Gain-of-function mutations in interleukin-7 receptor-α (IL7R) in childhood acute lymphoblastic leukemias

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Interleukin-7 receptor α (IL7R) is required for normal lymphoid development. Loss-of-function mutations in this gene cause autosomal recessive severe combined immune deficiency. Here, we describe somatic gain-of-function mutations in IL7R in pediatric B and T acute lymphoblastic leukemias. The mutations cause either a serine-to-cysteine substitution at amino acid 185 in the extracellular domain (4 patients) or in-frame insertions and deletions in the transmembrane domain (35 patients). In B cell precursor leukemias, the mutations were associated with the aberrant expression of cytokine receptor-like factor 2 (CRLF2), and the mutant IL-7R proteins formed a functional receptor with CRLF2 for thymic stromal lymphopoietin (TSLP). Biochemical and functional assays reveal that these IL7R mutations are activating mutations conferring cytokine-independent growth of progenitor lymphoid cells. A cysteine, included in all but three of the mutated IL-7R alleles, is essential for the constitutive activation of the receptor. This is the first demonstration of gain-of-function mutations of IL7R. Our current and recent observations of mutations in IL7R and CRLF2, respectively suggest that the addition of cysteine to the juxtamembranous domains is a general mechanism for mutational activation of type I cytokine receptors in leukemia.
Russell et al., 2009; Hertzberg et al., 2010; Yoda et al., 2010). These are either translocations into the immunoglobulin heavy locus or interstitial deletions creating chimeric P2RY8–CRLF2 transcripts. Additional mutations in the pathway, most commonly in JAK2 but occasionally in JAK1 or in CRLF2 itself, occur in approximately half of the patients. Overall, CRLF2 deregulation occurs in 5–10% of childhood ALL and in 60% of children with Down syndrome and BCP-ALL (DS-ALL; Israeli, 2010) and is associated with a worse prognosis (Cario et al., 2010; Harvey et al., 2010; Yoda et al., 2010; Ensor et al., 2011). Importantly, these leukemias may be sensitive to JAK inhibitors, suggesting the potential for a targeted therapy.

IL7R is constitutively expressed in BCP-ALL, and the formation of a TSLP receptor is plausible in cases with aberrant expression of CRLF2 (Hertzberg et al., 2010). Receptor association is functional because primary BCP-ALL cells expressing CRLF2 respond to TSLP, but not to IL-7, by phosphorylation of STAT5 and RPS6 (Tasian et al., 2010). We therefore hypothesized that IL7R might be mutationally activated in ALL samples expressing CRLF2. In addition to confirmation of this hypothesis, we also report the identification of such mutations in 10% of T-ALLs, thereby demonstrating a general involvement of the IL-7R in ALL.

RESULTS AND DISCUSSION

We screened DNA derived from 133 bone marrow samples of BCP-ALLs, including 62 DS-ALLs, with aberrant expression of CRLF2 and additional 153 BCP-ALL not expressing CRLF2 including 21 DS-ALLs. Nine leukemias with IL7R mutations were identified. The rate of mutations in the CRLF2 group (8/133; 6%) was significantly higher than the rest of BCP-ALLs (1/153; 0.6%; P = 0.01, Fisher’s exact test; Table I).

Two types of mutations in IL7R were identified (Fig. 1, A and B, and Table I). Replacement of serine with cysteine at position 185 at the extracellular domain in four patients and complex in-frame insertions and deletions resulted in the addition of 3–7 aa at the transmembrane domain in 5 patients. Whereas the inserted amino acids varied from patient to patient, cysteine was always included (Fig. 1 C).

All mutations were heterozygous and somatic, as they were absent in remission bone marrows. The mutated mRNA was expressed (Fig. 1 D and Fig. S1). JAK2 mutations were present in three samples, whereas no mutations in JAK1 were identified. Four patients relapsed. Examination of DNA derived from matched diagnostic and relapse samples revealed clonal diversification. The same IL7R mutation was present in both diagnosis and relapse in patient M61. IL7R mutation was present only at relapse in patient M122, whereas CRLF2 expression was already noted at diagnosis, suggesting that IL7R mutation was a progression event. Conversely, in two patients, M90 and M112 CRLF2 abnormalities and IL7R mutations were present at diagnosis and not in relapse, suggesting that relapse arose from a different subclone. These findings are consistent with the recent studies on the marked clonal diversity of B- and T-ALLs (Rothman et al., 2005; Anderson et al., 2011; Clappier et al., 2011).

We next tested whether the somatic mutations in IL-7R are gain-of-function mutations that cooperate with CRLF2 to form a constitutively active TSLP receptor. We used the IL-3–dependent mouse pro-B cells BaF3 (not expressing endogenous TSLP receptor; Fig. S2) to generate cell lines that express either WT or mutated IL7R alone or together with CRLF2. We expressed IL-7R S185C and the c.819 Ins12 (CCCCCGTGCCTA) 243 insertion (Ins) PPCL (herein “InsPPCL”) representing the two types of mutations. All proteins were expressed at the cell membrane as demonstrated by flow cytometry (Fig. 2 A). 1 wk after IL-3 withdrawal, only cells transduced with CRLF2 and mutated IL-7R survived (Fig. 2, B and C). Clos examination revealed two populations of surviving BaF3 cells transduced with CRLF2 and IL-7R InsPPLC, suggesting that IL-7R InsPPCL provided survival advantage by itself. Indeed, growth assays of BaF3 cells transduced with the IL-7R–InsPPCL construct in the absence of CRLF2 (Fig. 2 B) revealed robust cytokine-independent growth. In contrast, BaF3 cells expressing IL-7R S185C required CRLF2 co-expression for survival. Cells expressing only CRLF2, IL-7R S185C, or WT CRLF2 and WT IL-7R did not grow in the absence of cytokines.

**Table I.** Patients with B cell precursor ALL and somatic mutations in IL7R

| ID   | IL7R mutation DNA | IL-7R mutation protein | CRLF2         | JAK2 | Gender | Age at Dx (yr) | WBC/liter | Events               |
|------|-------------------|------------------------|---------------|------|--------|---------------|-----------|----------------------|
| DS46 | c.819 Ins 12      | 243 InsPPCL            | P2RY8–CRLF2   | mut  | M      | 4.9           | 30.5 × 10^9| first CCR            |
| DS92 | c.642 A>T         | S185C                  | IGH translocation | WT   | M      | 15.1          | 112.8 × 10^9| first CCR            |
| M90  | c.828 Ins7 Del T  | 246 InsKCH             | P2RY8–CRLF2   | WT   | M      | 1.7           | 123 × 10^9  | relapse              |
| M112 | c.642 A>T         | S185C                  | P2RY8–CRLF2   | mut  | F      | 14.3          | 9.7 × 10^9  | relapse              |
| M117 | c.814 Ins13 Del A | 241 InsFSCGP           | P2RY8–CRLF2   | mut  | F      | 10.3          | 7 × 10^9    | first CCR            |
| M122 | c.642 A>T         | S185C                  | P2RY8–CRLF2   | WT   | M      | 7.0           | 7.3 × 10^9  | relapse              |
| M124 | c.820 Ins10 Del C | 244 InsCHL             | High expression | WT   | M      | 4.9           | 8.9 × 10^9  | first CCR            |
| M223 | c.642 A>T         | S185C                  | P2RY8–CRLF2   | WT   | M      | 8.0           | 27 × 10^9   | first CCR            |
| M61  | c.820 ins21       | 244 InsPPVCST          | CRLF2 not expressed | WT   | M      | 10.7          | 152 × 10^9 | relapse              |

DS, Down syndrome; M, male; F, female; mut, mutated at JAK2 R683; CCR, continuous complete remission; Dx, Diagnosis; Del, Deletion; Ins, Insertion; WBC, white blood cells count.
Biochemical analysis of proteins extracted from BaF3 cell was consistent with the growth assays (Fig. 2 C). Both Stat5 and RPS6 were phosphorylated in the absence of cytokine in BaF3 cells transduced with the IL-7R InsPPCL alone, but not in cells expressing IL-7R, S185C or WT IL-7R. Co-expression of CRLF2 with each of these mutated IL-7R proteins, but not with the WT IL-7R, caused constitutive phosphorylation of Stat5 and RPS6. Together, the functional and biochemical assays demonstrate that the two types of somatic mutations in IL7R are activating mutations causing cytokine independent growth of mouse pro–B cells and constitutive activation of STAT and mTOR pathways. In accordance with the functional studies, the single patient in whom IL7R was mutated in the absence of CRLF2 expression (Table I, patient M61) had the insertion type of mutation with the CRLF2-independent activation phenotype.

To see if CRLF2 and mutated IL-7R form a functional TSLP receptor, BaF3 cells expressing CRLF2 and either WT or mutated IL-7R were starved from IL-3 and treated with 100 ng/ml TSLP for 25 min. As noted in Fig. 2 C, cells expressing the mutated receptor exhibited Stat5 and RPS6 phosphorylation in the absence of cytokine. Yet all cells responded to TSLP with a marked increase in Stat5 and RPS6 phosphorylation (Fig. 3 A). Thus, CRLF2 and mutated IL-7R form a functional TSLP receptor. To test if the presence of mutated IL-7R sensitizes BaF3 cells to TSLP, we treated BaF3 transduced with CRLF2 and either WT IL-7R or IL-7R S185C with increasing doses of TSLP in the absence of IL-3 (Fig. 3 B). Cells expressing CRLF2–IL-7R S185C grew in the absence of cytokines, but also demonstrated higher sensitivity to TSLP. As little as 0.1 ng/ml TSLP doubled the growth rate, but had no effect on BaF3 cells transduced with CRLF2–IL-7R WT. Similarly, cells transduced with IL-7R InsPPCL also responded to TSLP, in addition to marked self-activation of this allele (Fig. S3).

Loss-of-function mutations in IL-7R cause severe combined immunodeficiency characterized by the complete absence of T lymphocytes and the presence of B and NK cells (Puel et al., 1998). Both B- and T-ALLs express IL-7R and have been reported to respond to IL-7 (Touw et al., 1990). We have therefore hypothesized that gain-of-function mutations in IL-7R may also be detected in T-ALL. Accordingly, we screened 295 diagnostic childhood T-ALL samples treated on prospective BFM protocols (Kox et al., 2010) for IL7R mutations and identified 30 somatic mutations (10.5%). All the mutations were at the transmembrane domain encoded
by exon 6, and all but one were in-frame insertions and deletions (Table II); 27 of the 30 cases included an insertion of cysteine. The patients with mutations were younger and tended to have higher white blood cells counts at diagnosis (Table S1). There was no association between the presence of the mutations and response to therapy, and a similar proportion (10%) relapsed. Notch mutations, which hallmark ~50% of T-ALLs (Clappier et al., 2010; Kox et al., 2010), were detected in 64% of the IL7R-mutated leukemias compared with 48% of the T-ALL without IL7R mutations (P = 0.1, χ² test).

We and others (Hertzberg et al., 2010; Yoda et al., 2010) have recently reported an activating F232C mutation in CRLF2 that introduces cysteine in the juxtamembrane domain. All but three of the mutations observed in IL-7R included the addition of cysteine. To study whether the gain-of-function of the mutated IL-7R could be attributed to the addition of cysteine, we replaced the cysteines in the S815C and in the IL-7R InsPPCL alleles with glycine. These newly mutated receptors were expressed at the cell surface of transduced BaF3 cells (Fig. 4 A). Elimination of the cysteine abrogated the cytokine-independent growth (Fig. 4 B) and the constitutive phosphorylation of Stat5 (Fig. 4 C). Response to TSLP was not altered, confirming that “cysteine-lacking” mutated IL-7R formed a functional TSLP receptor with CRLF2 (Fig. 4 C). These results suggest that the presence of cysteine is critical for the gain-of-function phenotype both in the context of insertion of additional amino acids and as a point mutation.

The experimental insertion of cysteine into the transmembrane domain of the erythropoietin receptor (Constantinescu et al., 2001; Lu et al., 2006) activated the receptor by causing ligand-independent receptor dimerization. Indeed, we observed marked homodimerization of the IL-7R InsPPCL in protein analysis under nonreducing conditions (Fig. 4 D). Interestingly, CRLF2 was not present in these dimers, which is consistent with the observation that this mutant protein does not require CRLF2 for its activation and with the presence of these mutations in T-ALLs in which CRLF2 is not highly expressed. Our current and recent observations (Chapiro et al., 2010; Hertzberg et al., 2010; Yoda et al., 2010) of mutations in IL-7R and CRLF2, respectively, suggest that the addition of cysteine to the juxtamembranous domain is a general mechanism for mutational activation of type I cytokine receptors in leukemias.

These discoveries are a prime example of the interconnection of development and leukemia (Izraeli, 2004). Although loss-of-function mutations in IL-7R perturb lymphoid development, the novel gain-of-function mutations described here are associated with both BCP- and T-ALLs. Activating mutations of IL-7R are present in leukemias of the same lineage, for which it is developmentally required (T cells), or in B cell precursor leukemias that aberrantly express a T cell and monocytic receptor (CRLF2). The broad significance of this pathway in leukemia is further suggested by the aberrant deregulation of CRLF2-JAK–STAT pathway in BCP-ALL.

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**Figure 2.** *IL7R* mutations are gain-of-function mutations. (A) FACS analysis of BaF3 cells stably transduced with CRLF2 and either IL-7R WT, IL-7R S185C, or IL-7R InsPPCL in the presence of IL-3. The same cells were then grown without IL-3 for 1 wk and then analyzed again. (B) Cytokine withdrawal assay of BaF3 and BaF3-CRLF2 cells transduced with either IL-7R WT, IL-7R S185C, or IL-7R InsPPCL. Error bars represent SE. (C) Constitutive phosphorylation of Stat5 and ribosomal protein S6 (RPS6) in BaF3 and BaF3-CRLF2 cells expressing IL-7R mutants, after 5 h of cytokine deprivation. IL-3 + indicates cells harvested after 5 h of IL-3 deprivation followed by 20 min of IL-3 stimulation. All experiments were repeated four times.
activating mutations in T-ALL (Flex et al., 2008; Hornakova et al., 2010). Similar to mutational activations of other growth factor receptors in cancer (Gazdar, 2009), our observations have potential implications for targeted therapy.

(Bercovich et al., 2008; Constantinescu et al., 2008; Kearney et al., 2009; Mullighan et al., 2009; Russell et al., 2009; Hertzberg et al., 2010; Yoda et al., 2010) and by these newly described IL7R mutations and the previously reported JAK1 activating mutations in T-ALL (Flex et al., 2008; Hornakova et al., 2010). Similar to mutational activations of other growth factor receptors in cancer (Gazdar, 2009), our observations have potential implications for targeted therapy.

Table II.  IL7R mutations in T-ALL

| ID  | IL7R mutation DNA | IL-7R mutation protein |
|-----|------------------|------------------------|
| T1  | c.816 Ins 15 TTTTGCGGAAGGAC | 243 Ins FCRKD |
| T2  | c.815 Ins 7 GAGATGC Del 1 A | 243 Ins RC |
| T3  | c.818Ins 10 CTTGGCCCCCT Del 4 TAAC | 243 Ins PCPL |
| T4  | c.820 Ins 21 TGCCCAGAGGAGGCCCCA + point mutation c826 G->C | 244 Ins MPEQDCP +S246T |
| T5  | c.798 Ins 11 CCTCTCTGTGTC Del 17 AGATGGATCCTATCTTAA | 237 Ins ASWC |
| T6  | c.814 Ins 6 GCCCCC Del 6 TACTAA | 242 Ins CPP |
| T7  | c.817 Ins 11 GCTGCCCGTCC Del 2 TA | 243 Ins RCPS |
| T8  | c.815 Ins 16 CGACGTATTGGGGGGGTC Del 1 A | 242 Ins FDCIGV |
| T9  | c.822 Ins 10 CACCGGCGGT Del 4 ATCA | 245 Ins HRGC |
| T10 | c.817 Ins 12 CCTGCTGTCCG Del 3 TAA | 243 Ins PLC5A |
| T11 | c.849 Ins 9 GAGAGGCG  | 254 Ins GEA |
| T12 | c.817 Ins 19 CCATTATCGGTGCTTCT Del 4 TAAC | 243 Ins PIYRCVL |
| T13 | c.799 Ins 11 CCTCTCTGTGTC Del 17 AGATGGATCCTATCTTAA | 237 Ins ASWC |
| T14 | c.816 Ins 7 TAAGTGCT Del 1 A | 242 Ins FEC |
| T15 | c.815 Ins 11 TACCTGCCCCG Del 5 ACTAA | 243 Ins FTCS |
| T16 | c.816 Ins 11 TGCCCCCTCTC Del 2 CT | 243 Ins CPSP |
| T17 | c.850 Ins 6 AAAAG Del 3 CTC | 254 Ins EKV |
| T18 | c.823 Ins 12 GTACATGCGTCT Del 3 TCA | 245 Ins SHQPC |
| T19 | c.838 Ins 24 GTCAACACATACGATTGTTG | 250 Ins CTSISILS |
| T20 | c.832 Ins 7 GTCAAGCG Del 13 TAGAATTTTTC | 248 Ins QO |
| T21 | c.814 Ins 14 GTGTTATAAGGGAA Del 5 TACTA | 242 Ins CGIREI |
| T22 | c. 847 T>G  | V253G |
| T23 | c. 817 Ins 6 GCCTGT  | 243 Ins RC |
| T24 | c.817 Ins 6 GCTGTAG | 244 Ins GC |
| T25 | c.809 Ins 13 GTGCGGCTCCCAT Del 7 TATCTTA | 241 Ins CRPH |
| T26 | c.816 Ins 7 GCCTGTGA Del 1 C | 243 Ins GCI |
| T27 | c.821 Ins 12 GAGGCTCTGTGG | 245 Ins RPCG |
| T28 | c.821 Ins 15 ACTCCCTCCGCTTAC | 245 Ins LPCVF |
| T29 | c.814 Ins 10 GCTGGATGAA Del 1 T | 242 Ins CWMK |
| T30 | c.820 Ins 13 AAGAATGCACAAA Del 1 C | 244 Ins KKCTN |

Del, Deletion; Ins, Insertion. The amino acid cysteine included in 27 of 30 mutations is shown in bold.
We used intronic primers of human IL7R sequence (available from GenBank/EMBL/DDBJ under accession no. NM_002185.2; Table S1) to amplify exons 1–8 of the gene with PCR. Fast start Taq DNA polymerase (Roche) was used with the following thermal cycling conditions: 1 cycle of 94°C for 5 min, followed by 5 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C 30 s, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C 30 s, followed by 1 cycle of 72°C for 7 min. We then analyzed the fragments by denaturing high-performance liquid chromatography as described before (Bercovich et al., 2008; Hertzberg et al., 2010). Fragments with abnormal chromatography patterns were sequenced directly by the primer used for PCR, or cloned into TA vector pCR2.1 (Invitrogen). DNA from plasmid clones was extracted by AccuPrep Spin Miniprep kit (Bioneer) and sequenced.

**Site-directed mutagenesis.** The human IL7R cloned into the MSCVires-GFP retrovirus (encoding GFP) was used as a template for the generation of mutations by site-directed mutagenesis (QuickChange II XL; Stratagene). The plasmid sequences were verified by sequencing.

**Western blotting.** For Western blot analyses, 10% polyacrylamide linear gels were used. BaF3 cells were harvested after 5 h of IL-3 deprivation, and then stimulated with either 10 ng/ml IL-3 or 100 ng/ml TSLP for 25 min. The antibodies used were anti-phospho-STAT5 Tyr 694 (Epitomics), anti-STAT5, anti-S6 ribosomal protein and anti-Phospho-S6 ribosomal protein Ser 235/6 (Cell Signaling Technology). For Western blot analyses of disulfide-bonded dimers, 8% polyacrylamide linear gels were used. For nonreducing conditions, proteins were boiled in SDS buffer without 2-mercaptoethanol. Antibodies used were anti-human IL-7R (R&D Systems) and anti-human CRLF2 (BioLegend).
mutations. Table S2 shows primers used for genomic amplification of IL7R exons. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110580/DC1.

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