells to prednisolone. Additional experiments showed that this phenomenon largely depends on FYN-induced or LCK-induced tyrosine phosphorylation of annexin A2: knock-down of these Src kinases did not affect total annexin A2 protein levels, but severely inhibited annexin A2 phosphorylation, accompanied by the induction of prednisolone sensitivity in prednisolone-resistant *MLL*-rearranged ALL cells. Thus not *ANXA2* expression per se, but rather the presence of phosphorylated annexin A2 confers prednisolone resistance in *MLL*-rearranged ALL cells. In line with this, several known Src kinase inhibitors (including PP1, PP2, Imatinib, and AZD0530), with preferred specificity towards FYN and LCK, were also capable of sensitizing *MLL*-rearranged ALL cells to prednisolone.

Unfortunately, little is known about the effectiveness of the inhibitors PP1 and PP2 in acute leukemias. One study in acute myeloid leukemia (AML) carrying internal duplications (ITDs) in *FLT3* (FLT3-ITD+), demonstrated that PP2 reduced constitutive phosphorylation of both LYN and STAT5. Furthermore, PP2 treatment of mice transplanted with FLT3/ITD+ AML cells markedly inhibited disease onset and significantly decreased tumour size (27). Reports describing the efficacy of AZD0530 (or Saracatinib) in acute leukemias do not exist, but AZD0530 has been widely used in clinical trials focusing on varying types of human cancers, including metastatic breast cancer, adenocarcinoma, head and neck squamous cell carcinoma, and pancreatic cancer. All of these studies concluded that AZD0530 has no sufficient single-agent activity (28-31). We here showed that AZD0530 might be beneficial when implemented in current combination chemotherapy regimes designed for high-risk *MLL*-rearranged infant ALL patients being resistant to glucocorticoids.

Although imatinib was originally developed to specifically inhibit *BCR-ABL*-rearranged ALL and chronic myeloid leukemia (CML), it later became apparent that this agent also exhibited inhibitory activity towards Src kinases (24-25). At first site, these observations seemed promising as the simultaneous inhibition of Src and ABL kinases was proposed to be beneficial for patients with *BCR-ABL*· acute leukemia (32). Yet, the notion that leukemia patients treated with imatinib may acquire resistance to the inhibitor led to the development of more effective ABL kinase inhibitors (33). However, before clinical testing of Src kinase inhibitors as prednisolone-sensitizing drugs can be considered, solid data will need to be generated proving the concept in *in vivo* mouse models.

Based on our data, implementing an effective Src kinase (or annexin A2) inhibitor to overcome glucocorticoid resistance in *MLL*-rearranged ALL seems logic, but the importance of p11

(encoded by *S100A10*) should not be underestimated. We showed that the level of *S100A10* expression represents a better predictor of *in vitro* prednisolone resistance rather than the expression of *ANXA2*. Yet, S100A10 did not appear in our gene expression signature associated with prednisolone resistance in *MLL*-rearranged infant ALL. This may be explained by the fact that *S100A10* is highly expressed in all *MLL*-rearranged infant ALL patients: our recent gene expression profiling study showed that *S100A10* expression is highly discriminative between *MLL*-rearranged infant ALL and pediatric precursor B-ALL (26). Moreover, Krivtsov *et al.* recently showed that *S100A10* expression seems to be activated by the MLL fusion protein itself (34). Nevertheless, despite high-level mRNA expression of *S100A10* in *MLL*-rearranged ALL cells, the p11 protein, requires partnering with annexin A2 to become stable and protected from proteosome-dependent degradation (13). Thus, detectable levels of p11 protein may only exist in case sufficient annexin A2 protein is available. When that situation occurs, the presence of Src kinases like FYN and LCK are then required for effective annexin A2 phosphorylation, which, as shown in the present study, confers resistance to prednisolone in *MLL*-rearranged ALL. Therefore, we postulate that inhibition of p11 may well turn out to be the most effective way to dissociate the connection between Src kinase activity and annexin A2 phosphorylation. Interestingly, a recent publication by Reddy *et al.* reported on the development of 1-substituted 4-aroyl-3-hydroxy-5-phenyl-1H-pyrrol-2(5H)-one analogues that blocks the interaction between p11 and annexin A2, disrupting the physiological complex of annexin A2 and p11 in breast cancer cells (35). Thus, these specific inhibitors may be intriguing candidates worth testing for their prednisolone-sensitizing effects in *MLL*-rearranged ALL cells.

In conclusion, we here present a novel mechanism in which Src kinase-induced phosphorylation of annexin A2 plays a central role in maintaining prednisolone-resistance in *MLL*-rearranged infant ALL. Suppression of annexin A2, FYN, LCK, or the adaptor protein p11 (encoded by the *S100A10* gene) all result in the inhibition of annexin A2 phosphorylation, and restores susceptibility to prednisolone-induced leukemic cell death. Given the high-level expression of *S100A10* in *MLL*-rearranged infant ALL, we postulate that p11 protein represents an attractive therapeutic target for increasing glucocorticoid responses and with that improve prognosis for patients diagnosed with this aggressive type of leukemia.

REFERENCES

1. Ramakers-van Woerden NL, Beverloo HB, Veerman AJ, Camitta BM, Loonen AH, van Wering ER, *et al.* In vitro drug-resistance profile in infant acute lymphoblastic leukemia in relation to age, MLL rearrangements and immunophenotype. *Leukemia*. 2004 Mar;18(3):521-9.

2. Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, *et al.* Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants. *Leukemia*. 1998 Sep;12(9):1344-8.

3. Dordelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, *et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*. 1999 Aug 15;94(4):1209-17.

4. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, *et al.* A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007 Jul 21;370(9583):240-50.

5. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, *et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *J Clin Oncol*. 2003 Sep 1;21(17):3262-8.

6. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood*. 1998 Jul 1;92(1):259-66.

7. Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, *et al.* Association of high-level MCL1 expression with in vitro and in vivo prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia. *Blood*. 2010 Feb 4;115(5):1018-25.

8. Bao H, Jiang M, Zhu M, Sheng F, Ruan J, Ruan C. Overexpression of Annexin II affects the proliferation, apoptosis, invasion and production of proangiogenic factors in multiple myeloma. *Int J Hematol*. 2009 Sep;90(2):177-85.

9. Zhang F, Zhang L, Zhang B, Wei X, Yang Y, Qi RZ, *et al.* Anxa2 plays a critical role in enhanced invasiveness of the multidrug resistant human breast cancer cells. *J Proteome Res*. 2009 Nov;8(11):5041-7.

10. Braden AR, Kafka MT, Cunningham L, Jones H, Vishwanatha JK. Polymeric nanoparticles for sustained down-regulation of annexin A2 inhibit prostate tumor growth. *J Nanosci Nanotechnol*. 2009 May;9(5):2856-65.

11. Zheng L, Foley K, Huang L, Leubner A, Mo G, Olino K, *et al.* Tyrosine 23 phosphorylation-dependent cell-surface localization of annexin A2 is required for invasion and metastases of pancreatic cancer. *PLoS One*. 2011;6(4):e19390.

12. Hayes MJ, Moss SE. Annexin 2 has a dual role as regulator and effector of v-Src in cell transformation. *J Biol Chem*. 2009 Apr 10;284(15):10202-10.

13. He KL, Deora AB, Xiong H, Ling Q, Weksler BB, Niesvizky R, *et al.* Endothelial cell annexin A2 regulates polyubiquitination and degradation of its binding partner S100A10/p11. *J Biol Chem*. 2008 Jul 11;283(28):19192-200.

14. Dassah M, Deora AB, He K, Hajjar KA. The endothelial cell annexin A2 system and vascular fibrinolysis. *Gen Physiol Biophys*. 2009;28 Spec No Focus:F20-8.

15. Kaspers GJ, Veerman AJ, Pieters R, Broekema GJ, Huismans DR, Kazemier KM, *et al.* Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. *Br J Cancer*. 1994 Dec;70(6):1047-52.

16. Pieters R, Loonen AH, Huismans DR, Broekema GJ, Dirven MW, Heyenbrok MW, *et al.* In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. *Blood*. 1990 Dec 1;76(11):2327-36.

17. Spijkers-Hagelstein JA, Schneider P, Hulleman E, de Boer J, Williams O, Pieters R, *et al.* Elevated S100A8/S100A9 expression causes glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia. *Leukemia*. 2012 Jun;26(6):1255-65.

18. Benjamini Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Stat Soc B*. 1995;57(1):289-300.

19. Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP. GenePattern 2.0. *Nat Genet*. 2006 May;38(5):500-1.

20. Stam RW, den Boer ML, Schneider P, Nollau P, Horstmann M, Beverloo HB, *et al.* Targeting FLT3 in primary MLL-gene-rearranged infant acute lymphoblastic leukemia. *Blood*. 2005 Oct 1;106(7):2484-90.

21. Liu Y, Bishop A, Witucki L, Kraybill B, Shimizu E, Tsien J, *et al.* Structural basis for selective inhibition of Src family kinases by PP1. *Chem Biol*. 1999 Sep;6(9):671-8.

22. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, *et al.* Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem.* 1996 Jan 12;271(2):695-701.

23. Wong WS, Leong KP. Tyrosine kinase inhibitors: a new approach for asthma. *Biochim Biophys Acta.* 2004 Mar 11;1697(1-2):53-69.

24. Schenone S, Brullo C, Musumeci F, Botta M. Novel dual Src/Abl inhibitors for hematologic and solid malignancies. *Expert Opin Investig Drugs*. 2010 Aug;19(8):931-45.

25. Gnoni A, Marech I, Silvestris N, Vacca A, Lorusso V. Dasatinib: an anti-tumour agent via Src inhibition. *Curr Drug Targets*. 2011 Apr;12(4):563-78.

26. Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, *et al.* Gene expression profiling-based dissection of MLL translocated and MLL germline acute lymphoblastic leukemia in infants. *Blood*. 2010 Apr 8;115(14):2835-44.

27. Okamoto M, Hayakawa F, Miyata Y, Watamoto K, Emi N, Abe A, *et al.* Lyn is an important component of the signal transduction pathway specific to FLT3/ITD and can be a therapeutic target in the treatment of AML with FLT3/ITD. *Leukemia*. 2007 Mar;21(3):403-10.

28. Gucalp A, Sparano JA, Caravelli J, Santamauro J, Patil S, Abbruzzi A, *et al.* Phase II Trial of Saracatinib (AZD0530), an Oral SRC-inhibitor for the Treatment of Patients with Hormone Receptor-negative Metastatic Breast Cancer. *Clin Breast Cancer*. 2011 May 3.

29. Mackay HJ, Au HJ, McWhirter E, Alcindor T, Jarvi A, Macalpine K, *et al.* A phase II trial of the Src kinase inhibitor saracatinib (AZD0530) in patients with metastatic or locally advanced gastric or gastro esophageal junction (GEJ) adenocarcinoma: a trial of the PMH phase II consortium. *Invest New Drugs*. 2011 Mar 12.

30. Fury MG, Baxi S, Shen R, Kelly KW, Lipson BL, Carlson D, *et al.* Phase II study of saracatinib (AZD0530) for patients with recurrent or metastatic head and neck squamous cell carcinoma (HNSCC). *Anticancer Res.* 2011 Jan;31(1):249-53.

31. Renouf DJ, Moore MJ, Hedley D, Gill S, Jonker D, Chen E, *et al.* A phase I/II study of the Src inhibitor saracatinib (AZD0530) in combination with gemcitabine in advanced pancreatic cancer. *Invest New Drugs*. 2010 Dec 18.

32. Hu Y, Liu Y, Pelletier S, Buchdunger E, Warmuth M, Fabbro D, *et al.* Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. *Nat Genet*. 2004 May;36(5):453-61.

33. Manley PW, Cowan-Jacob SW, Mestan J. Advances in the structural biology, design and clinical development of Bcr-Abl kinase inhibitors for the treatment of chronic myeloid leukaemia. *Biochim Biophys Acta.* 2005 Dec 30;1754(1-2):3-13.

34. Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU, *et al.* H3K79 methylation profiles define murine and human MLL-AF4 leukemias. *Cancer Cell.* 2008 Nov 4;14(5):355-68.

35. Reddy TR, Li C, Guo X, Myrvang HK, Fischer PM, Dekker LV. Design, synthesis, and structure-activity relationship exploration of 1-substituted 4-aroyl-3-hydroxy-5-phenyl-1H-pyrrol-2(5H)-one analogues as inhibitors of the annexin A2-S100A10 protein interaction. *J Med Chem.* 2011 Apr 14;54(7):2080-94.

CHAPTER 6

Chemical genomic screening identifies LY294002 as a modulator of glucocorticoid resistance in *MLL*-rearranged infant ALL

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ABSTRACT

Successful treatment results for *MLL*-rearranged Acute Lymphoblastic Leukemia (ALL) in infants remain difficult to achieve. Significantly contributing to therapy failure is poor response to glucocorticoids (GCs), like prednisone. Thus, overcoming resistance to these drugs may be a crucial step towards improving prognosis.

We defined a gene signature that accurately discriminates between prednisolone-resistant and prednisolone-sensitive *MLL*-rearranged infant ALL patient samples. In the current study, we applied Connectivity Map analysis to perform an *in silico* screening for agents capable of reversing the prednisolone-resistance profile and induce prednisolone sensitivity. These analyses revealed that LY294002, a PI3K inhibitor, would potentially fulfill this task. Subsequent validation experiments demonstrated that indeed LY294002, and other known PI3K inhibitors, markedly sensitized otherwise resistant *MLL*-rearranged ALL cells to prednisolone *in vitro*. Using quantitative RT-PCR analyses, we validated the modulating effects of the PI3K inhibitors on the expression of the genes present in the prednisolone-resistance profile. Interestingly, prednisolone-sensitizing actions may be mediated by inhibition of *FCGR1B*. Moreover, only high-level expression of *FCGR1B* showed to be predictive for a poor prognosis and shRNA-mediated knock-down of *FCGR1B* led to *in vitro* prednisolone sensitization. Thus, implementing FDA-approved PI3K inhibitors in current treatments may potentially improve the GC-response and prognosis in patients with *MLL*-rearranged ALL.

INTRODUCTION

Despite the excellent efficacy of current combination chemotherapies used in the treatment of pediatric Acute Lymphoblastic leukemia (ALL), infants (<1 year of age) with ALL carrying leukemia-specific chromosomal translocations of the *Mixed Lineage Leukemia* (*MLL*) gene remain difficult to treat (1-2). While long-term survival rates for childhood ALL in general nowadays easily exceed 85% (3), the survival rates for *MLL*-rearranged infant ALL are only ~50% (2). An important factor contributing to this poor prognosis is cellular drug resistance, especially to glucocorticoids (GCs) like prednisolone (the biologically active metabolite of prednisone) and dexamethasone (4-5). Approximately one-third of all infant ALL cases show a so-called poor *in vivo* response to a 7-day window of prednisone mono-therapy (2). Yet, GCs remain essential drugs in the treatment of ALL and currently can neither be excluded nor replaced. Combination chemotherapy induces morphological complete remission in ~95% of the *MLL*-rearranged infant ALL cases (6-7). Unfortunately, most of these patients experience relapses on treatment within the first two years upon diagnosis. As both *in vitro* and *in vivo* GC resistance have been identified as poor prognostic factors (5, 8-9), overcoming resistance to these drugs should improve prognosis. Hence, the task at hand is to unravel the mechanisms underlying GC-resistance in *MLL*-rearranged infant ALL, and to develop strategies that improve the responses to these agents.

Recent work from our laboratory demonstrated differential gene expression in ALL samples from children older than 1 year of age (*i.e.* non-infants) either resistant or sensitive to prednisolone *in vitro* (10). Wei *et al.* recently investigated whether certain FDA-approved therapeutics were able to reverse a GC-resistance profile to a signature more consistent with that of GC-sensitive pediatric ALL samples (11). A large database of genome-wide expression profiles derived from a variety of human cancer cell lines individually treated with a broad spectrum of bioactive small molecules (known as the Connectivity Map) (12-13) was exploited, to identify agents that induce gene expression differences fitting GC sensitivity. This approach identified the mTOR inhibitor rapamycin as a modulator of GC resistance, inducing down-regulation of anti-apoptotic MCL1. Both, exposure to rapamycin as well as shRNA-based knock-down of MCL1, induced GC sensitivity in otherwise resistant pediatric precursor B-ALL cells (11). However, despite the association of high-level *MCL1* expression with GC resistance in *MLL*-rearranged infant ALL, we could not confirm these sensitizing effects of rapamycin in GC-resistant *MLL*-rearranged ALL cells and knock-down of MCL1 only moderately improved the in *vitro* GC response of *MLL*-rearranged ALL cells (14). This discrepancy may be easily explained by the unique genetic make-up of *MLL*-rearranged leukemias. Multiple gene expression profiling studies have showed that *MLL*-rearranged ALL cells display gene signatures that are significantly different from other ALL subtypes (15-17). Not surprisingly, we recently demonstrated that GC-resistance profiles in *MLL*-rearranged infant precursor B-ALL significantly deviate from GC-resistance signatures in non-infant pediatric precursor B-ALL (14). Our earlier GC-resistance profile in *MLL*-rearranged infant ALL was to some extent compromised by a relatively low accuracy due to the limited number of patient samples (14). Therefore, we recently produced a stronger and more accurate GC-resistance gene expression profile for *MLL*-rearranged infant

ALL (18). In the present study we used this gene signature in connectivity map analysis in order to identify agents that specifically modulate GC resistance in this distinct and aggressive type of ALL.

MATERIALS AND METHODS

Patient samples

Bone marrow or peripheral blood samples from untreated infants (*i.e.* children <1 year of age) diagnosed with *MLL*-rearranged ALL, were collected at Sophia Children's Hospital (Rotterdam, The Netherlands) as part of the international collaborative INTERFANT treatment protocol (2). Approval for these studies was obtained form the ErasmusMC Institutional Review Board. Informed consent was obtained according to the Declaration of Helsinki. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation, and contaminating non-leukemic cells removed using immunomagnetic beads as described before (19). All samples used in this study contained more than 90% of leukemic blasts.

In vitro prednisolone response

The *in vitro* prednisolone response was assessed by 4-day MTT assays as described elsewhere (20). Patient samples were characterized as prednisolone sensitive or resistant based on the LC_{50} value (*i.e.* the concentration of prednisolone lethal to 50% of the leukemic cells). Samples were typified as prednisolone-sensitive at LC_{50} values <0.1 µg/mL and prednisolone-resistant at LC_{50} values >150 µg/mL.

Connectivity map analyses

The Connectivity Map (build 02) represents a large collection of gene expression data derived from human cancer cell lines before and after 6-hour exposures to a broad selection of 1309 FDA-approved bioactive molecules (13). Connectivity map analysis utilizes a gene set enrichment metric (Kolmogorov-Smirnov statistic (13) to rank order individual treatment instances by their similarity to a given query signature; in this case, an *MLL*-rearranged infant ALL prednisolone-resistance signature previously described by us (18). The output consists of small-molecule compounds with assigned gene enrichment metric: *i.e.* the connectivity score. This relative score represents the correlation between the query signature and gene expression profiles from the individually treated cell lines compared to controls. In the present study we aimed to identify compounds that potentially reverse the prednisolone-resistance profile towards the profile associated with prednisolone sensitivity. In other words, candidate compounds should be able to modulate a substantial amount of genes over-expressed or under-expressed in prednisolone-resistant patients to induce a signature more comparable to that of prednisolone-sensitive patients. The entire analyses are carried out in the web-based tool provided at: http://www.broad.mit.edu/cmap/.

Quantitative RT-PCR analysis

Total RNA was extracted from a minimum of 5x10^6 leukemic cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified on a spectrophotometer. Next, RNA was reverse transcribed and the obtained cDNA was used to quantify mRNA expression of target genes relative to the housekeeping gene *B2M* using quantitative RT-PCR analysis. For this, PCR products were amplified using the DyNAmo SYBR Green qPCR kit (Finnzymes) according to the manufacturer's recommendations, using SYBR Green as a fluorophore to detect transcripts on an ABI Prism 7900 sequence detection system (Applied Biosystems). All oligonucleotide primers used for PCR amplification were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO) and purchased from Eurogentec (Seraing, Belgium). Differences in gene expression were statistically evaluated using the one-sided student *t*-test, and considered statistically significant at *P*-values *<0.05*.

Cell line cultures and *in vitro* assessment of prednisolone-sensitizing effects

The leukemia cell lines REH, NALM6, SEM and KOPN8 (Acute Lymphoblastic Leukemia; ALL), HL60 and MV4-11 (Acute Myeloid Leukemia; AML) were maintained as suspension cultures in RPMI 1640 supplemented with glutamax (Invitrogen, Life Technologies, Breda, the Netherlands), 10% (v/v) fetal calf serum (FCS), and 1% penicillin/streptomycin/fungizone (PSF; Invitrogen, Life Technologies, Breda, the Netherlands) at 37^{o}C in humidified air containing 5% CO_2. SEM and MV4-11 cells carry *MLL* translocation t(4;11) generating the MLL-AF4 fusion protein, KOPN8 cells are characterized by translocation t(11;19) producing the MLL-ENL fusion. REH, NALM6 and HL60 cells are negative for translocations of the *MLL* gene. All *MLL*-rearranged (18) and non-*MLL*-rearranged cell lines are resistant to prednisolone *in vitro*. Prednisolone-sensitizing effects were determined by performing conventional 4-day MTT- assays (20), either in the absence or presence of indicated compounds. In all instances, the observed prednisolone-sensitization was normalized against the effects of the co-administered agent by culturing control cells (not exposed to prednisolone) in the presence of these agents at similar concentrations.

shRNA-mediated RNA interference of *FCGR1B*

In order to knock-down *FCGR1B* expression by means of RNA interference, the *in vitro* prednisolone-resistant SEM cell line was virally infected by shRNA-expressing pLKO.1 vectors obtained from Sigma (St. Louis, MO, USA). shRNA target sequence for *FCGR1B* was 5'-GAA ATC TCT TTG GAT TCT GGT-3'. A vector with a similar backbone expressing a non-human targeting shRNA was used as a non-silencing control. Virus particles were produced by transient transfection of 293T cells with a mixture of psPAX-2, pMD2G-VSVG and pLKO.1. The virus-containing medium was harvested at 48 hours following transfection and filtrated prior infection of SEM cells. The expression of *FCGR1B* was determined by quantitative RT-PCR in pure populations. Subsequently, the *in vitro* prednisolone sensitivity in these cells was determined by 4-day MTT assays.

Calculation of synergy

A synergistic effect was tested by using the criteria described by Berenbaum (21). Briefly, a dose-response curve was constructed for each single drug and for combinations of 2 drugs together. Equipotent drug concentrations were then applied to the equation used by Berenbaum as follows: Synergy Factor (F_{syn}) = ([Drug $X_{in\ combination\ with\ Y}$] / [Drug X]) + ([Drug $Y_{in\ combination\ with\ X}$]/ [Drug Y]). F_{syn} less than 1 indicates synergy.

Statistical analysis

Differences in gene expression before and after depicted treatment were statistically evaluated using the paired student *t*-test. These analyses were all one-tailed, and differences were considered statistically significant at *P* values *<0.05* (indicated in experiments with asterisks). Log-rank test was performed to analyze differences in outcome between patient groups expressing high and low levels of *FCGR1B* or *D123*, with median expression values as cut-off values.

RESULTS

cMAP analysis identifies LY294002 as a modulator of prednisolone resistance in *MLL*-rearranged infant ALL

Connectivity mapping has been demonstrated to be a powerful tool for identifying FDA-approved compounds that potentially reverse glucocorticoid (GC) resistance in non-infant pediatric precursor B-ALL cells (11). Here we used connectivity map (cmap) analysis to search for therapeutic agents that reverse the *in vitro* prednisolone resistance signature of *MLL*-rearranged infant ALL. As the cmap was originally established using HU133A microarrays (Affymetrix), we were bound to deplete our HU133Aplus2.0-based signature from probe sets that are absent on the HU133A chip. Consequently, 24 probe sets needed to be excluded from our original prednisolone resistance signature (18). Nonetheless, the depleted signature (**Figure 1A**) remained discriminative between prednisolone-resistant and sensitive patients, albeit with slightly decreasing strength (**Figure 1B**). **Table 1** lists the top 10 compounds potentially inducing gene expression patterns similar to that of prednisolone-sensitive patient (positive enrichment), or similar to prednisolone-resistant patients (negative enrichment), as identified by cmap analysis. Only one agent, *i.e.* the PI3-kinase (PI3K) inhibitor LY294002, appeared to display a modest positive enrichment value accompanied by moderate specificity, indicating that this compound may reverse the expression of only a limited number of genes in our signature. Other agents either showed a positive enrichment value but with very low specificities, or displayed a negative enrichment (*i.e.* showing similarity to gene expression patterns observed in prednisolone-resistant patients). Interestingly, the compounds showing strong negative enrichment values included prednisolone. This may suggest that prednisolone itself potentially induces a gene expression pattern already present in prednisolone-resistant *MLL*-rearranged infant ALL cells, and as such may emphasize the relevance of the identified prednisolone-resistance profile.

Table 1. Connectivity Map defines LY-249002 as a modulator compound for reversing prednisolone resistance in *MLL*-rearranged infant ALL

Rank	Cmap name	Enrichment	Specificity	*P*-value
1	**LY-294002**	0.312	0.3020	0.00000
2	**Adiphenine**	-0.857	0.0403	0.00012
3	**Pindolol**	-0.794	0.0000	0.00074
4	**Mafenide**	-0.763	0.0000	0.00142
5	**Hexestrol**	0.822	0.0343	0.00169
6	**Prednisolone**	-0.763	0.0000	0.00262
7	**Troixysalen**	0.796	0.0087	0.00334
8	**Trazodone**	0.880	0.0373	0.00337
9	**Biperiden**	-0.715	0.1088	0.00411
10	**Amodiaquine**	-0.787	0.0066	0.00412

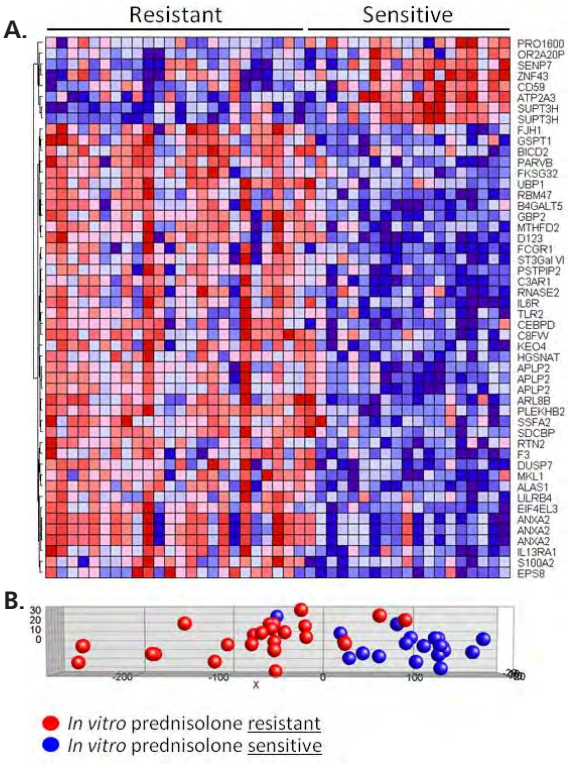


Figure 1. Trimmed prednisolone-resistance signature suitable for Connectivity Map analysis

A. Reduced heatmap, depleted from probe sets exclusively present on HU133plus2.0 but not on HU133A Affymetrix microarrays. A total of 24 needed to be removed from the original signature as presented in Spijkers-Hagelstein *et al.* (18) **B.** Principal Component Analysis (PCA) belonging to the trimmed prednisolone-resistance signature (50 probe sets), visualizing the degree of separation between prednisolone-resistant (red dots) and prednisolone-sensitive (blue dots) *MLL*-rearranged infant ALL samples.

The PI3K inhibitor LY294002 induces prednisolone-sensitization in *MLL*-rearranged ALL

Next we assessed the ability of the PI3K inhibitor LY294002 to modulate prednisolone resistance in *MLL*-rearranged ALL cells. First we determined the cytotoxic effects of LY294002 (4-day MTT assays) on the *MLL*-rearranged ALL cell lines SEM (carrying translocation *MLL-AF4*) and KOPN8 (carrying translocation *MLL-ENL*), the t(4;11)-positive AML cell line MV4-11, and the *MLL* translocation-negative cell lines REH, NALM6 (ALL) and HL60 (AML). As shown in **Figure 2A**, most of the cell lines showed pronounced cytotoxic responses to LY294002 with LC_{50} values ranging from 10-20 μM, indicating that this agent by itself exerts strong anti-leukemic effects. In contrast, the cell lines KOPN8 and HL60 appeared to be more resistant. Using the obtained LC_{50} values for LY294002 as guidance, we then determined the *in vitro* prednisolone response (MTT assays) in the presence of varying concentrations of LY294002 (10, 20 and 50 μM). In all *MLL*-rearranged leukemia cell lines, co-incubation with LY294002 led to more sensitive prednisolone responses, apparently in a dose-dependent manner (**Figure 2B-D**). Although we showed that LY294002 itself strongly affected the number viable cells, the observed sensitization cannot be ascribed to this phenomenon as all prednisolone responses were normalized against control cells exposed to corresponding concentrations of LY294002. Interestingly, the prednisolone sensitizing effects of LY294002 appeared to be restricted to *MLL*-rearranged leukemia cell lines (**Figure 2B-2D**), as in none of the *MLL* translocation-

negative leukemia cell lines modulation of prednisolone resistance was observed (**Figure 2E-2G**). As expected, none of the remaining compounds showing positive enrichment scores but weak specificities, including hexestrol, trioxysalen and trazadone (**Table 1**), were capable of sensitizing *MLL*-rearranged ALL cells to prednisolone (**Figure 3**).

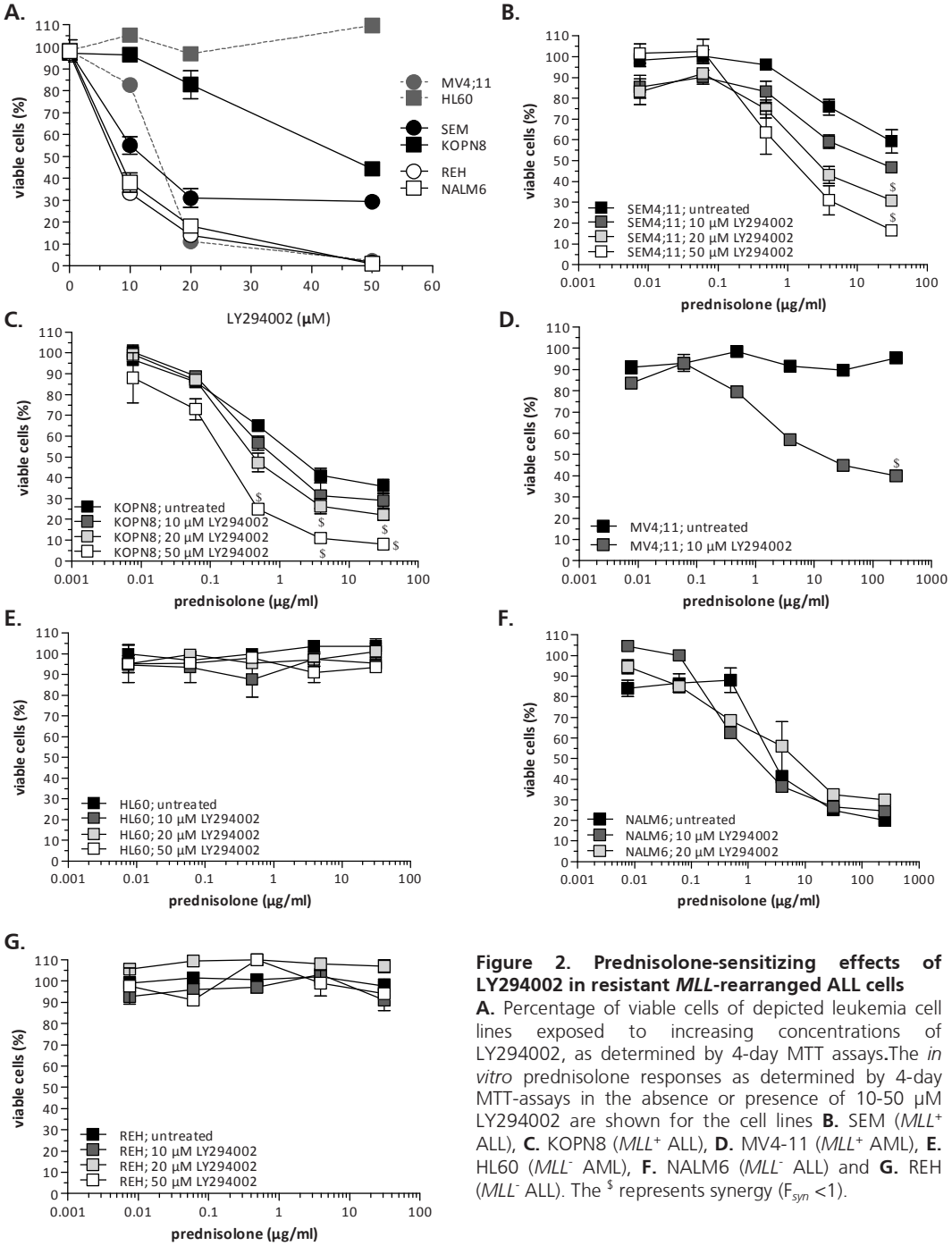


Figure 2. Prednisolone-sensitizing effects of LY294002 in resistant *MLL*-rearranged ALL cells
A. Percentage of viable cells of depicted leukemia cell lines exposed to increasing concentrations of LY294002, as determined by 4-day MTT assays.The *in vitro* prednisolone responses as determined by 4-day MTT-assays in the absence or presence of 10-50 µM LY294002 are shown for the cell lines **B.** SEM (*MLL*$^+$ ALL), **C.** KOPN8 (*MLL*$^+$ ALL), **D.** MV4-11 (*MLL*$^+$ AML), **E.** HL60 (*MLL*$^-$ AML), **F.** NALM6 (*MLL*$^-$ ALL) and **G.** REH (*MLL*$^-$ ALL). The $ represents synergy (F_{syn} <1).

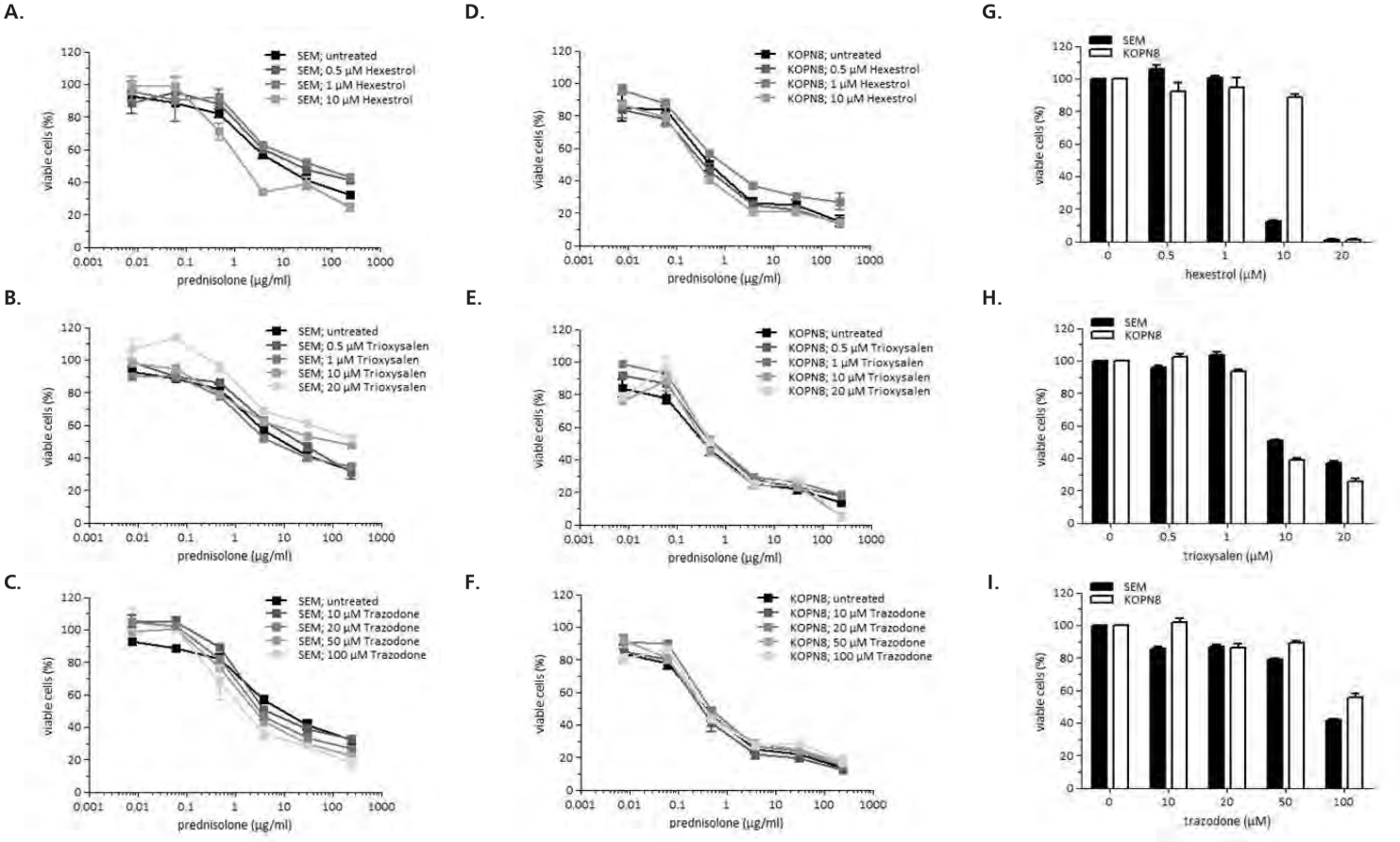


Figure 3. *In vitro* cytotoxicity testing of cMAP-determined drugs to reverse glucocorticoid resistance in the *MLL*-rearranged ALL cell lines.
In vitro cytotoxicity as determined by 4-day MTT assays in the glucocorticoid-resistant *MLL*-rearranged ALL cell lines **A-C.** SEM and **D-F.** KOPN8 in the absence or presence of 0.5-10 µM hexestrol, 0.5-20 µM trioxysalen and 10-100 µM trazodone. Cytotoxic dose-response curves are shown for prednisolone. The percentage of viable SEM cells after 4 days of culturing in the presence of **G.** hexestrol, **H.** trioxysalen or **I.** trazodone alone. All cytotoxic dose-response curves were corrected for the drug-specific cell death and only show normalized effects of prednisolone tested.

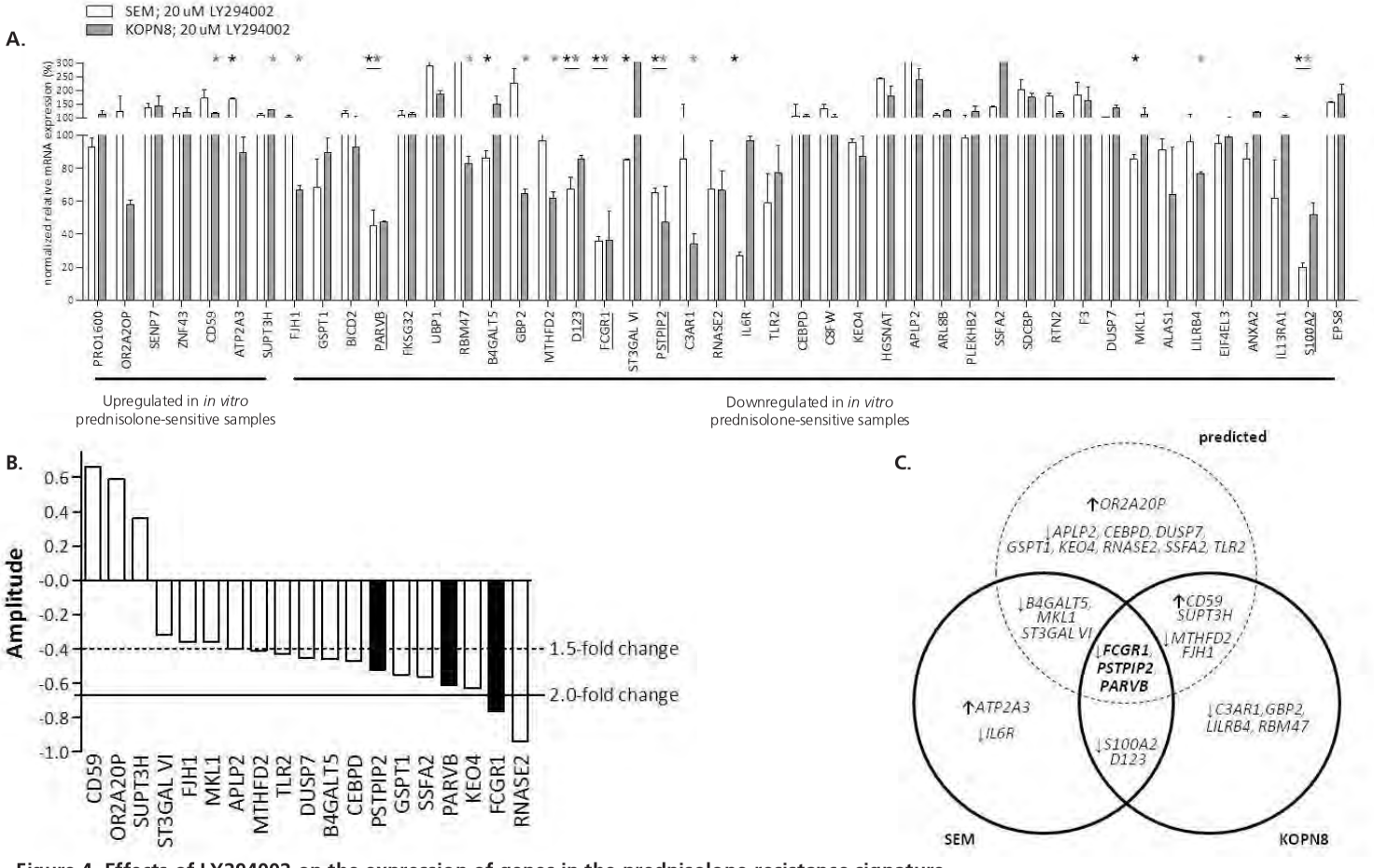


Figure 4. Effects of LY294002 on the expression of genes in the prednisolone-resistance signature

A. Relative expression levels of all genes in the trimmed prednisolone-resistance signature (Figure 2) measured in *MLL*-rearranged ALL cells (SEM and KOPN8) before and after 6 hours of exposure to LY294002. Gene expression was determined using quantitative RT-PCR analysis. Per gene, potential differences in expression before and after treatment were statistically evaluated using the student *t*-test (one-tailed) and were deemed significant at p-values *<0.05*. * Indicate significant alterations in expression. **B.** Genes in our prednisolone-resistance signature that are predicted to be modulated in HL60 cells in response to LY294002 exposure (connectivity map database) **C.** Venn diagram comparing predicted gene modulation in HL60 cells (cmap database) (dotted circle), and the significantly modulated genes in the *MLL*-rearranged ALL cell lines SEM and KOPN8 (closed circles) upon LY294002 treatment.

LY294002-induced specific alterations in the prednisolone-resistance profile

Next, we validated whether LY294002 induced gene expression differences that reverses a prednisolone-resistance signature towards a profile more comparable to that of prednisolone-sensitive patients. To this end, we incubated the *MLL*-rearranged ALL cell lines SEM and KOPN8 in the presence 20 μM LY294002 for the duration of 6 hours (based on a similar exposure period of individual treatment instances originally used to build the connectivity map database). Using quantitative RT-PCR we determined the expression of all genes of the prednisolone-resistance signature (Figure 1A) in both cell line models before and after LY294002 treatment (**Figure 4A**). As the connectivity map database consists of treatment/control signatures obtained in four human cancer cell lines, including one leukemia cell lines (*i.e.* the AML cell line HL60), we used the connectivity map results for LY294002 treatment in HL60 to compare with our data. The expression of three genes (*i.e. PARVB, FCGR1B,* and *PSTPIP2*) predicted to become down-regulated in response to LY294002 in HL60, appeared to be significantly ($p<0.05$) and consistently decreased in both SEM and KOPN8 (**Figure 4B**). Likewise, *RNASE2*, *GSPT1*, and *TLR2* expression was affected in both SEM and KOPN8, but did not reach statistical significance after 6 hours of LY294002 exposure (**Figure 4B**). In addition, two genes (*i.e. S100A2* and *D123*) were not predicted to become down-regulated in the HL60 cell line, but were significantly ($p<0.05$) suppressed in both of the *MLL*-rearranged ALL cell lines SEM and KOPN8 (**Figure 4**). Several other genes predicted to be affected based on the HL60 data (**Figure 4C**), appeared to be significantly down-regulated in only one of the *MLL*-rearranged ALL cell lines (either SEM or KOPN8) (**Figures 4**). Finally, connectivity map analysis predicted up-regulation of three genes (*CD59*, *OR2A20P*, and *SUPT3H*) highly expressed in prednisolone-sensitive, but not in prednisolone-resistant patient samples (Figure 1A and **Figure 4B**). Interestingly, modest trends towards elevated expression of *CD59* and *SUPT3H* were observed in response to 6 hours of exposures to LY294002 in both SEM and KOPN8, whereas *OR2A20P* expression was increased in SEM cells, but decreased in KOPN8 (**Figure 4A**). Overall these data show that LY294002 indeed modulates multiple genes in the prednisolone-resistance signature, establishing gene expression patterns more comparable to that of prednisolone-sensitive patient samples and inducing prednisolone sensitivity.

Prednisolone-sensitizing effects of other PI3K inhibitors

As LY294002 represents a well-known PI3K inhibitor, we finally explored the possibility that other PI3K inhibitors may also induce prednisolone sensitivity in otherwise resistant *MLL*-rearranged ALL cells. Again, the *MLL*-rearranged leukemia cell lines SEM, KOPN8 and MV4-11 were tested for *in vitro* prednisolone sensitivity in the absence or presence of potent PI3K inhibitors including wortmannin (5 μM) and PI-828 (5/10 μM). Like LY294002, the other PI3K inhibitors also induced prednisolone sensitivity in both SEM and KOPN8 cells, albeit to varying degrees. All prednisolone responses were normalized against control cells exposed to corresponding concentrations of wortmannin or PI-828 (**Figure 5A-B**). Clearly, the *MLL*-rearranged AML cell line MV4-11 appeared less susceptible to the prednisolone-sensitizing effects of these agents (**Figure 5C**). Remarkably, wortmannin is represented in the connectivity

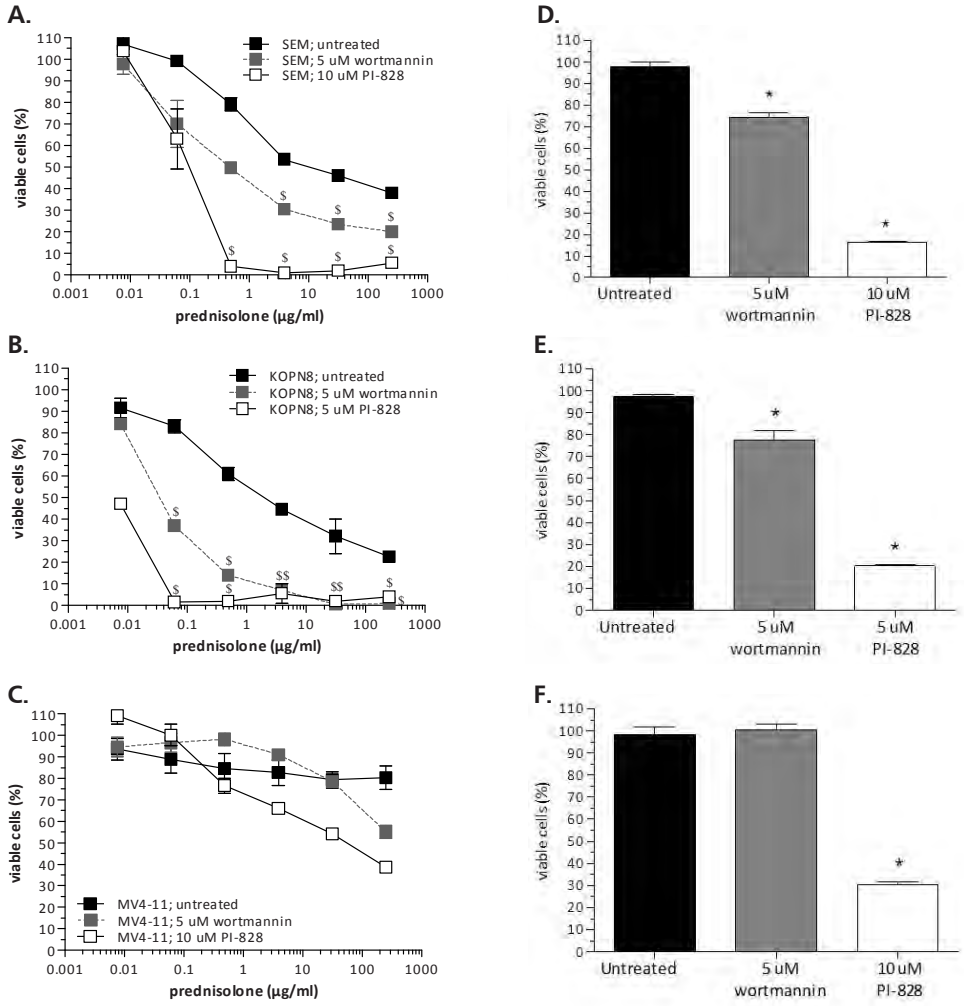


Figure 5. Prednisolone-sensitizing effects in *MLL*-rearranged cells by other PI3K inhibitors
In vitro prednisolone responses as determined by 4-day MTT-assays in the absence or presence of the PI3K inhibitors wortmannin (5 µM) or PI-828 (5/10 µM) are shown for the *MLL*-rearranged acute leukemia cell lines **A.** SEM, **B.** KOPN8, and **C.** MV4-11. The percentage of viable *MLL*-rearranged leukemia cell lines **D.** SEM, **E.** KOPN8, and **F.** MV4-11 after 4 days of culturing in the presence of wortmannin (5 µM) or PI-828 (5/10 µM). The $ represents synergy (F_{syn} <1).

map database, but was not identified as a potential modulator of prednisolone resistance. A possible explanation for this may be that this compound only modulates the expression of a small number of crucial (PI3K-dependent) genes in our signature, based on which the enrichment and/or specificity scores remained too low to become significant. In other words, PI3K inhibition may be sufficient to sensitize *MLL*-rearranged ALL cells to prednisolone, without the necessity of altering large numbers of genes in the prednisolone-resistance signature. Alternatively, this agent induces prednisolone via mechanisms less dependent on the genes in the prednisolone-resistance signature.

Interestingly, the most profound prednisolone-sensitizing effects were observed for PI-828 (**Figure 5A-C**), which in combination with 0.05 - 0.5 µg/mL of prednisolone basically eradicated the entire population of leukemic SEM and KOPN8 cells. At the same time, PI-828 also appeared to exhibit strong anti-leukemic effects in the absence of prednisolone, suggesting great potential in the treatment of *MLL*-rearranged ALL. However, PI-828 represents a rather novel PI3K inhibitor and little is known about the compound in terms of clinical safety and applications. As such, PI-828 has not (yet) been included in the connecitivity map database, and therefore could not be identified as a potential modulator of the prednisolone-resistance signature.

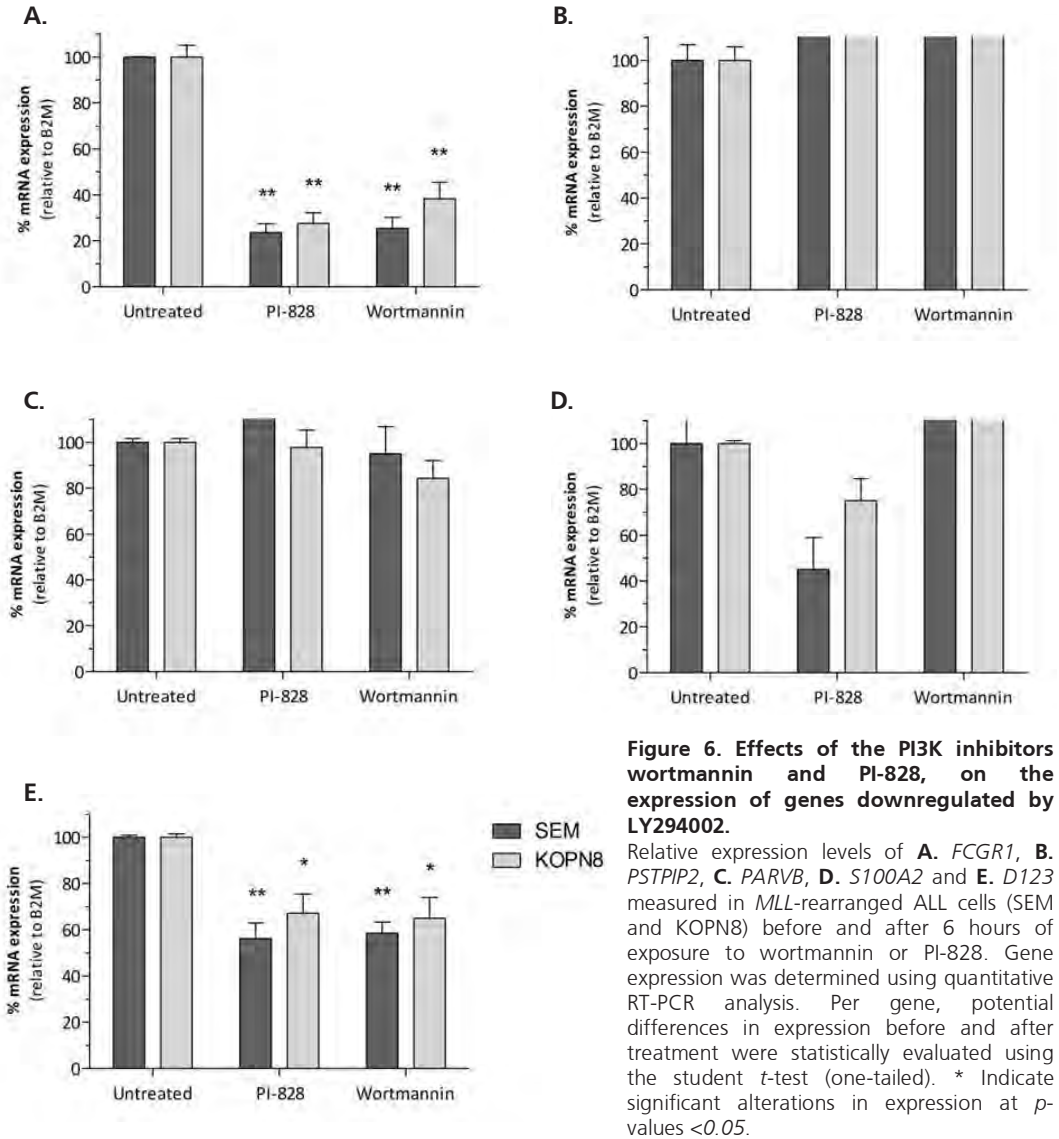


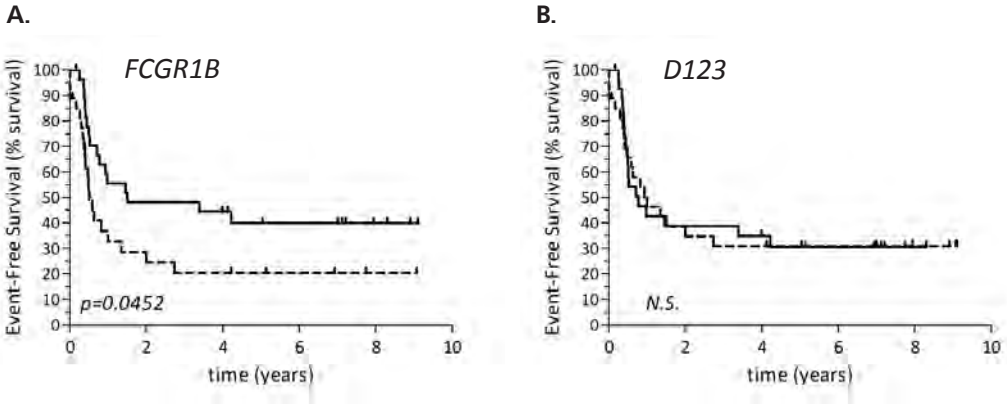
Figure 6. Effects of the PI3K inhibitors wortmannin and PI-828, on the expression of genes downregulated by LY294002.

Relative expression levels of **A.** *FCGR1*, **B.** *PSTPIP2*, **C.** *PARVB*, **D.** *S100A2* and **E.** *D123* measured in *MLL*-rearranged ALL cells (SEM and KOPN8) before and after 6 hours of exposure to wortmannin or PI-828. Gene expression was determined using quantitative RT-PCR analysis. Per gene, potential differences in expression before and after treatment were statistically evaluated using the student *t*-test (one-tailed). * Indicate significant alterations in expression at *p*-values *<0.05*.

To further investigate overlapping mechanisms by which wortmannin and PI-828 induce *in vitro* prednisolone sensitivity comparable to the PI3K inhibitor LY294002 in *MLL*-rearranged ALL cells, we treated the *MLL*-rearranged SEM and KOPN8 cell lines with either 5 µM wortmannin or 5-10 µM PI-828 for 6 hours and subsequently performed quantitative RT-PCR to search for differences in gene expression. For this, we only focused on the genes which were differentially expressed in these cell lines when treated with LY294002 (*FCGR1B, PSTPIP2, PARVB, S100A2* and *D123*). From this, only *FCGR1B* and *D123* were significantly downregulated in both *MLL*-rearranged cell lines after treatment with either wortmannin or PI-828 (**Figure 6**).

***FCGR1B*, a potential prednisolone-sensitizing target in *MLL*-rearranged leukemia cells**

Next, we determined the relation between VSN-normalized *FCGR1B* or *D123* expression and clinical outcome of *MLL*-rearranged infant ALL. We used the median expression as cut-off values to classify patients expressing either high-levels or low-levels of these genes. Interestingly, *FCGR1B*, but not *D123*, appeared predictive for clinical outcome (**Figure 7**). *MLL*-rearranged infant ALL patient samples expressing high-levels of *FCGR1B* had an inferior outcome when compared to patient samples expressing low-levels of *FCGR1B* (**Figure 7A**; *FCGR1B* Log-rank test *p<0.05* and **Figure 7B**; *D123* Log-rank test *p=0.966*). Subsequently, to determine the role of *FCGR1B* expression for *in vitro* prednisolone resistance in *MLL*-rearranged ALL cells, we virally transduced SEM cells with constructs stably expressing either shRNAs against human *FCGR1B* mRNA or expressing non-targeting shRNAs (non-silencing control: NSC). Using quantitative RT-PCR, we confirmed knock-down of *FCGR1B* (**Figure 7C**). Downregulation of *FCGR1B* mRNA induced a proliferation arrest (**Figure 7D**) and indeed led to prednisolone sensitization in prednisolone-resistant SEM cells (**Figure 7E and 7F**), suggesting a prominent role of *FCGR1B* in prednisolone resistance in *MLL*-rearranged infant ALL.



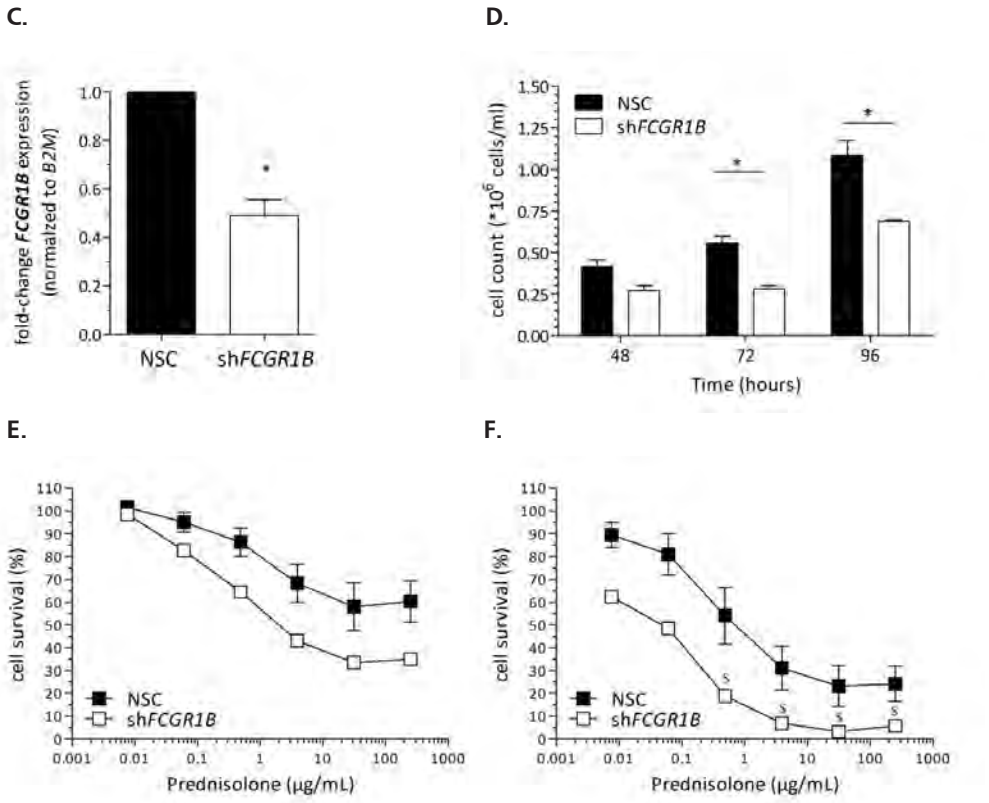


Figure 7. Role of *FCGR1B* in *in vitro* prednisolone resistance in *MLL*-rearranged infant ALL
Event-free survival analysis of *MLL*-rearranged infant ALL patients expressing either high (dashed lines) or low (solid lines) levels of **A.** *FCGR1B* or **B.** *D123*. The median expression value in the entire cohort was used as the cuf-off value. Differences in outcome were statistically analyzed using the Log-rank test. **C.** Quantitative RT-PCR showing the relative expression of *FCGR1B* in the *MLL*-rearranged cell line SEM after stably transduced with shRNAs directed against human *FCGR1B* on non-silencing control (NSC). * Indicate significant alterations in *FCGR1B* expression at *p*-values *<0.05*. **D.** Viable cell counts at indicated time points in SEM displaying knocked-down *FCGR1B*, or cells transduced with NSC shRNAs. *In vitro* prednisolone response, as determined by **E.** 2 days or **F.** 4 days MTT assays in SEM cells with or without suppressed *FCGR1B*.

DISCUSSION

Glucocorticoids (GCs), like prednisone and dexamethasone, are essential drugs in the treatment of childhood ALL, and both the *in vitro* and *in vivo* GC-response represent strong predictors of clinical outcome (5, 8-9). Infant patients diagnosed with *MLL*-rearranged ALL have a poor outcome that is related to GC resistance (4-5, 21). Therefore, this type of high-risk ALL may potentially benefit from additional therapeutic strategies circumventing resistance to these drugs. We here identified the PI3K inhibitor LY294002 to be a suitable candidate for this purpose. Apart from displaying strong anti-leukemic effects by itself, LY294002 significantly sensitized *MLL*-rearranged leukemia cells to prednisolone, while these effects were not observed for leukemic cells that do not carry *MLL* translocations. Moreover, the prednisolone sensitizing effects of LY294002 on two *MLL*-rearranged ALL cell lines appeared to be accompanied by consistent down-regulation of five genes, including *PARVB, D123, FCGR1B, PSTPIP2* and *S100A2*. All of these genes are highly expressed in prednisolone-resistant *MLL*-rearranged infant ALL samples, but not in prednisolone-sensitive samples. Thus, the basis for the prediction of LY294002 as a prednisolone-resistance modulating agent by connectivity map analysis may to a large extent rely on the down-regulation of these particular genes.

The fact that LY294002 represents a PI3K inhibitor (22,23), suggests the involvement of PI3K signaling in maintaining prednisolone resistance in *MLL*-rearranged infant ALL. Further supporting a role for PI3K in causing resistance to prednisolone in this specific type of leukemia is our observation that other known PI3K inhibitors (including wortmannin and PI-828), like LY294002, also induced prednisolone sensitivity. Contradictory, downstream inhibition of the PI3K/Akt/mTOR pathway by rapamycin (inhibitor of mTOR) has been shown to induce prednisolone sensitivity in resistant pediatric ALL cells (11), but appeared to have no effect on ALL cells carrying *MLL* translocations (14). Yet, here we show that inhibition of PI3K does sensitize *MLL*-rearranged ALL cells, suggesting that PI3K signaling independent of the PI3K/Akt/mTOR pathway is more important in this specific malignancy.

Evidence for a relation between the genes significantly down-regulated by LY294002 and PI3K signaling is lacking. Nonetheless, down-regulation of one of these genes enhanced leukemic cell death during co-exposures of LY294002 and prednisolone. *FCGR1* (or CD64), encoding the high affinity receptor for IgG (or FcγRI), is frequently expressed on Acute Myeloid Leukemia (AML) cells, in particular on AML cells carrying *MLL* translocations (24). Recombinant immunotoxins directed against CD64 effectively induced apoptosis in CD64+ AML cells (both cell lines and primary patient samples) *in vitro* (25). Moreover, anti-CD64 immunotoxins almost completely inhibited leukemic cell proliferation in a NOD/SCID AML mouse model (26). Thus, effective down-regulation of *FCGR1*B (CD64) by LY294002 may certainly provide additional therapeutic value in CD64-expressing prednisolone-resistant *MLL*-rearranged ALL cells. Taken together, LY294002-mediated down-regulation of *FCGR1B* provides plausible explanations for the cytotoxic effects on *MLL*-rearranged ALL cells by the compound itself, as well as for the observed sensitizing effects to prednisolone.

From a therapeutic point of view, using connectivity mapping to identify potentially favorable agents for a certain clinical application comes with an additional advantage: most of the

compounds present in the connectivity map are FDA-approved bioactive molecules. Therefore these agents can easily be implemented in current therapies or clinical trials. Interestingly, multiple reports already demonstrated positive effects on drug resistance for all compounds used in the present study, with the exception of the novel agent PI-828. For instance, both LY294002 and wortmannin have similar anti-proliferative effects of imatinib (Gleevec) on Chronic Myeloid Leukemia (CML) cells, even in case of imatinib-resistance (27). Moreover, LY294002 appeared to induce synergistic anti-leukemic effects with the cytidine analog Ara-C in primary AML (28) and acute megakaryocytic leukemia cells (29). In addition, wortmannin reversed the multi-drug resistance phenotypes in both acute and chronic leukemias (30-32). Hence, drug-sensitizing actions ascribed to widely used PI3K inhibitors are not uncommon in hematopoietic malignancies. Yet, to our knowledge, none of these agents had earlier been shown to modulate glucocorticoid resistance in leukemia.

In conclusion, we here demonstrated that PI3K inhibitors reversed prednisolone resistance in *MLL*-rearranged infant ALL. Studies on the implementation of PI3K inhibitors (*e.g.* LY294002) in current treatment regimes for this aggressive type of leukemia should be done and may lead to more favorable responses to glucocorticoids, and with that improving the prognosis.

REFERENCES

1. Greaves MF. Infant leukaemia biology, aetiology and treatment. *Leukemia*. 1996 Feb;10(2):372-7.

2. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, *et al.* A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007 Jul 21;370(9583):240-50.

3. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med*. 2004 Apr 8;350(15):1535-48.

4. Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, *et al.* Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants. *Leukemia*. 1998 Sep;12(9):1344-8.

5. Dordelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, *et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*. 1999 Aug 15;94(4):1209-17.

6. Frankel LS, Ochs J, Shuster JJ, Dubowy R, Bowman WP, Hockenberry-Eaton M, *et al.* Therapeutic trial for infant acute lymphoblastic leukemia: the Pediatric Oncology Group experience (POG 8493). *J Pediatr Hematol Oncol*. 1997 Jan-Feb;19(1):35-42.

7. Reaman GH, Sposto R, Sensel MG, Lange BJ, Feusner JH, Heerema NA, *et al.* Treatment outcome and prognostic factors for infants with acute lymphoblastic leukemia treated on two consecutive trials of the Children's Cancer Group. *J Clin Oncol*. 1999 Feb;17(2):445-55.

8. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, *et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *J Clin Oncol*. 2003 Sep 1;21(17):3262-8.

9. Kaspers GJ, Veerman AJ, Pieters R, Van Zantwijk CH, Smets LA, Van Wering ER, *et al.* In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. *Blood*. 1997 Oct 1;90(7):2723-9.

10. Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJ, Kazemier KM, *et al.* Gene expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *N Engl J Med*. 2004 Aug 5;351(6):533-42.

11. Wei G, Twomey D, Lamb J, Schlis K, Agarwal J, Stam RW, *et al.* Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. *Cancer Cell*. 2006 Oct;10(4):331-42.

12. Lamb J. The Connectivity Map: a new tool for biomedical research. *Nat Rev Cancer*. 2007 Jan;7(1):54-60.

13. Lamb J, Crawford ED, Peck D, Modell JW, Blat IC, Wrobel MJ, *et al.* The Connectivity Map: using gene expression signatures to connect small molecules, genes, and disease. *Science*. 2006 Sep 29;313(5795):1929-35.

14. Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, *et al.* Association of high-level MCL1 expression with in vitro and in vivo prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia. *Blood*. 2010 Feb 4;115(5):1018-25.

15. Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R, *et al.* Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell*. 2002 Mar;1(2):133-43.

16. Armstrong SA, Staunton JE, Silverman LB, Pieters R, den Boer ML, Minden MD, *et al.* MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat Genet*. 2002 Jan;30(1):41-7.

17. Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, *et al.* Gene expression profiling-based dissection of MLL translocated and MLL germline acute lymphoblastic leukemia in infants. *Blood*. 2010 Apr 8;115(14):2835-44.

18. Spijkers-Hagelstein JAP, Mimoso Pinhanços S, Schneider P, Pieters R, Stam RW. Src kinase-induced phosphorylation of annexin A2 mediates glucocorticoid resistance in *MLL*-rearranged infant acute lymphoblastic leukemia. *Leukemia*. 2013. 27(5):1063-1071.

19. Kaspers GJ, Veerman AJ, Pieters R, Broekema GJ, Huismans DR, Kazemier KM, *et al.* Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. *Br J Cancer*. 1994 Dec;70(6):1047-52.

20. Pieters R, Loonen AH, Huismans DR, Broekema GJ, Dirven MW, Heyenbrok MW, *et al.* In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. *Blood*. 1990 Dec 1;76(11):2327-36.

21. Ramakers-van Woerden NL, Beverloo HB, Veerman AJ, Camitta BM, Loonen AH, van Wering ER, *et al.* In vitro drug-resistance profile in infant acute lymphoblastic leukemia in relation to age, MLL rearrangements and immunophenotype. *Leukemia*. 2004 Mar;18(3):521-9.

22. Kong D, Yamori T. Phosphatidylinositol 3-kinase inhibitors: promising drug candidates for cancer therapy. *Cancer Sci.* 2008 Sep;99(9):1734-40.

23. Workman P, Clarke PA, Raynaud FI, van Montfort RL. Drugging the PI3 kinome: from chemical tools to drugs in the clinic. *Cancer Res*. 2010 Mar 15;70(6):2146-57.

24. Munoz L, Nomdedeu JF, Villamor N, Guardia R, Colomer D, Ribera JM, *et al.* Acute myeloid leukemia with MLL rearrangements: clinicobiological features, prognostic impact and value of flow cytometry in the detection of residual leukemic cells. *Leukemia*. 2003 Jan;17(1):76-82.

25. Tur MK, Huhn M, Thepen T, Stocker M, Krohn R, Vogel S, *et al.* Recombinant CD64-specific single chain immunotoxin exhibits specific cytotoxicity against acute myeloid leukemia cells. *Cancer Res.* 2003 Dec 1;63(23):8414-9.

26. Zhong RK, van de Winkel JG, Thepen T, Schultz LD, Ball ED. Cytotoxicity of anti-CD64-ricin a chain immunotoxin against human acute myeloid leukemia cells in vitro and in SCID mice. *J Hematother Stem Cell Res.* 2001 Feb;10(1):95-105.

27. Marley SB, Lewis JL, Schneider H, Rudd CE, Gordon MY. Phosphatidylinositol-3 kinase inhibitors reproduce the selective antiproliferative effects of imatinib on chronic myeloid leukaemia progenitor cells. *Br J Haematol*. 2004 May;125(4):500-11.

28. Grandage VL, Gale RE, Linch DC, Khwaja A. PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, Mapkinase and p53 pathways. *Leukemia*. 2005 Apr;19(4):586-94.

29. Edwards H, Xie C, LaFiura KM, Dombkowski AA, Buck SA, Boerner JL, *et al.* RUNX1 regulates phosphoinositide 3-kinase/AKT pathway: role in chemotherapy sensitivity in acute megakaryocytic leukemia. *Blood*. 2009 Sep 24;114(13):2744-52.

30. Neri LM, Borgatti P, Tazzari PL, Bortul R, Cappellini A, Tabellini G, *et al*. The phosphoinositide 3-kinase/AKT1 pathway involvement in drug and all-trans-retinoic acid resistance of leukemia cells. *Mol Cancer Res*. 2003 Jan;1(3):234-46.

31. Tabellini G, Cappellini A, Tazzari PL, Fala F, Billi AM, Manzoli L, *et al.* Phosphoinositide 3-kinase/Akt involvement in arsenic trioxide resistance of human leukemia cells. *J Cell Physiol*. 2005 Feb;202(2):623-34.

32. O'Gorman DM, McKenna SL, McGahon AJ, Knox KA, Cotter TG. Sensitisation of HL60 human leukaemic cells to cytotoxic drug-induced apoptosis by inhibition of PI3-kinase survival signals. *Leukemia*. 2000 Apr;14(4):602-11.

CHAPTER 7

Frequencies and prognostic impact of *RAS* mutations in *MLL*-rearranged acute lymphoblastic leukemia in infants

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ABSTRACT

Acute lymphoblastic leukemia (ALL) in infants represents an aggressive malignancy associated with a high incidence (~80%) of translocations involving the *Mixed Lineage Leukemia (MLL)* gene. Attempts to mimic MLL-fusion-driven leukemogenesis in mice raised the question whether these fusion proteins require secondary hits. *RAS* mutations are suggested as candidates. Earlier results on the incidence of *RAS* mutations in *MLL*-rearranged ALL are inconclusive. Therefore, we studied frequencies and relation with clinical parameters of *RAS* mutations in a large cohort of infant ALL patients.

Using conventional sequencing analysis, we screened *neuroblastoma RAS viral (v-ras) oncogene homolog gene (NRAS)*, *v-Ki-ras Kirsten rat sarcoma viral oncogene homolog gene (KRAS)*, and *v-raf murine sarcoma viral oncogene homolog B1 gene (BRAF)* for mutations in a large cohort (n=109) of infant ALL patients and studied the mutations in relation to several clinical parameters, and in relation to *Homeobox gene A9* expression and the presence of *ALL1 fused gene 4-Mixed Lineage Leukemia (AF4-MLL)*.

Mutations were detected in ~14% of all cases, with a higher frequency of ~24% in t(4;11)-positive patients (p=0.04). Furthermore, we identified *RAS* mutations as an independent predictor (p=0.019) for poor outcome in *MLL*-rearranged infant ALL, with a hazard ratio of 3.194 (95% confidence interval:1.211-8.429). Also, *RAS*-mutated infant ALL have higher white blood cell counts at diagnosis (p=0.013), and are more resistant to glucocorticoids *in vitro* (p<0.05). Finally, we demonstrate that *RAS* mutations, and not the lack of *Homeobox gene A9* expression nor the expression of *AF4-MLL* are associated with poor outcome in t(4;11)-rearranged infant ALL.

We conclude that the presence of *RAS* mutations in *MLL*-rearranged infant ALL is an independent predictor for a poor outcome. Therefore, future risk-stratification based on abnormal *RAS*-pathway activation and *RAS*-pathway inhibition could be beneficial in *RAS*-mutated infant ALL patients.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) in infants (<1 year of age) represents an aggressive, early onset type of leukemia characterized by high relapse rates during treatment, and an Unfavorable clinical outcome (1). This poor prognosis is associated with a high incidence of balanced chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene, which occur in ~80% of the infant ALL cases (1). The most common *MLL* translocation in infant ALL is t(4;11), in which the N-terminus of *MLL* (chromosome 11q23) fuses to the C-terminus of *AF4* (chromosome 4q23). As the joining of *MLL* and *AF4* occurs in-frame, the t(4;11) translocation generates a unique fusion gene encoding the chimeric, and supposedly oncogenic MLL-AF4 fusion protein. Other recurrent in-frame *MLL* rearrangements found among infant ALL patients are t(11;19) and t(9;11), giving rise to the fusion proteins MLL-ENL and MLL-AF9 respectively. The presence of an *MLL* translocation is the strongest independent predictor of an adverse outcome in infant ALL patients (2).

Over the past decades numerous studies provided important insights into the biology and pathogenesis of *MLL*-rearranged ALL, but so far *in vivo* validation of these achievements are hampered by the lack of genuine animal models accurately recapitulating this severe malignancy. Although various attempts have been made to develop mouse models mimicking leukemogenesis of human t(4;11)-positive ALL, these mice displayed propensities towards developing lymphomas or leukemia with phenotypes that significantly differ from those found in humans (3-5). Another discrepancy between murine MLL-AF4 models and t(4;11)-positive ALL in infants is disease latency. In human infants, *MLL* translocations arise *in utero* and rapidly lead to the development of overt leukemia, often at or shortly after birth (6). In contrast, most MLL-AF4 mouse models show mean latency periods of ~12-14 months (3, 5). Moreover, in *MLL*-rearranged infant ALL, short disease latency is strongly associated with a poor clinical outcome (2, 7).

Collectively, these inconsistencies form the basis of the question whether MLL fusion proteins (like MLL-AF4) alone are sufficient to induce ALL, or that these chimeric proteins require cooperative genetic lesions. Bursen *et al.* recently found that not MLL-AF4 but its reciprocal fusion protein AF4-MLL (independent of the presence of MLL-AF4) was capable of inducing pro-B ALL in mice, suggesting that in t(4;11)-positive ALL both fusions may function as cooperative oncoproteins (8). Tamai *et al.* showed that in a transgenic mouse model the latency period of MLL-AF4-induced B-cell leukemia/lymphoma can significantly be shortened by the addition of a *KRAS* mutation (9). Moreover, recent observations demonstrated that the MLL-AF4 fusion protein can activate Elk-1 through the *RAS*-pathway, which supports the involvement of *RAS* signaling in the pathogenesis of *MLL*-rearranged leukemia (10). Based on these findings it may be hypothesized that *RAS* mutations represent important secondary "hits". Recent studies on the incidence of *RAS* mutations in *MLL*-rearranged ALL demonstrate inconsistent results in limited patient groups. For instance, Liang *et al.* reported *RAS* mutations in 10/20 (50%) of the cases, while Mahgoub *et al.* could not identify *RAS* mutations among thirteen *MLL*-rearranged ALL samples (11-12). Besides, Tamai *et al.* speculate that the short latency in their *KRAS* mutation-positive mouse model is likely due to an acceleration of

leukemo-lymphomagenesis by a collaborative upregulation of *HOXA9* (9). *HOXA* over-expression is often believed to be a hallmark of *MLL*-rearranged leukemia and has recently been proposed to be required for leukemia survival of *MLL*-rearranged acute myeloid leukemia cells (13). Our recent gene expression profiling study revealed the presence of two distinctive subgroups of MLL-AF4 positive ALL cases; those with and those without *HOXA* expression, with patients lacking *HOXA* expression being at high risk of disease relapse (14). Based on this finding, as well as on the report demonstrating a prominent oncogenic role for AF4-MLL (8), and the results demonstrating accelerated MLL-AF4-driven leukemogenesis in the presence of a *KRAS* mutation, Tamai *et al.* proposed the following subdivision of t(4;11)-positive ALL: one group representing AF4-MLL-driven and *HOXA*-independent leukemogenesis, and another group displaying MLL-AF4 and *HOXA* dependence requiring additional genetic hits, such as *RAS* mutations, to accelerate leukemogenesis (9).

Yet, the precise frequencies and the potential role (in terms of disease aggressiveness) of *RAS* mutations in *MLL*-rearranged infant ALL, and their relation with *HOXA* expression and/or the presence of *AF4-MLL* remain unclear. Therefore, we screened a large cohort (n>100) of primary infant ALL samples for *NRAS, KRAS* and *BRAF* mutations. To further determine the clinical relevance, these mutations were studied in relation to several clinical parameters, as well as to *HOXA* expression and the presence of *AF4-MLL*.

DESIGN AND METHODS

Patient samples and cell lines

Bone marrow or peripheral blood samples from untreated infants (below one year of age) diagnosed with ALL were collected at the institutes participating in the international collaborative INTERFANT protocol (2). Informed consent was obtained according to the Helsinki declaration, and approved by the Institutional Review Board of the Erasmus University Medical Center. All samples were processed as described before (15). The t(4;11)-positive cell lines SEM, RS4;11, and MV4-11 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), BEL-1 was a kind gift from Dr. Tang (University Paris, France). The t(11;19)-positive cell line KOPN-8 was purchased from The Global Biosource Center (ATCC, Middlesex, UK). All cell lines were maintained as suspension cultures in RPMI 1640 with LAlanyl-L-Glutamine (Invitrogen Life Technologies, Breda, the Netherlands) supplemented with 10% FCS (Integro, Zaandam, the Netherlands).

DNA and RNA extraction

Genomic DNA and RNA were extracted from ~5x10^6 leukemic cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified on a Nanodrop ND-1000 spectrophotometer (Isogen). The integrity of DNA and RNA was assessed on standard 0.8% or 1.5% agarose gels, respectively.

Detection of *NRAS*, *KRAS* and *BRAF* mutations

Using PCR and sequence analysis, mutation hotspots were screened in *NRAS* and *KRAS* exon one and two, and in *BRAF* exon 15. (11, 16) Amplicons were generated on a 2720 Thermal cycler (Applied Biosystems, Foster City, CA, USA). A PCR mixture 25 µl containing 2.5 units of Amplitaq Gold polymerase (Applied Biosystems), PCR Buffer II (Applied Biosystems), 1.5 mM $MgCl_2$, 0.3 mM deoxyribonucleotide triphosphates (dNTPs) (Promega, Madison, WI, USA), 1 µM of forward and reverse primer, and ~40 ng of gDNA as a template was used. Cycling conditions were: polymerase activation at 94°C for 5 min, following 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and one additional hold at 72°C for 10 min. *NRAS* Exon 1 primers target the entire exon 1, with the exception of the first 2 nucleotides. *NRAS* Exon 2 primers target the entire exon 2, with the exception of the first 15 nucleotides. *KRAS* 1 and *KRAS* 2 primers target the entire exons 1 and 2.

Table 1. Sequences of primers

Primer	Sequence (5'-3')
***NRAS* Exon 1 Fw**	GTTTTCCCAGTCACGACGACTGAGTACAAACTGGTGG
***NRAS* Exon 1 Rv**	CAGGAAACAGCTAGTACTGCATAACTGAATGTATACCC
***NRAS* Exon 2 Fw**	GTTTTCCCAGTCACGACCAAGTGGTTATAGATGGTGAAACC
***NRAS* Exon 2 RV**	CAGGAAACAGCTATGACAAGATCATCCTTTCAGAGAAAATAAT
***KRAS* Exon 1 Fw**	GTTTTCCCAGTCACGACGGTGAGTTTGTATTAAAAGGTACTGGTG
***KRAS* Exon 1 Rv**	CAGGAAACAGCTATGACCCTGTATTGTTGGATCATATTCGTCC
***KRAS* Exon 2 Fw**	GTTTTCCCAGTCACGACGGATTCCTACAGGAAGCAAGTAGTAA
***KRAS* Exon 2 Rv**	CAGGAAACAGCTATGACCTATAATGGTGAATATCTTCAAATGATTTAGT
***BRAF* Exon 15 Fw**	GTTTTCCCAGTCACGACTCATAATGCTTGCTCTGATAGGA
***BRAF* Exon 15 Rv**	CAGGAAACAGCTATGACGGCCAAAATTTAATCAGTGGA
M13 Fw	CAGGAAACAGCTATGAC
M13 Rv	GTTTTCCCAGTCACGAC

Primer sequences were adapted from previous publications (11, 16) and modified by additional M13 tags (Table 1). Sequence analysis of both sense and antisense strands was carried out using M13 primers, and the BigDye terminator v1.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturers' recommendations, and analyzed on an Applied Biosystems 3130x/Genetic Analyzer. The CLC Workbench software (CLCbio, Aarhus, Denmark) was used to analyze the sequences; references are listed in Table 2. All mutations were confirmed in replicate sequences.

Table 2. Sequence references

Primer	References
NRAS Exon 1	ENSE00001364464
NRAS Exon 2	ENSE00001450282
KRAS Exon 1	ENSE00001189804
KRAS Exon 2	ENSE00000936617
BRAF Exon 15	ENSE00002324725

www.ensembl.org, release 59, date: 22th of April 2011

In vivo prednisone and *in vitro* prednisolone responses

In vivo prednisone responses, assessed during a prophase of one week of daily systemic prednisone (60 mg/m^2) administration before preceding combination chemotherapy, were available for a subset of patients. Responses are defined as good, when blast counts in the peripheral blood dropped below 1000 cells/μL, and poor when more than 1000 cells/μL remained detectable (2, 17). *In vitro* drug cytotoxicity of prednisolone (the active metabolite of prednisone) and dexamethasone was available for a subset of patients. The *in vitro* drug cytotoxcity was determined using 4-day MTT assays as described elsewhere (18).

Gene expression profiles

Due to our recently published gene expression profiling (GEP) study (14) microarray data (Affymetrix HU133plus2.0) was available for a part of the patient samples used in this study. Generation of these gene expression profiles has been described before (14). Data was deposited in the GEO database (19) under accession number GSE19475. Because of our interest in the relation of *HOXA* expression and *RAS* mutations, we extracted and studied the expression of *HOXA9* from the existing dataset (probe sets: 209905_at, and 214651_s_at). GEP data was available for 27 of the 38 t(4;11)-positive infant ALL cases.

Statistical Analysis

Fisher's Exact Test was used to compare mutation frequencies in different patient groups and Mann-Whitney *U*-Test to compare the median age at diagnosis. Event-free survival (EFS) and overall survival (OS) curves were estimated using the Kaplan-Meier method and analyzed by Log-rank (Mantel-Cox) tests. EFS is defined as time from diagnosis to death in induction, disease relapse, the emergence of secondary malignancies, or death in complete remission. OS is defined as time from diagnosis to death from any cause. Cumulative incidence of relapse (CIR) is defined as time from complete remission to disease relapse, adjusted for death as competing risk. Patients who did not achieve complete remission were allocated an event at time-point zero in the EFS and CIR analyses. Multivariate analysis of prognostic factors was performed by Cox regression model based on EFS and the Wald Backward Test (entry probability p=0.05 and removal probability p=0.10) was used for the joint analysis of age at diagnosis, white blood cell (WBC) counts, *in vitro* prednisolone response (LC_{50}: lethal concentration to 50% of the leukemic cells), *in vivo* prednisone response, and *RAS* mutations. *RAS* mutations and *in vivo* prednisone response analyzed as dichotomous variables, the other variables as continuous. Infant ALL patients without *MLL*-rearrangements were excluded from these analyses as the prognosis of these patients is significantly more favorable (2). CIR was computed with the statistical environment R version 2.14.0 using Bioconductor packages (R Development Core Team, 2011). The other analyses, were performed with SPSS Statistics version 17.0 (SPSS Inc. Chicago, IL, USA). All tests were two-tailed and a p-value less than 0.05 was considered significant.

RESULTS

RAS and *BRAF* mutations in infant ALL

RAS and *BRAF* mutation screening was performed in 109 primary infant ALL samples, as well as in an additional four matched relapsed samples. Patient's characteristics are listed in **Table 3**.

Table 3. Patient characteristics infant ALL patients

	No. of patients (%)
Sex (*n*=108)	
Male	43 (39.8)
Female	65 (60.1)
Age (median, range, days, *n*=108)	164 (1-360)
WBC counts at diagnosis x 10^9/L (median, range, *n*=101)	263 (1.4-1332)
Infant ALL (*n*=97 samples)	
pro-B	64 (66.0)
common B	8 (8.3)
pre-B	21 (21.7)
B-lineage not classified	1 (1.0)
Biphenotypic	1 (1.0)
T-cell	2 (2.0)
***MLL*-rearrangement (*n*=109)**	
t(4;11)	38 (34.9)
t(9;11)	11 (10.1)
t(11;19)	28 (25.7)
other 11q23*	14 (12.8)
germline-*MLL*†	18 (16.5)
***AF4*/ *MLL* (*n*=38)**	
positive	23 (60.5)
negative	15 (39.5)

*11q23; *MLL*-rearranged infant ALL patients, with unknown or rare partner gene (including one t(1;11)-, one t(3;11)-, and three t(10;11)-positive patients), common partner genes (t(4;11), and t(11;19)) were excluded by PCR,† Germline-*MLL*; infant ALL patients without *MLL*-rearrangement Abbreviations: WBC; white blood cell

Overall, in 15/109 (13.8%) of the patients a *RAS* mutation was detected, comprising 7/109 (6.4%) patients carrying an *NRAS* mutation, and 8/109 (7.2%) patients bearing a *KRAS* mutation (**Table 4** and **Figure 1**). No *BRAF* mutations were found. Among patients carrying *NRAS* mutations two harbored an exon one mutation at codon 12, and five an exon two mutation at codon 61. All observed *KRAS* mutations were located in exon one, of which four at codon 12 and four in codon 13. (**Table 4** and **Figure 1**). One mutation was found among the four matched relapse samples and displayed a *NRAS* Gln61Lys mutation that was not present in

the corresponding primary diagnosis sample. For the *MLL*-rearranged ALL cell lines, only KOPN-8 carried a *KRAS* mutation at exon one, at codon 12 (Gly12Asp) (**Figure 1E**).

Table 4. *RAS* mutations

Pt.	*MLL*	*AF4-MLL*	Gender	Age at Dx (mo.)	Immuno-phenotype	WBC at Dx ($x10^9$/L)	*NRAS* mutation	*KRAS* mutation
1	t(4;11)	Neg	male	5.5	pro-B	677		Gly12Val
2	t(4;11)	Neg	female	10.8	pre-B	813.7	Gly12Ser	
3	t(4;11)	Pos	male	1.9	pro-B	555		Gly13Asp
4	t(4;11)	Pos	female	4.1	pro-B	326	Gln61Arg	
5	t(4;11)	Neg	female		pro-B	1101.1	Gln61Lys	
6	t(4;11)	Pos	female	1.6	pro-B	358.3		Gly13Asp
7	t(4;11)	Pos	male	3.4	pro-B	348.6		Gly13Asp
8	t(4;11)	Pos	male	6.3	pro-B	204	Gln61Lys	
9	t(4;11)	Pos	female	2.3		204		Gly12Ser
10	t(11;19)		female	3.6	common	916		Gly12Val
11	t(11;19)		male	8.1				Gly12Asp
12	t(9;11)		male	0.8	pro-B	740	Gln61Lys	
13	11q23*		male	10.8	pro-B	5.1	Gln61Lys	
14	11q23*		female	11.8			Gly12Asp	
15	Germline-*MLL*†		female	11.0	common	1.4		Gly13Asp

*****11q23; *MLL*-rearranged infant ALL patients with unknown partner gene,† Germline-*MLL*; infant ALL patients without *MLL*-rearrangement. Mutation in patient 8 occurred in a relapse sample, which was not present in the corresponding diagnostic sample. Abbreviations: Gly; Glycine, Val; Valine, Ser; Serine, Asp; Aspartic acid, Gln; Glutamine, Lys; Lysine

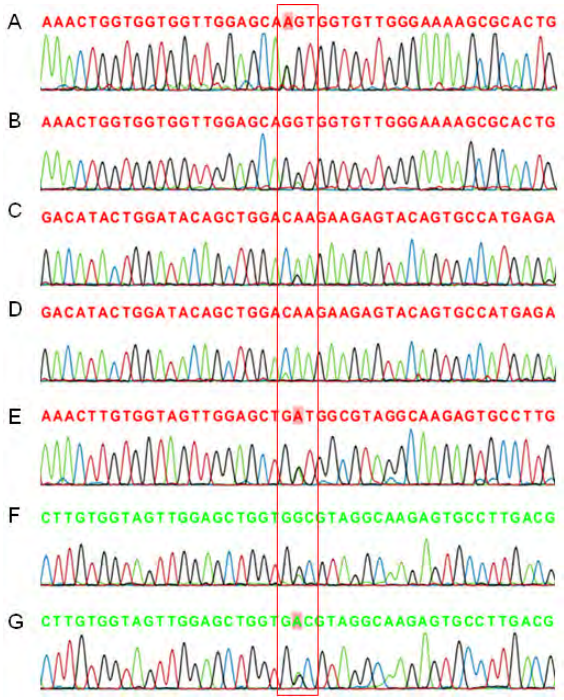

Figure 1. *RAS* mutations
A. *NRAS* exon 1 codon 12 (Gly>Ser) mutation, corresponding with patient 2, **B.** *NRAS* exon 1 codon 12 (Gly>Asp) mutation, corresponding with patient 14, **C.** *NRAS* exon 2 codon 61 (Gln>Arg) mutation, corresponding with patient 4, **D.** *NRAS* exon 2 codon 61 (Gln>Lys) mutation, corresponding with patient 13, **E.** *KRAS* exon 1 codon 12 (Gly>Asp) mutation, corresponding with KOPN-8 cell line, **F.** *KRAS* exon 1 codon 13 (Gly>Asp) mutation, corresponding with patient 6, **G.***KRAS* exon 1 codon 13 (Gly> Asp) mutation, corresponding with patient 7.

Frequency of *RAS* mutations among different infant ALL subtypes

Next we compared the frequencies of *RAS* mutations among different infant ALL subtypes including patients with t(4;11), t(11;19), t(9:11), and infant ALL patients without *MLL* translocations. Interestingly, we found a significantly higher frequency of 9/38 (23.7%) *RAS* mutations in t(4;11)-positive infants (p=0.04) compared to the remaining infant ALL cases, with a frequency of 6/71 (7.8%). In the other infant ALL subtypes the frequencies were not significantly different from the total patient cohort (**Table 5**).

Time of disease onset and *RAS* mutations

Early onset in *MLL*-rearranged infant ALL is associated with a poor clinical outcome (2, 7). The median age at diagnosis of primary *RAS* mutation-negative *MLL*-rearranged infant ALL patients (3.8 months; range 0.0–11.5 months) was not different from the *RAS*-mutated group (5.3 months; range 0.8-11.8 months) (p=0.89). Likewise, *RAS* mutations did not seem to affect disease latency when we analyzed t(4;11)-positive infant ALL patients alone. Also, dividing patients by their age at diagnosis in the following ordinal categories: <3 months, 3-6 months, 6-9 months, 9-12 months, demonstrated no increased frequencies in any of the age groups for neither the total *MLL*-rearranged cohort (p=0,51), nor for t(4;11)-positive patients (p=0.31).

Table 5. Frequencies of *RAS* mutations in *MLL*-subtypes of infant ALL

RAS mutation	t(4;11) (%)	t(9;11) (%)	t(11;19) (%)	11q23* (%)	Germline *MLL* (%)	Total *MLL*-R (%)	Total infant ALL
NRAS							
positive	4 (10.5)	1 (9.1)	0 (0)	2 (14.3)	0 (0)	7 (7.7)	7 (6.4)
negative	34 (89.5)	10 (89.9)	28 (100)	12 (85.7)	18 (100)	84 (92.3)	102 (93.6)
KRAS							
positive	5 (13.2)	0 (0)	2 (7.1)	0 (0)	1 (5.5)	7 (7.7)	8 (7.3)
negative	33 (86.8)	10 (100)	26 (92.9)	14 (100)	17 (94.5)	84 (92.3)	101 (92.7)
NRAS* and/ or *KRAS							
positive	9 (23.7)	1 (9.1)	2 (7.1)	2 (14.3)	1 (5.5)	14 (15.4)	15 (13.8)
negative	29 (76.3)	10(89.9)	26 (92.9)	12 (85.7)	17 (94.5)	77 (84.6)	94 (86.2)
***p*-values**	0.040	1.000	0.346	1.000	0.458		

Differences in frequencies of *RAS* mutations between *MLL*-subtypes. Patient groups were statistically analyzed using Fisher's Exact Test (two-sided) and *p*-value <0.05 was considered significant. *11q23; *MLL*-R (*MLL*-rearranged) infant ALL patients, with unknown or rare partner gene (including one t(1;11)-, one t(3;11)-, and three t(10;11)-positive patients, † Germline-*MLL*; infant ALL patients without *MLL*-rearrangement.

WBC count at diagnosis and *RAS* mutations

High WBC counts at diagnosis have previously been identified as a poor prognostic factor in infant ALL (2). Interestingly, *RAS*-mutated *MLL*-rearranged infants appeared to have significant higher WBCs at diagnosis (p=0.013). Approximately 82% (9/11) of the *RAS* mutation-positive cases showed WBCs higher than $300x10^9$ cells/L, compared to ~45% (33/73) of the *RAS* mutation-negative infants. Similarly, among t(4;11)-positive cases, WBCs higher than 300x109 cells/L were found in 87,5% (7/8) of the mutated cases, and in 41.4% (12/29) of the mutation-negative cases (p=0.018).

Drug resistance of *RAS*-mutated infant ALL patients

A poor *in vivo* response to prednisone represents an adverse prognostic factor in *MLL*-rearranged infant ALL (17), and *MLL*-rearranged infant ALL patients cells are highly resistant to prednisolone and dexamethasone *in vitro* (20). *MLL*-rearranged infant ALL cells bearing a *RAS* mutation at diagnosis appeared significantly (p<0.05) more resistant to both glucocortocoids (**Figure 2A-B**). For t(4;11)-positive samples alone a comparable trend was observed, although the differences did not reach statistical significance (**Figure 2C-D**). No differences were found comparing the *in vivo* prednisone response of *RAS*-mutated and non-*RAS*-mutated *MLL*-rearranged infant ALL patients (p=0.451), nor by comparing *RAS*-mutated and non-*RAS*-mutated t(4;11)-positive cases alone (p=0.635).

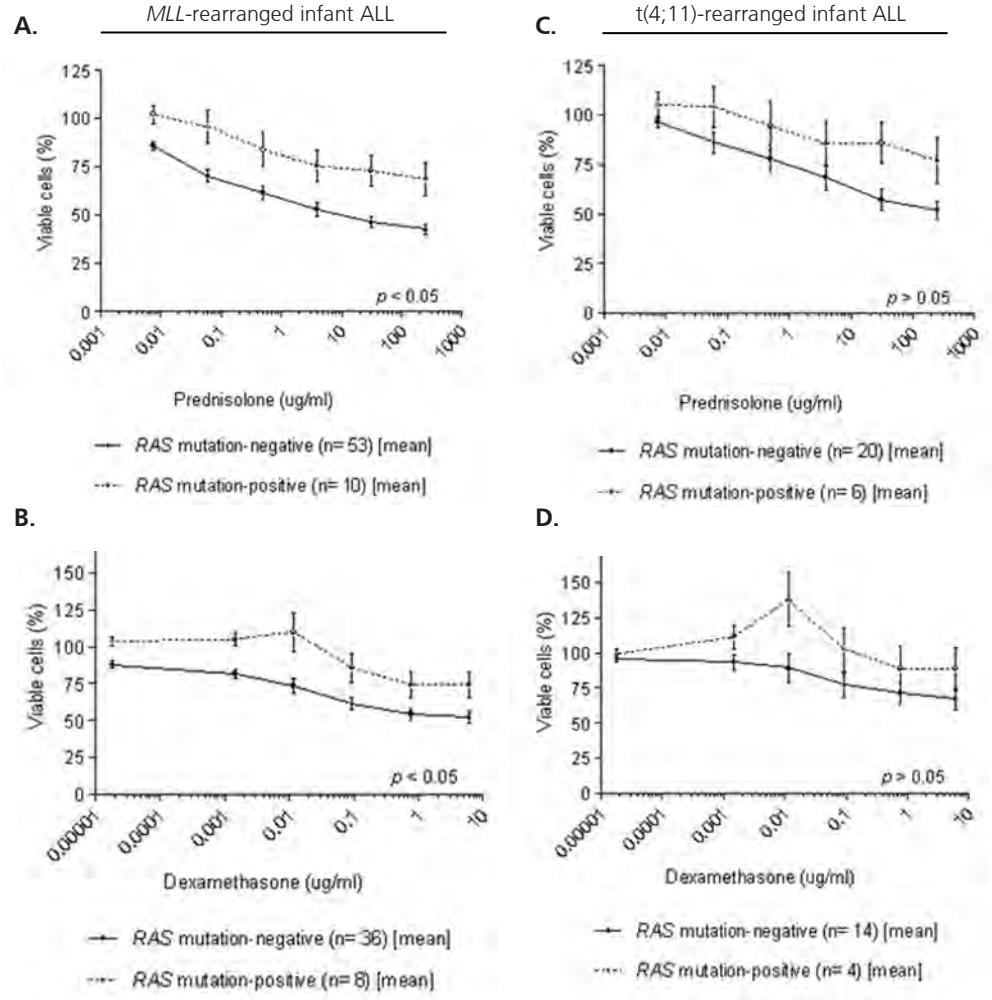

Figure 2: Drug Cytotoxicity of *RAS*-mutated and non-mutated infant ALL patients
A. *In vitro* prednisolone cytotoxicity in *MLL*-rearranged infant ALL patients, **B.** *In vitro* dexamethasone cytotoxicity in *MLL*-rearranged infant ALL patients, **C.** *In vitro* prednisolone cytotoxicity in t(4;11)-rearranged infant ALL patients, **D.** *In vitro* dexamethasone cytotoxicity in t(4;11)-rearranged infant ALL patients. Mean *in vitro* cytotoxicity responses in *RAS*-mutated and non-*RAS*-mutated infant ALL patients were statistically analyzed using Mann-Whitney *U*-test. Error bars represent standard error of the mean. Cytotoxicity data for prednisolone and dexamethasone was available for 63 and 44 *MLL*-rearranged infants ALL patients and 26 and 18 t(4;11)-rearranged infants, respectively.

Besides, studying the control cells (without glucocorticoid treatment) in our *in vitro* cytotoxicity assays, we found *RAS*-mutated *MLL*-rearranged infant ALL cells to display significantly (p=0.022) higher endogeneous viability (**Figure 3**).

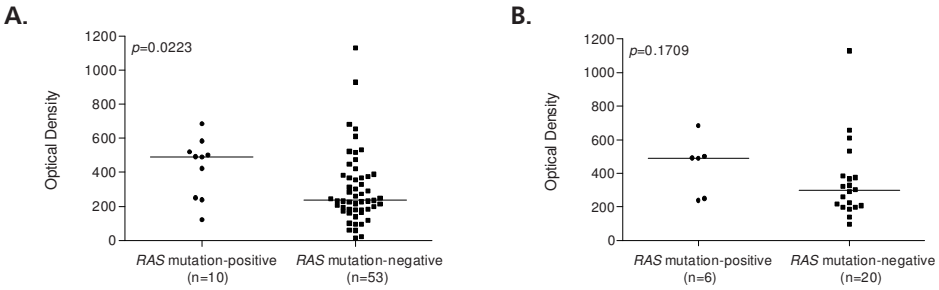

Figure 3. Optical Density (cell viability) of untreated primary infant ALL cells
In our *in vitro* cytotoxicity assays (*i.e.* MTT assays), leukemic cells are cultured in the presence of increasing drug concentrations. Cell viability is determined by measuring the optical density (OD) of the blue formazan derivative of the tetrazolium MTT, which is formed only by viable cells. Here we compared the ODs of control cells (*i.e.* cultured in the absence of drug) between *RAS*-mutated and non-*RAS*-mutated samples derived from **A.** *MLL*-rearranged infant ALL patients and **B.** t(4;11)-positive infant ALL patients. Differences in the ODs between *RAS*-mutated and non-*RAS*-mutated samples were statistically analyzed by the Mann-Whitney *U*-test. The horizontal lines represent the median OD in each group.

Furthermore, we asked whether exposure to glucocorticoids would invoke a positive selection for *RAS*-mutated cells in samples that ostensibly carry subclonal mutations. Therefore we performed a time lapse prednisolone exposure experiment and sequenced the *RAS* mutations in order to determine whether the sequence graphs revealed a positive selection of the mutated clone. However, we did not find any signs of positive selection in both patients: the intensity of the peak corresponding to the mutated nucleotide remained equal throughout the experiment (**Figure 4**). Suggesting that, either the subclone was stable during the experiment or that these mutations may not have been subclonal.

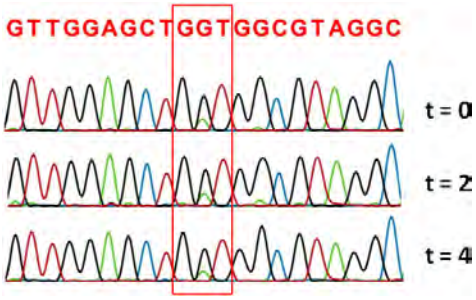

Figure 4. Representative sequences from time lapse *in vitro* prednisolone exposure experiment (*KRAS* exon 1 codon12 (Gly> Asp mutation).
Two infant ALL samples (that appeared to have subclonal *RAS* mutations) were exposed to various concentrations of prednisolone (0 µg/ml, 0.488281 µg/ml, and 250 µg/ml). Cells were harvested from different time-points (Day 0, Day 2, and Day 4) and sequenced for the *RAS* mutations.

Clinical outcome of *RAS*-mutated infant ALL patients

Clinical outcome data was available for 79 *MLL*-rearranged infant ALL cases, with 33 of them being t(4;11)-positive. The presence of a *RAS* mutation at diagnosis was associated with poor outcome in both the *MLL*-rearranged infant ALL patients, as well as in t(4;11)-positive cases alone. Among all *MLL*-rearranged infant ALL patients, the 5-year EFS rates for the *RAS* mutation-positive and *RAS* mutation-negative cases was 0.0±0.0 % vs. 32.7±6.0% (p=0.042), and the 5-year OS was 11.1±10.5% vs. 45.3±6.0% (p=0.08), respectively (**Figure 5A-B**).

CIR analysis showed a slight tendency towards a higher relapse risk for *RAS*-mutated cases, with a 3-year CIR of 66.7±15.7% vs. 48.1±6.1% in non-*RAS*-mutated patients (p=0.119) (**Figure 5C**). Among the t(4;11)-positive cases comparable, but more distinctive, results were found for the 5-year EFS (p=0.019), 5-year OS (p=0.020), and 3-year CIR (p=0.012) (**Figure 5D**, **5E** and **5F**).

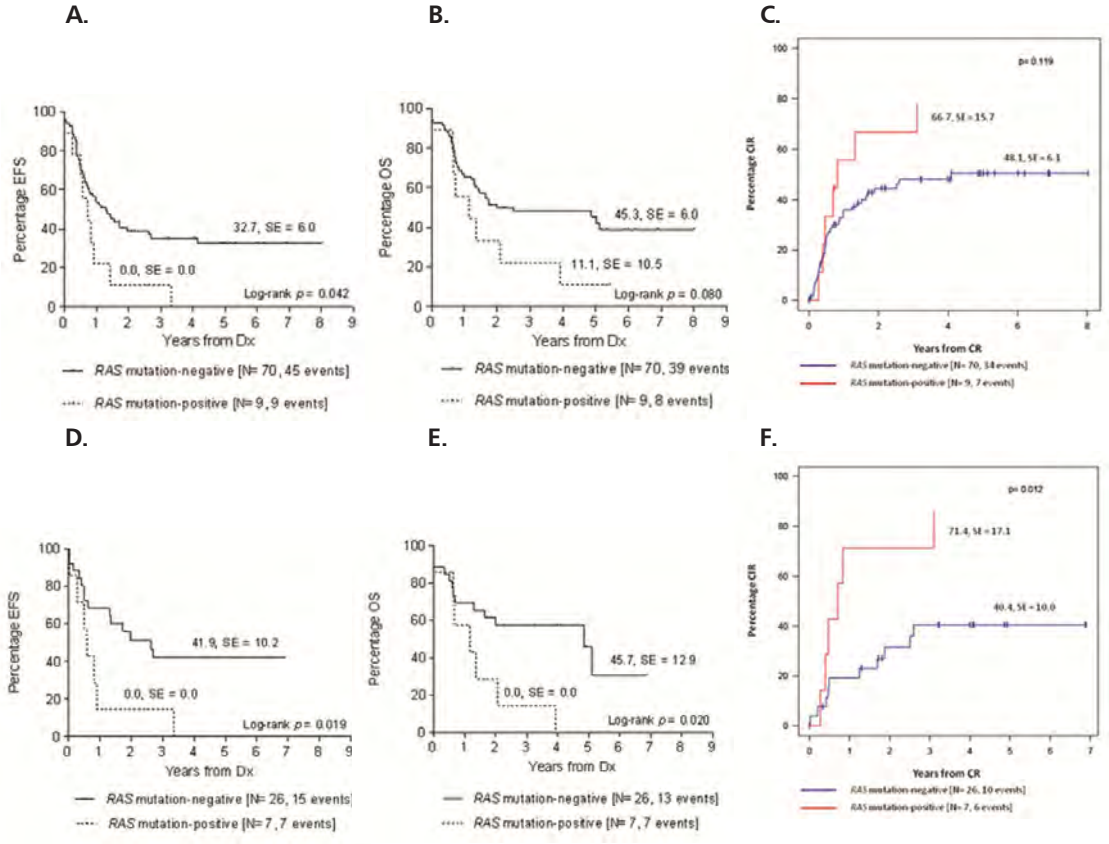

Figure 5. Survival of *RAS*-mutated and non-*RAS*-mutated infant ALL patients

A. 5-year event-free survival (EFS), **B.** 5-year overall survival (OS), **C.** 3-year cumulative incidence of relapse (CIR) for *RAS*-mutated *MLL*-rearranged infant ALL patients. Survival data was available for 79 of the 91 *MLL*-rearranged infant ALL cases. **D.** 5-year EFS, **E**. 5-year OS, **F.** 3-year CIR for *RAS*-mutated t(4;11)-positive infant ALL patients. Survival data was available for 33 of the 38 t(4;11)-positive infant ALL cases.

RAS mutations in relation to *AF4-MLL* and *HOXA* expression in t(4;11)-rearranged infants

We studied the relation between the presence of *AF4-MLL* and *HOXA9* expression in t(4;11)-positive infant ALL samples and the incidence of *RAS* mutations. The occurrence of *RAS* mutations did not differ significantly between cases with *AF4-MLL* (3/15) or without *AF4-MLL* (6/23). Re-analyzing our previously published gene expression profiling data we found that all *RAS* mutation-positive cases lacked *HOXA9* expression (**Figure 6**).

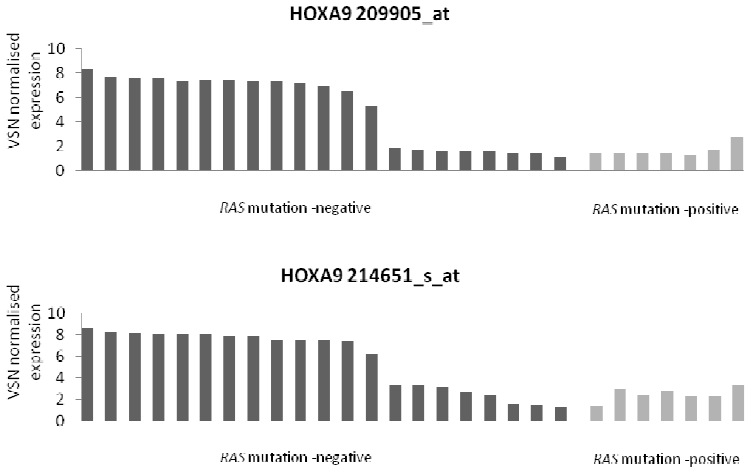

Figure 6. *HOXA9* expression of t(4;11)-rearranged infant ALL patients
HOXA9 mRNA expression (Affymetrix HU133plus2.0 microarray data) in t(4;11)-positive infant ALL patients. Differences in expression between *RAS*-mutated and non-mutated were statistically analyzed by the Mann-Whitney *U*-test, showing significant lower levels of *HOXA9* expression in *RAS*-mutated cases for both probesets tested: 209905_at (p=0.006) and 214651_s_at (p=0.020).

Our earlier observations suggested that t(4;11)-positive infants lacking *HOXA* expression have a worse prognosis than patients expressing high *HOXA* levels (14). However, when excluding the *RAS* mutation-positive cases from this analysis, the association of *HOXA* expression and clinical outcome was lost (p=0.857). Also, no association between *AF4-MLL* expression and clinical outcome was detected (p=0.354), even after excluding the *RAS*-mutated t(4;11)-positive infants (p=0.177). Thus, neither the level of HOXA nor the presence of *AF4-MLL* expression, but the presence of *RAS* mutations seems to dictate the poor prognosis in these patients. Next, we asked whether *RAS* mutations influenced the previously reported prognostic value of high-level *FLT3* expression as well (21). Therefore we studied the overlap between high *FLT3* expression and the presence of *RAS* mutations in our patient cohort, but we could not find any correlation between *FLT3* expression and *RAS* mutations at all. The *RAS*-mutated infants are equally divided between the patients with either *FLT3* high or low expression. Because this equal distribution we had no rational for reanalyzing the previous published prognosis data for *FLT3* expression in

the same manner as we did with the *HOXA* expression, where all *RAS*-mutated patients had low *HOXA* expression.

Multivariate analysis of *RAS* mutations and clinical parameters

Because the previously described clinical parameters in this study are interdependent we performed a Cox regression multivariate analysis, to evaluate the independent prognostic value of *RAS* mutations. This multivariate analysis was fitted on *MLL*-rearranged infants (n=50) from whom all prognostic variables were available. We identified the presence of a *RAS* mutation at diagnosis as an independent predictor (p=0.019) for poor outcome in *MLL*-rearranged infant ALL, with a hazard-ratio (HR) of 3.194 (95% confidence interval (CI): 1.211-8.429) (**Table 6**). Besides *RAS* mutations, low age at diagnosis was identified as an independent predictor (p=0.006, HR: 0.834, 95%-CI: 0.731-0.950) for poor outcome in our *MLL*-rearranged infant ALL cohort. Other variables in the final model were WBC counts at diagnosis (p=0.062, HR: 1.001, 95%-CI: 1.000-1.001) and *in vitro* prednisolone response (p=0.069, HR: 0.997, 95%-CI: 0.997-1.000) (**Table 6**).

Table 6. Univariate and multivariate analysis of prognostic factors of *MLL*-rearranged infant ALL patients

		Univariate analysis			Multivariate analysis	
	Patients	Events	5-year EFS (SE)	*p*-value	HR (95%-CI)	*p*-value
***RAS* mutation**				0.043	3.194 (1.211-8.429)	0.019
Negative	70	46	32.2% (5.9)			
Positive	9	9	0.0% (0.000)			
Age at diagnosis (months)				0.020	0.834 (0.731-0.950)*	0.006
< 3	26	22	15.4% (7.1)			
3-6	25	17	32.0% (9.3)			
6-9	17	10	38.6% (12.4)			
>9	11	6	39.8% (16.3)			
WBC count (x 10^9/ L)				0.022	1.001 (1.000-1.001)†	0.062
< 100	11	7	34.1% (15.0)			
100-300	27	15	40.0% (10.5)			
>300	39	31	19.0% (6.6)			
Response to prednisone prophase				0.602		
Good response (standard risk)	34	22	34.4% (8.3)			
Poor response (high risk)	28	17	36.7% (9.8)			
***In vitro* prednisolone response LC_{50}** (ug/ ul)				0.282	0.997 (0.994-1.000)§	0.069
≤ 0.100	19	13	33.7% (11.8)			
> 0.100 < 150	12	10	16.7% (10.8)			
≥ 150	27	17	35.3% (9.35)			

Univariate and multivariate analysis of the prognostic factors, including age at diagnosis, white blood cell (WBC) count at diagnosis, *in vivo* prednisone response, *in vitro* prednisolone response and *RAS* mutation status, in *MLL*-rearranged infant ALL patients. Multivariate analysis of prognostic factors was performed by Cox regression model based on EFS and the Wald backward test (entry probability p=0.05 and removal probability p=0.1). *RAS* mutations and *in vivo* prednisone response were in the Cox regression model analyzed as dichotomous variables, the other variables were analyzed continuous. This multivariate analysis was fitted on 50 *MLL*-rearranged infants from whom all variables were available. *Hazard-ratio (HR) per unit (months) increase of age, †HR per unit (1 x 10^9/L) increase of WBC, § HR per unit (1 ug/ ul) increase of *in vitro* prednisolone response.

DISCUSSION

Activating *RAS* mutations, resulting in a proliferative advantage, have been observed in several hematopoietic malignancies including, ALL, AML, chronic myelomonocytic leukemia, and juvenile chronic myelogenous leukemia (22-28). Here we report a *RAS* mutation frequency of ~14% in a large (*n*>100) cohort of infant ALL cases, and a frequency of ~24% in infant ALL patients carrying *MLL* translocation t(4;11). These results are not consistent with previously published studies that reported either high *RAS* mutation frequency of 50%, or a total absence of *RAS* mutations in *MLL*-rearranged ALL (11-12). The observed frequencies in these studies may have been compromised by the small patient numbers. However, these frequencies are in concordance with the previously reported frequencies of 6-20.8% *RAS* mutations found in childhood ALL (11, 29-31).

To determine the role of *RAS* mutations in respect of aggressiveness in *MLL*-rearranged infant ALL, we compared several clinical parameters in *RAS* mutation-positive and negative patients. Early onset of a *KRAS* mutation in a MLL-AF4-positive transgenic mouse model was associated with an early disease onset, and therefore suggested to represent a more aggressive leukemia (9). We could not confirm an association between the presence of *RAS* mutations and an early onset of *MLL*-rearranged infant ALL. However, our data showed that *RAS* mutations independently contribute to a poor outcome in *MLL*-rearranged infant ALL patients. Besides, *MLL*-rearranged infant ALL patients carrying *RAS* mutations also display significantly higher WBC counts at diagnosis, and appeared significantly more resistant to the glucocorticoids *in vitro*.

Although conventional Sanger sequencing certainly is not quantitative, 4/7 (57%) of the *NRAS* mutations and 5/8 (62%) of the *KRAS* mutations appeared to be subclonal in our sequencing graphs. Repeated sequence runs on these samples persistently showed that the peak corresponding to the mutated nucleotide remained markedly smaller than the wild-type nucleotide (*e.g.* Figure 1D). If indeed a relatively high number of *RAS* mutations is subclonal, suggesting that not all leukemic cells carry the genetic abnormality, it seems plausible that *RAS* mutations are acquired as secondary hits after the *MLL* fusions arise (for instance during a *MLL*-fusion-positive pre-leukemic state, or even during overt leukemia). An alternative explanation could be that *RAS* mutations are necessary for leukemogenesis and that patients harboring the wild-type *RAS* gene carry mutations in other genes supporting MLL-fusion-driven leukemogenesis. As we only use highly pure leukemic samples (>90% leukemic blasts), this supposed subclonality may not only indicate that a certain portion of the leukemic cells remained unaffected, but also it shows that these mutations are leukemia-specific and are unlikely to be present in germline. Unfortunately, we had no opportunity to validate this, as no germline samples were available. Nonetheless, although several of the identified *RAS* mutations may suggest subclonality, we did not find any differences in clinical parameters or outcome between patients harboring "subclonal" or "clonal" *RAS* mutation (data not shown). In order to confirm subclonality of the *RAS* mutations as implied by our Sanger sequencing results, we used TOPO® TA Cloning (Invitrogen Life Technologies, Breda, the Netherlands) to sequence single PCR-amplified DNA fragments in three patient samples. We found that in all patients the

number of mutated fragments was lower than the expected percentage of ~50% in case the mutation would have been clonal. Hence, these results demonstrate that *RAS* mutations in infant ALL patients can indeed be of a subclonal nature.

The observed presence of a *RAS* mutation in one of the relapse samples, which was not present in the patient-matched primary diagnostic sample, supports the hypothesis of *RAS* mutations as a secondary hit. In line with this, Case *et al.* recently demonstrated that in matched presentation/relapse samples of childhood ALL patients, *KRAS* mutations are predominantly found at relapse, and were observed at very low levels in the matched diagnostic samples (32). In combination, these data could suggest that *RAS* mutations represent secondary hits and that *RAS*-mutated clones may very well contribute to disease aggressiveness, progression, and relapse.

Finally, our data indicates that *RAS*-pathway inhibition could be beneficial for infant ALL. Therapy with specific *RAS* inhibitors would eradicate the *RAS*-mutated leukemic clones, but possibly leave the non-*RAS*-mutated *MLL*-rearranged leukemic cells, especially in the infant ALL that seem to harbor subclonal *RAS* mutations. Although, specific *RAS*-pathway inhibitors maybe not eradicate all leukemic clones, we strongly believe, based on our data, that targeting the *RAS*-mutated clones could lead to a less aggressive disease period and increased survival-rates. Therefore, we would not suggest *RAS*-pathway inhibition as a mono-therapy, but alongside the current infant ALL therapy. Interestingly, several *RAS*-pathway inhibitors, like tipifarib and sorafenib, are already available and currently studied in hematologic malignancies in phase I/II trials. Both compounds are well tolerated, however tipifarib activity did not seem to correlate with *RAS* mutations or *RAS*-pathway-dependent activation (33). On the other hand, phase I/II studies using sorafenib in AML and myelodysplastic syndrome patients, showed promising results and targeted inhibition of both ERK phosphorylation, as well as FLT3 signaling (34-36). A combined inhibitory effect on both RAS and FLT3 signaling may well be highly effective in the treatment of *MLL*-rearranged infant ALL, as the majority of these patients are also characterized by constitutive FLT3 activation (15).

In conclusion, we demonstrate that *RAS* mutations frequently occur in *MLL*-rearranged infant ALL cases and especially in t(4;11)-positive infant ALL patients, and their presence represents an independent poor prognostic factor. Therefore the *RAS*-signaling pathway could be a potential target for therapeutic intervention, but also provides a rationale for future risk-stratification strategies. However, although *RAS* mutation-positive patients are at high risk of relapse, the prognosis for *RAS* mutation-negative patients remains far from favorable. Thus, a continued search for additional mutations, for instance in other components of the *RAS* pathway, that typify an unfavorable outcome, may be beneficial.

REFERENCES

1. Greaves MF. Infant leukaemia biology, aetiology and treatment. *Leukemia*.1996;10(2):372-7.

2. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, *et al.* A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007;370(9583):240-50.

3. Chen W, Li Q, Hudson WA, Kumar A, Kirchhof N, Kersey JH. A murine Mll-AF4 knock-in model results in lymphoid and myeloid deregulation and hematologic malignancy.*Blood*. 2006;108(2):669-77.

4. Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU, *et al.* H3K79 methylation profiles define murine and human MLL-AF4 leukemias. *Cancer Cell*.2008;14(5):355-68.

5. Metzler M, Forster A, Pannell R, Arends MJ, Daser A, Lobato MN, *et al.* A conditional model of MLL-AF4 B-cell tumourigenesis using invertor technology. *Oncogene*. 2006;25(22):3093-103.

6. Greaves M. In utero origins of childhood leukaemia. *Early Hum Dev*. 2005;81(1):123-9.

7. van der Linden MH, Valsecchi MG, De Lorenzo P, Moricke A, Janka G, Leblanc TM, *et al.* Outcome of congenital acute lymphoblastic leukemia treated on the Interfant-99 protocol. *Blood*. 2009;114(18):3764-8.

8. Bursen A, Schwabe K, Ruster B, Henschler R, Ruthardt M, Dingermann T, *et al.* The AF4.MLL fusion protein is capable of inducing ALL in mice without requirement of MLL.AF4. *Blood*. 2010;115(17):3570-9.

9. Tamai H, Miyake K, Takatori M, Miyake N, Yamaguchi H, Dan K, *et al.* Activated K-Ras protein accelerates human MLL/AF4-induced leukemo-lymphomogenicity in a transgenic mouse model. *Leukemia*. 2011.

10. Ng MH, Ng RK, Kong CT, Jin DY, Chan LC. Activation of Ras-dependent Elk-1 activity by MLL-AF4 family fusion oncoproteins. *Exp Hematol*. 2010;38(6):481-8.

11. Liang DC, Shih LY, Fu JF, Li HY, Wang HI, Hung IJ, *et al.* K-Ras mutations and NRas mutations in childhood acute leukemias with or without mixed-lineage leukemia gene rearrangements. *Cancer*. 2006;106(4):950-6.

12. Mahgoub N, Parker RI, Hosler MR, Close P, Winick NJ, Masterson M, *et al.* RAS mutations in pediatric leukemias with MLL gene rearrangements. *Genes Chromosomes Cancer*. 1998;21(3):270-5.

13. Faber J, Krivtsov AV, Stubbs MC, Wright R, Davis TN, van den Heuvel-Eibrink M, *et al.* HOXA9 is required for survival in human MLL-rearranged acute leukemias. *Blood*. 2009;113(11):2375-85.

14. Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, *et al.* Gene expression profiling-based dissection of MLL translocated and MLL germline acute lymphoblastic leukemia in infants. *Blood*. 2010;115(14):2835-44.

15. Stam RW, den Boer ML, Schneider P, Nollau P, Horstmann M, Beverloo HB, *et al.* Targeting FLT3 in primary MLL-gene-rearranged infant acute lymphoblastic leukemia. *Blood*. 2005;106(7):2484-90.

16. Xu X, Quiros RM, Gattuso P, Ain KB, Prinz RA. High prevalence of BRAF gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines. *Cancer Res*. 2003;63(15):4561-7.

17. Dordelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, *et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*. 1999;94(4):1209-17.

18. Pieters R, Loonen AH, Huismans DR, Broekema GJ, Dirven MW, Heyenbrok MW, *et al.* In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. *Blood*. 1990;76(11):2327-36.

19. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*. 2002;30(1):207-10.

20. Ramakers-van Woerden NL, Beverloo HB, Veerman AJ, Camitta BM, Loonen AH, van Wering ER, *et al.* In vitro drug-resistance profile in infant acute lymphoblastic leukemia in relation to age, MLL rearrangements and immunophenotype. *Leukemia*. 2004;18(3):521-9.

21. Stam RW, Schneider P, de Lorenzo P, Valsecchi MG, den Boer ML, Pieters R. Prognostic significance of high-level FLT3 expression in MLL-rearranged infant acute lymphoblastic leukemia. *Blood*. 2007;110(7):2774-5.

22. Bar-Eli M, Ahuja H, Foti A, Cline MJ. N-RAS mutations in T-cell acute lymphocytic leukaemia: analysis by direct sequencing detects a novel mutation. *Br J Haematol*. 1989;72(1):36-9.

23. Bos JL, Verlaan-de Vries M, van der Eb AJ, Janssen JW, Delwel R, Lowenberg B, *et al.* Mutations in N-ras predominate in acute myeloid leukemia. *Blood*. 1987;69(4):1237-41.

24. Cogswell PC, Morgan R, Dunn M, Neubauer A, Nelson P, Poland-Johnston NK, *et al.* Mutations of the ras protooncogenes in chronic myelogenous leukemia: a high frequency of ras mutations in bcr/abl rearrangement-negative chronic myelogenous leukemia. *Blood*. 1989;74(8):2629-33.

25. Janssen JW, Steenvoorden AC, Lyons J, Anger B, Bohlke JU, Bos JL, *et al.* RAS gene mutations in acute and chronic myelocytic leukemias, chronic myeloproliferative disorders, and myelodysplastic syndromes. *Proc Natl Acad Sci U S A*. 1987;84(24):9228-32.

26. Miyauchi J, Asada M, Sasaki M, Tsunematsu Y, Kojima S, Mizutani S. Mutations of the N-ras gene in juvenile chronic myelogenous leukemia. *Blood*. 1994;83(8):2248-54.

27. Needleman SW, Kraus MH, Srivastava SK, Levine PH, Aaronson SA. High frequency of N-ras activation in acute myelogenous leukemia. *Blood*. 1986;67(3):753-7.

28. Rodenhuis S, Bos JL, Slater RM, Behrendt H, van 't Veer M, Smets LA. Absence of oncogene amplifications and occasional activation of N-ras in lymphoblastic leukemia of childhood. *Blood*. 1986;67(6):1698-704.

29. Lubbert M, Mirro J, Jr., Miller CW, Kahan J, Isaac G, Kitchingman G, *et al.* N-ras gene point mutations in childhood acute lymphocytic leukemia correlate with a poor prognosis. *Blood*. 1990;75(5):1163-9.

30. Wiemels JL, Zhang Y, Chang J, Zheng S, Metayer C, Zhang L, *et al.* RAS mutation is associated with hyperdiploidy and parental characteristics in pediatric acute lymphoblastic leukemia. *Leukemia*. 2005;19(3):415-9.

31. Yokota S, Nakao M, Horiike S, Seriu T, Iwai T, Kaneko H, *et al.* Mutational analysis of the N-ras gene in acute lymphoblastic leukemia: a study of 125 Japanese pediatric cases. *Int J Hematol*. 1998;67(4):379-87.

32. Case M, Matheson E, Minto L, Hassan R, Harrison CJ, Bown N, *et al.* Mutation of genes affecting the RAS pathway is common in childhood acute lymphoblastic leukemia.*Cancer Res*. 2008;68(16):6803-9.

33. Lancet JE, Duong VH, Winton EF, Stuart RK, Burton M, Zhang S, *et al.* A phase I clinical-pharmacodynamic study of the farnesyltransferase inhibitor tipifarnib in combination with the proteasome inhibitor bortezomib in advanced acute leukemias. *Clin Cancer Res*. 2011;17(5):1140-6.

34. Crump M, Hedley D, Kamel-Reid S, Leber B, Wells R, Brandwein J, *et al.* A randomized phase I clinical and biologic study of two schedules of sorafenib in patients with myelodysplastic syndrome or acute myeloid leukemia: a NCIC (National Cancer Institute of Canada) Clinical Trials Group Study. *Leuk Lymphoma*. 2010;51(2):252-60.

35. Ravandi F, Cortes JE, Jones D, Faderl S, Garcia-Manero G, Konopleva MY, *et al.* Phase I/II study of combination therapy with sorafenib, idarubicin, and cytarabine in younger patients with acute myeloid leukemia. *J Clin Oncol*. 2010;28(11):1856-62.

36. Borthakur G, Kantarjian H, Ravandi F, Zhang W, Konopleva M, Wright JJ, *et al.* Phase I study of sorafenib in patients with refractory or relapsed acute leukemias. *Haematologica*. 2011;96(1):62-8.

CHAPTER 8

General Discussion

Glucocorticoid resistance in
MLL-rearranged infant acute lymphoblastic leukemia

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ABSTRACT

Glucocorticoids (GCs) are the most important drugs in the treatment of Acute Lymphoblastic Leukemia (ALL). Unfortunately, *MLL*-rearranged infant ALL patients (<1 year of age at diagnosis) who have a relatively poor outcome, are frequently resistant to these drugs. Although GC resistance mechanisms in pediatric ALL have been extensively investigated, resistance mechanisms in infant ALL remain largely unidentified.

This review focuses on established and novel GC resistance mechanisms in *MLL*-rearranged infant ALL. Inhibition of GC-induced cytosolic calcium mobilization by the calcium-binding S100 family member proteins and the involvement of apoptotic pathway members of the BCL-2 family herein are discussed as a mechanism of GC resistance. Furthermore, Src kinases which form a complex with the GR, and dissociate from GR upon GC treatment, play an important role in glucocorticoid resistance in *MLL*-rearranged infant ALL, most likely by phosphorylation of annexin A2. Additionally, we here describe the role of PI3K inhibitors in inducing prednisolone sensitivity in *MLL*-rearranged infant ALL by targeting *FCGR1B*. Finally, the association between miRNAs and GC resistance in *MLL*-rearranged infant ALL are discussed, with emphasis on miR-128b and miR-708. Treatment with small molecule inhibitors targeting aforementioned GC-resistance mechanisms offer promising perspectives to improve the outcome of *MLL*-rearranged infant ALL.

GENERAL INTRODUCTION

Glucocorticoids (GCs) belong to the class of steroid hormones, and affect metabolism, differentiation, proliferation and cell survival, and exert these functions by binding to the glucocorticoid receptor (GR). GCs are being used for treating hematological malignancies including leukemia (Kofler 2000, Distelhorst 2002). GCs are more efficient for the treatment of acute lymphoblastic leukemias when compared to myeloid leukemias, which are largely resistant to GC therapy. GC-based therapies resulted in great improvements in treating childhood acute lymphoblastic leukemia (ALL) over the last decades, reaching survival rates of 85-90%. Unfortunately, infants (<1 year of age) carrying a translocation of the *MLL* (*Mixed Lineage Leukemia*) gene (representing ~80% of all infant ALL) (Greaves 1996, Biondi *et al.* 2000) have survival rates of only ~50% (Pieters *et al.* 2007). An important factor contributing to the treatment failure is cellular drug resistance. Infant ALL cells are ~500 fold more resistant *in vitro* to GCs (prednisolone and dexamethasone), when compared to ALL cells from older children with ALL (Pieters *et al.* 1998, Ramakers-van Woerden *et al.* 2004). Moreover, *MLL*-rearranged infant ALL patients are more often resistant to GCs *in vivo* when compared to pediatric ALL; 30% versus 10% of patients respectively show a poor response to prednisone *in vivo* (Dördelmann *et al.* 1999).

Despite the overall GC-resistant character of *MLL*-rearranged infant ALL, GC-based therapies induce morphologically complete remission in ~95% of the cases (Frankel *et al.* 1997, Reaman *et al.* 1999). Unfortunately, 30-50% of these patients experience relapse on treatment within the first two years after diagnosis. Since both *in vitro* and *in vivo* GC responses are predictive markers for the outcome in pediatric ALL (Riehm *et al.* 1987, Pieters *et al.* 1991, Kaspers *et al.* 1998, Dördelmann *et al.* 1999, Den Boer *et al.* 2003), overcoming GC resistance might improve outcome in these very young children with ALL. Hence, the task at hand is to unravel the mechanisms underlying GC-resistance in *MLL*-rearranged infant ALL, and to develop strategies to augment the cellular response to these drugs.

Previously, we demonstrated that pediatric ALL patients resistant to prednisolone *in vitro* displayed genomic differences compared to patients that are *in vitro* sensitive to this drug (Holleman *et al.* 2004). This prednisolone-resistant gene expression signature could not be extrapolated to *MLL*-rearranged infant ALL patient samples (Stam *et al.* 2010), indicating that other GC resistance mechanisms play a role in *MLL*-rearranged infant ALL. This review will focus on both established and novel GC resistance mechanisms in *MLL*-rearranged infant ALL.

1. MCL1 AND THE APOPTOTIC PATHWAY MEMBERS OF THE BCL-2 FAMILY

Gene expression profile studies of *in vitro* GC-resistance in pediatric ALL revealed an elevated expression of the anti-apoptotic BCL-2 family member, *MCL1* (Holleman *et al.* 2004, Wei *et al.* 2006). We confirmed that high *MCL1* expression is one of the few overlapping characteristics of the gene expression profile related to GC resistance signatures in both pediatric and *MLL*-rearranged infant ALL (Stam *et al.* 2010).

Abrogating MCL1 activity by the mTOR inhibitor rapamycin restored GC sensitivity in otherwise GC-resistant ALL cells (Wei *et al.* 2006). Furthermore, GC-resistant ALL cells were sensitized to GCs by the BCL-2 inhibitor obatoclax, inducing necroptosis rather than apoptosis (Bonapace *et al.* 2010). However, both studies did not show that *MLL*-rearranged ALL cells were sensitized to prednisolone by either rapamycin or obatoclax. Treatment of *MLL*-rearranged ALL cells with rapamycin resulted in a marginal downregulation of MCL1 but increased levels of phosphorylated MCL1, which primes cells for apoptosis initiation (Opferman 2006, Spijkers-Hagelstein *et al.* 2014). Knock-down of *MCL1* by RNAi in prednisolone-resistant *MLL*-rearranged ALL cells induced only moderate sensitization to prednisolone (Stam *et al.* 2010), suggesting that high-level expression of MCL1 alone is not solely responsible for GC resistance in *MLL*-rearranged infant ALL. Apart from MCL1, there are also other members of the BCL-2 protein family that are associated with GC-induced apoptosis in ALL (Salomons *et al.* 1997, Haarman *et al.* 1999, Broome *et al.* 2002, Casale *et al.* 2003, Zhao *et al.* 2011). These proteins exhibit either pro-survival or pro-apoptotic functions. GC resistance is generally associated with high expression of the pro-survival proteins BCL-2 or BCL-X (Casale et al.2003, Ploner *et al.* 2008). Although inhibition of MCL1 by the low molecular weight drugs rapamycin or obatoclax did not induce prednisolone sensitivity in *MLL*-rearranged ALL cells, the pan-BCL-2 inhibitors gossypol and AT-101 showed a high sensitization potential to prednisolone. Whereas this effect was not observed in non-*MLL*-rearranged precursor B-cell ALL cell lines (Spijkers-Hagelstein *et al.* 2014). Notably, these pan-BCL-2 inhibitors did not reduce the levels of the pro-survival proteins BCL-2, BCL-X or MCL1, but rather induced the expression of the pro-apoptotic proteins BAD, BID, BIM and NOXA. BID and BIM might be the key initiators in prednisolone sensitivity, as these were induced at earlier time points during the exposure to prednisolone (Spijkers-Hagelstein *et al.* 2014). Furthermore, GC-sensitive lymphoid cells consistently showed induction of *BIM* when exposed to GC (Wang *et al.* 2003, Medh *et al.* 2003, Planey *et al.* 2003, Abrams *et al.* 2004, Zhang *et al.* 2004, Zhao *et al.* 2011), and RNAi directed against *BIM* in GC-sensitive cells resulted in protection against GC-induced apoptosis (Lu *et al.* 2006, Ploner *et al.* 2008). Interestingly, no basal upregulation of *BIM* was detected in GC-resistant cells (Zhao *et al.* 2011). However, for BID-deficient or BAX-deficient knock-out mice it was demonstrated that GC-induced apoptosis is not dependent on these proteins (Knudson *et al.* 1995, Yin *et al.* 1999). Thus, these data suggest that BIM is a predominant causal factor for GC resistance in *MLL*-rearranged infant ALL in contrast to MCL1.

2. CALCIUM-INDUCED GLUCOCORTICOID SENSITIVITY AND THE ROLE OF CALCIUM-BINDING PROTEINS

One of the important molecules mediating GC-induced signaling responses is calcium. Calcium represents a key second messenger molecule in a multitude of cellular signaling pathways, such as differentiation, proliferation and apoptosis.

About 30 years ago, Kaiser and Edelman (1977) already observed that GC-induced lymphocytolysis is associated with increased calcium release from the endoplasmic reticulum (ER) into the cytosol. Furthermore, this mechanism was confirmed in thymocytes treated with dexamethasone (McConkey *et al.* 1989). In addition, GC treatment in both lymphocytes and thymocytes induced activation of phospholipase C (PLC) via several tyrosine kinases (Schieven *et al.* 1993, Yao *et al.* 1993), which eventually results in cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to the IP_3-receptor (IP_3R) localized on the endoplasmic reticulum (ER), which leads to release of calcium stored in the ER into the cytosol. These cytosolic calcium waves subsequently increase mitochondrial calcium signals, initiating cytochrome c release and inducing apoptotic cell death (Schieven *et al.* 1993, Krebs *et al.* 1998). MCL1 inhibits mitochondrial calcium signaling when triggered by apoptotic stimuli and thereby forestalling apoptotic cell death (Minagawa *et al.* 2005). However, down-regulation of MCL1 by shRNA-mediated knockdown or by specific MCL1 inhibitors (Wei *et al.* 2006, Stam *et al.* 2010) only showed a modest sensitization towards prednisolone, suggesting that other mechanisms might be more important in prednisolone resistance in *MLL*-rearranged infant ALL.

It has been demonstrated that high-level expression of the calcium-binding proteins *S100A8* and *S100A9* also affects the calcium signaling, acting as calcium modulators upstream of MCL1. S100A8 and S100A9 proteins belong to the S100 calcium-binding protein family which comprises more than 15 members (Donato 1991, Donato 2003, Heizmann 2002a, 2002b). Most S100 family members are located at chromosome 1q21: duplications of this region are known to be involved in carcinogenesis. However, *MLL*-rearranged ALL cells do not contain duplications of chromosome 1q21, therefore this mechanism is not responsible for the high expression of *S100A8* and *S100A9* in GC-resistant *MLL*-rearranged ALL cells (Spijkers-Hagelstein *et al.* 2012). S100 proteins share the highly conserved calcium-binding EF-hand motif. Although calcium binding is required for the functionality of S100A8 and S100A9, other S100 family members (*e.g.* S100A10, S100A11) act independently of calcium binding. S100A8 and S100A9 induce prednisolone resistance in otherwise prednisolone-sensitive cells by scavenging GC-induced free-cytosolic calcium release. Conversely, *in vitro* prednisolone resistance was more dependent on MCL1 in cells expressing low-levels of both *S100A8/S100A9* (Spijkers-Hagelstein *et al.* 2012), which is in concordance with the modest sensitization of MCL1 in prednisolone-resistant cells (Stam *et al.* 2010). This supports the idea that MCL1 might function more downstream of S100A8/S100A9 in inhibiting calcium signaling. Recently, Qazi *et al.* demonstrated that *S100A8* and *S100A9* were discriminative between infant ALL cells and non-infant ALL cells (Qazi *et al.* 2010). In general, infant ALL cells express higher levels of *S100* genes compared to non-infant ALL cells, which is especially true for *S100A4*, *S100A8*, *S100A9*,

S100A10 and *S100A12* (Spijkers-Hagelstein *et al.* 2012). We showed that transduction of prednisolone-sensitive *MLL*-rearranged ALL cells with eukaryotic expression vectors encoding either S100A8 or S100A9 induced a moderate resistance to prednisolone, but cells transduced with both S100A8 and S100A9 together became highly resistant to this drug (Spijkers-Hagelstein *et al.* 2012). Re-expression of *S100A8* and *S100A9* in prednisolone-sensitive cells was accompanied by a reduction in GC-induced free-cytosolic calcium. Although prednisolone resistance in *MLL*-rearranged infant ALL is correlated with a high expression of both *S100A8* and *S100A9*, other S100 proteins such as *S100A10* were also highly expressed in prednisolone-resistant infant ALL patients (Spijkers-Hagelstein *et al.* 2012), suggesting a prominent role for these S100 family member proteins in GC resistance as well.

Other calcium-binding proteins apart from S100 proteins have been shown to play a role in GC resistance in *MLL*-rearranged infant ALL. Recent studies have revealed high annexin A2 (encoded by *ANXA2*) expression in GC-resistant *MLL*-rearranged infant ALL, which is a member of the phospholipid/calcium-binding annexin protein family (Spijkers-Hagelstein *et al.* 2013a). Interestingly, annexin family members are dependent on association with S100 family members for protein stability. For instance, annexin A1 has a high preference to associate with S100A11, whereas annexin A2 binds to S100A10; in both cases this leads to stabilization and thus activation of the annexin protein (Miwa *et al.* 2008, Rintala-Dempsey *et al.* 2008). Knock-down of annexin A2 or its adaptor-protein S100A10 leads to prednisolone sensitization in *MLL*-rearranged ALL cells. Interestingly, the expression of *ANXA2* highly correlates with specifically *S100A10* and not with *S100A8/S100A9* expression (Spijkers-Hagelstein *et al.* 2013a). GC resistance mediated via annexin A2 is dependent on binding to S100A10; upon binding to this adaptor-molecule, annexin A2 is phosphorylated by Src kinases leading to cell survival (He *et al.* 2008, Hayes *et al.* 2009, Dassah *et al.* 2009, Zheng *et al.* 2011, Spijkers-Hagelstein *et al.* 2013a). Remarkably, high-level expression of *ANXA2/S100A10* did not correlate with high-level expression of *S100A8/S100A9*, suggesting that GC resistance can be mediated via a diversity of S100-dependent mechanisms, even independent of each other.

3. KINASE-DEPENDENT GLUCOCORTICOID RESISTANCE – involvement of PI3K an Src kinase

Phosphatidylinositol 3-kinase (PI3K)/Akt pathway activation is important for cell proliferation, cell growth, cell cycle and metabolism. Rapamycin induces GC sensitivity in ALL by inhibiting the downstream target of the PI3K/Akt/mTOR pathway, MCL1 (Wei *et al.* 2006). Whereas MCL1 is involved in GC resistance in both pediatric ALL and *MLL*-rearranged infant ALL, the cause of high-level expression of MCL1 still needs to be elucidated. Interestingly, PI3K can indirectly regulate MCL1 expression (Mills *et al.* 2008); the downstream target of PI3K, GSK3, phosphorylates MCL1 and primes MCL1 for degradation. However, *MLL*-rearranged infant ALL cells did not respond to GC-induced apoptosis in the presence of the mTOR inhibitor rapamycin; also, no degradation of MCL1 was observed after rapamycin treatment (Spijkers-Hagelstein *et al.* 2013c).

Using connectivity map analysis, an *in silico* approach to screen for FDA-approved bioactive small molecules that reverse the GC-resistant profile into a GC-sensitive profile, revealed the potential of the PI3K inhibitor LY294002 to induce this effect in *MLL*-rearranged infant ALL (Spijkers-Hagelstein *et al.* 2013b). GC resistance in *MLL*-rearranged ALL cells was indeed reversed upon LY294002 treatment. Other known PI3K inhibitors (wortmannin and PI-828) also induced prednisolone sensitivity, implying a role of PI3K in GC resistance independent of the PI3K/Akt pathway. Interestingly, GC-sensitizing effects in *MLL*-rearranged ALL cells by the PI3K inhibitors LY294002, wortmannin or PI-828 were mainly associated by the suppression of *FCGR1B* expression (Spijkers-Hagelstein *et al.* 2013b).

Other kinases involved in GC resistance in *MLL*-rearranged infant ALL might be the Src kinases. Src kinases belong to a family of non-receptor tyrosine kinases which comprises eight highly homologous family members (c-Src, Lyn, Fyn, c-Yes, Fgr, Hck, Lck and Blk). They are involved in the activation of numerous signaling processes, regulating cell proliferation, differentiation, gene expression, metabolism and cytoskeletal architecture. Interestingly, Src kinases bind to HSP90, a co-chaperone of the GR complex. Upon GC stimulation, GR is not only released from its co-chaperones, but the associated Src kinases also dissociate from the GR-complex. Src kinases in general have pro-survival activities, *e.g.* activating the PI3K/Akt pathway (Karni *et al.* 2005). Inhibition of Src kinase by selective Src kinase inhibitors (PP1 or PP2), overcomes GC resistance in lymphoma cells and primary T cells (Sade *et al.* 2004, Spokoini *et al.* 2010). Furthermore, inhibition of Src kinases led to GC-sensitization of *in vitro* prednisolone-resistant *MLL*-rearranged ALL cells, but not in non-*MLL*-rearranged ALL cells (Spijkers-Hagelstein *et al.* 2013a). Moreover, Src kinases bind to the cytosolic site of the phospholipid membrane fraction, where it phosphorylates many downstream targets, like vinculin, paxillin, FAK, β-catenin and annexin A2 (Parsons *et al.* 1997, Thomas *et al.* 1997, Abram *et al.* 2000, He *et al.* 2008, Dassah *et al.* 2009, Hayes *et al.* 2009). As previously mentioned, annexin A2 is highly expressed in *MLL*-rearranged infant ALL patient samples resistant to prednisolone (Spijkers-Hagelstein *et al.* 2013a). Moreover, Src-induced phosphorylation of annexin A2, which occurs in the presence of S100A10 (Hayes *et al.* 2009, Zheng *et al.* 2011) led to prednisolone resistance (Spijkers-Hagelstein *et al.* 2013a). Specific downregulation of the Src kinases FYN or LCK by Src kinase inhibitors and RNAi led to prednisolone sensitivity in otherwise prednisolone-resistant *MLL*-rearranged ALL cells (Spijkers-Hagelstein *et al.* 2013a). Targeting other Src kinase family members (e.g. src, c-yes, lyn) did not induce GC sensitivity in *MLL*-rearranged ALL cells (Spijkers-Hagelstein *et al.* 2013 unpublished data), suggesting a specificity for FYN and LCK in GC resistance in *MLL*-rearranged infant ALL.

Src kinases also activate the PI3K/Akt pathway by binding to PI3K itself. Interestingly, the PI3K inhibitor LY294002 also demonstrated to affect the phosphorylation of annexin A2 (Spijkers-Hagelstein *et al.* 2013, unpublished data), suggesting that PI3K and Src kinases might collaborate; thus targeting both proteins might overcome GC resistance in *MLL*-rearranged infant ALL.

4. miRNA AND GLUCOCORTICOID RESISTANCE

MiRNAs bind to their associated mRNA and suppress protein expression by inhibition of mRNA-mediated protein translation or by mRNA degradation. Recently, the expression of specific miRNAs has been linked to *in vitro* drug resistance and increased relapse rates in childhood ALL (Kotani *et al.* 2009, Kotani *et al.* 2010, Schotte *et al.* 2011, Han *et al.* 2011). For instance, miR-196b is highly expressed in *MLL*-rearranged ALL cells, but is not associated with *in vitro* prednisolone responses (Schotte *et al.* 2010). However, Schotte *et al.* demonstrated a relation between miRNA expression and vincristine, daunorubicin and L-asparaginase resistance. Interestingly, other miRNAs *i.e.* miR-128b and miR-221, were lower expressed in *MLL*-rearranged ALL compared to other ALL cases (Kotani *et al.* 2009). Re-expression of both miR-128b and miR-221 sensitized *MLL-AF4* rearranged cell lines to GCs (Kotani *et al.* 2009). Moreover, miR-128b downregulates *MLL, AF4, MLL-AF4* and *AF4-MLL* fusion gene expression. Interestingly, a novel mutation found in miR-128b, significantly reduces the expression of this miRNA, contributing to GC resistance in *MLL*-rearranged infant ALL (Kotani *et al.* 2010). However, it remains yet unclear whether miR-128b and miR-211 induce GC resistance separately or when co-expressed.

Han *et al.* performed genome-wide miRNA microarray analysis to determine relapse-associated miRNAs patterns and discovered that low expression of miR-708, miR-223 and miR-27a correlate with an adverse clinical outcome in pediatric ALL (relapse-free survival). Interestingly, only miR-708 expression levels seem to be directly associated with GC resistance, since *in vivo* prednisone good responders express elevated levels of miR-708 (Han *et al.* 2011). Overall, there is upcoming evidence that miRNAs play a role in GC resistance, but how still needs to be clarified.

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Infant ALL is characterized by a high incidence of rearrangements of the *MLL* gene and a poor prognosis. Resistance towards glucocorticoids (GCs), is a major contributor to this poor outcome. Therefore, overcoming GC resistance might be a major step to improve outcome. Gene expression profile studies from GC resistance in *MLL*-rearranged infant ALL provided novel mechanistic insights into this, including the role of GC-induced cytosolic calcium inhibition by the calcium-binding proteins S100A8/S100A9, Src kinase-induced phosphorylation of annexin A2 and PI3K-induced GC resistance. Modulation of aforementioned proteins involved in GC resistance, by either Src kinase inhibitors (inhibition of S100A8/S100A9 and annexin A2 expression), the use of FDA-approved PI3K inhibitors or pan-BCL-2 family member inhibitors (*e.g.* gossypol and AT-101) showed to overcome GC resistance in *MLL*-rearranged infant ALL, and are interesting potential strategies for inducing GC sensitivity; thus improving the outcome of *MLL*-rearranged infant ALL.

REFERENCES

Abram CL, Courtneidge SA. Src family tyrosine kinases and growth factor signaling. *Experimental Cell Research*. 2000. 254(1):1-13.

Abrams MT, Robertson NM, Yoon K, Wickstrom E. Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM mRNA with small interfering RNA and short hairpin RNA. *Journal of Biological Chemistry*. 2004. 279(53):55809-55817

Arancibia S, Benítez D, Núńez LE, Jewell CM, Langjahr P, Candia E, Zapata-Torres G, Cidlowski JA, González MJ, Hermoso MA. Phosphatidylinositol 3-kinase interacts with the glucocorticoid receptor upon TLR2 activation. *Journal of Cellular and Molecular Medicin*. 2011. 15(2):339-349.

Armstrong SA, Staunton JE, Silverman LB, Pieters R, den Boer ML, Minden MD, Sallan SE, Lander ES, Golub TR, Korsmeyer SJ. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nature Genetics*. 2002. 30(1):41-47.

Biondi A, Cimino G, Pieters R, Pui CH. Biological and therapeutic aspects of infant leukemia. *Blood*. 2000. 96:24–33.

Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, Niggli FK, Schafer BW, Schrappe M, Stanulla M, Bourquin JP. Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. *Journal of Clinical Investigation*. 2010. 120(4):1310-1323

Broome HE, Yu AL, Diccianni M, Camitta BM, Monia BP, Dean NM. Inhibition of Bcl-XL expression sensitizes T-cell acute lymphoblastic leukemia cells to chemotherapeutic drugs. *Leukemia Research*. 2002. 26:311-316.

Casale F, Addeo R, D'Angelo V, Indolfi P, Poggi V, Morgera C, Crisci S, Di Tullio MT. Determination of the *in vivo* effects of prednisone on Bcl-2 family protein expression in childhood acute lymphoblastic leukemia. *International Journal of Oncology*. 2003. 22(1):123-128.

Dassah M, Deora AB, He K, Hajjar KA. The endothelial cell annexin A2 system and vascular fibrinolysis. *General Physiology and Biophysics*. 2009;28(SPEC): F20–F28.

Den Boer ML, Harms DO, Pieters R, *et al*. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *Journal of Clinical Oncology*. 2003. 21:3262-3268.

Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. *Cell Death and Differentiation*. 2002. 9(1):6-19.

Donato R. Perspectives in S-100 protein biology. Review article. *Cell Calcium*. 1991. 12(10):713-726.

Donato R. Intracellular and extracellular roles of S100 proteins. *Microscopy Research and Technique*. 2003. 60(6):540-551.

Dördelmann M, Reiter A, Borkhardt A, Ludwig WD, Götz N, Viehmann S, Gadner H, Riehm H, Schrappe M. Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*. 1999. 94(4):1209-1217.

Frankel LS, Ochs J, Shuster JJ, Dubowy R, Bowman WP, Hockenberry-Eaton M, Borowitz M, Carroll AJ, Steuber CP, Pullen DJ. Therapeutic trial for infant acute lymphoblastic leukemia: the Pediatric Oncology Group experience (POG 8493). *Journal of Pediatric Hematology/oncology*. 1997. 19:35–42.

Greaves MF. Infant leukaemia biology, aetiology and treatment. *Leukemia*. 2000. 10:372–377.

Haarman EG, Kaspers GJ, Pieters R, Van Zantwijk CH, Broekema GJ, Hahlen K, Veerman AJ. BCL-2 expression in childhood leukemia versus spontaneous apoptosis, drug induced apoptosis, and *in vitro* drug resistance. *Advances in Experimental Medicin and Biology*. 1999. 457:325-333.

Han BW, Feng DD, Li ZG, Luo XQ, Zhang H, Li XJ, Zhang XJ, Zheng LL, Zeng CW, Lin KY, Zhang P, Xu L, Chen YQ. A set of miRNAs that involve in the pathways of drug resistance and leukemic stem-cell differentiation is associated with the risk of relapse and glucocorticoid response in childhood ALL. *Human Molecular Genetics*. 2011. 20(24):4903-4915.

Hayes MJ, Moss SE. Annexin 2 has a dual role as regulator and effector of v-Src in cell transformation. *Journal of Biological Chemistry*. 2009. 284(15):10202-10210.

He KL, Deora AB, Xiong H, Ling Q, Weksler BB, Niesvizky R, Hajjar KA. Endothelial cell annexin A2 regulates polyubiquitination and degradation of its binding partner S100A10/p11. *Journal of Biological Chemistry*. 2008. 283(28):19192-19200.

Heizmann CW. The multifunctional S100 protein family. *Methods in Molecular Biology*. 2002a. 172:69-80.

Heizmann CW, Fritz G, Schäfer BW. S100 proteins: structure, functions and pathology. *Frontiers in Bioscience*. 2002b. 7:d1356-1368.

Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJ, Kazemier KM, Pei D, Cheng C, Pui CH, Relling MV, Janka-Schaub GE, Pieters R, Evans WE. Gene expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *New England Journal of Medicin*. 2004. 351(6):533-542.

Kaiser N, Edelman IS. Calcium dependence of glucocorticoid-induced lymphocytolysis. *PNAS USA*. 1977. 74(2):638-642.

Karni R, Gus Y, Dor Y, Meyuhas O, Levitzki A. Active Src elevates the expression of beta-catenin by enhancement of cap-dependent translation. *Molecular Cell Biology*. 2005. 25(12):5031-9.

Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood*. 1998. 92:259-266.

Kimura M, Murakami T, Kizaka-Kondoh S, Itoh M, Yamamoto K, Hojo Y, Takano M, Kario K, Shimada K, Kobayashi E. Functional molecular imaging of ILK-mediated Akt/PKB signaling cascades and the associated role of beta-parvin. *Journal of Cell Science*. 2010. 123(Pt 5):747-755.

Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*. 1995. 270:96-99.

Kofler. The molecular basis of glucocorticoid-induced apoptosis of lymphoblastic leukemia cells. *Histochemistry and Cell Biololgy*. 2000. 114(1):1-7.

Kotani A, Ha D, Hsieh J, Rao PK, Schotte D, den Boer ML, Armstrong SA, Lodish HF. miRNA-128b is a potent glucocorticoid sensitizer in MLL-AF4 acute lymphoblastic leukemia cells and exerts cooperative effects with miR-221. *Blood*. 2009. 114(19):4169-4178.

Kotani A, Ha D, Schotte D, den Boer ML, Armstrong SA, Lodish HF. A novel mutation in the miR-128b gene reduces miRNA processing and leads to glucocorticoid resistance of MLL-AF4 acute lymphoblastic leukemia cells. *Cell Cycle*. 2010. 9(6):1037-1042.

Krebs J. The role of calcium in apoptosis. Biometals. 1998. 11(4):375-82.

Li H, Chen L, Zhang Y, Lesage G, Zhang Y, Wu Y, Hanley G, Sun S, Yin D. Chronic stress promotes lymphocyte reduction through TLR2 mediated PI3K signaling in a β-arrestin 2 dependent manner. *Journal of Neuroimmunology*. 2011. 233(1-2):73-79.

Lu J, Quearry B, Harada H. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. *FEBS Letter*. 2006. 580(14):3539-3544.

McConkey DJ, Nicotera P, Hartzell P, Bellomo G, Wyllie AH, Orrenius S. Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca2+ concentration. *Archives of Biochemistry and Biophysics*. 1989. 269(1):365-370.

Medh RD, Webb MS, Miller AL, Johnson BH, Fofanov Y, Li T, Wood TG, Luxon BA, Thompson EB. Gene expression profile of human lymphoid CEM cells sensitive and resistant to glucocorticoid-evoked apoptosis. *Genomics*. 2003 Jun;81(6):543-555.

Mills JR, Hippo Y, Robert F, Chen SM, Malina A, Lin CJ, Trojahn U, Wendel HG, Charest A, Bronson RT, Kogan SC, Nadon R, Housman DE, Lowe SW, Pelletier J. mTORC1 promotes survival through translational control of MCL1. *PNAS USA*. 2008. 105(31):10853-8.

Minagawa N, Kruglov EA, Dranoff JA, Robert ME, Gores GJ, Nathanson MH. The anti-apoptotic protein MCL1 inhibits mitochondrial Ca2+ signals. *Journal of Biological Chemistry*. 2005. 280(39):33637-33644.

Miwa N, Uebi T, Kawamura S. S100-annexin complexes-biology of conditional association. *FEBS Journal*. 2008. 275(20):4945-4955.

Nicolas E, Ramus C, Berthier S, Arlotto M, Bouamrani A, Lefebvre C, Morel F, Garin J, Ifrah N, Berger F, Cahn JY, Mossuz P. Expression of S100A8 in leukemic cells predicts poor survival in de novo AML patients. *Leukemia*. 2011. 25(1):57-65.

Opferman JT. Unraveling MCL1 degradation. *Cell Death and Differentiation*. 2006. 13(8):1260-1262.

Parsons JT, Parsons SJ. Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. *Current Opinion in Cell Biology*. 1997. 9(2):187-92.

Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, van Wering ER, Veerman AJ. Relation between age, immunophenotype and *in vitro* drug resistance in 395 children with acute lymphoblastic leukemia–implications for treatment of infants. *Leukemia*. 1998. 12:1344–1348.

Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, Hovi L, LeBlanc T, Szczepanski T, Ferster A, Janka G, Rubnitz J, Silverman L, Stary J, Campbell M, Li CK, Mann G, Suppiah R, Biondi A, Vora A, Valsecchi MG. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007. 370(9583):240-250.

Planey SL, Abrams MT, Robertson NM, Litwack G. Role of apical caspases and glucocorticoid-regulated genes in glucocorticoid-induced apoptosis of pre-B leukemic cells. *Cancer Research*. 2003. 63(1):172-178.

Ploner C, Rainer J, Niederegger H, Eduardoff M, Villunger A, Geley S, Kofler R. The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. *Leukemia*. 2008. 22(2):370-7.

Qazi S, Uckun FM. Gene expression profiles of infant acute lymphoblastic leukaemia and its prognostically distinct subsets. *British Journal of Haematology*. 2010. 149(6):865-73.

Ramakers-van Woerden NL, Beverloo HB, Veerman AJ, Camitta BM, Loonen AH, van Wering ER, Slater RM, Harbott J, den Boer ML, Ludwig WD, Haas OA, Janka-Schaub GE, Pieters R. *In vitro* drug-resistance profile in infant acute lymphoblastic leukemia in relation to age, MLL rearrangements and immunophenotype. *Leukemia*. 2004. 18:521–529.

Reaman GH, Sposto R, Sensel MG, Lange BJ, Feusner JH, Heerema NA, Leonard M, Holmes EJ, Sather HN, Pendergrass TW, Johnstone HS, O'Brien RT, Steinherz PB, Zeltzer PM, Gaynon PS, Trigg ME, Uckun FM. (1999) Treatment outcome and prognostic factors for infants with acute lymphoblastic leukemia treated on two consecutive trials of the Children's Cancer Group. *Journal of Clinical Oncology*. 1999. 17:445–455.

Riehms H, Reiter A, Schrappe M, Berthold F, Dopfer R, Gerein V, Ludwig R, Ritter J, Stollmann B, Henze G. Corticosteroid-dependent reduction of leukocyte count in blood as a prognostic factor in acute lymphoblastic leukemia in childhood (therapy study ALL-BFM 83). *Klinische Padiatrie*. 1987. 199:151–160.

Rintala-Dempsey AC, Rezvanpour A, Shaw GS. S100-annexin complexes--structural insights. FEBS Journal. 2008. 275(20):4956-4966.

Sade H, Krishna S, Sarin A. The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. *Journal of Biological Chemistry*. 2004. 279(4):2937-2944.

Salomons GS, Brady HJM, Verwijs-Janssen M, Van den Berg JD, Hart AAM, Van den Berg H, Behrendt H, Hälhen K, Smets LA. The Bax:Bcl-2 ratio modulates the response to dexamethasone in leukaemic cells and is highly variable in childhood acute leukaemia. *International Journal of Cancer*. 1997. 71:959-965.

Schieven GL, Kirihara JM, Gilliland LK, Uckun FM, Ledbetter JA.Ultraviolet radiation rapidly induces tyrosine phosphorylation and calcium signaling in lymphocytes. *Molecular Biology of the Cell*. 1993. 4(5):523-30.

Schotte D, Lange-Turenhout EA, Stumpel DJ, Stam RW, Buijs-Gladdines JG, Meijerink JP, Pieters R, Den Boer ML. Expression of miR-196b is not exclusively MLL-driven but is especially linked to activation of HOXA genes in pediatric acute lymphoblastic leukemia. *Haematologica*. 2010. (10):1675-82.

Schotte D, De Menezes RX, Akbari Moqadam F, Mohmmadi Khankahdani L, Lange-Turenhout E, Chen C, Pieters R, Den Boer ML. MicroRNA characterize genetic diversity and drug resistance in pediatric acute lymphoblastic leukemia. *Haematologica*. 2011. 98(5):703-711.

Spijkers-Hagelstein JAP, Schneider P, Hulleman E, de Boer J, Williams O, Pieters R, Stam RW. Elevated S100A8/S100A9 expression causes glucocorticoid resistance in *MLL*-rearranged infant acute lymphoblastic leukemia. *Leukemia*. 2012. 26(6):1255-1265.

Spijkers-Hagelstein JAP, Mimoso Pinhanços S, Schneider P, Pieters R, Stam RW. Src kinase-induced phosphorylation of annexin A2 mediates glucocorticoid resistance in *MLL*-rearranged acute lymphoblastic leukemia. *Leukemia*. 2013a. 27(5):1063-1071.

Spijkers-Hagelstein JAP, Mimoso Pinhanços S, Schneider P, Pieters R, Stam RW. Chemical genomic screening identifies LY294002 as a modulator of glucocorticoid resistance in *MLL*-rearranged infant ALL. *Leukemia*. 2013b.

Spijkers-Hagelstein JAP, Schneider P, Mimoso Pinhanços S, Pieters R, Stam RW. Glucocorticoid sensitization in *MLL*-rearranged ALL cells by pan-BCL-2 family member inhibitors gossypol and AT-101. *submitted.* 2014.

Spokoini R, Kfir-Erenfeld S, Yefenof E, Sionov RV. Glycogen synthase kinase-3 plays a central role in mediating glucocorticoid-induced apoptosis. *Molecular Endocrinology*. 2010. 24(6):1136-50.

Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, de Lorenzo P, Sallan SE, Brady HJ, Armstrong SA, Pieters R. Association of high-level MCL1 expression with *in vitro* and *in vivo* prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia. *Blood*. 2010. 115(5):1018-1025.

Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. *Annual Review of Cell and Developmental Biology*. 1997. 13:513-609.

Wang Z, Malone MH, He H, McColl KS, Distelhorst CW. Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. *Journal of Biological Chemistry*. 2003 Jun 27;278(26):23861-23867.

Wei G, Twomey D, Lamb J, Schlis K, Agarwal J, Stam RW, Opferman JT, Sallan SE, den Boer ML, Pieters R, Golub TR, Armstrong SA. Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. *Cancer Cell*. 2006. 10(4):331-342.

Yao Y, Parker I. Inositol trisphosphate-mediated Ca2+ influx into Xenopus oocytes triggers Ca2+ liberation from intracellular stores. *Journal of Physiology*. 1993. 468:275-95.

Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R, Behm FG, Raimondi SC, Relling MV, Patel A, Cheng C, Campana D, Wilkins D, Zhou X, Li J, Liu H, Pui CH, Evans WE, Naeve C, Wong L, Downing JR. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell*. 2002. 1(2):133-143.

Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA, Korsmeyer SJ. Bid-deficient mice are resistant to FAS-induced hapatocellular apoptosis. *Nature*. 1999. 400:886-891.

Zhang L, Insel PA. The pro-apoptotic protein Bim is a convergence point for cAMP/protein kinase A- and glucocorticoid-promoted apoptosis of lymphoid cells. *Journal of Biological Chemistry*. 2004. 279(20):20858-20865.

Zhao YN, Guo X, Ma ZG, Gu L, Ge J, Li Q. Pro-apoptotic protein BIM in apoptosis of glucocorticoid-sensitive and -resistant acute lymphoblastic leukemia CEM cells. *Medical Oncology*. 2011. (4):1609-17.

Zheng L, Foley K, Huang L, Leubner A, Mo G, Olino K, Edil BH, Mizuma M, Sharma R, Le DT, Anders RA, Illei PB, Van Eyk JE, Maitra A, Laheru D, Jaffee EM. Tyrosine 23 phosphorylation-dependent cell-surface localization of annexin A2 is required for invasion and metastases of pancreatic cancer. *PLoS One*. 2011. 6(4):e19390.

CHAPTER 9

Summary

In the Netherlands, the annual incidence of cancer in children aged between 0 and 18 years is of approximately 650 cases. From these young patients, 100-120 children are presented with acute lymphoblastic leukemia (ALL); with incidence peaks at 3-5 years of age. Although treatment strategies for childhood leukemias have vastly improved over the past decades, *Mixed Lineage Leukemia* gene (*MLL)*-rearranged leukemias, particularly in infants (< 1 year of age), form an exception to this. As of yet, the overall 5-year survival rate of *MLL*-rearranged infant ALL does not exceed 50%. Cellular drug resistance is thought to be a significant contributing factor to this dismal prognosis, especially resistance to glucocorticoids (GCs) such as prednisone and dexamethasone. Both *in vitro* and *in vivo* prednisone responses have been identified as prognostic markers for therapy success (Kaspers *et al.* 1998, Dördelmann *et al.* 1999, Den Boer *et al.* 2003). In order to improve the outcome of *MLL*-rearranged infant ALL, we focused our investigation on *in vitro* prednisolone resistance mechanisms.

Chapter 2 describes the *in vitro* drug response data of *MLL*-rearranged infant ALL patient cells, as determined by *in vitro* cytotoxicity testing (MTT assay). *MLL*-rearranged infant ALL patients which are *in vitro* resistant to prednisolone, vincristine and L-asparaginase (also known as PVA) showed to have a worse prognosis when compared to patients that were *in vitro* sensitive to PVA. Interestingly, the only cross-resistances observed was for prednisolone and vincristine with both dexamethasone and daunorubicin. Moreover, we showed that established clinical parameters, *i.e.* high white blood cell count and age at diagnosis, correlated primarily with *in vitro* drug response to prednisolone in *MLL*-rearranged infant ALL. Because of the primarily involvement of *in vitro* prednisolone response in outcome and it's correlation with clinical parameters, we searched for novel targets of *in vitro* prednisolone resistance by defining a specific prednisolone-resistance gene expression signature of *MLL*-rearranged infant ALL patients (see Chapter 5).

MCL1 is an anti-apoptotic BCL-2 family member that is highly expressed in both GC-resistant pediatric leukemia cells (Holleman *et al.* 2004) as well as in GC-resistant *MLL*-rearranged infant leukemia cells (Stam *et al.* 2010a). In **Chapter 3** we validated the ability of MCL1-inhibiting agents – gossypol, AT-101, rapamycin, SU9516 and obatoclax – to sensitize GC-resistant *MLL*-rearranged ALL cells to prednisolone via modulation of the apoptotic pathway. In this chapter we demonstrated that only gossypol and AT-101 induced prednisolone sensitivity in *MLL*-rearranged ALL cells by increasing the expression of the pro-apoptotic proteins BAD, BID, BIM and NOXA, but not via inhibition of MCL1 expression. Furthermore, we showed that both agents impaired cell proliferation and decreased cell viability only upon co-treatment with prednisolone, but not on their own, suggesting a synergistic effect of gossypol or AT-101 with prednisolone. Although MCL1 expression remained unaltered, the function of MCL1 might be affected by these agents. In 1970s, Kaiser and Edelmann already described that GC-induced apoptosis is characterized by calcium release into the cytosol by the endoplasmic reticulum (ER) (Kaiser and Edelman 1977) and that elevated cytosolic calcium subsequently increases mitochondrial calcium signals, initiating cytochrome c release leading to apoptotic cell death (Schieven *et al.* 1993, Krebs *et al.* 1998). Interestingly, it has been demonstrated that MCL1 inhibits mitochondrial calcium, subsequently inhibits cytochrome c release and blocks apoptosis

(Minagawa *et al.* 2005). Interestingly, in the next paragraph we describe another mechanism of cytosolic calcium modulation which might require more attention in preserving prednisolone resistance in *MLL*-rearranged infant ALL, than MCL1.

In **Chapter 4** we investigated the role of two members of the S100 family of calcium-binding proteins (*S100A8* and *S100A9*) which are highly expressed in *MLL*-rearranged infant ALL cells resistant to prednisolone *in vitro*, while being low expressed in *MLL*-rearranged infant *in vitro* sensitive to prednisolone, ALL without *MLL* rearrangements or other pediatric ALL patient samples. We show that *S100A8* and *S100A9* induce GC resistance by inhibition of GC-induced calcium release from the ER. By restoring the expression of both *S100A8* and *S100A9* in prednisolone-sensitive cells we were able to increase resistance to prednisolone, an effect which was accompanied by inhibition of free-cytosolic calcium levels. Interestingly, we found that the Src kinase inhibitor PP2 which inhibits S100A8/S100A9 release from neutrophils (Ryckman *et al.* 2004), also inhibited the expression of S100A8 and S100A9 in prednisolone-resistant *MLL*-rearranged ALL cells. As described in the previous paragraph, MCL1 is able to inhibit mitochondrial calcium and forestall apoptosis. Therefore, we subsequently analyzed whether the expression of *MCL1* or *S100A8/S100A9* alone or the combination of *MCL1* and *S100A8/S100A9* is necessary for *in vitro* prednisolone resistance. Interestingly, the expression of *MCL1* is only essential in inducing *in vitro* prednisolone resistance in *MLL*-rearranged ALL cells with low-level expression of *S100A8/S100A9*.

Apart from the calcium-scavenging protective effect of *S100A8/S100A9*, we also discovered that high expression of another calcium-binding protein annexin A2 seemed to play a role in GC resistance (**Chapter 5**). Annexin A2 is activated via phosphorylation by Src kinases. Concomitantly, inhibition of Src kinases by both Src kinase inhibitors specific for FYN or LCK or shRNA-mediated silencing of *FYN* or *LCK* expression induced *in vitro* prednisolone sensitization. This also reduced both proliferation as well as annexin A2 phosphorylation, but did not affect total annexin A2 expression. Annexin A2 phosphorylation by Src kinases is facilitated by the adaptor protein S100A10. Similar to the expression of *S100A8* and *S100A9*, *S100A10* is highly expressed prednisolone-resistant *MLL*-rearranged infant ALL samples; S100A10 was lower expressed in pediatric ALL or infant ALL without *MLL* translocations (Stam *et al.* 2010b, Spijkers-Hagelstein *et al.* 2012). Although *S100A8*, *S100A9* and *S100A10* belong to the same S100 protein family, these proteins were shown to function upon different stimuli. While the expression of *S100A10* strongly correlated with *ANXA2*, both *S100A8* and *S100A9* did not. Moreover, specific down-regulation of S100A10 by shRNA interference also showed an inhibition in proliferation, and subsequently *in vitro* prednisolone sensitization in *MLL*-rearranged ALL cells. Interestingly, this sensitization effect was also accompanied by reduced phosphorylation of annexin A2; while total annexin A2 remained unaltered. Recently, peptides which specifically inhibit the S100A10 and annexin A2 complex formation were developed (Reddy *et al.* 2011) and might be promising to induce prednisolone sensitivity in otherwise prednisolone-resistant *MLL*-rearranged infant cells, thus improving the prognosis of this high-risk subgroup of pediatric ALL. Preliminary data showed that these peptides indeed were able to induce prednisolone sensitivity in *MLL*-rearranged cells. Whether this prednisolone

sensitization is due to a reduction in S100A10/Annexin A2 complex formation remains to be investigated.

To gain even more insights in GC resistance in *MLL*-rearranged infant ALL, we generated a specific *in vitro* prednisolone-resistance signature, as the drug resistance signatures used for pediatric ALL could not be applied to in *MLL*-rearranged infant ALL patient samples (see introduction). In **Chapter 6,** we used the specific *in vitro* prednisolone-resistance signature (Chapter 5) to perform connectivity map analyses, in order to search for small molecule inhibitors able to reverse this signature into a more sensitive profile. Connectivity map analysis indicated that the PI3K inhibitor LY294002 might be able to induce prednisolone sensitivity in prednisolone-resistant *MLL*-rearranged ALL cells. Indeed, treatment of *MLL*-rearranged cells with LY294002 induced prednisolone sensitivity by inhibiting proliferation. Moreover, other PI3K inhibitors like wortmannin and PI-828, also sensitized *MLL*-rearranged ALL cells to prednisolone, suggesting a prominent role of PI3K in prednisolone resistance. To test which genes were responsible for inducing prednisolone sensitivity when co-incubated with either LY294002, wortmannin or PI-828, we performed quantitative RT-PCR analysis before and after treatment. From this, *FCGR1B* was predicted to be downregulated after LY294002 treatment by the connectivity map analysis. *FCGR1B* was indeed significantly downregulated in prednisolone-resistant *MLL*-rearranged ALL cell line models after these were incubated with LY294002. Furthermore, wortmannin and PI-828 also reduced *FCGR1B* expression levels. Downregulation of *FCGR1B* by shRNA interference induced *in vitro* prednisolone sensitization in *MLL*-rearranged ALL cells, demonstrating a crucial role of *FCGR1B* in preserving prednisolone resistance.

In **Chapter 7** we screened infant ALL samples for the presence of *RAS* mutations (*K-RAS*, *N-RAS* and *B-RAF*) and demonstrated that ~14% of these samples contained *RAS* mutations mostly comprised of *K-RAS* and *N-RAS* mutations (15/109), but no *B-RAF* mutations. Interestingly, infant ALL patients with a t(4;11)-translocation made up for 60% of the *RAS*-mutated group (9/15 *RAS*-mutated *MLL*-rearranged infant ALL). Additionally, when comparing *RAS*-mutated versus non-*RAS*-mutated *MLL*-rearranged infant ALL samples in correlation to aforementioned prognostic parameters (*i.e.* white blood cell count, age at diagnosis, *in vitro* and *in vivo* prednisone response), showed that *RAS*-mutated *MLL*-rearranged infant ALL have higher white blood cell counts at diagnosis and displayed *in vitro* resistance towards prednisolone. This suggests a pivotal role for *RAS* in prednisolone resistance in *MLL*-rearranged infant ALL.

Chapter 8 reviews the current status of the knowledge on GC resistance in *MLL*-rearranged infant ALL. Herein we discuss the involvement of *S100A8/S100A9*, Annexin A2/S100A10 protein complex, PI3K pathway or BCL-2 family member proteins and the involvement of miRNAs in GC resistance in *MLL*-rearranged infant ALL.

In conclusion, in this thesis we describe novel prednisolone resistance mechanisms in *MLL*-rearranged infant ALL. Treatment with inhibitors like PP2 (targeting S100A8/S100A9 and Annexin A2 phosphorylation), PI3K inhibitors LY294002, wortmannin or PI-828, the pan-BCL-2 family member inhibitors gossypol or AT-101, offer great perspectives to improve the outcome of *MLL*-rearranged infant ALL.

REFERENCES

Dördelmann M, Reiter A, Borkhardt A, Ludwig WD, Gotz N, Viehmann S, *et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*. 1999. 94(4):1209-1217.

Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, *et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *Journal of Clinical Oncology*. 2003. 21(17):3262-3268.

Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJ, Kazemier KM, Pei D, Cheng C, Pui CH, Relling MV, Janka-Schaub GE, Pieters R, Evans WE. Gene expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *New England Journal of Medicin*. 2004. 351(6):533-542.

Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood*. 1998. 92:259-266.

Krebs J. The role of calcium in apoptosis. Biometals. 1998. 11(4):375-82.

Minagawa N, Kruglov EA, Dranoff JA, Robert ME, Gores GJ, Nathanson MH. The anti-apoptotic protein MCL1 inhibits mitochondrial Ca2+ signals. *Journal of Biological Chemistry*. 2005. 280(39):33637-33644.

Reddy TR, Li C, Guo X, Myrvang HK, Fischer PM, Dekker LV. Design, synthesis, and structure-activity relationship exploration of 1-substituted 4-aroyl-3-hydroxy-5-phenyl-1H-pyrrol-2(5H)-one analogues as inhibitors of the annexin A2-S100A10 protein interaction. *Journal of Medical Chemistry*. 2011. 54:2080-2094.

Ryckman C, Gilbert C, de Medicis R, Lussier A, Vandal K, Tessier PA. Monosodium urate monohydrate crystals induce the release of the proinflammatory protein S100A8/A9 from neutrophils. *Journal of Leukocyte Biology*. 2004. 76(2):433-440.

Schieven GL, Kirihara JM, Gilliland LK, Uckun FM, Ledbetter JA. Ultraviolet radiation rapidly induces tyrosine phosphorylation and calcium signaling in lymphocytes. *Molecular Biology of the Cell*. 1993. 4(5):523-30.

Spijkers-Hagelstein JAP, Schneider P, Hulleman E, de Boer J, Williams O, Pieters R, Stam RW. Elevated *S100A8/S100A9* expression causes glucocorticoid resistance in *MLL*-rearranged infant acute lymphoblastic leukemia. *Leukemia*. 2012. 26(6):1255-1265.

Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, de Lorenzo P, Sallan SE, Brady HJ, Armstrong SA, Pieters R. Association of high-level *MCL1* expression with *in vitro* and *in vivo* prednisone resistance in *MLL*-rearranged infant acute lymphoblastic leukemia. *Blood*. 2010a. 115(5):1018-1025.

Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, de Lorenzo P, Valsecchi MG, Pieters R. Gene expression profiling-based dissection of *MLL* translocated and *MLL* germline acute lymphoblastic leukemia in infants. *Blood*. 2010b. 115(14):2835-2844.

CHAPTER 10

Nederlandse Samenvatting

Jaarlijks worden in Nederland ongeveer 650 kinderen (0-18 jaar) gediagnosticeerd met kanker. Circa 20% van deze kinderen met kanker ontwikkelt acute lymfatische leukemie (ALL), welke vooral voorkomt rond de kleuterleeftijd. Bij leukemie is er sprake van een ongeremde groei van onrijpe witte bloedcellen. Hoewel de overlevingskansen voor kinderen met leukemie door verbeterde behandelingsmethoden is gestegen van 10% (1960-1965) naar inmiddels ongeveer 85%, is de overlevingskans voor zuigelingen (jonger dan 1 jaar) met ALL slechts 50%. Tachtig procent van de zuigelingen met leukemie hebben een herschikking van het "*Mixed Lineage Leukemia" (MLL)* gen. Deze genherschikking zorgt voor de slechte prognose van ALL bij zuigelingen: aangezien de dragers hiervan een overlevingskans hebben van ~50% terwijl de overlevingskans bij zuigelingen met ALL zonder een *MLL* herschikking ~80% is. Een factor die een belangrijke bijdrage levert aan deze lage overleving is ongevoeligheid van de ALL cellen voor chemotherapeutica, waaronder voornamelijk de glucocorticoïden zoals prednison. Zowel *in vitro* als *in vivo* prednisongevoeligheid voorspellen de overlevingskans. Om de lage overlevingskans van zuigelingen met ALL met een *MLL*-genherschikking te verbeteren hebben we onderzocht waarom deze patiënten ongevoelig zijn voor prednisolon (het actieve bestandsdeel van prednison).

In **hoofdstuk 2** hebben we *in vitro* chemotherapierespons van zuigelingen met *MLL*-herschikte ALL gemeten. Zuigelingen met ALL patiënten die ongevoelig zijn voor de chemotherapeutica prednisolon, vincristine en L-asparaginase (PVA) blijken een slechtere prognose te hebben dan patiënten die gevoelig zijn voor deze geneesmiddelen. Verder was ongevoeligheid voor prednisolon of vincristine geassocieerd met een kruisongevoelighed voor dexamethason en daunorubicine. Daarnaast bleek resistentie voor prednisolon gekoppeld te zijn aan andere parameters die een slechtere overlevingskans voorspellen, zoals de hoeveelheid witte bloedcellen bij diagnose en de leeftijd bij diagnose. Dit alles leidt tot onderzoek van prednisolon-ongevoeligheid bij zuigelingen met ALL en een *MLL* herschikking.

Recentelijk is beschreven dat *MCL1*, een eiwit dat celdood remt, hoog tot expressie komt in cellen die ongevoelig zijn voor prednisolon. Dit was het geval bij zowel kinderen met ALL als zuigelingen met ALL met een *MLL* herschikking hebben. In **hoofdstuk 3** zijn een aantal mogelijke MCL1 remmers onderzocht die prednisolon-gevoeligheid induceren. Hiervan bleken alleen gossypol en AT-101 in staat om prednisolon-gevoeligheid te induceren in *MLL*-herschikte ALL cellen. Echter ging dit niet gepaard met een remming van het MCL1 eiwit, maar met activatie van pro-apoptotische eiwitten BAD, BID, BIM en NOXA. Beide chemotherapeutica waren in staat de celdeling te remmen en celdood te induceren, maar enkel wanneer deze cellen gelijktijdig blootgesteld werden aan prednisolon. Hoewel MCL1 zelf niet geremd wordt, kan wel de functie van MCL1 geremd worden. MCL1 is namelijk in staat om calcium in het mitochondrium te binden en vervolgens de afgifte van cytochroom c te remmen. In het volgende hoofdstuk beschrijven we een ander mechanisme dat de calciumconcentraties in de cel kan veranderen en onafhankelijk van MCL1 functioneert en een belangrijke rol speelt bij het in stand houden van prednisolon-ongevoeligheid in *MLL*-herschikte ALL cellen. In **hoofdstuk 4** hebben we de rol van twee calciumbindingseiwitten *S100A8* en *S100A9*. Deze genen komen hoog tot expressie in ALL cellen van zuigelingen die prednisolon-ongevoelig zijn en een *MLL*

herschikking hebben. Leukemiecellen van zuigelingen zonder een *MLL* herschikking of van oudere kinderen hadden een veel lagere expressie van *S100A8* en *S100A9*. De eiwitproducten van deze genen waren in staat om het calcium te remmen, dat onder invloed van prednisolon vanuit het endoplasmatisch reticulum in de cel wordt vrijgegeven. De functie van MCL1 is dus alleen belangrijk in *MLL*-herschikte ALL cellen wanneer de expressie van *S100A8* en *S100A9* laag is en hun calciumbinding niet kunnen uitvoeren. Het herintroduceren van S100A8 en S100A9 in prednisolon-gevoelige cellen leidde ertoe dat deze cellen ongevoelig werden voor prednisolon. In een eerdere studie is beschreven dat de Src kinase remmer PP2 de afgifte van S100A8/S100A9 door neutrofiele cellen remt. In hoofdstuk 4 wordt beschreven dat PP2 ook in staat is om de expressie van S100A8/S100A9 in prednisolon-resistente cellen te remmen wat tot een verhoogde calciumconcentratie in de cel en uiteindelijk ook tot prednisolon-gevoeligheid leidde.

Een ander calciumbindingseiwit – annexine A2 – komt ook hoog tot expressie in leukemische cellen van zuigelen met ALL en een *MLL* herschikking. Echter, de activiteit van annexine A2 is onafhankelijk van de calciumconcentratie in de cel (**hoofdstuk 5**). De activatie van annexine A2 (gevormd door het gen *ANXA2*) wordt gereguleerd door Src kinase-gemedieërde fosforylatie. Remming van de Src kinases FYN of LCK, door enerzijds specifieke chemische remmers of door anderzijds shRNA-gemedieërde remming leidde in beide gevallen tot prednisolon-gevoeligheid in prednisolon-ongevoelige cellen. Dit fenomeen ging gepaard met een remming in celdeling en een verminderde fosforylatie van annexine A2 (actief annexine A2). Annexine A2 kan enkel door Src kinases worden gefosforyleerd in de aanwezigheid van het calciumbindingseiwit S100A10. Net zoals *S100A8* en *S100A9*, is ook de expressie van *S100A10* erg hoog in zuigelingen met ALL en een *MLL* herschikking. Hoewel deze eiwitten tot dezelfde familie van eiwitten behoren, hebben ze niet allen dezelfde functie. In tegenstelling tot *S100A8* en *S100A9* is de expressie van *S100A10* sterk gecorreleerd met de expressie van *ANXA2*. shRNA-gemedieërde remming van S100A10 leidde to prednisolon-gevoeligheid, welke gepaard ging met een verminderde fosforylatie van annexine A2.

In **hoofdstuk 6** is gebruik gemaakt van de zogenoemde "connectivity map analyse". Het genexpressieprofiel dat specifiek is voor prednisolon-resistentie in *MLL*-herschikte ALL bij zuigelingen is geanalyseerd, met als doel om geneesmiddelen te vinden die het prednisolon-ongevoelige profiel kunnen omzetten in een prednisolon-gevoelig profiel. Uit deze analyse bleek de PI3K remmer LY294002 een sterke kandidaat te zijn. *MLL*-herschikte cellen die werden behandeld met LY294002 werden geremd in hun celdeling en werden uiteindelijk ook prednisolon-gevoelig. Ook andere PI3K remmers – wortmannin en PI-828 – bleken in staat te zijn om prednisolon-gevoeligheid te induceren. In een poging de genen te identificeren die het meest betrokken zijn bij prednisolon-resistentie, hebben we prednisolon-ongevoelige cellen met een *MLL* herschikking blootgesteld aan enkel LY294002, wortmannin of PI-828 om zo alle genen binnen het prednisolon-ongevoeligheidsprofiel te valideren. Hieruit bleek dat de expressie van het *FCGR1B* gen in alle drie de behandelingen werd geremd. Een specifieke shRNA-gemedieërde remming van *FCGR1B* leidde ook tot prednisolon-gevoeligheid, wat

suggereert dat *FCGR1B* een prominente rol speelt in het behouden van het prednisolon-ongevoelige karakter in ALL cellen met een *MLL* herschikking.

In **hoofdstuk 7** is de gehele zuigelingen populatie met ALL gescreend op *RAS* mutaties. *K-RAS-*, *N-RAS-* en *B-RAF* mutaties zijn de meest voorkomende mutaties in andere soorten tumoren. Ongeveer 14% van alle zuigelingen ALL met een *MLL* herschikking hadden *RAS* mutaties van voornamelijk *K-RAS* of *N-RAS*, maar niet van *B-RAF*. Het merendeel van de *RAS* mutaties bleek voornamelijk voor te komen bij zuigelingen met ALL die een MLL-AF4 herschikking hebben. De aanwezigheid van een *RAS* mutatie bij *MLL*-herschikte ALL bij zuigelingen bleek gecorreleerd te zijn aan hoge aantallen witte bloedcellen bij diagnose en ongevoeligheid voor prednisolon. Deze waarneming oppert mogelijk een rol voor *RAS* signalering in prednisolon-resistentie.

Tenslotte zijn in **hoofdstuk 8** de voornaamste prednisolon-resistentie mechanismen in zuigelingen met ALL met een *MLL* herschikking beschreven en bediscussieerd, waaronder de functie van S100A8/S100A9, Annexine A2 en S100A10, PI3 kinase en de rol van miRNAs in dit resistentie mechanisme.

In conclusie: in dit proefschrift zijn meerdere nieuwe mechanismen beschreven die betrokken zijn bij prednisolon-resistentie in *MLL*-herschikte zuigelingen ALL. Het behandelen van zuigelingen ALL met een *MLL* herschikking met PP2 (remming van S100A8/S100A9, defosforylatie van annexine A2), PI3K remmers, gossypol en/of AT-101 bieden mogelijk betere overlevingskansen voor deze patiënten. Dit zal in toekomstige klinische studies moeten worden aangetoond.

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Thanks to all the **infant group members** for all their expertise and the fun trips we did (midgetgolf, bowling, going to Blijdorp Zoo, etc.).

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Pauline, zowel als hoofdanaliste van het gehele oncologielab, alsook allround-analist binnen de infantgroep ben onmisbaar en lever je een bijzondere bijdrage. Geen vraag om hulp was jou te veel. Dank voor je praktische en persoonlijke steun die jij mij de laatste maanden van mijn AIO periode in het bijzonder hebt geboden, vooral bij de vele Western blots (50 stuks, of zelfs meer!?). En hopelijk kunnen we in de toekomst elkaar bij een hapje en een drankje blijven zien.

Patricia, it was nice to start your first weeks together in the lab. This really was the foundation of a good colleagueship but even better friendship. Thank you for all your input in our infant group. You are an excellent scientist with a broad knowledge. I hope to read your work soon in the many publications to come. You are truly inspiring, not the least in your excellent help during my last writings. I appreciated all your work and comments!

Marieke en Emma, ik vond het altijd heel erg gezellig met jullie. Emma, ik vond het toch wel erg toevallig dat wij samen op de zelfde dag bevielen van twee schatten van kinderen. Geweldig! Heel veel succes met jullie (laatste loodjes van jullie) promotie en succes met jullie verdere carriere.

Eddy, als postdoc kwam jij het epigenetische deel van het infantgroepje ondersteunen. Inmiddels ben je betrokken bij meerdere projecten. Veel succes met je onderzoek en sterkte als één van de weinige mannen op het lab.

Merel, als startende analist weet jij wat aanpakken is en jij weet je ook al goed binnen de groep te passen. Niets is jou te veel, je helpt wanneer je helpen kunt. Vooral bedankt voor je hulp bij mijn laatste experimenten! Nannet, Isabelle, Sander en Rianne, dank voor al jullie hulp en inzet tijdens jullie stages. Ik vind het heel leuk dat er vanuit de Hogeschool Heerlen nu eindelijk meer studenten hun stage "buiten Limburg" uitvoeren. Veel succes met jullie verdere studie/loopbaan!

Dominique, hoewel je nu niet meer werkzaam bent in Rotterdam behoor je voor mij nog altijd tot de infant groep. Dank voor al jouw interesse en steun. Door je goede presentatietechnieken en translationele kennis ben jij bent een ware inspiratie voor mij en de overige AIO's. Fantastisch hoe jij het allemaal hebt weten af te ronden! Heel veel succes als kinderarts in opleiding in Utrecht. Dit gaat je vanzelfsprekend lukken!

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Jean, bedankt voor het ontwikkelen van al die primersets (cmap paper) en de bijbehorende optimalisaties. Wat een klus, om dit voor maar liefst 45 (!) genen te doen! Veel succes met je nieuwe baan!

De studenten die ik heb mogen begeleiden **Nannet**, **Isabelle**, **Sander** en **Rianne**: dank voor jullie inzet. Een aantal door-jullie-uitgevoerde experimenten zijn terug te vinden in verscheidene hoofdstukken. Ik ben benieuwd waar jullie zullen eindigen.

Bedankt aan alle **werkgroepleiders**, **kinderoncologen**, **AIO´s** (in de kliniek), **specieel lab**, **secretaressen** en alle **anderen die tot de afdeling kinderoncologie behoren**, maar in het bijzonder dank aan: grote dank gaat uit naar **Jules**, jij wist tijdens besprekingen ook altijd datgene te vragen wat nog extra uitwerking nodig hadden. Dank voor je kritische maar altijd opbouwende noot! Als onderzoeker heb ik enorm veel van jou geleerd (en al helemaal in mijn huidige functie)! Succes met je succesvolle en groeiende T-ALL groep!

Dear **Dr O. Williams** and **Dr J. de Boer**, dear Owen and Jasper; thank you for letting me visit your lab. Almost five years ago, I had the great opportunity to travel to University College London; I stayed at your department for one month. Thank you for teaching me how to perform proper stable cellular infections. And indeed, after a while trying in our lab, we finally managed it (chapters 5 and 6). Thank you for your enthusiasm, your guidance and hospitality during my stay and the pleasant email communications afterwards. Hopefully you enjoyed the Dutch beer!? Good luck to the both of you and to the leukemia group!

Lab kindergeneeskunde: iedereen (!) bedankt voor alle leerzame en gezellige momenten. Grappig dat doordat ik enkele vlaaien had meegebracht, het toen vergane initiatief om een taartbak-, en de volgende koekjesbak-, borrelhapjeswedstrijden etc. te starten opnieuw heeft aangewakkerd.

In het bijzonder dank aan **Marcel**, **Ingrid** en **Silvia E**. Naast jullie kennis en bereidwilligheid dit te delen, heb ik ook mogen genieten van jullie ideeën over koken. **Ingrid**, de vele jaren werken bij het lab kindergeneeskunde heb jij mogen ruilen voor je pensioen. Wat een prestatie. Geniet ervan! Jammer dat we geen recepten meer kunnen uitwisselen. **Marcel**, jammer dat er nu nog steeds geen wijnproeverij op het lab is geweest. Dat had ik graag willen meemaken. Veel plezier in je nieuwe functie! **Silvia E**., kamergenootje. Gelukkig voelde je niet geïntimideerd door al die onco's om je heen. Ik vond het erg gezellig om jouw achterbuurvrouw te mogen zijn. Merci voor de vele croissantjes! Veel plezier met de nieuwe lichting kamergenootjes.

Dank gaat uit naar de **(oud)postdocs** binnen de kinderoncologie: **Astrid, Kirsten, Yun-Lei, Dirk, Elnaz, Maghban, Marjon, Judith, Esther, Lieke en Mirjam** voor de bijdrage die jullie hebben geleverd gedurende deze periode.

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Natuurlijk gaat ook veel dank uit aan alle **(oud)analisten** binnen de kinderoncologie: **Susan, Nicola, Lonneke, Jessica, Wilco, Clarissa, Mathilde, Monique, Ellen, Marieke, Carla, Rosanna** en **Pieter**. Jullie vormen een heel divers groepje met elk zijn eigen expertise. Veel succes, binnen of buiten de kinderoncologie.

Alle **promovendi binnen de kinderoncologie** bedankt voor jullie vakinhoudelijke bijdrage en gezelligheid tijdens onze eigen variatie van *get out of your lab days* (of beter gezegd: *hours*). **Diana, Iris, Irene** en **Brian**, elk van jullie heeft het AIO-schap naar een goed einde weten te brengen en zijn daarmee een voorbeeld voor de toekomstige AIO's. Veel succes met jullie opleidingen. Well done!

Floor, bedankt voor de gemeenschappelijke treinreizen; er is geen stil moment met jou geweest! Het leuke initiatief dat je nam om te koken voor Diana, Dominique en mij is zeer gewaardeerd. Veel plezier met je functie binnen de Klinische Genetica; je zult hier vast je draai kunnen vinden!

Andrica, Marjolein, Trudy, Eva, Anna, Malou, Farhad, Stefanie, João, Janet, Imbritt, Maartje, Rui, Laura, Wing en **Lizet**: succes met jullie promotie en/of nieuwe baan! Farhad, João and Rui, hopefully you can read the previous sentence. That's right, it is in Dutch!

Jenny en **Ingrid**, bedankt voor al jullie gezellige koffietijd- en dus kletsmomentjes. Ze waren erg hard nodig. Leuk dat we het initiatief hebben genomen om ook buiten werktijd af te spreken. Ik vind het nog steeds heel gezellig om voor elkaar te koken of samen te gaan uiteten. **Jenny**, veel succes met je laatste loodjes en met je nieuwe start om weer aan je studie (co-schappen) te beginnen. Je kunt het! **Ingrid**, ooch ich mis dich al, veural als "pred-AIO-maatje-oes-Limbeurrig". Ich how het dankwoad al lang jeschjreave, wos ich veul dats du nog ieder promoveerts wie ich. Ich how dich namelijk veul succes mit ding litste paar moand vaan dië jaanse leuke ongerseuk jeweunscht! Mer ich woal dich lotte wisse dats du inge jowwe ongerseuker bis, en dat tse ding promotie vlot en hiel jot has jedao! Du keunst der waal, dat wees ich sicher!!! Veul succes en plezeer in Boston ;-)

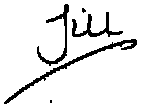
Arian, de laatste maanden mocht ik met jou aan mijn zijde doorbrengen. Na het vertrek van Linda, heb jij haar plaats ingenomen. Nou ja, meer dan alleen de plaats van haar, daar was de benodigde rode tape welk de grens van onze bureauruimten aangaf het duidelijkst voorbeeld van. Hoe vaak deze toch nog altijd werd overschreden! Ik heb een mooie tijd met jou als buurman beleefd en heb er leuke herinneringen aan overgehouden. Bedankt voor je belangstelling in werk- en niet-werkrelateerde dingen. Heel veel succes met jouw laatste loodjes om het proefschrift af te ronden (binnenkort mag jij je proefschrift uitdelen) en als AGNIO in Antwerpen!

Linda, je bent als laatste van de hele 'bups' hier beschreven, zodat ik wat meer tekst en dankbetuiging aan je kan wijden als je 'buurvrouw', maar zeer zeker als een hele goede vriendin. Ik vond het wel grappig om te lezen dat mensen zeiden dat wanneer "je Linda zag, je mij ook zag". Zo zagen anderen ons, en inderdaad vanaf het begin was er een juiste klik, we voelden elkaar goed aan. Vooral wanneer er even behoefte was om te kletsen, of juist niet! We hebben lief en leed gedeeld. We hebben samen veel gereisd en veel leuke en interessante dingen ondernomen. Ik vond het heel bijzonder om jou als mijn bruidsmeisje op mijn bruiloft te hebben, en nu als paranimf naast mijn zijde. Bedankt voor alle leuke lach-, huil- en kletsbuien. Inmiddels werk je weer een behoorlijke tijd bij Somantix als postdoc waar je je goed kunt ontwikkelen. Veel succes met jouw laatste loodjes voor je promotieonderzoek en je verdere onderzoekscarriere! Tot snel!

Velen van jullie zijn naast goede collegae ook goede vrienden geworden; op naar een volgende gezamenlijk etentje! Sushi?

Tenslotte, wil ik toch nog de gelegenheid nemen om **vrienden** en **familie** te bedanken. Het zijn er teveel om op te noemen, daarom doe ik het zo: **vrienden uit de Randstad**, **vrienden uit het Zuiden**, **Anique**, **Michiel** en jullie dochtertje **Lena**, **Emile** en **Paula**, dank voor al jullie interesse en steun die jullie hebben gegeven. En grote dank aan mijn ouders en zusje: **pap**, **mam** en **Joyce**, dank voor jullie onvoorwaardelijke liefde, steun en al het andere gedurende deze niet-altijd-even-gemakkelijke periode (het promotietraject, maar ook mijn verhuizing naar Utrecht: en uiteindelijk weer wat zuidelijker). Het wordt enorm gewaardeerd!

Last but (definitely) not least. **Léon**, hoewel jij een niet-werkgerelateerd dankwoord afgezaagd vindt, wil ik toch ook graag mijn dank ook naar jou toe betuigen. Schjattie, bedankt voor je onvoorwaardelijke steun die ik van jou als echtgenoot, *soulmate*, maatje en ook als mede-AIO heb mogen ontvangen naast natuurlijk jouw oprechte liefde! Ik ben heel trots op jou en je prestaties! Hehe, ben je alsnog eerder gepromoveerd! Maar ik ben vooral blij met ons gezinnetje welk met Lisa voor belangrijke mate is uitgebreid! **Lisa**, jij bent papa's en mama's mooiste en fijnste ervaring tijdens deze reis geweest. De laatste stelling is daarom ook aan jou opgedragen. Dikke pütschj voor jullie beiden!



About the Author

LIST OF PUBLICATIONS

Spijkers-Hagelstein JAP, Pieters R, Stam RW. Glucocorticoid resistance in *MLL*-rearranged infant acute lymphoblastic leukaemia. *Submitted* 2014.

Spijkers-Hagelstein JAP, Driessen EM, Schneider P, Garrido Castro P, den Boer ML, Pieters R, Stam RW. Relation between *in vitro* drug responses and prognostic markers in *MLL*-rearranged infant acute lymphoblastic leukemia. *Submitted* 2014.

Spijkers-Hagelstein JAP, Schneider P, Mimoso Pinhancos, Garrido Castro P, Pieters R, Stam RW. Glucocorticoid sensitization in *MLL*-rearranged ALL cells by the pan-BCL-2-family member inhibitors gossypol and AT-101. *In revision at European Journal of Cancer.* February 2014 (impact factor 5.061).

Spijkers-Hagelstein JAP, Mimoso Pinhancos, Schneider P, Pieters R, Stam RW. Gene expression-based *in silico* screening for glucocorticoid-sensitizing therapeutics in *MLL*-rearranged infant ALL identifies PI3K inhibitors as potential modulators of prednisolone resistance. *Leukemia***.** August 2013. (impact factor 10.164)

Driessen EMC, van Roon EHJ, Spijkers-Hagelstein JAP, Schneider P, de Lorenzo P, Valsecchi MG, Pieters R, Stam RW. Frequencies and prognostic impact of *RAS* mutations in *MLL*-rearranged acute lymphoblastic leukemia in infants. *Haematologica***.** Feb 12. 2013. (impact factor 6.424)

Spijkers-Hagelstein JAP, Mimoso Pinhancos, Schneider P, Pieters R, Stam RW. Src kinase-induced phosphorylation of annexin A2 mediates glucocorticoid resistance in *MLL*-rearranged acute lymphoblastic leukemia. *Leukemia***.** Dec 28. 2012 (impact factor 9.561)

Spijkers-Hagelstein JAP, Schneider P, Hulleman E, de Boer J, Williams O, Pieters R, Stam RW. Elevated *S100A8/S100A9* expression causes glucocorticoid resistance in *MLL*-rearranged infant acute lymphoblastic leukemia. *Leukemia*. Jan 27. 2012 (impact factor 9.561)

Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, Menezes RX, de Lorenzo P, Valsecchi MG, Pieters R. Gene expression profiling-based dissection of *MLL* translocated and *MLL* germline acute lymphoblastic leukemia in infants. *Blood*. Apr 8. 2010 (impact factor 9.898)

Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein JA, Valsecchi MG, de Lorenzo P, Sallan SE, Brady HJ, Armstrong SA, Pieters R. Association of high-level MCL1 expression with *in vitro* and *in vivo* prednisone resistance in *MLL*-rearranged infant acute lymphoblastic leukemia. *Blood*. Feb 4. 2010 (impact factor 9.898)

Pijnenborg JM, Wijnakker M, Hagelstein J, Delvoux B, Groothuis PG. Hypoxia contributes to development of recurrent endometrial carcinoma**.** *International Journal of Gynecology Cancer*. Aug 17. 2007 (impact factor 2.18)

PHD PORTFOLIO

Name PhD student:	Jill AP Spijkers-Hagelstein, MSc
EMC department:	Pediatric Oncology
Research School:	Molecular Medicine
PhD Period:	1 September 2007- 1 September 2011
Promotor:	Prof. dr. R. Pieters
Supervisor:	Dr. RW. Stam

1. PhD training	Year	Workload (ECTS)
General Courses		
Biomedical English Writing and Communication	2009	4
Classical methods for data-analysis (CC02)	2007-2008	5.7
Specific Courses		
Molecular Medicine	2008	1.8
Analysis of microarray gene expression data using R/Bio C and web tools	2008	1.4
Basic and Translational Oncology	2007	1.8
Seminars and Workshops		
Annual Molecular Medicine Day	2007-2011	1.2
Workshop "Browsing genes and genomes with Ensembl"	2007	0.6
Oral Presentations		
7th Bi-annual I-BFM Leukemia Symposium, Antalya, Turkey	2010	1.6
51th ASH Annual Meeting, New Orleans, USA	2009	0.6
Yearly department presentations (3/year)	2007-2011	2.4
Yearly workgroup presentations (inclusive journal club)	2007-2011	2
Poster Presentations		
17th Congress of EHA, Amsterdam, The Netherlands (2x)	2012	2
8th Bi-annual I-BFM Leukemia Symposium, Santiago, Chili (2x)	2012	2
14th Annual Molecular Medicine Day, Rotterdam	2010	1
Annual Pediatric Research Meeting, Rotterdam	2009	0.6
International Conferences		
AACR Conference, Amsterdam, The Netherlands	2010	1
51th ASH Annual Meeting, New Orleans, USA	2009	1
BSH conference, Glasgow, UK	2008	0.6
50th ASH Annual Meeting, San Francisco, USA	2008	1
Other		
Visiting Researcher at University College London Institute of Child Health and Great Ormond Street Hospital for Children, London, UK		
Department of Molecular Haematology and Cancer Biology under supervision of Dr. O. Williams and Dr. J. de Boer		

2. Teaching	Year	Workload (ECTS)
Supervising Rianne Strik, student of Biomedical Laboratory School, Life Sciences, Hogeschool Zuyd (Heerlen) during her 5-month traineeship on "Prednisolone resistance in *MLL*-rearranged infant ALL - involvement of Src kinases" (Bachelor's theses; Sept 2010 - Jan 2011)	2010-2011	10
Supervising Isabelle Meijssen, student of Biomedical Laboratory School, Life Sciences, Hogeschool Zuyd (Heerlen) during her 8-month traineeship on "*In vitro* L-asparaginase (L-ASP) resistance in *MLL*-rearranged infant ALL - the role of sphingolipids" (Bachelor's theses; Nov 2008 - June 2009)	2008-2009	10
Supervising Nannet van der Schoot, student Medicine, University of Utrecht (Utrecht) during her 9-month traineeship on "The expression of asparagine synthetase (ASNS) related to L-asparaginase resistance in *MLL*-rearranged infant ALL" (Bachelor's theses; Sept 2008 - June 2009)	2008-2009	10
Total		**62.3**

CURRICULUM VITAE

Jill Spijkers-Hagelstein werd geboren op 5 augustus 1983 te Heerlen, Limburg. In 2001 behaalde zij haar VWO diploma in Kerkrade-West (Sancta Maria College). Hier werd ook al duidelijk dat zij graag onderzoek naar kanker wilde verrichten. De logische stap om haar studie te vervolgen aan de bacheloropleiding Biologisch en Medisch Laboratoriumonderzoek te HsZuyd was een feit. Gedurende deze opleiding heeft Jill 9 maanden stage gelopen bij de afdeling Pathologie van het Academisch Ziekenhuis Maastricht/Universiteit Maastricht onder supervisie van dr. Patrick Groothuis. Hier heeft Jill onderzoek gedaan naar de invloed van p53 op de celcyclus in endometriumcarcinomen. Na het behalen van haar bachelordiploma (2005) zette zij haar studiecarričre voort met een Masteropleiding aan de Universiteit Maastricht/Transnationale Universiteit Limburg. Gedurende de studie Klinische Moleculaire Wetenschap werd haar interesse in kankeronderzoek verder aangewakkerd door twee stages. Tijdens de zes maanden durende minor stage onderzocht Jill het effect van hypoxie op de tumorigenese bij de afdelingen Moleculaire Genetica, Moleculaire Celbiologie en MaastRO onder supervisie van dr. Jan-Willem Voncken, prof. dr. Bradley Wouters, dr. Ton Hopman en dr. Bert Schutte. Maar tijdens haar acht maanden durende major stage bij de afdeling Gezondheidsrisico Analyse en Toxicologie (GRAT), onder supervisie van dr. Sahar Khosrovani-van Barjesteh van Waalwijk en dr. Roger Godschalk, ontwikkelde zij een voorkeur voor specifiek leukemieonderzoek, alwaar zij ook een nieuwe assay heeft opgezet om het herstel van DNA dubbelstrengsbreuken te kunnen meten. Na het behalen van haar Masterdiploma (2007) is ze haar promotieonderzoek aan het Erasmus Medisch Centrum te Rotterdam gestart en verhuisde hiervoor naar Utrecht. Gedurende haar promotieonderzoek onderzocht Jill aan de afdeling Kinderoncologie de cellulaire mechanismen die betrokken zijn bij prednisolon-ongevoeligheid bij zuigelingen met acute lymfatische leukemie met een *MLL* herschikking onder het promotorschap van prof.dr. Rob Pieters en co-promotorschap van dr. Ronald W. Stam. Het resultaat van dit onderzoek is gepresenteerd in dit proefschrift en is veelvuldig gecommuniceerd op internationale congressen. Na haar promotieonderzoek heeft Jill nog een jaar als postdoc gewerkt aan dezelfde afdeling. Gedurende deze periode heeft ze de zogenoemde "synthetic lethality screening" opgestart, met het vinden van nieuwe mechanismen op prednisolon-ongevoeligheid in zuigelingen met leukemie tot doel, maar ook om tumorigenetisch-specifieke mechanismen voor *MLL*-herschikte leukemie te achterhalen. Vanaf oktober 2013 is Jill werkzaam als investigator bij Netherlands Translational Research Center (NTRC) BV te Oss, onder leiding van dr. Guido Zaman en dr. Rogier Buijsmans. Haar hobby's omvatten zaalvoetbal, dansen, tekenen en knutselen en genieten van haar gezinnetje. Ze woont samen met haar echtgenoot Léon in Utrecht en is moeder van een dochter Lisa (2012).