Tumor-induced thymic atrophy: Alteration in interferons and Jak/Stats signaling pathways

ROBERTO CARRIO[1], MARTA TORROELLA-KOURI[1],
VIJAYA IRAGAVARAPU-CHARYULU[2] and DIANA M. LOPEZ[1]

[1]Department of Microbiology and Immunology, University of Miami, Miller School of Medicine, Miami, FL 33136 and [2]Department of Basic Science, Charles E. Schmidt College of Medicine, Florida Atlantic University, Boca Raton, FL 33431, USA

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Abstract. The thymus is the major site of T cell differentiation and a key organ of the immune system. Thym atrophy has been observed in several model systems including aging, and tumor development. Previous results from our laboratory have reported that the thymic atrophy seen in mammary tumor bearers is associated with a severe depletion of $CD4^+CD8^+$ double positive immature cells and changes in the levels of cytokines expressed in the thymus microenvironment. Cytokines regulate numerous aspects of hematopoiesis via activation of the Jak/Stat pathways. In the present study we have used our mammary tumor model to investigate whether changes in the levels of cytokines in the thymus could affect the normal expression of the aforementioned pathways. RNA and protein analysis revealed an overexpression of the different members of interferons, a downregulation of most of the Jak/Stat pathways, and an increased expression of several suppressors of cytokine signaling (SOSC) in the thymuses of tumor bearers. Together, our data suggest that the impaired Jak/Stat signaling pathways observed in the whole thymus of tumor-bearing mice could be contributing to the abnormal T cell development and apoptosis observed during the tumor-induced thymic atrophy.

Introduction

The thymus is the primary lymphoid organ where bone marrow-derived T cells precursors differentiate and proliferate, ultimately leading to migration of positively selected thymocytes to the peripheral organs where they respond to antigen stimulation and undergo effector differentiation (1,2). It has been well defined that the thymus provides an optimal and essential microenvironment for T cell development and maturation (3). Atrophy in the thymus has been identified as one of the key events that precede inefficient functioning of the immune system as well as an irreversible, inevitable age-related deterioration process of this organ (4,5). Thymic atrophy has been observed in several other model systems, including graft-vs-host-disease, bacterial and viral infections and tumor development (6-8), however, the exact mechanisms involved in this phenomenon remain to be elucidated.

Using a murine mammary adenocarcinoma originally induced in BALB/c mice by dimethylbenzanthracene (D1-DMBA-3) (9), we have previously described a profound progressive thymic atrophy associated with tumor development (10). This thymic involution is accompanied by a severe depletion of the most abundant subset of thymocytes; $CD4^+CD8^+$ double positive (DP) immature cells and an increase in the percentages of $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive populations and $CD4^-CD8^-$ double negative population (11). We have investigated several possible mechanisms leading to this thymic atrophy. In this regard, previous results from our laboratory have shown that the severe thymic atrophy and the impaired T cell development seen in mammary tumor bearers are associated with an arrest in at least two steps of T cell differentiation (12,13), changes in the levels of crucial cytokines expressed in the thymus microenvironment (14), and a progressive increase in apoptosis during the tumor development, mainly due to downregulation of important molecules that control programmed cell death (13). Moreover, we have also shown that mice bearing D1-DMBA-3 mammary tumors develop many changes in their cytokine production in peripheral T splenocytes (15,16).

In the present study we have examined other possible causes leading to the impaired T cell development observed during this thymic atrophy. Using protein analysis and defined pathway-specific cDNA arrays, we found an up-regulation of the different interferon molecules, together with a general down-regulation of the Jak/Stat pathways and an increased expression in several negative regulators of the Jak/Stat pathway in the whole thymus of tumor bearers. Collectively, our data suggest that the previously described

Correspondence to: Dr Diana M. Lopez, Department of Microbiology and Immunology, Miller School of Medicine, University of Miami, P.O Box 016960, Miami, FL 33101, USA
E-mail: dlopez@med.miami.edu

Abbreviations: NM, normal mice; TBM, tumor-bearing mice

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changes in the levels of cytokines expressed in the thymus microenvironment as well as the up-regulation of suppressor of cytokine signaling could be causing the down-regulation of the Jak/Stat pathways, leading to the impaired T cell development and apoptosis observed during the tumor-induced thymic atrophy.

Materials and methods

Mice and tumor. Male and female BALB/c mice were bred and housed under barrier conditions in the Division of Veterinary Resources at the University of Miami, Miller School of Medicine. Female BALB/c mice of 10 to 14 weeks of age were used for tumor transplantation. The D1-DMBA-3 transplantable mammary adenocarcinoma (9) was maintained and used as previously described (10). Briefly, the immunogenic D1-DMBA-3 tumor is routinely transplanted in BALB/c mice by s.c. injection of $1x10^6$ tumor cells. Palpable tumor is apparent ~8 days following implantation and the mice normally die between 4 and 6 weeks after tumor inoculation. Our institutional animal care and use committee (IACUC) approved the animal experiments.

Thymocytes collection and culture. Mice were euthanized and the thymuses were carefully dissected from the chest cavity and placed in Petri dishes containing 1X Hanks' balanced salt solution, 1% calf serum, 10 mM Hepes, pH 7.2, to prevent drying. The thymic lobes were placed in a cell strainer in a Petri dish with a drop of medium on the top, and gently compressed with the base of a 3 ml syringe followed by a wash with cold media and transfer to polypropylene tubes. Cells from the different mice were resuspended in RPMI, counted and cultured in 24-well tissue culture plates (Costar, Cambridge, MA) at a concentration of 10^6 cell/ml in RPMI-1640 containing 10% FCS, glutamine (30 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2-ME ($5x10^{-5}$ M) (CM) and stimulated with αCD3 (1 μg/ml) and IL-2 (10 ng/ml; Peprotech, Inc., Rocky Hill, NJ) for 48 h. Supernatants were collected and stored at -80˚C. Thymocytes activated with anti-CD3 and IL-2 were harvested, washed three times with RPMI-1640, and $1x10^6$ cells/well were cultured in 1 ml of CM in the presence of IL-2 (10 ng/ml) in 24-well culture plates for two additional days. Supernatants were also collected.

Cytokine ELISA. The amounts of IFN-γ and IL-10 present in the supernatants from CD3/IL-2-stimulated and also of the IL-2-cultured T lymphocytes were measured by ELISA according to the manufacturer's instructions. IFN-γ and IL-10 were analyzed using standard ELISA kits (BD Biosciences, San Diego, CA) as previously described (16). A standard curve was generated by plotting the OD of the standards vs their known cytokine concentration.

mRNA analysis. Mouse Common Cytokines Gene Arrays systems and Mouse JAK/STAT Signaling cDNA Pathway Gene Array systems were purchased from SuperArray Bioscience Corporation (Frederick, MD). Total RNA was extracted from the entire thymus with TRIzol (Life Technologies, Grand Island, NY) using a tissue homogenizer from OMNI International (Marietta, GA) as previously described (14). cDNA was prepared from this total RNA and hybridized to the arrayed filters according to the manufacturer's instructions. The resulting hybridization signal was visualized by chemiluminescence. Data were subjected to densitometric analysis using Scion Image Software (Scion, Frederick, MD). RNA levels were expressed as relative OD measurement after normalizing to the hybridization signals to ß-actin as previously described (14,17).

Results

As we have shown earlier, the growth of D1-DMBA-3 mammary tumor leads to extreme thymic atrophy in the host (10-14) which has been associated, among other, with changes in the levels of RNA expression of several molecules in the thymic microenvironment (14). For example, we have reported that whole thymuses from tumor-bearing mice are characterized by an up-regulation in the IFN-γ RNA expression when compared to those of normal mice (13). In the current study, in order to determine whether this up-regulation also takes place at the protein level, we analyzed IFN-γ expression in thymocytes from tumor bearers and normal mice by ELISA.

Thymocytes from normal and tumor-bearing mice were activated *in vitro* during 48 h in the presence of anti-CD3 and IL-2 as described in Material and methods. As shown in Fig. 1A, the expression of IFN-γ was significantly elevated in thymocytes from tumor-bearing mice when compared to thymocytes from normal mice. To determine whether this major expression in thymocytes from tumor bearers could be related more to the increased numbers of mature thymocytes in these thymuses than an altered function, *in vitro* activated thymocytes from normal and tumor-bearing mice were washed, and the same cell number were recultured in the presence of IL-2 as described in Material and methods. As shown in Fig. 1B, thymocytes from tumor bearers revealed a continuous increased expression of IFN-γ in comparison with the levels from normal mice.

Because IL-10 is a potent inhibitor of IFN-γ expression (18), and our present data show upregulated IFN-γ in the thymus, we determined whether the overexpression of IFN-γ is due to downregulated IL-10 expression. Thus, activated thymocytes from normal and tumor-bearing mice were also analyzed to detect the expression of this cytokine by ELISA. As described above, thymocytes from the different mice were activated *in vitro* during 48 h in the presence of anti-CD3 and IL-2. Interestingly, however, as shown in Fig. 2A, the expression of IL-10 was also extensively elevated in thymocytes from tumor-bearing mice. Furthermore, *in vitro* activated thymocytes from normal and tumor-bearing mice were also washed, and the same cell numbers were recultured in the presence of IL-2. As shown in Fig. 2B, thymocytes from tumor bearers showed a continuous elevated expression of IL-10 in comparison with the levels from normal mice.

Upregulation of different interferon family members during the tumor-induced thymic involution. Interferons are proteins made and released by lymphocytes in response to the presence of pathogens, such as viruses, bacteria, or parasites or tumor cells (19-22). They allow communication between cells to trigger the productive defenses of the immune system to

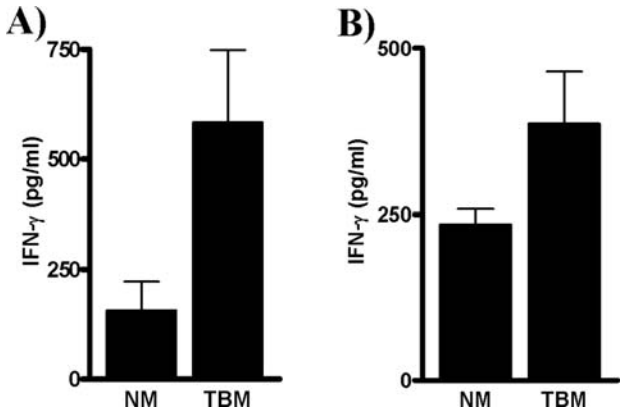

Figure 1. IFN-γ overexpression in thymocytes from tumor-bearing mice. (A) Thymocytes from normal mice (NM) and tumor-bearing mice (TBM) were activated *in vitro* in the presence of anti-CD3 and IL-2. (B) Activated thymocytes from the different mice were *in vitro* cultured in the presence of IL-2. Supernatants were collected and IFN-γ expression was assessed by ELISA. Data shown are the mean ± SD of 3-4 mice per group from three experiments.

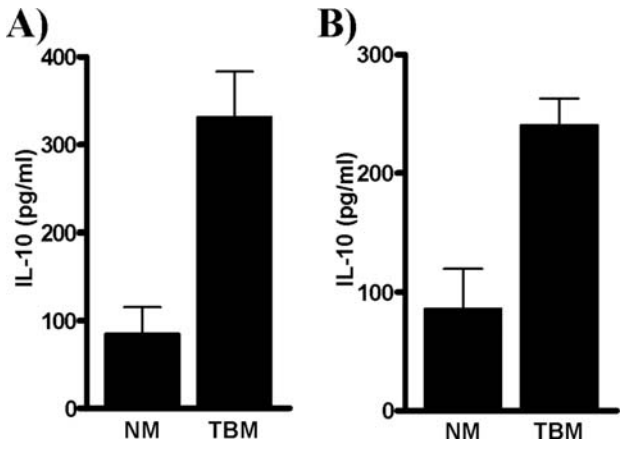

Figure 2. IL-10 expression in tumor bearer T lymphocytes. (A) Thymocytes from normal mice (NM) and tumor-bearing mice (TBM) were activated *in vitro* and (B) *in vitro* cultured as described in Fig. 1. Supernatants were collected and IL-10 expression was assessed by ELISA. Data shown are the mean ± SD of 3-4 mice per group from three independent experiments.

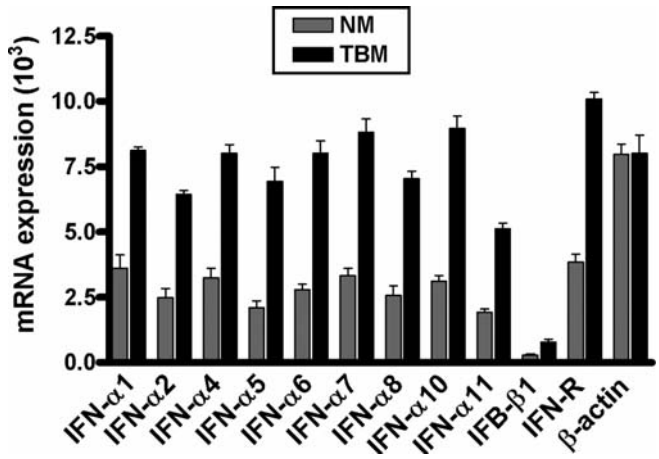

Figure 3. Interferon family members are up-regulated in thymuses of tumor-bearing mice. Mouse Common Cytokines Gene Arrays were hybridized with cDNA probes that were reverse-transcribed from the total RNA derived from whole thymuses of normal mice (NM) and tumor-bearing mice (TBM) as described in 'Material and methods'. Relative RNA of selected gene was normalized to ß-actin expression as described in 'Materials and methods'. Data shown are the mean ± SD of three independent experiments.

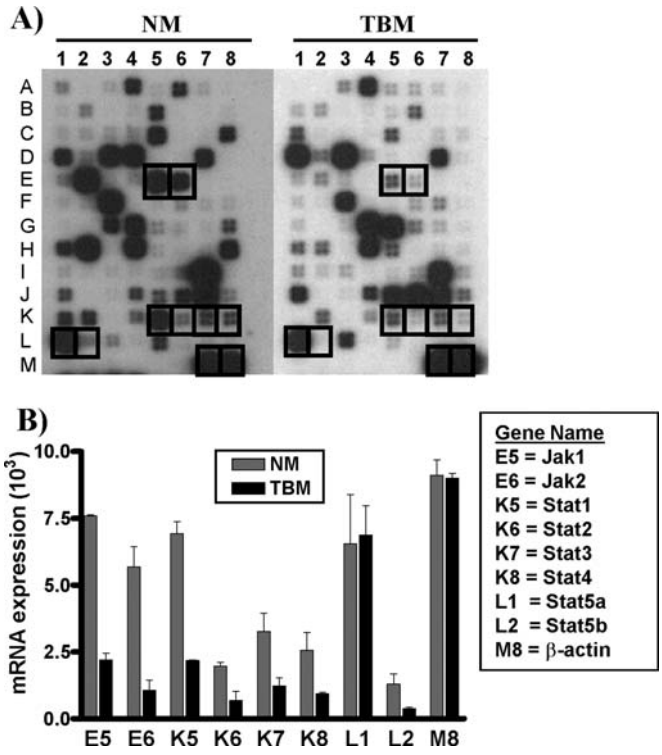

Figure 4. Altered Jak/Stat signaling pathway in tumor bearer T lymphocytes. Jak/Stat signaling Pathways Gene Arrays were hybridized with cDNA probes that were reverse-transcribed from the total RNA derived from whole thymuses of normal mice (NM) and tumor-bearing mice (TBM). (A) Representative hybridized arrays. (B) Relative RNA of selected Jak/Stat gene was normalized to ß-actin expression as described in 'Materials and methods'. Data shown are the mean ± SD of three independent experiments.

eradicate pathogens or tumors (22). In order to evaluate the presence of other members of the interferon family, we evaluated their mRNA expression in the whole thymus from normal and tumor-bearing mice using the Mouse Common Cytokines Gene Array system (SuperArray Bioscience Corporation). Microarray analysis of the different experiments revealed that most of the IFN-α members are up-regulated in the thymuses of tumor-bearing mice when compared to those from normal mice. As shown in Fig. 3, IFN-α1, IFN-α2, IFN-α4, IFN-α5, IFN-α6, IFN-α7, IFN-α8, IFN-α10, IFN-α11 are up-regulated by at least 2- to 3-fold. In addition, IFN-ß1 and IFNR are also differentially up-regulated.

Thymic atrophy in tumor-bearing mice is associated with a downregulation on Jak/Stats signaling pathway genes. In previous studies, we have demonstrated that the thymic involution observed in tumor-bearing mice is also associated with a downregulation in the gene expression of several

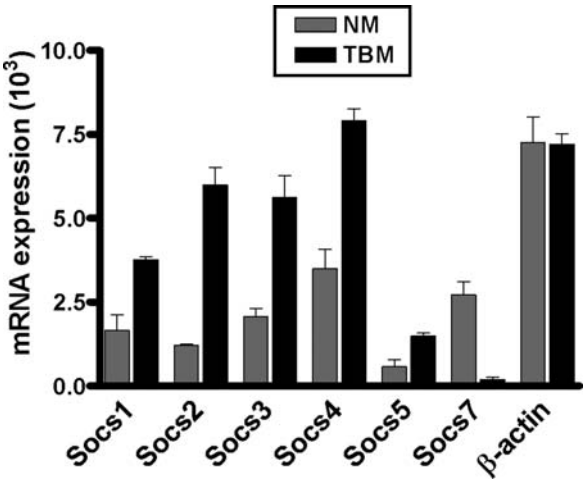

Figure 5. Differential expression of suppressor of cytokine signaling gene during the thymic involution of tumor-bearing mice. Gene Array analysis were performed using RNA derived from the whole thymuses of normal mice (NM) and tumor-bearing mice (TBM) as described in 'Materials and methods'. Relative RNA of the different suppressor of cytokine signaling genes were normalized to ß-actin expression as described in 'Materials and methods'. Data shown are the mean ± SD of three independent experiments.

crucial cytokines, such as IL-7 and IL-15, which are normally expressed in the thymic microenvironment (14). As we mentioned above, cytokines regulate numerous aspects of hematopoiesis and the immune response via activation of the Jak/Stat pathways (23,24). To begin to investigate whether these pathways could be affected during thymic atrophy, we analyzed the expression of mRNAs from Jak/Stat pathways in the whole thymuses of normal mice and tumor bearers using the Mouse JAK/STAT Signaling cDNA Pathway Gene Array system (SuperArray Bioscience Corporation). Microarray analysis (Fig. 4A) of the different experiments revealed that mostly all the Jak/Stat mRNAs are downregulated in the thymuses of tumor-bearing mice when compared to those from normal mice. As shown in Fig. 4B, Jak1, Jak2, Stat1, Stat2, Stat3, Stat4 and Stat5b are differentially downregulated by at least 3-fold. However, interestingly, only Stat5a appears to be expressed at very similar levels in both thymuses from normal and tumor-bearing mice.

Differential expression of the negative regulators of the Jak/Stat pathway during the thymic involution of tumor-bearing mice. Suppressors of cytokine signaling (SOCS) are important negative feedback regulators of the JAK/STAT signaling pathway, and have been recently investigated for their role in the development of different cancers (25). Densitometric analysis of the Jak/Stat microarray experiments also revealed that several suppressors of cytokine signaling (SOCS) were overexpressed in whole thymuses from tumor-bearing mice. As shown in Fig. 5, SOCS1, SOCS2, SOCS3, SOCS4 and SOCS5 were up-regulated by at least 3-fold when compared to those from normal mice. Interestingly, in contrast to the other members, SOCS7 was the only member downregulated in thymocytes from tumor bearers.

Discussion

The immune system originates from bone marrow-resident hematopoietic stem cells which lead to the generation of all lineages of mature blood cells. Whereas the majority of hematopoietic lineages mature in the marrow, T cell development takes place in a specialized organ, the thymus (3). Here, immature progenitor cells are guided through the differentiation and selection steps required to generate a T-cell repertoire

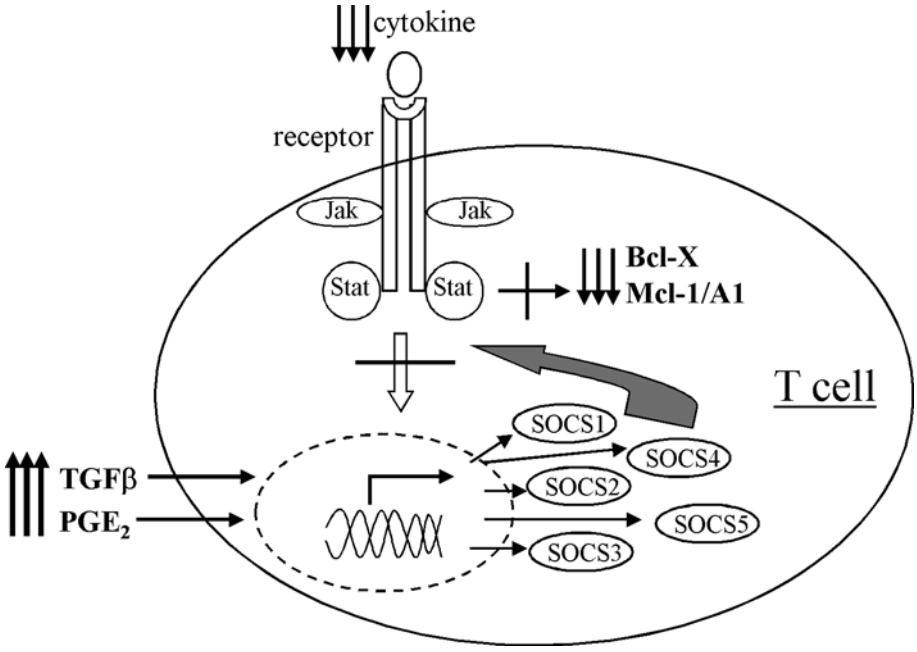

Figure 6. Model proposed to explain the impaired T cell development and increased apoptosis observed during the tumor-induced thymic involution.

that is both self-tolerant and binds to self major histocompatibility complex components (26). In mice, thymic atrophy (or involution) begins from the time of sexual maturity. Although the thymus remains functional, the export rate is insufficient to replace naive T cells lost daily from the periphery, homeostatic proliferation is triggered, and the memory pool expands (27). With time these changes are associated with an increased incidence of autoimmunity, a reduced capacity for immune surveillance and measurable decline in T-cell function (28). Although thymic atrophy has been identified as an irreversible, inevitable age-related deterioration process of this organ (4,5), it has been also observed in several other conditions including tumor development. However, the mechanisms involved in this phenomenon remain to be elucidated.

We have previously reported that the involution and impaired T cell development observed in the thymuses of D1-DMBA-3 mammary tumor-bearing mice are associated with an arrest in at least two steps of T cell differentiation (12,13). Also, changes in the levels of crucial cytokines expressed in the thymus microenvironment (14), and a progressive increase in apoptosis during the tumor development have been described (13). Our earlier studies suggest that thymic stromal cells from tumor bearers may not produce the necessary cytokines for appropriate T cell development and also show an impaired expression of molecules that regulate numerous aspects of hematopoiesis. In this regard, in a previous publication, we showed an up-regulation in the IFN-γ RNA expression in the whole thymuses from tumor-bearing mice (13). In this report we explored whether this IFN-γ up-regulation is observed at protein levels, as well and if it is also produced by thymocytes from tumor bearers. To this aim, cells isolated from the thymuses of normal and tumor-bearing mice were activated with αCD3 and IL-2 and activated cells were further expanded with IL-2. These experiments revealed that thymocytes from tumor-bearing mice are characterized by an elevated expression of IFN-γ. The correlation between IFN-γ overexpression and our previously observed increase in the percentages of $CD4^+CD8^-$ and $CD4^-CD8^+$ single populations during the tumor-induced thymic involution agrees with a previous report in which an increase of IFN-γ production through aging correlates with an expanded peripheral $CD8^+$ T cell population (29).

IFN-γ has numerous effects on hematopoietic and tumor cells (19,30,31). One of the consequences of IFN-γ treatments is the induction of apoptosis (20,32). Moreover, Lester *et al* (33) showed that an ever greater augmentation of IFN-γ production could be caused by neutralization of IL-10 activity. Interestingly, an apparently paradoxical finding in our studies is the fact that in our tumor model, in addition to IFN-γ, thymocytes from tumor-bearing mice also express high levels of IL-10. The possibility exists that both IFN-γ and IL-10 could be produced in order to increase a specific function. Indeed, Haringer *et al* (34) have previously identified and characterized an IL-10/IFN-γ-producing effector-like T cells with regulatory function in human blood. Furthermore, in recent unpublished data from our laboratory, using thymuses from normal and tumor-bearing mice, an increase in the naturally occurring regulatory cells during the thymic involution was detected (data not shown). In addition, in this report we also have shown an increased mRNA expression of the different members of the interferon family. IFNs are capable of modulating a variety of cellular responses, including cell growth and apoptosis (35,36). Several studies have shown that IFNs induce a strong and direct apoptotic response in primary malignant cells and in tumor cell lines *in vitro* (37-39). Our results indicate that the progressive increase in apoptosis observed during the tumor-induced thymic involution could be produced in part by an overexpression in the different members of the interferon family.

One characteristic intrinsic to the immune response is that cytokines regulate numerous aspects of hematopoiesis and T cell response via the activation of the Jak/Stat pathways (23,24). In order to simultaneously analyze the mRNA expression patterns of the different Jak/Stat genes, we utilized cDNA microarray technology. When compared to thymuses from normal mice, thymuses from tumor-bearing mice showed a downregulated expression of mostly all the Jak/Stat mRNAs. Only Stat5a is expressed to similar level in thymuses from normal and tumor-bearing mice. The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is prominent both in normal hematopoiesis and in hematological malignancies. Stats are phosphorylated on tyrosine residues via JAK kinases and on serine residues by a variety of serine/threonine kinases. Stats then dimerize, translocate to the nucleus and bind DNA, initiating the transcription of target genes. STAT proteins mediate cell growth, differentiation, apoptosis, transformation, and other fundamental cell functions (40). In this regard, Yun *et al* (41) showed that the inhibition of several Jak proteins decreased the expression of STAT3 downstream target genes such as Bcl-X and MCL-1/A1. We have also previously described the downregulation of these antiapoptotic proteins during the thymic involution of tumor-bearing mice (13). Moreover, Soldevila *et al* (42) also showed that the absence of Jak/Stat pathways affects T-cell development, not only through an impaired interleukin-7 receptor (IL-7R)-mediated signaling, but also through impaired chemokine-mediated responses, which are crucial for thymocyte migration and differentiation. However, in a previous publication, we showed a profound downregulation of IL-7 and IL-15 in the thymuses of mice bearing mammary tumors (14). Although the activity of the Jak/Stat proteins has not been analyzed, our data suggest that the downregulated cytokine expression may be leading to the lower expression of the Jak/Stat mRNAs. Rane *et al* (43) showed that certain cytokines such as granulocyte colony stimulating factor (G-CSF) dramatically induces the expression of Jak3 at mRNA level.

In addition to JAK/STAT pathway effectors, negative regulators exist: suppressors of cytokine signaling (SOCS). SOCS proteins inhibit components of the cytokine signaling cascade via direct binding or by preventing access to the signaling complex (44-46). Thus, the expression of the SOCS family was also evaluated in the whole thymuses of tumor-bearing mice. Interestingly, except SOCS7, most of the members of the SOCS family were up-regulated in the thymuses from tumor-bearing mice when compared to thymuses from normal mice. Although SOCS are induced by STAT proteins, and hence act like classic feedback inhibitors (25,47), the exact mechanisms involved in this phenomenon remain to be

elucidated. Several groups have recently suggested that an additional level of regulation is provided by an E3 ubiquitin-ligase complex bound to the SOCS box motif, which ubiquitinates the associated proteins targeting them for proteasomal degradation (48).

Taking all these facts into consideration, we suggest the following model (Fig. 6). Previous studies from our laboratory indicated the downregulation of crucial cytokines in the thymus microenvironment, which control numerous aspects of the T cell development. Importantly, the existence of insufficient mRNA expression of Jak/Stats pathways, together with an overexpression of negative regulators of these important pathways, members of the SOCS family, are contributing factors to the impaired T cell development in the thymuses of tumor bearers. We also suggest that other stimuli could be helping the overexpression of these SOCS family members. For example, the mammary tumor cells used in our studies secrete several molecules, TGB-ß and PGE2 among others, which have been shown to have effects in various compartments of the immune system (49,50). In this regard, previous studies have shown that some tumor-associated factors such as TGB-ß and PGE2 induce several of these suppressors of cytokine signaling (51,52). Moreover, the downregulation of several Jak/Stat genes could explain the decreased expression of antiapoptotic genes such as Bcl-X and A1, previously described (13). Collectively, these factors may be contributing to the impaired T cell development and increased apoptosis observed during the tumor-induced thymic involution.

Acknowledgements

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