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Reduced STAT3 activity in mice mimics clinical disease syndromes

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Abstract

Phosphorylation on Y705 is obligatory for STAT3 activation, but full transcriptional activity of this widely expressed protein also requires phosphorylation on S727. We described earlier the STAT3 SA/− mice (SA, S727A allele) on a Black 6 (Bl6) background that showed 75% perinatal lethality and early growth retardation presumably due to the decreased transcription supported by STAT3 S727A. We now report additional analyses of long-term surviving SA/− animals which show no important tissue abnormalities. However, we have found a much greater susceptibility to doxorubicin-induced heart failure in the SA/− mice. Also we introduced the SA allele into strain 129 and found the SA/− mice showed greater susceptibility to LPS-induced toxicity. These results suggest a continued need for normal STAT3 transcriptional activity to resist two different noxious challenges that mimic the conditions necessary to induce adult diseases.

Keywords: STAT3; Transcription; S727A mutation

Activation of STAT (signal transducers and activators of transcription) proteins occurs by tyrosine phosphorylation in response to many different cytokine, growth factor, and peptide ligands binding to cell surface receptors. Phosphorylated STAT dimers accumulate in the nucleus to drive transcription of target genes [1].

Stat3 is ubiquitously expressed and is transiently activated by a large number of different ligands, including cytokines of the IL-6 family such as IL-6, ciliary neurotrophic factor (CNTF), oncostatin M (OSM), leukemia inhibitory factor (LIF), as well as non-cytokine ligands such as growth factors EGF, PDGF, HGF, granulocyte colony-stimulating factor (G-CSF), and growth hormone and IGF-1 [2–5]. Stat3−/− animals die around day E7.0 [6], thus cell-specific targeted knockouts are required to observe the function of STAT3 in various adult tissues. Consistent with the widespread expression of STAT3, such tissue-specific knockouts of STAT3 reveal the requirement of this protein in many adult tissues ([7–18], reviewed by [2,3]). In all these experiments it is the complete removal of the STAT3 protein that is under study. In addition, recent work has demonstrated that STAT3 as a non-phosphorylated, and potentially much less active transcriptional factor, may also have a function [19].

In addition to obligatory tyrosine phosphorylation, a single serine phosphorylation (residue 727 in STAT1 and STAT3) in the C-terminal transactivation domain is required for maximum transcriptional activation of STAT1, STAT3, STAT4, and STAT5A and STAT5B [20,21]. Previously we described the generation of a STAT3 S727A mutant mouse strain on Black 6 background (Bl6, Black 6). This mutation leaves the STAT3 protein present in normal amounts in all cells but fibroblasts of SA/− genotype had 25% or less of the STAT3 transcriptional response of wild-type cells [22]. This decrease of STAT3 transcriptional activity in the SA/−

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mice resulted in 75% perinatal lethality, and in the survivors, initial growth retardation and other negative effects in adult tissues, including increased apoptosis in thymocytes [22].

Because of the widespread expression and various routes of STAT3 activation in different tissues, we continued to follow the STAT3SA/− mice with time and have uncovered increased susceptibility to doxorubicin-induced cardiac failure in the SA/− male mice. In addition, when both the STAT3 SA and the null alleles were bred onto the 129/Sv mouse strain thus making examination of SA/− 129/Sv animals possible, the same phenotype of partial perinatal death was observed. However, in 129 SA/− survivors a much greater susceptibility to LPS-induced toxic shock was found. Both clinical syndromes mimicked by these two phenotypes, heightened susceptibility to cardiac failure and to toxic shock, are of great clinical importance.

Materials and methods

Animals. The generation of the STAT3 S727A knock-in mouse line on the C57 Black 6 background was described previously [22]. The STAT3+/− mouse line on this background was also described before [23]. In addition, both STAT3SA/+ and STAT3+/− strains were backcrossed to 129/Sv wild-type mice (Taconic) for at least 7 generations. The genotyping was performed as described previously. All animals were housed and maintained under regular conditions according to the guidelines approved by the Laboratory Animal Research Center of the Rockefeller University.

Histological examination. Mice were euthanatized by carbon dioxide inhalation. Complete postmortem evaluations were performed on all mice. All tissues were fixed in 10% neutral buffered formalin with the exception of the skull, vertebral column, and rear leg which were fixed in Decalcifier I (Surgipath Medical Industries, Richmond, IL) overnight. All tissues were processed by routine methods and embedded in paraffin wax. Sections (5 μm) were stained with hematoxylin and eosin (HE), and evaluated with an Olympus BX45 light microscope (New York/New Jersey Scientific, Middlebush, NJ).

All the major tissues and organs were examined, including: tongue and diaphragm, skeletal muscle with peripheral nerve, trachea, esophagus, thyroid gland, parathyroid gland, thymus, heart, lung, kidney and adrenal glands, salivary glands and lymph nodes, stomach, duodenum, ileum, cecum, colon, pancreas, spleen, liver, urinary bladder, skin, brown adipose tissue, sternum, stifle and tarsus with bone marrow and attached skeletal muscle, coronal sections of the head including calvarium, eye, haderian glands, nose, olfactory lobes of the brain, teeth, cerebrum, hippocampus, cerebellum, brainstem and bullae, longitudinal and transverse sections of vertebral column with spinal cord, bone marrow and attached skeletal muscles, as well as male reproductive organs (testes, epididymides, seminal vesicles, coagulating glands, and bulbourethral gland) or female reproductive organs (ovaries, uterus and vagina, and mammary gland).

Hematology. Whole blood was collected by cardiac puncture following euthanasia. Complete blood counts were performed on a portion of EDTA anticoagulated whole blood (Cell-Dyn 3700, Abbott Laboratories, Abbott Park, IL). Following coagulation of the remaining whole blood at room temperature for 30 min, the clotted blood was centrifuged at 3000 rpm for 5–10 min. at 4 °C. Serum was submitted to a commercial reference laboratory (IDEXX Laboratories, Totowa, NJ) for determination of biochemical parameters.

Treatment of animals. For the doxorubicin treatment, 9–12 months old, sex matched STAT3SA/− mice and littermate control mice (Bl6) were injected intraperitoneally with doxorubicin (Sigma) at a single dose of 15 mg/kg. The animals were bred normally and survival was checked twice daily.

For the LPS challenge, age and sex matched STAT3SA/− mice and littermate control mice of the Bl6 strain or 129/SVE strain were injected intraperitoneally with LPS (055:B5, Sigma L2880), at a single dose of 25 mg/kg. The animals were bred normally and survival was checked every 12 h for two weeks.

Statistical analysis. Results were analyzed by a two-tailed Student's *t* test. A P value less than 0.05 was considered to be statistically significant.

Results and discussion

STAT3 SA/− survivors show no obvious defects

Since STAT3 is ubiquitously expressed and can be activated by many ligands, we continued to follow the transcriptionally compromised STAT3SA/− mice with time and performed a thorough histologic analysis of all the major organs and tissues from animals (6–9 months of age). No regular occurrence of abnormalities was observed and as reported earlier the SA/− animals recovered from initial growth retardation to be of approximately normal size and weight.

Requirement of full STAT3 transcription for normal heart muscle function in males in response to doxorubicin treatment

STAT3 is the major transcription factor activated by the gp130 receptor which in turn is required for cardiac myocyte survival and in the cardiac hypertrophy that occurs in response to work overload [24,25]. In addition, overexpression of STAT3 in transgenic animals results in cardiac hypertrophy but at the same time protects against doxorubicin-induced cardiomyopathy [26]. Consistent with these results, mice with cardiomyocyte-restricted knockout of STAT3 develop age-associated heart dysfunction [17]. Thus, wild-type STAT3 in heart muscle serves a crucial function(s).

We therefore investigated cardiac muscle function in SA/− animals 9–12 months old. Both mutant and control mice (sex matched littermates) were injected intraperitoneally with doxorubicin (Dox) at a single dose of 15 mg/kg. This antitumor drug induces cardiac toxicity presumably due to apoptosis. Only 22% (2/9) of male SA/− mice survived compared with 77% (10/13) of the male SA/+ mice four weeks after Dox treatment ($P < 0.01$, Fig. 1A). The affected mice exhibited massive bilateral pleural effusion and ascites, indicating death due to congestive heart failure [26]. In contrast, both SA/+ and SA/− female mice showed survival comparable to the SA/+ male mice (Fig. 1B).

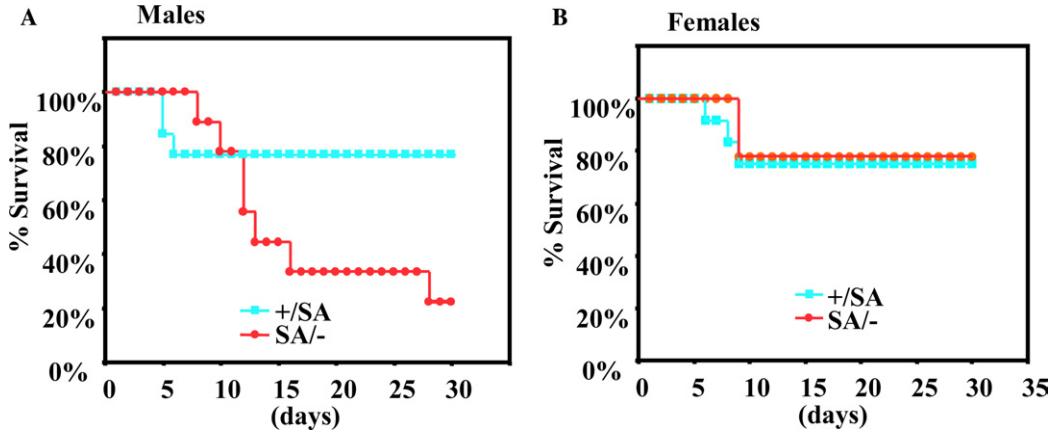

Fig. 1. SA/− male mice are more sensitive to doxorubicin treatment. Sex and age matched STAT3SA/− mice and littermate control mice (9–12 months) were injected intraperitoneally with doxorubicin at a single dose of 15 mg/kg. (A) Males SA/+, total of 13 animals; SA/−, total of 9 animals. (B) Females SA/+, total of 12 animals; SA/−, total of 9 animals.

Thus, a lack of full transcriptional activity of STAT3 in the SA/− male mice was associated with an event of aging that affects men more than women, namely, cardiac failure.

Strain-specific requirement for STAT3 transcription in controlling toxic shock

Various in vitro and in vivo studies have suggested important role of STATs in the regulation of myeloid differentiation [1,27]. Mice lacking STAT3 in hematopoietic progenitors produce excess myeloid-lineage cells including neutrophils [11] and macrophages, and are deficient in dendritic cells [10]. To examine the effect of diminished transcription in the STAT3 S727A mutants in hematopoietic cells, complete blood counts and differentials were performed on the whole blood of the SA/− and littermate control mice (Table 1). Neutrophils, lymphocytes, and myeloid cells were present in normal numbers in both SA/+ and SA/− animals. Also, no significant difference was found in mean corpuscular volume or mean cell hemoglobin of erythrocytes, and platelet counts were normal.

STAT3 is the main mediator of IL-6 signaling and is essential for liver acute phase responses [9]. We previously found little or no change in inflammatory response genes in liver in response to turpentine or lipopolysaccharide (LPS) injection in SA/− Bl6 animals [22]. LPS, an endotoxin derived from Gram-negative bacteria, triggers systemic inflammation and at high dose can result in lethal endotoxin shock in mice (a mouse model of "toxic shock"). Mice with STAT3 deletion in macrophages become much more susceptible to LPS-induced septic shock, indicating an anti-inflammatory function of IL-10 through STAT3 in macrophages [7]. Similarly, endothelial cells require STAT3 for protection against endotoxin-induced inflammation [12]. STAT3α is the full-length molecule while STAT3β, an alternatively spliced form, lacks the –COOH transcription activation domain. Mice lacking STAT3β only are viable but also show impaired recovery from LPS shock [28,29]. To investigate the effect of diminished STAT3 stimulated

Table 1
WBC differentiation count and hematological parameters

	SA/+ (M)	SA/− (M)	SA/+ (F)	SA/− (F)
WBC differentiation count				
Neutrophils (%)	27 ± 5	34 ± 5	19 ± 5	19 ± 4
Lymphocytes (%)	72 ± 5	63 ± 4	79 ± 4	78 ± 2
Macrophages (%)	ND	ND	ND	ND
Monocytes (%)	1 ± 1	2 ± 2	2 ± 0	3 ± 2
Eosinophils (%)	1 ± 1	1 ± 1	ND	ND
Hematological parameters				
Hematocrit (%)	44 ± 2	41 ± 2	43 ± 1	42 ± 1
Hemoglobin (g/dl)	15.0 ± 0.6	14.0 ± 0.7	15.0 ± 0.2	15.0 ± 0.3
WBC (K/ul)	6.4 ± 0.7	7.8 ± 2.5	12.3 ± 3.2	11.9 ± 3.3
Platelets (K/ul)	925 ± 35	938 ± 104	807 ± 55	810 ± 74

Whole blood was counted for granulocyte cell types and hematological parameters were determined as described in Materials and methods. Shown are average results with standard derivation from four 9-month-old male or female littermate SA/+ and SA/− mice.

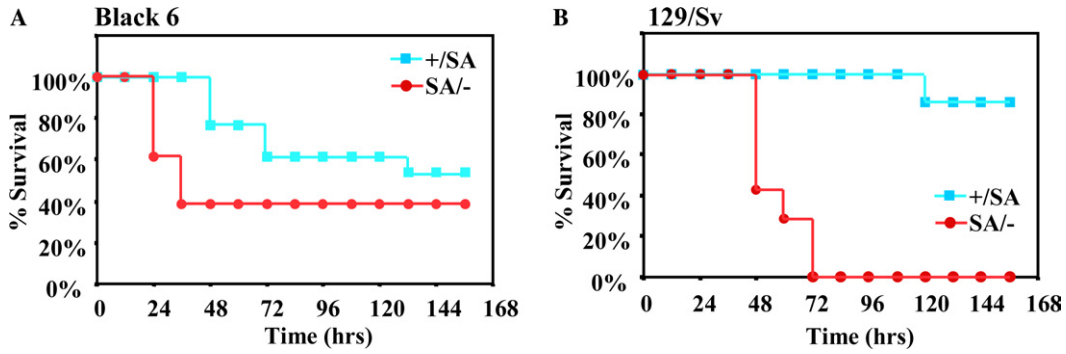

Fig. 2. Susceptibility of the SA/− mice to LPS-induced endotoxin shock. (A) Survival of the Bl6 strain. Four to seven months age and sex matched SA/− mice and littermate control SA/+ mice (Bl6 strain, 13 animals each) were injected intraperitoneally with LPS at a single dose of 25 mg/kg. Mice were monitored for two weeks, with no additional mortality after one week. (B) Survival of the 129/Sv strain. As for (A) 3–6 months age and sex matched STAT3SA/− mice and littermate control SA/+ mice (129/Sv strain, 7 animals each) were treated.

transcription on septic shock, the SA/− and SA/+ (Bl6) littermates were challenged with a high dose of LPS (intraperitoneal injection) and followed for two weeks. SA/− mice and control mice showed similar overall lethality to the LPS challenges (8 out of 13 SA/− mice versus 6 out of 13 SA/+ animals died within 6 days, Fig. 2A).

To test the importance of STAT3 serine phosphorylation on a different genetic background, we crossed both the STAT3SA allele and STAT3+/− allele onto strain 129/Sv (at least 7 generations for each strain). The SA/− offspring showed the previously recognized partial perinatal lethality (21 SA/− and 77 SA/+ progeny from the 129/Sv SA/SA to 129/Sv STAT3+/− breeding, i.e., ~73% perinatal death) as well as the early growth retardation (data not shown), just as did the SA/− Bl6 mice [22]. However, there was a major difference in LPS sensitivity in the SA/− (129/Sv) animals (Fig. 2). While only 1 out of 7 SA/+ mice died 4 days after LPS challenge, 7 out of 7 SA/− (129 strain) animals died within 3 days when challenged with LPS ($P < 0.0001$). Further investigation of the different responses of the 129/Sv and Bl6 SA/− mice in septic shock should reveal critical factors in this important physiological response.

The results in this report help sharpen the focus of STAT3 as a transcriptional regulator compared to total knockout experiments. In the SA/− phenotype, there is a normal amount of STAT3 but it is deficient in driving transcription on different target genes. Thus, defects or deficiencies in the SA/− mouse can be clearly attributed to decreased transcriptional effectiveness. This leads to our main conclusion that there are tissues—cardiac muscle and bone marrow—where full transcriptional activity of activated STAT3 is required to prevent defective responses to stress that produces conditions in mice that mimic severe human disease. These results provide a mouse strain in which induction of these diseases should be easier to study. Finally because of the diminished STAT3 transcriptional activity in the whole animal this mouse strain should be of great value in testing the potential toxicity of anti-STAT3 compounds. Such compounds are being widely sought since persistently active STAT3 is required for transformed cell survival in a wide range of human tumors [30].

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