

response, and HTLV-1-mediated malignant and inflammatory diseases.

STATUS OF HTLV-1 EXPRESSION *IN VIVO*

The understanding of HTLV-1 expression *in vivo* has caused much confusion, largely owing to two reasons: (a) HTLV-1 proteins are not detectable in infected cells in the peripheral blood of HTLV-1 carriers; (b) two types of ATL cases exist, and while HTLV-1 expression in ATL cells is conserved in some cases, this expression is lost in other cases (Figure 1).

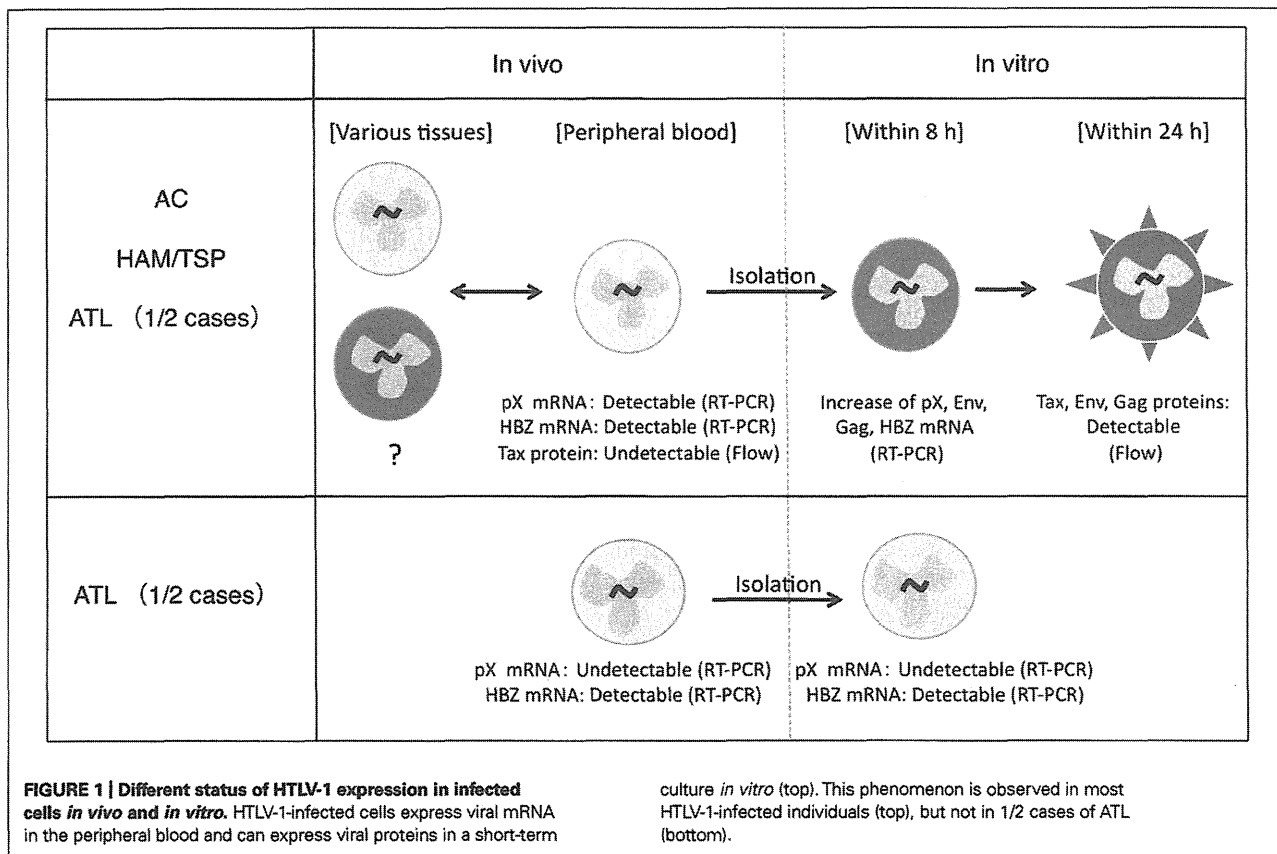
EXPRESSION OF HTLV-1 PROTEINS *IN VIVO*

HTLV-1 mRNA but not proteins are detectable in the peripheral blood mononuclear cells (PBMCs) of HTLV-1-infected individuals including ATL patients (Kinoshita et al., 1989). The presence of HTLV-1 mRNA was also reported in other tissues such as muscle in myositis patients (Tangy et al., 1995; Ozden et al., 2004) or the spinal cord in HAM/TSP patients (Lehky et al., 1995). Therefore, HTLV-1 gene expression does occur *in vivo* at least at a transcriptional level. Furthermore, based on the findings that HTLV-1-infected individuals maintain serum antibodies directed to the HTLV-1 structural Env and Core proteins as well as Tax-specific T-cells, HTLV-1 expression must be occurring also at a protein level *in vivo*. This notion is further supported by the emergence of Tax-specific CTL responses in ATL patients who received hematopoietic stem cells from HTLV-1-negative donors

(Harashima et al., 2004, 2005). In these cases, the CTL responses are presumably triggered by the *de novo* exposure of donor-derived T-cells to Tax antigen in the recipients, and resemble an acute infection. These findings suggest that the sensitivity of T-cells recognizing HTLV-1 antigen may be much higher than the detection by serological means such as flow cytometry or immunoblotting, which are dependent on antibody binding. The conflicting arguments concerning HTLV-1 expression might thus continue until more sensitive protein detection methods are developed.

TWO TYPES OF ATL WITH OR WITHOUT HTLV-1 EXPRESSION

HTLV-1 expression in ATL cells immediately after isolation from peripheral blood is very low, and becomes significantly induced only after *in vitro* cultivation (Hinuma et al., 1982). This phenomenon is observed in about half of the ATL cases, regardless of the severity of the disease (Kurihara et al., 2005). A similar induction of viral expression after *in vitro* culture has also been observed in PBMCs from HAM/TSP patients and ACs (Hanon et al., 2000). Recent analysis using quantitative RT-PCR methods confirmed this phenomenon in PBMCs from both ATL and HAM/TSP patients. The data further showed that levels of Tax/Rex mRNA were increased as early as 4 h after initiation of culture, and peaked at 8 h, followed by an increase in Env, Gag/Pol, and other mRNAs (Rende et al., 2011). This finding is consistent with the critical roles of Tax and Rex proteins for viral expression through



transcriptional transactivation, regulation of RNA splicing, and nuclear export of the mRNAs, which were described in previous studies (Yoshida, 2001; Younis and Green, 2005). The rapid induction of viral expression in culture further suggests the presence of a common mechanism transiently suppressing HTLV-1 expression *in vivo*, irrespective of the disease.

In the remaining half of the ATL cases, however, such viral induction does not occur, even after *in vitro* culture. This may be due to genetic and epigenetic changes in the viral genome (Tamiya et al., 1996; Takeda et al., 2004). The malignant phenotype of ATL cells in these cases is presumably attributed to other mechanisms acquired at additional steps of leukemogenesis, independently of HTLV-1 expression.

EXPRESSION OF HBZ IN INFECTED CELLS

In uncultured PBMCs from HTLV-1-infected individuals, expression of the HTLV-1 genome is suppressed as noted above, whereas mRNA of HBZ, the minus-strand HTLV-1-encoded gene, is continuously expressed, irrespective of the disease (Satou et al., 2006). Transcription of HBZ in the absence of Tax implies an indispensable role of HBZ in HTLV-1-infected cells. Interestingly, mice carrying an HBZ transgene under the control of the CD4 promoter often develop lymphoproliferative disease with frequent Foxp3 expression and inflammatory skin lesions (Satou et al., 2011). These features partly resemble the characteristics of ATL.

However, expression of HBZ at a protein level is still controversial. In a study analyzing mRNA kinetics during the initial culture of PBMC from infected individuals, Tax/Rex and other positive-strand transcripts were promptly exported to the cytoplasm after transcriptional induction, while HBZ mRNA was mostly retained in the nucleus (Rende et al., 2011). In addition, HBZ-specific CTLs induced in human leukocyte antigen (HLA)-A2-transgenic mice lysed HBZ peptide-pulsed HLA-A2-positive target cells but not HTLV-1-infected HLA-A2-positive cells (Suemori et al., 2009). These observations suggest that expression of HBZ at a protein level in HTLV-1-infected cells might be limited, even though substantial amounts of HBZ mRNA are expressed. Nevertheless, the presence of T-cells responding to HBZ peptides have been reported in HAM/TSP patients at a low frequency, implying a small amount of HBZ protein synthesis *in vivo* (Hilburn et al., 2011).

HTLV-1 EXPRESSION IN HAM/TSP PATIENTS

HTLV-1 expression is detectable at the transcriptional, but not the protein level in uncultured PBMCs, and such basal levels of mRNA differ among diseases. An early study showed that the pX mRNA/DNA ratio was lower in ATL patients compared with ACs or HAM/TSP patients (Furukawa et al., 1995). This might partly reflect that in 50% of ATL cases the cells lost viral gene expression, as mentioned above. A recent study using a real-time quantitative PCR analysis indicated that HTLV-1 mRNA levels are significantly higher in HAM/TSP patients compared with ACs (Yamano et al., 2002). This is in agreement with high levels of serum antibodies to HTLV-1 in HAM/TSP patients (Dekaban et al., 1994). The detection of HTLV-1-specific antibodies in the cerebrospinal fluid and the pX mRNA in the spinal cord were also reported in HAM/TSP patients (Lehky et al., 1995; Gessain, 1996). These observations

indicate that HTLV-1 expression is elevated in HAM/TSP patients generally, and also in the spinal cord.

Little is known about the difference in the levels of HTLV-1 expression between tissues in humans. In transgenic mice carrying the pX gene driven by the HTLV-1 long terminal repeat (LTR), RNA expression of the transgene is detectable only in selected organs, including the central nervous system, eyes, salivary glands, and joints (Iwakura et al., 1991). Transgenic mice and rats with the pX gene often develop arthritis and other collagen-vascular inflammatory conditions (Iwakura et al., 1991; Yamazaki et al., 1997). This is partly explained by Tax-mediated activation of NF- κ B, a key transcription factor for multiple inflammatory cytokine production. In addition, a certain WKAH strain exhibits HAM/TSP-like symptoms after HTLV-1 infection. This rat model of HAM/TSP shows increased Tax mRNA expression in the spinal cord before the manifestation of the symptoms, suggesting that viral expression in the spinal cord may be the primary event (Tomaru et al., 1996). Reduced IFN- γ production in the spinal cord has been suggested in this particular rat strain (Miyatake et al., 2006).

DIFFERENT HTLV-1-SPECIFIC T-CELL RESPONSES BETWEEN DISEASES

ANTI-TUMOR AND ANTI-VIRAL SURVEILLANCE BY HTLV-1-SPECIFIC T-CELLS

The strength of the host T-cell response against HTLV-1 differs among diseases. CD8⁺ HTLV-1-specific CTL responses are activated in HAM/TSP patients but not in ATL patients (Jacobson et al., 1990; Parker et al., 1992; Arnulf et al., 2004; Takamori et al., 2011). These CTLs mainly recognize HTLV-1 Tax and kill HTLV-1-infected cells *in vitro* (Jacobson et al., 1990) (Kannagi et al., 1991). The HTLV-1 envelope protein is also a major target, especially for CD4⁺ CTLs (Goon et al., 2004). Other viral antigens, including polymerase (Elovaara et al., 1993), TOF, ROF (Pique et al., 2000), and HBZ, (Macnamara et al., 2010) have also been shown to be targets of CTLs. Elimination of CD8⁺ cells from the PBMCs from HAM/TSP patients induces HTLV-1 expression during subsequent cell culture (Asquith et al., 2005), clearly indicating that CD8⁺ HTLV-1-specific CTLs contribute to the control of HTLV-1-infected cells.

A series of experiments using a rat model of HTLV-1-infected T-cell lymphoma indicated that inhibition of the T-cell response accelerated tumor development (Hanabuchi et al., 2000), and further showed that vaccination with a Tax-encoding DNA or peptides corresponding to a major epitope for Tax-specific CTLs lead to the eradication of such tumors (Ohashi et al., 2000; Hanabuchi et al., 2001). In a different rat model of HTLV-1 infection, oral HTLV-1 infection induced HTLV-1-specific T-cell tolerance and caused an elevation in the proviral load, while re-immunization of these rats resulted in the recovery of HTLV-1-specific T-cell responses and caused a reduction in the proviral loads (Hasegawa et al., 2003; Komori et al., 2006). Similarly, patients carrying HTLV-1 developed ATL after liver transplantation, when immunosuppressants were administered (Kawano et al., 2006; Suzuki et al., 2006). These findings suggest that HTLV-1-specific T-cells, especially Tax-specific CTLs, play important roles in anti-tumor and anti-viral surveillance in HTLV-1 infection.

The pathological significance of HTLV-1-specific T-cells activated in HAM/TSP patients remains controversial (Jacobson, 1995; Osame, 2002). Activated CTLs produce IFN- γ or TNF- α , which might potentially participate in inflammation in HAM/TSP. However, activation of Tax-specific CTLs could also merely be a result of elevated viral expression in these individuals. HLA-A02-positive individuals in HAM/TSP patients are less frequent compared with the control population, indicating a protective role of HLA-A02 against HAM/TSP. Since HLA-A02 can present a major epitope of HTLV-1 Tax, the strong CTL response induced is thought to mediate the protective effect of HLA-A02 (Jeffery et al., 1999). The association of the protective HLAs with epitopes favoring HBZ-specific CTLs has also been suggested (Macnamara et al., 2010).

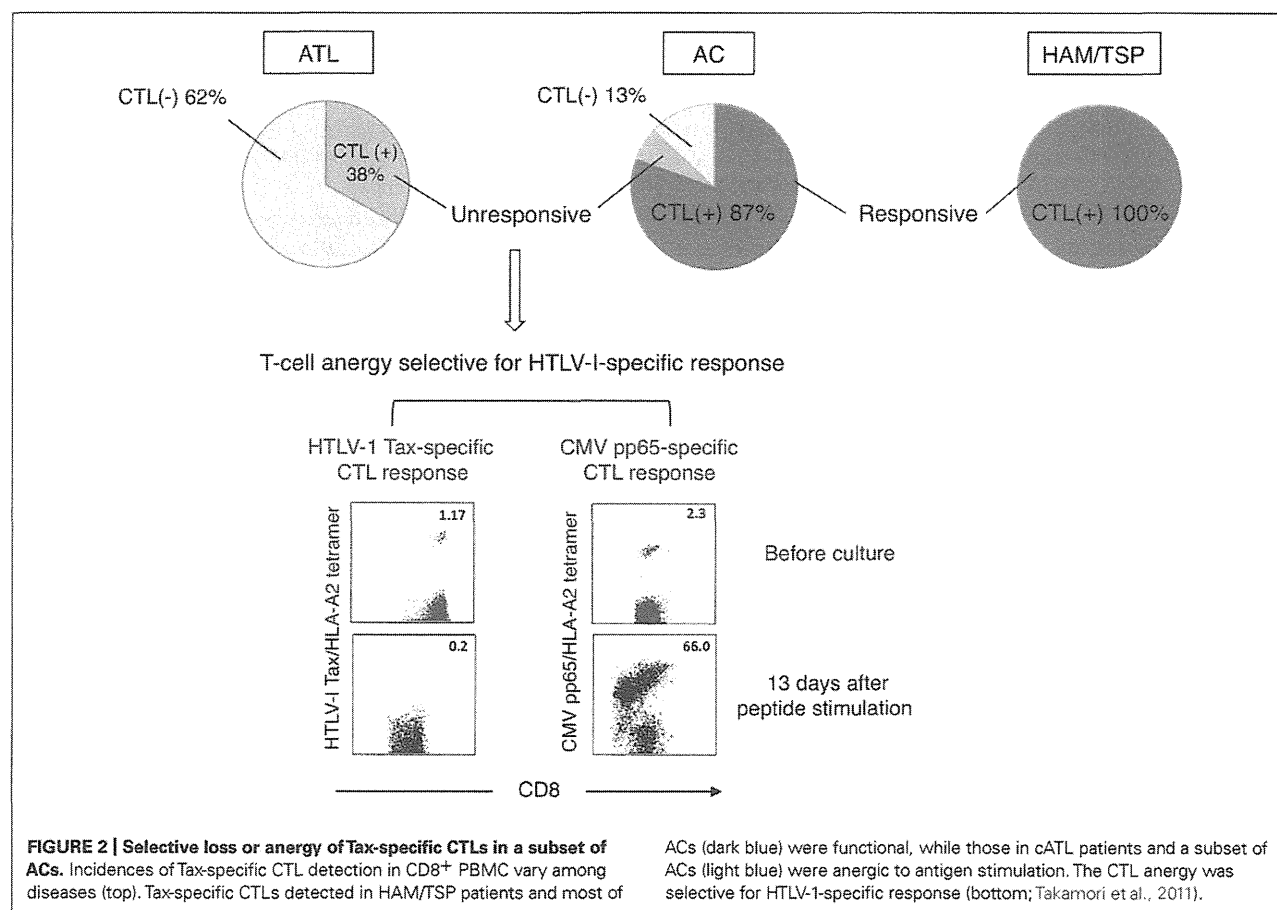
SELECTIVE IMPAIRMENT OF HTLV-1-SPECIFIC T-CELLS IN EARLY STAGES OF ATL, A POTENTIAL RISK FOR ATL

We previously identified some major epitopes recognized by HTLV-1-specific CTLs presented by HLA-A2, -A11, or -A24 through analysis of CTLs collected from ATL patients after HSCt or collected from HAM/TSP patients (Kannagi et al., 1992; Harashima et al., 2004, 2005). The identification of these epitopes allowed us to monitor HTLV-1-specific CTLs and analyze their functions *ex vivo* by using antigen/HLA tetrameric complexes. In

our study using Tax-specific tetramers on HLA-A2, -A11, or -A24-positive individuals, we detected Tax-specific CTLs in 100% of HAM/TSP patients, 87% of ACs, and 38% of chronic ATL (cATL) patients tested (Figure 2; Takamori et al., 2011).

It is interesting that Tax-specific CTLs were detectable also in cATL patients, although their frequency among peripheral CD8⁺ cells is low. However, these CTLs were anergic as they neither proliferated nor produced IFN- γ upon peptide stimulation. In contrast, Tax-specific CTLs in HAM/TSP patients were highly active even without stimulation, and their response was further enhanced by stimulation.

Amongst ACs, Tax-specific CTLs are detectable in the majority but not a small subset of individuals. Although Tax-specific CTLs detected in ACs are mostly functional, sporadic AC cases with impaired CTL responses to peptide-stimulation analogous to ATL patients have been identified. Interestingly, such functional impairment of CTLs seems selective to HTLV-1-specific responses, as cytomegalovirus (CMV)-specific CTLs remain functional (Figure 2; Takamori et al., 2011). Similar dysfunctions of Tax-specific but not CMV-specific CTLs are found in smoldering ATL, an early stage of ATL without clinically apparent lymphoproliferation. These findings suggest that the scarcity and/or dysfunction of Tax-specific CTLs are not merely the result of ATL, but represent host conditions in a subset of HTLV-1 carriers at



asymptomatic stages. A reduced number and/or dysfunction of Tax-specific CTLs could thus represent an underlying risk factor for the development of ATL.

Epidemiological studies indicated that increased numbers of abnormal lymphocytes or HTLV-1 proviral loads are risk factors for the development of ATL (Tajima, 1990; Hisada et al., 1998). However, elevated HTLV-1 proviral loads are also detected in HAM/TSP patients, and do not discriminate risks for ATL and HAM/TSP (Nagai et al., 1998). The immunological studies described above suggested that insufficiency in host T-cell responses against HTLV-1 might be another risk factor for ATL. We therefore suggest that the combination of elevated proviral loads and low HTLV-1-specific T-cell responses may represent a more selective indicator for the risk of developing ATL.

MECHANISMS OF T-CELL SUPPRESSION IN HTLV-1 INFECTION

It is known that ATL is often associated with severe immune suppression (Tashiro et al., 1992), and a small number of studies reported general immune suppression also in ACs (Imai and Hinuma, 1983). The mechanism of general immune suppression in these individuals is not known. ATL cells are positive for CD4, CD25, CCR4, and frequently express Foxp3, all of which match the phenotype of regulatory T-cells. If ATL cells function as Treg cells, this would be a strong reason for the observed general immune suppression (Karube et al., 2004). There are reports of increased numbers of Foxp3-expressing Treg cells in the HTLV-1-negative cell population in HAM/TSP patients (Toulza et al., 2008). Recent studies reported that HBZ is potentially involved in immune suppression by enhancing TGF- β signaling and suppressing Th1 cytokine production (Zhao et al., 2011; Sugata et al., 2012).

As mentioned above, the insufficient Tax-specific CTL response observed in the early stages of ATL and in a subpopulation of ACs was selective for the response to HTLV-1, and did not affect CMV-targeting CTLs. From this differential effect we deduce that other HTLV-1-specific suppressive mechanisms are active, in addition to the general suppression. In general, antigen-specific T-cell suppression can be induced by immune tolerance and T-cell exhaustion. In HTLV-1 infection, vertical infection could be a reason for T-cell tolerance. In animal models, oral HTLV-1 infection induces T-cell tolerance to HTLV-1, resulting in increased levels of proviral loads (Hasegawa et al., 2003). Since vertical infection of HTLV-1 is established mainly through breast feeding (Kinoshita et al., 1987), it may induce both new-born tolerance and oral tolerance. This might partly explain the epidemiological finding that vertical HTLV-1 infection is one of the risk factors for ATL (Tajima, 1990).

Besides immune tolerance, T-cell suppression can also be caused by T-cell exhaustion, which may be a consequence of continuous expression of HTLV-1 antigens *in vivo*. Expression of PD-1 in Tax-specific CTLs has been reported (Kozako et al., 2009), although the involvement of this molecule in the suppression of CTLs in HTLV-1-infected individuals is still controversial (Takamori et al., 2011). The relevance of other antigens remains unknown, as for example recent studies revealed that Tax-specific CTLs in HAM/TSP patients express reduced levels of Tim3, one of

the T-cell exhaustion markers, despite high viral gene expression in these patients (Abdelbary et al., 2011; Ndhlovu et al., 2011).

IMPACT OF TYPE-I IFNs IN CONTROLLING HTLV-1 EXPRESSION

INDUCTION OF TYPE-I IFN RESPONSE BY HTLV-1 INFECTION

Various viruses induce type-I IFN responses. In HTLV-1 infection, however, the number of studies investigating a putative HTLV-1-induced type-I IFN response is limited. One of the reasons is that efficient HTLV-1 infection is mediated mainly through cell-cell contact. A recent report indicated that addition of cell-free HTLV-1 particles propagated using an HTLV-1 molecular clone to plasmacytoid dendritic cells (pDCs) induced a type-I IFN response through Toll-like receptor 7 (TLR7; Colisson et al., 2010). pDCs are a major producer of type-I IFNs, and are reported to be susceptible to HTLV-1 infection (Hishizawa et al., 2004; Jones et al., 2008). In ATL patients, the number of pDCs is decreased, and the remaining pDCs lack the ability to produce IFN- α (Hishizawa et al., 2004).

At cell-cell contacts between HTLV-1-infected T-cells and stromal cells, we found that HTLV-1 induced a type-I IFN response in the stromal cells, suggesting an involvement of pattern recognition molecules other than TLR7. However, the precise mechanisms of HTLV-1-induced type-I IFN responses remain to be clarified.

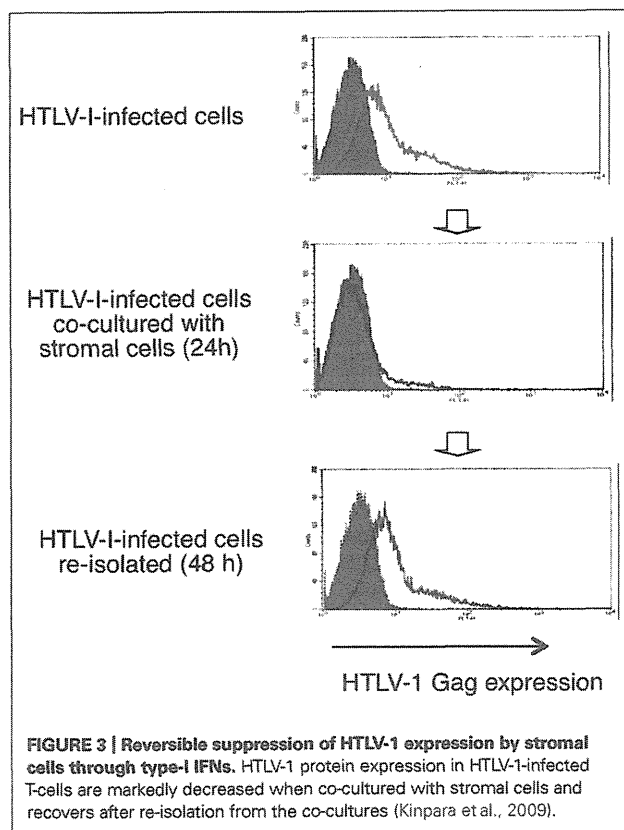
SUPPRESSION OF HTLV-1 EXPRESSION BY TYPE-I IFNs

HTLV-1 mRNA and protein expression in HTLV-1-infected T-cells are markedly decreased when co-cultured with stromal cells such as epithelial cells and fibroblasts. This suppression of HTLV-1 expression is inhibited by blocking the IFN- α/β receptor, and is therefore thought to be mediated through type-I IFN responses (Figure 3; Kinpara et al., 2009).

Interestingly, when infected cells were re-isolated from the co-cultures, viral expression was restored to the original level over the following 48 h (Figure 3). This phenomenon resembles the induction of HTLV-1 expression in freshly isolated ATL cells after culture *in vitro*. Type-I IFNs might therefore explain the long-puzzling observation that HTLV-1 expression is suppressed *in vivo*. In support of this notion, viral expression in HTLV-1-infected cells was significantly suppressed when injected into wild-type mice but not into IFN regulatory factor-7-knockout mice, which are deficient in most type-I IFN responses (Kinpara et al., 2009).

In general, type-I IFNs suppress viral replication mostly at post-transcriptional level. Since HTLV-1 transcription is regulated by transactivation of its own LTR, mainly through cyclic AMP (cAMP) response element-like repeats by the Tax protein (Fujisawa et al., 1985; Sodroski et al., 1985), limitation of this protein below a certain level will efficiently reduce HTLV-1 expression to a basal level. Involvement of inducible cAMP early repressor (ICER) and transducer of regulated CREB protein 2 (TORC2) in the inhibition of HTLV-1 transactivation has also been suggested (Newbound et al., 2000; Jiang et al., 2009).

Addition of IFNs alone also elicits suppressive effects in HTLV-1 expression. However, the levels of suppressive effects differ among studies. In HTLV-1-infected cell lines, it has been reported that IFN- α 2a decreased HTLV-1 assembly and viral release but not viral protein synthesis (Feng et al., 2003).



RESISTANCE OF HTLV-1 AGAINST TYPE-I IFN SIGNALING

As is the case with many other viruses, HTLV-1 has developed strategies to evade IFN responses. It has been reported that HTLV-1 infection reduces the phosphorylation of tyrosine kinase 2 (TYK2) and signal transducer and transcriptional activator 2 (STAT2; Feng and Ratner, 2008), and that Tax inhibit the induction of IFN-stimulated genes (ISGs) by competing with CREB binding protein/p300 (Zhang et al., 2008). Recent reports also suggest that Tax-mediated up-regulation of suppressor of cytokine signaling 1 (SOCS1) inhibits IFN signaling (Oliere et al., 2010; Charoenthongtrakul et al., 2011). However, expression levels of Tax protein are low *in vivo*, and it is unclear to what extent the evading mechanisms observed *in vitro* are effective *in vivo*.

It has been reported that a combination therapy of AZT and IFN- α is effective for the treatment of ATL (Hermine et al., 1995), indicating that HTLV-1-infected cells retain some susceptibility to IFNs *in vivo*. Intriguingly, this combination of AZT/IFN- α does not affect HTLV-1-infected cells *in vitro* (Bazarbachi et al., 2000), and the mechanistic effect of this therapy is not known. The discrepancy in the therapeutic effects *in vivo* and *in vitro* is presumably due to the different status of HTLV-1-infected cells in the two systems.

AZT/IFN- α is not a radical therapy, and ATL relapses are frequently observed after cessation of the therapy (Hermine et al., 2002), suggesting that AZT/IFN- α may not be cytotoxic but rather has static effects on infected cells. Another combination therapy

of arsenic trioxide and IFN- α shows more favorable therapeutic effects *in vivo*, and also shows proteolysis of Tax in HTLV-1-infected cells *in vitro* (El Hajj et al., 2010). IFN- α or β alone appears less effective for the treatment of HAM/TSP, but does show some therapeutic effects, especially during the early stages of HAM/TSP (Izumo et al., 1996; Saito et al., 2004).

IFN RESPONSES IN HTLV-1-INFECTED INDIVIDUALS

A recent study revealed up-regulation of SOCS1 in CD4⁺ cells of HAM/TSP patients, which caused enhanced viral expression through inhibition of type-I IFN signaling (Oliere et al., 2010). At the same time, a different study showed that HTLV-1 Tax up-regulates SOCS1 (Charoenthongtrakul et al., 2011). These findings indicate that up-regulation of SOCS1 might be a result and/or cause of enhanced viral expression in HAM/TSP. Another recent study using gene expression array analysis reported up-regulation of a subset of ISGs, including STAT1, CD64, FAS, and CXCL10, especially in the neutrophil and monocyte fractions from peripheral blood of HAM/TSP patients (Tattermusch et al., 2012). This suggests that type-I IFN responses were induced in these cell populations directly or indirectly by HTLV-1, although type-I IFN production in these cells was not clear. The strong HTLV-1-specific T-cell response in these patients might also cause such effects through IFN- γ production.

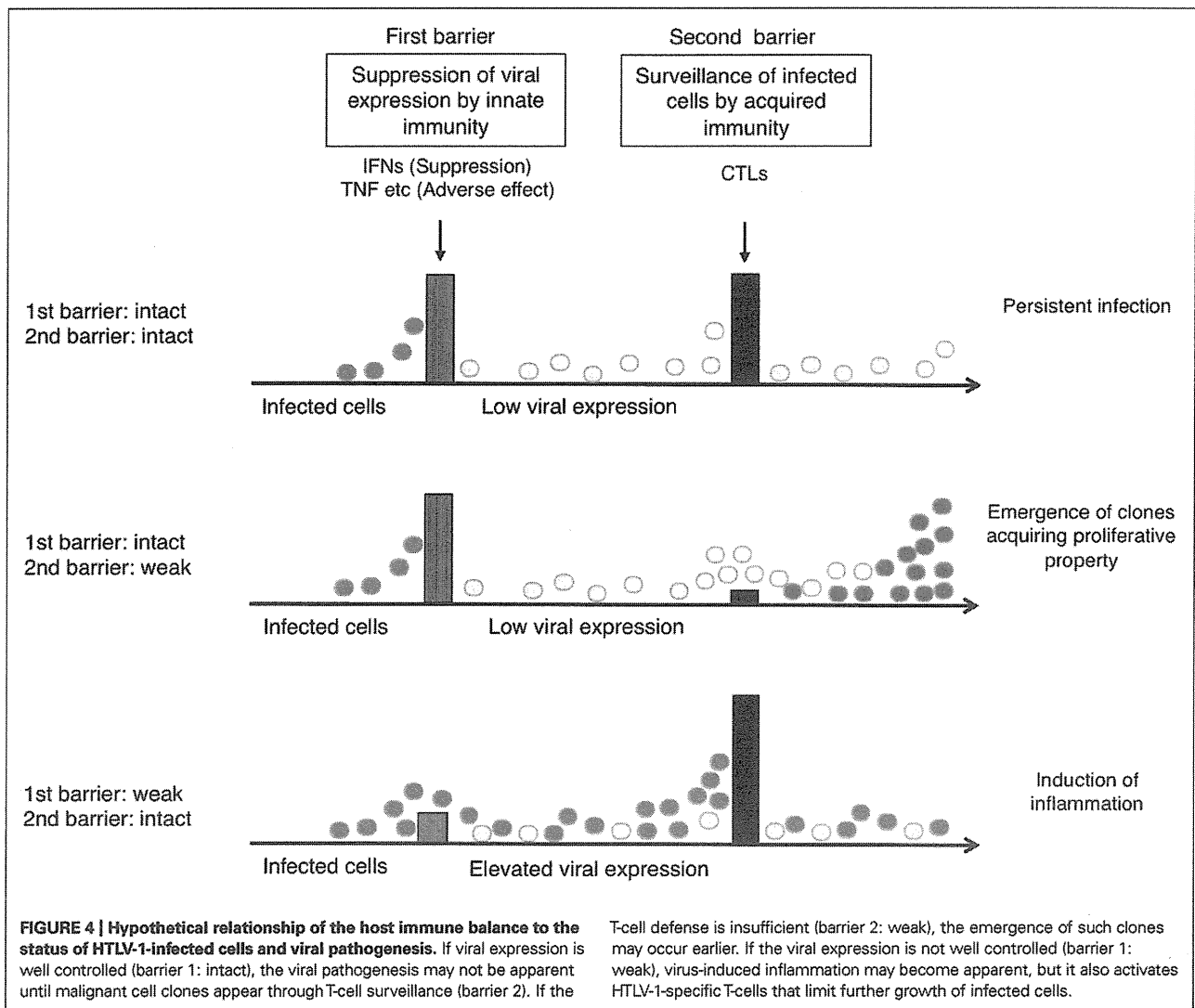
The signature of IFN responses in the peripheral blood of HAM/TSP patients left an unanswered question what enhances the basal level of viral expression in these patients. Increased inflammatory cytokines in HAM/TSP patients might be candidates to enhance viral expression, but again these could be a result and/or cause of enhanced HTLV-1 expression.

THE RELATIONSHIP AMONG VIRAL EXPRESSION, HOST IMMUNITY, AND VIRAL PATHOGENESIS

It is speculated that the status of viral expression and host immunity may differ among various tissues *in vivo*. Therefore, it is difficult to estimate HTLV-1 status in the entire body based on the information gained only from peripheral blood. Nevertheless, the recent findings about innate immunity described above provide clues as to how the current knowledge around HTLV-1 expression and host immunity can be integrated, especially when they so closely interact and have both causes and effects on each other.

Type-I IFNs are likely to be the representative factor to control HTLV-1 expression, and HTLV-1-specific T-cells survey infected cells to limit their growth. The suppression of viral expression might interfere with the efficacy of HTLV-1-specific T-cells by reducing the levels of target molecules, even in hosts with a functional HTLV-1-specific T-cell response. The resulting low efficiency of T-cell surveillance would be one of the mechanisms behind persistent HTLV-1 infection, although it seems that T-cells would still contribute to the control of HTLV-1-infected cell growth to some extent.

Despite the negative impact on T-cell surveillance, the suppression of viral expression is important for the host to reduce viral pathogenesis since Tax has a strong ability to activate NF- κ B, which is critical for the induction of inflammation or cell growth signaling.



Supposing that the suppression of viral expression is the first barrier and T-cell surveillance of infected cells is the second barrier in the host defense, the balance of these barriers would influence the status of HTLV-1-infected cells *in vivo*. A conceivable scenario is as follows (Figure 4).

If viral expression is well controlled, the viral pathogenesis may not be apparent until malignant cell clones appear through the process of clonal evolution in the infected cell reservoir. This might explain the long incubation time for ATL development. In the absence of effective T-cell responses, the emergence of such clones may occur earlier, as clonal survival may be more likely.

In contrast, if the suppression of viral expression is insufficient, either by insufficient IFN response or increased inflammatory cytokines, viral pathogenesis will become apparent and symptoms will be exhibited, especially in the tissues where viral proteins reach functional levels. In this case, however, the elevated levels of viral expression would also activate HTLV-1-specific T-cells, which potentially limit further growth of infected cells.

CONCLUSION

The status of HTLV-1-specific T-cell response has been shown to be a determinant of HTLV-1-mediated diseases because of its anti-tumor and anti-viral effects. The selective impairment of HTLV-1-specific T-cell responses in early stages of ATL patients implies the presence of HTLV-1-specific suppressive mechanisms. The combination of insufficient HTLV-1-specific T-cell response and elevated proviral load may allow the identification of a group with a high risk for the development of ATL. In addition, vaccines that augment HTLV-1-specific T-cell responses may prove beneficial in reducing the risk in such a subpopulation.

The status of HTLV-1 expression can be another determinant of HTLV-1-mediated diseases. Suppression of viral expression contributes to reduced viral pathogenesis, although it may, at the same time, partially interfere with T-cell surveillance. Host innate immunity, especially type-I IFN, is a candidate for the regulation of viral expression.

Thus, both acquired and innate immunity can be host determinants that modulate HTLV-1-associated diseases. The involvement of the two control systems and their partially conflicting effects on one another may explain why the same virus can cause different diseases after a long incubation

time. Further studies will elucidate the precise mechanisms for the regulation of host immunity and viral expression, and thereby provide insights for the prediction of disease risks, as well as new targets for the prevention of HTLV-1-mediated diseases.

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The *Mycobacterium tuberculosis* Stress Response Factor SigH Is Required for Bacterial Burden as Well as Immunopathology in Primate Lungs

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(See the editorial commentary by Kernodle, on pages 1186–8.)

Background. Sigma H (sigH) is a major *Mycobacterium tuberculosis* (*Mtb*) stress response factor. It is induced in response to heat, oxidative stress, cell wall damage, and hypoxia. Infection of macrophages with the Δ -sigH mutant generates more potent innate immune response than does infection with *Mtb*. The mutant is attenuated for pathology in mice.

Methods. We used a nonhuman primate (NHP) model of acute tuberculosis, to better understand the phenotype of the Δ -sigH mutant in vivo. NHPs were infected with high doses of *Mtb* or the mutant, and the progression of tuberculosis was analyzed in both groups using clinical, pathological, microbiological, and immunological parameters.

Results. Animals exposed to *Mtb* rapidly progressed to acute pulmonary tuberculosis as indicated by worsening clinical correlates, high lung bacterial burden, and granulomatous immunopathology. All the animals rapidly succumbed to tuberculosis. On the other hand, the NHPs exposed to the *Mtb*: Δ -sigH mutant did not exhibit acute tuberculosis, instead showing significantly blunted disease. These NHPs survived the entire duration of the study.

Conclusions. The *Mtb*: Δ -sigH mutant is completely attenuated for bacterial burden as well as immunopathology in NHPs. SigH and its regulon are required for complete virulence in primates. Further studies are needed to identify the molecular mechanism of this attenuation.

Tuberculosis is responsible for the deaths of >1.7 million people annually [1]. This situation is exacerbated by the emergence of drug-resistant *Mycobacterium tuberculosis* (*Mtb*) [2, 3], AIDS coinfection [4], and the failure of the BCG vaccine [5]. Development of efficacious treatments

and vaccines against tuberculosis will require better understanding of the pathogenesis of *Mtb*.

Alternative sigma (σ) factors allow bacteria to respond to changes in the extracellular environment by modulating the expression of specific sets of genes [6]. The temporal expression of specific regulons controlled by the induction of ≥ 1 of the 10 alternate σ factors [7] encoded by its genome may allow *Mtb* to survive in diverse environments encountered by it in vivo. Sigma H (sigH) is an alternate σ factor induced by various stress conditions, phagocytosis, cell wall damage, enduring hypoxia, and reoxygenation and possibly plays a role in reactivation [8–19]. The Δ -sigH mutant fails to induce granulomatous pathology in spite of bacterial replication in mice [8]. Infection of nonhuman primate (NHP) bone-marrow macrophages with *Mtb*: Δ -sigH results in a significantly enhanced monocyte chemotaxis and

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apoptosis relative to cells infected with *Mtb* [20]. Thus, sigH appears to code for functions crucial for modulating immune response.

Due to their physiological and immunological similarity to humans, NHPs are excellent experimental models of tuberculosis [21, 22]. Two key NHP models of tuberculosis exist. One model is based on infected cynomolgus macaques with virulent *Mtb* via the intratracheal route [22]. The other model is based on infection of rhesus macaques with virulent *Mtb* via the aerosol route mimicking the natural method of exposure [23–25]. By modulating the number of infectious aerosols presented, it is possible to model either acute [23, 24] or latent [25] tuberculosis in these NHPs. The number of presented aerosols is standardized by plethysmography immediately prior to infection. The acute model is used to understand bacterial pathogenesis, whereas the latent model is used to study the various mediators of latency, reactivation, and tuberculosis/AIDS coinfection [25]. We employed the acute model to address whether sigH is important for growth and replication of *Mtb* as well as immunopathology in primates.

MATERIALS AND METHODS

Animals and Infection

Animals were cared for according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee guidelines. Aerosol infection of 13 Indian-origin rhesus macaques (Table 1) was performed as described elsewhere

[23, 25]. Seven animals were exposed to a high-dose (approximately 5000 colony-forming units [CFUs]) of *Mtb* CDC1551. Six NHPs were similarly exposed to the *Mtb*: Δ -sigH mutant. Blood, bronchoalveolar lavage (BAL), and peripheral lymph node (LN) samples were collected periodically. Clinical assessment of disease, C-reactive protein (CRP) assay, chest radiography (CXR), tuberculin skin test (TST), necropsy, and histopathology procedures, including the analysis of percentage of the lung area involved in tuberculosis-like pathology, have been described in NHPs [23, 25, 26].

Bacterial Burden

Viable CFUs were compared in periodic BAL fluid and LN homogenates as well as random lung section homogenates at necropsy as described elsewhere [23, 25], to measure the burden of viable tubercle bacilli. For confocal microscopy based detection of the bacilli in the lung lesions, a polyclonal anti-*Mtb* antibody raised in rabbit (Abcam ab905) was used as described elsewhere [23–26].

Comparison of Granulomatous Gene-Expression Upon Infection With *Mtb* and *Mtb*: Δ -sigH

DNA microarray experiments were performed as described elsewhere [24]. Transcripts isolated from 3 randomly chosen NHPs from each group were profiled, relative to normal rhesus lung tissue.

Immunohistochemistry and Confocal Microscopy

CD3⁺, FoxP3⁺, and CD25⁺ cells in tissues were counted by immunohistochemistry as described elsewhere [27]. The following

Table 1. Animals Infected With *Mycobacterium tuberculosis* (*Mtb*) or the *Mtb*: Δ -sigH Mutant and Their Tuberculin Skin Test Results

ID	Infecting Agent	Age at Infection (Years)	Weight at Infection (kg)	Time to Death (Days)	Time to Experiment Death (Days)	TST (Preinfection)	TST (Week 3)	TST (Week 9)
CG58	<i>Mtb</i>	7.50	11.40	75		NNN	PPP	P ^a
DJ57	<i>Mtb</i>	5.80	12.65	75		NNN	PPP	P ^a
HB38	<i>Mtb</i>	3.20	5.00	71		NNN	PPP	NC
HB43	<i>Mtb</i>	3.20	5.30	56		NNN	NPP	NC
GN05	<i>Mtb</i>	4.02	6.30	57		NNN	PPP	NC
GK47	<i>Mtb</i>	4.10	6.70	49		NNN	NPP	NC
GM97	<i>Mtb</i>	4.02	5.70	79		NNN	PPP	PPP
CC41	<i>Mtb</i> : Δ -sigH	7.75	14.20		76	NNN	PPP	P ^a
DM75	<i>Mtb</i> : Δ -sigH	5.80	12.55		76	NNN	PPP	P ^a
EE62	<i>Mtb</i> : Δ -sigH	8.13	14.00		70	NNN	NPP	PPP
DE35	<i>Mtb</i> : Δ -sigH	9.17	13.30		70	NNN	NPP	PPP
GM51	<i>Mtb</i> : Δ -sigH	4.03	5.50		71	NNN	PPP	PPP
HC50	<i>Mtb</i> : Δ -sigH	3.17	5.60		71	NNN	NNN	PPP

Shown are the unique Tulane National Primate Resource Center–assigned IDs, age and weight at infection, and time to death or experimental euthanasia following infection. Results from eyelid TST are also described for 3 different time points: preinfection and weeks 4 and 8 postinfection [33]. Results were measured at 24, 48, and 72 hours postadministration.

Abbreviations: N, negative; NC, not conducted; NNN, a negative TST was recorded at each of the 3 time points (24, 48, and 72 hours); NPP, a positive TST was recorded at the 48- and 72-hour time points only; P, positive; PPP, a positive TST at each of the 3 time points; TST, tuberculin skin test.

^a PRIMAGAM, an interferon γ release assay, was substituted for TST [33].

antibodies were used: anti-CD3 (Dako-A0452, rabbit, 1:50); antiFoxP3 (Vector Labs- VP-C340, mouse, 1:50) and anti-CD25 (eBioscience-14-4776-82, rat, 1:50). Multilabel confocal immunofluorescence was performed using previously described protocols [23–26] that used the following antibodies: interferon γ (IFN- γ) (1:10, mouse, BD Biosciences 551221); CD3 (1:10; mouse, Dako M7254), and FoxP3 (1:500; rabbit, Abcam ab10901).

Recruitment of Monocytes Derived From Bone Marrow Upon Infection

In vivo bromodeoxyuridine (BrdU) pulse-labeling and flow-cytometric analysis were performed as described elsewhere [28].

RESULTS

Comparison of Clinical Correlates of Infection in NHPs Infected With *Mtb*: Δ -sigH, Relative to Those Infected With *Mtb*

All 13 NHPs were negative for simian immunodeficiency virus (SIV), simian T-lymphotropic virus (STLV), simian retrovirus (SRV), and hepatitis B virus. All NHPs converted to a positive TST within 3–5 weeks postinfection, indicating successful infection (Table 1). TST was read at 24, 48, and 72 hours postadministration. Positive TST result was obtained for all 13 NHPs at 72 hours and for most NHPs at 48 and 24 hours. The animals infected with *Mtb* began exhibiting clinical features associated with acute tuberculosis within 3–4 weeks postinfection. In total, 6 of 7 NHPs in this group had elevated temperatures of $>2^{\circ}\text{F}$ higher than average preinfection temperatures at the week 3 postinfection time point, whereas none of the 6 NHPs exposed to *Mtb*: Δ -sigH experienced a significant increase in body temperature at any time during the study (Figure 1A).

The *Mtb*-infected NHPs exhibited significant weight loss over the study period, losing 5%–25% of their body weight over the duration of the study (Figure 1B). In fact, by week 7 postinfection, the NHPs in this group had lost an average of approximately 12% in body weight. In contrast, the NHPs in the mutant-infected group gained an average of 7% body weight during the study (Figure 1B).

NHPs infected with *Mtb* are known to exhibit increased serum levels of acute-phase proteins such as CRP. We therefore studied serum levels of CRP to measure systemic inflammation, over the course of infection [23, 25–27]. The serum CRP levels spiked in each of the 7 *Mtb*-infected NHPs between 3 and 6 weeks postinfection (Figure 1C) and were significantly elevated relative to preinfection baseline for each of the 7 NHPs (Figure 1C). At week 3 postinfection, the CRP values for the NHPs in this group ranged from 3.26 to 44.75 mg/L, as compared with preinfection values ranging from 0.0 to 0.4 mg/L. The mutant-infected NHPs did not exhibit elevated CRP values. The maximal CRP value for the mutant-infected NHPs throughout the study was 2.5 mg/L. Only 2 animals in this group exhibited higher than baseline

CRP values at any time point but with significantly lower magnitude. The differences between the 2 groups were significant from week 3 postinfection onward (Figure 1C).

CXRs from these NHPs were assigned a subjective score on a scale of 0–3, using the following scoring criterion: no involvement, 0; minimal disease, 1; moderate disease, 2; and severe/miliary disease, 3 [23, 25]. All NHPs infected with *Mtb* exhibited gradually increasing CXR scores that were significantly higher than baseline scores at weeks 3 and 7 postinfection. The mutant infected animals indicated significantly lower CXR scores (Figure 1D). These results corroborate the lack of progression of tuberculosis in NHPs infected with *Mtb*: Δ -sigH, relative to NHPs infected with *Mtb*, in this high-dose, acute disease model.

Survival Differences Between the 2 Groups of NHPs

Differences in the progression of tuberculosis in NHPs infected with *Mtb* vs *Mtb*: Δ -sigH were apparent in the survival of the 2 groups. All 7 NHPs infected with *Mtb* succumbed to acute, pulmonary tuberculosis within 13 weeks postinfection. The median time to death for this group was 65 days (Figure 1E). None of the animals infected with the mutant died during this period due to disease. These NHPs were experimentally killed. The survival proportions between the 2 groups were statistically significant (Figure 1E).

Bacterial Burden in NHPs Infected With *Mtb* and *Mtb*: Δ -sigH

We analyzed bacterial burden in BAL temporally at 3, 5, 9, and 11 week postinfection. A gradual increase in the number of viable *Mtb* CFUs was observed. In NHPs exposed to *Mtb*, viable CFU load was detected beginning week 5 postinfection, peaking at week 9 postinfection (average, $\sim 2 \times 10^3$ CFUs). No viable CFUs could be detected in the BAL fluid obtained from mutant-infected NHPs 5 week postinfection. Significantly low numbers of CFUs were recovered in the BALs of some NHPs in this group ($1\text{--}2 \times 10^2$ CFU) relative to the *Mtb* group at weeks 9 and 11 (Figure 2A).

Similarly, significantly high bacillary load was observed in the LNs of *Mtb*-infected NHPs, relative to mutant-infected NHPs. A higher load was observed for both groups at week 9 postinfection, relative to week 3 postinfection. The levels of *Mtb*: Δ -sigH in LNs were significantly lower relative to *Mtb* throughout the study (Figure 2B).

Finally, we assessed the *Mtb* load in lungs at the time of necropsy (Figure 2C). Average lung CFU values in *Mtb*-infected NHPs ranged from 7×10^3 to 8×10^4 CFUs/g. In contrast, the average lung CFUs in the mutant-infected NHPs were significantly lower, ranging from 0 to 3.3×10^2 CFUs/g (Figure 2C). The *Mtb*: Δ -sigH mutant exhibits a remarkably reduced bacterial burden in tissues compared with *Mtb*, in the NHP model.

The CFU results from BAL and lung were corroborated by immunofluorescence-based detection of bacilli in the lungs

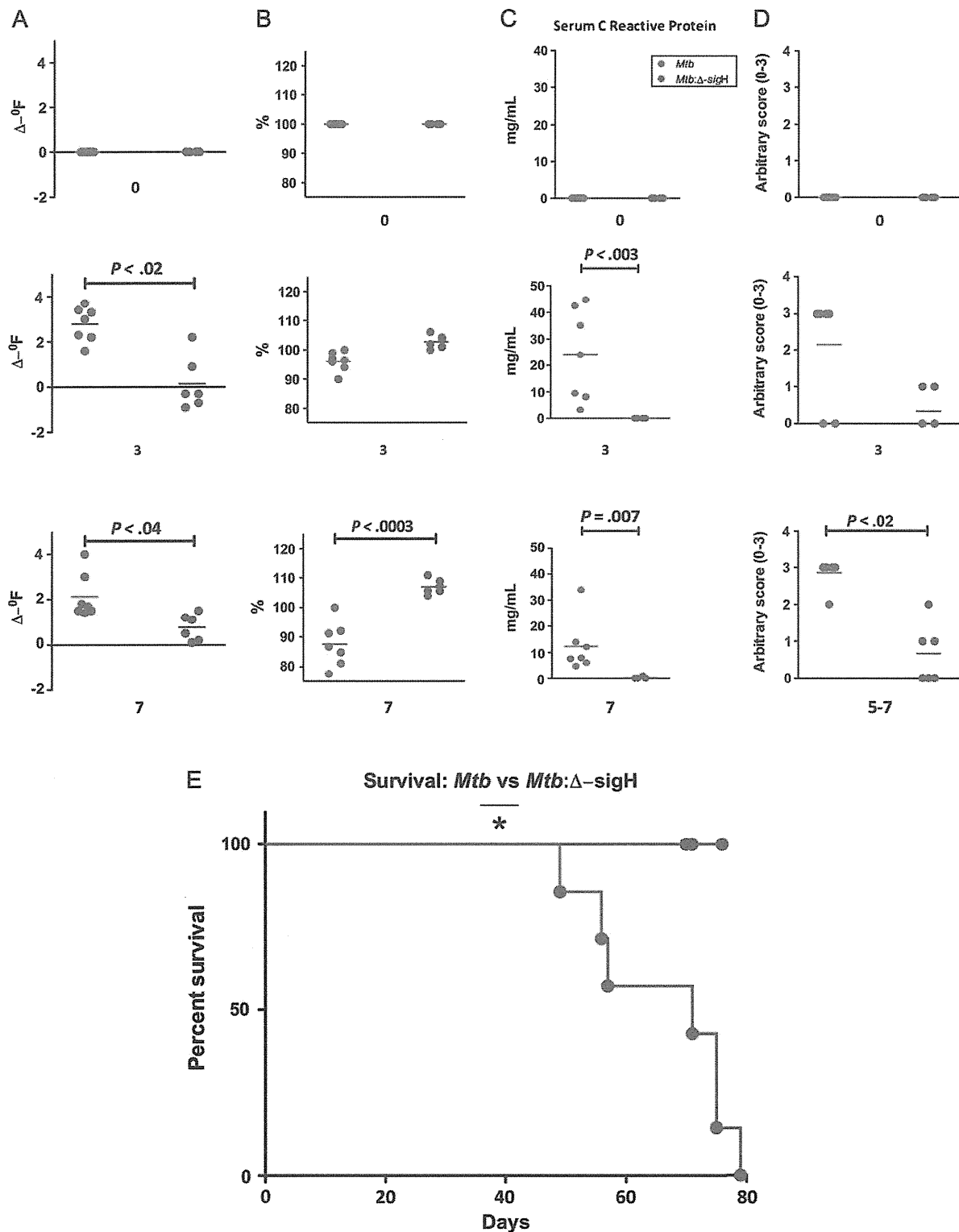


Figure 1. Clinical data from nonhuman primates (NHPs) infected with *Mycobacterium tuberculosis* (*Mtb*) as well as the *Mtb*: Δ -sigH mutant. *A*, Changes in body temperature, expressed in Δ -°F. *B*, Changes in body weight, expressed as percentage of total weight at the time of *Mtb* infection. *C*, Changes in serum C-reactive protein (CRP) levels. *D*, Changes in arbitrary chest radiographic (CXR) scores. *E*, Survival proportions. Data are shown for week 0 (preinfection) as well as weeks 3 and 7 postinfection for *A*, *B*, *C*, and *D*. The x-axis values represent week postinfection. Red circles denote NHPs infected with *Mtb*, whereas blue circles denote NHPs infected with the *Mtb*: Δ -sigH mutant. Significant differences are shown wherever detected.

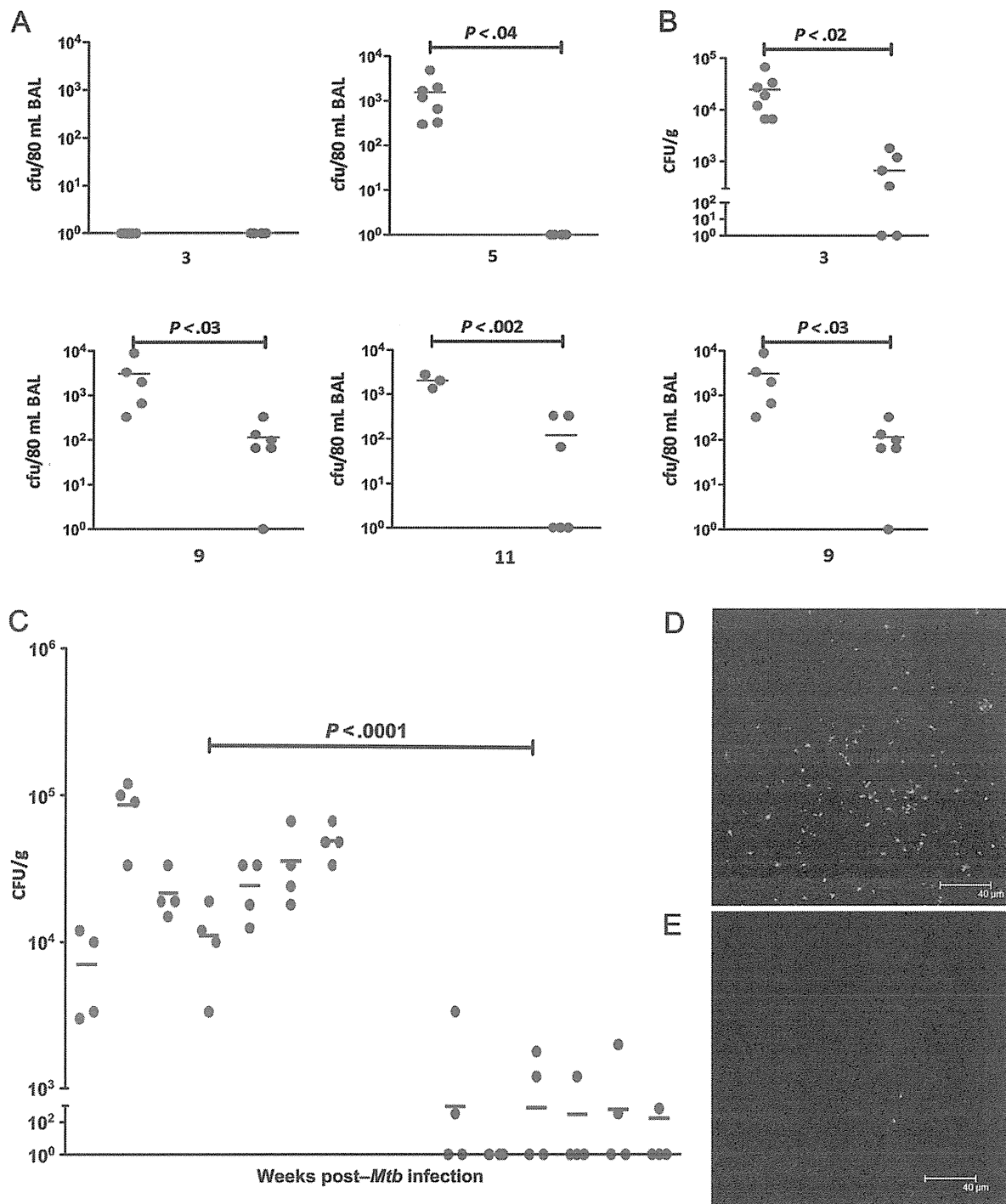


Figure 2. Bacterial burden in the 2 groups of nonhuman primates (NHPs). Temporal *Mycobacterium tuberculosis* (*Mtb*) colony-forming units (CFUs) are shown per 80 mL bronchoalveolar lavage (BAL) samples obtained from both groups of NHPs at weeks 3, 5, and 9 postinfection. **A**, Temporal *Mtb* CFUs are shown per gram of bronchial lymph node tissue obtained from both groups of NHPs at weeks 3 and 7 postinfection. **B**, *Mtb* colony-forming units (CFUs) are also shown per gram of lung tissue obtained at necropsy from both groups of NHPs. Each lung was randomly sectioned at necropsy and 10 sections from each of the 2 lungs were pooled into 2 groups (right lung 1, right lung 2; left lung 1, left lung 2). Results are shown for all 4 of these lung samples for each of the 13 NHPs. The x-axis values represent the week postinfection. Red circles denote NHPs infected with *Mtb*, and blue circles denote NHPs infected with the *Mtb*: Δ -sigH mutant. Confocal microscopy shows the extent of bacterial presence in the lungs of a representative NHP infected with *Mtb* (**D**) relative to a representative NHP infected with *Mtb*: Δ -sigH (**E**).

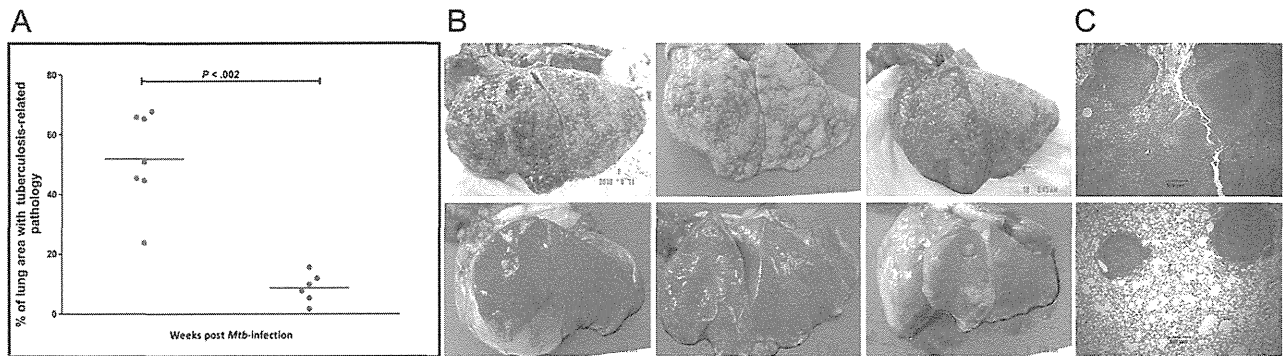


Figure 3. Postnecropsy pathology data from the 2 groups of nonhuman primates (NHPs). The percentage of lung area with tuberculosis-related pathology was estimated in both groups of NHPs (A) using methods described elsewhere [23, 25]. The x-axis values represent week postinfection. Red circles denote NHPs infected with *Mycobacterium tuberculosis* (*Mtb*), whereas blue circles denote NHPs infected with the *Mtb:Δ-sigH* mutant. Lines correspond to mean values. The differences between 2 groups were statistically significant. Gross pathology is shown for 3 representative NHPs each, infected with *Mtb* (top panel) and *Mtb:Δ-sigH* (bottom panel) (B). Histopathologic analysis of hematoxylin and eosin–stained lung samples from 1 representative NHP each, infected with *Mtb* (top panel) and *Mtb:Δ-sigH* (bottom panel) (C).

of *Mtb*- (Figure 2D) and mutant-infected NHPs (Figure 2E). Significantly higher levels of bacterial infection could be observed in the lungs of NHPs infected with *Mtb* (Figure 2D), whereas lungs of NHPs infected with the mutant were largely devoid of any bacilli (Figure 2E).

Lung Pathology in NHPs Infected With *Mtb:Δ-sigH*, Relative to Those Infected With *Mtb*

The NHPs infected with *Mtb* exhibited extensive pulmonary granulomatous immunopathology. The observed disease pathology was similar to an earlier study in which NHPs were exposed to a high dose of *Mtb* transposon mutants [23]. The extent of diseased tissue involved in tuberculous lesions (necrosis, granulomas, edema, etc) was calculated as a percentage of the total area (Figure 3). The average percentage of involvement in the lungs of *Mtb* and *Mtb:Δ-sigH* infected NHPs were 56.6% and 8.6%, respectively. Significant differences were apparent in lung lesions of the 2 groups (Figure 3A). Although the lungs of NHPs infected with high-dose *Mtb* exhibited multifocal and confluent granulomas (Figure 3B), the animals infected with the mutant exhibited relatively few and widely scattered lesions (Figure 3B). Histopathologic analysis using hematoxylin and eosin–stained lung tissues confirmed that the lesions observed in the NHPs infected with both *Mtb* and *Mtb:Δ-sigH* exhibited classical tuberculoïd pattern with central necrosis and a peripheral infiltrate consisting of histiocytes, lymphocytes, and multinucleated giant cells (Figure 3C). NHPs infected with *Mtb* also exhibited acute inflammation with hemorrhage and neutrophilic infiltration suggesting concurrent chronic and rapid progression. Therefore, the infection with a high dose of *Mtb:Δ-sigH* produced a significantly and severely blunted tuberculosis disease.

Granulomatous Immune Response to Infection of NHPs With *Mtb:Δ-sigH*, Relative to *Mtb*

Using global transcriptomics, lung tubercular lesions from the 2 groups of NHPs were used to study changes in the pattern of immune response to infection [24]. Because we have already reported the transcriptome of primate lesions in response to *Mtb* infection at both the acute and the chronic stages [24], here we focused on genes that exhibit maximal significance in the magnitude of gene expression between the 2 groups, using multiple-hypothesis corrected *P* value (Figure 4A).

The expression of MMP9 (150-fold), CCL5 (28-fold), LTA (10-fold), and FOSB (4-fold) genes was induced to significantly higher levels in the lesions of *Mtb*-infected NHPs but remained at normal levels in lesions from the mutant-infected NHPs (Figure 4A). On the other hand, the expression of SOCS3 (8-fold), FOXJ1 (6-fold), BAX (5-fold), and CCL14 (3-fold) was induced to significantly higher levels in the lesions of mutant-infected NHPs, relative to *Mtb*-infected NHPs.

Because the expression of several proinflammatory genes was induced to higher levels in *Mtb* lesions, we studied the expression of the prototypical proinflammatory molecule, IFN- γ , by immunofluorescence. Lesions derived from *Mtb*-infected NHPs exhibited significantly high IFN- γ levels (Figure 4B), relative to lesions from mutant-infected NHPs (Figure 4C). We also studied the expression of the regulatory T cell (Treg) marker FoxP3 on T cells in these lesions by immunohistochemistry (Figure 5A–C) and immunofluorescence (Figure 5D and 5E). The total number of (CD3⁺) T cells was significantly higher in the mutant lesions, relative to *Mtb* lesions (Figure 5A and 5E). This result is consistent with the fact that animals in the *Mtb* group exhibited acute pathology. However, the percentage of T cells that also exhibited FoxP3 expression was slightly higher in the mutant group,

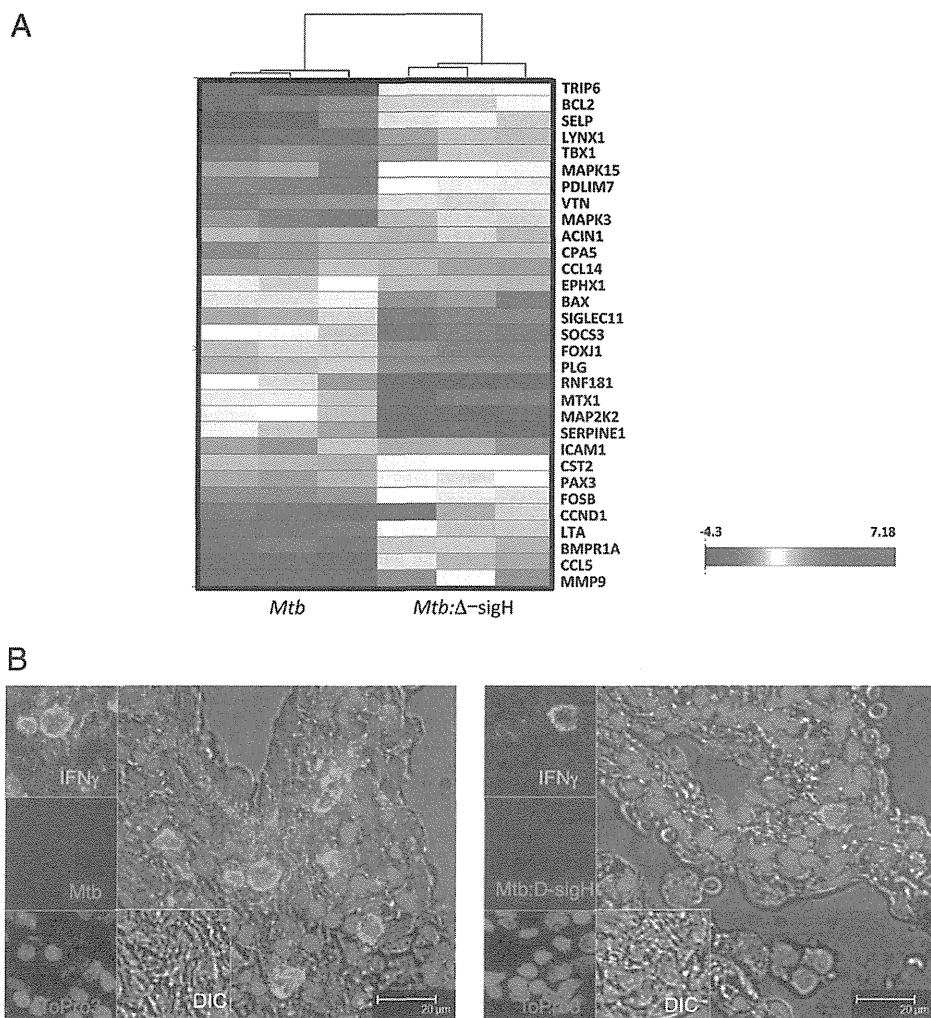


Figure 4. Immune response analysis using microarrays and confocal microscopy. Triplicate RNA samples derived from tuberculosis lesions of 3 nonhuman primates (NHPs) of each group were profiled on Agilent Rhesus Macaque 4×44 microarrays. The derived expression changes were sorted based on significance. Data are shown as heat-map clusters for genes with highly significantly differential expression in NHPs infected with *Mycobacterium tuberculosis* (*Mtb*) or the *Mtb:Δ-sigH* relative to the other group (A). The color schematics for the heat map are as follows: blue, lower expression relative to normal lung; white, comparable expression relative to normal lung; red, higher expression relative to normal lung. The intensity of red and blue color corresponds to the extent of induction or repression and is shown as a color bar (A). Multilabel confocal microscopy shows differential expression of the foremost anti-*Mtb* Th1 type proinflammatory cytokine interferon γ (IFN- γ) in the lung lesion of an NHP infected with *Mtb* (B) and *Mtb:Δ-sigH* (C). Eukaryotic cells were detected using a nuclear stain (TO-PRO3) (blue signal), and *Mtb* was detected using an *Mtb*-specific antibody (red signal), both as described elsewhere [23–26]. IFN- γ was detected using a specific antibody (green signal) as described in the "Materials and Methods" section.

although this difference was not significant (Figure 5B and 5F). The mutant-derived lesions also showed slightly higher number of T cells positive for CD25, an activation marker typically expressed on Tregs, but this difference was also not significant (Figure 5C).

Recruitment of Monocytes Derived From Bone Marrow During Infection With *Mtb* and *Mtb:Δ-sigH*

We compared the dynamic recruitment of monocytes in response to infection with *Mtb*, relative to infection with the

mutant, in the 2 groups of NHPs at different stages of infection by in vivo BrdU labeling and flow cytometry. BrdU was injected 24 hours prior to bleeds [28]. Four animals in each group were used for these assays. No differences in BrdU incorporation was observed in the 2 groups preinfection (Figure 6A). However, at 4–5 weeks postinfection, the level of BrdU⁺ monocytes was significantly higher in the blood of *Mtb*-infected NHPs, relative to the mutant-infected group (Figure 6B). Only 2 NHPs in the mutant group exhibited an increase in the incorporation of BrdU in CD14⁺ over

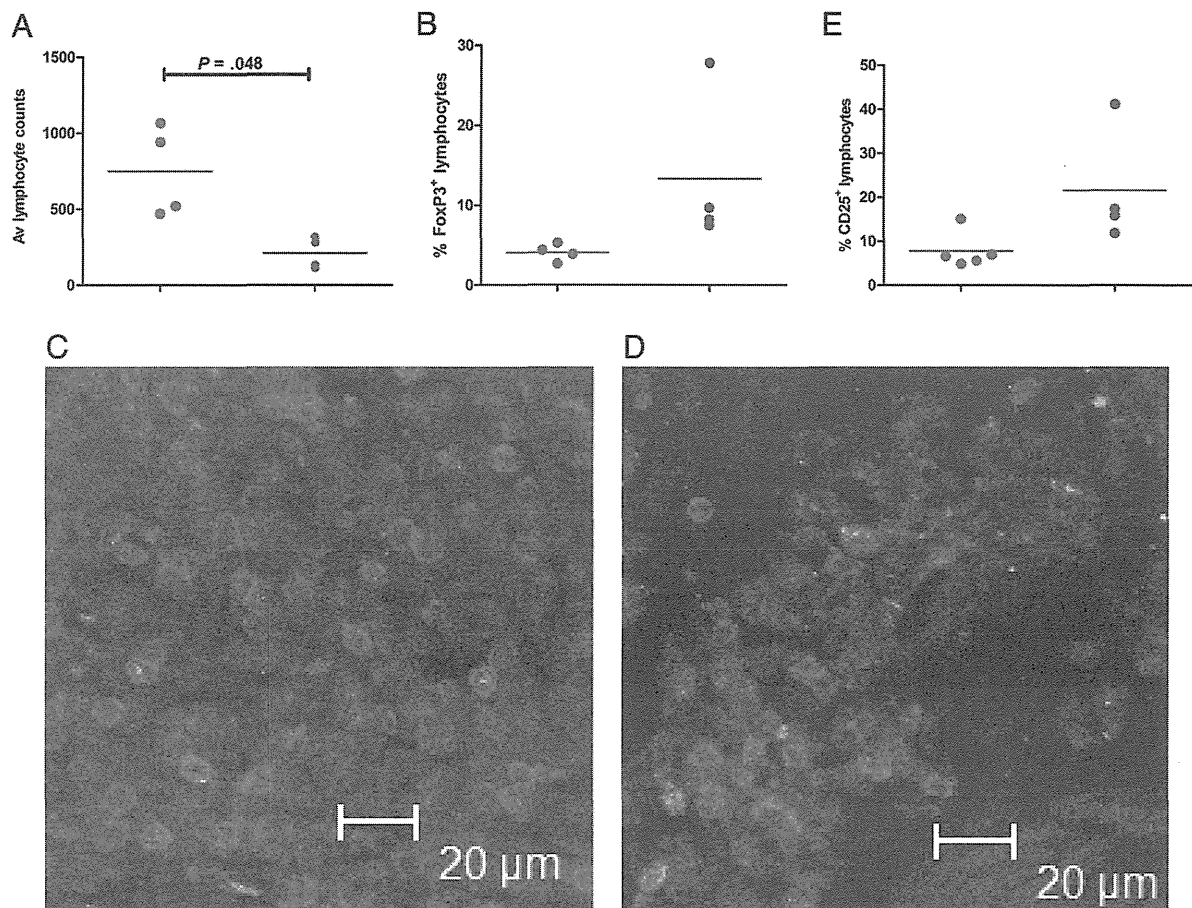


Figure 5. Identification of regulatory T cells (Tregs) in the tubercle lesions of the 2 groups of nonhuman primates (NHPs). Immunohistochemistry (A–C) and immunofluorescence were performed to identify total CD3⁺ T cells (A, D), CD3⁺ FoxP3⁺ Tregs (B, E), and CD3⁺CD25⁺ activated Tregs (E). For immunohistochemical determination of total numbers, 6 fields per slide were counted for 4 NHPs in each group at ×20 magnification using a Leica DMLB scope and a SPOT Insight Color 3.2.0 camera. Images were taken with SPOT 3.4.5 software and counted using Image Pro-Plus 4.5.0.19.

baseline values, but the magnitude of this increase was substantially lower than that observed in all 4 NHPs from the *Mtb* group. These differences were highly significant.

DISCUSSION

The work of several laboratories has established that sigH is a major stress response factor of *Mtb* [8–10] that is either directly or indirectly involved in coordinating the pathogen's response to a wide variety of stress conditions [8–19]. The *Mtb*:Δ-sigH mutant is attenuated for pathology but not *Mtb* replication in mice [8], and more susceptible to a variety of stress conditions [9, 10, 15]. A significantly higher number of *Mtb* mutants are attenuated in NHPs, compared with murine lungs [23]. We therefore hypothesized that the *Mtb*:Δ-sigH mutant may be attenuated for bacterial growth in the NHP model.

The *Mtb*:Δ-sigH mutant elicits a more robust immune response upon infecting host macrophages, relative to infection

with *Mtb* [20]. This greater immune response is characterized by significantly higher levels of β-chemokine secretion and chemotaxis of naive monocytes, and a higher degree of apoptosis [20]. Similar results have been reported for SigE, a related alternate σ factor [29]. It is therefore conceivable that SigH and SigE induce the expression of molecule(s) that interact with and modulate chemotaxis and apoptosis by host macrophages. Conceivably, this would aid *Mtb* in its persistence and dissemination of the initial infection, because chemotaxis is necessary for activated immune cells to arrive at the initial site of infection [30], and apoptosis is a key mechanism for the innate clearance of *Mtb* [31]. Therefore, we also hypothesize that infection of NHPs with the *Mtb*:Δ-sigH mutant will elicit a much stronger immune response than with *Mtb* alone. This scenario is consistent with the hypothesis that increased SigH and other antioxidative mechanisms are responsible for reduced immunogenicity of the BCG vaccine strains [32, 33]. We have devised experiments to test whether

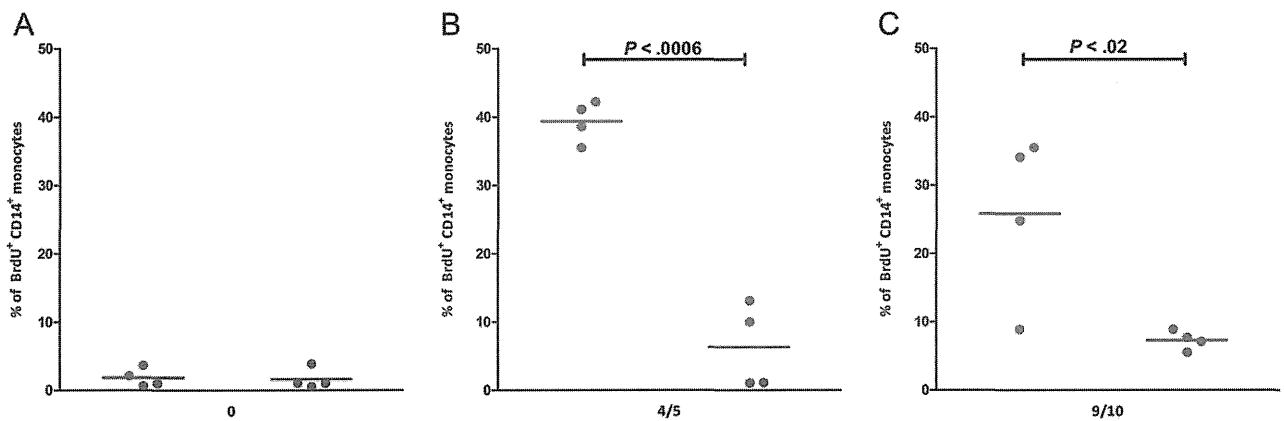


Figure 6. In vivo bromodeoxyuridine (BrdU) labeling and flow cytometry. Four animals from each group were injected with BrdU (10 mg/kg body weight) as described elsewhere [28], prior to infection (A) and 4–5 weeks postinfection (B). Blood was collected 24 hours after BrdU inoculation. The percentages of BrdU-positive cells in CD14⁺ monocytes were analyzed by flow cytometry as described elsewhere [28].

the *Mtb*: Δ -sigH mutant is attenuated for both bacterial infection in the NHP model.

Clinical, microbiological, and pathological assays show that the *Mtb*: Δ -sigH mutant is attenuated for both bacterial burden and immunopathology. Although NHPs infected with *Mtb* developed acute tuberculosis and died within weeks, the NHPs infected with the mutant remained completely devoid of disease. Therefore, the *Mtb* sigH regulon is required for full virulence in primates. Specific mechanisms behind this significant attenuation are presently unclear. It is possible that the rate of intrinsic replication of the mutant is reduced in vivo. On the basis of the known functions of SigH, however, and the fact that *Mtb*: Δ -sigH exhibits no effect of the mutation during in vitro growth, this is not the likeliest of possibilities. Alternatively, it is possible that the mutant is relatively more susceptible to host innate immunity. SigH is strongly induced in response to redox stress and phagocytosis and may play a role in countering oxidative burst [8–10]. It is therefore plausible that the reduced burden of the mutant reflects greater susceptibility to phagocyte oxidative burst. Another possibility is that the *Mtb*: Δ -sigH strain is incapable of immunomodulating adverse host responses. Our in vitro results that phagocytes infected with *Mtb*: Δ -sigH exhibit higher levels of chemotaxis and apoptosis, raise the possibility that antigen(s) regulated by SigH negatively modulate innate phagocytic immune responses and the loss of SigH in the mutant strain unmasks this effect. The current experimental design is insufficient to differentiate between these possibilities. The use of technologies such as unstable plasmids with finite rate of exclusion will allow us to differentiate between replication rate and bacterial burden in vivo [34]. We are now studying the temporal immune response to infection with these strains to better understand the mechanism of attenuation. We are also trying to identify

the bacterial factors involved in potentially modulating phagocyte responses.

The induction of MMP9, which promotes granulomatous pathology [35, 36], CCL5, involved in chemotaxis, LTA, involved in the control of chronic *Mtb* infection [37], and FOXB, known to be induced in a tumor necrosis factor–dependent manner [38], is consistent with our current understanding. However, proteins that are relatively more abundant in mutant lesions may be more important in understanding the mechanism of attenuation. The proapoptotic BCL2 family member BAX is involved in cell death induced by cytotoxic T cells [39]. Its increased levels in the mutant lesions may be due to increased cytotoxic lymphocyte activity in the lungs of macaques infected with the mutant. Interestingly, modifications that reduce the production of *Mtb* antioxidants (eg, sigH or sodA) enhance CD8 T-cell responses [32, 40]. In the future, we will design experiments to specifically address this hypothesis.

Alternatively, the attenuation with *Mtb*: Δ -sigH may result from the unmasking of Tregs that reduce overexuberant proinflammatory response. Oxidants are reported to significantly reduce tissue inflammation [41]. We observed that the expression of SOCS3, which targets STATs [42], and FOXJ1, which modulates inflammatory reactions by interfering with the NF κ B pathway [43], was significantly higher in mutant lesions. Recent reports suggest that in an NHP model of tuberculosis, instead of acute disease, the frequency of Tregs was correlated directly to the ability to control infection in a latent state [44]. Because our expression analysis supported the hypothesis that the attenuation of *Mtb*: Δ -sigH may be at least in part from reduced inflammation due to Treg activity, we performed experiments to measure Tregs in the lung lesions of the 2 groups. Not surprisingly, total CD3⁺ cells were higher in *Mtb* lesions. However, slightly higher

Treg numbers were seen in the mutant lesions (Figure 5). Moreover, the activation status of Tregs (measured by CD25 staining) was also slightly higher in the mutant group. Interestingly, FoxP3 staining was also observed in the mutant group in CD3⁻ cells (Figure 5E). Although we do not know the identity of these cells at the present time, FoxP3⁺ macrophages have been recently reported to play roles in modulating inflammation [45]. These results suggest that active suppression of inflammation may be of benefit to the host.

BrdU is a reliable marker for dividing cells. Because blood monocytes are unable to proliferate in circulation, they do not incorporate BrdU. Thus, BrdU-labeled monocytes can be considered to have recently migrated from the bone marrow [28]. In vivo BrdU labeling indicates massive destruction of lung macrophages during active tuberculosis. These results are corroborated by the 150-fold induction of MMP9 levels in these lungs.

When the *Mtb*: Δ -sigH mutant was used to infect mice, bacterial burden comparable to *Mtb* was observed in lungs, in the absence of typical pathology [8]. These results in the NHP model are different. In response to *Mtb*, NHPs generate highly organized, humanlike granulomatous lesions, unlike mice [46]. It is thus conceivable that anti-*Mtb* responses (eg, hypoxia, oxidative stress) are more effective at the cellular level in the lungs of NHPs and are able to exert a greater degree of immune pressure on the bacilli. This could potentially explain why a mutant may be completely attenuated in the NHP model but only partially attenuated in the mouse model. In an unrelated study of the survival of hundreds of *Mtb* transposon mutants in NHP lungs, one-third of the mutants failed to survive in the NHP model [23]. In contrast, only 6%–10% of these mutants failed to survive in mice. Moreover, differences in primate vs murine immune system may account for the differential phenotypes of the SigH mutant in these species. In particular, our observations may directly result from the fact that reactive oxygen species play a greater role in primate immune system relative to the murine immune system where nitric oxide is more important. The importance of nitric oxide in primate tuberculosis is a matter of controversy reviewed by Tufariello et al [47]. It is possible that the loss of SigH regulon renders the mutant more susceptible to oxidants rather than to nitric oxide. We are now studying the early features of the immune response generated during early infection with the mutant and comparing it to the response generated by *Mtb*. Because the *Mtb*: Δ -sigH mutant is completely attenuated in NHPs, if a more robust immune response is observed, then this mutant may emerge as a candidate antitubercular vaccine. Interestingly, vaccination with a mutant in the related protein sigE protects against challenge with *Mtb* [48].

Notes

Author contributions. D. K. and A. A. L. were responsible for the funding; S. M. and D. K. were responsible for the research design; S. M., N. A. G., K. J. S., X. A., C. J. R., S. K. S., C. S., and A. H. performed the research; L. A. D., K. E. R.-L., and J. L. B. were responsible for the veterinary medicine; P. J. D. and A. A. L. were responsible for veterinary pathology; D. K., S. M., and M. J. K. performed the data analysis; D. K. wrote the study with input from S. M. and A. A. L.

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