Lethal Cutaneous Disease in Transgenic Mice Conditionally Expressing Type I Human T Cell Leukemia Virus Tax*

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Type I human T cell leukemia virus (HTLV-I) is etiologically linked with adult T cell leukemia, an aggressive and usually fatal expansion of activated CD4+ T lymphocytes that frequently traffic to skin. T cell transformation induced by HTLV-I involves the action of the 40-kDa viral Tax transactivator protein. Tax both stimulates the HTLV-I long terminal repeat and deregulates the expression of select cellular genes by altering the activity of specific host transcription factors, including cyclic AMP-responsive element-binding protein (CREB)/activating transcription factor, NF-κB/Rel, and serum response factor. To study initiating events involved in HTLV-I Tax-induced T cell transformation, we generated “Tet-off” transgenic mice conditionally expressing in a lymphocyte-restricted manner (EμSReα promoter-enhancer) either wild-type Tax or mutant forms of Tax that selectively compromise the NF-κB (M22) or CREB/activating transcription factor (M47) activation pathways. Wild-type Tax and M47 Tax-expressing mice, but not M22-Tax-expressing mice, developed progressive alopecia, hyperkeratosis, and skin lesions containing profuse activated CD4+ T cell infiltrates with evidence of deregulated inflammatory cytokine production. In addition, these animals displayed systemic lymphadenopathy and splenomegaly. These findings suggest that Tax-mediated activation of NF-κB plays a key role in the development of this aggressive skin disease that shares several features in common with the skin disease occurring during the preleukemic stage in HTLV-I-infected patients. Of note, this skin disease completely resolved when Tax transgene expression was suppressed by administration of doxycycline, emphasizing the key role played by this viral oncoprotein in the observed pathology.

Infection with the type I human T cell leukemia virus (HTLV-I)2 is etiologically linked with adult T cell leukemia (ATL), a malignancy of activated CD4+ T lymphocytes (1). ATL develops in 1–3% of infected individuals but only after a very prolonged period of latency often lasting between 20 and 50 years (2). The long latency between HTLV-I infection and the development of frank ATL suggests that multiple genetic events may be required for the full evolution of T cell transformation (3). HTLV-I infection has also been linked with a broader spectrum of diseases, including HTLV-I-associated myelopathy or tropical spastic paraparesis (HAM/TSP), HTLV-I infective dermatitis, HTLV-associated arthropathy, uveitis, and polymyositis (2, 4–6).

Dermatological lesions ranging from infective dermatitis to cutaneous lymphoma are commonly observed in HTLV-I-infected individuals who ultimately develop one of the four subtypes of ATL (smoldering, chronic, acute, or lymphoma) (2, 7–12). Localized or widespread skin lesions are commonly observed in these conditions associated with circulating atypical lymphocytes characterized by a convoluted or flower-shaped nucleus (9). Of note, in some patients with the smoldering or chronic subtype of ATL, skin lesions form the only manifestation of disease (13–16). Primary histological features of these cutaneous lesions include profuse infiltration of neoplastic cells primarily into the epidermis, dermis, and subcutaneous tissues. Lymphadenopathy, hepatosplenomegaly, and hypercalcaemia may also be present. A high proportion of infective dermatitis patients subsequently progress to ATL or HAM/TSP (5, 17–19). It is currently believed that the chronic or smoldering forms of ATL may represent a preleukemic stage of disease that will in time progress to full-blown acute ATL. This evolution of disease is often associated with an initial polyclonal expansion of CD4+ T cells supplanted later by a monoclonal expansion of fully transformed CD4+ T cells.

The molecular basis for these HTLV-I-induced diseases remains rather poorly understood. However, the Tax gene product of HTLV-I encoded within the 3’ pX region of the virus appears importantly involved. Tax alone is sufficient to promote oncogenic effects in many different cellular environments, including various transgenic animal models (20–26). However, full recapitulation of ATL-like malignancies comprised of CD3ε, CD4+ IL-2Ra+, and HLA-DR+ tumor cells has not yet been achieved in these transgenic models. HTLV-I Tax does not act by binding directly to DNA; rather it alters the activity of various host transcription factors through protein-protein interactions. Tax action promotes increased activity of the integrated HTLV-I long terminal repeat and altered expression of select subset of cellular genes (reviewed in Ref. 27). Specifically, Tax enhances HTLV-I gene expression by directing the assembly of a quaternary complex composed of CREB, Tax, and CBP that stimulates transcription from the HTLV-I long terminal repeat. Many cellular genes are transcriptionally activated through the effects of Tax on the NF-κB/Rel family of enhancer-binding proteins. Tax induction of NF-κB appears to involve its assembly with the NF-κB essential modulator component of the IκB kinase complex leading to constitutive activation of the IκB kinases and sustained induction of IκBα phosphorylation and proteasome-mediated degradation of this NF-κB inhibitor (28). These events liberate the NF-κB het-

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erodimer (p50/RelA), allowing for its rapid translocation into the nucleus where it engages cognate κB enhancers and increases the expression of select cellular target genes.

Using wild-type (wt) and mutant forms of Tax that selectively compromise stimulation of the CREB (M47 Tax) or NF-κB (M22 Tax) transcription pathways (29, 30), many investigators have studied the transforming properties of Tax in vivo. Two studies implicated a role for the CREB/activating transcription factor pathway in the Tax-mediated transformation (31, 32). Conversely, three groups found that Tax-mediated induction of NF-κB is critical for cellular transformation (33–35). Finally, the clonal transformation of primary CD4 T lymphocytes by Tax required the concomitant activation of both the CREB/activating transcription factor and NF-κB pathways (36). These in vitro findings highlight the current uncertainty that surrounds the precise mechanism by which Tax exerts its oncogenic effects.

In this study, we have attempted to explore early Tax-induced events involved in lymphocyte transformation occurring in vivo. For this analysis, transgenic mice conditionally expressing wt Tax (NF-κB+/CREB−), M47 Tax (NF-κB+/CREB−), and M22 Tax (NF-κB+/CREB−) were prepared. Expression of these various Tax analogues was confined to the lymphocyte compartment through the use of EμSra-tTA to drive the Tet-O-Tax transgenes. Expression of the Tax transgenes could be suppressed in these animals by administration of doxycycline, which blocks the stimulatory action of tTA (Tet-off system). We observed that wt Tax and M47 Tax, but not M22 Tax, transgenic mice develop a progressive and ultimately lethal skin disease resembling the dermatological abnormalities present in various preleukemic stages of HTLV-I infection. Suppression of Tax expression led to complete resolution of this aggressive skin disease, indicating that continuous expression of this viral transactivator is required for progression of this florid dermatopathology. Additionally, we observed profuse infiltration of the involved skin with activated, Tax-expressing T cells and increased local expression of inflammatory cytokines in the skin of these Tax transgenic mice. Together, these findings suggest that Tax induction of the observed skin disease involves intense infiltration of the dermis and epidermis with Tax-expressing T cells leading in turn to NF-κB-dependent, sustained expression of inflammatory cytokines. These cytokines likely serve as key effector molecules producing the severe, progressive skin disease.

**Experimental Procedures**

**Transgenic Mice—EμSra-tTA/Tet-O-wt/M22/M47 Tax (tTA/Tax) mice** were generated in two steps. First, independent Tet-O-wt, M22, and M47 Tax (Tax) mice were generated by pronuclear microinjection of DNA fragments containing the Tet- operon and wt, M22, and M47 Tax cDNA into freshly fertilized mouse oocytes. The Tet- operon contains the Tet-responsive element comprised of seven copies of the 42-bp Tet operator sequence positioned upstream of the minimal cytomegalovirus promoter. Subsequently, Tet-o-Tax mice were bred with EμSra-tTA “driver” mice (tTA) (37) to generate bigenic mice expressing Tax under the control of tetracycline (Tet-off). Mice were housed under specific pathogen-free conditions with frequent monitoring.

**Skin Cell Preparation and Flow Cytometry**—Shaved skin from individual mice was removed and scraped free of subcutaneous tissue with a scalpel. Subsequently, the skin was cut into small pieces and incubated in 2.5 mg/ml collagenase II and IV (Invitrogen) and 0.1 mg/ml DNase I (Sigma) for 45 min at 37°C (38, 39). Skin cell suspensions were then filtered through a 100-μm mesh. Flow cytometric studies were performed using a FACSCalibur (BD Biosciences) on skin cell suspensions previously immunostained with directly conjugated antibodies from BD PharMingen specific for various cell surface markers, including CD3 (145–2C11), CD4 (RM4–5), CD8 (53–6.7), TCRβ (H57–597), and TCRγδ (GL3). Green fluorescent protein expression in Tax expressing cells transduced with lentiviruses containing 5× NF-κB or HTLV-I long terminal repeat d1EGFP was also analyzed by flow cytometry (see supplemental data).

**Western Blotting**—The cells were washed with phosphate-buffered saline and lysed in Western lysis buffer containing 0.5% Nonidet P-40, 10 mM Tris-Cl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Calbiochem). Equivalent amounts of protein extracts were loaded on 10% SDS-polyacrylamide gel. After electrophoretic separation, the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) in buffer containing 30 mM Tris, 200 mM glycine, and 10% methanol for 1 h. The membranes were blocked by incubation in phosphate-buffered saline containing 5% milk for 1 h and then incubated overnight at 4°C or 2 h at room temperature with anti-Tax (40), anti-VP16 (3844-1; Clontech), anti-actin (Sigma), and anti-tubulin (Sigma) antibodies as indicated. After serial washing in phosphate-buffered saline, the membranes were reacted with a peroxidase-conjugated secondary anti-mouse or rabbit antibody (Amersham Biosciences) and visualized using an enhanced chemiluminescence detection system (ECL; Amersham Biosciences).

**Histology and Immunohistochemistry**—Skin biopsies were preserved in O.C.T. (Tissue-Tek) for frozen sections and 10% buffered formalin for paraffin-embedded fixed sections. Select samples were stained with hematoxylin and eosin in the Gladstone Microscopy Core. Histology and immunohistochemistry analyses of skin cryosections were performed using a VectaStain ABC kit (Vector Laboratories) with primary antibodies directed against CD3 (500A2), CD4 (RM4–5), CD8 (53–6.7), B220 (RA3–6B2), Gr-1 (RB6–8C5), I-A/I-E (2G9), ICAM (3E2, PharMingen), interferon-γ (IFN-γ; MAB1152, Chemicon), and IL-1β (AF-401-NA)/IL-10 (AF519) from R & D according to the manufacturer’s instructions. Fluorescent immunostaining was performed on formalin-fixed paraffin-embedded sections using antibodies specific for keratin 6 (K6, PRB-169P), keratin 14 (K14; PRB-155P), and loricrin (PRB-145P) from Covance and p65 (C-20; Santa Cruz) in combination with Alexa 488-conjugated secondary antibody (Molecular Probes). Histology and immunohistochemistry on formalin-fixed paraffin-embedded sections were performed with proliferating cell nuclear antigen (MS106-p0) stained with AEC system from Lab Vision Corp. Antigen retrieval was performed when required. The sections were also counterstained to define tissue structure and nuclei with tetramethyl rhodamine B isothiocyanate-conjugated phallolidin (Sigma) and Hoechst (Molecular Probes). The slides were analyzed using an Arcturus PixCell II, Nikon Eclipse TE300, or Olympus BX60 confocal microscope with the Bio–Rad Radiance 2000 Laser Scanning System.

**RNA Protection Assays and Quantitative Real Time Reverse Transcription-PCR**—Total RNA was extracted using an RNAeasy mini or micro kit (Qiagen) from Tax-expressing Jurkat cells (see supplemental data) or the skin of mice and then subjected to RNA protection assay according to the manufacturer’s instructions using the hAPO3 (for Jurkat cells, see supplemental data), mCK1b, mCK2b, mCK3, and mCR5 multi-probe sets (BD PharMingen). The resulting protected RNAs were separated on 5% denaturing polyacrylamide gel and exposed to x-ray film. Total RNA was extracted as described above from thymocytes, skin cells, CD3+, and CD3− cells present in the skin of mice. RNA was analyzed by real time reverse transcription-PCR. The RNA was treated with RNase-free DNase (RQ1 DNase; Promega) and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) following the
manufacturer’s instructions. The resulting cDNAs were amplified with the HOTaq PCR mix (McLaff, South San Francisco, CA). The real time reaction mix was as recommended by the manufacturer with 400 nm primers (see below), 200 nm probe (see below), and 50 ng of linear acrylamide (Ambion). The sequences of primers and probe for tTA were: forward primer, 5'-TCG AAC CCT TAG CCA TTG A-3'; reverse primer, 5'-TTG CCA GCT TTC CCC TTC T-3'; probe, 5'-VIC-TGT TAG ATA GCC ACC ATA CTC ACT TTG GCC CTT-TAMRA-3'. 18 S rRNA was used to normalize each PCR. Primers and probes for the 18 S rRNA were obtained commercially (ABI) and used according to the manufacturer’s instructions. Amplifications were performed using the ABI-Prism 7700 sequence detection system.

Genomic DNA PCR—Genomic DNA was isolated from sorted CD3+/CD4+ T cells present in mouse skin for analysis of TCRB rearrangement by PCR using a DNeasy kit (Qiagen). PCRs were prepared with genomic DNA in PCR mixture containing 1 X Pfx buffer, 0.3 mM dNTP (Amersham Biosciences), 0.1 μCi/μl (α-32P)dCTP (Amersham Biosciences), 1 mM MgSO4, 0.2 μM primers (see below), and 0.25 units of Platinum Pfx (Invitrogen). Primers used for rearrangement were: for DJ1-J1, TCRB-D1U-S and TCRB-J12-D; for DJ2-DJ1, TCRB-D2U-S and TCRB-J2D-A; for Vβ1-Jβ2, TCRB-V1-S and TCRB-J2D-A; for Vβ2-Jβ2, TCRB-V2-S and TCRB-J2D-A; for Vβ6-Jβ2, TCRB-V6-S and TCRB-J2D-A; for Vβ10-Jβ2, TCRB-V10-S and TCRB-J2D-A; and for IgM control, IgM-S and IgM-A (41, 42). The PCR mixture was subjected to 30 cycles of denaturation for 15 s at 94 °C, annealing for 30 s at 59 °C, and polymerization for 1 min 30 s at 68 °C, and the resulting PCRs were resolved on a 5% denaturing polyacrylamide gel and exposed to x-ray film.

RESULTS

Transgenic Mice Conditionally Expressing HTLV-I Tax in Lymphocytes Develop Progressive Skin Disease—To study early events involved in the initiation of Tax-mediated T cell transformation and to discern which host transcription factor pathway(s) are required for these events, we generated transgenic mice conditionally expressing HTLV-I wt, Tax hereafter) are shown in Fig. 1A. Lanes 1 and 2 show mice containing only the tTA or Tax transgenes respectively, whereas the mice shown in

![FIGURE 1. Screening and skin disease phenotype of TTA/Tax mice. A, genomic DNA isolated from tTA (lane 1), Tax (lane 2), TTA-Tax (lane 3), and TTA/Tax in the presence of Dox for 10 days, (lane 4) mice was subjected to multiplex genomic DNA PCR. Arrows indicate amplified PCR products for GFAP (loading control), Tax, and TTA. B, whole cell lysates of thymocytes isolated from each of the four mice shown in A were subjected to Western blot analysis with anti-Tax and anti-ITT (VP16 component) antibodies. Arrows indicate Tax and ITT proteins. C, western blot analysis of tax and actin antibodies. Arrows indicate Tax and actin proteins. D, skin disease occurring in a TTA/Tax animal progressing to involve the entire body at 8 months. E, splenomegaly occurring in a representative TTA/Tax mouse with skin disease (top panel) compared with a TTA animal (bottom panel). F, running of TTA/Tax mice. The size of a representative TTA/Tax animal is shown in comparison with a control littermate TTA animal (bottom panel). Skin sections from a control ITT mouse (G) and from TTA/Tax mice (H-L) were stained with hematoxylin and eosin. Blue lines in G and H demarcate the epidermis. In H, skin sections from TTA/Tax mice reveal hyperkeratosis (thickened keratin layer) and acanthosis (epidermal thickening) designated by an asterisk and a blue line, respectively. I, TTA/Tax mice also exhibit parakeratosis (thickened keratin with nuclear remnants) marked with an asterisk. Skin sections from TTA/Tax mice also reveal evidence of increased cellularity in the dermis (J) and infiltration of mononuclear cells (K). L, immunohistochemically identified infiltrating cells as CD3+ T cells. White arrows indicate Pautrier’s abscesses in the epidermis. Immunofluorescent microscopy shows an increase in T cells in the skin of a TTA/Tax mouse (N) compared with a control TTA mouse (M). White dotted lines outline the junction between the dermis and epidermis. The original magnification of slides was 100×.](http://www.jbc.org/content/journal/jbc/280/42/35715.full.html)
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lanes 3 and 4 are bigenic, containing both the tTA and Tax transgenes. When Tax protein expression was evaluated in the thymocytes of mice by immunoblotting with anti-Tax antibodies (Fig. 1B), the bigenic mouse shown in lane 3 expressed readily detectable quantities of Tax. However, when a bigenic littermate mouse shown in lane 4 was fed with Dox chow (200 mg/kg) for 10 days prior to analysis, expression of Tax was no longer detectable. Similar Dox-regulatable expression of Tax was observed with bigenic mice from each of the 18 founder lines. Down-regulation of Tax expression occurred as early as 3 days following Dox treatment (data not shown). These findings underscore the conditional and highly regulatable nature of Tax expression in these transgenic animals.

Strikingly, beginning at 4 months of age, mice from three wt Tax and four M47 Tax bigenic lines (referred to as tTA/Tax mice hereafter) exhibited progressive alopecia and exfoliation of the skin. These skin lesions first appeared around the neck area and progressed to involve nearly the entire body at 8 months (Fig. 1D). Many of these tTA/Tax mice also exhibited marked splenomegaly and lymphadenopathy (Fig. 1E and data not shown). The genetic penetrance of the skin disease phenotype in wt Tax and M47 Tax transgenic mice was 25.9 and 23.2%, respectively. None of the 124 M22 Tax transgenic mice examined developed skin disease. Wide time lags of 3–17 weeks of age were observed for the onset of the skin disease phenotype. These affected mice remained fertile, but litter size was reproducibly small. After inbreeding, the successive generations developed more pronounced skin disease that appeared as early as 3 weeks after birth. This acceleration of disease likely reflected increasing homozygosity of the two transgenes. Animals developing skin disease as early as 3 weeks after birth died within 4 weeks. Thymic atrophy was observed in Tax transgenic mice as shown in prior Tax transgenic mice (43). Both spleen and liver appeared pale in the Tax transgenic mice, and red blood cell counts were 2-fold lower compared with control mice. The absolute number of circulating lymphocytes in the Tax transgenic mice was 4.3-fold lower than control mice; however, the Tax transgenic mice displayed neutrophilia (absolute number of circulating neutrophils in Tax transgenic mice was three times higher than in control mice). All other organs and tissues in the Tax transgenic mice were histologically unremarkable. Affected tTA/Tax mice could be distinguished even earlier than 3 weeks based on their smaller size compared with healthy littersmates (Fig. 1F). To rule out the possibility that the absence of skin disease in the M22 Tax transgenic mice was due to impaired M22 Tax expression, levels of this Tax mutant were compared with that of wt Tax in thymocytes (Fig. 1C). As shown in lanes 2 and 4, comparable amounts of M22 Tax were detected relative to wtTax. Thus, the absence of the skin disease phenotype in these animals cannot be attributed to reduced expression of the M22 Tax protein. Of note, such skin disease was never observed in any of the M22 Tax transgenic mouse lines where Tax induction of NF-κB is defective. These findings reveal an interesting pathological condition occurring in multiple founder lines of animals expressing either wild-type Tax or M47 Tax (NF-kB+/CREB−).

Microscopic analysis of the skin from tTA/Tax mice revealed marked hyperkeratosis (thickened keratin layer; Fig. 1H, asterisk), acanthosis (epidermal thickening; Fig. 1H, blue line), and parakeratosis (thickened keratin with nuclear remnants; Fig. 1I, asterisk) compared with control skin (Fig. 1G). Increased cellularity in the dermal layer was also evident (Fig. 1J). More importantly, pronounced lymphocyte-like cell infiltrates were evident in the skin of these mice that were similar in character to the histological changes observed in the skin of cutaneous ATL patients including the presence of epidermal Pautrier’s abscesses (Fig. 1K). Based on immunohistochemical staining, these cellular infiltrates were mainly comprised of CD3+ T cells (Fig. 1L). When control skin was analyzed (Fig. 1M) only scattered CD3+ cells, possibly corresponding to CD3+ dendritic epidermal cells (44), were detected. In contrast, bright staining of multiple CD3+ cells was present in the skin of Tax transgenic mice (Fig. 1N).

Deregulation of Keratinocyte Development and Tax Expression in the Skin of tTA/Tax Mice—Skin from tTA/Tax mice displayed a sharp increase in the thickness of the epidermal layer. This finding prompted us to examine the pattern of epidermal proliferation and differentiation using immunofluorescence staining of various keratin gene products. Compared with normal controls, the skin of tTA/Tax mice displayed abnormal patterns of expression of K14 indicative of keratinocyte proliferation throughout the epidermal layer as well as an abnormally expanded pattern of expression of keratin 10 (K10) and loricrin, which are markers of keratinocyte differentiation (Fig. 2, A–C). Specifically, suprabasal expression of K14 was observed in the epidermis of skin from tTA/Tax mice (Fig. 2A, right panel), whereas expression of this keratin was confined, as expected, to the basal epidermal layer in control skin (Fig. 2A, left panel). K10 is usually expressed within the granular layer of epidermis; however, K10 was coexpressed with K14 in the suprabasal layer of affected skin from the tTA/Tax mice (Fig. 2B). Loricrin also displayed an abnormal and more diffuse pattern of epidermal expression (Fig. 2C). Interestingly, expression of keratin 6, which is a marker for activated keratinocytes and is often associated with hyperproliferation, was also up-regulated (Fig. 2D). Increased proliferation of keratinocytes was confirmed by proliferating cell nuclear antigen staining (Fig. 2E). These results reveal marked abnormalities in the normal pattern of both epidermal proliferation and differentiation in the skin of tTA/Tax mice.

Of note, Tax expression was limited to thymocytes of tTA/Tax mice that failed to develop skin disease; conversely, Tax expression in skin as well as in spleen (data not shown) was observed in the mice developing dermatopathological changes (Fig. 2F, lanes 4 and 5 compared with lane 3). Thus, peripheral expression of Tax in the spleen and skin correlated with subsequent development of progressive skin disease. As expected, purified CD3+ T cell in the skin expressed Tax (Fig. 2G, lane 4). However, Tax expression was also evident in the CD3− subset of cells (Fig. 2G, lane 3) and in keratinocytes (data not shown). This result prompted examination of tTA transactivator expression in CD3− cells from the skin by real time PCR. As shown in Fig. 2H, tTA mRNA was expressed at a 2.5-fold lower level in the skin compared with the level in thymocytes in tTA mice (lane 2 compared with lane 1). As expected, there was 3-fold increase in tTA expression in the skin of tTA/Tax mice, which likely reflects the sharply increased number of infiltrating T cells in the Tax-expressing mice (Fig. 2H, lane 3 compared with lane 2). However, the levels of tTA mRNA were approximately two times as high in the CD3+ cells, which includes the keratinocytes, compared with the CD3− cells from the skin of tTA/Tax mice (Fig. 2H, lanes 4 and 5). This finding led us to consider the possibility that the skin phenotype was due to the aberrant action of Tax in the keratinocytes. However, when nuclear translocation of NF-κB p65 was evaluated in the keratinocytes, p65 was exclusively localized in the cytoplasm (Fig. 2I). These findings support the notion that although Tax may be expressed in the keratinocytes, it is not functionally active in these cells because it fails to induce NF-κB activation. The nature of the other cell types expressing tTA or Tax within the CD3− population of cells from inflamed skin lesion and whether Tax is indeed functional in these cells is currently under study. However, because keratinocytes represent the great majority of the CD3− population, we conclude that Tax expression within the CD3− T cell subset likely plays a key role in the observed skin disease.
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**Increase in Immune Cells in the Skin of tTA/Tax Mice**—Based on the observed microscopic changes, perturbations in epidermal proliferation and differentiation, and the presence of increased numbers of infiltrating Tax-expressing T cells in both dermis and epidermis, we further characterized the various cells present in the skin of the tTA/Tax mice. Immunohistological analysis of the skin from tTA/Tax mice revealed the presence of activated CD4+ and to a much lesser extent CD8+ T cells expressing major histocompatibility complex II and ICAM1 (Fig. 3, A–H). These cells often appeared in clusters. Other immune cells including macrophages and B cells were present as well. Immunophenotyping of the lymphocytic infiltrates in the skin revealed expression of CD3+, confirming their identity as T cells and a sharp increase in CD4+ and to a lesser extent CD8+ T cells compared with control mice (CD3+/CD4+ cells, 37.7% versus 11.5%; CD3+/CD8+ cells, 2.98% versus 0.27%; Fig. 3, I and J). Furthermore, flow cytometric analyses revealed that the relative number of CD3+ cells bearing TCRαβ was moderately increased in the skin of the TTA/Tax mice (86.4% versus 55.3%; Fig. 3L), whereas the percentage of γδ T cells was greatly decreased (6.9% versus 42.3%; Fig. 3K).

**Is the Expansion of CD4+ T Cells in the Skin of tTA/Tax Mice Polyclonal or Monoclonal?**—Clonal expansion of HTLV-I-infected CD4+ T cells is a characteristic of ATL. Clonal rearrangement of T cell receptor β gene has been demonstrated in lymphocytes infiltrating the skin of HTLV-I seropositive patients (16, 45). Accordingly, we assessed the pattern of T cell receptor β rearrangement occurring in CD3+/CD4+ T cells from skin of tTA/Tax mice using a DNA-PCR assay. This approach is reliable, requires only small quantities of genomic DNA, and provides a semiquantitative profile of TCR rearrangements (Fig. 4A and Ref. 46). When the primers detecting Dβ2-Jβ2 and Dβ1-Jβ1 TCR gene rearrangement were used, the CD3+/CD4+ T cells present in the skin of tTA/Tax mice displayed very similar levels of Dβ-JB rearrangement as control thymocytes, suggesting that the skin T cell infiltrate in these animals was polyclonal in nature (Fig. 4B). When different primer sets for Vβ-DJβ rearrangement were used, slightly different patterns of PCR amplification from skin infiltrating CD3+/CD4+ T cells of the tTA/Tax mice were occasionally apparent compared with the CD3+/CD4+ T cells isolated from control skin (Fig. 4C). Taken together, all of these data suggest that the infiltrating skin T cells are polyclonal or perhaps in some cases oligoclonal in nature. These results do not support the presence of monoclonal tumor cells infiltrating the skin of these Tax-expressing animals. Consistent with this conclusion, the infiltrating skin T cells were not significantly labeled with any of the anti-Vβ antibodies present in the TCR screening panel (Pharmingen; data not shown).

**Increase in Inflammatory Cytokine Expression in the Skin of tTA/Tax Mice**—Because we strongly suspected the presence of an abnormal inflammatory response in the skin of the TTA/Tax animals, RNase pro-

**FIGURE 2.** Deregulation of keratinocyte development and Tax expression in the skin of TTA/Tax mice. Immunofluorescent staining of skin biopsies from control TTA (left panels) and TTA/Tax mice (right panels) was performed using various markers of keratinocyte development. Skin biopsies from TTA/Tax mice showed deregulated expression of K14 (a marker for keratinocyte proliferation, A), K10 (a marker of keratinocyte differentiation, B), loricrin (a second marker of keratinocyte differentiation, C), and K6 (a marker of keratinocyte inflammation and proliferation, D). Green, keratin markers; red, phalloidin; blue, nucleus. E, increase in proliferation of keratinocytes in the skin of TTA/Tax mice was also shown by immunohistochemical staining with anti-proliferating cell nuclear antigen antibodies. White dotted lines outline the junction between the dermis and epidermis. The original magnification of the slides was 200×. F, Tax expression in the skin of TTA/Tax mice parallels the development of skin disease. Lysates from skin preparation of control TTA (lane 1), control Tax (lane 2), TTA/Tax (lane 3), without skin disease, and TTA/Tax (lanes 4 and 5, with skin disease) were subjected to Western blotting analysis with anti-actin and anti-Tax antibodies. G, Tax expression in both CD3+ and CD3− cells from the skin of TTA/Tax mice. Lane 1, skin from TTA mouse; lane 2, skin from TTA/Tax mouse; lane 3, CD+ cells from the skin of TTA/Tax mice; lane 4, CD3− cells from the skin of TTA/Tax mice; lane 5, CD+ cells of the skin from TTA/Tax mouse; lane 6, CD3+ cells of the skin from TTA/Tax mouse. H, increase in TTA mRNA expression in the skin of TTA/Tax mice shown by real time PCR compared with control TTA mice. Lane 1, positive control from thymocytes of TTA mice; lane 2, skin from TTA mice; lane 3, skin from TTA/Tax mice; lane 4, CD3+ cells from the skin of TTA/Tax mice; lane 5, CD3− cells of the skin from TTA/Tax mice; lane 6, negative control. I, NF-κB p65 is predominantly localized in the cytoplasm of keratinocytes isolated from TTA/Tax mice. Green, p65; blue, nucleus; red, phalloidin. The original magnification of the slides was 400×. Red and blue lines indicate epidermis and dermis, respectively. Note that NF-κB p65 remains cytoplasmic in the keratinocytes of TTA/Tax mice.
tection assays were performed to profile the level of inflammatory cytokine mRNA expression occurring in the skin of these mice versus controls. These studies revealed marked increases in various inflammatory cytokine mRNAs including tumor necrosis factor-α, IL-6, IL-1α, IL-1β, lymphotoxin-β, and IFN-γ in the affected skin of tTA/Tax mice (Fig. 5, A–C). Of note, many of these genes are direct targets of NF-κB, a finding consistent with the absence of skin abnormalities in mice expressing M22 Tax, which fails to activate NF-κB. NF-κB-inducible chemokines and chemokine receptors also play important roles in in vivo spread and tissue localization of various tumors, including ATL (47–49). When chemokine receptor expression was examined in the skin of tTA/Tax mice (Fig. 5D), marked increases in CCR1, CCR2, and CCR5 mRNA levels were observed compared with the control skin. These data raise the possibility that the recruitment of T cells to the skin involves Tax-induced expression of specific chemokine receptor(s). Once targeted to skin, these Tax-expressing T cells promote a local NF-κB-dependent inflammatory response involving deregulated cytokine expression that alters the proliferation and differentiation of the resident keratinocytes.
In addition to these proinflammatory cytokines, increased expression of transforming growth factor-β1 and IL-10 mRNA was detected.

Regression of Skin Disease Following Suppression of Tax Transgene Expression—Finally, to test whether this progressive skin disease was dependent upon the continuous expression of Tax, tTA/Tax mice manifesting skin lesions were fed with Dox-containing chow and monitored over time. Administration of Dox to tTA/Tax mice exhibiting florid skin disease resulted in a major clearing of the skin lesions and regrowth of hair over a period of 1 month (Fig. 6A). Additionally, the mice gained weight and returned to normal activity levels. However, one potentially confounding issue relates to the potential antibacterial effects of doxycycline. Prior studies have shown that HTLV-I-associated infective dermatitis often responds to treatment with antibiotics. Because Dox is a derivative of tetracycline and exerts antibiotic activity, it was formally possible that resolution of the skin disease could reflect successful treatment of a superficial bacterial infection of the skin rather than the concomitant suppression of Tax transgene expression. To investigate this possibility, a cohort of tTA/Tax mice with skin disease were treated with trimethoprim-sulphamethoxazole, an antibiotic currently used in the treatment of patients with HTLV-I infective dermatitis (50). However, in contrast to Dox treatment, administration of trimethoprim-sulphamethoxazole did not alter the progression of the skin disease (Fig. 6, B and C). Regression of skin disease after Dox, but not trimethoprim-sulphamethoxazole, treatment supports the notion that the effect of Dox was through suppression of Tax transgene expression and that continued Tax expression is required for maintenance and progression of the skin disease. We next investigated whether the skin disease reemerged when Dox was removed from the diet after 14 days (Fig. 6 B). The skin disease reappeared 7 months after the withdrawal of Dox (Fig. 6B, red box). This finding further implicates Tax expression as a key factor underlying the observed skin disease.

DISCUSSION

We demonstrate that transgenic mice conditionally expressing the HTLV-I Tax oncoprotein in the lymphocyte compartment develop a sporadic, hyperproliferative, and progressive inflammatory skin disease. This skin disease is associated with the presence of profuse infiltration of the dermis and epidermis with Tax-expressing CD4+ T cells and an increase in production of a variety of inflammatory cytokines. Of note, such skin disease was not observed in any of the six founder lines of mice.
expressing the M22 Tax mutant, which lacks the ability to induce NF-κB. Histologically the affected skin revealed the presence of acanthosis, hyperkeratosis, and parakeratosis. In addition, mice developing this skin disease displayed splenomegaly and lymphadenopathy, whereas littermates not developing skin disease lacked such changes. Suppression of Tax expression in these animals by administration of doxycycline resulted in rapid clearing of the skin disease and a return of the animals to full health emphasizing the central role Tax played in this progressive skin disease.

HTLV-I infection is associated with many different abnormalities in the skin. One manifestation is cutaneous ATL where the proliferation of tumor cells is confined to the skin (13–16). Although less severe and not reflecting the infiltration of frank tumor cells, TSP/HAM patients also may develop progressive dermatological lesions (51). Indeed, the histology of the cutaneous lesions occurring in the tTA/Tax mice very closely resembles many histopathological features previously described in HTLV-I-infected patients (16, 51–53). These patient skin biopsies exhibit varying degrees of T cell infiltration associated with hyperkeratosis and acanthosis like that observed in the skin sections from the affected tTA/Tax mice (51, 54). Similar histological features have been described in the skin of patients with cutaneous T cell lymphoma, including cutaneous ATL (55). Patients with cutaneous ATL display
activated CD4+ T cells expressing CD25+ and major histocompatibility complex II within the dermis and epidermis (16). As shown in Fig. 3, clusters of activated CD3+/CD4+/major histocompatibility complex II+ T cells were present in the dermis and epidermis of the tTA/Tax mice. Indeed, Pautrier's abscesses, a histological hallmark of cutaneous T cell lymphomas corresponding to clusters of T cells in the epidermis, were frequently detected in the tTA/Tax mice. However, an important distinction is that the dermal infiltrating T cells in ATL patients are clonal with respect to TCR rearrangement (16, 45). In contrast, the infiltrating CD4+ T cells present in the skin of tTA/Tax mice represent polyclonal/oligoclonal populations of cells (Fig. 4). This finding indicates that the cutaneous lesions observed in the tTA/Tax mice do not correspond to cutaneous ATL. Of note, there are several case reports describing the infiltration of the skin with polyclonal or oligoclonal expansions of activated CD4+ T cells in earlier clinical stages of ATL (15, 16, 56, 57). These reports argue that an early polyclonal proliferation of HTLV-I-infected T cells occurs in the skin, setting the stage for subsequent genetic events that promote the emergence of fully transformed monoclonal tumors (11, 53). Thus, it is possible that the polyclonal population of activated CD4+ T cells infiltrating the skin of the tTA/Tax mice could correspond to such a preleukemic population of T cells.

It is possible that the latency period for tumor development in these mice, like HTLV-I-infected ATL patients, is quite long. In this regard, epidermal polyclonal T cell infiltrates have also been detected in skin lesions developing in HTLV-I-infected rabbits. However, only after 3.5 years did this HTLV-I skin disease progress to cutaneous T cell lymphoma (13, 58). We have attempted to follow our Tax-expressing mice for longer periods of time; however, the severe and progressive nature of their skin disease consistently results in early death, precluding such analysis.

Our finding that skin disease did not occur in animals expressing M22 Tax (NF-κB+/CREB+) suggests that Tax-mediated induction of NF-κB may play a key role in the development of the observed skin disease. Of note, similar pathological changes in skin have been observed in various animal models where NF-κB signaling is up-regulated, including mice lacking IκBα or RelB (59–61). One possible consequence of NF-κB action in these infiltrating T cells is an increased production of various cytokines that in turn may alter the normal pattern of keratinocyte proliferation and differentiation. Indeed, as shown in Figs. 1 and 3, infiltration of the skin with T cells was also a prominent component in this disease model. The presence of such infiltrating T cells raised the possibility that deranged production of cytokines by these cells might underlie the development of skin disease. In this regard, we have detected the expression of a broad array of inflammatory cytokines in the tTA/Tax mice, including tumor necrosis factor-α, IL-6, IL-1α, IL-1β, lymphotxin-β, and IFN-γ (Fig. 5). Interestingly, transgenic mice overexpressing a variety of different cytokines, including IL-1α, IL-2, IL-7, and IFN-γ manifest similar skin diseases (38, 62–66). Among these cytokines, we have observed increases in IL-1α and IFN-γ in the skin of tTA/Tax mice. Whether all of these cytokines are directly secreted by infiltrating T cells or secondarily by keratinocytes as a consequence of effects of the skin infiltrating T cells cannot be determined by the RNase protection assays we have performed. However, increased production of IL-1, IFN-γ, and tumor necrosis factor-α and has been detected in ATL cells, HTLV-I-infected cells, and/or Tax-expressing T cells (67–73).

The observation that the presence of infiltrating activated T cells expressing Tax was correlated with the development of the progressive skin disease in the absence of nuclear NF-κB translocation in the kera-

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