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The IL-15 cytokine system provides growth and survival signals in Hodgkin lymphoma and enhances the inflammatory phenotype of HRS cells

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Classical Hodgkin lymphoma (HL) is a common lymphoid malignancy that displays unique features with respect to morphology, tumor biology and clinical presentation. Histologically, cHL is characterized by large mononuclear Hodgkin and multinucleated Reed-Sternberg (HRS) cells. A hallmark of HL cells is the highly inflammatory phenotype. Typically, the malignant HRS cells represent only a minor fraction of the affected lymph nodes and are surrounded by numerous benign reactive cells, including lymphocytes, macrophages and granulocytes. These inflammatory cells are attracted by cytokines and chemokines secreted by the malignant cell population.[1,2] The complex interactions between HRS cells and bystander cells create a specific microenvironment that is thought to be critical for growth and survival of HRS cells. A detailed analysis of signals evoked by these interactions does not only contribute to a better understanding of HL pathogenesis, but might also provide a basis for the development of new treatment strategies. Here, we describe an aberrant expression and activity of the interleukin 15 (IL-15) cytokine/cytokine receptor system in HL, resulting in mitogenic and anti-apoptotic signals as well as the enhanced expression of inflammatory factors by HRS cells. IL-15 is a pro-inflammatory cytokine that was originally identified through its ability to induce T cell proliferation, displaying IL-2-like properties.[3] Indeed, both cytokines act through a heterotrimeric receptor that shares the IL-2 receptor β(IL-2Rβ, also referred to as IL-15Rβ, CD122) and the common γ chain (IL-2Rγ, CD132). However, high affinity binding specificity for each ligand is conferred by a unique subunit, namely IL-2Rα and IL-15Rα, respectively.[3] Functionally, IL-15 plays a pivotal role in the differentiation and/or survival of natural killer (NK) cells, NK T cells as well as intraepithelial lymphocytes (IELs) and is critical for the maintenance of $CD8^+$ memory T cells.[4]

To identify factors that are involved in the formation of the HL-specific tumor microenvironment, we screened microarray gene expression profiles of HRS and B non-Hodgkin cell lines with a special focus on cytokines and cytokine receptors. In addition to several factors known to be highly expressed by HRS cells, such as IL-6, IL-13 and IL-13Rα, we identified an up-regulation of IL-15 and the corresponding receptor components IL-15Rα,

IL-2Rβ/IL-15Rβ and IL-2Rγ in Hodgkin-derived cell lines (Supplementary Figure 1). Given the role of IL-15 as a prominent pro-inflammatory cytokine and its growth-supporting effects on several lymphoid cell populations, we decided to investigate the role of IL-15 in HL in more detail. First, we examined the expression status of IL-15 and its receptor components in a broad panel of lymphoma-derived cell lines. As determined by quantitative PCR, most HL cell lines showed a strong up-regulation of *IL-15* mRNA compared to non-Hodgkin B cell lines (Figure 1a). For *IL-15Rα* and *IL-2Rβ/IL-15Rβ* mRNA, we observed a similar overexpression in HL cell lines, whereas the mRNA for the common γ chain (*IL-2Rγ*) was ubiquitously expressed in all cell lines tested (Figure 1a). Subsequently, the expression of the IL-15 receptor components was analyzed on protein level by flow cytometry, corroborating enhanced expression of IL-15Rα and IL-2/IL-15Rβ on HRS cell lines (Figure 1b).

To investigate the expression of IL-15 in primary HL cases, we performed immunohistochemical staining on frozen sections of control and HL tissue samples. Immunohistochemistry of non-malignant reactive tonsils demonstrated specific staining for IL-15 predominantly in cells displaying macrophage or dendritic cell morphology, a feature that was particularly apparent in germinal centers, most likely corresponding to follicular dendritic cells,[5] whereas GC B cells themselves were negative (Figure 1c). Moreover, immunohistochemistry revealed IL-15 expression in HRS cells in a series of 8 primary HL cases with variable percentages of positive cells (Figure 1c; Supplementary Table 1). In addition, cells of the tumor microenvironment stained positive for IL-15, including cells with monocytic/dendritic morphology and endothelial cells, which is in accordance with previous studies describing IL-15 expression in these cell types.[6-8] These observations suggest that two sources of IL-15 exist in HL-affected lymph nodes which might act either in an autocrine or paracrine manner on HRS cells.

Next, we investigated the functional role of IL-15 signaling in HRS cells. To this end, HRS cell lines were stimulated with recombinant human (rh) IL-15 and the activation status of intracellular signaling pathways, which have been described to respond to IL-15 in other

cellular systems,[9] was monitored in a time- and dose-dependent manner. Stimulation with rhIL-15 induced the phosphorylation of the MAP kinases ERK1/2 as well as STAT5 in HRS cell lines, showing the most pronounced effects on STAT5 activation (Figure 2a). Furthermore, we observed an increased proliferation of KM-H2 and L591 HRS cells after IL-15 stimulation as determined by [^{3}H]-thymidine incorporation (Figure 2b). Next, we analyzed whether IL-15 mediates survival signals for HRS cells, especially in the context of cellular stress evoked by treatment with chemotherapeutic agents. For this purpose, KM-H2 cells were pre-stimulated with rhIL-15 and subsequently treated with etoposide or doxorubicin, which are part of HL treatment regimens, or geldanamycin, a pharmacologic inhibitor of heat shock protein 90 (HSP90) and NF-κB signaling.[10] Measuring the percentage of viable and apoptotic cells by annexin V-FITC/propidium iodide (PI) staining and flow cytometry, we observed that pre-treatment with rhIL-15 is able to mitigate drug-induced cell death in the KM-H2 HRS cell line (Figure 2c). Furthermore, cells were stained in parallel with an antibody that recognizes active caspase 3, which is an indicator of ongoing programmed cell death, revealing a lower percentage of apoptotic cells in rhIL-15 pre-treated KM-H2 cells (Figure 2c).

To gain insight into the molecular mechanisms of IL-15 signaling in HRS cells, we performed oligonucleotide microarray analysis of KM-H2 cells following rhIL-15 stimulation. Gene expression profiles of KM-H2 cells were determined at 0, 4, 10 and 24 hours of rhIL-15 treatment and normalized to profiles of PBS-treated KM-H2 cells that were analyzed in parallel to control for expression changes induced by growth in cell culture *per se*. This approach identified a specific set of genes that showed a significant regulation by rhIL-15 at the respective time points, with 43 genes being differentially expressed at 24 hours (with a fold change of at least $\log2^{0.75}$; Supplementary Table 2). To identify biological processes regulated by IL-15, we used the DAVID classification tool that classifies gene lists into functionally related gene groups. In accordance with our functional data, we observed a significant enrichment of gene ontology (GO) terms associated with cellular proliferation and survival within our set of rhIL-15-regulated genes (Supplementary Table 3). Remarkably, we

also found a prominent enrichment of clusters related to inflammatory processes, such as cytokine production and activity, receptor-ligand or cell-cell interactions and lymphocyte activation (Supplementary Table 3). A number of genes that were differentially expressed in the microarray experiments were selected from the functional groups for verification by quantitative PCR. Indeed, we could corroborate a significant regulation for the cell-cycle regulator *CCND2* (*cyclin D2*), for the transcription factors *ETV5* and *BCL6* as well as for several cytokines/chemokines, including *IL-1*α, *IL-6*, *IL-9*, *IL-12*β, *CCL3* (*MIP-1*α) and the receptor *IL-2R*α (Figure 2d), supporting the notion that IL-15 has a significant impact on mediators of inflammatory responses.

Taken together, our data demonstrate that the IL-15 cytokine/cytokine receptor system is up-regulated in HL and that stimulation of Hodgkin cells with rhIL-15 results in activation of MAP kinase and JAK/STAT5 signal transduction pathways. In addition, functional studies indicate that IL-15 promotes proliferation and apoptosis resistance of Hodgkin tumor cells, thereby extending previous reports that have described mitogenic and anti-apoptotic effects of IL-15 on lymphoid tumor cells of different entities, e.g. multiple myeloma, T cell large granular lymphocyte leukemia and cutaneous T cell lymphomas.[11-13] A role of IL-15 signaling in HL tumor biology is further supported by published gene expression data demonstrating that IL-15 and the corresponding receptors are not only up-regulated in primary HRS cells compared to various non-malignant and malignant B cell populations (Supplementary Table 5), but are also prominent components of deregulated functional pathways in HRS cells.[14,15] As a major feature of the cellular response to IL-15 stimulation, we identified a significant induction of cytokines and chemokines in HRS cells. Several of these factors, such as IL-6, IL-9 and CCL3, in turn may have the potential to mediate growth and survival signals for HRS cells as well as to attract or stimulate cells of the tumor microenvironment.[1,2] In summary, our study identifies IL-15 as part of the complex interactions between tumor cells and the microenvironment in HL that provides growth and survival signals for HRS cells.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary information is available at Leukemia's website.

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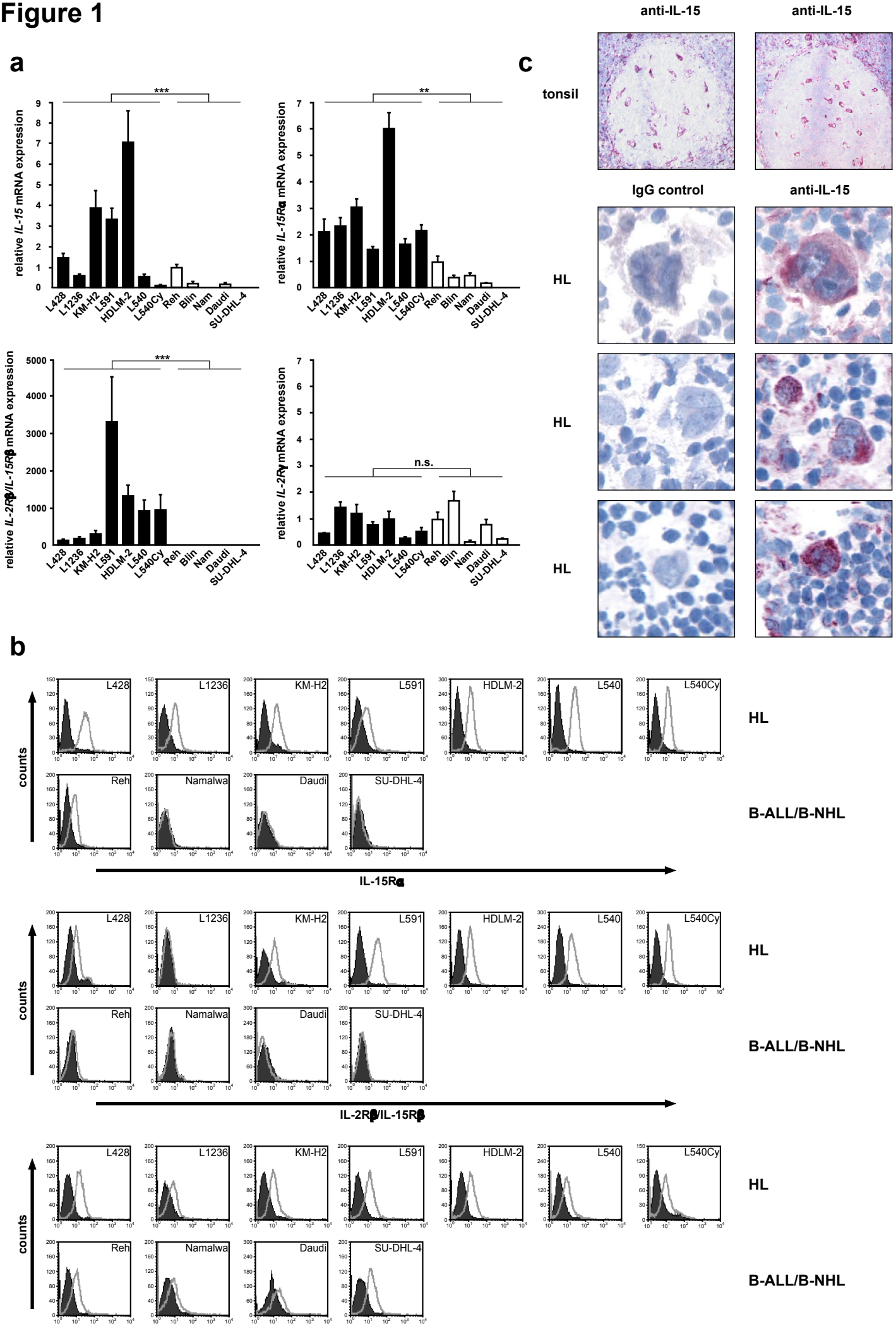
Figure legends

Figure 1. Expression of IL-15 and IL-15 receptor subunits in human B cell-derived cell lines and primary HL tissue. (**a**) Quantitative PCR analysis of *IL-15*, *IL-15Rα*, *IL-2Rβ/IL-15Rβ* and *IL-2Rγ* (*common gamma chain,* γ_c) mRNA in HRS (L428, L1236, KM-H2, L591, HDLM-2, L540, L540Cy) and B-ALL/B-NHL cell lines (Reh, Blin, Namalwa, Daudi, SU-DHL-4). *GAPDH* mRNA expression served as input control. Relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method with the cell line Reh defined as 1. Error bars show 95% confidence intervals. For statistical analysis, *P*-values were determined by a two-sided Welch's *t*-test using the ΔCt values of the two groups (HRS vs. non-Hodgkin). n.s. denotes not significant; ** *P*<0.01; *** *P*<0.001. (**b**) Protein expression of IL-15Rα, IL-2Rβ/IL-15Rβ and IL-2Rγ on HRS and B-ALL/B-NHL cell lines was determined by flow cytometry. Cells were stained with anti-IL-15Rα, anti-IL-2Rβ/IL-15Rβ and anti-IL-2Rγ antibodies, respectively, and primary antibodies were detected with PE-conjugated secondary antibodies (open histograms). Filled histograms indicate isotype-matched controls. (**c**) Immunohistochemistry for IL-15 in fresh frozen samples of reactive tonsils and primary HL tissue. Antigen detection was carried out with an anti-IL-15 mouse monoclonal antibody or a corresponding isotype control. Bound antibody was detected with polyclonal rabbit anti-mouse immunoglobulin and visualized using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method.

Figure 2. Functional activity of the IL-15/IL-15 receptor system in HRS cell lines. (**a**) HRS cell lines (L428, L1236, KM-H2, L591) were starved in medium with 1 % FBS overnight and treated with rhIL-15 (25 ng/ml or 50 ng/ml) or PBS control. Cells were harvested and analyzed for phosphorylation of ERK1/2 and STAT5 by Western blotting. Expression levels of total ERK1/2 and STAT5 protein were determined to control for equal loading. The IL-2/IL-15 responsive cutaneous T cell lymphoma cell line Se-Ax was used as positive control. (**b**) KM-H2 and L591 cells were treated with rhIL-15 (50 ng/ml) or PBS control, and [^{3}H]-

thymidine incorporation was determined for 24 hours after stimulation. Measurements were performed in triplicates and data are indicated as means ± s.d. One representative out of five independent experiments is shown. *P*-values were determined by a one-sided Welch's *t*-test. * *P*<0.05; ** *P*<0.01 (**c**) KM-H2 cells were pre-stimulated with rhIL-15 or PBS for 18 hours and subsequently treated with dimethyl sulfoxide (DMSO) or H_2O control, etoposide, doxorubicin or geldanamycin, as indicated. The percentage of viable cells, defined by flow cytometry as double-negative for annexin V-FITC and PI, is shown after treatment for 72 hours (upper panel). Measurements were performed in triplicates and data are indicated as means ± s.d. One representative out of three independent experiments is shown. *P*-values were determined by a one-sided Welch's *t*-test. n.s., not significant; * *P*<0.05; ** *P*<0.01. In parallel, KM-H2 cells were stained with an anti-active caspase 3-PE antibody (lower panel). The percentage of active caspase 3-positive cells is indicated. One out of three independent experiments is shown. (**d**) HRS KM-H2 cells were stimulated with rhIL-15 or PBS, harvested after 4, 10 and 24 hours of stimulation before being subjected to gene expression profiling using Illumina HumanHT-12 v4 Expression BeadChips. To verify the differential expression of selected genes following rhIL-15 treatment, quantitative PCR was performed for *CCND2* (encoding cyclin D2), *ETV5*, *BCL6*, *IL-1*α, *IL-6*, *IL-9*, *IL-12*β, *CCL3* (*MIP-1*α) and *IL-2R*α. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with the respective control sample (0 hours, PBS control) defined as 1. Error bars show 95% confidence intervals. *P*-values were determined by a one-sided Welch's *t*-test using the ΔCt values of time-matched rhIL-15- vs. PBS-treated samples. n.s., not significant; * *P*<0.05; ** *P*<0.01; *** *P*<0.001. Primer sequences are listed in Supplementary Table 4.

Figure 1


a
relative IL-15 mRNA expression
relative IL-15Rα mRNA expression
relative IL-2Rβ/IL-15Rβ mRNA expression
relative IL-2Rγ mRNA expression
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n.s.
L428
L1236
KM-H2
L591
HDLM-2
L540
L540Cy
Reh
Blin
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SU-DHL-4
c
anti-IL-15
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L428
L1236
KM-H2
L591
HDLM-2
L540
L540Cy
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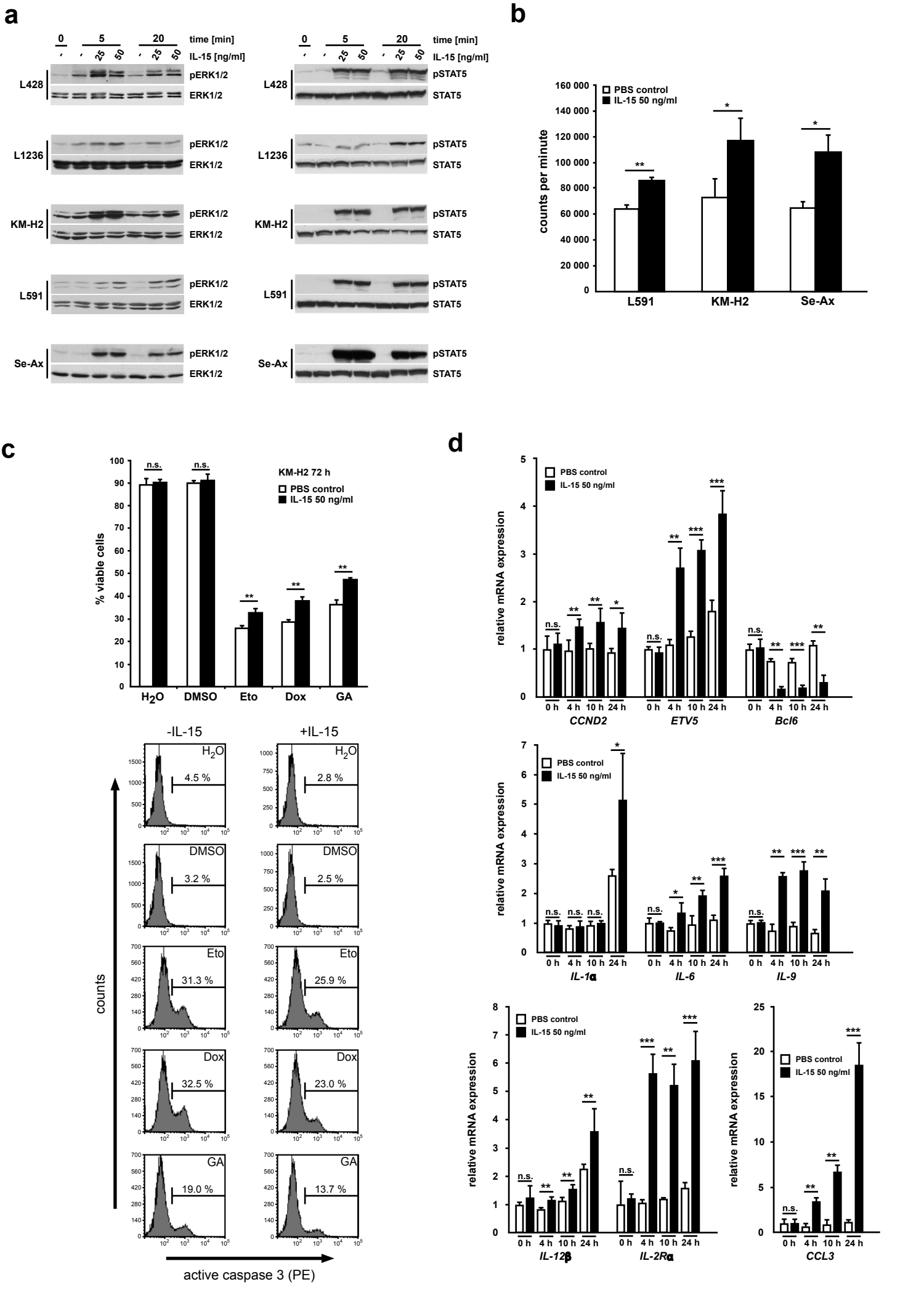
B-ALL/B-NHL
IL-15Rα
IL-2Rβ/IL-15Rβ
IL-2Rγ

Figure 2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STAT5
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PBS control
IL-15 50 ng/ml
counts per minute
c
KM-H2 72 h
% viable cells
H2O
DMSO
Eto
Dox
GA
-IL-15
+IL-15
counts
active caspase 3 (PE)
d
relative mRNA expression
CCND2
ETV5
Bcl6
IL-1α
IL-6
IL-9
IL-12β
IL-2Rα
CCL3