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Stat5 Synergizes with T Cell Receptor/Antigen Stimulation in the Development of Lymphoblastic Lymphoma

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Abstract

Signal transducer and activator of transcription (STAT) proteins are latent transcription factors that mediate a wide range of actions induced by cytokines, interferons, and growth factors. We now report the development of thymic T cell lymphoblastic lymphomas in transgenic mice in which Stat5a or Stat5b is overexpressed within the lymphoid compartment. The rate of lymphoma induction was markedly enhanced by immunization or by the introduction of TCR transgenes. Remarkably, the Stat5 transgene potently induced development of $CD8^+$ T cells, even in mice expressing a class II–restricted TCR transgene, with resulting $CD8^+$ T cell lymphomas. These data demonstrate the oncogenic potential of dysregulated expression of a STAT protein that is not constitutively activated, and that TCR stimulation can contribute to this process.

Key words: Stat5 • TCR • lymphoma • DNA microarray • $CD8^+$ T cell

Introduction

The Janus family tyrosine kinase (JAK)–signal transducer and activator of transcription (STAT)* pathway is a major signaling pathway used by cytokines and interferons (1–3). STAT proteins have also been linked to oncogenesis (4). Constitutive activation of STATs, as evidenced by constitutive tyrosine phosphorylation and DNA binding activity, has been found associated with cellular transformation (5–8) and in various human tumors (4, 9, 10), and correspondingly, a constitutively activated form of Stat3 is oncogenic (11). Recently, retroviral insertional mutagenesis (RIS) in the Stat5a gene was found in a murine pre-B cell lymphoma that also exhibits constitutive STAT activation (12), and we have found RISs associated with Stat5b as well as Stat5a in two cases of murine AKXD Burkitt-like B cell lymphoma (online supplemental Fig. S1). Interestingly, in one form of acute promyelocytic leukemia, Stat5b is part of a retinoic acid receptor (RAR) fusion oncoprotein, but this lacks the Stat5b SH2 domain and acts based on its ability to repress normal RAR function and enhance IL-6–mediated activation of Stat3 (13, 14).

We now report the development of T cell lymphoblastic lymphomas in transgenic mice expressing nonactivated (HA-tagged WT) forms of either Stat5a or Stat5b and thereby provide direct evidence for the role of Stat5 in oncogenesis. Moreover, we demonstrate in these mice that a marked synergistic effect in the rate of tumor development is conferred by antigen-activation or by coexpression of either MHC class I– or class II–restricted TCR transgenes.

Materials and Methods

Generation of Mice. Stat5a and Stat5b transgenic mice were generated and bred onto $Stat5a^{-/-}$, $Stat5b^{-/-}$, or $Stat5a^{-/-}Stat5b^{-/-}$ double knockout backgrounds as part of a series of experiments to evaluate functional similarities and differences between Stat5a and Stat5b (15), as well as onto WT C57BL/6 and BALBc back-

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**Abbreviations used in this paper:* EMSA, electrophoretic mobility shift assay; STAT, signal transducer and activator of transcription.

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grounds. Stat5 transgenic mice were also mated with mice expressing a TCR transgene specific for a peptide of pigeon cytochrome c (amino acids 81–104) presented by the MHC class II molecule I-Ea/k (TCR-Cyt-5C.C7-I/RAG2$^{-/-}$ mice; Taconic). We analyzed CD4/CD8 and CD44/IL-2Rα (CD25) profiles (gated on CD4$^-$/CD8$^-$ [DN]) of mice that were heterozygous for the Stat5b and 5C.C7 TCR transgenes and RAG2 on the H-2$^{k/d}$ or H-2$^{k/b}$ MHC backgrounds. Similarly, Stat5b transgenic mice were mated with HY TCR transgenic to generate mice that were heterozygous for the Stat5b and HY TCR transgenes and RAG2 on the H-2$^{b/b}$ MHC background. Stat5b transgenic, 5C.C7 TCR transgenic, and 5C.C7 TCR/Stat5b transgenic mice were immunized intraperitoneally with 100 μg of cytochrome c (Sigma-Aldrich) mixed with 100 μl CFA (Pierce Chemical Co.).

Mice lacking expression of both the common cytokine receptor γ chain (γ_c) and RAG2 (Taconic) were injected subcutaneously with a mixture of 10^6 fresh thymic and cervical lymph node tumor cells. Tissues (thymus, spleen, and lymph nodes) were fixed by immersion in 10% formalin and embedded in paraffin by standard procedures. Sections were stained with hematoxylin and eosin. All experiments were performed under protocols approved by the NIH Animal Use and Care Committee and followed the NIH guidelines "Using Animals in Intramural Research."

Flow Cytometric Analysis. Single-cell suspensions from thymus, spleen, and lymph node were stained and analyzed using a FACSort® with CELLQuest™ software (Becton Dickinson). Anti-CD4-FITC, -PE, and -Cy-Chrome; anti-CD8-PE and -APC; anti-IL-2Rα (CD25)-FITC and-PE; anti-CD44-Cy-Chrome; anti-IL-2Rβ-FITC and-PE; anti-pan-NK cells (DX5)-FITC; anti-Vβ2–6, 8, 10, 11-FITC and-PE; Vα11-FITC; and anti-CD3-APC were from BD Biosciences.

Western Blotting. Whole cell extracts (5–20 μg/sample) were fractioned on 8% SDS polyacrylamide gels (Invitrogen/NOVEX™) and transferred to Immobilon-P membranes (Millipore). After blocking with 5% milk, the blots were incubated with rabbit anti-phospho-Stat5 (Tyr694), anti-phospho-Stat3 (Tyr705; Cell Signaling Technology) or mouse anti-HA (Santa Cruz Biotechnology, Inc.), washed, and incubated with a horseradish peroxidase-conjugated anti–rabbit or anti–mouse antibody (Nycomed Amersham). Blots were developed with an enhanced chemiluminescent substrate (Pierce Chemical Co.).

Electrophoretic Mobility Shift Assays. Electrophoretic mobility shift assays (EMSAs) were performed using nuclear extracts and a β-casein probe as described previously (16). For supershifting assays, nuclear extracts were preincubated for 20 min with antibodies to Stat3 or Stat5b (Zymed Laboratories).

RNA Purification and Affymetrix Gene Chip Analysis. RNA was isolated using a kit (RNeasy®; QIAGEN) and processed to cRNA probes for GeneChip® analysis according to the manufacturer's instructions (Affymetrix, Inc.). The probes were hybridized to U75A GeneChip® (Affymetrix, Inc., with oligonucleotides corresponding to 12,489 transcripts per microarray), washed, and scanned (Hewlett Packard Gene Array scanner G2500A) according to procedures outlined by the manufacturer (Affymetrix, Inc.).

Data were analyzed using "unsupervised" hierarchical clustering (17, 18). This approach "seeks structure inherent in the data and assumes no a priori classification of genes or samples, with the goal of identifying related expression pathways of genes or samples"(17). Results corresponding to 4372 genes whose expression was detected ('present'; see online supplemental material) in all groups (WT spleen [WT^S], Stat5b transgenic spleen [TG^S], WT CD8 splenocytes [CD8WT^S], Stat5b transgenic CD8 T splenocytes [CD8TG^S], WT thymus [WT^T], Stat5b transgenic thymus [TG^T], lymphoma spleen [ONCO^S], and lymphoma thymus [ONCO^T]) were expressed as a dendogram (samples with the most similar expression profiles are directly connected and those less similar are connected through additional "branches" of the dendogram "tree"). Further analysis was done using 7195 genes whose expression was detected ('present') in splenic and/or thymic groups. We identified 1512 genes that were more highly expressed in ONCO^S or ONCO^T than in at least one of the other groups listed above for further examination. The raw data and a complete list of the genes will be available at www.nhlbi.nih.gov/labs/supplements.

To examine gene expression patterns over time, 5C.C7 TCR transgenic mice, with and without the Stat5b transgene, were killed at 2, 3, 6, 12 or 13 wk of age. CD8$^+$ thymocytes were enriched using CD4 paramagnetic beads and an autoMACS (Miltenyi Biotec). RNA was extracted and hybridized to U75A GeneChips®, as described above, and data were analyzed using hierarchical clustering (18). Analysis was performed using unsupervised hierarchical clustering (as above) for 6250 genes whose expression was detected ("present"; see online supplemental material) in any of four groups (5C.C7 single transgenic [group 1], Stat5b/5C.C7 double transgenic 2–3 wk old [group 2], Stat5b/5C.C7 double transgenic 6–8 weeks old [group 3], Stat5b/5C.C7 double transgenic 12–13 wk old [group 4]) were presented as a dendogram. In addition, genes that were found to be increased in lymphomas in Stat5b transgenic mice (see Fig. 4 C) were examined over time in 5C.C7 and Stat5b/5C.C7 mice.

Isolation and Stimulation of Enriched CD4$^+$/CD8$^+$ DP Thymocytes. CD4$^+$/CD8$^+$ (DP) thymocytes were enriched using CD8α paramagnetic beads and an autoMACS (Miltenyi Biotec), and 4×10^7 cells were cultured in 10 ml for 16 h with phorbol-12-myristate-13-acetate (PMA, 0.2 ng/ml) + ionomycin (0.2 μg/ml; Calbiochem). Cells were then washed and cultured in RPMI 1640 medium containing 10% FBS and 2 mM L-glutamine, and antibiotics (complete medium) for 48 h (day 3 cells; reference 19).

Proliferation Assays. Fresh thymocytes were cultured for 48 h in complete medium, with or without 1 nM IL-7, in 96 well flat-bottom plates (2×10^5 cells/well) with and without soluble anti-CD3 (2 μg/ml) plus anti-CD28 (2 μg/ml). Wells were pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; NEN Life Science Products) for the last 9 h of culture. Thymocytes from heterozygous TCR-Cyt-5C.C7-I/RAG2$^{+/-}$ cytochrome c TCR transgenic expressing the Stat5b transgene, with and without lymphoma, were incubated with cytochrome c (200 μg/ml; Sigma-Aldrich) for 72 h. Wells were pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; NEN Life Science Products) for the last 9 h of culture.

Online Supplemental Material. Supplemental Fig. S1 shows RISs in the Stat5a/Stat5b genomic locus in two mice with AKXD Burkitt-like lymphoma. This suggests the oncogenic potential of RISs in either the Stat5a or Stat5b loci. Supplemental Table S1 shows a complete listing of genes from the microarray analyses presented in Fig. 4, B and C. Supplemental Table S2 shows a complete listing of genes from the microarray analyses presented in Fig. 7 E. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20021548/DC1.

Results

Mice Overexpressing Stat5 Develop CD8$^+$ Lymphoblastic Lymphoma. Stat5a and Stat5b are highly-related proteins that are required for normal lactation, growth, hemato-

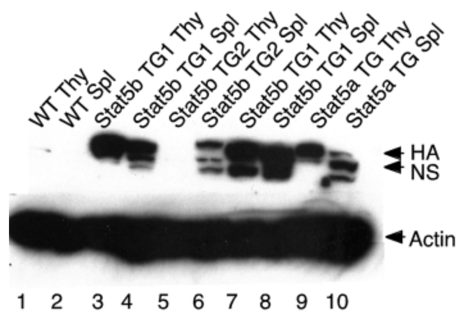

Figure 1. Anti-HA Western blotting of splenic and thymic lysates from two Stat5b transgenic lines (TG1 and TG2) and one Stat5a line (TGA). WT lysates are included as controls.

poiesis, and immune function (20–26). We generated Stat5a and Stat5b transgenic mice, using a vector containing the H-2K^b promoter and IgM enhancer, in order to evaluate distinctive versus overlapping actions of these highly homologous proteins (>90% identical; reference 27) within the lymphoid system. Two Stat5b founder lines (TG1, TG2) and one Stat5a line that expressed the appropriate transgene in the immune system were evaluated (Fig. 1; reference 15). Both Stat5a and Stat5b transgenic mice have increased splenic CD8$^+$ T cells at the earliest point examined (12 d; reference 15). Unexpectedly, as the mice aged, many developed striking lymphadenopathy that we noted at a median age of 205 d (range 78–456 d, Table I and Fig. 2, A and B), with enlarged thymuses (unpublished

Table I. *Lymphomas in Stat5 Transgenic Mice*

Transgene	Genetic background[a]	Mouse ID[b]	Age days[c]	Tumor phenotype[d]		TCR Vβ[d]
				$CD4^+CD8^+$	$CD4^-CD8^+$	
Stat5b TG1[e]	$Stat5a^{+/-}$ $Stat5b^{+/-}$ (C57BL/6 × 129)	Onco 1	92	ND	ND	ND
		Onco 2	155 (E)	+	+	Vβ10
		Onco 3	241 (E)	−	+	Vβ4
		Onco 4	270 (E)	+	+	Vβ3
		Onco 5	353	ND	ND	ND
		Onco 6	370 (E)	−	+	Vβ6
	$Stat5a^{+/-}$ (C57BL/6 × 129)	Onco 7	78	ND	ND	ND
		Onco 8	337	ND	ND	ND
	$Stat5a^{-/-}$ $Stat5b^{-/-}$ (C57BL/6 × 129)	Onco 9	133 (E)	+	+	ND
	WT (C57BL/6)	Onco 10	113 (E)	+	+	Vβ10
		Onco 11	162 (E)	+	+	ND
		Onco 12	196 (E)	+	+	Vβ2
		Onco 13	380 (E)	+	+	Vβ2
		Onco 14	456	ND	ND	ND
Stat5b TG2[e]	$Stat5a^{-/-}$ (C57BL/6)	Onco 15	253 (E)	+	+	ND
Stat5a TG[e]	$Stat5a^{-/-}$ (C57BL/6)	Onco 16	116	ND	ND	ND
		Onco 17	234	ND	ND	ND
		Onco 18	289	ND	ND	ND
	$Stat5b^{-/-}$ (C57BL/6 × 129)	Onco 19	207 (E)	+	+	ND
	WT (C57BL/6)	Onco 20	162 (E)	+	+	Vβ3

Characteristics of the first 20 Stat5a and Stat5b transgenic mice that developed lymphoblastic lymphoma, including genetic background, age, and cell surface phenotypes.
[a]Background is either C57BL/6, or mixed C57BL/6 × 129; with varied Stat5 knockout backgrounds as indicated.
[b]ID = arbitrary mouse number, mice are referred to as Onco (oncogenesis), and grouped for convenience.
[c]E = euthanasia at stated age; other mice were found dead at the stated age.
[d]Determined by cell surface staining.
[e]Rate of tumor formation is 25% for TG1, 5% for TG2, and 8% for the Stat5a TG.

data), and spleens (Fig. 2 C). Normal lymph node architecture was disrupted by neoplastic lymphocytes (Fig. 2 D) with cytologic features consistent with the diagnosis of T cell lymphoblastic lymphoma (Fig. 2 E). The first 20 lymphomas occurred in 15 Stat5b transgenic mice (14 from the TG1 and one from the TG2 transgenic lines), and five Stat5a transgenics (Table I). The Stat5b transgenic line with the highest expression within the thymus (TG1) had the highest rate of tumor formation (25% of 60 mice over 4 mo old), the Stat5a transgenic line had intermediate rate of tumor formation (8% of 65 mice over 4 mo old), while Stat5b TG2 line had only 1 lymphoma out of 20 mice over 4 mo old, correlating thymic Stat5 transgenic expression (Fig. 1, lanes 3, 5, and 9) and lymphoma formation. Initially, mice were bred onto Stat5a$^{-/-}$, Stat5b$^{-/-}$, or Stat5a$^{-/-}$/Stat5b$^{-/-}$ backgrounds as part of a study to compare the actions of Stat5a and Stat5b, but lymphomas have also developed on a WT background (Table I).

Of the 12 lymphomas from Table I analyzed by flow cytometry, 10 had populations of both CD4$^+$CD8$^+$ double positive and CD8$^+$ single positive cells (Table I, Onco 2, 4, 9–13, 15, 19, and 20; Fig. 3 A, Onco 12, panels a, b, and c) and two had predominantly CD8$^+$ cells (Table I, Onco 3 and 6; Fig. 3 A, Onco 3, panels e, f, and g) in thymus, spleen, and lymph nodes. Of eight mice examined for TCR-β usage, one exhibited two prominent populations and seven a single prominent population based on Vβ staining (e.g., Onco 12 cells stained only for Vβ2 (Table I and Fig. 3 B, panels a versus b), while Onco 3 had only Vβ4$^+$ staining (Table I, and Fig. 3 B, panels c versus d). We confirmed the monoclonal nature of the tumor in two lymphomas by PCR analysis of TCRβ rearrangements (reference 28; unpublished data). Subcutaneous injection of malignant cells into γ_c/RAG2 double KO mice resulted in tumor formation at the site of injection; cells from these tumor masses had flow cytometric profiles indistinguishable from the original transplanted donor cells (Fig. 3 A, panels d versus a, b, c and h versus e, f, g; and Fig. 3 B, panels g versus a, and h versus d). Thus, both Stat5a and Stat5b transgenes predisposed to development of T cell lymphoblastic lymphoma.

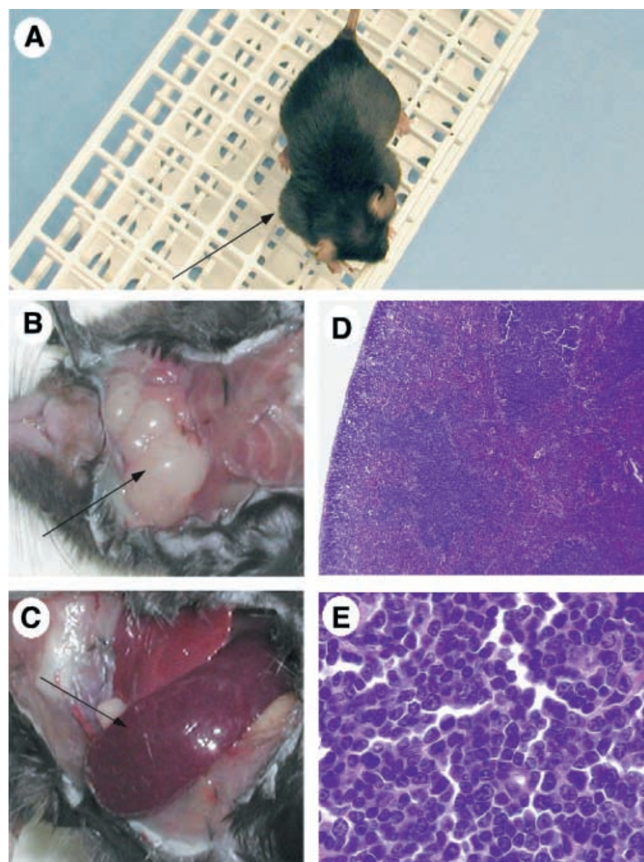

Figure 2. Lymphoblastic lymphoma in a representative Stat5b transgenic mouse. Prominent cervical adenopathy (A and B) and splenomegaly (C) were evident. (D and E) A representative cervical lymph node with round nuclei with stippled chromatin, prominent nucleoli, and frequent mitotic figures.

Neither Stat5 Nor Stat3 Is Constitutively Active in the Lymphoma Tissues. Oncogenesis has been correlated previously with the constitutive expression of activated STAT proteins (4, 11, 29). Surprisingly, however, we detected little if any constitutively-activated Stat5 or Stat3 in lymphoma tissues by anti-phospho-Stat5 Western blotting (Fig. 3 C, lanes 4–7), by anti-phospho-Stat3 (Fig. 3 D, lanes 2–5), or by electrophoretic mobility shift assays (EMSAs; Fig. 3 E, lanes 4, 7, 12, 14, and 17). This is consistent with our transgene being a WT (i.e., not constitutively activated) form of Stat5. However, stimulation of lymphocytes in vitro with IL-2 or IL-7, two cytokines that can activate Stat5, potently augmented DNA binding activity in Stat5b tumor lysates to levels higher than seen in lysates from WT mice (Fig. 3 E, lanes 5, 6, 13, and 18 versus 2, 3, 9, 16, and 22). As expected, Stat5b was a major protein in the binding complex based on supershifting with anti-Stat5b (lane 20), whereas anti-Stat3 had no effect (lane 19). Immunoprecipitation with anti-HA, followed by anti-phospho-Stat5 Western blotting, confirmed phosphorylation of the transgene in lymphoid tissues from Stat5b transgenic mice with and without lymphoma (reference 15, and unpublished data).

DNA Microarray Indicates the Lymphomatous Cells Have Characteristics of Immature Thymocytes. To investigate if the transgene augmented or repressed expression of certain genes, we next performed microarray analysis of mRNAs isolated from WT and Stat5b transgenic mice. Despite the effects of Stat5 on peripheral CD8$^+$ T cell homeostasis (15), there was little difference between the splenic gene expression patterns of WT and Stat5b transgenic mice (Fig. 4 A, "splenic cluster"). In contrast, the expression pattern seen in Stat5b transgenic lymphomatous spleens was more similar to that of WT thymuses (Fig. 4 A, "thymic cluster"). The augmented expression in the tumors of genes such as TCRγ, RAG1, RAG2, and TdT (terminal deoxytransferase) that are involved in T cell development (Fig. 4 B) is consistent with the diagnosis of precursor T cell lymphoblastic lymphoma (30). Microarray analysis also identified a group of genes whose increased expression appeared

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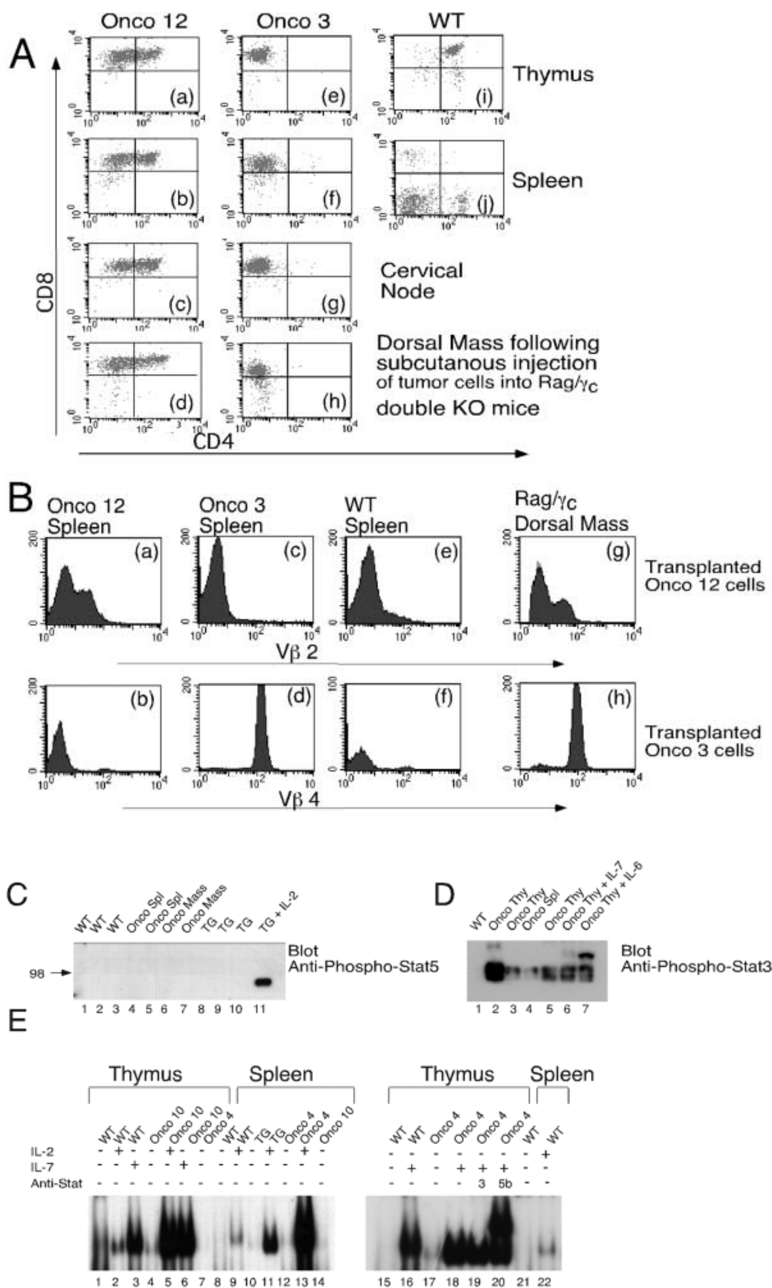

Figure 3. Analysis of lymphoblastic lymphoma cells from Stat5 transgenic mice. (A) Augmented $CD4^+/CD8^+$ and/or $CD8^+$ T cells in Stat5b transgenic mice. Flow cytometric analysis of cells from thymus (panels a, e, and i), spleen (panels b, f, and j), and cervical lymph node (panels c and g). Also shown is the CD4/CD8 profile of subcutaneous cervical lesions in a RAG/γ_c double KO mouse 3 wk after subcutaneous injection of fresh tumor cells (panels d and h). (B) Vβ2-FITC and Vβ4-PE staining in two representative tumors demonstrating a single population of positively staining cells. Although Onco 12 had a substantial Vβ2-negative population, staining for Vβ3–6, 8, 10, 11, and Vα11 was negative. Tumor masses resulting from the injection of lymphoma cells into RAG/γ_c double KO mice maintained the same expression profile as the donor cells (panels g vs. a and h vs. d). (C) No constitutive phosphorylation of Stat5a or Stat5b, as evaluated by anti-phospho-Stat5 Western blotting, is seen in the tumor lysates (lanes 4–7), whereas the positive control (Stat5b transgenic splenocytes stimulated with IL-2 for 30 min) confirmed the function of the anti-phosphoStat5 antibody (lane 11). (D) No constitutive phosphorylation of Stat3 was detected by anti-phospho-Stat3 Western blotting in tumor lysates (lanes 2–5), whereas the positive control (tumor lysates stimulated with IL-6 for 30 min) confirmed the function of the anti-phospho-Stat3 antibody (lane 7). (E) EMSAs using a β-casein probe and the indicated samples. Cells from WT (lanes 1–3, 8, 9, 15, 16, 21, 22), transgenic (TG; lanes 10, 11), or mice with tumors (Onco; lanes 4–7, 12–14, 17–20) were not stimulated or stimulated with IL-2 or IL-7, and DNA binding activity measured. Supershifting with anti-Stat5 or Stat5b is shown in lanes 19 and 20, respectively.

to be distinctive for the lymphoma tissue regardless of tissue source (Fig. 4 C).

Stat5b Transgenic Thymocytes Exhibit Enhanced Activation and Proliferation. Because of the thymic origin of the lymphoma, we further evaluated thymocytes from WT and Stat5b transgenic mice in which lymphomas had not yet developed. After stimulation with PMA plus ionomycin (PI; Fig. 5 A), or anti-TCRβ plus anti-CD2 (unpublished data), both WT and Stat5b transgenic thymocytes had an increase in the percentage of $CD8^+$ single positive T cells by day 1 (Fig. 5 A, panel c versus a and d versus b), and this increase was preferentially sustained in Stat5b transgenic mice until at least day 3 (Fig. 5 A, panel f versus e). The percentage of $CD4^+$ cells was also increased in response to PI, but little if any difference was seen between WT and Stat5b transgenic mice (Fig. 5 A, panels d versus c and f versus e). The thymocyte differentiation/activation markers CD44 and IL-2Rα (CD25; reference 31) were more highly expressed on thymocytes from Stat5b transgenic mice as compared with WT mice (Fig. 5 B, panels d versus c and f versus e), and these Stat5b transgenic thymocytes proliferated more vigorously than wild type thymocytes in response to anti-CD3 plus anti-CD28, IL-7, or a combination of these stimuli (Fig. 5 C), or to stimulation with anti-TCRβ plus anti-CD2 (unpublished data). Thus, after various exogenous stimuli, Stat5b thymoctes, as compared with WT thymocytes, proliferated more vigorously and exhibited an increase in the number

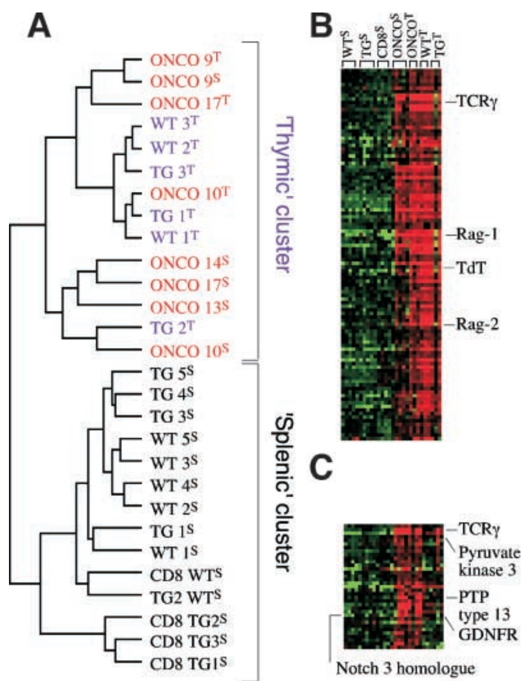

Figure 4. Oligonucleotide (GeneChip®) microarray analysis indicating that the lymphomas are of thymic origin. (A) Shown is a dendogram corresponding to 4372 genes whose expression was detected in all groups (WT^S, TG^S, $CD8WT^S$, $CD8TG^S$, WT^T, TG^T, $ONCO^S$, $ONCO^T$). The "Thymic" and "Splenic" clusters refer to samples whose expression patterns were most similar to those found in normal thymus and spleen, respectively (i.e., they "coclustered"). Note that the pattern observed for $CD8^+$ T cell enriched splenocytes was similar to that observed in total splenocytes. The mice with lymphomas are designated as in Table I. (B) A selection of genes that were similarly expressed in the lymphomas and thymic tissue. (C) Genes that were more highly expressed (>1.4-fold increased) in lymphomas than in any of the WT or TG tissue samples. Green squares correspond to genes with relatively low level of expression and red squares to genes with relatively high level of expression (e.g., in one of the TG^T samples [second from the right] fewer genes were highly expressed). The raw data and a complete list of the genes will be available at www.nhlbi.nih.gov/labs/supplements.

of $CD8^+$ T cells and the percentage of these cells that were activated.

Antigen Stimulation Increases Rate of Lymphoma Formation. Because of the effect of anti-CD3 stimulation on the proliferation and activation of Stat5b transgenic thymocytes, we hypothesized that TCR activation might promote the growth and/or malignant transformation in Stat5b transgenic mice, consistent with a suggested role for immune receptor stimulation in certain lymphoid neoplasms (32, 33). In this regard, coexpression of a TCR transgene greatly augmented thymic $CD8^+$ T cell development in the Stat5b transgenic mice. Remarkably, this occurred not only with the class I–restricted HY TCR trangene (unpublished data), but also with the 5C.C7 class II–restricted TCR trangene (Fig. 6 A, panels b versus a), even though class II restricted TCRs normally direct development in the CD4 pathway. Interestingly, expression of the 5C.C7 TCR transgene increased the percentage of DN1 cells ($CD4^-/CD8^-$ double negative cells that are $CD44^{hi}IL\text{-}2R\alpha^-$; panel d versus c). After immunization, Stat5b/5C.C7 TCR transgenic mice had more $CD8^+$ T cells than mice that expressed only the 5C.C7 TCR transgene (Fig. 6 B, panel b versus a), and there was an increase in the percentage of these $CD8^+$ T cells that were activated ($CD44^{hi}IL\text{-}2R\alpha^+$; Fig. 6 B, panel e versus d). The $CD8^+$ T cells were $V\alpha11^+/V\beta3^+$ (Fig. 6 B, panels g and h), confirming that their selection was via the cytochrome c-specific 5C.C7 TCR (34).

Strikingly, Stat5b transgenic mice coexpressing a TCR transgene developed lymphoma at a much younger age (57 and 70 d, respectively, for HY and 5C.C7 transgenic mice) than Stat5b transgenic mice on a non-TCR transgenic background (median of 205 d; Fig. 6 C, and unpublished data). Stat5b/5C.C7 trangenic mice, whether immunized with cytochrome c in CFA (Fig. 6 B, panel c and f), or not immunized, developed lymphomas that were $V\alpha11^+/V\beta3^+$ (Fig. 6 B, panel i) and vigorously proliferated to IL-7 (Fig. 6 D). Because of the effect of the TCR transgenes, we immunized 6–8-wk-old Stat5b transgenic mice lacking the TCR transgene with ovalbumin/CFA. Remarkably, tumors were evident by 3 mo of age (median 72 d; Fig. 6 C), whereas this was not observed in WT controls. Immunization of Stat5b/5C.C7 double trangenic mice did not alter the age at which these mice developed lymphoma (Fig. 6 C). Thus, malignant transformation in Stat5b transgenic mice was increased by antigenic/adjuvant stimuli. Most of the thymic tumors arising in younger mice were detected before obvious splenic involvement, as evaluated by CD4/CD8 and $V\alpha/V\beta$ profiles.

Analysis of Stat5b/5C.C7 Double Transgenic Mice Over Time. To investigate the mechanism underlying the oncogenic transformation, we analyzed Stat5b/5C.C7 double transgenic mice over time. As noted above, these mice develop lymphoblastic lymphoma at a median age of 70 d, and all double transgenic mice over 3 mo of age we have studied had lymphoma.

Unlike their 5C.C7 littermates, Stat5b/5C.C7 double transgenic mice exhibit an expansion of $CD8^+$ T cells over time (Fig. 7, A and B). This expansion of $CD8^+$ T cells was evident at two weeks of age (Fig. 7 B, panel g versus a) and was marked in the older double transgenic mice (Fig. 7 B, panels i and j). 5C.C7 mice without the Stat5b transgene exhibited either of two phenotypes: an age-dependent deletion of thymocytes (Fig. 7 B, panels c and d, note the low cellularity) or an expansion of $CD4^+$ cells (Fig. 7 B, panels e and f).

Proliferation of thymocytes to anti-CD3 stimulation was greater in the Stat5b/5C.C7 mice than in 5C.C7 littermates (Fig. 7 C). This was evident in mice as young as 2 wk old and increased over time until 8 wk of age. The older double transgenic mice (12–13 wk old) had enlarged thymuses consistent with lymphoblastic lymphoma, and some of these mice had more vigorous thymocyte proliferation in response to anti-CD3 stimulation, while others had a more vigorous response to IL-7 (Fig. 7 C, and unpublished data).

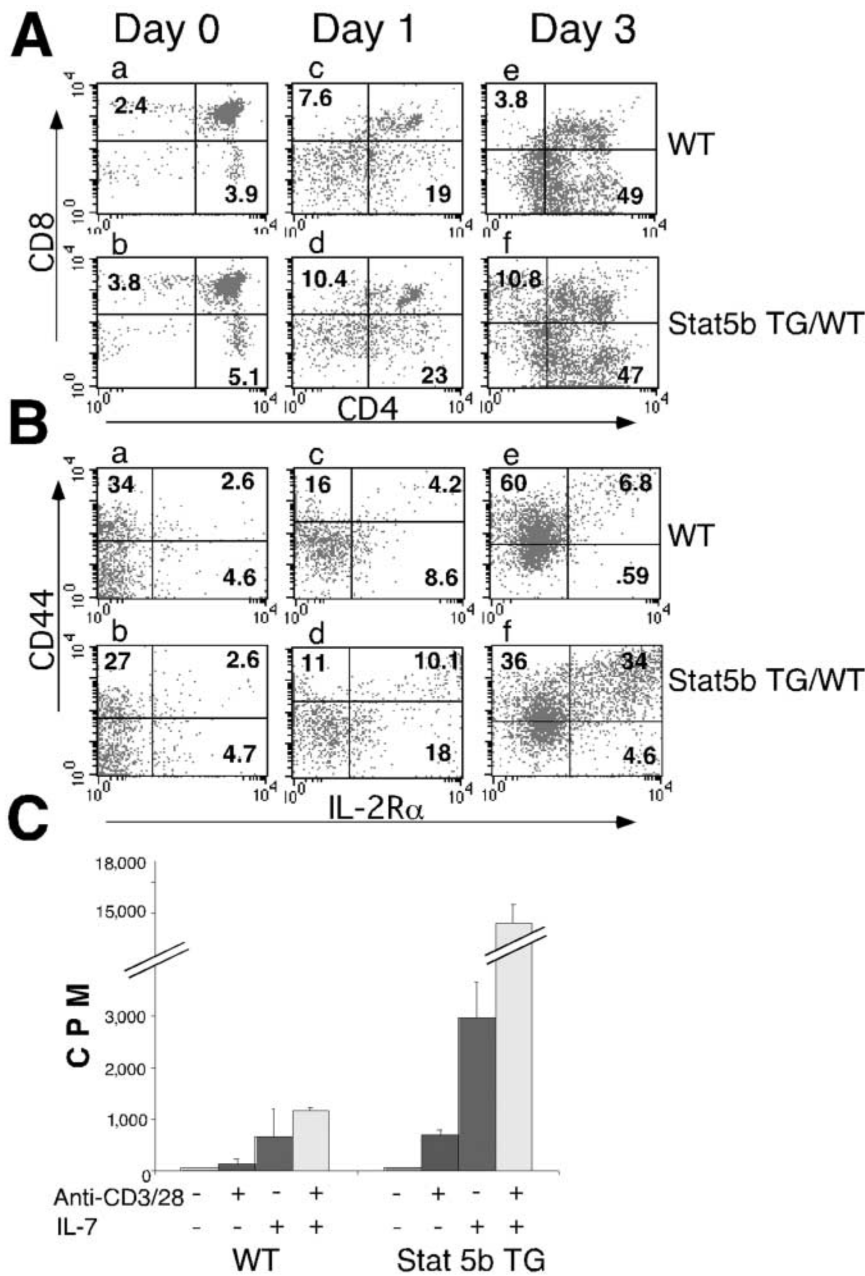

Figure 5. Effect of the Stat5b and TCR transgenes on CD4/CD8 flow cytometric profiles, activation state, and proliferative potential of thymocytes. (A) CD4/CD8 profiles for wild type vs. Stat5b transgenic thymocytes. $CD4^+$/$CD8^+$ (DP) thymocytes (day 0) were cultured with PMA + ionomycin for 24 h (day 1) followed by 48 h in RPMI (day 3 cells; reference 19). Similar results were obtained if anti-TCRβ (H57–597) + anti-CD2 (RM2–5) was used instead of PMA + ionomycin. (B) CD44/IL-2Rα profiles (gated on total population) for the same cells as in A. (C) Stat5b transgenic thymocytes exhibit augmented proliferation to IL-7, to anti-CD3 + anti-CD28, or to both stimuli.

We next evaluated gene expression in $CD8^+$ thymocytes over time using microarrays. Unsupervised hierarchical clustering revealed that the expression profiles of $CD8^+$ T cells from 5C.C7 single transgenic mice, regardless of age, were similar and formed a distinct cluster (Fig. 7 D). Conversely, the expression profiles of $CD8^+$ T cells from the Stat5b/5C.C7 double transgenic correlated with age in that the expression profiles of 'premalignant' $CD8^+$ cells of mice between 2 and 8 wk were similar, whereas the profiles of mice 12–13 wk of age with overt tumors formed a separate group in the clustering analysis. In this longitudinal study we also examined the expression over time of those 35 genes that were shown in Fig. 4 C to be more highly expressed in mice with lymphoblastic lymphoma in the WT background. We found that 18 of these 35 genes were more highly expressed in Stat5b/5C.C7 double transgenics than in 5C.C7 single transgenic mice. These 18 genes appeared to fall into two major groups; the expression of 12 genes (Fig. 7 E, group a) was not increased until 12–13 wk of age, a time when the mice exhibited overt evidence of lymphoblastic lymphoma, whereas the other 6 genes (Fig. 7 E, group b) were more highly expressed in Stat5b/5C.C7 double transgenic $CD8^+$ thymocytes from an early age. One gene that was increased in young Stat5b/5C.C7 double transgenic thymocytes, as well as in the lymphoblastic lymphoma in Stat5b/WT mice, is the gene encoding protein tyrosine phosphatase non receptor type 13 (Ptpn13). Interestingly, this gene was not increased in Stat5b trans-

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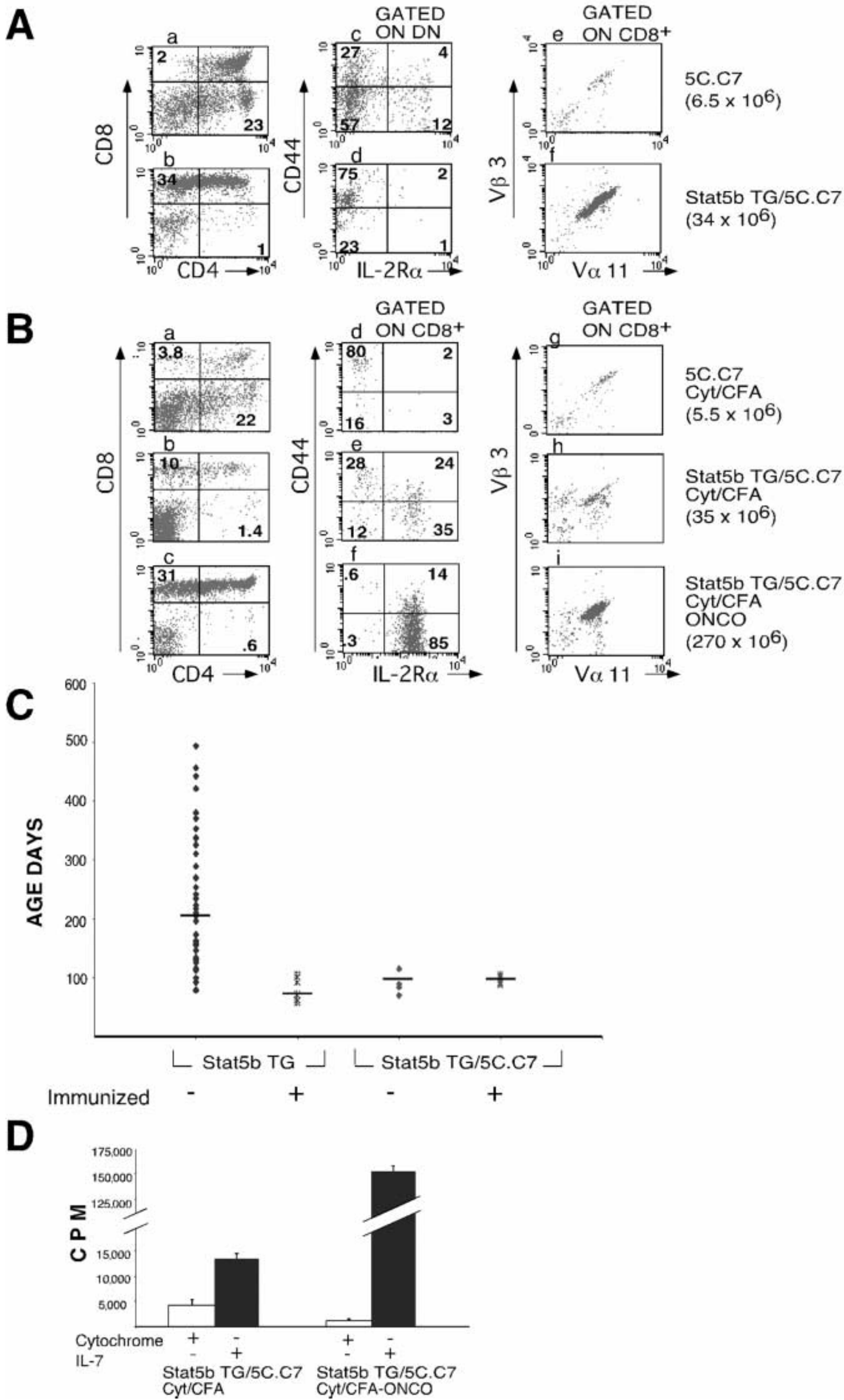

Figure 6. Stat5 transgenic mice are more prone to malignant transformation in the presence of a TCR transgene. (A) Mice that were heterozygous for the Stat5b and 5CC7 TCR transgenes (b and d) exhibited an increase in activated $CD8^+$ T cells as compared with mice only carrying the 5CC7 transgene (a and c). CD4/CD8 (a and b), CD44/IL-2Rα (gated on $CD4^-/CD8^-$ [DN], c and d), and Vβ3/Vα11 (e and f) profiles of mice that were heterozygous for the 5C.C7 TCR transgene and RAG2 (panels a, c, and e) and also for the Stat5b transgene (panels b, d, and f) are shown. (B) 5C.C7 TCR transgenic mice (panels a and d) and 5C.C7 TCR/Stat5b transgenic mice (panels b, c, e, and f) were immunized intraperitoneally with cytochrome c/CFA, and CD4/CD8 (panels a, b, and c) and CD44/IL-2Rα (gated on $CD8^+$, panels d, e, and f) profiles were analyzed upon sacrifice 3 wk later. The mouse represented by panels b, e, and h had no evidence of lymphoma whereas the mouse represented by panels c, f, and i had an enlarged thymus consistent with lymphoblastic lymphoma. In A and B, total thymocyte numbers are shown in parentheses on the far right. (C) Age of diagnosis of lymphomas. Shown are Stat5b transgenic mice, not immunized or immunized with ovalbumin/CFA, and Stat5b/5C.C7 double transgenic mice that were either not immunized or immunized with cytochrome c/CFA. Each point represents a single mouse, and the median for each group is indicated by the horizontal bar. (D) Proliferation of thymocytes from mice that were heterozygous for both the Stat5b and 5CC7 TCR transgenes, with and without lymphoma, after incubation with cytochrome c (200 μg/ml) or 1 nM IL-7 for 72 h.

genic mice on a WT background until the development of lymphoma. Ptpn13 (35) is also known as Fas-associated phosphatase-1 (FAP-1; reference 36) and is a negative regulator of FAS-mediated apoptosis in human cancer cells (37).

Discussion

We previously found that overexpression of Stat5 proteins can increase peripheral $CD8^+$ T cell numbers by augmenting proliferation and cell survival (15), but we did not anticipate that such overexpression would predispose to malignant transformation. The dysregulated expression of the transgenic Stat5 proteins could theoretically result in a state of hyperresponsiveness to cytokines, and in fact the level of Stat5 DNA binding activity in response to IL-2 or IL-7 was higher than seen in WT mice. However, Stat5 was not constitutively activated, and our DNA microarray gene expression studies revealed relatively few differences

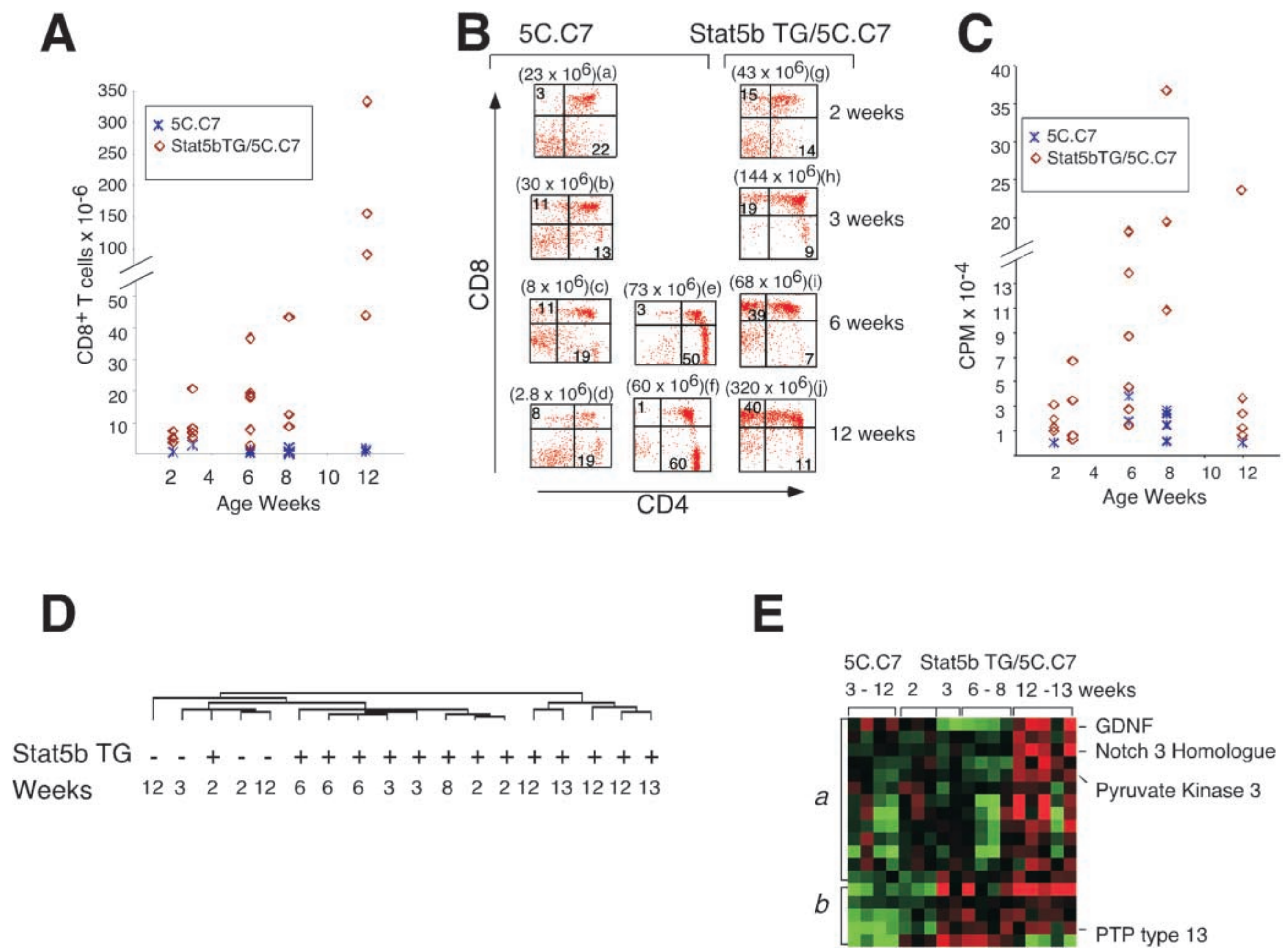

Figure 7. Time-course analysis of Stat5b/5C.C7 and 5C.C7 mice. (A) CD8$^+$ thymocyte numbers increase over time in Stat5b/5C.C7 double transgenic mice. Each point represents a single mouse. (B) CD4/CD8 profile of Stat5b/5C.C7 and 5C.C7 transgenic mice over time. Representative profiles are shown for the time points indicated (with total cellularity indicated above in parentheses). The 5C.C7 single transgenic mice either have significant loss of thymocytes over time (panels c and d) or an expansion of CD4$^+$ cells with time (panels i and j), whereas the Stat5b/5C.C7 double transgenic mice have an expansion of CD8$^+$ cells. (C) Anti-CD3 induced proliferation of total thymocytes. Each point represents a single mouse. (D) Shown is a dendogram resulting from unsupervised clustering of 6250 genes that were expressed "present" in any group. (E) Expression of genes that were found to be more highly expressed in Stat5b transgenic mice with lymphoma (see Fig. 4 C and online supplemental material), were also analyzed over time in 5C.C7 and in Stat5b/5C.C7 mice. In A and C, the 12-wk-old group includes all mice 12–13 wk old.

in gene expression between T cells from WT mice and mice overexpressing Stat5b but lacking lymphoma. Nevertheless, it is possible that transient cytokine-mediated activation at critical time points plays an important role in tumorigenesis in these animals. Interestingly, there was an increased rate/incidence of lymphomas in Stat5b transgenic mice expressing a TCR transgene, or after immunization. This is potentially consistent with antigen/adjuvant-mediated cytokine production (38), which in turn could, at least transiently, activate the transgenic Stat5. However, sustained Stat5 activation was not observed. Nevertheless, as shown in Fig. 7 E in Stat5b/5C.C7 double transgenic mice, a number of genes were induced over time. Additional work is needed to clarify the potential role of these genes in oncogenesis. In addition to the Stat5 transgene acting in a classical tyrosine phosphorylation-dependent manner, alternative mechanisms are possible. For example, Stat1 has been shown to affect expression of certain genes by a mechanism independent of its tyrosine phosphorylation (39). Such a mechanism could potentially apply to other STAT proteins as well.

Given that Stat5 can be activated in D10 cells (a T helper cell clone) after TCR signaling (40), and that T cells from Stat5a$^{-/-}$Stat5b$^{-/-}$ double knockout mice fail to proliferate in response to anti-CD3 stimulation (25, 41) and have defective ERK activation (our unpublished observations), we hypothesize that Stat5 transgenic mice might be more susceptible to the effects of TCR-stimulation in vivo. As CD8$^+$ T cell expansion and CD8$^+$ lymphomas are seen even in mice expressing the class II–restricted 5C.C7 TCR transgene, this indicates a particularly potent effect of the Stat5 transgene in controlling CD8$^+$ T lineage develop-

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ment. Moreover, the fact that the tumors arise earlier in mice expressing a transgenic TCR or after immunization indicates a role for TCR signaling and possibly selection in the earlier development of these tumors.

In contrast to systems that have correlated constitutively activated STAT proteins with oncogenesis, our Stat5a and Stat5b transgenic models demonstrate that overexpression of a WT (HA-tagged) STAT protein can also predispose to malignant transformation. Because the transgenic vector we used directs high level expression in lymphoid tissue, it is not surprising that the tumors that developed in our mice were lymphomas. However, given the ubiquitous expression of Stat5 proteins and the fact that they are activated by many different cytokines and growth factors, we hypothesize that the oncogenic potential of Stat5a and Stat5b may not be restricted to the immune system, and that overexpression in other tissues might cause other forms of neoplasia. Accordingly, Stat5a and Stat5b could prove to be rational target molecules for manipulating the growth of a number of different tumors. Finally, the fact that TCR/antigen stimulation could augment the rate of lymphoma formation in Stat5 transgenic mice has implications related to tumor and vaccine immunology.

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