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Allograft Rejection Requires STAT5a/b-Regulated Antiapoptotic Activity in T Cells but Not B Cells[1]

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STATs play key roles in immune function. We examined the role of STAT5a/b in allograft rejection. STAT5a/b-deficient mice showed a 4-fold increased survival time of heart allografts ($p < 0.01$). Unlike wild type, purified STAT5a/b$^{-/-}$ T cells transferred to Rag1$^{-/-}$ recipients failed to mediate heart allograft rejection until supplemented with STAT5a/b$^{-/-}$ B cells. In vitro, STAT5a/b$^{-/-}$ T cells did not proliferate in response to Con A or alloantigens but entered apoptosis within 48 h (95%). Activated STAT5a/b$^{-/-}$ T cells showed increased expression of proapoptotic (caspases, DNA repair genes, TNF/TNFR-associated factor family genes) and decreased antiapoptotic mRNAs in microarrays, while Western blots confirmed reduced antiapoptotic Bcl-2 and elevated proapoptotic Bax protein expression. Interestingly, at 24 h postactivation, STAT5a/b$^{+/+}$ and STAT5a/b$^{-/-}$ T cells produced similar levels of IL-2, IL-4, IL-10, and IFN-γ mRNA; ELISPOT assay showed an equivalent number of IL-4- and IFN-γ-producing T cells in both STAT5a/b$^{+/+}$ and STAT5a/b$^{-/-}$ splenic populations. Sera from STAT5a/b$^{+/+}$ and STAT5a/b$^{-/-}$ rejectors had donor-specific IgM, IgG1, IgG2a, and IgG2b Ab, while STAT5a/b deficiency had no impact on B cell survival or proliferation in response to LPS. Compared with allografts from STAT5a/b$^{+/+}$ recipients, heart allografts from STAT5a/b$^{-/-}$ recipients had markedly reduced infiltration by CD4 and CD8 T cells but increased infiltration by B cells and dense endothelial deposition of C4d, a marker of humoral rejection. Thus, activated STAT5a/b$^{-/-}$ T cells produce cytokines prior to entering apoptosis, thereby promoting differentiation of B cells yielding donor-specific IgM and IgG Ab that mediate allograft rejection. *The Journal of Immunology,* **2006, 176: 128–137.**

Activation of T cells is initiated by a sequential cascade of molecular events triggered after the engagement of the TCR/CD3 by specific Ags (signal 1). TCR-driven signals amplified by costimulatory molecules B7-1/CD28 and CD40/CD154 (signal 2) induce the synthesis of multiple cytokines including IL-2 (1). Finally, cytokine/cytokine receptor-driven signals instigate proliferation and differentiation of T cells (signal 3) (2, 3). For example, IL-2 binding to the affinity-conferring IL-2Rα heterotrimerizes with two additional receptor chains, IL-2Rβ and the common γ-chain (γ_c),[3] and recruits the corresponding receptor-associated JAK1 and JAK3, respectively (4, 5). Next, JAK3 catalyzes tyrosine phosphorylation of the IL-2Rβ chain, allowing recruitment and activation of other signaling elements, including STAT5a and STAT5b (6). After docking through Src homology 2 domains to selected phosphotyrosines, STAT5a/b are tyrosine phosphorylated by JAK or *src* kinases on defined residues, initiating dissociation from receptors of STAT5a/b (7, 8), their homodimerization or heterodimerization, and migration of such duplexes to the nucleus, where they bind to the promoter sites on multiple genes that control cell growth and differentiation (9, 10).

STAT transcription factors have well-established roles in immune functions (10). Mice deficient in STAT1 were highly sensitive to viral and bacterial infections due to lack of responsiveness to IFN-γ and IFN-α (11, 12), whereas mice void of STAT4 or STAT6 displayed a loss of Th1 and Th2 cell function, respectively (13, 14). However, deficiency of STAT4 or STAT6 had no impact on the tempo of allograft rejection (15). Highly homologous STAT5a, originally identified as a prolactin-responsive mammary gland factor, and STAT5b proteins (16, 17) are activated in response to multiple cytokines, including IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-13, IL-15, IL-21, thrombopoietin, erythropoietin, growth hormone, and GM-CSF (18). Despite 96% homology, STAT5a and STAT5b play distinct roles during development: lack of STAT5a alone in female mice affected development of lobuloalveolar region and reduced milk production in response to prolactin (19); STAT5b knockout alone caused retarded growth, as in the Laron dwarfism syndrome (20). Although lack of STAT5a or STAT5b has no impact on the immune response, double STAT5a/b deficiency caused impaired proliferation in response to IL-2 and halted cell cycle progression of mature T cells (18). STAT5a/b-deficient mice also have reduced levels of cyclins A, D2, D3, E, and Cdk6 proteins, supporting the notion that STAT5a/b promotes cell cycle progression (21, 22). Prosurvival genes also are regulated by STAT5a/b, including c-*myc*, *bcl-x*,

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[3] Abbreviations used in this paper: γ_c, common γ-chain; CSR, class switch recombination; antisense ODN, antisense phosphothioate-2′-*O*′-methoxyethyloligonucleotide; RT, room temperature; Stam, signal-transducing adaptor molecule; Treg, regulatory T.

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bcl-2 (23), and *pim-1* (24). When expression of STAT5a/b proteins was inhibited by antisense phosphothioate-2′-*O*-methoxyethyl-oligodeoxynucleotides (antisense ODN), the majority of activated T cells entered apoptosis (25). Thus, STAT5a/b are both required in response to IL-2 and other cytokines in T cells acting predominantly as antiapoptotic survival transcription factors, and perhaps only secondarily participate in the regulation of cell cycle progression.

Despite the fact that several publications reported the importance of STAT5a/b in T cell functions (9, 10), their role in allograft rejection is not clear (26). Our present experiments explored the question about function of STAT5a/b in T and B cells during allograft rejection. Activated STAT5a/b-deficient T cells produced cytokines, but failed to proliferate and instead readily entered apoptosis. This apoptotic activity correlated with the decreased expression of antiapoptotic and increased expression of proapoptotic genes. STAT5a/b deficiency had no impact on B cell functions, with STAT5a/b knockout recipients producing donor-specific IgM and IgG Ab that mediated allograft rejection. These results suggest that targeting STAT5a/b may uncouple T cells, but not B cells, serving as a target molecule to regulate allograft rejection.

Materials and Methods

Animals

Mixed background BALB/c/C57BL/6 STAT5a/b$^{-/-}$ mice generously provided by J. Ihle (St. Jude Children's Research Hospital, Memphis, TN) as well as BALB/c STAT4$^{-/-}$, STAT6$^{-/-}$, and STAT4/6$^{-/-}$ mice obtained from The Jackson Laboratory were bred in our facility. BALB/c, C57BL/6, and C3H mice (4–6 wk old) were purchased from Harlan Sprague Dawley. All mice were kept in microisolators with free access to sterilized food and water.

Heart transplantation

Heart transplantation was performed by a modified method (27). In brief, hearts harvested from donors were perfused with chilled saline and immediately transplanted to recipients under full anesthesia. Suture 12-0 (Ethicon) was used to perform anastomosis of aorta to aorta and pulmonary artery to vena cava. Heartbeat was evaluated daily, and the day of complete cessation was considered rejection. Some recipients were sensitized by skin transplantation performed on their back with patches of donor abdominal skin sutured with 7-0 sutures; rejection was considered with 50% damage.

Cell culture

Purity of T and B cells (99%), obtained by negative isolation method on magnetic beads coated with anti-CD5 or anti-Thy-1/2 Ab, respectively (Dynal Biotech), was confirmed by FACS with specific Abs (BD Biosciences). T cells were stimulated with 5 μg/ml Con A (Sigma-Aldrich), and B cells stimulated with 5 μg/ml LPS (Sigma-Aldrich) were cultured for 48 h in RPMI 1640 supplemented with 10% FCS (Invitrogen Life Technologies), 2 mM L-glutamine (50 IU/ml), and penicillin-streptomycin (50 mg/ml. Proliferation was measured by pulsing cultures for the last 6 h with 0.5 μCi/well [^{3}H]thymidine. Harvested and dried on nitrocellulose paper, samples were counted for activity in a beta counter, and the results were expressed in counts per minute.

Adoptive transfer experiments

Pure T cells were isolated from STAT5a/b$^{+/+}$ and STAT5a/b$^{-/-}$ splenocytes in two steps. In the first step, mouse T cell-negative isolation kit was used with a mixture of rat anti-mouse mAb to deplete CD45$^+$ (B220), CD11b$^+$ (Mac1), Ter-199$^+$, and CD16$^+$/32$^+$ cells by magnetic beads coated with anti-rat IgG Ab (Dynal Biotech). In the second step, purified T cell population was coated with rat anti-mouse IgM/IgG-FITC Ab (BD Pharmingen) and selected by flow cytometry (BD Biosciences), achieving a purity of 99.9%. Such pure T cells were injected i.v. into C57BL/6$^{Rag1-/-}$ recipients of C3H heart allografts. Pure B cells were purified in one step using Dynal mouse B cell-negative isolation kit to deplete CD43$^+$ (Ly-48), Ter-119$^+$, and CD4$^+$ cells with a purity of 95%. In some experiments, a mixture of T (70%) and B cells (30%) was injected i.v. into C57BL/6$^{Rag1-/-}$ recipients of C3H heart allografts.

Detection of IgM and IgG

As previously described, sera were analyzed for the presence of donor-specific binding alloantibodies (28). Diluted sera (1/2, 1/8, and 1/32) with HBSS and 0.5% BSA (Sigma-Aldrich) were added in equal volume to donor or syngeneic 3×10^3 lymph node cells, achieving a final dilution of 1/4, 1/16, or 1/64. Following incubation (45 min at 4°C), cells were washed thrice; stained with fluorescinated rat anti-mouse IgM (5 μg/ml), IgG1 (10 μg/ml), IgG2a (10 μg/ml), IgG2b (5 μg/m), or IgG3 (5 μg/ml) Abs (50 μL; BioSource International); and incubated for 20 min at 4°C. After washing three times, cells were fixed with 400 μl of 1% paraformaldehyde, and the intensity of staining was evaluated by FACS (BD Biosciences). Results are expressed as the mean channel shift with SD. Statistical significance was calculated by T-Test from triplicate experiments.

TUNEL assay

STAT5a/b$^{+/+}$ and STAT5a/b$^{-/-}$ spleen cells were Con A activated for 48 h and then washed with PBS and fixed in prechilled 1% formaldehyde/PBS at 4°C for 20 min. After washing twice with PBS, cells were permeabilized by 70% ice-cold ethanol and then stained for fragmented DNA with TdT in the presence of FITC-dUTP label (BD Clontech) at 37°C for 60 min and intact DNA with 0.5 μg/ml propidium iodide/RNase in PBS at room temperature for another 20 min. The percentage of apoptotic cells was analyzed by flow cytometry based on green fluorescence at 520 ± 20 nm.

Western blot analysis

Pure T or B cells from STAT5a/b$^{+/+}$ or STAT5a/b$^{-/-}$ mice were stimulated with Con A (5 μg/ml) or LPS (5 μg/ml), respectively. Cells harvested at different time points were resuspended in 100 μl of lysing buffer with proteinase inhibitor (Roche), and protein concentration was measured for each sample. Equivalent protein (10 μg) samples were separated by SDS-PAGE gel electrophoresis (Bio-Rad), transferred to Immobilon-P membrane (Millipore), and blotted with rabbit anti-mouse Bcl-2 (1:500; Santa Cruz Biotechnology), rabbit anti-mouse Bax (1/500; BD Pharmingen), or mouse anti-mouse β-actin (1/5000; Sigma-Aldrich) Abs. Donkey anti-rabbit or sheep anti-mouse Abs (1/1000; Amersham Biosciences) coupled to HRP were used to visualize bands by an ECL kit (Amersham Biosciences) and x-ray film (Kodak).

Measurements of cytokine mRNAs

Total RNA isolated by RNA-Bee kit (Tel-Test) and treated with DNase was used for standard procedure (SuperScript II RNase H Reverse Transcriptase; Invitrogen Life Technologies) (29). Briefly, the mixture of 500 ng of oligo(dT), 3 μg of total RNA, and dNTP was heated (65°C for 5 min) and quickly chilled on ice. After adding reverse-transcriptase buffer, DTT RNAsin, and SuperScript reverse transcriptase, the mixture was incubated for 50 min at 42°C, followed by 15 min at 70°C. Next, 5 μl of cDNA was used for RT-PCR (BioSource International), according to the manufacturer's instruction. The cDNA samples were mixed with PCR buffer and specific primers (a mixture of primers for detection of IL-2, IL-4, IL-10, IFN-γ, and GAPDH). The PCR was hot started with TaqDNA polymerase (Invitrogen Life Technologies) in a GeneAmp PCR System 2700 (Applied Biosystems) with the programmed time-temperature profile, as follows: an initial denaturing step at 94°C for 4 min, 2 cycles at 96°C for 1 min and at 57°C for 4 min, followed by 35 cycles at 94°C for 1 min and at 57°C for 2.5 min. At the final step, samples were incubated at 70°C for 10 min and maintained at 25°C. The PCR products were detected on agarose gel electrophoresis by ethidium bromide staining. The final gel was examined with a UV transilluminator.

Microarray analysis

Total RNA was isolated by the standard TRIzol method (Invitrogen Life Technologies), and content was evaluated by absorbance readings (Bio-Rad). The GEArray gene expression array (SuperArray Bioscience) was used to examine 96 apoptosis-related genes, according to manufacturer's recommendations. After the membrane was exposed to x-ray film, the results were analyzed using a SuperArray analysis program.

ELISPOT assay

The ELISPOT assay was performed using the previously described method (30). A single cell suspension was prepared from spleens of normal or experimental mice. ELISPOT plates (Mabtech) were coated by overnight incubation (4°C with 1 μg/well) with anti-mouse IL-4 mAb or anti-mouse IFN-γ mAb (Mabtech). After five washings with PBS, plates were incubated for 30 min in 200 μl/well blocking medium containing 10% FCS at

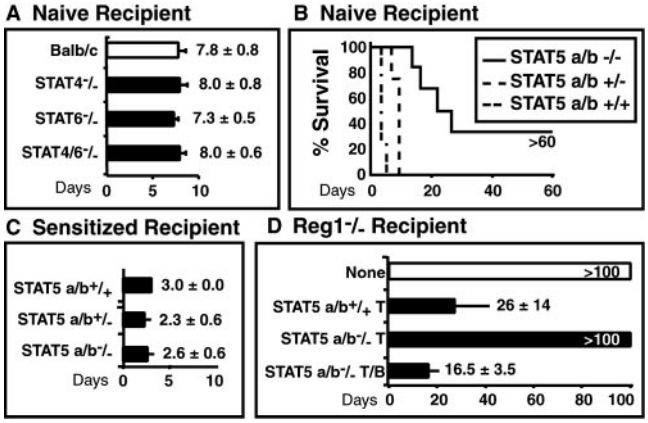

FIGURE 1. Impact of STAT5a/b or STAT4/6 deficiency on survival of primary and secondary heart allografts. *A*, Normal as well as STAT4$^{-/-}$, STAT6$^{-/-}$, and STAT4/6$^{-/-}$ mice (all with BALB/c background) were transplanted with C57BL/6 heart allografts (n = 4–5). *B*, STAT5a/b$^{-/-}$, STAT5a/b$^{+/-}$, and STAT5a/b$^{+/+}$ mice (all with a mixed BALB/c/C57BL/6 background) were transplanted with C3H heart allografts (n = 5–7). *C*, STAT5a/b$^{-/-}$, STAT5a/b$^{+/-}$, and STAT5a/b$^{+/+}$ mice that rejected C3H skin allografts were transplanted with C3H heart allografts. *D*, C57BL/6$^{Rag-/-}$ recipients of C3H heart allografts received none or 5–10 × 10^6 purified T cells from naive STAT5a/b$^{+/+}$ or STAT5a/b$^{-/-}$ mice (n = 3–4) or a mixture of 10–20 × 10^6 purified T (70%) and B (30%) cells from naive STAT5a/b$^{-/-}$ mice (n = 2). Heart function was evaluated daily, and the day that the heart ceased beating was considered the day of rejection. Skin allografts were placed on same recipients for sensitization and were evaluated daily; the rejection was considered when destruction of at least 50% skin graft occurred (9.8 ± 0.8 days).

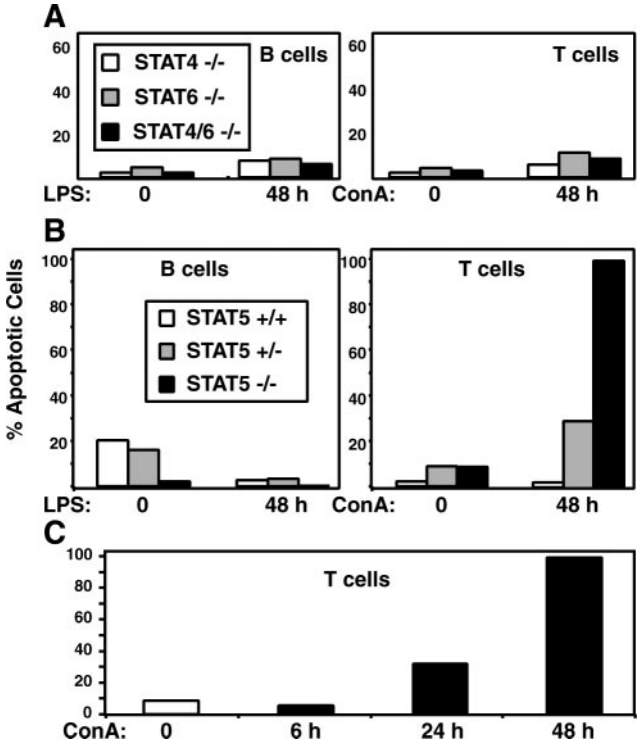

FIGURE 3. STAT5a/b deficiency induces apoptosis in T, but not B cells. Purified B or T cells were harvested from spleens of STAT4$^{-/-}$, STAT6$^{-/-}$, or STAT4/6$^{-/-}$ mice (*A*), as well as STAT5a/b$^{+/+}$, STAT5a/b$^{+/-}$, and STAT5a/b$^{-/-}$ mice (*B*). Purified T cells were harvested from spleens of STAT5a/b$^{-/-}$ mice (*C*). Purified T cells stimulated with Con A (5 μg/ml) and purified B cells with LPS (5 μg/ml) for 0 or 48 h were evaluated for apoptosis by TUNEL assay (*A* and *B*). Purified T cells stimulated with Con A for 0, 6, 24, or 48 h were evaluated for apoptosis by TUNEL assay (*C*). Results represent four independent experiments with similar outcomes. For additional details, see *Materials and Methods*.

room temperature, and cells (2 × 10^5/well) were added with Con A (5 μg/ml). Plates were then incubated for 24 h after Con A stimulation (37°C in 5% CO_2) in complete RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 200 μg/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine, 5 × 10^{-5} M 2-ME, 10 mM HEPES, and 10% FCS (HyClone). After culture, plates were washed five times in PBS (200 μl, 4°C), and appropriate biotinylated Abs (1 μg/well) were added to each well (RT/2 h), namely, anti-mouse IL-4 mAb or anti-mouse IFN-γ mAb (Mabtech). Following the next five washings, 100 μl of streptavidin-HRP (1:1000) was added to each well (RT/1 h). After an additional five washings (200 μl/well), 100 μL of substrate solution (5-bromo-4-chloro-3-indolyl phosphate/NBT; Mabtech) was added, and the spots were developed for ~5 min; plates were washed under tap water, dried, and read using an ELISPOT reader (Zellnet Consulting). The average ± SD for each group was calculated from triplicate wells; the frequency was normalized based on 10^5 total cells.

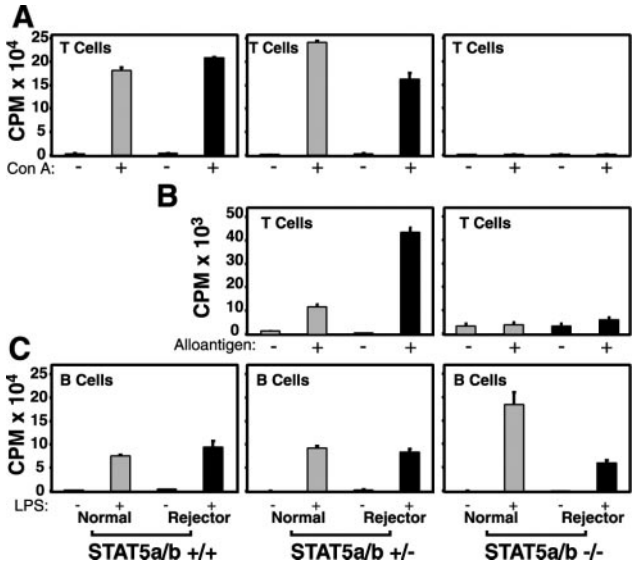

FIGURE 2. STAT5a/b deficiency prevents proliferation of T, but not B cells. Spleen cells from STAT5a/b$^{+/+}$, STAT5a/b$^{+/-}$, or STAT5a/b$^{-/-}$ naive mice (normal) or recipients of heart allografts (rejector) were stimulated for 48 h with Con A (5 μg/ml; *A*); spleen cells from STAT5a/b$^{+/-}$ and STAT5a/b$^{-/-}$ naive or sensitized mice (rejector) were stimulated with irradiated (2000 rad) allogenic spleen cells for 72 h (*B*); spleen cells from STAT5a/b$^{+/+}$, STAT5a/b$^{+/-}$, or STAT5a/b$^{-/-}$ naive mice or recipients of heart allografts (rejector) were stimulated with LPS for 48 h (5 μg/ml; *C*). Proliferation was evaluated by [^{3}H]thymidine incorporation over the final 6 h. The experiments were repeated four times with nearly identical results.

Immunopathology

Heart allografts were collected for immunohistologic evaluation at day 7 posttransplant from STAT5a/b$^{-/-}$, STAT5a/b$^{+/-}$, and STAT5a/b$^{+/+}$ recipients. Samples (two to four per group) were snap frozen, and cryostat sections were stained using mAbs to murine CD4, CD8, and CD22, or isotype-matched control mAbs (BD Pharmingen). C4d deposition was assessed using a rat anti-mouse C4 mAb (Novus Biologicals), which recognizes C4, C4b, and C4d complement components. Primary Abs, plus control mAbs and rabbit IgG, were localized using an Envision kit (DakoCytomation) (31).

Results

Impact of STAT5a/b deficiency on allograft rejection

Previously published work documented that STAT4$^{-/-}$ and STAT6$^{-/-}$ mice rejected heart allografts in an identical tempo as normal controls (15). In this study, we explored the impact of

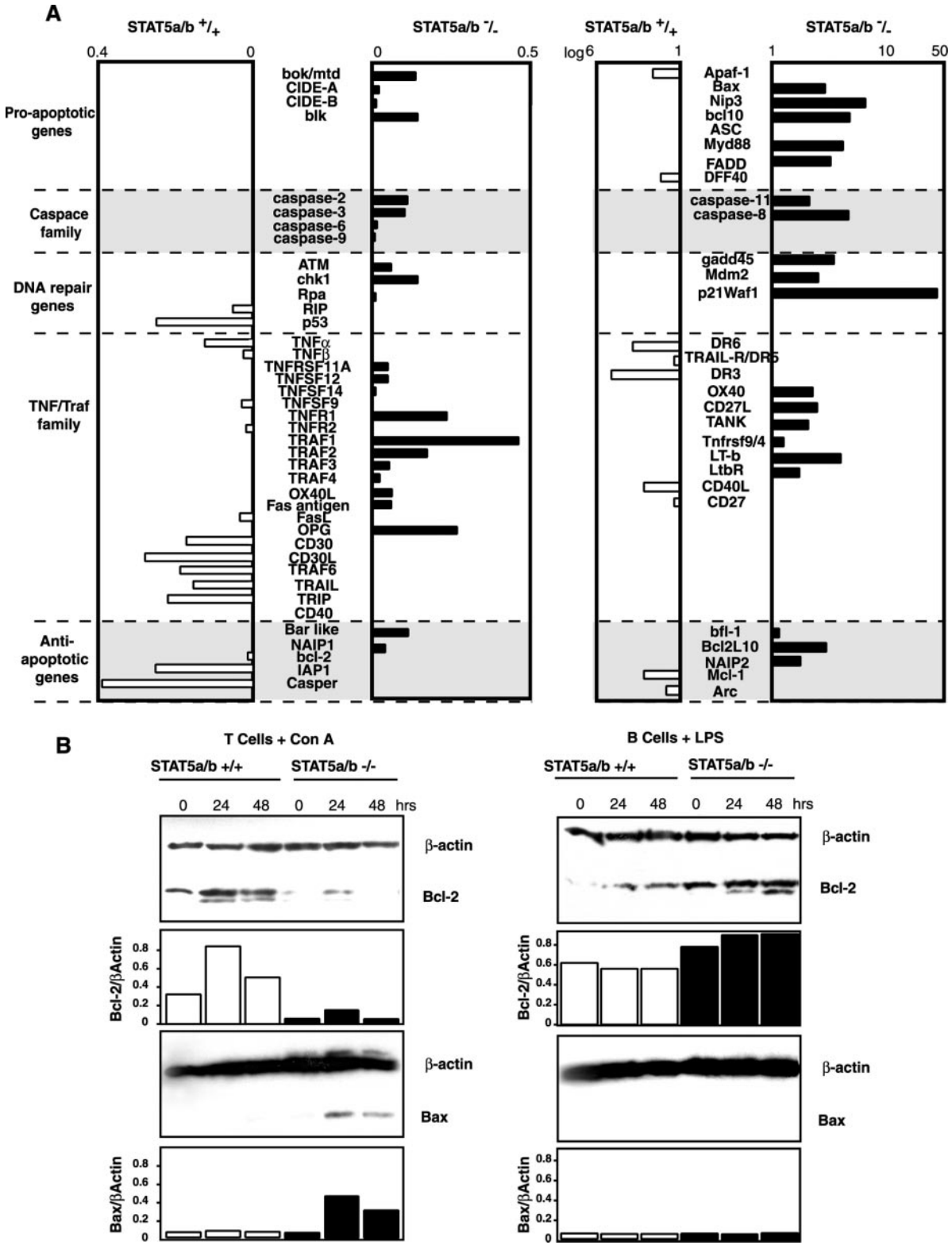

FIGURE 4. STAT5a/b transcription factors regulate anti- and proapoptotic activity in T cells. *A*, Purified STAT5a/b$^{+/+}$ or STAT5a/b$^{-/-}$ T cell populations were stimulated with Con A for 36 h and examined for mRNA expression of 96 different apoptosis-related genes using microarray (SuperArray). *B*, Purified STAT5a/b$^{+/+}$ or STAT5a/b$^{-/-}$ T cells were stimulated with Con A for 0, 24, or 48 h (*left panel*), or purified STAT5a/b$^{+/+}$ or STAT5a/b$^{-/-}$ B cells were stimulated with LPS for 0, 24, or 48 h (*right panel*). Expression of Bcl-2, Bax, and β-actin proteins was measured by Western blot method; densitometry analysis for each sample between Bcl-2 or Bax and β-action is presented.

double STAT4/6 deficiency on the tempo of heart allograft rejection. Normal as well as STAT4$^{-/-}$, STAT6$^{-/-}$, and STAT4/6$^{-/-}$ BALB/c recipients acutely rejected C57BL/6 heart allografts (Fig. 1*A*). A different picture emerged when we explored the impact of double deficiency of STAT5a/b on rejection of heart allografts. Because STAT5a and STAT5b exhibit 96% homology (18), we have examined their role in allograft rejection using double-knockout mice with a mixed BALB/c and C57BL/6 background (32).

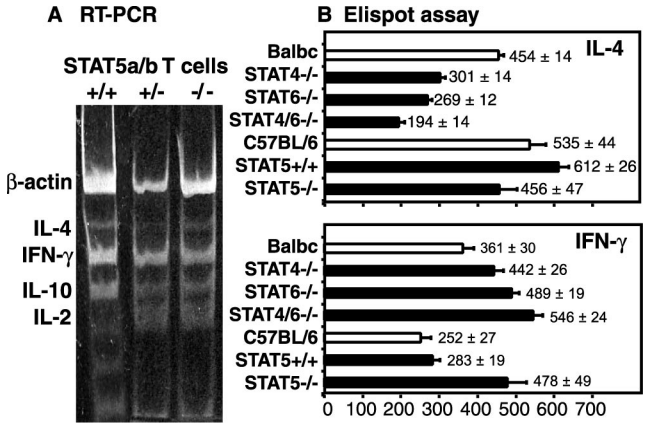

FIGURE 5. Activated STAT5a/b-deficient T cells produce significant amounts of cytokines. *A*, Purified STAT5a/b$^{-/-}$, STAT5a/b$^{+/-}$, and STAT5a/b$^{+/+}$ T cells stimulated with Con A for 24 h were examined for expression of IL-2, IFN-γ, IL-4, and IL-10 mRNAs by RT-PCR method with specific primers ($n = 3$). *B*, Purified T cells from STAT4$^{-/-}$, STAT6$^{-/-}$, STAT4/6$^{-/-}$, STAT5a/b$^{-/-}$, STAT5a/b$^{+/-}$, and STAT5a/b$^{+/+}$ mice were stimulated for 24 h with Con A and examined for the numbers of IL-4- and IFN-γ-producing clones by ELISPOT assay ($n = 4$). For additional details, see *Materials and Methods*.

Naive STAT5a/b$^{+/+}$ (7.3 ± 0.5 days; $n = 4$) as well as STAT5a/b$^{+/-}$ (9.6 ± 0.6 days; $n = 4$) recipients acutely rejected C3H heart allografts. In contrast, STAT5a/b$^{-/-}$ mice showed significantly extended survivals of heart allografts (32.6 ± 21.5 days; $n = 6$; $p < 0.01$; Fig. 1*B*), with two of six recipients displaying significant function loss by day 25, but remained beating when sacrificed at day 60. However, sensitized recipients, which previously had rejected skin allografts, subsequently destroyed heart allografts in accelerated fashion within 2–3 days independent of STAT5a/b expression (Fig. 1*C*). Thus, double STAT5a/b deficiency delays heart allograft rejection by naive, but not sensitized, recipients.

To directly examine the role of STAT5a/b in allograft rejection, C57BL/6$^{Rag1-/-}$ recipients (lacking both T and B cells) of C3H heart allografts were transferred with doubly purified T cells from naive STAT5$^{+/+}$ and STAT5$^{-/-}$ mice (Fig. 1*D*). Although C57BL/6$^{Rag1-/-}$ recipients maintained heart allografts for >100 days, adoptive transfer of purified STAT5a/b$^{+/+}$ T cells (5–10 × 10^6) induced acute heart allograft rejection (26 ± 14 days; $n = 4$). In contrast, the same number of purified STAT5a/b$^{-/-}$ T cells failed to mediate rejection of heart allografts (>100 days; $n = 6$). When purified T cells were transferred as a mixture of 70% T and 30% B cells (10–20 × 10^6), they fully restored the ability of C57BL/6$^{Rag1-/-}$ recipients to mediate heart allograft rejection (Fig. 1*D*). Thus, STAT5a/b transcription factors are necessary for in vivo T cell- but not T/B cell-mediated allograft destruction.

STAT5a/b is required for proliferation and survival of T cells but not B cells

To explain the mechanism of delayed rejection by STAT5a/b$^{-/-}$ mice, we examined the functions of purified T and B cell populations. In vitro, T cells from STAT5a/b$^{-/-}$, but not from STAT5a/b$^{+/+}$ or STAT5a/b$^{+/-}$ mice failed to proliferate in response to Con A (Fig. 2*A*) or alloantigens (Fig. 2*B*). In contrast, B cells from normal and STAT5a/b-deficient mice proliferated in a similar fashion after LPS stimulation (Fig. 2*C*). There was no impact on T or B cell proliferative responses among STAT4-, STAT6-, and STAT4/6-deficient mice (data not shown). Next, we examined the impact of activation on apoptosis in STAT5a/b-deficient lymphocytes. As shown by TUNEL analysis, activated STAT4$^{-/-}$,

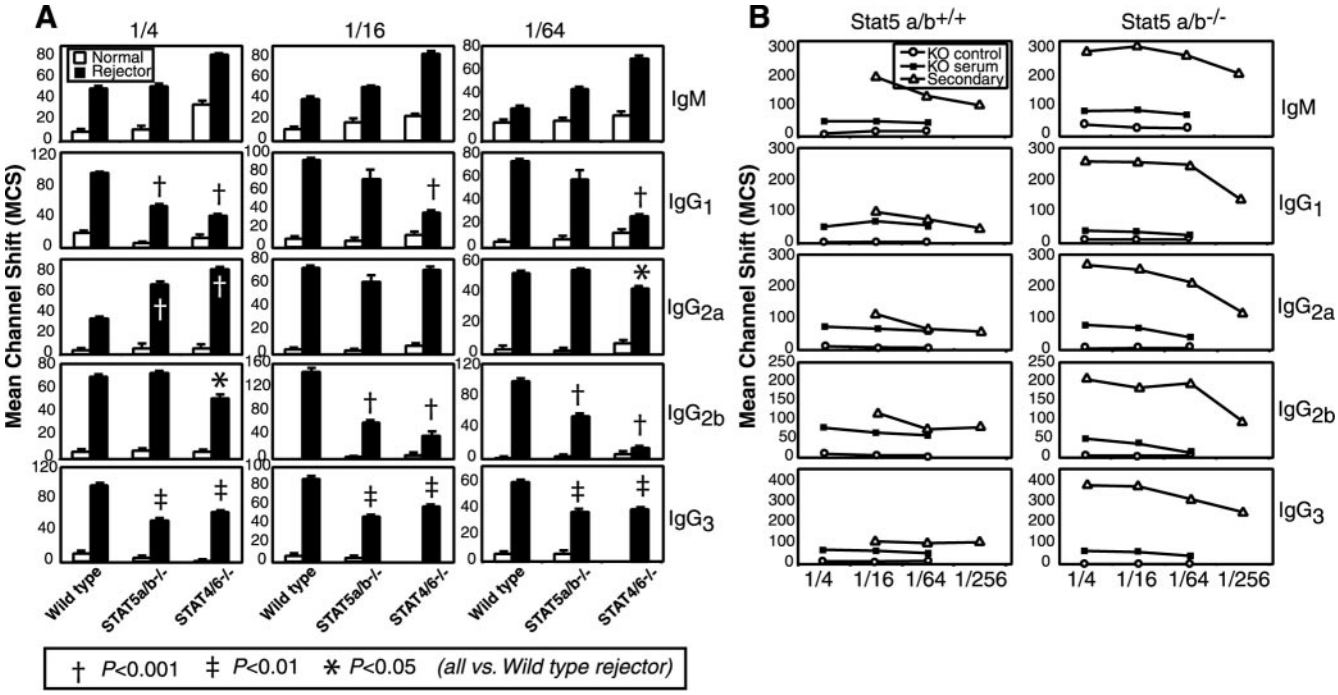

FIGURE 6. STAT5a/b-deficient recipients produce significant amounts of donor-specific IgM or IgG Abs. *A*, Sera from STAT5a/b$^{+/+}$, STAT5a/b$^{-/-}$ recipients that had rejected C3H heart allografts (days 15–30) as well as STAT4/6$^{-/-}$ recipients that had rejected C57BL/6 heart allografts (days 15–20) were examined for the presence of donor-specific IgM, IgG1, IgG2a, IgG2b, and IgG3 alloantibodies. Results are presented as mean channel shift (MCS) ± SD from triple experiments; *p* values were calculated by *t* test. *B*, Sera from STAT5a/b$^{+/+}$ (*left panel*) and STAT5a/b$^{-/-}$ (*right panel*) recipients that had rejected primary or secondary C3H heart allografts were examined for the presence of donor-specific IgM, IgG1, IgG2a, IgG2b, and IgG3 alloantibodies. FACS analysis was used to determine binding to donor lymph node cells. One of four independent experiments is shown.

STAT6$^{-/-}$, or STAT4/6$^{-/-}$ B and T cells showed minimal apoptosis (Fig. 3*A*). Indeed, while LPS-activated STAT5a/b$^{-/-}$ B cells had no signs of apoptosis, Con A-activated STAT5a/b$^{-/-}$ T cells displayed 30 and 95% TUNEL-positive cells at 24 and 48 h, respectively (Fig. 3, *B* and *C*). Thus, STAT5a/b is most likely fundamental for the expansion and survival of activated mature T, but not B cells.

STAT5a/b regulate expression of apoptotic genes in T cells

To determine which genes regulate this apoptotic event, purified STAT5a/b$^{+/+}$ or STAT5a/b$^{-/-}$ T cells stimulated with Con A for 36 h were examined for mRNA expression of 96 different apoptosis-related genes (Fig. 4*A*). Multiple mRNAs of proapoptotic genes, caspases, and DNA repair genes were inducibly up-regulated almost exclusively in activated STAT5a/b$^{-/-}$ T cells, compared with STAT5a/b$^{+/+}$ T cells (Fig. 4*A*). In addition, several proapoptotic effectors within the TNF/TNFR-associated factor family genes also were expressed in activated STAT5a/b$^{-/-}$ T cells, but not in STAT5a/b$^{+/+}$ T cells (Fig. 4*A*). Interestingly, although STAT5a/b$^{+/+}$ T cells expressed some antiapoptotic genes (Bcl-2, IAP1, Casper, Mcl-1, and Arc), other antiapoptotic genes remained expressed in STAT5a/b$^{-/-}$ T cells (Bar like, NAIP1, bfl-1, Bcl2L10, and Mcl-1), suggesting a complex interplay among these pathways. We also examined the kinetics of expression of the antiapoptotic Bcl-2 and proapoptotic Bax proteins in T and B cells. Con A-activated STAT5a/b$^{+/+}$ T cells increased expression of Bcl-2 over 24–48 h (Fig. 4*B*, *left panel*); activated STAT5a/b$^{-/-}$ T cells had low levels of detectable Bcl-2 at 24 h, but none at 48 h, while expressing the proapoptotic Bax protein at 24 and 48 h. Identical analysis showed that STAT5a/b deficiency had no impact on the expression of Bcl-2 in LPS-activated purified B cells (Fig. 4*B*, *right panel*). These findings suggest that STAT5a/b regulate antiapoptotic and proapoptotic activities in T cells. Whether STAT5a/b directly or indirectly regulates Bcl-2 and Bax, as well as other apoptotic-related genes, remains to be examined.

STAT5a/b deficiency does not affect T cell cytokine production

We also examined how STATs' deficiencies affect cytokine production by T cells. A RT-PCR analysis showed that Con A-stimulated T cells from STAT5a/b$^{+/+}$, STAT5a/b$^{+/-}$, and STAT5a/b$^{+/-}$ mice expressed (at 24 h) similar amounts of IL-2, IL-4, IL-10, and IFN-γ mRNA (Fig. 5*A*). These results were confirmed by the ELISPOT assay, in which purified STAT4$^{-/-}$, STAT6$^{-/-}$, and STAT4/6$^{-/-}$ T cells stimulated overnight with Con A produced lower numbers of IL-4-producing clones, compared with STAT5a/b$^{+/+}$ and STAT5a/b$^{-/-}$ T cells (Fig. 5*B*). Identical Con A stimulation generated higher numbers of IFN-γ-producing STAT4$^{-/-}$, STAT6$^{-/-}$, and STAT4/6$^{-/-}$ T cells, compared with STAT5a/b$^{+/+}$ and STAT5a/b$^{-/-}$ T cells (Fig. 5*B*). Interestingly, STAT4/6$^{-/-}$ T cells had the fewest numbers of IL-4-producing T cells and the greatest numbers of IFN-γ-producing T cells. Overall, these results document that before entering apoptosis, STAT5a/b-deficient T cells can produce normal quantities of cytokines, compared with controls.

STAT5a/b-deficient mice produce both IgM and IgG Abs

To explain the mechanism of delayed graft destruction by STAT5a/b-deficient mice, sera of normal, as well as STAT4/6$^{-/-}$ and STAT5a/b$^{-/-}$ recipients that had rejected allografts were examined for donor-specific IgM and IgG Abs. FACS analysis of this sera with donor target cells revealed that, immediately after heart allograft rejection, all examined recipients had circulating anti-donor IgM and IgG Abs (Fig. 6*A*). Sera from STAT4/6$^{-/-}$ rejectors showed reduced production of IgG1, IgG2b, and IgG3, whereas sera from STAT5a/b$^{-/-}$ rejectors showed somewhat decreased production of IgG2b and IgG3. We also tested the production of donor-specific Abs in recipients that were sensitized by skin allografts and then rejected heart allografts in an accelerated fashion (Fig. 1*D*). Interestingly, sera of STAT5a/b$^{-/-}$ recipients showed higher levels of donor-specific IgM and all subclasses of IgG Abs compared with STAT5a/b$^{+/+}$ (Fig. 6*B*). Thus, although STAT5a/b deficiency may affect the production of some IgG subclasses, STAT5a/b$^{-/-}$ mice produce large amounts of donor-specific IgM and IgG during primary and secondary allograft rejection.

The prominent role of humoral reactants in STAT5a/b$^{-/-}$ recipients was confirmed by the immunoperoxidase staining of heart allografts harvested from STAT5a/b$^{+/+}$ and STAT5a/b$^{-/-}$ recipients. When examined at postgrafting day 7, heart allografts from STAT5a/b$^{+/+}$ recipients showed the expected mixture of host CD4^{+} and CD8^{+} T cells (31), along with infrequent numbers of B cells and low intensity staining for the humoral reactant C4d (Fig. 7, *left panel*). In contrast, heart allografts harvested at postgrafting day 7 from STAT5a/b$^{-/-}$ recipients showed 80–90% fewer CD4 and CD8 T cells, but had a florid infiltrate of CD22^{+} mature B and plasma cells (Fig. 7, *right panel*) (31). In addition, endothelial cells throughout the same allografts displayed moderate to dense staining for C4d/C4b (Fig. 7, *right panel*), consistent with local deposition of C fragments. Thus, heart allografts in STAT5a/b$^{-/-}$ recipients were rejected predominantly by donor-specific Abs.

Discussion

The present studies demonstrate that STAT5a/b transcription factors act as survival proteins in T cells and that highly purified STAT5a/b$^{-/-}$ T cells alone are unable to mediate allograft rejection. Con A or alloantigen activation of STAT5a/b$^{-/-}$ T cells induced production of multiple cytokines, followed by entry into apoptosis. These events in activated STAT5a/b-deficient T cells correlated with elevated expression of multiple proapoptotic genes and reduction of antiapoptotic genes. Specifically, activated STAT5a/b$^{-/-}$ T lacked the antiapoptotic Bcl-2 protein and showed an induction of the proapoptotic Bax protein. In contradistinction, lack of STAT5a/b did not affect several B cell functions, including cell proliferation in response to LPS, IgM to IgG class switching, or synthesis of donor-specific Abs. Such a turn of events had a dramatic impact on the kinetics and mechanism of allograft rejection. Indeed, STAT5a/b deficiency extended the survival time of primary heart allografts but had no effect on the survival of secondary heart allografts by sensitized recipients. Activated T cells produced cytokines (e.g., IL-4) that most likely promote proliferation and differentiation of B cells, regulating their ability to produce donor-specific IgM and IgG Abs that mediate allograft rejection (Fig. 8). To the best of our knowledge, this is the only model of allograft rejection involving T-B cell collaboration and predominantly Ab-mediated allograft destruction. Because purified STAT5a/b$^{-/-}$ T cells could not mediate destruction of heart allografts unless supplemented with B cells, we postulate that Abs may significantly contribute to the process of acute rejection.

The major observation of this study is that STAT5a/b transcription factors are critical for T cell survival, which supports our recently published work (25). Indeed, massive T cell apoptosis was induced after depletion of STAT5a/b (via antisense ODN) in PHA-primed human T cells in contradistinction to unprimed human T cells or non-IL-2-responsive Jurkat cells. The apoptotic process was associated with the activation of proapoptotic caspase-8 and -9 effectors (25). Our present experiments extended this observation by a microarray analysis that found overexpression of multiple

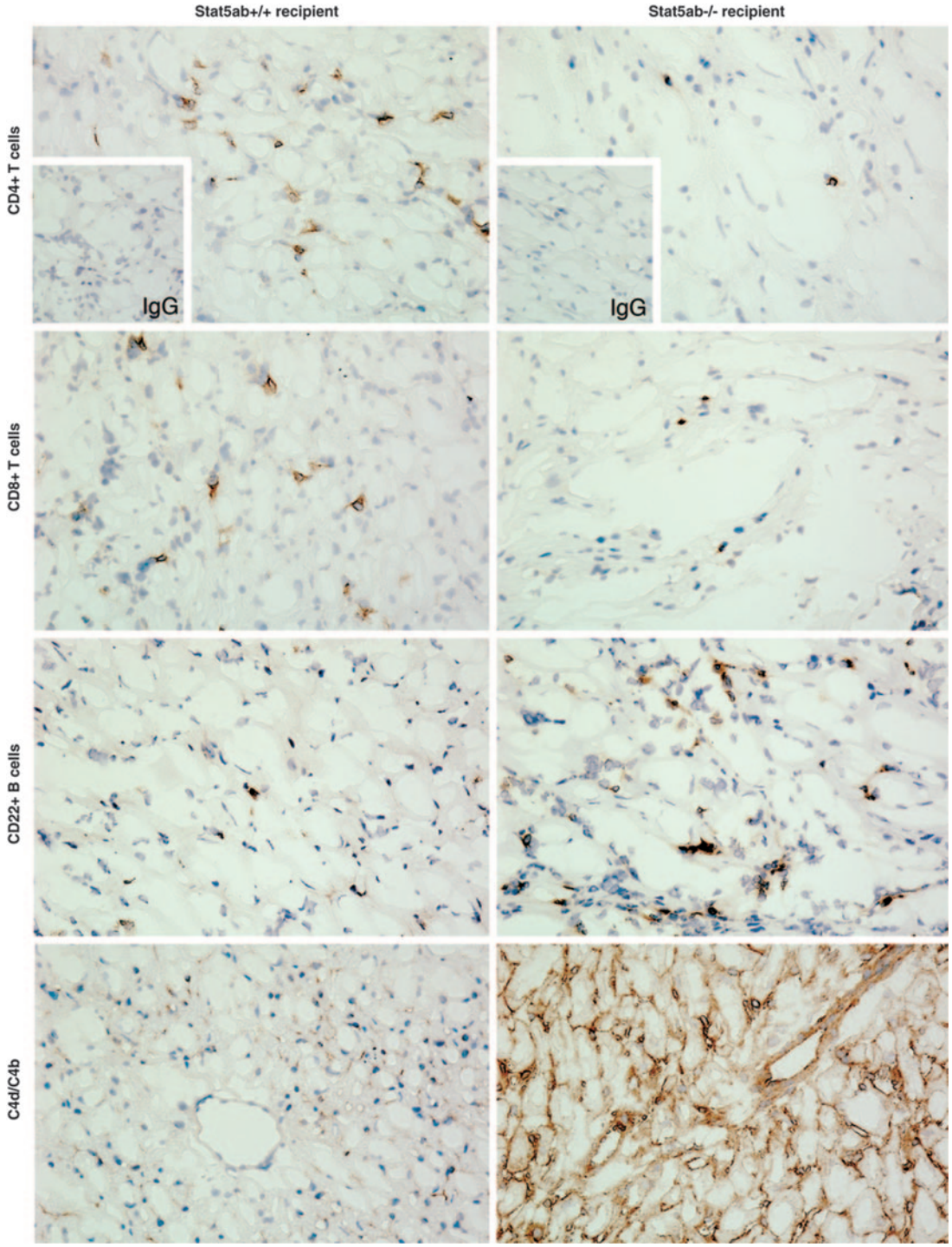

FIGURE 7. Evidence that heart allografts in STAT5a/b-deficient recipients are rejected predominantly by Abs. Immunostaining of heart allografts from STAT5a/b$^{+/+}$ recipients (*left panels*) showed a prominent infiltrate of CD4$^+$ and CD8$^+$ T cells, an occasional infiltrate of CD22$^+$ B cells, and weak staining for C4d/C4b. In contrast, heart allografts from STAT5a/b$^{-/-}$ recipients (*right panel*) showed few CD4$^+$ and CD8$^+$ T cells, a numerous infiltrate of CD22$^+$ B cells, and widespread endothelial deposition of C4d/C4b. Hematoxylin-counterstained cryostat sections are ×500 magnifications, whereas control inserts for IgG are ×250 magnifications; data are representative of findings in two to four grafts/group.

genes including 10 proapoptotic genes, 6 caspases, and 6 DNA repair genes in activated STAT5a/b$^{-/-}$ T cells. Furthermore, whereas antiapoptotic Bcl-2 protein was elevated in normal T cells, it was depressed in T cells lacking STAT5a/b with the appearance of the proapoptotic Bax protein. Thus, our present and recently published results support the hypothesis that STAT5a/b transcription factors protect the survival of activated T cells.

The antiapoptotic activity of STAT5a/b also has been documented during the terminal stages of myeloid differentiation (33). During hemopoiesis, a dominant-negative STAT5 construct blocked myeloid cell maturation and induced massive cell apoptosis. Parallel experiments showed this apoptotic process was prevented by cytokine-independent overexpression of Bcl-2 or Bcl-x in bone marrow cells transfected with dominant-negative STAT5

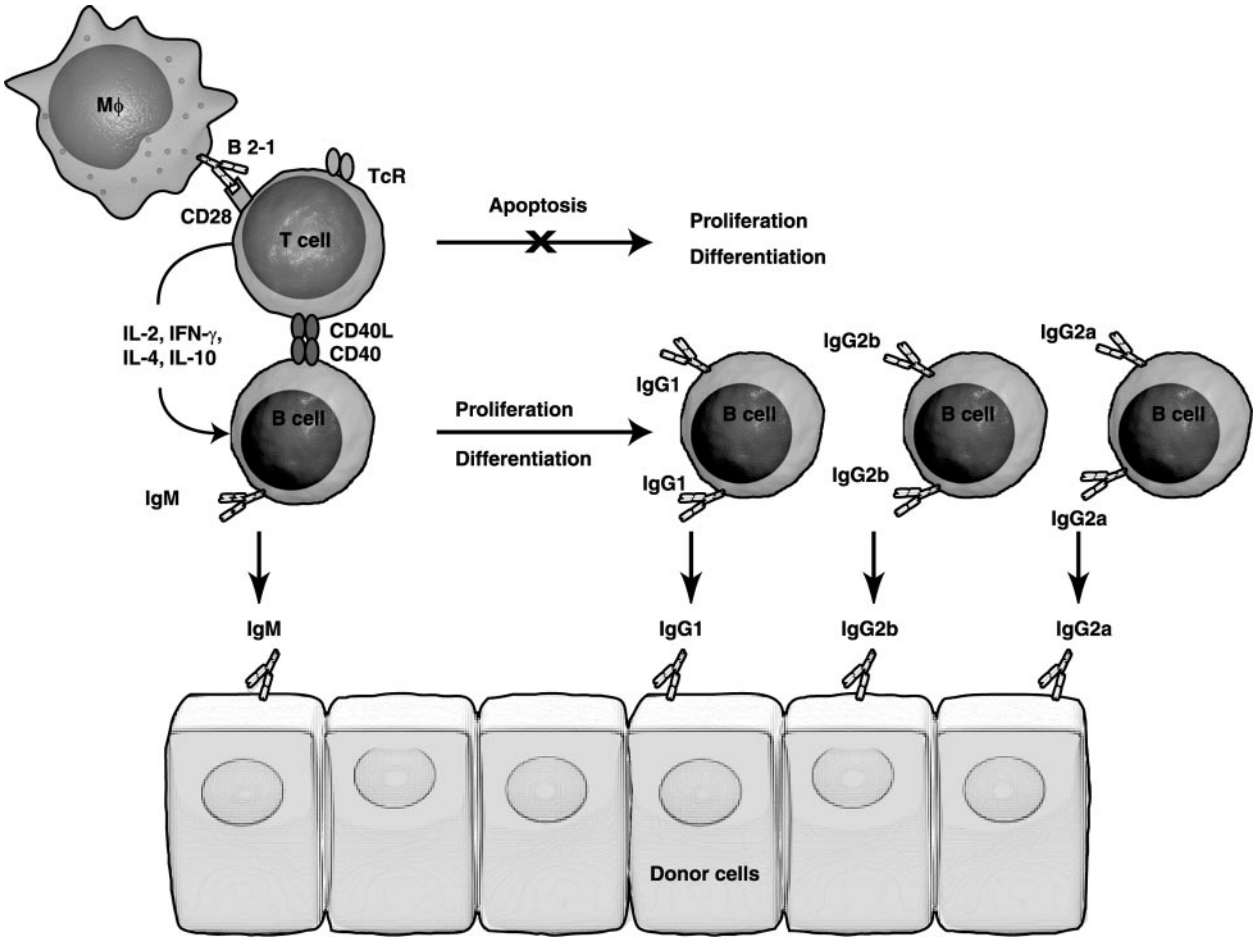

FIGURE 8. Model of allograft rejection by STAT5a/b-deficient mice. Activated STAT5a/b$^{-/-}$ T cells produce cytokines prior to entering apoptosis. Cytokines contribute to activation of STAT5a/b$^{-/-}$ B_{IgM} cells, which proliferate and differentiate into B_{IgG1}-, B_{IgG2a}-, B_{IgG2b}-, and B_{IgG3}-producing Ab that subsequently mediate allograft rejection.

variant, suggesting that STAT5 is fundamental in the survival of these cells (33). It also is apparent that STAT5a/b may be critical for the maintenance of self-tolerance: double STAT5a/b-deficient mice displayed alterations in several bone marrow progenitors concomitant with cellular infiltration affecting internal organs such as colon, liver, and kidneys. Because IL-2Rβ chain-deficient mice exhibited similar hemopoietic abnormalities as STAT5a/b$^{-/-}$ mice, IL-2/STAT5-dependent signaling may be important to control self-tolerance by CD4$^+$CD25$^+$ regulatory T (Treg) cells (33). Another recent study also examined the role of CD4$^+$CD25$^+$ Treg cells in inhibiting autoreactive T cells (34). Indeed, transient activation of STAT5a/b in these cells boosted the number of Treg cells even in IL-2-deficient mice (possibly mediated by other γ_c cytokines), suggesting that STAT5a/b are essential in the regulation of self-tolerance (34). Moreover, lymphocytes harvested from mice bearing s.c. introduced mammary adenocarcinoma tumors were unable to generate cellular and humoral responses (35). Purified T and B cells from these mice had reduced STAT5a/b protein levels (36). Similarly, HIV-1-infected patients displayed increased T cell apoptosis and reduced STAT5a/b protein levels. Taken together, these reports suggest that STAT5a/b transcription factors are critical for T cell survival activity. Although the STAT5a/b null mice used in our studies were generated in a distinct manner from those presented in a recent publication, the overall conclusions confirmed that STAT5a/b are critical for T cell survival (37).

Although a variety of signal molecules may regulate survival in the early stages of hemopoiesis, their identity remains unclear. Potential candidate regulators include signal-transducing adaptor molecule (Stam)1 and Stam2, which were found to be tyrosine phosphorylated upon stimulation with cytokines recruiting the γ_c/JAK3 signaling pathway (38). Systemic double Stam1/2 mutations proved embryonically lethal in mice, whereas mice with the T cell-selective Stam1/2 mutations of phosphotyrosine residues displayed a significant reduction of peripheral mature T cells. Therefore, Stam1/2 phosphotyrosines are most likely indispensable for T cell development and may act in STAT5a/b$^{-/-}$ mice to protect T cell progenitors against apoptosis during cytokine/γ-chain/JAK3 signaling.

A different picture has emerged in STAT5a/b-deficient B cells. There was little difference in cellular proliferation between LPS-stimulated STAT5a/b$^{-/-}$ and STAT5a/b$^{+/+}$ B cells. Similarly, both B cell populations demonstrated the ability to produce alloantigen-specific IgM and IgG Abs. In fact, because pure STAT5a/b$^{-/-}$ T cells did not promote rejection, destruction of allografts by STAT5a/b$^{-/-}$ recipients must be mediated predominantly by donor-specific IgM and IgG Abs. These observations are consistent with previous reports that found little detectable difference in differentiation and function between normal and STAT5a/b-deficient B cells (18). However, recent work found that IL-5-dependent class switch recombination (CSR) from μ to γ1 as well as IgM and IgG1 productions were influenced by a lack of STAT5a/b (39). It was shown that IL-5 activated STAT5 (40) and induced CSR from μ to γ1 (41). Furthermore, IL-5Rα mutant had impaired B cell

development, reduced IgG3 and IgM levels, and reduced IL-5-mediated B cell proliferation and CSR function (42). These results may be similar to our in vitro studies that revealed that STAT5a/b-deficient recipients produced somewhat reduced quantities of IgG2b and IgG3, compared with control recipients. However, STAT5a/b deficiency had no impact on B cell survival or anti-apoptotic activity in B cells. To explain this difference, we suggest that other cytokines and signaling pathways may become involved in B cell survival and CSR in B cells. Indeed, IL-21 has emerged recently as a potent regulator of B cells (43), as IL-21R knockout mice had deficient IgG1 and increased IgE production. Another newly described cytokine, IL-27, induced expression of IgG2a, but not IgG1 (44). These observations also are confirmed by difficulties in the development of chemical agents to selectively block Ab-mediated allograft rejection.

There is increasing experimental and clinical evidence to support the notion that donor-specific Abs contribute to acute allograft rejection (45–47). Emerging criteria for such rejection include circulating donor-specific IgG Abs and biopsy-confirmed changes with C4d fragment deposition and neutrophil infiltration into peritubular capillaries, acute tubular injury, or arterial fibrinoid necrosis (48–51). Recently, C4d staining combined with relevant pathological changes and graft dysfunction was described in recipients of ABO blood group-incompatible kidney transplants (52). In a passive transfer model to Ig-deficient mice, donor-specific C-binding IgG2 mAb restored acute allograft rejection of heart allografts, otherwise surviving >14 days (47). Although donor-specific non-C-binding IgG1 mAb failed to restore acute rejection, the combination of both IgG1 and IgG2 mAb was the most effective. Our present results demonstrate that sera of STAT5a/b$^{-/-}$ mice had donor-specific IgM and IgG Abs (Fig. 6), and that only a mixed T and B cell population mediated heart allograft rejection. Immunohistology of heart allografts from STAT5a/b$^{-/-}$ recipients confirmed their predominant infiltration with B cells and plasma cells with endothelial deposits of C4d, consistent with humoral rejection (Fig. 7). Thus, we propose that, after T-B cell collaboration, Abs may be predominant contributors to allograft destruction (Fig. 8).

In conclusion, the present findings show a crucial role for STAT5a/b in mediating T cell survival. However, STAT5a/b-deficient T cells can possibly initiate the process of allograft rejection by producing critical T cell cytokines to drive B cell-dependent processes, including the ability to synthesize donor-specific Abs. This work suggests that targeted inhibition of STAT5a/b may facilitate the induction of transplantation tolerance by promoting the elimination of alloreactive T cell clones. For mature B cells, STAT5a/b transcription factors may be dispensable for their survival, proliferation, class switching, and production of IgM and IgG Ab.

Disclosures

The authors have no financial conflict of interest.

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