A Small-Molecule c-Rel Inhibitor Reduces Alloactivation of T Cells without Compromising Antitumor Activity

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ABSTRACT
Preventing unfavorable GVHD without inducing broad suppression of the immune system presents a major challenge of allogeneic hematopoietic stem cell transplantation (allo-HSCT). We developed a novel strategy to ameliorate GVHD while preserving graft-versus-tumor (GVT) activity by small molecule–based inhibition of the NF-κB family member c-Rel. Underlying mechanisms included reduced alloactivation, defective gut homing, and impaired negative feedback on interleukin (IL)-2 production, resulting in optimal IL-2 levels, which, in the absence of competition by effector T cells, translated into expansion of regulatory T cells. c-Rel activity was dispensable for antigen-specific T-cell receptor (TCR) activation, allowing c-Rel–deficient T cells to display normal GVT activity. In addition, inhibition of c-Rel activity reduced alloactivation without compromising antigen-specific cytolysis of human T cells. Finally, we were able to demonstrate the feasibility and efficacy of systemic c-Rel inhibitor administration. Our findings validate c-Rel as a promising target for immunomodulatory therapy and demonstrate the feasibility and efficacy of pharmaceutical inhibition of c-Rel activity.

SIGNIFICANCE: Chemical inhibition of c-Rel diminishes alloactivation while preserving antigen-specific TCR activation, revealing the redundancy of c-Rel in T cell–mediated antitumor activity of both mouse and human T cells. Our study provides a highly innovative immunomodulatory approach that has true potential for drug development and clinical application with broad therapeutic implications, including allo-tolerance induction after allo-HSCT, as well as antitumor therapies. Cancer Discov; 4(5); 578–91. ©2014 AACR.

INTRODUCTION
Allogeneic hematopoietic stem cell transplantation (allo-HSCT) represents a potent therapy for malignant and nonmalignant hematologic diseases (1). In the care of patients with malignant diseases, it was initially developed to follow high-dose chemotherapy/radiotherapy to rescue from therapy-related bone marrow (BM) failure; however, the emphasis has now shifted toward allo-HSCT as a strategy to facilitate graft-versus-tumor (GVT) activity (2). GVHD remains a major complication of allo-HSCT, resulting in significant morbidity and mortality (3, 4), and strategies to suppress GVHD are often associated with broad suppression of the immune system, leading to immune deficiency and compromised antitumor activity (5). Molecular pathways that have been targeted in preclinical studies to accomplish separation of GVHD from GVT activity include the mTOR,

histone deacetylases, and NF-κB (via proteasome inhibition; ref. 6). The NF-κB family member c-Rel is a transcription factor that regulates lymphocyte survival and proliferation following antigen receptor triggering and plays a dominant role in inflammation, autoimmunity, and alloimmunity (7–10). c-Rel regulates expression of many inflammatory cytokines and is expressed in T and B cells as well as monocytes/macrophages and dendritic cells (11). Immune defects secondary to c-Rel deficiency have been attributed to impaired activation of lymphocytes, particularly T cells. In T cells, the main target gene of c-Rel is interleukin (IL)-2, a cytokine required for normal T-cell proliferation and differentiation. Previous reports demonstrated that c-Rel–deficient T cells showed reduced Th1 but normal Th2 responses (12, 13) and are compromised in the generation of anti-inflammatory natural regulatory T cells (Treg; refs. 14–18) and proinflammatory Th17 cells (18, 19). Given the limitations of existing immunosuppression-based therapies for GVHD and the pivotal role of c-Rel in T-cell proliferation and function, we used strategies targeting the c-Rel pathway, including small molecule–based inhibition of c-Rel activity (20, 21), to modulate T-cell responses in the context of GVHD and malignant diseases.

RESULTS

C-Rel Expression Is Upregulated during Allo-HSCT
We first analyzed the biologic significance of c-Rel for hematopoietic reconstitution and T-cell activation after allo-HSCT. We performed studies assessing c-Rel expression in T cells in the setting of radiation-induced injury as well as GVHD (Fig. 1A and Supplementary Fig. S1). After irradiation and especially during GVHD, IL-2, CD25 (IL-2Rα), and

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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c-Rel were upregulated, consistent with lymphocyte activation. c-Rel expression was analyzed by both intracellular and intranuclear staining (22), and the results were closely correlated (Fig. 1B and C). Hematopoietic stem cell functions, including the development of lymphoid and myeloid lineages, are not known to be dependent on c-Rel activity. To confirm this, we performed an allo-HSCT using c-Rel−/− donor BM in an MHC-disparate allo-HSCT model. c-Rel−/− BM engrafted and reconstituted recipients as efficiently as wild-type (WT) BM (Fig. 1D). We also sought to determine whether c-Rel activity in cells other than donor T cells played a role for GVHD development by using different combinations of c-Rel−/− BM and T cells as the donor source (Fig. 1E), as well as using c-Rel−/− mice as recipients (Fig. 1F). c-Rel

Figure 1. c-Rel expression in donor T cells is increased after allo-HSCT. A, sublethally or nonirradiated BALB/c recipients were transplanted with C57BL/6 T cell depleted (TCD) BM cells with or without 1 × 10⁵ C57BL/6 wild-type (WT) T cells. Levels of IL-2, CD25, and c-Rel are shown after gating on either donor or residual recipient T-cell populations on day 7. Values, mean ± SEM (n = 5). XRT, irradiation; *, P < 0.05; **, P < 0.01; MFI, mean fluorescence intensity. B and C, splenocytes were stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 5 hours and analyzed for intracellular and intranuclear c-Rel expression by flow cytometry. B, intracellular c-Rel staining was performed following fixation and permeabilization (eBioscience kit). C, cytoplasm was removed from c-Rel-stained cells by 0.03% saponin with nuclear isolation media (NP-40, nonyl phenoxypolyethoxylethanol) to confirm nuclear localization of c-Rel. D, lethally irradiated BALB/c recipients were transplanted with C57BL/6/WT or c-Rel−/− TCD BM cells. Thymus, BM, and spleen were analyzed on day 28 after HSCT. Values, mean ± SEM (n = 4). Data shown in A to D are representative of two independent experiments. DC, dendritic cells; MDSCs, myeloid-derived suppressor cells; NK, natural killer. E, lethally irradiated C57BL/6/wild-type (WT) or c-Rel−/− recipients were transplanted with B10 BR TCD BM cells with or without 2 × 10⁵ B10 BR T cells. Survival curve is shown. F, lethally irradiated C57BL/6 WT or c-Rel−/− recipients were transplanted with B10 BR TCD BM cells with or without 2 × 10⁵ B10 BR T cells. Survival curve is shown. Data in E and F are combined from three independent experiments, and values represent mean ± SEM (n = 21). ***, P < 0.001; n.s., not significant.
deficiency in donor BM or in the recipient did not affect survival or GVHD scores.

**Recipients of c-Rel–Deficient T Cells Exhibit Increased IL-2 Levels Associated with Expansion of Tregs**

A recent study found evidence for a role of c-Rel in donor T cells during acute GVHD in mice (10). We were able to confirm these observations by using c-Rel−/− donor T cells in mouse models of MHC-disparate as well as a more clinically relevant minor antigen-mismatched allo-HSCT, which in both cases resulted in significant amelioration of GVHD as determined by survival, weight loss, histopathology of GVHD target organs, and clinical GVHD scores (Supplementary Fig. S2 and data not shown). Moreover, we evaluated the profiles of donor T cells in the spleens of recipient mice on day 7, revealing that the effector-naïve T-cell (Teff/T naïve) ratios were decreased for both CD4+ and CD8+ T cells, and we observed lower CD8+ T cells/Tregs ratios in mice receiving c-Rel−/− T cells compared with recipients of WT T cells (Fig. 2A). We also found increased thymic cellularity on day 14 in recipients of c-Rel−/− T cells compared with WT T cells, indicating reduced thymic GVHD (Fig. 2B). Furthermore, c-Rel−/− T cells expressed significantly lower levels of the intestinal homing marker LPAM-1 on T cells (Fig. 2C), consistent with decreased expression of CD25 by c-Rel−/− T E cells (Fig. 2D). We also found increased thymic cellularity on day 14 and histologic examination was performed. For immunohistochemistry, small intestines are fixed with 4% paraformaldehyde, embedded in paraffin, and stained with anti-mouse FoxP3 antibody. Three slide sections from each mouse were stained with the antibody and blinded quantitative histologic analysis was performed in 5-mm length of longitudinally sectioned small intestine tissue. Mean values and SEM are presented (n = 4). n.s., not significant.
Increased IL-2 production in recipients of c-Rel-deficient T cells is associated with promoted expansion of Tregs. A to E, lethally irradiated BALB/c recipients were transplanted with C57BL/6 WT or c-Rel−/− T cells. One of three independent experiments is presented. Values, mean ± SEM (n = 5). *P < 0.05; **P < 0.01. A, serum levels of IL-2 on day 7 after HSCT are shown. Dotted line, signaling threshold of IL-2. B, secretion of IL-2 from donor CD4+ cells in spleen on day 7. C, pSTAT5 levels in IL-2-stimulated splenocytes from WT or c-Rel−/− T cells transplanted recipients after cytokine stripping with glycine. Expression levels of pSTAT5 in CD25+ donor T cells are shown. D, time-course analyses of absolute numbers of donor CD4+CD25+FoxP3+ cells are shown. E, expression level of CD25 in donor Tregs in spleen on day 14. F, schematic diagram of proposed c-Rel/IL-2 interaction pathways during GVHD. G, lethally irradiated BALB/c recipients were transplanted with C57BL/6 TCD BM cells with 1 × 10^6 C57BL/6 WT or FoxP3-DTR T cells. T cells were treated with c-Rel inhibitor or control vehicle for 24 hours before transplantation. Diphtheria toxin (DT) was administered on day 13 after HSCT to eliminate donor Tregs in recipients transplanted with FoxP3-DTR T cells (n = 5–8). H, lethally irradiated BALB/c recipients were transplanted with C57BL/6 TCD BM cells and 0.5 × 10^6 C57BL/6 WT T cells with or without 0.5 × 10^6 C57BL/6 Tregs or c-Rel−/− Tregs. Control recipients were transplanted with C57BL/6 TCD BM only. Survival curve is shown. Data are combined from two independent experiments (n = 16–18). *, P < 0.05. MFI, mean fluorescence intensity; n.s., not significant.

**T Cells Treated with a Small-Molecule c-Rel Inhibitor Compound Cause Less GVHD**

Some natural compounds and their synthetic derivatives inhibit Rel or NF-κB via specific interaction with the cysteine residues critical for binding to the specific κB-DNA sequence (20). For example, dehydroxymethylepoxyquinomicin (DHMEQ) was shown to inhibit NF-κB binding activity; however, the complex structures and poor pharmacokinetics due to reactive sites found in natural products made them unsuitable for further development. To develop direct Rel inhibitors, we established an assay [fluorescence polarization (FP) assay; for more details, see Methods] for high-throughput screening. Screening of a library of 15,000 compounds yielded 20 hits. We conducted structure–activity relationship studies of these initial hits using electrophoretic mobility shift assay (EMSA) and finally identified the hydrophobic small-molecule pyrimidinetrione and its derivatives as potent and highly specific inhibitors of c-Rel activity, having a 20- to 200-fold higher inhibitory effect on c-Rel and NF-κB than on other transcription factors such as OCT1 and AP1 (Fig. 4A and data not shown). These compounds bind c-Rel directly and change the conformation of the protein, inhibiting DNA binding and transcriptional activity. We developed several generations of...
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Figure 4. Pretreatment of donor T cells with a c-Rel antagonist prevents GVHD in allogeneic HSCT. A, results of FP as well as EMSA are shown (for more details, see Methods). c-Rel inhibitor compound IT-603 at 2-fold dilutions (2,000 to 0.33 nmol/L) was mixed with CD28RE-FITC (10, 3.3, 1.1, 0.33, and 0.11 nmol/L) in FP buffer for 30 minutes. Data for 10 and 0.33 nmol/L are shown. EMSA for c-Rel, OCT1, and AP1 at different inhibitor concentrations are shown. B to E, CD5+ positively selected C57BL/6 splenocytes were treated with two different types of c-Rel antagonists for 24 hours and analyzed after anti-CD3/CD28 stimulation for another 24 to 48 hours. Data are representative of more than three independent experiments. B, representative flow cytometric analysis of intracellular c-Rel and IL-2 expressions after 24 hours of anti-CD3/CD28 stimulation and incubation with inhibitor compound IT-603 at four different concentrations. C, representative flow cytometric analysis of intracellular c-Rel and IL-2 expressions after 24 hours of anti-CD3/CD28 stimulation and incubation with inhibitor compound IT-603 at four different concentrations. Data for 10 and 0.33 nmol/L are shown. EMSA for c-Rel, OCT1, and AP1 at different inhibitor concentrations are shown. D, representative flow cytometric analysis of intracellular c-Rel and IL-2 expressions after 24 and 48 hours of anti-CD3/CD28 stimulation and incubation with inhibitor compounds IT-603, IT-901, or empty vehicle. Percentages of live/dead cells and Annexin-V–positive proapoptotic cells are shown. E and F, lethally irradiated BALB/c recipients received C57BL/6 TCD BM cells with 0.5 × 10^6 C57BL/6 WT T cells after 24 hours of pretreatment with c-Rel inhibitor compound IT-603 or with empty vehicle solution as a control. Data are representative of two independent experiments. F, various populations of splenocytes on days 4 and 14 are shown and expressed as the ratio to the total numbers of transplanted T cells (0.5 × 10^6). CD4+ and CD8+ T cells are gated on donor-derived cells. Mean values and SEM are presented (n = 5). *, P < 0.05; **, P < 0.001. G, survival curve and body weight (BW) changes (n = 5–10). ***, P > 0.001. H, plasma samples were analyzed at 30 minutes, 1, 2, 4, 6, and 16 hours after intraperitoneal administration (12 mg/kg) of c-Rel inhibitor compound IT-603. To assess the level of IT-603 in blood, samples were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS-MS; for more details, see Methods). n.s., not significant.
Pyrimidinetrione derivatives to further optimize the inhibitory effect and found that treatment of activated T cells with one of those compounds (IT-603) resulted in most efficient inhibition of c-Rel activity as well as IL-2 expression in vitro (Fig. 4B–D) without altering cell viability after 24 hours of incubation (Fig. 4E). IT-603 was used for all subsequent experiments involving chemically induced c-Rel inhibition. Basic characteristics of IT-603 are summarized in Table 1.

We next tested the capacity of IT-603–pretreated T cells to induce GVHD. By day 4 after allo-HSCT, T cells pretreated with IT-603 expanded less compared with T cells treated with empty vehicle [dimethyl sulfoxide (DMSO); Fig. 4F], and even though the inhibitory effect of the compound was temporary and c-Rel activity returned to normal by day 4 after allo-HSCT (data not shown), those T cells caused significantly reduced GVHD (Fig. 4G). We also observed higher IL-2 secretion from donor CD4<sup>+</sup>T cells on day 4 in this experiment (Supplementary Fig. S4). Of note, we recently developed a DMSO-free lipid-based formulation of IT-603 using the U.S. Food and Drug Administration (FDA)–approved nonionic surfactant Cremophor. Intraperitoneal injection of this formulation was well tolerated and allowed us to study pharmacokinetics (Table 1 and Fig. 4H), in a first step toward development of a c-Rel inhibitor drug.

### Treatment of T Cells with a c-Rel Inhibitor Compound Does Not Compromise Antitumor Activity

A recent study found evidence indicating that in recipients of c-Rel<sup>−/−</sup> T cells, GVT activity against A20-TGL mouse lymphoma cells can be intact (10). We confirmed these findings and were, moreover, able to demonstrate that this effect can be sustained with T-cell doses as low as 25% of the standard dose (data not shown), and when targeting less immunogenic A20 tumor cells as well as a solid tumor RENCA (renal cell carcinoma; data not shown).

In addition, c-Rel<sup>−/−</sup> T cells displayed strong antitumor activity against EL4-TGL T-cell lymphoma cells even in the absence of T-cell alloactivation (Fig. 5A). In this syngeneic model, the level of CD25 on c-Rel<sup>−/−</sup> CD8<sup>+</sup>T cells on day 7 after transplantation was similar to that of WT T cells (Fig. 5B), in striking contrast with the allo-HSCT setting, in which CD25 levels in c-Rel<sup>−/−</sup> T cells were significantly decreased (Fig. 5C). We performed a similar experiment using B16-TGL melanoma tumor cells and melanoma-specific syngeneic donor T cells from Pmel1<sup>+/+<sup> mice after incubating those T cells with the c-Rel inhibitor compound. Pmel1<sup>+/+<sup> T cells retained normal GVT activity under c-Rel–deficient conditions (Fig. 5D), indicating that inhibition of c-Rel activity is not sufficient to prevent antigen-specific T-cell receptor (TCR) activation. To further dissect the role of c-Rel in antigen-specific activation versus alloactivation of T cells, we analyzed the expression levels of c-Rel in Pmel1<sup>+/+<sup> T cells stimulated by B16 tumor cells and compared them with those of WT C57BL/6 T cells stimulated with MHC-mismatched splenocytes (Fig. 5E and F). We found significantly lower levels of c-Rel in Pmel1<sup>+/+<sup> T cells specifically stimulated with target antigen, suggesting that c-Rel activity is less required for antigen-specific T-cell activation. Moreover, T-cell stimulation in the presence of the c-Rel inhibitor compound resulted in

### Table 1. Characteristics of c-Rel inhibitor compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;-EMSA &amp; μmol/L</th>
<th>Tumor cell growth inhibition in vitro&lt;sup&gt;a&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt;, μmol/L)</th>
<th>Optimum concentration for in vitro T-cell treatment</th>
<th>Duration of inhibitory effect&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Plasma half-life&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone</td>
<td>392.46</td>
<td>N/A</td>
<td>N/A</td>
<td>&gt;20</td>
<td>CD5&lt;sup&gt;+&lt;/sup&gt;-splenocytes were treated with IT-603 for 24 hours and transferred to lethally irradiated recipients. Analysis of c-Rel activity was performed by flow cytometry.</td>
<td>&lt;96 h</td>
<td>2.25 h</td>
</tr>
<tr>
<td>DHMEQ</td>
<td>261.23</td>
<td>40</td>
<td>7</td>
<td>20 μmol/L for 24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IT-603</td>
<td>329.16</td>
<td>3</td>
<td>18</td>
<td>20 μmol/L for 24 h</td>
<td>&lt;96 h</td>
<td>2.25 h</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MW, molecular weight; N/A, not applicable.

<sup>a</sup>Human diffuse large B-cell lymphoma cell line Ly3 was used.

<sup>b</sup>CD5<sup>+</sup>-splenocytes were treated with IT-603 for 24 hours and transferred to lethally irradiated recipients. Analysis of c-Rel activity was performed by flow cytometry.

<sup>c</sup>Plasma samples were analyzed by LC/MS-MS at various time points after 12 mg/kg intraperitoneal injection of IT-603.
c-Rel Inhibition Separates GVHD from GVT Activity

Fig. S5). As a result of our ongoing efforts to establish less-toxic compound formulations, we recently succeeded in developing a regimen for systemic administration of the inhibitor compound as GVHD therapy, and we were able to establish in vitro efficacy (Fig. S5). Although the administration of empty vehicle was not associated with any signs of toxicity, daily intraperitoneal administration of c-Rel inhibitor solution at a dose of 24 mg/kg for more than 2 weeks resulted in mild diarrhea and ruffled fur. Importantly, c-Rel deficiency (as a result of p210 BM) did not have a negative impact on hematopoiesis and immune reconstitution (Fig. 1D and data not shown).

Our inhibitor compound effectively inhibited c-Rel activity of human T cells (Fig. 5K). Moreover, in vitro cytotoxicity analysis of human cytomegalovirus (CMV)-specific T cells (Fig. 5L; ref. 30) as well as Wilms tumor 1 (WT1) and...
Epstein–Barr virus (EBV)–specific T cells (Fig. 5M and data not shown) demonstrated that inhibition of c-Rel activity did not impair antigen-specific TCR-mediated killing. Alloreactivity of human T cells was on the other hand dramatically reduced when human peripheral blood mononuclear cells (PBMC) were cultured with HLA-mismatched stimulators in the presence of the inhibitor compound (Fig. 5M). These data reinforce the notion of separation of GVHD from GVT activity through inhibition of c-Rel activity even in human T cells.

**DISCUSSION**

The NF-κB/Rel transcription factor family is composed of five members of interacting proteins: c-Rel, p50, p65, p52, and RelB. c-Rel is involved in the pathway downstream of antigen-stimulated canonical NF-κB signal transduction and is crucial for T-cell proliferation and differentiation (7, 8). Studies in mouse models revealed unique roles for c-Rel in the pathophysiology of allergic reactions, autoimmunity, and allogeneic transplantation (10, 11, 31, 32). Our study revealed that deficiency of c-Rel in donor T cells ameliorated GVHD due to impaired alloactivation and proliferation of T<sub>H</sub> decreased homing of donor c-Rel<sup>−/−</sup> T<sub>H</sub> to the small intestine, and increased Treg-mediated suppression.

Surprisingly, levels of IL-2, one of the key target genes of c-Rel, were increased in recipients of c-Rel<sup>−/−</sup> T cells on day 7 after allo-HSCT. We identified a feedback mechanism that resulted in reduced downregulation of IL-2 production by c-Rel<sup>−/−</sup> T cells during periods of T-cell activation in the setting of acute GVHD. In addition to the relative increase of IL-2 at a critical time period during early GVHD, we also...
found that serum levels of Th2-type cytokines, such as IL-4, IL-5, and IL-13, were significantly increased on day 7 after allo-HSCT, and that GATA-3 expression of c-Rel−/− T cells was increased, whereas expression of T-bet was decreased (Supplementary Fig. S6), suggestive of Th2 polarization in recipients of c-Rel−/− T cells (12, 13). The availability of optimal IL-2 levels in the absence of competition by effector T cells resulted in expansion of c-Rel-deficient natural Tregs and, more importantly, induction of Tregs. These findings can certainly be considered counterintuitive, given the role of c-Rel for IL-2 as well as Treg generation under physiologic conditions. However, our experiments reveal a differential outcome of the IL-2–STAT5 pathway in WT compared with c-Rel-deficient T cells during GVHD. In the setting of GVHD, pSTAT5 in c-Rel-deficient donor T cells is decreased, which over time leads to increased IL-2 levels (due to decreased negative feedback) and renders T*E at the same time noncompetitive (due to decreased CD25 expression), while allowing Tregs, a T-cell population that expresses CD25 by definition, to outcompete T*E. Our findings as well as a recently described similar concept of competition for IL-2 between T*E and Tregs (23, 24) indicate that this mechanism may indeed have significant implications for alloactivation and GVHD, and it will be an important line of future research to evaluate the kinetics of T*E and Tregs in response to interventions modulating the IL-2 pathway (33). Reported roles of c-Rel in T-cell responses and transplantation immunology in comparison with our findings during GVHD are summarized in Table 2.

Inhibition of c-Rel activity did not impair GVT activity, as demonstrated by various tumor models in the setting of allogeneic and syngeneic HSCT. This phenomenon was most evident when using c-Rel−/− T cells as opposed to inhibitor compound-treated T cells, which can be attributed to expected potential limitations of any chemical strategy to inhibit protein function (such as limited duration of the effect, potential off-target effects, and nonspecific toxicities). Furthermore, antigen-specific T-cell activation was associated with significantly less c-Rel expression than allostimulation. The redundancy of c-Rel activity for antigen-specific TCR activation provides an underlying molecular mechanism for the observed separation of GVHD from GVT activity: upon only antigen-specific activation (but not alloactivation), c-Rel-deficient T cells can differentiate into activated effector T cells that exhibit normal cytotoxicity responses. In addition, we found that in the setting of c-Rel deficiency, Treg-mediated suppression of GVHD represents an important additive factor without equally affecting GVT activity. Although it is possible that this phenomenon is more pronounced in experimental models in which the kinetics of tumor eradication can be much different from real-life scenarios, it is important to keep in mind that there is accumulating evidence that Tregs can preferentially affect GVHD while preserving protective immunity (34, 35). Our findings in the setting of an MHC-matched allo-HSCT indicate that c-Rel activity is also involved in T-cell activation mediated by minor transplantation antigens. This and other important questions about the differential requirements for c-Rel activity for TCR triggering will need to be addressed in more depth in future studies; however, our current evidence that inhibition of c-Rel activity separates GVHD from GVT activity at the level of TCR signaling has important clinical implications. Administration of donor lymphocyte infusions containing high levels of allogeneic CTL precursors (allo-CTLp) in the early posttransplant period correlates with a high risk of GVHD, whereas infusion of antigen-specific T cells with low levels of allo-CTLp never causes GVHD. Using limited dilution analysis, we were able to show that pretreatment of PBMCs with our inhibitor compound prevents activation of alloreactive T cells and results in a 20-fold decrease of allo-CTLp to levels comparable with allo-CTLp levels detected in EBV CTLs that are safely used for adoptive therapy for EBV lymphoproliferative disorders (36).

Table 2. Roles of c-Rel in T-cell responses and transplantation immunology

<table>
<thead>
<tr>
<th>T-cell response</th>
<th>c-Rel−/− mice (steady state/autoimmune/infection)</th>
<th>GVHD (c-Rel−/− or inhibitor treated donor T cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>Defective-normal</td>
<td>Decreased</td>
</tr>
<tr>
<td>Th2</td>
<td>Normal</td>
<td>Increased</td>
</tr>
<tr>
<td>Th17</td>
<td>Defective</td>
<td>12, 13</td>
</tr>
<tr>
<td>nbreg</td>
<td>Defective</td>
<td>12, 19</td>
</tr>
<tr>
<td>iTreg</td>
<td>Defective</td>
<td>14–17</td>
</tr>
<tr>
<td>Graft survival</td>
<td>Normal</td>
<td>17</td>
</tr>
<tr>
<td>GVHD survival</td>
<td>Normal</td>
<td>Increased</td>
</tr>
<tr>
<td>IL-2</td>
<td>Decreased</td>
<td>31, 32</td>
</tr>
<tr>
<td>IL-2 ratio</td>
<td>Decreased (in vitro)</td>
<td>N/A</td>
</tr>
<tr>
<td>T-cell activation</td>
<td>Defective (in vitro)</td>
<td>Increased</td>
</tr>
<tr>
<td>T-cell proliferation</td>
<td>Defective (in vitro)</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

Abbreviations: iTreg, induced Treg; nbreg, natural Treg; N/A, not applicable.

Summary of our findings.
Two recent studies have validated possible uses of a proteasome inhibitor and an IκB kinase inhibitor to target NF-κB (37, 38). These pan–NF-κB inhibitors have broad effects on the downstream signaling pathways and have significant potential to cause serious adverse effects such as increased radiation-induced epithelial damage (39). However, because c-Rel activity is restricted to a small number of hematopoietic lineages, a c-Rel–specific inhibitor will likely have a better safety profile than a pan–NF-κB inhibitor. Indeed, we have evidence that systemic administration of the c-Rel inhibitor compound discovered by us is feasible, safe, and effective. Furthermore, Rel/NF-κB factors are also known for their roles as proto-oncogenes by contributing to tumor growth, survival, drug resistance, and metastasis of lymphoid malignancies and breast, head, and neck cancers (9, 40). We found in a preliminary experiment that intraperitoneal c-Rel inhibitor compound administration displayed antineoplastic activity in a xenograft model of human diffuse large B-cell lymphoma (data not shown).

In conclusion, our data provide evidence, for the first time, for a differential role of c-Rel for alloactivation versus antigen-specific T-cell activation: although c-Rel activity is critically important for T-cell activation during GVHD, it is dispensable for antigen-specific TCR activation. As a result, inhibition of c-Rel activity reduces the severity of GVHD without compromising antitumor activity of T cells. Our findings validate c-Rel as a highly promising therapeutic target, and we demonstrate biologic benefits of inhibition of c-Rel activity in both mouse and human T cells with a highly specific small-molecule compound. Drug development studies are currently under way in an effort to translate this technology from bench to bedside.

METHODS

Mice and BM Transplantation

We obtained female C57BL/6 (B6, H-2b), LP/J (H-2b), B10.BR (H-2b), and BALB/c (H-2a) mice from The Jackson Laboratory. B6 mice carrying the c-Rel gene null mutation (c-Rel<sup>−/−</sup>) were originally generated by Shono et al. Foxp3<sup>−/−</sup>/DTR transgenic B6 mice that expressed Foxp3 promoter (27) as well as Pmel1<sup>+/-</sup> transgenic B6 mice (41) were maintained at Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY) in accordance with the Institutional Animal Care and Use Committee Standards. Mice used for experiments were 6 to 9 weeks old. Mouse HSCT experiments were performed as previously described (42), with 850 cGy split-dosed lethal irradiation of BALB/c recipients transplanted with BM (5 × 10<sup>6</sup>), T cell depleted (TCD) with anti-Thy-1.2 and low-TOX-M rabbit complement (Cedarlane Laboratories), or with 1,100 cGy split-dosed lethal irradiation of B6 or LP recipients transplanted with TCD BM (5 × 10<sup>6</sup>) as well. Donor T cells were prepared by harvesting donor splenocytes and enriching T cells by Miltenyi MACS purification of CD5 (routinely >90% purity). For the Treg transfer experiment, highly enriched (routinely >90% purity) CD4<sup>+</sup>CD25<sup>+</sup> T cells were obtained by positive selection with the Miltenyi MACS magnetic sorting system. In GVHT experiments, animals received tumor cells intravenously in a separate injection on day 0.

Small-Molecule c-Rel Inhibitor Compounds

Pyrimidinetrione derivatives were previously identified as small-molecule c-Rel inhibitor compounds (20, 21). c-Rel inhibitory activity of the compounds was confirmed by FP as well as EMSA using the DNA-binding property of the c-Rel protein. Detailed methods for these analyses were described previously (7, 20, 43). Unless otherwise indicated, we used IT-603 [(5Z)-5-[(5-bromo-2-hydroxy-3-methoxyphenyl)methylidene]2-sulfanylidenemida-zolidin-4-one; molecular weight = 329.1 g/mol; obtained from ChemDiv] by incubating cells with the compound for 24 hours at a concentration of 20 μM/L. For high-throughput screening, we used an FP assay that uses the DNA-binding property of the c-Rel protein. Specifically, c-Rel binds with high affinity to the CD28 responsive element (CD28RE) in the IL-2 promoter. The differential signals of free CD28RE versus bound CD28-Rel complex were used to screen for compounds that disrupted CD28-Rel interaction.

Assessment of GVHD and GVT; In Vivo BLI and Cell Lines

Mice were monitored daily for survival and weekly for GVHD clinical scores (44). Small intestine, large intestine, liver, and skin samples were evaluated histologically for evidence of GVHD and scored as previously described (45). In GVT experiments, we determined the bioluminescent signal intensity (BLI) of tumor-bearing mice twice weekly as described previously (46). We superimposed pseudocolor images showing the whole-body distribution of BLI on grayscale photographs and determined total flux (photons s<sup>−1</sup>) for individual mice. We determined the cause of death (tumor vs. GVHD) by necropsy and histopathology as previously described (45). All cell lines used in our experiments were originally obtained from the American Type Culture Collection (ATCC) in 2006. All cells were maintained and propagated according to the recommendations of ATCC and were validated as Mycoplasma-negative. All cell lines underwent authentication testing at ATCC; in addition, the histologic origin of tumors derived from cancer cell lines in our animal experiments was confirmed by histopathology.

Serum Cytokines Analyses

Blood was collected into microcentrifuge tubes, allowed to clot, and centrifuged, and the supernatant was collected. Multiplex ELISA was conducted as per the manufacturer’s instructions (Millpore). Results were acquired with a Luminex 200 instrument and analyzed with xPONENT software (Luminex Corporation).

Antibodies and Flow Cytometry

All antibodies other than the anti–c-Rel antibody (Santa Cruz Biotechnology) and the anti–CD44 antibody (BioLegend) were obtained from BD Biosciences–Pharmingen. For cell analysis of surface markers, cells were stained for 20 minutes at 4°C in PBS with 0.5% bovine serum albumin (PBS/BSA) after Fc block, washed, and resuspended in 4’-6-diamidino-2-phenylindole (DAPI) in PBS/BDA. IL-2 secretion analyses were performed using the IL-2 Secretion Assay Kit as per the manufacturer’s instructions (Miltenyi Biotec). c-Rel expression was analyzed by intracellular staining after cells were stimulated for 5 hours with Cell Stimulation Cocktail (ebiScience) unless otherwise indicated. Cell surface staining was followed by intracellular staining with the eBioscience kit as per the manufacturer’s instructions. Dead cells were excluded with the LIVE/DEAD Fixable Dead Cell Stain kit (Invitrogen). Intracellular pSTAT5 was detected as described previously (24). Briefly, mice were sacrificed and splenocytes were instantaneously exposed to different concentrations of mouse IL-2 for 10 minutes at 37°C, followed by fixation with 1.6% paraformaldehyde (PFA) and permeabilization with 90% methanol. During this process, membrane-bound IL-2 was stripped from cell surfaces by a 2-minute incubation with 0.1 mol/L glycine buffer equilibrated at pH 4.0, followed by a 5-minute wash in RPMI before the exposure to exogenous IL-2. All flow cytometry was performed on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo (TreeStar Software).
c-Rel Inhibition Separates GVHD from GVT Activity

**Pharmacokinetics**

Plasma samples were analyzed at 30 minutes, 1, 2, 4, 6, and 16 hours after intraperitoneal administration (12 mg/kg) of c-Rel inhibitor compound IT-603. To assess the level of IT-603 in blood, samples were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS-MS) as previously described (47, 48). Calibration curves were determined for IT-603 to permit conversion of peak areas to the drug amounts against external reference standards. The MS-MS detector (Model ABI/Sciex API 4000; Applied Biosystems) permitted verification of peak identity as well as a quantitative assessment of the compounds in the samples.

**Human T-Cell Cytotoxicity and Limited Dilution Assays**

*In vitro* cytotoxicity analyses of human CMV-specific T cells as well as human WT1 and human EBV-specific T cells were performed as previously described (30). In brief, CMV-specific T cells were generated from PBMCs of a healthy donor by repeated * in vitro stimulations with an autologous EBV-transformed B-cell line (EBV BLCL) loaded with a pool of CMVpp65-derived pentapeptides overlapping by 11 amino acids and including the entire sequence of the protein. T cells were tested on day 34 of culture in a standard 51Cr release assay against a panel of targets including autologous dendritic cells without CMVpp65 peptide, autologous dendritic cells loaded with a CMVpp65 peptide, and fully HLA-mismatched allogeneic BLCL. The effectortarget ratio was 25:1. We compared unmodified T cells with control- and compound-treated T cells (pretreatment for 24 hours in media containing IT-603 compound at 20 μmol/L).

The frequencies of EBV-specific T cells and allogeneic T cells were measured in the PBMCs from a normal EBV seropositive donor by limited dilution analysis as previously described (36). Briefly, the aliquots of PBMCs were stimulated on day 0 with either autologous EBV BLCL or allogeneic HLA-mismatched BLCL after coinoculation with the inhibitor compound IT-603 or with the control solution (DMSO). The stimulated PBMCs were plated at serial dilutions and cultured in the presence of IL-2 for 14 days. The CTL precursor frequencies were measured in a 51Cr assay against either autologous BLCL or allogeneic HLA-mismatched BLCL. The HLA type of the PBMC donor was: A0201 A3601 B44WRJ B1510 C0501 C04JERF DRB1 0401 DRB1 0301 DQB1 0301 DQB1 0201.

**Statistical Analysis**

Data are presented as mean ± SEM. Survival data were analyzed with the Mantel-Cox log-rank test. For nonsurvival pointwise analyses, an unpaired t test was used for comparisons between two experimental groups, and the nonparametric Mann-Whitney U test was used for non-Gaussian distributions, and ANOVA was used for comparisons of more than two groups. All statistical analyses were performed using GraphPad Prism 5. A P value of less than 0.05 was considered statistically significant.

**Disclosure of Potential Conflicts of Interest**

H.-C. Liou is employed as the CEO of ImmuneTarget, Inc. J.L. Zakrzewski is a consultant/advisory board member of ImmuneTarget, Inc. No potential conflicts of interest were disclosed by the other authors.

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