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Immunology

Early-onset multifocal inflammation in the transforming growth factor β1-null mouse is lymphocyte mediated

(severe combined immunodeficient mice/cardiac hyperplasia)

RONALD J. DIEBOLD*†, MICHAEL J. EIS*, MOYING YIN*, ILONA ORMSBY*, GREG P. BOIVIN‡, BRUCE J. DARROW§, JEFFREY E. SAFFITZ§, AND THOMAS DOETSCHMAN*

Departments of *Molecular Genetics, Biochemistry and Microbiology and ‡Comparative Pathology, University of Cincinnati College of Medicine, Cincinnati, OH 45267; and §Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110

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ABSTRACT **Transforming growth factor β1 (TGFβ1)-null mice die from complications due to an early-onset multifocal inflammatory disorder. We show here that cardiac cells are hyperproliferative and that intercellular adhesion molecule 1 (ICAM-1) is elevated. To determine which phenotypes are primarily caused by a deficiency in TGFβ1 from those that are secondary to inflammation, we applied immunosuppressive therapy and genetic combination with the severe combined immunodeficiency (SCID) mutation to inhibit the inflammatory response. Treatment with antibodies to the leukocyte function-associated antigen 1 doubled longevity, reduced inflammation, and delayed heart cell proliferation. TGFβ1-null SCID mice displayed no inflammation or cardiac cell proliferation, survived to adulthood, and exhibited normal major histocompatibility complex II (MHC II) and ICAM-1 levels. TGFβ1-null pups born to a TGFβ1-null SCID mother presented no gross congenital heart defects, indicating that TGFβ1 alone does not play an essential role in heart development. These results indicate that lymphocytes are essential for the inflammatory response, cardiac cell proliferation, and elevated MHC II and ICAM-1 expression, revealing a vital role for TGFβ1 in regulating lymphocyte proliferation and activation, which contribute to the maintenance of self tolerance.**

Transforming growth factor β1 (TGFβ1) is the prototypic member of the larger TGFβ superfamily of proteins that affect growth and differentiation of many cell types (1, 2). Numerous studies have shown TGFβ1 to be a potent modulator of immune responses (3) and mice lacking TGFβ1 develop an early-onset multifocal inflammation culminating in death around 3 weeks of age (4, 5). Further analysis of these mice has revealed elevated levels of peripheral lymphocytes and immature neutrophils (4), increased expression of major histocompatibility complex (MHC) molecules (6), enhanced adhesive properties of TGFβ1-null leukocytes (7), increased numbers of proliferating cells in mutant spleens and lymph nodes, and up-regulated thymic interleukin 2 expression (8). Since the onset of inflammation is detectable within the first week of life (9) it is unclear whether the deficiency in TGFβ1 or the inflammatory cells and their cytokines are the primary cause of the mutant phenotypes. To more accurately assess the *in vivo* functions of TGFβ1, we sought to suppress the chronic inflammation that invariably develops in these animals.

MATERIALS AND METHODS

PCR Genotyping. Newborn mice were genotyped using an upstream primer, 5′-GAGAAGAACTGCTGTGTGCG-3′, and a downstream primer, 5′-GTGTCCAGGCTCCAAATATAGG-3′, corresponding to TGFβ1 exon 6 sequences flanking the *neo* insert in the TGFβ1-null allele. Capillary PCR was performed on a Corbett (American Biotechnologies) cycler in a 20-μl volume at 95°C for 20 sec, 55°C for 50 sec, and 72°C for 1 min for 30 cycles in a reaction mixture of 1× PCR buffer, 2.5 mM dNTPs, 10 μM primer, 0.4 unit of *Taq* polymerase (all from Boehringer Mannheim), 0.5% formamide, and PCR dye (0.4% cresol red in 60% sucrose).

Anti-CD11α Therapy. CD11α is the α_L subunit of the $\alpha_L\beta_2$ integrin, known as leukocyte function-associated antigen 1. Pups were injected s.c. with anti-murine CD11α clone M17 (Genentech) at 60 μg per injection every other day from day 2 until weaning.

Derivation of TGFβ1-Null Severe Combined Immunodeficient (SCID) Mice. C3H-HeJ mice homozygous for the SCID mutation (10) (Harlan Laboratories, Haslett, MI) were bred with mice heterozygous for the TGFβ1-null allele. Failure to detect serum immunoglobulin was initially used to screen for SCID homozygotes. Splenocytes from immunodeficient TGFβ1-null mice (homozygous for SCID and TGFβ1-null alleles) consistently lacked the B- and T-cell markers B220 and TCRα/β, as determined by flow cytometry on a FACStar instrument (Becton Dickinson).

Immunohistology and BrdUrd Labeling. For horseradish peroxidase immunohistochemical staining, the Vectastain ABC peroxidase system (Vector Laboratories) was used as described by the manufacturer on 5-μm frozen sections. All primary biotinylated antibodies (Abs) were diluted 1:1000 except anti-intercellular adhesion molecule 1 (anti-ICAM-1) Abs, which were applied as an undiluted hybridoma cell supernatant. Abs used were rat anti-murine ICAM-1 clone YN1-1.7.4 (American Type Culture Collection CRL 1878), rabbit biotinylated anti-rat IgG (Vector Laboratories), and biotinylated anti-murine I-A^k, I-E^k, B220, and αβTCR (all from PharMingen). For fluorescence-activated cell sorting analysis, phycoerythrin- or fluorescein isothiocyanate-conjugated hamster anti-murine CD45R (B220) or αβTCR (PharMingen) was utilized. The BrdUrd labeling protocol was adapted from Aiken and Roth (11). Animals were injected i.p. with 120 mg of BrdUrd (Sigma) per kg of body weight 90 min prior to fixing the tissue in Bouin's solution. Paraffin-embedded tissue sections (5 μm) were incubated overnight at 4°C with goat anti-BrdUrd at a 1:1000 dilution (gift from Kevin Roth, Washington University) or rabbit anti-myosin at a 1:100 dilution (gift from Stacy Smith, Washington University). After rinsing, tissues were incubated with a 1:200 dilution of secondary Ab, CY3-conjugated donkey anti-goat or fluorescein isothiocyanate-conjugated anti-rabbit (Jackson ImmunoResearch). For nuclear staining tissues were incubated with

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Abbreviations: TGFβ1, transforming growth factor β1; SCID, severe combined immunodeficient/immunodeficiency; MHC, major histocompatibility complex; Ab, antibody; ICAM-1, intercellular adhesion molecule 1.

†Present address: Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, MI 48109.

Hoechst 33258 (0.4 μg/ml in phosphate-buffered saline) for 10 min.

RESULTS

Ab therapy aimed toward LFA-1 (CD11α) and its ligand ICAM-1 has been reported to prevent cardiac graft rejection (12). Since ICAM-1 expression is elevated in the TGFβ1-mutant hearts (Fig. 1*A* and *B*), we initiated neonatal injections of Abs against CD11α to suppress cardiac inflammation. Mutants injected s.c. with CD11α antibodies (60 μg per injection) every other day from day 2 until weaning exhibited a significantly increased lifespan compared to TGFβ1-null mice treated with saline (39 ± 7 days, n = 12, for anti-CD11α-treated mice vs. 20 ± 6 days, n = 9, for saline-treated mice, $P < 0.01$), and the occurrence of inflammatory cells was greatly diminished following anti-CD11α treatment in older animals (>37 days) (Fig. 1 *C* and *D*).

Microscopic examination of the Ab-treated TGFβ1-null hearts revealed a hypercellular phenotype. This was confirmed utilizing BrdUrd labeling of anti-CD11α-treated wild-type and mutant animals. Fig. 2 *A* and *B* show BrdUrd nuclear labeling of sections of cardiac tissue isolated from 5-week-old Ab-treated wild-type and mutant animals. The latter lack detectable inflammatory lesions. The TGFβ1-null heart displays a significant increase in nuclear labeling compared to the control (109.1 ± 15.2 per high-power field for TGFβ1-null mice vs. 18.5 ± 5.5 per high-power field for wild-type mice, $P < 0.01$). The preponderance of labeled cells appears to be cardiac interstitial cells. However, labeled nuclei were detected in cells that stained positive for myosin heavy chain (Fig. 2 *C* and *D*), indicating that some cardiac myocytes were undergoing proliferation. Myocyte-specific nuclear labeling was significantly higher in treated mutants compared to treated controls (8.9 ± 1.8 per high-power field for mutant mice vs. 1.1 ± 0.4 per high-power field for wild-type mice, $P < 0.05$).

Hematological analysis of anti-CD11α-treated mutants revealed an increased number of circulating total white blood cells, including lymphocytes, monocytes, and neutrophils, compared to anti-CD11α-treated controls (Table 1). No differences were detected between treated and untreated control animals (data not shown).

Although most anti-CD11α-treated mutant mice had little or no detectable inflammation in the organs analyzed, the presence of occasional small inflammatory foci did not allow us to rule out inflammation as the causative agent for hyperproliferation in the heart and blood. To more completely suppress the TGFβ1-null inflammatory phenotype, the TGFβ1 and SCID mutations were combined.

Immunodeficient TGFβ1-null animals survived well into adulthood (>80 days). No inflammatory cell infiltrate was detected in any organs, including the heart (Fig. 3 *A* and *B*), liver, pancreas, stomach, and diaphragm (Table 2). In two instances, inflammation associated with pulmonary bacterial lesions was found in the lungs of immunodeficient TGFβ1-null animals. However, these were unlike the perivascular inflammation normally observed in immunocompetent TGFβ1-null mice (4, 5, 9). No other immunodeficient TGFβ1-null mutants displayed inflammatory lesions in the lung. Cardiac tissue from adult immunodeficient TGFβ1-null mice revealed no elevated expression of MHC II (Fig. 3 *C* and *D*) or ICAM-1 (Fig. 3*E*, compare to Fig. 1*B*).

Since TGFβ1 has been reported to inhibit the proliferation of many cell types, including fibroblasts, myocytes, and cells of

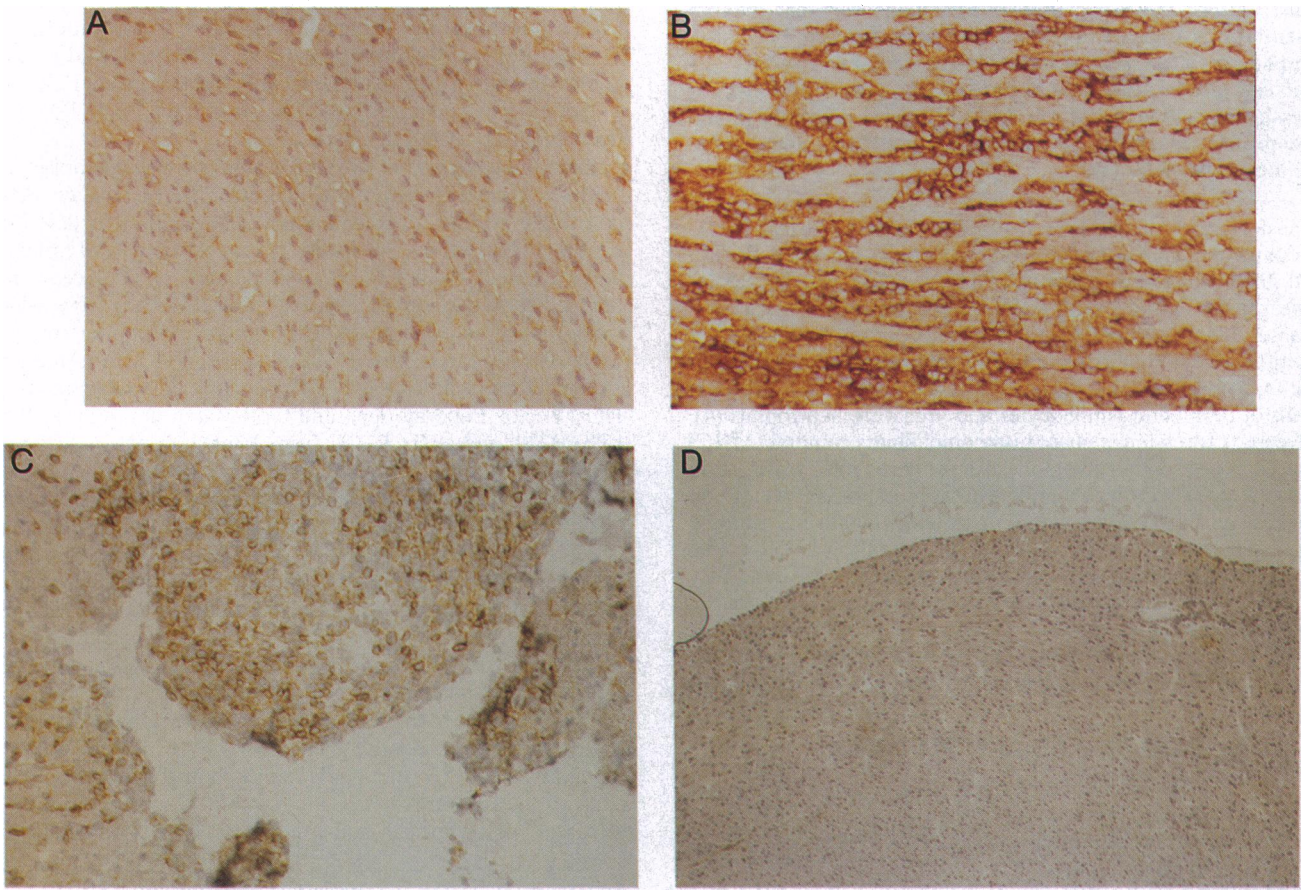

FIG. 1. Anti-CD11α treatment suppresses cardiac inflammation of TGFβ1-null mice. (*A*) Wild-type cardiac tissue stained with anti-ICAM-1. (*B*) TGFβ1-null heart stained with anti-ICAM-1 revealing elevated levels of ICAM-1 expression. (*C*) TGFβ1-null heart stained with anti-TCRα/β revealing the presence of T lymphocytes. (*D*) Anti-TCRα/β-stained heart from anti-CD11α-treated TGFβ1-null mouse. Note the lack of lymphocytic infiltration in the anti-CD11α-treated tissue. (*A–C*, ×95; *D*, ×45.)

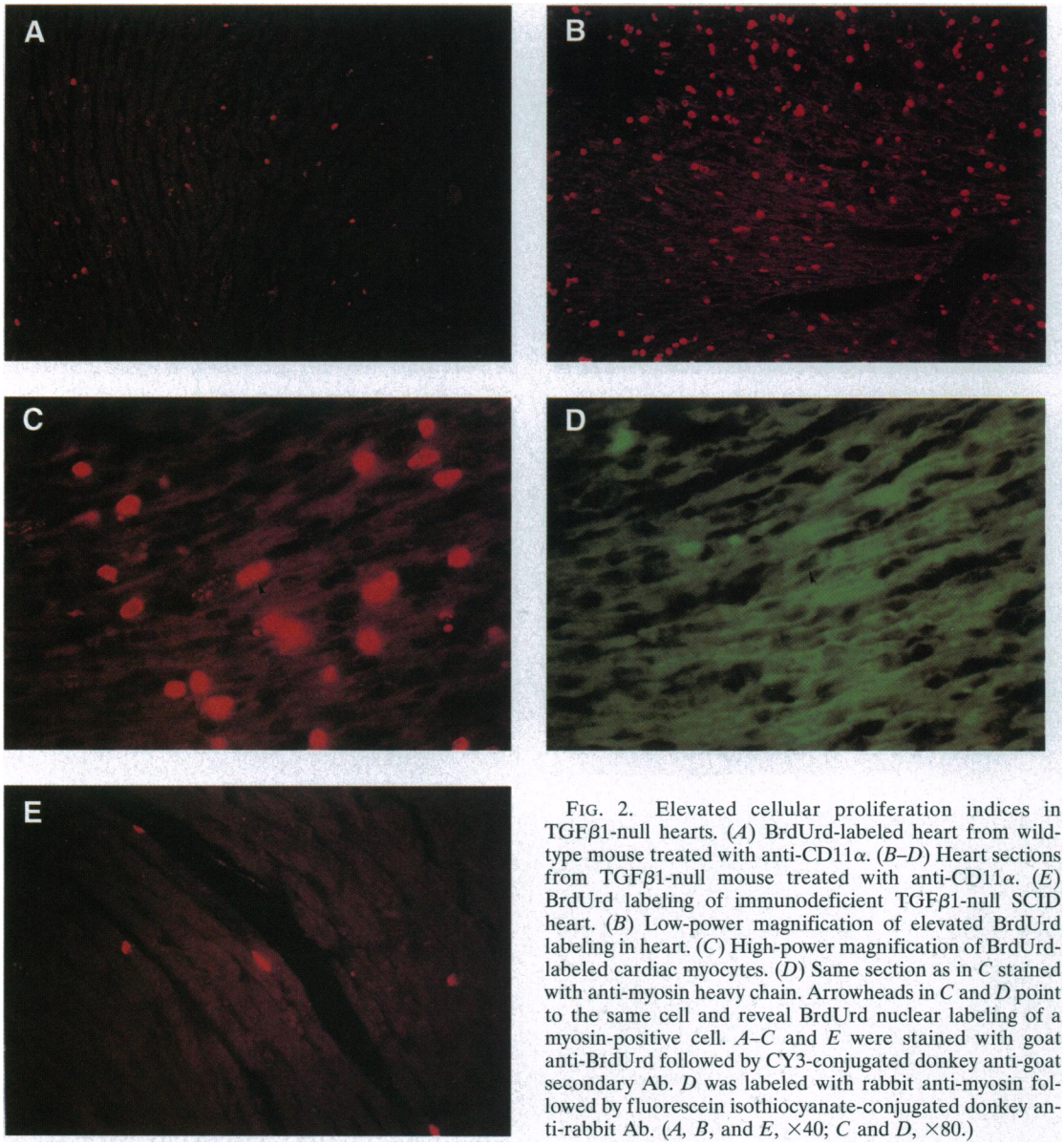

FIG. 2. Elevated cellular proliferation indices in TGFβ1-null hearts. (*A*) BrdUrd-labeled heart from wild-type mouse treated with anti-CD11α. (*B–D*) Heart sections from TGFβ1-null mouse treated with anti-CD11α. (*E*) BrdUrd labeling of immunodeficient TGFβ1-null SCID heart. (*B*) Low-power magnification of elevated BrdUrd labeling in heart. (*C*) High-power magnification of BrdUrd-labeled cardiac myocytes. (*D*) Same section as in *C* stained with anti-myosin heavy chain. Arrowheads in *C* and *D* point to the same cell and reveal BrdUrd nuclear labeling of a myosin-positive cell. *A–C* and *E* were stained with goat anti-BrdUrd followed by CY3-conjugated donkey anti-goat secondary Ab. *D* was labeled with rabbit anti-myosin followed by fluorescein isothiocyanate-conjugated donkey anti-rabbit Ab. (*A*, *B*, and *E*, ×40; *C* and *D*, ×80.)

the hematopoietic system (13–15), it seemed reasonable to suggest that the cardiac hypercellularity and elevated white blood cell counts of the TGFβ1-null mice were simply due to the absence of TGFβ1. To determine whether this was the case, cardiac cell proliferation was measured in immunodeficient TGFβ1-null mice in which there was an absence of lymphocytic infiltration. The total cellular BrdUrd-labeling index was not significantly elevated in cardiac tissue from immunodeficient null mutants (Fig. 2*E*). Similarly, no differences were detected in myocyte-specific labeling (data not shown). Hematological analysis revealed comparable levels of total white blood cell counts and leukocyte populations between TGFβ1-competent and TGFβ1-null SCID mice (Table 1).

To address the requirement for maternally derived TGFβ1 in the developing mouse embryo, immunodeficient TGF-β1-null females were bred with SCID males. One adult TGFβ1-null female became pregnant and carried a litter to term. Five pups were born, three of which were heterozygous and two

Table 1. Hematological profile of anti-CD11α-treated TGFβ1-null and nontreated TGFβ1-null SCID mice

Cells	Anti-CD11α-treated		Nontreated SCID	
	Control	Mutant	Control	Mutant
Total WBCs	2089 ± 746	4644* ± 1652	2257 ± 746	2114 ± 654
Lymphocytes	1481 ± 647	3042* ± 1202	586 ± 223	495 ± 201
Total neutrophils	525 ± 149	1352** ± 984	1603 ± 533	1566 ± 495
Immature neutrophils	44 ± 35	230** ± 221	51 ± 15	49 ± 33
Monocytes	72 ± 51	251* ± 184	52 ± 40	51 ± 17

Data are presented as the mean absolute number of cells per mm^3 of blood (±SD). Control refers to wild-type or heterozygous animals, whereas mutant refers to mice homozygous for the TGFβ1-null allele. Anti-CD11α refers to animals treated with Abs to CD11α. Significant differences of $P < 0.01$ (*) and $P < 0.05$ (**) were determined by *t* test of paired comparisons between age-, parent-, and, whenever possible, sex-matched mutant and control animals within anti-CD11α (nine pairs) or SCID (seven pairs) sets. No significant differences were noted between TGFβ1 wild-type SCID and mutant SCID animals. WBCs, white blood cells.

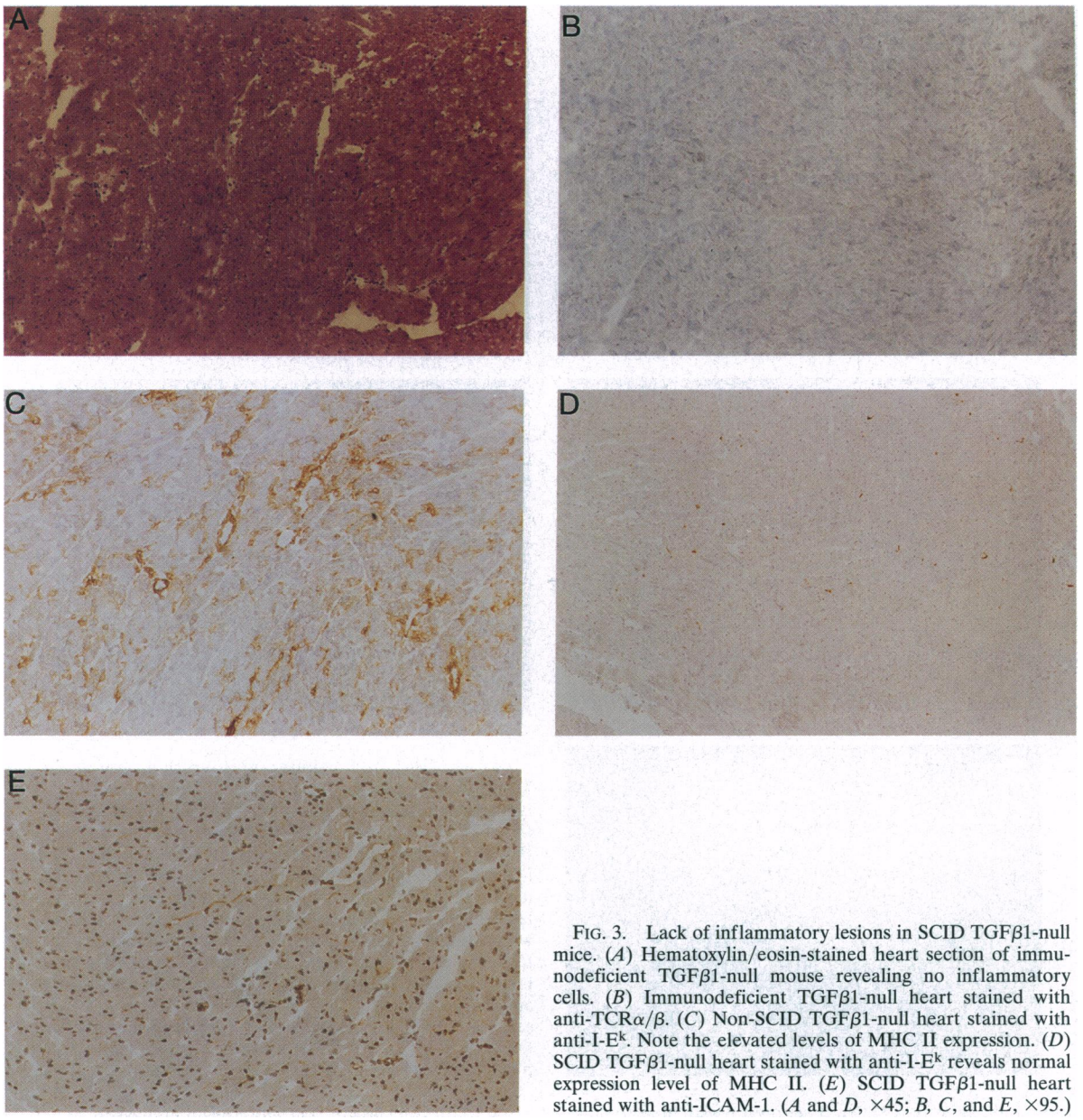

FIG. 3. Lack of inflammatory lesions in SCID TGFβ1-null mice. (*A*) Hematoxylin/eosin-stained heart section of immunodeficient TGFβ1-null mouse revealing no inflammatory cells. (*B*) Immunodeficient TGFβ1-null heart stained with anti-TCRα/β. (*C*) Non-SCID TGFβ1-null heart stained with anti-I-E^k. Note the elevated levels of MHC II expression. (*D*) SCID TGFβ1-null heart stained with anti-I-E^k reveals normal expression level of MHC II. (*E*) SCID TGFβ1-null heart stained with anti-ICAM-1. (*A* and *D*, ×45; *B*, *C*, and *E*, ×95.)

homozygous for the TGFβ1-null allele. The two homozygous null pups were indistinguishable from their littermates and at day 18 were analyzed. No abnormalities were found in the heart in terms of gross morphology (Fig. 4*A*) or disorganized ventricular muscle (Fig. 4 *B* and *C*). Similarly, no other organs of the TGFβ1-null pups born to the TGFβ1-null mother were found to exhibit gross abnormalities or pathological lesions.

Table 2. Occurrence of inflammation in TGFβ1-null SCID mice

Mice	Heart	Lung	Liver	Stomach	Diaphragm	Pancreas
TGFβ1(−/−) SCID	0/15	2*/15	0/15	0/15	0/15	0/15
TGFβ1(−/−) non-SCID	6/9	5/9	4/9	5/9	6/9	5/9

Data is presented as the number of animals scored positive for inflammation per number of animals analyzed. Ages of TGFβ1(−/−) non-SCID mice ranged from 14 to 17 days, during which time the animals displayed a moribund appearance. TGFβ1(−/−) SCID mice ranged from 17 to 148 days and did not exhibit a moribund phenotype. *Two animals developed bacterial pneumonia and were analyzed at day 21.

DISCUSSION

The immunodeficient TGFβ1-null mice reveal an essential role for lymphocytes in the early-onset inflammation that invariably occurs in the immunocompetent mutants. Further, these results indicate a vital *in vivo* function for TGFβ1 in regulating lymphocytic activation and proliferation, which contribute to the maintenance of self tolerance. Although immunodeficient TGFβ1-null mice survive into adulthood, they are routinely 50–80% the size of their TGFβ1-expressing SCID littermates and do not thrive. This general lack of vigor implicates other important physiological homeostatic functions for TGFβ1. Nonetheless, immunodeficient TGFβ1-null mice will be useful for reconstituting the inflammatory phenotype and for analyzing the molecular and cellular effector mechanisms giving rise to widespread inflammation in the absence of TGFβ1.

The elevated numbers of circulating leukocytes reported here, along with the histopathologic analysis of the heart, suggest that anti-CD11α therapy inhibits the extravasation of inflammatory cells that are, nonetheless, proliferating in the TGFβ1-null mutants. The apparent lack of elevated numbers of circulating

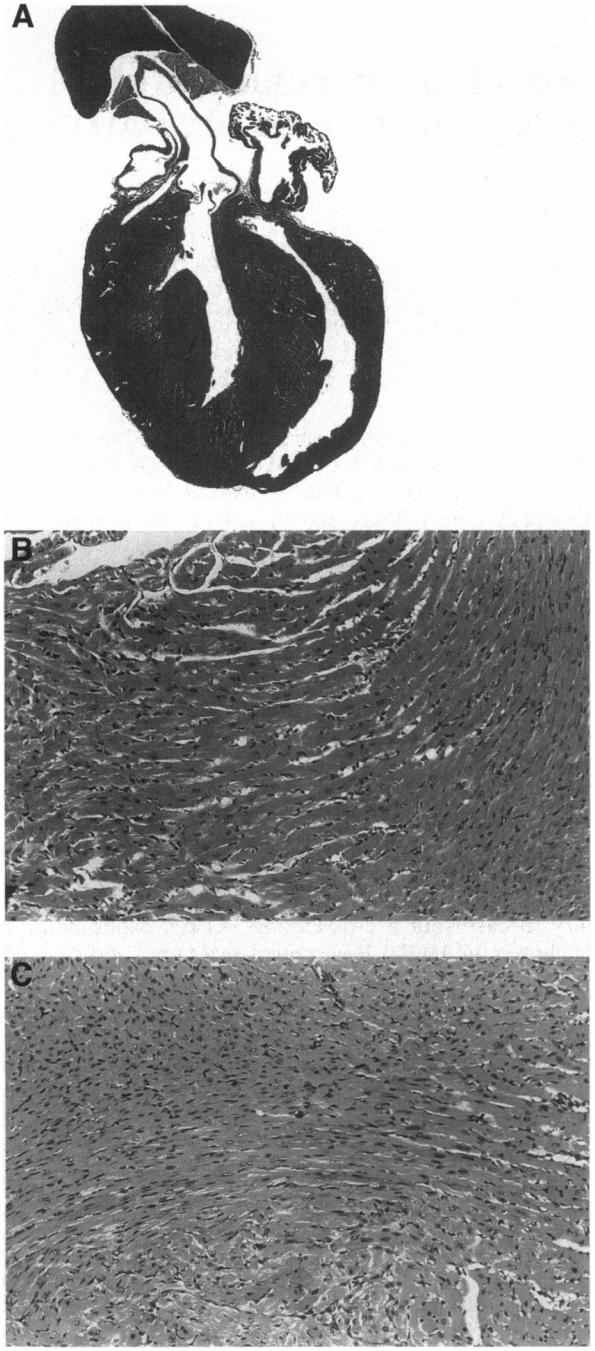

FIG. 4. TGFβ1-null pups born to a TGFβ1-null dam do not display any gross cardiac morphological abnormalities. (*A* and *B*) Hematoxylin/eosin (H&E)-stained heart from an 18-day-old TGFβ1-null pup born to TGFβ1-null dam. (*C*) H&E-stained heart from an 18-day-old TGFβ1-positive littermate. (*A*, ×30; *B* and *C*, ×95.)

lymphocytes and mature neutrophils in untreated mutants as previously reported (4) is consistent with extravasation and migration of these cells into surrounding tissues. The anti-CD11α-treated mutants also indicate that the enhanced cellular proliferation observed in the heart and blood of the TGFβ1 mutants is a consequence of inflammation. This proliferative response likely results, in part, from the production of cytokines synthesized by TGFβ1-null lymphocytes and/or by an increased sensitivity to such factors by peripheral tissues lacking TGFβ1. Some obvious candidate cytokines whose activities and expression are modulated by TGFβ1, and which are elevated in the TGFβ1-null mice, include tumor necrosis factor α, interferon γ, and interleukin 1β (4).

Proinflammatory cytokines could also contribute to the elevated expression of cell adhesion (16) and MHC molecules (17). The observations described here indicate that the levels of MHC II and ICAM-1 are not elevated in inflammation-free hearts of adult immunodeficient TGFβ1-null mice, although they undoubtedly contribute to the progressive pathology observed in the immunocompetent mutants.

Contrary to a previous report (18), we find no gross abnormalities in TGFβ1-null pups born to a TGFβ1-null immunodeficient female. In the earlier study the mother had been kept alive by dexamethasome treatment which diminished, but did not eliminate, tissue inflammation similar to what is observed with anti-CD11α treatment. This suggests that inflammation and/or steroid treatment during pregnancy may have contributed to the congenital heart defects observed in the TGFβ1-null pups. Just as TGFβ1 modulates the inflammatory effects on cardiac growth in adults, so may it carry out a similar function *in utero* to protect the fetus from chronic inflammation in the mother. Thus it would appear that, at least on the strain C3H background, TGFβ1 is not essential for heart development.

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