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Diverse Transcriptional Response of CD4$^+$ T Cells to Stromal Cell-Derived Factor (SDF)-1: Cell Survival Promotion and Priming Effects of SDF-1 on CD4$^+$ T Cells[1]

Yasuhiro Suzuki,* Monzur Rahman,[†] and Hiroaki Mitsuya[2]*[‡]

Stromal cell-derived factor (SDF)-1 is a ligand for the chemokine receptor CXCR4 which is broadly expressed in lymphocytes, but the effects of SDF-1 on T cells are largely unknown. When examined using complementary DNA microarray, up-regulation of genes which are associated with DNA repair, detoxification, apoptosis, cell morphology, cell adhesion, and signal transduction was seen in CD4$^+$ T cells upon SDF-1 exposure. SDF-1 was shown to promote CD4$^+$ T cell survival through the phosphatidylinositol 3-kinase (PI3K)- and mitogen-activated protein kinase (MAPK)-cascades without cell cycle progression. The proapoptotic Bcl-2 antagonistic of cell death protein was also seen inactivated by the SDF-1-mediated activation of MAPK-extracellular signal-regulated kinases (MEK)-extracellular signal-regulated kinase-ribosomal S6 kinases- and PI3K-pathways. Moreover, the genes known to be associated with cell survival were up-regulated upon SDF-1 exposure and were linked to the MAPK-MEK and PI3K-pathways. Thus, SDF-1 promotes cell survival by two mechanisms: posttranslational inactivation of the cell death machinery and an increased transcription of cell survival-related genes. SDF-1 also primed resting CD4$^+$ T cells for cytokine- and TCR-mediated stimuli. These data suggest that the SDF-1-mediated cell survival combined with its priming function would set T cells to respond to immunologic challenges. *The Journal of Immunology,* **2001, 167: 3064–3073.**

T he chemokine receptor, CXCR4, a receptor for stromal cell-derived factor (SDF)[3]-1 and a coreceptor for HIV-1 cellular entry, is broadly expressed in lymphoid cells, including B cells and both naive and memory CD4$^+$ T cell subpopulations (1). SDF-1 stimulates proliferation of B cell progenitors in vitro and is constitutively expressed in bone marrow-derived stromal cells (2, 3). There are reports that mice lacking either SDF-1 or CXCR4 die perinatally and the numbers of B cell and myeloid progenitors in such embryos are severely reduced in bone marrow (4–7). These data strongly suggest that SDF-1 and CXCR4 are responsible for B cell lymphopoiesis and bone marrow myelopoiesis. Such SDF-1- or CXCR4-deficient mice also had defects in vascular development, cardiogenesis, and cerebellar development; however, T cell development and functions including trafficking to lymphoid organs were apparently unaffected in those mice (6, 7).

From these data, one could postulate that SDF-1 does not exert significant effects on T cells. However, Nanki and Lipsky (8) have recently reported that SDF-1 plays a role as a costimulator in anti-CD3-induced CD4$^+$ T cell activation. They showed that the concomitant exposure of SDF-1 and anti-CD3 Ab up-regulates the expression of early activation markers such as CD25 and CD69 and proliferation of CD4$^+$ T cells. SDF-1 also has been reported to cause apoptosis in neuronal cells in culture (9, 10), and it is thought that CXCR4-mediated neuronal cell death contributes to the CNS abnormalities in patients with HIV-1 infection. However, such an apoptosis-related activity of SDF-1 has not been shown in CD4$^+$ T cells.

In the present study, we examined large-scale genome-wide effects of SDF-1 on CD4$^+$ T cells using the cDNA microarray technique and demonstrate that SDF-1 promotes CD4$^+$ T cell survival through the phosphatidylinositol 3-kinase (PI3K)- and mitogen-activated protein kinase (MAPK) signal transduction cascades without affecting cell cycle progression and that SDF-1 also exerts priming function to set CD4$^+$ T cells to respond to immunologic stimuli.

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Materials and Methods

Reagents

An inhibitor of MAPK-extracellular signal-regulated kinases (MEK) activity, PD098059, and a PI3K-specific inhibitor, LY294002, were purchased from Biomol (Plymouth Meeting, PA) and New England Biolabs Beverly, MA), respectively. Recombinant human IL-2, IL-4, IL-6, IL-15, and SDF-1 (SDF-1β was used throughout this study) were purchased from R&D Systems (Minneapolis, MN). The following mAbs were used for the stimulation of T cells: anti-CD3 (UCTH1; Beckman Coulter, Fullerton, CA) and anti-CD28 (LewTM-28; BD Biosciences, Mountain View, CA). The following mAbs were purchased from BD PharMingen (San Diego, CA) and used for staining surface Ags: anti-CD3-FITC, anti-CD4-FITC and -PE, anti-CD8-FITC, anti-CD14-FITC, anti-CD16-FITC, anti-CD20-FITC, anti-HLA-DR-FITC, anti-CD25-FITC, anti-CD69-FITC, and anti-CXCR4-PE (12G5). mAbs against human CD8, CD20, CD25, and HLA-DR were produced, purified, and used for the purification of dense resting CD4$^+$ (drCD4$^+$, see below) T cells. Anti-human CD16 (LNK16)

Ab was purchased from Serotec (Oxford, U.K.). For Western blotting, Abs against various proteins were purchased from New England Biolabs. Expression vectors for ribosomal S6 kinase (Rsk) proteins (pMT2-HA-RSK2-WT, pMT-HA-RSK2-KN, and a control vector) were kind gifts from Dr. M. E. Greenberg (Children's Hospital, Harvard Medical School, Cambridge, MA). For transfection of Jurkat cells, a green fluorescent protein expression vector (Clontech Laboratories, Palo Alto, CA) and Gene-PORTER transfection reagents (Gene Therapy Systems, San Diego, CA) were used.

Chemotaxis assays

Chemotactic activity of SDF-1 was determined using microchemotaxis plates (NeuroProbe, Gaithersburg, MD) as previously described (11). Two pore size (5- and 10-μm) filters were used for drCD4$^+$ T and Jurkat cells, respectively.

Cell culture and cDNA microarray experiments

The Jurkat (clone E6-1; ATCC TIB-152) cell line was used throughout this study. Cells ($\sim$7 $\times$ 10^5/ml) were maintained in RPMI 1640 containing 1 mM L-glutamine supplemented with 10% (v/v) heat-inactivated FCS (Bio-Whittaker, Walkersville, MD) at 37°C in a 5% CO$_2$ containing humidified air. To study the effects of SDF-1 on CD4$^+$ T cells as cultured in the absence of FCS, cells were first maintained in RPMI 1640 containing 0.5% FCS for 36 h, thoroughly washed with FCS-free RPMI 1640, divided into two flasks, and incubated further in FCS-free medium for 6 h to deactivate the PI3K and MAPK signal transduction cascades to an undetectable level. Cells in one flask were stimulated with SDF-1 and harvested at 45 min, 3 h, 6 h, and 12 h for isolation of mRNA. mRNA was also isolated from SDF-1-unexposed cells in another flask and served as a reference for each time point. Preparation of mRNA and the labeling of the probe and hybridization conditions are described in detail at http://nciarray.nci.nih.gov/reference/index.shtml. In brief, total mRNA was isolated using RNeasy kit (Qiagen, Chatsworth, CA) and 25 μg of mRNA was used for generating fluorescence-labeled cDNA probes using Cy3- and Cy5-dUTP (Amersham, Arlington Heights, IL), and the resulting cDNA was subjected to hybridization on microarray slides. Methods used for hybridization, fluorescein scanning, and image analysis are described in detail at http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/img_analysis.html. All measurements were stored in a computer database for analyses and interpretations. The microarray slides used contained 2304 elements including 2140 human cDNAs which represented 1847 different genes (the complete list of the genes can be seen at http://nciarray.nci.nih.gov/cgi-bin/gipo) and were obtained from the Advanced Technology Center, National Cancer Institute.

RNase protection assays

RNase protection assays were conducted using RiboQuant MultiProbe RNase Protection Assay kits (BD PharMingen) according to the manufacturer's instructions. Total mRNA was isolated from SDF-1 (0.38 μg/ml)-exposed cells cultured in 10% FCS-containing medium for 3, 6, 12, 24, and 36 h and the expression of 79 selected genes encoding cytokines, chemokines, apoptosis-related genes, and angiogenesis factors (hCK-1, hCK 2, hCK-3, hCR-1, hCR-5, hCR-6, hAPO-2c, hAPO-3c, hAPO-4, hSTRESS-1, hAngio-1) was examined.

Isolation of drCD4$^+$ T cells

drCD4$^+$ T cells were isolated from PBMC obtained from healthy donors as previously described by Sun et al. (12). In brief, PBMC were isolated over Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) and subjected to nylon wool fiber column (Polysciences, Warrington, PA) separation to deplete B cells and macrophages. The nonadherent cells were overlaid on a discontinuous Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient and the dense T cells lodged in the interface between 60 and 65% Percoll were collected. CD4$^+$ T cells were purified through negative selection using a magnetic cell separation system (Dynal MPC-1 column; Dynal Biotech, Great Neck, NY) with a mixture of mAbs specific for CD8, CD16, CD20, CD25, and HLA-DR. Unstained cells were harvested with a magnetic column. The purity and the cell surface expression of various molecules were determined using a calibrated FACScan flow cytometer and were analyzed by CellQuest software (BD Biosciences). The purity of the drCD4$^+$ T cells thus harvested routinely consisted of >97% CD4$^+$ cells, <1.2% CD25$^+$ cells, and <1.1% HLA DR$^+$ cells.

Apoptosis and T cell proliferation assays

The percentages of apoptotic cells were determined using Annexin V^{FITC} and propidium iodide (PI; BD PharMingen) costaining according to the

manufacturer's instructions and flow cytometry. Jurkat cells (10^4) or drCD4$^+$ T cells (10^5) were cultured in the presence of various Abs or cytokines in 96-well flat-bottom microtiter culture plates. After 48 or 72 h in culture, each cell preparation was pulsed for 16 h with [^{3}H]thymidine (1 μCi for drCD4$^+$ T cells and 0.5 μCi for Jurkat cells). To determine whether SDF-1-exposed drCD4$^+$ T cells progressed in the cell cycle, they were exposed to SDF-1 (0.38 μg/ml) for 72 h and their 5-bromo-2'-deoxyuridine (BrdU) incorporation and Ki-67 expression were determined using a BrdU Flow kit (BD PharMingen) and Ki-67 Ab kit (BD PharMingen), respectively.

Western blot analysis

Whole cell lysates derived from Jurkat, MAGI, and drCD4$^+$ T cells were examined using 4–20% gradient polyacrylamide gels (Pharmacia Biotech). For Western blotting, 1/3000 and 1/10000 dilutions were used for the first Abs and a secondary HRP-conjugated anti-rabbit IgG (New England Biolabs), respectively. Proteins were visualized using a SuperSignal West Pico Chemiluminescent kit (Pierce, Rockford, IL) and Biomax-MR film (Kodak, Rochester, NY).

In vitro kinase assay

Jurkat cells were cultured with FCS (0.5%) containing RPMI 1640-based culture medium for 36 h, then in FCS-free medium for 6 h, and exposed to SDF-1 (0.38 μg/ml) for 10 min. Some cells were exposed to PD098059 (50 μM) or LY294002 (20 μM). Akt1 and Rsk2 were immunoprecipitated by Abs that are specific for Akt1 (New England Biolabs) and Rsk2 (Upstate Biotechnology, Lake Placid, NY, respectively). The immunoprecipitated kinases were subjected to in vitro kinase reaction with 200 μM ATP along with 1 μg of the substrate recombinant Bcl-2 antagonist of cell death (BAD; Upstate Biotechnology). In vitro kinase assays were performed using a kit purchased from New England Biolabs according to the manufacturer's instructions. Immunoblotting was performed using Abs that were specific for BAD phosphorylated at Ser^{112} or Ser^{136} and an Ab that was reactive with BAD regardless of phosphorylation, as described above.

Results

Induction of diverse transcriptional response in Jurkat T cells upon SDF-1 exposure

To examine the profile of gene expression in CD4$^+$ T cells upon SDF-1 exposure, we first determined the SDF-1 concentration that induced a maximal migration response. When Jurkat and drCD4$^+$ T cells were exposed to serially diluted SDF-1 (0, 0.12, 0.38, 1.1, 3.3, and 10 μg/ml), the maximum migration occurred in the presence of 0.38 μg/ml SDF-1. We treated Jurkat cells with SDF-1 (0.38 μg/ml) in the presence and absence of FCS, since the PI3K- and MAPK-dependent signal pathways, known to be activated by SDF-1, are also activated by FCS (13–16). Total mRNA was isolated from Jurkat cells at several time points following SDF-1 exposure and fluorescence-labeled cDNA probes were prepared using Cy3- and Cy5-dUTP as described in *Materials and Methods*. The Cy3- and Cy5-labeled cDNA probes were hybridized onto a cDNA microarray slide containing 2140 cDNA clones. Following thorough washing, the array was scanned and the fluorescence ratios were determined.

The transcriptional response upon SDF-1 exposure occurred within 3 h (Fig. 1, *A* and *B*). The initial response was much greater in cells exposed to SDF-1 in the absence of FCS than in those treated similarly but in the presence of FCS. In 6 h and beyond, the pattern of gene expression was comparable in the two cell preparations, but the ratios were greater in the cells cultured in the absence of FCS than in those cultured in the presence of FCS (Fig. 1, *A* and *B*). To corroborate these gene expression data and to confirm its reproducibility, we chose 79 genes (see *Materials and Methods*) encoding cytokines, chemokines, apoptosis, and angiogenesis factors and examined their gene expression profiles in the SDF-1-exposed cells using the MultiProbe RNase protection assay. The levels of expression of these selected genes were comparable to the ratio changes observed in the microarray assays (data not shown).
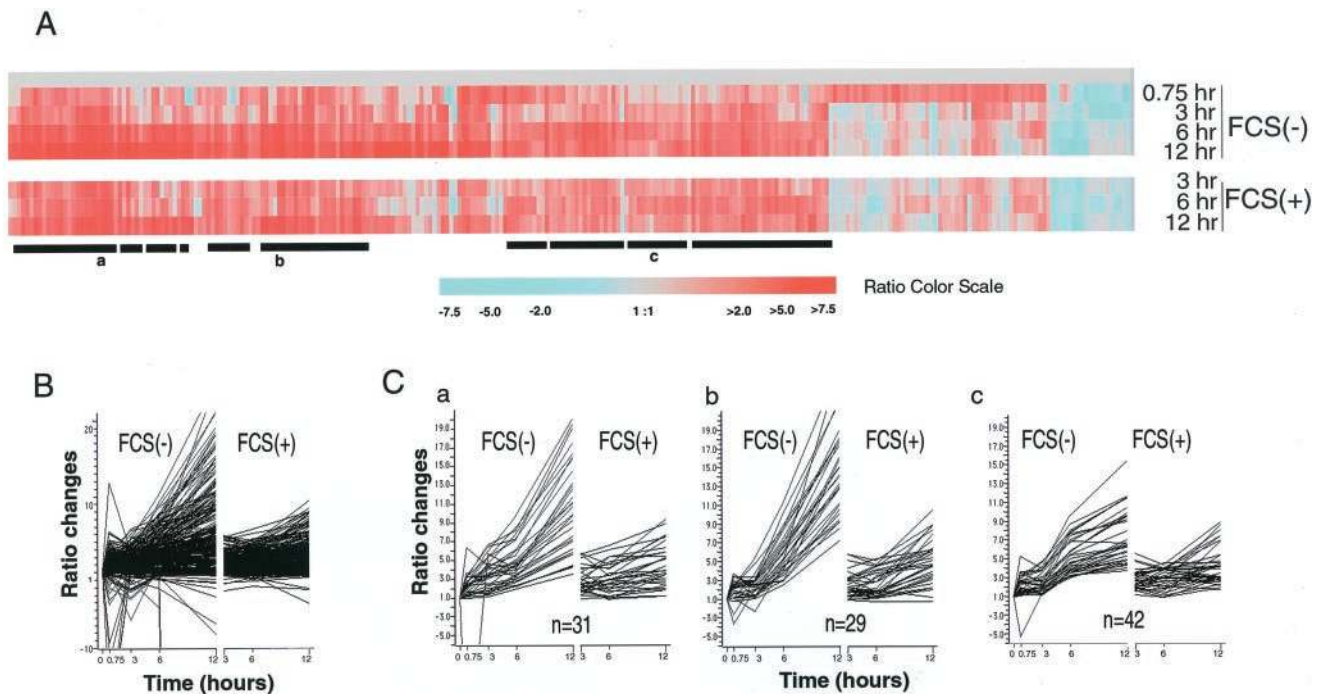
FIGURE 1. Gene expression profiles of SDF-1-treated CD4$^+$ T cells. *A*, mRNA was harvested from SDF-1 (0.38 μg/ml)-exposed CD4$^+$ Jurkat cells at indicated times and was used to prepare a cDNA probe labeled with Cy5-dUTP; a reference mRNA was similarly prepared from SDF-1-unexposed Jurkat cells and its cDNA probe was labeled with Cy3-dUTP. The expression pattern of each gene in this set is displayed as a transverse strip. For each gene, the ratio of mRNA levels in SDF-1-exposed Jurkat cells to those in unexposed Jurkat cells is represented by a color according to the color scale at the bottom. Genes which exhibited significant ratio changes over 2.5 (the genes which were abundantly expressed) or below 0.3 (the genes which were suppressed) in response to SDF-1 exposure were chosen and clustered using the tree program. *B*, The mRNA expression ratio changes in SDF-1-exposed and -unexposed Jurkat cells are shown in line graphs. *C*, Among the gene clusters identified, three clusters (indicated by *a*–*c*) whose ratios continued to increase throughout the culture following SDF-1 exposure. *n*, Number of genes within the cluster.

Genes that exhibited significant ratio changes upon SDF-1 exposure were selected and clustered using the tree program (Genespring software; Silicon Genetics, Mountain View, CA). Among such 292 genes consisting of 269 cDNA species, 3 conspicuous clusters were identified which contained 101 genes in total (121 including overlapped among the clusters) whose ratios continued to increase throughout the 12-h culture following SDF-1 exposure (Fig. 1, *A* and *C*). Such genes included detoxification (members of cytochrome P450 subfamilies)-, DNA repair (XRCC5, XRCC3, ERCC1, RAD54L, MSH6, TDG)-, and antiapoptosis (defender against apoptotic death-1 (DAD-1) PPEF-1, PPEF-2, Nak1)-related genes (Fig. 2). We presumed that these genes were associated with the promotion of cell survival. Genes associated with TCR signaling (CD3γ, Fyn, SLP-76, HS1, Nck1), inflammatory cytokine receptors and related proteins, IFN receptors, and downstream molecules (IFN regulatory factor 6 and IFN regulatory factor 3) were also identified, leading us to postulate that the expression of these genes represented immunologic response in CD4$^+$ T cells upon SDF-1 exposure. Moreover, the genes encoding cell adhesion molecules (members of integrins, CD36, CD33, protocadherin 42), kinases involved in the reorganization of actin cytoskeleton (ROCK1, MDK), actin-binding proteins (HS1, GAS2, PKD1, filamin A, CDC10), chemokine receptors (STRL33.1), and the genes involved in the remodeling of extracellular matrix and metastasis (MMP-11) were identified (Fig. 2). In addition, RNase protection assays using the RiboQuant MultiProbe RNase Protection kit (hCR-5) revealed that the expression level of CCR8 which was not included in the microarray increased 20.8-fold in 36 h in Jurkat cells after SDF-1 exposure (data not shown). Thus, 6% of the 101 genes were related to DNA repair enzymes, 5% detoxification en-

zymes, 15% signal transduction molecules, and 18% cytokinesis/cell morphology/cell adhesion molecules.

SDF-1 promotes cell survival without affecting cell cycle

In an attempt to examine whether the genes expressed seen in the microarray technique were also phenotypically expressed, we conducted functional assays for apoptosis and T cell signal transduction using highly purified drCD4$^+$ T cells as well as CD4$^+$ Jurkat cells. The drCD4$^+$ T cells are largely in the G$_0$ phase of the cell cycle and do not produce detectable amounts of cytokines, making SDF-1-specific effects easily identified. It was found that the removal of FCS from the culture of drCD4$^+$ T cells and Jurkat cells induced apoptosis, but when cultured with SDF-1, the percentage of apoptotic drCD4$^+$ T cells was greatly decreased in the absence of FCS to the level observed in the presence of FCS (Fig. 3, *Aa* and *B*). The SDF-1-induced suppression of apoptosis was also observed in Jurkat cells, although the suppression was not complete (Fig. 3*C*).

Similarly, γ-irradiation-induced apoptosis in drCD4$^+$ T and Jurkat cells was suppressed by the addition of SDF-1 in culture (Fig. 3*D, a* and *b*). These results strongly suggest that SDF-1 blocks apoptosis in CD4$^+$ T cells. Considering that CXCR4 is abundantly expressed in resting CD4$^+$ T cells, it is likely that SDF-1 contributes to the maintenance and protection of unstimulated CD4$^+$ T cells. By contrast, high-affinity receptors for a number of cytokines such as IL-2 and IL-4 appear only upon immunologic stimulation of T cells, and such cytokines elicit antiapoptotic activity, cell cycle progression, and differentiation in CD4$^+$ T cells. Thus, the antiapoptotic effects of SDF-1 seem to differ from the antiapoptotic activities of cytokines.
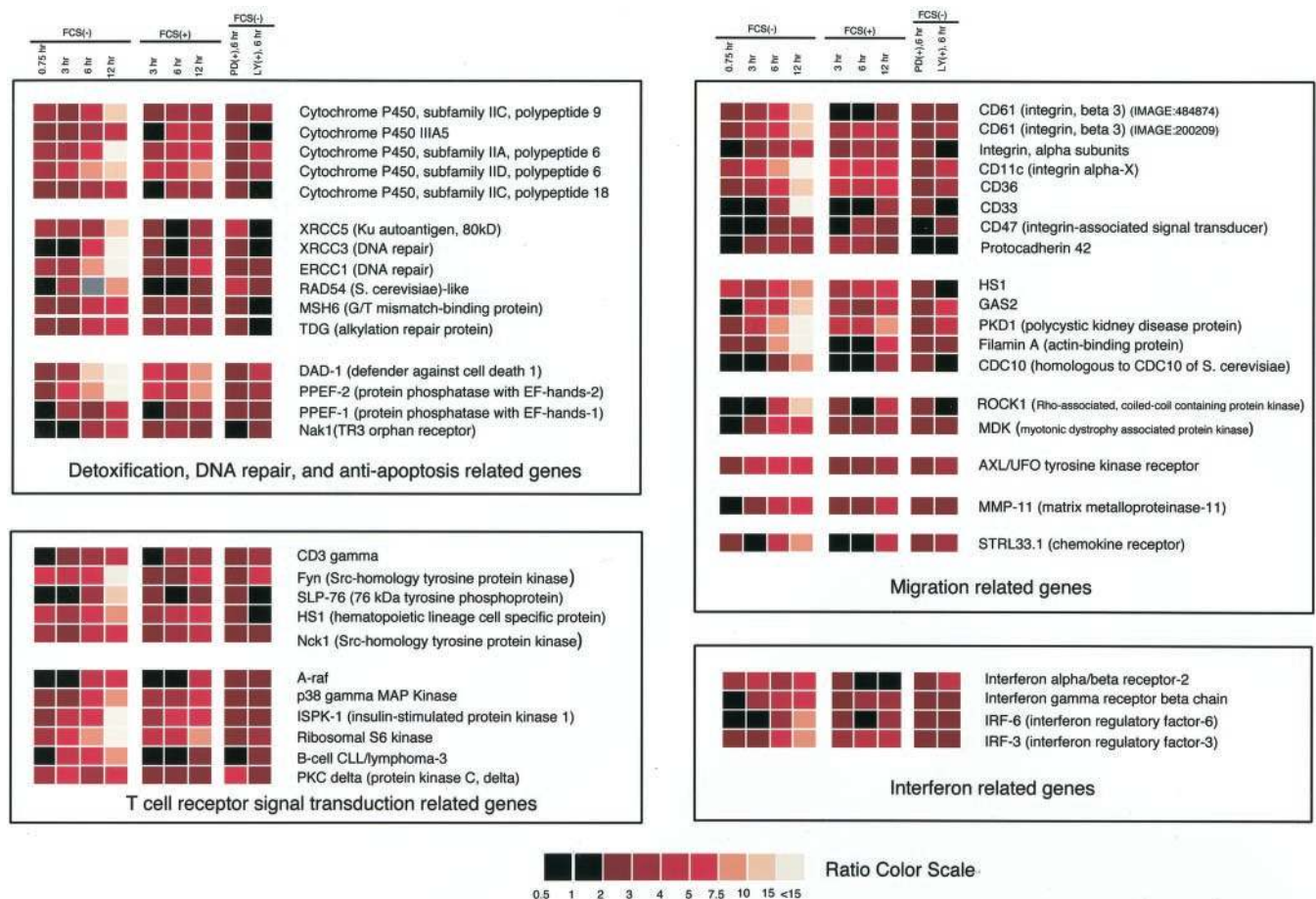
FIGURE 2. Transcriptional response of selected cellular genes in Jurkat T cells upon SDF-1 exposure. Among the genes which showed a continuous increase in their expression following SDF-1 exposure were those involved in detoxification, DNA repair, apoptosis, TCR signal transduction, cytokinesis, cell adhesion molecules, chemokine receptors, and IFN-related molecules. The effects of MEK and PI3K inhibitors (50 μM PD098059 (20, 21) and 20 μM LY294002 (22), respectively) on the expression of these genes are also shown. Jurkat cells were exposed to SDF-1 in the presence of the inhibitors for 6 h without FCS. Reference mRNA was obtained from cells exposed to the inhibitors but not to SDF-1. The expression ratios were determined as described in the legend to Fig. 1.

Next, we examined whether drCD4$^+$ T cells were immunologically activated upon SDF-1 exposure. There was no expression of activation markers CD25 or CD69 in drCD4$^+$ T cells or Jurkat cells upon SDF-1 exposure, whereas those markers were readily detected following stimulation with anti-CD3 Ab (Fig. 3E). We also failed to detect alterations in replication rates or cell sizes in Jurkat cells following SDF-1 exposure (data not shown). Moreover, we asked whether drCD4$^+$ T cells progressed to the G$_1$ phase using a [^{3}H]thymidine incorporation assay (Fig. 3F), flow cytometry for BrdU incorporation, DNA content determination (Fig. 3Ga), and the assay for expression of Ki-67, a protein seen in all stages except the G$_0$ phase of the cell cycle (Fig. 3Gb). We found no evidence for the progression of drCD4$^+$ T cells to the G$_1$ phase or beyond. Thus, we concluded that SDF-1 promotes survival of drCD4$^+$ T cells without forwarding them in the cell cycle.

Cell survival is promoted by SDF-1 through MEK- and PI3K-dependent pathways

Cell survival factors such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor 1 activate the PI3K- and/or MAPK-mediated signaling pathways and transduce cell survival signals (17–19). Recently, Vicente-Manzanares et al. and others (13–16) reported that SDF-1 activates the MAPK-MEK- and PI3K-serine/threonine kinase Akt (protein kinase B) pathways to induce chemotaxis in CD4$^+$ T cells. We therefore examined

whether the activation of these two pathways preceded the promoted survival in SDF-1-exposed CD4$^+$ T cells. To this end, we used PD098059, an agent that blocks MEK activity (20, 21), and LY294002, a PI3K-specific inhibitor (22). Both inhibitors almost completely suppressed the SDF-1-induced survival in drCD4$^+$ T cells and Jurkat cells (Fig. 3, Ab and C), suggesting that the activation of the MAPK-MEK and PI3K signaling pathways is required for the SDF-1-induced promoted survival of CD4$^+$ T cells. For biochemical confirmation of the involvement of the MAPK-MEK and PI3K signaling pathways, we asked whether the signal transduction molecules (MEK, extracellular signal-regulated kinase (ERK), Rsks (p90), and a transcription factor CREB) in the MAPK signaling pathway were phosphorylated in Jurkat cells upon SDF-1 exposure. When examined using the immunoblotting technique, all such molecules were found to be phosphorylated (Fig. 4A). When Jurkat cells were cultured with PD098059, however, a substantial suppression in the phosphorylation of ERK, Rsks, and CREB was observed (Fig. 4A).

It has been reported that Rsks represent key factors for neural cell survival which BDNF induces through the activation of CREB (17, 23, 24). In fact, we found that an overexpression of a dominant-negative Rsk2 (Rsk2-KN) (17) in Jurkat cells resulted in the suppression of SDF-1-induced promoted cell survival (Fig. 4B). To determine whether CREB phosphorylation follows the phosphorylation of Rsks, we used transiently Rsk2-KN-overexpressing
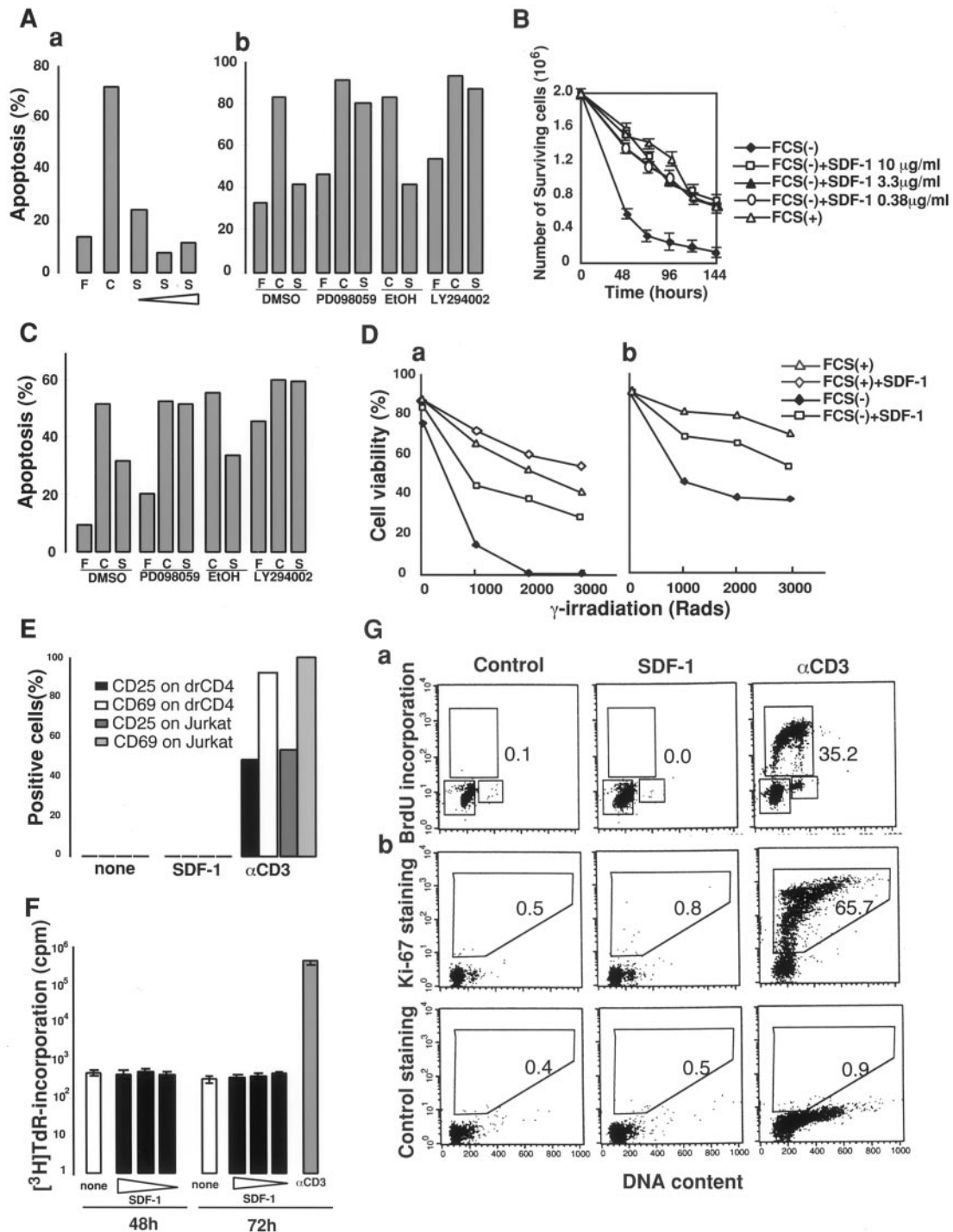
FIGURE 3. The MAPK- and PI3K-dependent pathways are involved in SDF-1-induced promoted survival of drCD4$^+$ T and Jurkat cells. *A* and *C*, letters F, C, and S denote FCS (10%)-containing medium, FCS-free medium, and FCS-free medium with SDF-1 exposure, respectively. The data shown are representatives of 6 (*A*), 2 (*B–D* and *G*), and 3 (*E* and *F*) independent assay data. *A, a,* Immediately after isolation, drCD4$^+$ T cells (10^5/ml) were cultured in FCS-free medium for 1 h at 37°C and exposed to SDF-1 (0.38, 3.3, and 10 μg/ml). *A, b,* The effects of the MEK inhibitor (PD098059, 50 μM) and PI3K inhibitor (LY294002, 20 μM) on the SDF-1 (0.38 μg/ml)-induced cell survival were examined. The vehicles of the inhibitors (DMSO and ethanol for PD098059 and LY294002, respectively) served as controls. The percentages of apoptotic cells were determined using Annexin V^{FITC} and PI costaining and flow cytometry in 48 h of culture in the presence of SDF-1. *B,* Numbers of surviving cells in SDF-1-exposed drCD4$^+$ T cells were determined using the trypan blue exclusion assay. *C,* Jurkat cells were cultured in FCS (0.5%)-containing medium for 36 h, then in FCS-free medium for 6 h, and exposed to SDF-1 (0.38 μg/ml), and the effects of PD098059 (50 μM) and LY294002 (20 μM) were examined. The percentages of apoptotic cells were determined using Annexin V^{FITC} and PI costaining in 48 h of culture in the presence of SDF-1. *D,* SDF-1-induced inhibition of γ-irradiation-induced apoptosis in drCD4$^+$ T (*a*) and Jurkat cells (*b*) was examined. Following SDF-1 (0.38 μg/ml) exposure for 12 h, drCD4$^+$ T and Jurkat cells were γ-irradiated and the viability of the cells was determined in 24 h using the Annexin V^{FITC}/PI costaining. *E,* The cells were exposed to SDF-1 (0.38 μg/ml) or anti-CD3 Ab (UCTH1: 40 μg/ml plate bound) for 24 h and the expression of CD25 and CD69 was determined. *F,* Following SDF-1 (0.38 μg/ml) exposure, the [^{3}H]thymidine incorporation by drCD4$^+$ T cells (10^5/well) was determined. *G,* Cell cycle profiles (*a*) and Ki-67 expression (*b*) after SDF-1 exposure (72 h) in drCD4$^+$ T cells. BrdU incorporation and Ki-67 expression were determined. The number shown in each box represents the percentage of cells in the S phase (*G, a*) and that of Ki-67-positive cells (*G, b*).
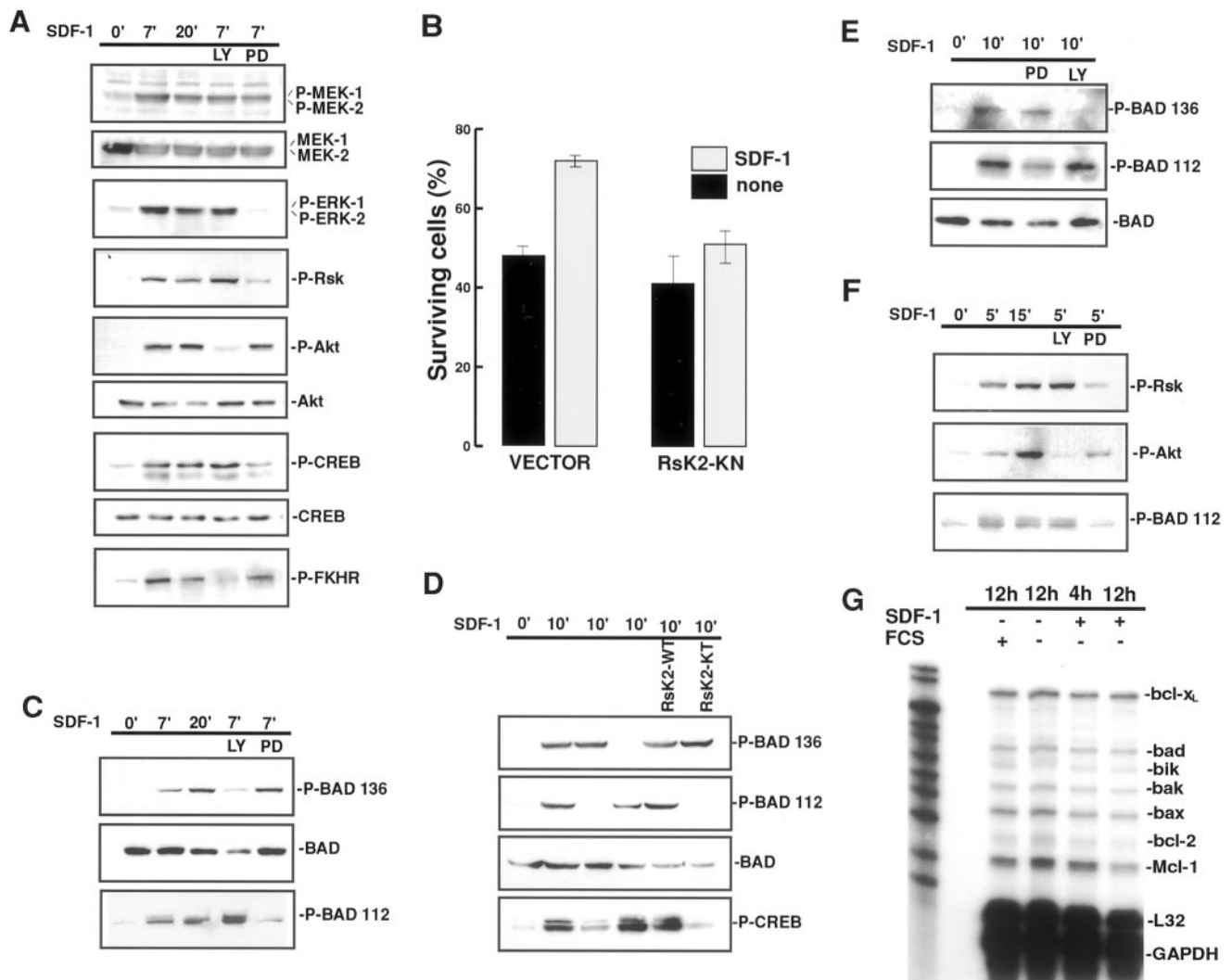
FIGURE 4. SDF-1 promotes CD4^{+} T cell survival through transcription-dependent and -independent mechanisms. *A,* SDF-1-induced phosphorylation of CREB at Ser[133] and FKHR at Ser[256] in the MEK-Rsk and PI3K-Akt pathways, respectively. Jurkat cells were cultured with FCS (0.5%)-containing medium for 36 h, then in FCS-free medium for 6 h, and exposed to SDF-1 (0.38 μg/ml) for 7 or 20 min. Some cells were exposed to the MEK inhibitor (PD098059, 50 μM) or PI3K inhibitor (LY2940020, 20 μM). Immunoblotting was performed using Abs that specifically recognized phosphorylated forms of MEK 1/2, ERK, Rsks, Akt, CREB (at Ser[133]), and FKHR (at Ser[256]) or with Abs that recognized MEK1/2, Akt, and CREB regardless of phosphorylation. *B,* Requirement of endogenous Rsks for SDF-1-induced cell survival. Jurkat cells were transfected with plasmids expressing Rsk2-KN or a control plasmid along with a green fluorescent protein expression vector. One day after transfection, cultures were deprived of FCS, and the cells were exposed to 0.38 μg/ml SDF-1. Two days later, the cells were stained with PI, and the percentage of apoptotic cells in the green fluorescent protein-expressing cells was determined by flow cytometry. *C,* SDF-1-induced phosphorylation of BAD at Ser[112] and Ser[136]. Immunoblotting of Jurkat cell lysates was performed using Abs that are specific for BAD phosphorylated at Ser[112] or Ser[136] and an Ab that recognizes BAD regardless of phosphorylation. *D,* SDF-1 induced phosphorylation of BAD at Ser[112] and Ser[136] through the MEK-Rsk and PI3K-Akt pathways, respectively. CXCR4^{+} MAGI cells were transfected with expression vectors containing Rsk2-WT, Rsk2-KN, or BAD. One day after transfection, cultures were deprived of FCS for 8 h and exposed to SDF-1 (0.38 μg/ml) for 10 min. Cells transfected with a BAD expression vector were incubated with PD098059 (50 μM) or LY2940020 (20 μM), exposed to SDF-1 (0.38 μg/ml), and the cell lysates were subjected to immunoblotting. *E,* Phosphorylation of BAD by Akt1 and Rsk2. Jurkat cells were cultured in FCS (0.5%)-containing medium for 36 h, then in FCS-free medium for 6 h, and exposed to SDF-1 (0.38 μg/ml) for 10 min. Some cells were exposed to PD098059 (50 μM) or LY2940020 (20 μM). Rsk2 and Akt1 were immunoprecipitated and subjected to in vitro kinase reaction assays. The results of BAD phosphorylation mediated by Akt1 and Rsk2 are shown in the *top* and *middle panels*, respectively. The *bottom panel* represents the blot shown in the *middle panel* but reprobed with polyclonal anti-BAD Ab, confirming that an equal amount of protein was loaded in each of four lanes. *F,* SDF-1-induced phosphorylation of Akt, Rsks, and BAD in drCD4^{+} T cells. Immediately after isolation, drCD4^{+} T cells were cultured in FCS-free medium for 3 h and exposed to SDF-1 (0.38 μg/ml) for 5 or 15 min. *G,* SDF-1 exposure did not affect the transcriptional regulation of the Bcl-2 family genes in Jurkat cells. Total mRNA was isolated from SDF-1-exposed and -unexposed Jurkat cells and the level of expression of the Bcl-2 family genes was determined.

CXCR4[+] fibroblasts (MAGI cells) (25). With respect to the use of MAGI cells, the efficiency of transfection of dominant-negative protein Rsk2-KN in Jurkat was very poor (only ~8% of the cells became positive for Rsk2-KN) and it was not possible to reliably perform the immunoblotting assays. Therefore, we chose CXCR4-expressing MAGI cells, which we found was a more transfection-

efficient (~70% of the cells became positive) cell line, enabling us to study the effect of SDF-1. In this respect, we had learned that SDF-1 exposure induced the phosphorylation of Rsks and CREB in both Jurkat and MAGI cells, and that the MEK inhibitor PD098059 inhibited the SDF-1-induced phosphorylation of Rsks and CREB in both cell lines as shown in Fig. 4, *A* and *D*. These

data suggest that SDF-1 exposure induces the phosphorylation of Rsks and CREB through the MEK-dependent pathway(s) in both Jurkat and MAGI cells. When the Rsk2-KN-overexpressing CXCR4$^+$ MAGI cells were exposed to SDF-1, no phosphorylation of CREB was seen (Fig. 4D). In this respect, it has been shown that ERK serves as a substrate for MEK and the activated ERK subsequently induces Rsk phosphorylation (17, 23, 24). As shown above, it appears that the SDF-1-induced activation of CREB occurred through the MEK-ERK-Rsk pathway(s) (Fig. 4, A and D). Taken together, it is likely that the SDF-1-induced cell survival promotion seen in this study occurs through the MEK-ERK-Rsk-CREB cascade.

Since the PI3K-Akt-induced phosphorylation and inactivation of Forkhead family transcription factors are reportedly involved in cell survival (26, 27), we also asked whether Akt and FKHR (a member of the Forkhead family) were involved in SDF-1-induced signaling pathways. Upon SDF-1 stimulation, both Akt and FKHR were found phosphorylated and the PI3K-specific inhibitor, LY294002, inhibited the phosphorylation of these two molecules (Fig. 4A). These data strongly suggest that FKHR inactivation (phosphorylation) was mediated through the PI3K-Akt pathway.

We also asked whether SDF-1 stimuli were transduced through pathways other than the MAPK and PI3K signaling pathways and examined the activation of three signal transduction molecules (p38 MAPK, c-Jun N_2-terminal kinase 1/2, and IκB) associated with other signal transduction pathways, but no phosphorylation was detected in any of these three molecules (data not shown). Furthermore, we examined whether the genes associated with DNA repair, detoxification, and apoptosis, which we identified to be up-regulated upon SDF-1 exposure in the microarray assays, were linked to the MAPK-MEK and PI3K signaling pathways using PD098059 or LY294002, respectively, in Jurkat cells (Fig. 2). These two inhibitors blocked the up-regulation of those genes, suggesting that the two pathways are involved in the SDF-1-induced transcription.

SDF-1 exposure induces BAD phosphorylation

The Bcl-2 family proteins consist of molecules that enhance cell survival as well as those that promote apoptosis (28, 29). Recently, one such Bcl-2 family protein, BAD, which is bound to Bcl-2 and/or Bcl-x$_L$ and exerts a proapoptotic function, has been shown to be phosphorylated at Ser^{112} and Ser^{136} by Rsks and Akt and to be involved in cell survival (17–19). We therefore examined whether the phosphorylation of BAD occurred in SDF-1-exposed CD4$^+$ Jurkat cells using the immunoblotting assay with Abs that recognized phosphorylated BAD. It was revealed that the MEK inhibitor PD098059 decreased BAD phosphorylation at Ser^{112} and the PI3K inhibitor LY294002 the phosphorylation at Ser^{136} (Fig. 4C), suggesting that SDF-1 causes the phosphorylation of BAD at Ser^{112} or Ser^{136} via the MEK and PI3K pathways, respectively. We next overexpressed both Rsk2 and BAD in CXCR4$^+$ MAGI cells and examined whether BAD phosphorylation was enhanced upon SDF-1 exposure. With the overexpression of wild-type Rsk2 (Rsk2-WT), BAD phosphorylation at Ser^{112} was clearly enhanced (Fig. 4D), whereas when the dominant-negative Rsk2-KN was overexpressed such phosphorylation was suppressed. Moreover, to determine whether BAD is a substrate for Rsks and/or Akt, we employed an in vitro kinase assay using the cell lysates of SDF-1-exposed Jurkat cells. It was then shown that Rsk2, which was immunoprecipitated from the lysates of SDF-1-exposed cells, clearly phosphorylated BAD protein at Ser^{112} (Fig. 4E). When Jurkat cells were incubated concomitantly with SDF-1 and PD098059, Rsk2-induced phosphorylation of BAD at Ser^{112} was reduced, whereas Rsk2-induced BAD phosphorylation was not

suppressed by LY294002 treatment. We also determined the in vitro kinase activity of Akt1, which was immunoprecipitated from the cell lysates of SDF-1-exposed Jurkat cells. Immunoprecipitated Akt1 phosphorylated BAD at Ser^{136}; however, treatment of the cells with LY294002, not with PD098059, substantially reduced Akt1 kinase activity to phosphorylate BAD at Ser^{136}. Therefore, immunoprecipitated Rsk2 and Akt1, phosphorylated upon SDF-1 exposure, caused the phosphorylation of recombinant BAD protein, indicating that BAD protein served as a substrate for both kinases.

We further asked whether the phosphorylation of Rsks, Akt, and BAD occurred via the MEK and PI3K pathways in drCD4$^+$ T cells exposed to SDF-1. In immunoblotting assays, we found that PD098059 but not LY294002 decreased the phosphorylation of Rsk and Ser^{112} of BAD while LY294002 but not PD098059 suppressed the phosphorylation of Akt (Fig. 4F). These findings suggest that the activation of MEK-ERK-Rsk and PI3K-Akt causes BAD phosphorylation, leading to the promoted survival of SDF-1-exposed CD4$^+$ T cells.

Bonni et al. (17) reported that BDNF-mediated nerve cell protection was associated with the phosphorylation of CREB and an enhanced expression of Bcl-2. We therefore asked whether the expression level of the Bcl-2 family mRNA was altered upon SDF-1 exposure. As assessed by the RNase protection assay, no changes in any of detectable Bcl-2 family mRNA were observed in CD4$^+$ Jurkat T cells (Fig. 4G).

SDF-1 primes CD4$^+$ T cells for TCR-CD3-mediated stimulation

Finally, we examined whether anti-CD3-induced proliferation of drCD4$^+$ T cells was affected in the presence of SDF-1. When drCD4$^+$ T cells were stimulated with plate-bound anti-CD3 at 10, 20, and 40 μg/ml, CXCR4 down-regulation occurred in 52 $\pm$ 2, 62 $\pm$ 2, and 75 $\pm$ 3% of those cells, respectively, in agreement with reports by Jourdan et al. and others (30–32). Our kinetic studies of CXCR4 expression showed that the anti-CD3-induced CXCR4 down-regulation started as early as 6 h in culture and reached a maximum level at around 16 h. Once the anti-CD3-induced down-regulation of CXCR4 occurred, it generally took >7 days to fully regain CXCR4 on the cellular surface (data not shown).

Interestingly, when drCD4$^+$ T cells were pretreated with SDF-1 and then exposed to anti-CD3, a significant level of proliferation was observed (Fig. 5A). When drCD4$^+$ T cells were concomitantly stimulated with anti-CD3 and SDF-1, an enhanced cell proliferation was also observed. However, the anti-CD3 response was less than that of the cells pre-exposed to SDF-1 (Fig. 5A). The percentage of CD69$^+$ cells in SDF-1-pre-exposed, anti-CD3-exposed drCD4$^+$ T cells was greater than the percentage in concomitantly SDF-1/anti-CD3-exposed drCD4$^+$ T cells (~82 vs ~58%) as assessed in 16 h following the exposure, whereas the viability was comparable in the two drCD4$^+$ T cell populations, corroborating the data illustrated in Fig. 5A that the SDF-1-pre-exposed drCD4$^+$ T cells underwent a greater magnitude of activation. The observation that once exposed to SDF-1, the presence of SDF-1 in culture was not required for the enhanced activation and proliferation of drCD4$^+$ T cells suggests that SDF-1 primed CD4$^+$ T cells for stimuli incoming through the TCR-CD3 complexes rather than promoted their survival.

We also asked whether SDF-1 modified the effects of other immunologic stimuli. When drCD4$^+$ T cells were concomitantly exposed to SDF-1 and IL-4 or IL-6, no significant cell proliferation occurred regardless of the presence of SDF-1. However, when the cells were exposed to SDF-1 and IL-2 or IL-15, modest proliferation was elicited, whereas the cells that were pretreated with
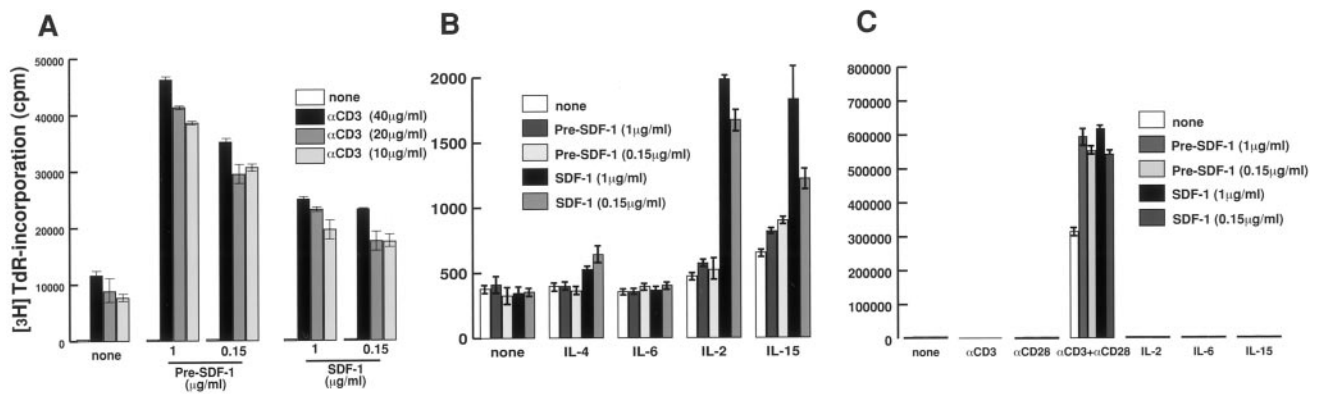
FIGURE 5. SDF-1 enhances cell proliferation triggered by TCR- or cytokine-mediated stimulation. *A* and *B*, Following SDF-1 pre-exposure for 12 h (pre-SDF-1) and thorough washing, drCD4$^+$ T cells were exposed to anti-CD3 (UCTH1: 40, 20, 10 μg/ml plate bound), IL-2 (1 ng/ml), IL-4 (0.5 ng/ml), IL-6 (1.5 ng/ml), or IL-15 (4 ng/ml), and the [^{3}H]thymidine incorporation by drCD4$^+$ T cells was examined in 72 h of culture. In addition, the same preparations of the cells were concomitantly exposed to SDF-1 and one of the cytokines. *C*, SDF-1 effects on Jurkat cells exposed to anti-CD3 and/or cytokines were determined. Jurkat cells were similarly treated as described above. In this assay, an anti-CD28 (LewTM-28: 20 μg/ml) Ab was also used. The data shown are representatives of three independent assays.

SDF-1 and subsequently exposed to IL-2 or IL-15 failed to proliferate (Fig. 5*B*). The combination of anti-CD3 and anti-CD28, however, enhanced the proliferation of Jurkat cells when they were exposed to SDF-1 (Fig. 5*C*). These data suggest that the mechanism of the cell proliferation enhancement with IL-2 or IL-15 along with SDF-1 is different from that of anti-CD3-mediated cell proliferation (Fig. 5, *A* and *B*).

Discussion

SDF-1 is a protein that belongs to the α-chemokine (C-X-C) family of cytokines and is a ligand for the chemokine receptor CXCR4, which is also known as a coreceptor for HIV-1 and is expressed largely in various B cell and CD4$^+$ T cell subsets (1). In addition to its role as a chemoattractant for T cells, B cells, monocytes, and neutrophils, SDF-1 is known to be essential for viability of the embryo and to play a critical role in bone marrow hemopoiesis, cardiogenesis, and B lymphopoiesis (4–6).

Well-defined studies by Nagasawa et al. and others (4–6) have shown that mice genetically deficient in SDF-1 or CXCR4 suffer profound defects in the hemopoietic and nervous systems. However, in such SDF-1-deficient mice, no alterations were identified in T cell development or in T cell trafficking to lymphoid organs (6, 7), leading to an assumption that SDF-1 may not have significant effects on T cells. It is possible, however, that the specific role of SDF-1 on T cells could not be examined since the embryo died so early in its development. Recently, Nanki and Lipsky (8) reported that SDF-1 serves as a costimulating factor with anti-CD3 for CD4$^+$ T cell activation, proliferation, and production of IL-2, IFN-γ, IL-4, and IL-10, although SDF-1 alone reportedly does not induce CD4$^+$ T cell activation or cytokine production. In this study, we used the complementary DNA microarray and examined whether certain genes were induced in CD4$^+$ T cells when they were exposed to SDF-1.

Following the exposure of CD4$^+$ T cells with SDF-1, significantly altered expression profiles of ~269 genes were seen (Fig. 1*A*). Among the genes that exhibited significant ratio changes upon SDF-1 exposure, three conspicuous clusters, whose ratios continued to increase throughout a 12-h culture following SDF-1 exposure, were identified (Fig. 1, *A* and *C*). These genes were those associated with DNA repair, apoptosis, detoxification, cell morphology, cell adhesion, and signal transduction (Fig. 2). The number of drCD4$^+$ T cells we can obtain from a single donor is limited and their use for the microarray assay could not be chosen since at

least 4 × 10^8 drCD4$^+$ T cells yielding ~25 μg of total mRNA were required. Therefore, in the present study we used Jurkat T cells for the microarray experiment for the assay. However, we found that SDF-1 exposure caused the phosphorylation of the proteins involved in the two major cascades, MEK- and PI3K-dependent pathways, in both Jurkat T cells and drCD4$^+$ T cells (Fig. 4, *A*, *C*, and *F*). Moreover, the phosphorylation of such proteins and the expression of the genes induced upon SDF-1 exposure were blocked by the MEK and/or PI3K inhibitors (Figs. 2 and 4), suggesting that the signals from CXCR4 receptors are transduced to the nucleus via the MEK and/or PI3K pathways. Considering that the activation of the same cascades were seen in SDF-1-exposed Jurkat T cells and drCD4$^+$ T cells (Fig. 4, *A* and *C*), it seems that the gene induction seen in SDF-1-exposed Jurkat T cells is likely to occur in drCD4$^+$ T cells as well. Indeed, using Multiple-Probe RNase Protection kits with SDF-1-treated drCD4$^+$ T cells, we found that SDF-1 exposure also induced DAD-1 mRNA expression in drCD4$^+$ T cells (data not shown). Thus, it appears that certain antiapoptotic genes are induced in normal CD4$^+$ T cells by SDF-1 exposure.

Although no significant effects of SDF-1 on CD4$^+$ T cells have been reported (6, 7), as described above, the involvement of a large number of genes linking to CD4$^+$ T cell functions was seen upon SDF-1 exposure, which was rather unexpected. With this respect, Onai et al. (33) recently reported an impaired T cell maturation in the thymus of SDF-1-transduced and SDF-1-intrakine-transduced mice. They hypothesized that SDF-1 is associated with the migration of T cell progenitors to thymus and the ensuing intrathymic selection of T cells. Our data show that SDF-1 also plays a significant role in T cell functions. We found that 1) activation of MEK-ERK-Rsk and PI3K-Akt causes BAD phosphorylation, leading to inactivation of its proapoptotic function and that 2) the expression of cell survival-related genes was associated with SDF-1-activated-MAPK-MEK and PI3K signaling (Figs. 2, 3, *Ab* and *C*, and 4), suggesting that SDF-1-induced survival promotion occurred through at least two mechanisms: posttranslational inactivation of the cell death machinery and an increased transcription of cell survival-related genes. With this respect, Kaul and Lipton (10) reported that SDF-1 induced apoptosis in neuronal cells through the p38 MAPK signaling pathway. However, we detected no phosphorylation of p38 MAPK protein in SDF-1-stimulated Jurkat T cells (Y. Suzuki, unpublished data). It is possible that different

signal transduction pathways used in those different types of cells have brought about the different outcomes in response to SDF-1.

We also observed that the anti-CD3-stimulated proliferation of CD4$^+$ T cells was greatly enhanced when the cells had been pre-exposed to SDF-1 for 12 h and thoroughly washed to remove SDF-1 from culture (Fig. 5A). We thought that SDF-1 induced signal transduction molecules and preset CD4$^+$ T cells to readily and effectively transduce the incoming TCR/CD3 stimuli to the nucleus. Unexpectedly, the magnitude of CD4$^+$ T cell proliferation was substantially reduced when the cells were concomitantly exposed to anti-CD3 and SDF-1 (Fig. 5A). We presume that with the anti-CD3 stimulation, the down-regulation of CXCR4 occurred and SDF-1 binding to CD4$^+$ T cells was substantially prevented throughout the 3-day culture, although SDF-1 could bind to CD4$^+$ T cells to some extent at the beginning of the culture. Indeed, a profound down-regulation of CXCR4 occurred as early as 6 h after anti-CD3 stimulation and once the anti-CD3-induced down-regulation of CXCR4 occurred, it generally took >7 days to fully recover CXCR4 in drCD4$^+$ T cells. It should be noted that anti-CD3 stimulation does not induce CXCR4 down-regulation in Jurkat CD4$^+$ T cells and this may explain why the magnitude of proliferation of Jurkat T cells stimulated with anti-CD3 plus anti-CD28 was comparable regardless of SDF-1 pretreatment or concomitant exposure (Fig. 5C). We thus conclude that SDF-1 exerts priming functions on CD4$^+$ T cells for stimuli incoming through the TCR-CD3 complexes but does not serve as a costimulation factor to anti-CD3 stimulation. There is a possibility that upon SDF-1 pre-exposure, a certain population of drCD4$^+$ T cells remained more robust than others and were ready to receive activation stimuli because of the survival-promoting property of SDF-1. Regarding the viability of drCD4$^+$ T cells, the experiment illustrated in Fig. 5A was conducted in the presence of FCS; therefore, as expected virtually all drCD4$^+$ T cells (>90%) were viable in any of those drCD4$^+$ T cell populations in the initial 24 h of culture. Furthermore, the percentage of CD69$^+$ cells in SDF-1-pre-exposed and anti-CD3-exposed drCD4$^+$ T cells was greater than the percentage in concomitantly SDF-1/anti-CD3-exposed drCD4$^+$ T cells, whereas the viability was comparable in the two drCD4$^+$ T cell populations, corroborating the data illustrated in Fig. 5A that the SDF-1-pre-exposed drCD4$^+$ T cells underwent a greater magnitude of activation. Thus, it seems likely that the priming effect of SDF-1 rather than its survival-promoting property was involved.

The data we generated in this study in SDF-1-exposed CD4$^+$ T cells led us to postulate that SDF-1, beyond its known functions as a chemoattractant, plays a critical role in the CD4$^+$ T cell-mediated immune response. SDF-1 is known to be produced in a variety of tissues including lymphoid organs, liver, lung, and mesenchymal cells surrounding endothelial cells (5). Thus, one can assume that CD4$^+$ T cells constantly receive SDF-1 signal and gain their survival and are primed at a low, basic level. Indeed, a single i.v. infusion of a CXCR4 antagonist AMD3100 reportedly caused dose-related elevation of white blood cell counts (polymorphonuclear leukocytes, lymphocytes, and monocytes) within 2 h, suggesting that circulating blood cells constantly receive signal(s) from CXCR4 and lodge onto endothelia cells (34). However, when CXCR4 receptors were blocked by AMD3100, a "demargination" effect occurred in which white blood cells including lymphocytes were released from attachment to the endothelial cell surface into circulation. Thus, it appears that SDF-1 undertakes the housekeeping or maintenance role for lymphocytes. On the other hand, once inflammation occurs, various cells, attracted by chemoattractants, migrate into regions where immunologic and other related stimuli are imposed, and generate oxygen radicals, NO, and numerous tissue-destructive factors. In such an environment, SDF-1-medi-

ated cell survival promotion, DNA damage repair, and priming function would become critical for the effective defense. It is also worth noting that with regard to the pathogenesis of HIV-1 infection, when HIV-1 or its envelope glycoprotein gp120 binds to CXCR4 receptors on CD4$^+$ T cells and blocks the binding of SDF-1 to CXCR4 and/or down-regulates CXCR4 expression, a decrease or cancellation of the effect of SDF-1 to maintain and protect CD4$^+$ T cells ensues, thus contributing to the premature death of CD4$^+$ T cells.

Acknowledgments

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