Abstract

Purpose: The dual BCR-ABL/SRC kinase inhibitor dasatinib entered the clinic for the treatment of chronic myeloid leukemia and Ph+ acute lymphoblastic leukemia. Because SRC kinases are known to play an important role in physiologic T-cell activation, we analyzed the immunobiological effects of dasatinib on T-cell function. The effect of dasatinib on multiple T-cell effector functions was examined at clinically relevant doses (1-100 nM); the promiscuous tyrosine kinase inhibitor staurosporine was used as a comparator.

Experimental Design: Purified human CD3+ cells and virus-specific CD8+ T cells from healthy blood donors were studied directly ex vivo; antigen-specific effects were confirmed in defined T-cell clones. Functional outcomes included cytokine production (interleukin-2, IFNγ, and tumor necrosis factor α), degranulation (CD107a/b mobilization), activation (CD69 up-regulation), proliferation (carboxyfluorescein diacetate succinimidyl ester dilution), apoptosis/necrosis induction, and signal transduction.

Results: Both dasatinib and staurosporine inhibited T-cell activation, proliferation, cytokine production, and degranulation in a dose-dependent manner. Mechanistically, this was mediated by the blockade of early signal transduction events and was not due to loss of T-cell viability. Overall, CD4+ T cells seemed to be more sensitive to these effects than CD8+ T cells, and naïve T cells more sensitive than memory T-cell subsets. The inhibitory effects of dasatinib were so profound that all T-cell effector functions were shut down at therapeutically relevant concentrations.

Conclusion: These findings indicate that caution is warranted with use of this drug in the clinical setting and provide a rationale to explore the potential of dasatinib as an immunosuppressant in the fields of transplantation and T-cell–driven autoimmune diseases.

Tyrosine kinase (TK) inhibitors such as imatinib (Glivec, STI571, Novartis) have entered the clinic as specific cancer treatments. Imatinib is now first-line therapy for chronic myeloid leukemia (1, 2) and is increasingly used in conjunction with allogeneic hematopoietic stem cell transplantation (3–5). Recently, in vitro data have highlighted the inhibitory effects of imatinib on leukemia-specific T cells (6). However, its effect on graft-versus-host disease, graft-versus-leukemia, or other immune-mediated processes affecting transplant outcome are not completely understood. We and others have found that imatinib exerts T-cell–suppressive effects in vitro, possibly via LCK inhibition (7–9). LCK is a major player in proximal T-cell signaling: CD3/T-cell receptor (TCR)–mediated signal transduction leads to activation of the SRC family TKs LCK and FYN, which are associated with the coreceptors CD4 and CD8 (10). Activated LCK and/or FYN, in turn, phosphorylates the immunoreceptor tyrosine–based activation motifs located within the CD3 and ζ chains of the TCR/CD3 complex itself. The phosphorylated immunoreceptor tyrosine–based activation motifs serve as a docking site for ZAP70, which is also a substrate for LCK. ZAP70, in turn, phosphorylates the T-cell–specific adapters LAT and SLP-76, which generate the secondary messenger effectors of T-cell activation and proliferation. Patients can become resistant to imatinib, and thus there is a demand for alternative kinase inhibitors. Several TK inhibitors, which are often less specific than imatinib, are currently undergoing preclinical and clinical development or have recently been approved. However, whereas these agents show much promise, there are concerns that concurrent immunosuppressive effects mediated through inhibition of TKs involved in the antigen receptor signaling pathways of T cells as
Dasatinib Inhibits T Cells

Materials and Methods

Reagents. Dasatinib was synthesized according to the published procedure (14). Imatinib was extracted from a commercially available 400-mg imatinib mesylate tablets as described (9). The identity and purity of dasatinib and imatinib were established as described (9). Staurosporine was obtained from Biomol. All TK inhibitors were dissolved in DMSO at stock concentrations of 100 μmol/L (staur- osporine), 10 μmol/L (dasatinib), and 10 nmol/L (imatinib). The biological activities of the extracted imatinib, synthesized dasatinib, and commercially available staurosporine were tested in a cell death titration assay on BA/F3 bcr-abl+ cells as described (18).

Cell culture and activation. Peripheral blood mononuclear cells were collected from healthy volunteer donors at the Frankfurt Red Cross Transfusion Medicine department in Germany after obtaining written informed consent. Jurkat T cells and primary human T cells from healthy blood donors were cultured in RPMI 1640 (PAN) containing 10% FCS, 2 mmol/L L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (R10). Primary T cells were purified by Ficoll-Hypaque (PAN) density gradient centrifugation, followed by negative selection with magnetic beads coated with an antibody mix and an LS magnetic-activated cell sorting column according to the manufacturer’s instructions (Pan T Cell Kit Untouched, Miltenyi Biotec). CD3+ T-cell purity, determined by flow cytometry, ranged from 80% to 97.5% (median, 90.5%); viability was always >90% as determined by trypan blue staining (Trypan Blue Solution 0.2%, Sigma). After overnight rest, the cells were incubated with or without TK inhibitors for 1 h and then stimulated with 5 μg/mL OKT3 (Orthoclone, Janssen-Cilag). Unstimulated T cells as a negative control and DMSO diluted 1:10 in R10 as a solvent control were included in all assays.

Antibodies and basic flow cytometry. All antibodies for flow cytometry were obtained from BD Pharmingen. Stained samples were collected on a four-color FACSCalibur flow cytometer (BD Immunocytometry Systems). List mode files were analyzed using CellQuest software (BD Pharmingen). In all cases, at least 30,000 events were collected for analysis. Gating was set on live cells and cells to tetramer CD3+ cytotoxic (CD3+CD8+), and helper (CD3+CD4+) T cells, as well as naïve (CD4- or CD8-CD45RO-CD27-), and memory (CD4- or CD8-CD45RO-CD27+; CD4- or CD8-CD45RO-CD27+) T-cell subsets, to evaluate proliferative responses to OKT3, and on CD8+ T cells to evaluate responses to CMV or EBV peptides. Activation of T cells was evaluated by analysis of CD69 expression on gated cells after 24-h stimulation with 5 μg/mL OKT3 in the presence or absence of TK inhibitors. The following antibodies were used: CD3-FITC/allophycocyanin (UCHT1), CD4-phycocerythrin (RPA-T4), CD4-peridinin chlorophyll protein (PerCP; SK3), CD8-FITC (RPA-78), CD8-PerCP (SK1), CD27-phycocerythrin (M-T271), CD45RO-allophycocyanin (UCHL1), CD69-phycocerythrin (FN50), CD69-PerCP (L78), CD107a- FITC (H443), CD107b-FITC (H4B8), Annexin V-phycocerythrin, INFγ-phycocerythrin (B27), and tumor necrosis factor α (TNFα)-phycocerythrin (Mab11); 7-amo-actinomycin D (7-AAD) was used to discriminate live from dead cells.

Carboxyfluorescein diacetate succinimidyl ester proliferation assay. Purified human T cells were suspended in PBS (1 × 10^6/mL) and labeled with the vital dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) at a final concentration of 0.25 μmol/L as described (9). After overnight rest, CFSE-labeled cells were preincubated with TK inhibitors as indicated for 1 h, and then cultured for 4 d in the presence of 5 μg/mL OKT3 and antibodies against the costimulatory molecules CD28 and CD49d (1 μg/mL each; BD Pharmingen). In washout experiments, T cells were incubated for 24 h with dasatinib or staurosporine, then harvested and washed twice before assay.

Apoptosis assay. T cells (1 × 10^6/mL) were stimulated with 5 μg/mL OKT3 and antibodies against the costimulatory molecules CD28 and CD49d (1 μg/mL each), with or without 50 nmol/L dasatinib or staurosporine, for 4 d after overnight rest in R10, and then harvested and stained with Annexin V-phycocerythrin and 7-AAD. Apoptotic cells were defined by flow cytometry as 7-AAD+/Annexin V-; necrotic cells were defined as 7-AAD+/Annexin V+.

Cytokine secretion, degranulation, and proliferation of epitope-specific CD8+ T cells stimulated with HLA A*0201–restricted CMV and EBV peptides. T cells from HLA A2* CMV+ and/or EBV IgG seropositive healthy donors were magnetic-activated cell sorted and CFSE labeled as described above. Purified cells were stimulated with 2 μmol/L of the HLA A*0201–restricted peptides (purity >70%; IPT Peptide Technologies GmbH) CMV pp65495-503 (NVP-LVMPTV) or EBV BMFL159-267 (GLCTLVAML) in the presence of antibodies against the costimulatory molecules CD28 and CD49d (1 μg/mL each). Proliferation of CMV- or EBV-specific CD8+ cells was determined after 6 d of culture by staining with anti-CD8-PerCP and allophycocyanin-labeled HLA A*0201 tetrameric complexes (tetramers) refolded with CMV pp65495-503 or EBV BMFL159-267 peptides (19); tetramers were produced as previously described (20). Expression of the degranulation markers CD107a/b, together with intracellular production of INFγ and TNFα, was evaluated in 5-h assays using GolgiStop and Cytofix/ Cytoperm (BD Pharmingen), with and without dasatinib or staurosporine, at the indicated concentrations as previously described (21). The percentages of antigen-specific CD8+ cells secreting cytokine and expressing CD107a/b were determined by flow cytometry using a FACSCalibur instrument with CellQuest software.

Interleukin-2 ELISA. Purified T cells (2 × 10^6 per well) were plated in 96-well plates with or without TK inhibitors at the indicated
concentrations for 18 h and stimulated with 5 μg/mL OKT3 in the presence of antibodies against the costimulatory molecules CD28 and CD49d (1 μg/mL each). Interleukin-2 (IL-2) was measured with a commercially available ELISA kit according to the manufacturer’s instructions (OptEIA Human IL-2; BD Biosciences).

**Analysis of TCR signal transduction in Jurkat T cells.** Jurkat T cells (1 × 10^6/mL) were incubated for 1 h with dasatinib at the indicated concentrations and stimulated for 5 min with 5 μg/mL OKT3. Cells were lysed in 1-mL lysis buffer containing 1% Triton X-100. Protein concentrations were adjusted by detergent-compatible protein assay kit according to the manufacturer’s instructions (Bio-Rad). For each sample, 20-μg protein was loaded on a 10% Bis-Tris Gel (Invitrogen) and transferred to nitrocellulose. Western blots were incubated with the indicated antibodies in PBS containing 3% to 4% milk/Tween 0.1%. Antibodies were as follows: mouse monoclonal IgG2b antibody clones PY20 and PY99 (Santa Cruz Biotechnology) specific for phosphotyrosine mixed 1:1, a polyclonal antibody specific for LCK (Cell Signaling), and a monoclonal antibody specific for β-actin (clone AC-74, Sigma). Blots were developed by enhanced chemiluminescence.

**Cell culture of antigen-specific CD8<sup>+</sup> and γδ T-cell lines.** The CD8<sup>+</sup> T-cell clones EBV-C, ILA-1, and Mel-13 were isolated from peripheral blood mononuclear cells of healthy donors as described (22). EBV-C is specific for the HLA A*0201–restricted EBV-derived BMLF1-encoded epitope GLCTLVAML; ILA-1 recognizes the HLA A*0201–restricted human telomerase reverse transcriptase epitope ILAFLGILTV. The γδ line AJH is specific for the HLA A*0201–restricted EBV-derived MelanA epitope GLCTLVAML; ILA-1 recognizes the HLA A*0201–restricted EBV-derived FLAFLGILTV. The γδ line AJH is >99% Vγ9/Vδ2<sup>+</sup> and was generated as described (23). Antigen-specific CD8<sup>+</sup> T-cell clones and the γδ T-cell line were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies), 2 mMol/L l-glutamine (Life Technologies), 100 units/mL penicillin (Life Technologies), 100 μg/mL streptomycin (Life Technologies), 5% Cellkines (Helvetia Healthcare), 200 units/mL IL-2 (Chiron), and 25 ng/mL IL-15 (Peprotech). Clones and lines were periodically restimulated using 5 μg/mL phytohemagglutinin with irradiated allogeneic peripheral blood mononuclear cells from at least three individuals as feeder cells.

**Cytometric bead arrays.** EBV-C CD8<sup>+</sup> T cells (2 × 10<sup>5</sup>) or AH-1 γδ T cells (1 × 10<sup>5</sup>) were treated with either dasatinib or staurosporine for 1 h at the concentrations indicated. EBV-C CD8<sup>+</sup> T cells were then stimulated with 2.5 × 10<sup>4</sup> C1R A2 B cells that had been prepulsed with the indicated concentrations of specific peptide (GLCTLVAML) for 1 h and washed twice with R10 as above. The AH-1 γδ line was stimulated by the addition of 1 μg/mL OKT3. Cells were incubated in a 96-well U-bottomed plate for 4 h, then pelleted by centrifugation. The supernatant was harvested and assayed with the human TH1/TH2 cytokine kit (BD Biosciences) according to the manufacturer’s instructions. Analysis was done with a FACS Calibur flow cytometer.

**Cell-surface TCR expression assays.** EBV-C, ILA-1, or Mel-13 CD8<sup>+</sup> T cells (1 × 10<sup>5</sup>) were resuspended in 100 μL of R10 as above. Cells were then treated with either dasatinib or staurosporine at 0, 2, 10, or 100 nmol/L for 3 h. Following incubation with TK inhibitors, cells were stained with anti-α/β TCR-FTTC (Serotec), anti-CD8–allophycocyanin (BD PharMingen), and 7-AAD for 30 min on ice, washed twice in PBS, and resuspended in 200-μL PBS before analysis.

**Statistical analyses.** Results were expressed as mean ± SD. Statistical significance was determined by the two-tailed Student t test or one-way ANOVA and considered statistically significant at P < 0.05. The mean IC<sub>50</sub> was calculated with GraphPad Prism software (sigmoidal dose response, variable slope).

**Results**

All assays described herein were done at clinically relevant doses of dasatinib between 1 and 100 nmol/L according to the published serum levels achieved in patients taking 70 mg dasatinib twice daily (24). Positive, negative, and solvent controls (DMSO) were included in all assays. The effects of the dual SRC/BCR-ABL inhibitor dasatinib were compared with the promiscuous natural TK inhibitor staurosporine.

**Dasatinib and staurosporine inhibit T-cell proliferation in a dose-dependent manner.** To investigate the effects of dasatinib and staurosporine on induced T-cell proliferation, we applied the murine monoclonal antibody OKT3, which cross-links the CD3 component of the CD3/TCR complex. To visualize cell proliferation, we labeled the cells with the vital dye CFSE. This dye is retained in the cytoplasm and is diluted out with each cell division, thereby allowing visualization of successive cell divisions by flow cytometry. A dose-dependent inhibition of T-cell proliferation was detected with almost complete inhibition (96%) occurring at a concentration of 20 nmol/L dasatinib (IC<sub>50</sub>, 11 nmol/L; Fig. 1). T cells that were incubated with dasatinib for 24 hours and then removed from dasatinib proliferated as well as untreated T cells over a period of 4 days (P = 0.156; n = 5); this argues for a reversible blockade of T-cell proliferation. In contrast, staurosporine led to a dose-dependent, but irreversible, inhibition of proliferation at a concentration of 10 nmol/L (mean of 90% inhibition; P = 0.027; n = 4).

**Dasatinib and staurosporine inhibit T-cell activation and IL-2 release in a dose-dependent manner.** We further investigated the functional effects of dasatinib and staurosporine on T-cell activation and cytokine release. A statistically significant inhibition of OKT3-induced up-regulation of the early T-cell activation marker CD69 was observed at concentrations as low as 10 nmol/L, and almost complete inhibition (99%)
occurred after 24 hours at a concentration of 50 nmol/L (IC50, 11 nmol/L; Fig. 2); corresponding effects for staurosporine occurred at IC50 of 4 to 5 nmol/L (data not shown). In line with the inhibition of CD69 up-regulation, we also observed a profound reduction of IL-2 secretion measured by ELISA in T cells treated with dasatinib and staurosporine (n = 6; IC50, 2 nmol/L for both; Fig. 2).

Dasatinib and staurosporine do not increase apoptosis in T cells at clinically relevant concentrations. To investigate whether the induction of apoptosis contributes to the dose-dependent inhibition of T-cell activation and proliferation induced by dasatinib and staurosporine, we measured apoptosis and necrosis by Annexin V and 7-AAD staining after 4 days of stimulation with OKT3 and antibodies against the costimulatory molecules CD28 and CD49d. Neither dasatinib nor staurosporine increased activation-induced cell death in T cells (n = 5). Instead, the addition of dasatinib and staurosporine led to a statistically significant increase in necrosis compared with stimulated T cells alone (n = 5; Supplementary figure). Overall, naïve CD8+ T cells develop more readily into effector T cells after short-term primary stimulation than naïve CD4+ T cells (25). In our assays, CD4+ T cells were marginally more sensitive than CD8+ T cells to the inhibitory effects of staurosporine on activation and proliferation (data not shown). The same held true for dasatinib; the IC50 for inhibition of activation was 10 nmol/L for CD4+ cells and 15 nmol/L for CD8+ cells (Fig. 2), whereas the IC50 for inhibition of proliferation was 10 nmol/L for CD4+ cells and 13 nmol/L for CD8+ cells (data not shown).

Naïve T cells are more sensitive than memory T cells to the inhibitory effects of dasatinib. Murine cytotoxic memory T cells are more sensitive than naïve CD8+ T cells to the inhibitory effects of imatinib (26, 27). In contrast, we observed that naïve T cells were more sensitive to the inhibitory effects of dasatinib (activation: IC50, 16 nmol/L for naïve CD8+ cells, 12 nmol/L for naïve CD4+ cells; proliferation: IC50, 15 nmol/L for naïve CD8+ cells, 9 nmol/L for naïve CD4+ cells) than memory subsets (activation: IC50,
Virus-specific CD8+ T-cell responses are suppressed by dasatinib and staurosporine in a dose-dependent manner. Because CD8+ T-cell–mediated immunity is essential for the long-term control of persistent DNA viruses, we evaluated the effect of dasatinib and staurosporine on antigen-specific T-cell responses to CMV and EBV. Proliferation of both CMV-specific (n = 4) and EBV-specific (n = 3) CD8+ T cells was suppressed in a dose-dependent manner by both TK inhibitors, with complete inhibition observed at 100 nmol/L dasatinib and 50 nmol/L staurosporine (Fig. 3). We also observed a dose-dependent inhibition of IFNγ and TNFα secretion as well as an inhibition of CD107a/b mobilization (CMV n = 3, EBV n = 2); surface expression of CD107a/b follows activation-induced degranulation and is thus a necessary precursor to perforin/granzyme–mediated cytolysis (Fig. 4). These results were confirmed with an EBV-specific CD8+ T-cell clone (Fig. 5A).

Dasatinib and staurosporine inhibit γδ T-cell activation. Interestingly, dasatinib was also shown to inhibit γδ T-cell activation. The secretion of both TH1 (IFNγ, TNFα, and IL-2) and TH2 (IL-4) cytokines was inhibited to the same extent (Fig. 5B).

Dasatinib and staurosporine inhibit TCR and CD8 down-regulation from the surface of cytotoxic T cells. Both dasatinib and staurosporine exhibited dose-dependent effects on the levels of TCR and CD8 expressed on the surface of T-cell clones. Indeed, expression levels of TCR and CD8 at the cell surface of the antigen-specific T-cell clone EBV-C increased by ~15% and 40%, respectively, after 3 hours of exposure to 50 nmol/L staurosporine or dasatinib (Fig. 5C). These observations suggest that dasatinib and staurosporine block TCR down-regulation from the cell surface (28), one of the initial events that occur after TCR engagement.

Dasatinib inhibits proximal transduction components of the CD3/TCR complex and decreases LCK phosphorylation. To
dissect the mechanism by which dasatinib and staurosporine inhibit all T-cell effector functions, we stimulated Jurkat T cells in the presence or absence of dasatinib or staurosporine, or left untreated, for 1 h in a total volume of 100 μL. C1R B cells that express HLA A*0201 were pulsed with GLCVTLVAML peptide at concentrations of 0 to 100 μmol/L for 1 h, then washed twice. Pulsed target cells (2.5 × 10⁶) were then added to the previously treated EBV-C CD8⁺ T cells in a final volume of 200 μL and incubated for 4 h at 37°C. After pelleting the cells by centrifugation, supernatant was collected and assayed for IL-2, TNFα, and IFNγ levels using a TH1/TH2 cytokine kit (BD). A, AJH γδ T cells (1 × 10⁵) were treated with either 10 or 50 nmol/L dasatinib, or left untreated, for 1 h at 37°C. After addition of 1 μg/mL OKT3, cells were incubated for a further 4 h at 37°C. Supernatant was then harvested and analyzed by cytometric bead array. B, cells (1 × 10⁵) of the CD8⁺ T-cell clones EBV-C, MEL-13, or ILA-1 were either left untreated or treated with either dasatinib or staurosporine at concentrations of 2, 10, or 50 nmol/L for 3 h at 37°C and analyzed for TCRα and CD8 expression as described in Materials and Methods.

Discussion

In this study, we examined the effects of the dual BCR-ABL/SRC kinase inhibitor dasatinib on multiple T-cell effector functions. Using the promiscuous TK inhibitor staurosporine
marginal differences in antigen sensitivity between CD8+ T cells and naïve T cells, and LCK expression (29), and differential requirements for LCK during primary and memory CD8+ T-cell activation (30). Indeed, it is known that antigen sensitivity of CD8+ T cells is a Src kinase with an important role in proximal signal transduction. Observation of delayed antigen presentation might reflect differential expression levels of LCK, being more sensitive than memory T-cell subsets. This latter response to multiple intracellular pathogens including EBV infection, and recent studies have suggested a role for γδ T cells in the control of human and murine CMV viruses in vivo (16, 17). Therefore, inhibition of γδ T-cell activation could increase host susceptibility to viral infection.

The in vitro assays used here may not completely reproduce in vivo conditions, but our data still suggest that dasatinib and staurosporine attenuate T-cell function at the level of effector cytokine secretion and degranulation at experimentally relevant concentrations (24). These findings suggest that dasatinib could be considered as a potential therapeutic strategy in T-cell-mediated autoimmune disorders or as an immunosuppressant in the transplantation setting. Inevitably, the observation that both cytokine production and degranulation are impaired is a concern from the clinical standpoint given the potential increased susceptibility to infections. Indeed, in clinical trials conducted to evaluate the safety and efficacy of dasatinib before approval for the treatment of imatinib-refractory chronic myeloid leukemia and Ph+ acute lymphoblastic leukemia, a small percentage of patients developed infections, especially pneumonia, which might reflect not only myelosuppression but also inhibition of T-cell effector functions (11–13). However, as our washout experiments show, the effects of dasatinib on T cells are transient; thus, dose reduction or treatment interruption could rapidly reverse any dasatinib-induced immune insufficiency, which is in line with what is observed in current clinical practice. Furthermore, we observed no obvious increase in activation-induced cell death with dasatinib. Apoptosis is one mechanism through which immune responses are controlled, and LCK is required for activation-induced cell death to occur in T cells (36). In line with the literature, we observed a decreased rate of activation-induced cell death in T cells treated with dasatinib and activated with OKT3 and costimulatory molecules. However, in contrast to the effects of imatinib on apoptosis (6, 9), the apoptosis rate of OKT3-stimulated T cells in the presence of dasatinib was higher than in the untreated T cells, thereby arguing for a potential interaction of dasatinib with other apoptosis pathways.

Mechanistically, the inhibitory effects of dasatinib documented above seem to be mediated primarily by inhibition of LCK. We observed profound dephosphorylation of LCK in Jurkat T cells (Fig. 6); furthermore, the striking inhibition of CD3/TCR and CD8 down-regulation on the surface of antigen-specific T-cell clones attests to the potency with which very proximal events are inhibited by dasatinib (Fig. 5C).

Based on our findings, close monitoring of patients undergoing treatment with TK inhibitors seems to be warranted with respect to reactivation of persistent viral infections and newly acquired opportunistic infections. However, these findings also indicate a potential role for dasatinib as an immunosuppressant in the fields of transplantation and autoimmunity. Of note, a recent case report showed the efficacy of dasatinib in the treatment of a patient with thymoma (37). Thus, whereas caution is advised with respect to the clinical use of dasatinib, the data presented here also suggest new therapeutic uses for this novel drug.

Fig. 6. Dasatinib inhibits signal transduction through the CD3/TCR complex. Jurkat T cells were stimulated with OKT3 for 5 min in the presence or absence of dasatinib. Whole-cell lysates were analyzed by Western blotting for tyrosine phosphorylation (PTyr). To quantify LCK expression and to control for protein loading, the antiphosphotyrosine antibody was stripped off and the membrane was reprobed with an LCK-specific antibody.

References


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