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Irina V. Lyadova; ... et. al

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Comparative Analysis of T Lymphocytes Recovered from the Lungs of Mice Genetically Susceptible, Resistant, and Hyperresistant to *Mycobacterium tuberculosis*-Triggered Disease[1]

Irina V. Lyadova,[2]* Evgenyi B. Eruslanov,* Sergei V. Khaidukov,† Vladimir V. Yeremeev,* Konstantin B. Majorov,* Alexander V. Pichugin,* Boris V. Nikonenko,* Tatiana K. Kondratieva,* and Alexander S. Apt*

Genetic control of susceptibility to tuberculosis (TB) is being intensively studied, and immune responses to mycobacteria are considerably well characterized. However, it remains largely unknown which parameters of response distinguish resistant and susceptible TB phenotypes. Mice of I/St and A/Sn inbred strains and (A/Sn × I/St)F_1 hybrids were previously categorized as, respectively, susceptible, resistant, and hyperresistant to *Mycobacterium tuberculosis*-triggered disease. In the present work we compared parameters of lung T cell activation and response following *M. tuberculosis* challenge. In all mice, the disease progression was accompanied by a marked accumulation in the lungs of activated $CD4^+$ ($CD44^{high}/CD45RB^{low}$) and $CD8^+$ ($CD44^{high}/CD45RB^+$) T cells capable of secreting IFN-γ and of activating macrophages for NO production and mycobacterial growth inhibition. However, significantly more $CD8^+$ T cells were accumulated in the lungs of resistant A/Sn and F_1 compared with I/St mice. About 80% A/Sn and F_1 $CD8^+$ cells expressed $CD44^{high}/CD45RB^+$ phenotype, while about 40% I/St $CD8^+$ cells did not express CD45RB marker at week 5 of infection. In contrast, in susceptible I/St mice lung $CD4^+$ cells proliferated much more strongly in response to mycobacterial sonicate, and a higher proportion of these cells expressed CD95 and underwent apoptosis compared with A/Sn cells. Unseparated lung cells and T cells of I/St origin produced more IL-5 and IL-10, respectively, whereas their A/Sn and F1 counterparts produced more IFN-γ following infection. F_1 cells overall expressed an intermediate phenotype between the two parental strains. Such a more balanced type of immune reactivity could be linked to a better TB defense. ***The Journal of Immunology,*** **2000, 165: 5921–5931.**

Tuberculosis (TB)[3] is a major cause of mortality worldwide; thus, elucidation of immunologic mechanisms of the host resistance against *Mycobacterium tuberculosis* infection and of the pathogenesis of TB is a high priority objective. Studies in mice bearing disrupted genes for several cytokines have shed some light on this problem. The type 1 cytokines IFN-γ and IL-12 were found to be essential for the development of protective immunity (1–4), confirming the concept that IFN-γ-producing $CD4^+$ and $CD8^+$ T cells are crucial for the control of mycobacterial infections (5–10). These experimental data are in agreement with recent findings in humans. Thus, it was shown that individuals lacking functional IFN-γ receptors (11, 12) or IL-12 receptors (13, 14) due to mutations in corresponding genes are particularly susceptible to mycobacterial infections. The mechanism by which IFN-γ mediates anti-mycobacterial activity is thought to be macrophage activation for the production of NO, the metabolite that plays a pivotal role in mycobacterial intracellular killing (15). Another type 1 cytokine that is able to up-regulate NO production by macrophages, TNF-α, apparently displays a dual role in the course of *M. tuberculosis*-triggered disease. Besides the host-protective anti-mycobacterial activity of TNF-α (16), its ability to cause tissue damage may contribute to lung pathology and to a more rapid death of the host (17, 18).

Although murine experimental systems based upon genetic disruption of genes encoding central elements of host defense have proven to be extremely useful analytical tools, they possess at least one major intrinsic disadvantage for modeling the spectrum of genetic and immune variability existing in the general population. Gene targeting that results in the complete abrogation of any key function of the immune system leads to a defect in protection against infection which is extreme. Given that such defects are normally rapidly eliminated from a population by natural selection, they hardly could account for the much more common, modestly susceptible phenotypes. Genetic and immunologic mechanisms underlying the expression of the latter could be more rationally studied by the traditional means of segregation genetic analysis and interstrain comparison of several parameters of the immune response. It should be emphasized, however, that quantitative interstrain differences concerning, for example, cytokine production or the degree of cell activation are in most cases under polygenic

*Department of Immunology, Central Institute for Tuberculosis of Russian Academy of Medical Sciences; and †Laboratory for Immunochemistry, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of Russian Academy of Sciences, Moscow, Russia

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[2] Address correspondence and reprint requests to Dr. Irina V. Lyadova, Laboratory for Immunogenetics, Central Institute for Tuberculosis, Yauza Alley 2, Moscow 107564, Russia. E-mail address: asapt@aha.ru

[3] Abbreviations used in this paper: TB, tuberculosis; 7AAD, 7-amino-actinomycin D; BrdU, bromodeoxyuridine; RNI, reactive nitrogen intermediates; CD95L, CD95 ligand.

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control. Thus, the interpretation of results obtained by comparison between conventional mouse strains is usually far more ambiguous than that of results from experiments with gene-targeted animals.

Nevertheless, using these traditional approaches we demonstrated the impact of several *H-2* and non-*H-2* genetic loci on the course of a lethal TB infection and clarified several aspects of the immune response to mycobacterial Ags following Calmette-Guérin bacillus vaccination and infection (19–22). In particular, it was found that the severe course of the disease caused by i.v. *M. tuberculosis* H37Rv injection in mice of the I/St inbred strain contrasts sharply with its relatively mild development in mice of the A/Sn inbred strain (22, 23). Genome-wide linkage analysis of the severity of TB showed a significant linkage with microsatellite loci on distal chromosome 3 and proximal chromosome 9 in females and suggestive linkages for both sexes with loci on chromosomes 5, 8, 10, and 17 (23). Despite the fact that chromosome regions surrounding corresponding quantitative trait loci contain several genes that regulate the function of cells of the immune system (candidate genes), the physiologic basis for the difference in susceptibility to TB between I/St and A/Sn mouse strains remains unknown. Interestingly, it was found that in (I/St $\times$ A/Sn)F_1 hybrids both mortality and body weight loss following tuberculous challenge were significantly postponed compared even to resistant A/Sn parental mice (20, 23, 24). Thus, the combination of resistant A/Sn, susceptible I/St, and hyperresistant F_1 mice probably reflects the spectrum that exists in the general population and provides a useful tool to study functional aspects of TB control.

To address this issue, we have started to study the properties of immune T cells that reside in the lungs of infected mice, assuming that their responses to mycobacteria play the pivotal role in both protection against and pathology of *M. tuberculosis*-triggered disease. It was demonstrated that after the onset of infection lung T cells of susceptible I/St mice rapidly acquire the $CD44^+$/$CD45RB^{-/low}$ activated surface phenotype. These cells readily proliferate in the presence of mycobacterial Ags and produce several type 1 and type 2 cytokines, with a bias toward IL-10 synthesis (25). Here we report on the comparative ex vivo study of T lymphocytes recovered from the lungs of susceptible I/St, resistant A/Sn, and hyperresistant (I/St $\times$ A/Sn)F_1 mice following tuberculous challenge.

Materials and Methods

Animals

I/StYCit (I/St), A/JSNYCit (A/Sn), and (A/Sn $\times$ I/St) F_1 mice were bred at the animal facilities of the Central Institute for Tuberculosis (Moscow, Russia) according to the rules of Russian Academy of Medical Sciences, with water and food provided ad libitum. Female mice, 2–4 mo of age, were used.

Infection

Mice were infected i.v. with 10^5, 10^4, or 10^3 CFUs of mid-log phase *M. tuberculosis* strain H37Rv (collection of the Central Institute for Tuberculosis) in 0.5 ml of saline. The method of establishment of clump-free, mid-log phase mycobacterial preparations was described previously in detail (22, 25). To assess mycobacterial load in spleens and lungs, 0.2 ml of serial 10-fold dilutions of organ homogenates was plated onto Dubos agar, and colonies were counted after 18–20 days of incubation at 37°C.

Lung, spleen, and lymph node cell suspensions

Two, 5, and 8 wk following challenge, mice were euthanized by injection of an overdose of thiopental (Biochemie, Vienna, Austria), and suspensions of spleen, auxiliary lymph nodes, and lung cells were prepared individually. Lung cells were isolated using the method described by Holt et al. (26) with our modifications (25). Briefly, blood vessels were washed out, and repeated bronchoalveolar lavage was performed using 0.02% EDTA-HBSS solution. Lung tissue was sliced into 1- to 2-mm^3 pieces and incubated at 37°C in RPMI 1640 containing 5% FCS, antibiotics, 10 mM HEPES (all from HyClone, Carlington, The Netherlands), 200 U/ml collagenase, and 50 U/ml DNase (Sigma, St. Louis, MO). Single-cell suspensions were obtained by vigorous pipetting. Cells (hereafter referred to as unseparated cells) were washed and resuspended in culture medium, i.e., RPMI 1640 supplemented with 5% FCS, 10 mM HEPES, 2 mM L-glutamine, 1% nonessential amino acids, pyruvate, 5×10^{-5} 2-ME, antibiotics (all from HyClone). T lymphocyte enrichment was achieved by sequential elimination of plastic-adherent and nylon wool-adherent cells, which resulted in approximately 75% $CD3^+$ cell purity (25). The viability of cells, as determined by trypan blue exclusion, was >93%.

Cloning of lung T cells

To estimate the efficacy of lung T cell cloning, T-enriched lung cells were cloned by limiting dilutions as described previously (25). Briefly, lung T lymphocytes obtained at week 5 postinfection were enriched in mycobacteria-specific cells by stimulation in vitro with H37Rv sonicate; the latter were isolated by centrifugation on Lympholyte M gradient (Cedarlane, Ontario, Canada) and cloned in the presence of H37Rv sonicate, irradiated splenic APCs, and conditioned medium, as a source of cytokines. Positive wells were restimulated in situ or were split into new wells every 10–14 days. The efficacy of T cell cloning in response to Con A (2.5 μg/ml; Sigma) was estimated for freshly isolated T-enriched lung cells from either infected or naive mice. Cells were stimulated once with Con A, APC, and cytokines at the beginning of experiment, and the number of positive wells was estimated microscopically between days 7 and 12 of culture.

Proliferative response

T-enriched lung and spleen cells (10^5) mixed with 3×10^5 syngenic APC or 3×10^5 bulk lymph node cells were cultured in a well of 96-well flat-bottom plate (Costar, Badhoevedorp, The Netherlands) at 37°C in 5% CO_2 in the presence of 10 μg/ml H37Rv sonicate. All cultures were performed in triplicate, and nonstimulated wells served as controls. Cultures were pulsed with 0.5 μCi of [^{3}H]thymidine for the last 18 h of a 48- to 72-h incubation. The label uptake was measured in a liquid scintillation counter (Wallac, Turku, Finland) after harvesting the well's contents onto fiberglass filters using a semiautomatic cell harvester (Scatron, Oslo, Norway).

Staining of cell surface molecules

Lung cells ($3–5 \times 10^5$) were washed twice in PBS containing 0.01% NaN_3 and 0.5% BSA and were incubated for 5 min at 4°C in the presence of CD16/CD32 mAbs (clone 2.4G2, PharMingen, San Diego, CA) to block Fc receptors. Cells were then double or triple stained with directly conjugated Abs according to the manufacturer's instructions. All Abs except FITC-anti-CD11α (clone I21/7, Sigma) were purchased from PharMingen: FITC-anti-CD4 (clone H129.19), PE-anti-CD8a (clone 53-6.7), PE-anti-CD44 (clone IM7), FITC-anti-CD45RB (clone 16A), PE-anti-CD28 (clone 37.51), FITC-anti-CD152 (CTLA-4, clone UC10-4F10-11), FITC-anti-CD95 (clone Jo2), and FITC-anti-CD95 ligand (anti-CD95L; clone MFL3). Stained cells were washed twice, fixed with 1% paraformaldehyde, and analyzed by flow cytometry.

Apoptosis evaluation

Unseparated lung cells recovered from the lungs of infected mice were cultured in 24-well plates (10^6/well) in the presence or the absence of 10 μg/ml H37Rv sonicate for 40 h. Cells were harvested and stained with PE-labeled anti-CD4 or anti-CD8 mAbs (Caltag, South San Francisco, CA), followed by staining with FITC-annexin V and 7-amino-actinomycin D (7AAD; PharMingen), according to the manufacturer's instructions. 7AAD stain was used because, unlike propidium iodide, its fluorescent spectrum allows gating of PE-stained cell subsets.

Assessment of T cell proliferation by bromodeoxyuridine (BrdU) incorporation

At week 5 postinfection, mice were injected with 2 mg of BrdU (Sigma) i.p. Two hours later, auxiliary lymph nodes were extracted, single-cell suspensions were prepared individually, and BrdU incorporation was assessed as described previously by Esin et al. (27). Cells were washed in mouse tonicity HBSS supplemented with 5% FCS and 20 U/ml DNase I (Sigma). CD4 and CD8 membrane markers were stained with PE-labeled mAbs. Cells were washed in supplemented mouse tonicity HBSS and fixed with 1% paraformaldehyde in PBS containing 0.01% Tween 20. After 96 h of incubation at 4°C, cells were washed and incubated with DNase solution (4.2 mM $MgCl_2$, 10 μM HCl, and 50 U/ml DNase in 0.15 M NaCl) at 37°C for 60–120 min. After washing, cells were stained with FITC-anti-BrdU mAbs (Becton Dickinson, San Jose, CA), washed in PBS/FBS/Tween 20

(PBS supplemented with 0.5% Tween 20 and 5% FBS), resuspended in PBS, and analyzed by flow cytometry.

Flow cytometry of stained cells

An EPICS Elite flow cytometer (Coulter, Miami, FL) equipped with a Cyonics argon laser (Uniphase, San Jose, CA) with excitation at 488 nm and 15-mW power, and barrier filters at 488BK, 550DL, 525BP, 625DL, and 575BP, was used throughout the experiments. At least 10^4 cells from each sample were analyzed, and the data were processed by means of MultiGraph software (Coulter). Unstained cell controls were analyzed at each time point.

Cytokine assays

ELISAs were used to detect IL-4, IL-5, IL-10, IL-12, TNF-α, and IFN-γ in 48-h culture supernatants. Capture and detecting (biotinylated) mAbs specific for mouse cytokines were purchased from PharMingen: for IFN-γ, clones R4-6A2 and XMG1.2 (sensitivity, 312 pg/ml); for IL-4, clones 11B11 and BVD6-24G2 (sensitivity, 62 pg/ml); for IL-5, clones TRFK5 and TRFK4 (sensitivity, 24 pg/ml); for IL-10, clones JES5-2A5 and JES5-16E3 (sensitivity, 312 pg/ml); for IL-12, clones C 17.8 and C 15.6 (sensitivity, 250 pg/ml); and for TNF-α, clone MP6-XT22 and polyclonal Abs (sensitivity, 125 pg/ml). ELISAs were performed following the manufacturer's instructions. A standard curve for each assay was generated with known concentrations of mouse rIL-4, rIL-5, rIL-12, and rTNF-α (all from PharMingen), rIL-10 (Sigma), and rIFN-γ (Genzyme, Boston, MA).

Antimycobacterial activity of macrophages

To assess the anti-mycobacterial activity of macrophages, we used the method described by Stach et al. (28) with modifications (25). Briefly, peritoneal exudate cells (15×10^6) were incubated for 1 h on 60-mm petri dishes (Costar) in 3 ml of antibiotic-free cultural medium at 37°C. Plastic nonadherent cells were removed, and plastic adherent cells were detached by incubation in 2 mM EDTA-PBS. Plastic adherent cells (6×10^4; >90% of nonspecific esterase-positive cells) were put in a well of a flat-bottom 96-well plate (Costar) and 12×10^4 live-filtered *M. tuberculosis*/well were added. Infected macrophages were cocultured with 6×10^4/well lung or spleen T cells freshly isolated from infected mice. T cell-free cultures of mycobacteria-loaded macrophages, supplemented or not with 100 U/ml recombinant murine IFN-γ (Genzyme), served as positive and negative controls, respectively. Multiplication of mycobacteria was assessed by [^{3}H]uracile uptake exactly as previously described (25, 28). Results are expressed as counts per minute.

NO production by infected macrophages

NO production was assessed as described previously (29). Briefly, peritoneal macrophages were loaded with *M. tuberculosis* and cultured with or without addition of T cells as described above. Thirty-six hours later, 100 μl of Griess reagent was added to 100-μl supernatant aliquots in the wells of a round-bottom plate, and plates were incubated for 10 min at room temperature. Absorbance was measured at 550 nm in a micro-ELISA reader (Sigma), using a 620-nm reference filter.

Statistical analysis

The significance of the differences was estimated by Student's *t* test, Wilcoxon test, and Mann-Whitney test. $p < 0.05$ was considered statistically significant.

Results

Mycobacterial growth in lungs and spleens of infected mice

Groups of I/St, A/Sn, and F_1 female mice were challenged i.v. with 10^5 *M. tuberculosis* H37Rv CFUs, and individual whole-organ homogenates of lungs and spleens were plated in serial dilutions onto agar dishes to determine mycobacterial recovery. As shown in Fig. 1, during the first 2 wk of infection mycobacterial multiplication was prominent in spleens of all infected mice; at least 20 times more mycobacteria were recovered compared with the number injected. An approximately 5-fold difference between susceptible I/St and two other mouse strains was observed (Fig. 1*A*). The size of the mycobacterial population residing in lungs 2 wk postinfection was considerably smaller than that in spleens, and mycobacterial burden was 10-fold higher in I/St compared with A/Sn and F_1 mice (Fig. 1*B*). At week 5 following challenge, the number of mycobacteria in spleens of susceptible I/St mice remained constant, whereas in spleens of both A/Sn and F_1 mice mycobacterial load decreased to a significantly lower ($p = 0.03$) level (Fig. 1*A*). In the lungs, the enlargement of the mycobacterial population between weeks 2 and 5 was registered in all three mouse strains ($p < 0.01$ compared with week 2). Again, the mycobacterial population reached about 10-fold higher numbers in I/St compared with A/Sn ($p = 0.01$) and F_1 ($p = 0.02$) mice (Fig. 1*B*). At week 8 postinfection, the mycobacterial population diminished in size in spleens (Fig. 1*A*) and reduced the speed of growth in lungs (Fig. 1*B*) of A/Sn and F_1 mice. In contrast to the generally good health of all resistant animals at week 8 postinfection, only 4 of 11 I/St mice survived (one, one, and two in three independent experiments), which did not allow us to reliably estimate the bacterial load in their organs. These results indicate that our earlier conclusions concerning interstrain differences in susceptibility to infection (20, 22) and the dominant inheritance of resistance by F_1 hybrids (24) are applicable to the dynamics of mycobacterial growth in organs of infected mice.

Lung T cell subsets following infection

The total cell yield from the lungs of naive and infected mice of the three strains was estimated by microscopy of unseparated lung cell suspensions. The proportions of $CD3^+$, $CD4^+$, $CD8^+$, and NK-

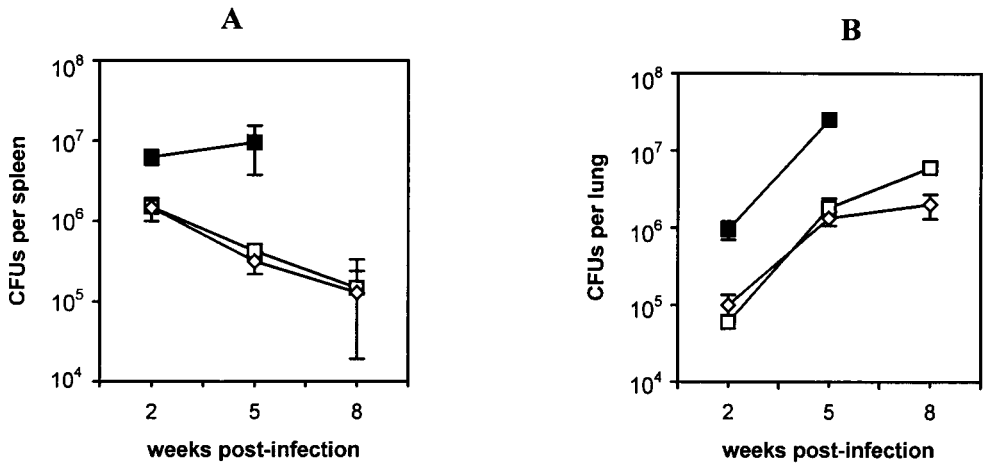

FIGURE 1. Dynamics of mycobacterial multiplication in spleens (*A*) and lungs (*B*) of infected I/St, A/Sn, and F_1 mice. Groups of mice (n = 4–6) were infected i.v. with 10^5 CFUs of *M. tuberculosis*. Two, 5, and 8 wk following the challenge, serial dilutions of whole organ homogenates were plated individually onto Dubo agar for CFU assessment. The number of CFUs per organ are shown. ■, I/St; □, A/Sn; ◇, F_1.

1.1^+ cells were assessed in T-enriched cell suspensions by flow cytometry (see the footnote to Table I).

In naive mice there were no apparent interstrain differences with respect to the total number of cells recovered from lungs. The total cell yield was relatively low ($8–10 \times 10^6$/mouse), and the CD4/CD8 ratio in the CD3-positive population was uniformly close to 1.3:1 (data not shown). As shown in Table I, the cellularity of lungs of all three strains was markedly elevated ($p < 0.05–0.01$) throughout the infectious course compared with that in uninfected syngenic controls. Accumulation of bulk $CD3^+$ T cells following infection was more pronounced in the lungs of A/Sn and F_1 mice compared with that in I/St mice. The most notable difference between susceptible I/St and resistant A/Sn and F_1 mice was that significantly higher numbers of $CD8^+$ T cells were recovered from lungs of the latter two strains throughout infection ($p < 0.05$). In addition, in F_1 hybrids the total lung cellularity as well as the number of $CD4^+$ T cells increased earlier than in either parental strain (Table I, second week, $p < 0.05$). NK-1.1^+ cells were scarcely present in the lungs of all mice (~1.5% positive cells; data not shown).

Proliferation of lung and spleen T cells in the presence of mycobacterial Ag

It was of interest to find out whether the proliferative capacity of lung T cells is essential for the expression of the resistant phenotype. We also wished to clarify whether a more pronounced accumulation of these cells in resistant animals is the consequence of their capacity to proliferate more vigorously in response to mycobacterial Ags. Thus, we assessed the level of lung T cell proliferation in the presence of syngenic splenic APCs and H37Rv sonicate as a source of Ag. Unexpectedly, I/St lung T cells started to proliferate earlier following challenge and retained a higher proliferative capacity ($p = 0.02$) throughout the infectious course than their A/Sn counterparts (Fig. 2*A*). In four of five experiments A/Sn lung T cell did not proliferate at all at 2 wk postinfection. Furthermore, lung T cells from F_1 mice exhibited intermediate levels of proliferation. Similar results were obtained when the proliferation of cells from auxiliary lymph nodes at 5 wk postinfection was measured (Fig. 2*B*). Taken together, these results suggest that neither a more pronounced accumulation of T cells in the lungs nor the ability of the host to better control the disease is due to Ag-induced local T cell proliferation in the vicinity of tuberculous foci.

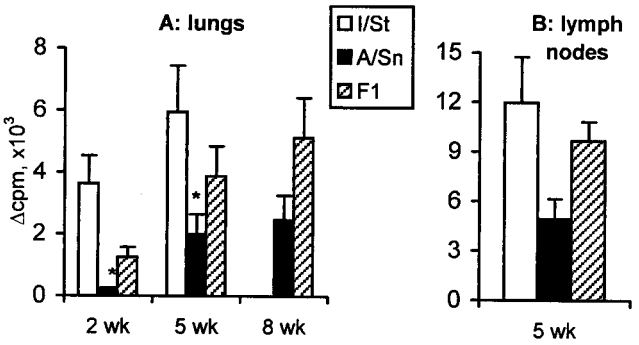

FIGURE 2. Ag-specific proliferative response of T-enriched lung cells (*A*) and lymph node cells (*B*) to *M. tuberculosis* sonicate. Mice were infected with 10^5 CFUs of H37Rv i.v. Proliferation was assessed 2, 5, and 8 (lung cells) or 5 (lymph node cells) wk following challenge and is expressed as [^{3}H]thymidine uptake (lung cells: cpm ± SD for four independent experiments; lymph node cells: counts per minute ± SD for three individual mice). Only 4 of 11 I/St mice survived longer than 8 wk (1, 1, and 2 in three independent experiments), which did not allow us to estimate reliably their response. *, Significant differences between A/Sn and I/St .

A more vigorous proliferation of I/St T cells could simply reflect a higher mycobacterial load in these mice throughout infection (Fig. 1) or could be due to their intrinsic capacity to develop higher levels of mycobacteria-specific and/or nonspecific T cell responses. To distinguish between these two possibilities, groups of I/St mice were infected with decreasing doses of mycobacteria and compared with A/Sn mice infected with a standard dose (10^5 CFUs) with respect to mycobacterial load in their lungs and capacity of lung T cells to respond to the mycobacterial sonicate. As shown in Table II, I/St lung T cells responded to the Ag almost regardless of the challenging dose of H37Rv. Moreover, when I/St mice were infected with 10^3 CFUs and the mycobacterial load in their lungs did not differ from that of A/Sn mice challenged with 10^5 CFUs, a dramatic interstrain difference in proliferative response was still present (Table II).

The suggestion that lung T cells of infected I/St mice proliferate more vigorously in the course of infection was further confirmed in

Table I. *Cell yield (10^6/mouse) from lungs of infected I/St, A/Sn, and F_1 mice*[a]

	I/St	A/Sn	F_1
Total			
2 wk	14.1 ± 4.7	16.7 ± 2.3	**21.8 ± 0.2**
5 wk	15.7 ± 4.9	25.2 ± 10.5	**29.4 ± 9.2**
8 wk	ND	40.7 ± 7.8	31.6 ± 21.3
CD3			
2 wk	2.7 ± 0.9	4.0 ± 1.3	**6.1 ± 1.6**
5 wk	3.5 ± 1.3	7.0 ± 3.2	**6.6 ± 0.4**
8 wk	ND	7.4 ± 5.4	7.3 ± 1.8
CD4			
2 wk	1.6 ± 0.8	1.9 ± 1.0	**3.8 ± 0.8**
5 wk	2.1 ± 0.6	3.1 ± 1.6	3.6 ± 1.5
8 wk	ND	4.6 ± 3.4	4.1 ± 0.6
CD8			
2 wk	1.0 ± 0.4	**1.9 ± 0.6**	**2.4 ± 1.0**
5 wk	1.5 ± 0.4	**3.5 ± 1.0**	**2.8 ± 0.2**
8 wk	ND	2.9 ± 1.9	3.3 ± 1.6

[a] Cells were obtained by enzymatic disruption of lungs from four mice in each group, pooled, and counted (total). Percentages of $CD3^+$, $CD4^+$, and $CD8^+$ cells were estimated by flow cytometry of T-enriched cell suspensions, and the yield of corresponding T cell subsets was calculated by multiplying the percentage by the total yield of nylon wool-nonadherent cells. Values are means from three to four independent experiments ± SD. Values for A/Sn and F_1 cells that differ significantly from those for I/St cells are in bold ($p < 0.05$ as determined by Student's *t* test and Wilcoxon's *U* criterion). Only 4 of 11 I/St mice survived longer than 8 wk (1, 1, and 2 in three independent experiments), which did not allow us to estimate reliably the cellular counts.

Table II. *Interstrain difference in capacity of lung T cells to proliferate in response to mycobacterial sonicate does not depend on mycobacterial burden*[a]

Mouse Strain and Size of Inoculum	Lung CFUs/Mouse	Δ cpm
I/St		
10^3	$8.1 \pm 0.9 \times 10^4$	2226 ± 245
10^4	$2.6 \pm 0.9 \times 10^5$	2730 ± 190
10^5	$9.5 \pm 0.3 \times 10^5$	3511 ± 320
A/Sn		
10^5	$6.3 \pm 0.5 \times 10^4$	<200

[a] Groups of I/St and A/Sn mice ($n = 4$) were infected i.v. with indicated doses of *M. tuberculosis*; 2 wk postinfection, lungs were extracted and weighed. Approximately one-third of the lung from each mouse was homogenized and plated in serial dilutions onto Dubos agar for individual CFU counting. CFU represent calculated numbers per whole organ. The rest of the lung was enzymatically digested, cells from four mice were pooled, and suspensions were enriched for T cells and used for proliferative response assessment as described in *Materials and Methods*.

T cell cloning experiments. As shown in Tables III and IV, I/St and A/Sn lung T cells extracted at 5 wk postinfection showed strikingly different patterns of growth under identical limiting dilution conditions (described previously (25)). During the initial three stimulation/rest cycles, the efficacy of cloning (the number of positive wells) for I/St lung T cells was at least 10- fold higher than that for A/Sn cells (Table III). Moreover, a few initially established A/Sn T cell clones ceased to proliferate at the fifth to sixth stimulation round, while several I/St clones were successfully expanded.

To determine whether the difference in cloning efficacy was due to the inherently poor T cell clonal growth in A/Sn mice or was secondary to differently developing lung disease, we have estimated parameters of clonal growth in the presence of the T cell mitogen Con A. Lung T cells were obtained from infected and naive I/St and A/Sn mice and cloned by limiting dilutions in a single Con A stimulation round. The frequency of Con A-responding precursors was identical in the lungs of naive I/St and A/Sn mice (Table IV). However, the size of all proliferating cell populations of A/Sn origin by day 12 of culture was limited to 20–40 cells, whereas the vast majority of I/St-positive wells contained several hundred blast-like cells. The difference was even more striking when T cells were obtained from infected mice. In addition to the manyfold bigger size of individual I/St clones, a higher frequency of positive wells in I/St cultures was apparent (Table IV). These results suggest that I/St lung T cells are more readily activated for further proliferation both in vivo by infection and in vitro by Con A stimulation than their A/Sn counterparts.

To confirm that the interstrain difference in the proliferative capacity of T cells was not restricted to the in vitro experimental system, we assessed the proliferation of T cells in situ by BrdU incorporation. At 5 wk postinfection, I/St and A/Sn mice were injected i.p. with BrdU, and its incorporation into $CD4^+$ and $CD8^+$ lung and lymph node T cells was individually analyzed by flow cytometry. As shown in Fig. 3, among lymph node cells extracted from I/St mice, about twice as many ($p < 0.05$, by Mann-Whitney t test; $n_1 = 4$; $n_2 = 4$) $CD4^+$ and $CD8^+$ cells incorporated BrdU compared with A/Sn cells, thus confirming the results obtained in vitro. The proportion of lung T cells incorporating BrdU was too low to draw conclusions.

Lung T cell surface phenotype before and after infection

Given the differences in the ability of I/St and A/Sn T cells to proliferate in vitro in response to mycobacterial Ags, we wondered whether the expression of T cell activation markers and of costimulatory molecules also differ in the lungs of these mice. T-enriched lung cell suspensions were triple stained with Abs against either CD4 or CD8 subset marker in combination with anti-(CD44, CD45RB) Abs, triple stained with the combination of anti-(CD4, CD8) Abs with either anti-CD95 or anti-CD95L, or double stained with anti-CD4 and anti-CD8 Abs in reciprocal combinations with anti-CD28, anti-CD152, or anti-CD11-α Abs.

Table III. *Clonal growth (percent of positive wells) of lung T cells extracted at week 5 postinfection after stimulation with H37Rv[a] sonicate*

Initial Number of Cells/Well	Mouse Strain	Stimulation Round 1	2	3
500	I/St	47	53	65
	A/Sn	1	0	0
1500	I/St	83	91	>95
	A/Sn	6	9	1
4500	I/St	>95	NS[b]	NS[b]
	A/Sn	42	17	26

[a] See *Materials and Methods* for details.
[b] NS, Nonstimulated, due to the polyclonal growth.

Table IV. *Clonal growth (% of positive wells) of lung T cells after stimulation with Con A[a]*

Number of Cells/Well		Lung T Cells from Naive mice	Infected mice
10	I/St	0	ND
	A/Sn	0	ND
50	I/St	3	23
	A/Sn	1	3
250	I/St	25	83
	A/Sn	24	21
1250	I/St	ND	>95
	A/Sn	ND	36

[a] See *Materials and Methods* for details.

No major interstrain differences were found in the expression of CD44/CD45RB molecules on T cells before challenge (Fig. 4, *plates 1–3*). Regardless of the mouse strain, 30–45% $CD4^+$ cells and 15–30% $CD8^+$ cells expressed the $CD44^{low}/CD45RB^+$ phenotype of resting/naive cells. Approximately 20–40% $CD4^+$ and 70–80% $CD8^+$ cells expressed the phenotype of activated cells ($CD44^+/CD45RB^{low}$ and $CD44^+/CD45RB^+$, respectively). The relative abundance of activated lung T cells before challenge is most likely due to routine stimulation of the respiratory tract with inhaled antigenic substances. Since the expression of CD44 and CD45 markers is known to vary naturally between mouse strains (30), it was important to show that there was no major intrinsic genetic variation with respect to this trait in the strains under study. As early as 2 wk postinfection, the percentage of CD44-positive activated lung T cells markedly increased in both major subsets (data not shown) and remained remarkably high throughout infection. At 5 wk only about 10–20% of the cells retained the $CD44^{low}$ phenotype and up to 90% were expressing CD44 activation marker (Fig. 4, *plates 4–6* and *10–12*).

The most prominent difference between mouse strains was in CD45RB expression at 5 wk following challenge. Activated $CD4^+$ cells are known to strongly reduce CD45RB expression (31), whereas activated $CD8^+$ cells are thought to continue to express this molecule (32, 33). Between weeks 2 and 5 postinfection, there

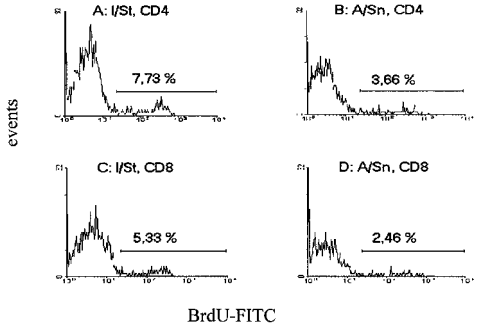

FIGURE 3. BrdU incorporation in vivo by $CD4^+$ (*A* and *B*) and $CD8^+$ (*C* and *D*) cells extracted from auxiliary lymph nodes at 5 wk postinfection. Mice were given BrdU i.p. 2 h before being euthanized. Auxiliary lymph nodes cells were stained with PE-anti-CD4 or PE-anti-CD8 mAbs followed by staining with FITC-anti-BrdU mAbs and were assessed by flow cytometry. Four mice of each strain (two independent experiments) were analyzed individually, and the results of one experiment are shown.

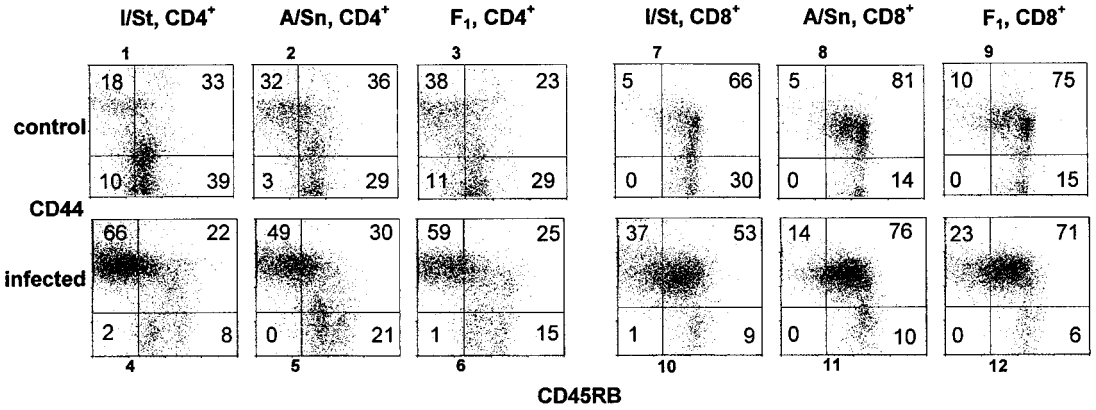

FIGURE 4. Expression of CD44/CD45RB molecules by $CD4^+$ (*panels 1–6*) and $CD8^+$ (*panels 7–12*) lung T cells. Cells were isolated from lungs of control or infected mice and were analyzed by flow cytometry after gating $CD4^+$ or $CD8^+$ cells. Numbers represent the percentage in each quadrant. Two independent experiments gave similar results.

was a progressive decrease in CD45RB expression by CD4-positive cells, and by week 5 this decrease was more pronounced in I/St mice. As shown in Fig. 4, in I/St lungs the total number of $CD45RB^+$ $CD4^+$ cells did not exceed 30% (Fig. 4, *plate 4*) compared with 50% in A/Sn lungs (Fig. 4, *plate 5*). Cells from F_1 mice occupied an intermediate position between those from parental strains (Fig. 4, *plate 6*). Among $CD8^+$, the $CD44^+/CD45RB^+$ activated phenotype was expressed by >70% A/Sn (Fig. 4, *plate 11*) and F_1 (Fig. 4, *plate 12*) cells, but only by approximately 50% I/St (Fig. 4, *plate 10*) cells. Making an inventory of the interstrain differences in total numbers of lung $CD8^+$ cells (Table I) following the challenge, the number of $CD8^+$ $CD44^+/CD45RB^+$ cells was about 4-fold lower in I/St compared with A/Sn and F_1 mice (0.7, 2.7, and 2.0 million/mouse, respectively).

Neither $CD4^+$ nor $CD8^+$ lung T cells from all three mouse strains expressed CTLA-4 (CD152) molecule, but both subsets readily and uniformly expressed CD28 molecule. In all mice, the proportion of CD28-positive cells increased from 40–50% at 2 wk to 90–95% at 5 wk postinfection. Analogously, infection was accompanied by a substantial increase in the proportion of cells expressing the α-chain of LFA-1 (CD11-α) integrin (from 45–60% before infection to 90–99% at 5 wk postinfection).

Since a significantly higher proliferative response of T cells in the lungs of infected I/St mice (Fig. 2) did not lead to their accumulation in the organ (Table I), we compared the expression of the most common apoptotic marker, CD95 (Fas), on lung T cells, anticipating that more I/St cells could undergo apoptosis during the infectious course. In all mice CD95 was scarcely present on the surface of lung T cells at 2 wk postinfection (data not shown). As shown in Fig. 5, at 5 wk following challenge, a significantly higher proportion of I/St lung $CD4^+$ T cells carried the CD95 marker compared with A/Sn (58 and 30%; $p < 0.05$). Interestingly, in all three mouse strains the proportion of $CD8^+$ lung T cells expressing CD95 was always higher than that of $CD4^+$ cells, although no interstrain differences were found (Fig. 5). To ensure ourselves that Fas expression is not a simple consequence of cell activation but is functionally linked to apoptosis, we have evaluated the proportion of $CD4^+$ and $CD8^+$ apoptotic cells from I/St and A/Sn infected lungs. Unseparated lung cells were stained with annexinV/7AAD following stimulation in vitro with mycobacterial sonicate. As shown in Fig. 6, fewer I/St $CD4^+$ cells remained alive, and more underwent apoptosis compared with A/Sn $CD4^+$ cells (16 vs 26% and 83 vs 64%, respectively). As with CD95 expression, a higher proportion of $CD8^+$ compared with $CD4^+$ cells was subjected to apoptotic death, and no interstrain differences were found in this T cell subset.

Cytokine production by lung cells

Cytokine production was determined in lung, spleen, and lymph node cell culture supernatants of two different types. First, unseparated cells alone were cultured in the presence of H37Rv sonicate, and the contents of six different cytokines in supernatants were determined. Second, T-enriched lung or spleen cells were cocultured with irradiated syngenic splenic APC and the Ag, and the contents of IFN-γ, IL-4, IL-5, and IL-10 were assessed. Since

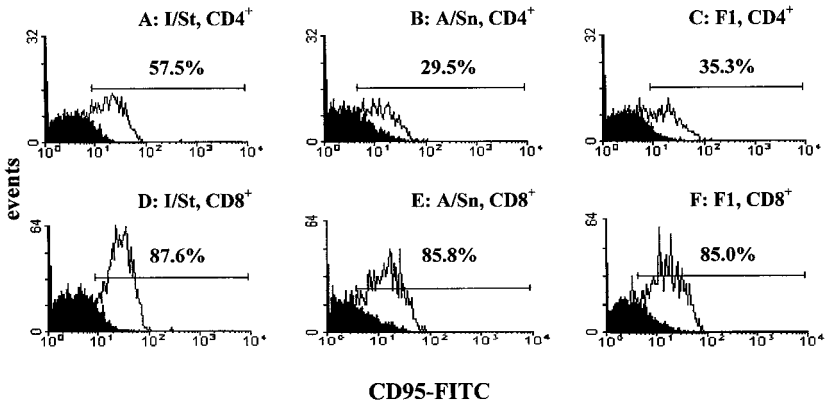

FIGURE 5. CD95 molecule expression by $CD4^+$ (*A–C*) and $CD8^+$ (*D–F*) lung T cells at 5 wk postinfection.

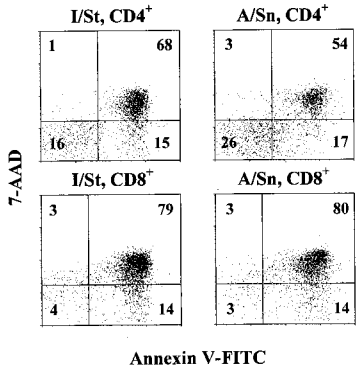

FIGURE 6. Apoptosis is induced in a higher proportion of I/St compared with A/Sn lung $CD4^+$ cells. Unseparated lung cells were stimulated in vitro with H37Rv sonicate for 40 h, harvested, and triple stained with PE-anti-CD4 or PE-antiCD8 mAbs combined with FITC-annexin V and 7-AAD. CD4 and CD8 subsets were gated and analyzed separately. Quadrants 2, 3, and 4 correspond to, respectively, late apoptotic plus necrotic, early apoptotic, and live cells.

TNF-α and IL-12 are synthesized predominantly by macrophage-like cells, we did not measure the contents of these two products in the second type of cultures.

At week 2 postinfection, I/St bulk lung cells produced significantly ($p < 0.05$) less IFN-γ than their A/Sn and F_1 counterparts. At this time purified lung T cells from all mice produced similar levels of IFN-γ, but always lower levels compared with unseparated cells, suggesting that non-T lung cells contribute much to IFN-γ production/induction, especially in resistant mice (Table V). The most potent IFN-γ inducer, IL-12, was also synthesized by unseparated lung cells of all three mouse strains. At week 2 postinfection I/St and F_1 cells exhibited identical IL-12 production, whereas at week 5 postinfection I/St cells synthesized lower amounts of IL-12. A 2-fold difference in TNF-α levels occurred at weeks 2 and 5 postinfection between I/St and resistant mice. Thus, the production of three major type 1 cytokines by the lung cells was moderately higher in resistant mice.

IL-10 was equally well produced by unseparated lung cells of all mice at 2 and 5 wk; however, its production by T-enriched lung cells differed between mouse strains. I/St T cells produced IL-10 in larger quantities than A/Sn and F_1 T cells, and this was particularly clear late in the infectious course (Table V). These differences, however, were not statistically significant between parental strains due to variations in the levels of cytokine production in distinct experiments. Thus, in four experiments IL-10 levels in I/St cultures varied between 300 and 3500 pg/ml. A level of 600 pg/ml was detected in A/Sn supernatant in one experiment, but only trace quantities were spotted in three others. However, the difference between I/St and F_1 mice was significant ($p < 0.01$). In agreement with the results obtained in lungs, IL-10 was synthesized by auxiliary lymph node cells from infected I/St mice (500–800 pg/ml; three mice were assessed individually at week 5), but was not produced by A/Sn lymph node cells. That I/St lung cells more readily produce type 2 cytokines, as suggested by IL-10 data, was confirmed by IL-5 assessment. Unseparated lung cells from I/St mice produced measurable amounts of IL-5 at weeks 2 and 5, whereas only trace amounts were found in A/Sn supernatants (Table V). F_1 mice showed intermediate inheritance of this phenotype. Interestingly, no production of IL-5 was detected in cultures of T-enriched lung cells, suggesting that non-T cells residing in infected lungs are responsible for IL-5 production and/or induction.

Taken together, these results indicate that there is a moderate bias toward activation of a type 2-like cell response in the lungs of TB-susceptible I/St mice. However, polarization of response is incomplete (i.e., the absence of IL-4 and readily detectable levels of IFN-γ in I/St supernatants) and is masked when unseparated lung cells are studied, apparently due to abundant production of several cytokines by non-T lung cells.

Stimulation of anti-mycobacterial activity of macrophages by lung and spleen cells from infected mice

Given that IFN-γ production by I/St lung T cells was somewhat lower than that by A/Sn and F_1 cells and anticipating that not only IFN-γ potentiates macrophage activation, we examined whether T

Table V. *Cytokine production by T-enriched and unseparated lung cells*[a]

	2 wk			5 wk		
Cytokine (pg/ml)	I/St	A/Sn	F_1	I/St	A/Sn	F_1
Unseparated cells						
IFN-γ	17,000[b]	30,000	30,000	15,600	17,700	21,200
	3,600	4,300	2,900	950	1,250	1,100
IL-12	1,050	2,500	1,500	820	1,000	1,800
	900	1400	1,150	1,100	840	1,300
TNF-α	270	455	542	350	735	650
	0	0	0	0	0	0
IL-5	780	50	290	200	0	70
	150	0	0	ND	ND	40
IL-10	3,065	1,950	2,750	2,650	3,200	3,000
	2,985	400	580	1,150	1,470	740
T-enriched cells						
IFN-γ	7,600	8,500	6,600	11,500[b]	18,400	30,000
	0	0	0	400	520	780
IL-10	1,900	625	1,050	2,920[c]	600	0
	0	0	0	0	0	0

[a] T-enriched lung cells (1.5×10^6) plus irradiated syngenic APC (0.5×10^6) or unseparated (2×10^6) lung cells alone were cultured in the presence or absence of 10 μg/ml H37Rv sonicate for 60 h. Cytokine content was measured by ELISA. The results of one representative cytokine determination of three to five sets of supernatants obtained in independent experiments are presented. SD among triplicate determinations was <10%. 0, Concentration lower than the sensitivity of corresponding ELISA (see *Materials and Methods*). There was no measurable IL-4 in all supernatants, IL-5 was undetectable in supernatants of T-enriched cultures. In each data set, the upper value is sonicate-stimulated and the lower value is spontaneous cytokine production.

[b] $p < 0.05$ compared with A/Sn and F_1 values as calculated for the mean of four independent experiments.

[c] $p < 0.01$ compared with F_1 values as calculated for the mean of three independent experiments.

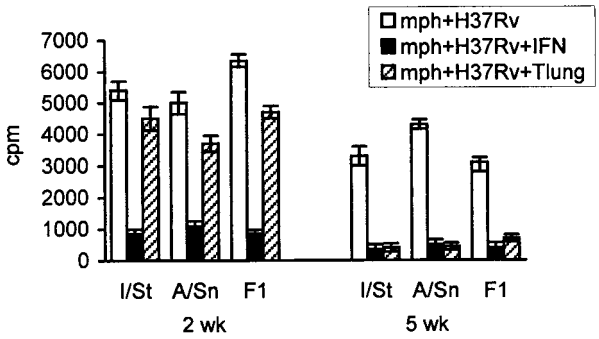

FIGURE 7. Stimulation of macrophage (mph) anti-mycobacterial activity by lung T cells from infected mice. Peritoneal macrophages (6×10^4/well) were loaded with 12×10^4 live mycobacteria, and 10^5 freshly isolated lung T cells were added to cultures. Following 96 h of incubation, mycobacterial growth was measured as [^{3}H]uracil uptake (mean cpm ± SD for triplicate determinations). The results of one of two similar experiments are shown. □, Macrophages and H37Rv; ■, macrophages, H37Rv, and rIFN-γ; ▨, macrophages, H37Rv, and lung T cells.

cells from these mice differ with respect to their capacity to activate anti-mycobacterial macrophage function. To address this issue, peritoneal macrophages from I/St, A/Sn, and F_1 mice were cocultured in vitro with live H37Rv mycobacteria and syngenic T-enriched lung cells obtained from infected mice. Mycobacterial growth was assessed by [^{3}H]uracil incorporation (28). When cultured in antibiotic-free culture medium, alone or within macrophages, mycobacteria readily incorporate [^{3}H]uracil (25). As shown in Fig. 7, addition of exogenous rIFN-γ to macrophage/mycobacteria cocultures (positive control) caused a profound inhibition (85–95%) of mycobacterial growth regardless of the mouse strain. Addition of lung T cells extracted 2 wk postinfection from I/St, A/Sn, and F_1 mice moderately and equally inhibited mycobacterial multiplication (20–30% inhibition in different experiments). Five weeks after challenge the ability of T cells of all three strains to stimulate anti-mycobacterial activity of syngeneic macrophages significantly increased; up to 90% inhibition of mycobacterial growth was registered. Thus, no major interstrain differences in the ability of T cells to stimulate the restriction of mycobacterial growth in vitro were found.

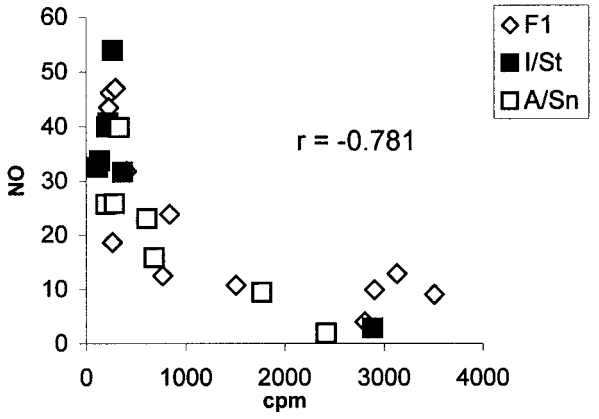

FIGURE 8. Reverse correlation between mycobacteria growth and RNI production by peritoneal macrophages in the presence of lung T cells. Mycobacteria, macrophages, and T cells were cocultured as described in Fig. 5. Forty-eight hours later the supernatants of three wells were harvested, and the levels of NO production were determined. Mycobacterial growth was assessed by determining [^{3}H]uracil uptake. $r = -0.781$.

It is widely accepted (34–36) that the main mechanism of intracellular mycobacterial killing by murine macrophages is production of reactive nitrogen intermediates (RNI). To determine the role of RNI in our system and to evaluate the correctness of measuring mycobacterial growth by [^{3}H]uracil incorporation, we assessed the content of NO in supernatants of mycobacteria-loaded cell cultures. Individual NO concentrations, as measured in all types of cell cultures (with and without addition of lung T cells or rIFN-γ and with cells obtained from all three mouse strains) were plotted against the levels of [^{3}H]uracil incorporation (counts per minute) in corresponding cultures. As shown in Fig. 8, there was a good inverse correlation between the two measurements ($r = -0.781$), suggesting the pivotal role of RNI in mycobacterial killing in our system.

Discussion

In this paper we characterize immune reactivity in the lungs of mice of I/St and A/Sn inbred strains and their F_1 hybrids in the course of TB infection. The two parental strains were previously categorized as polar extremes of susceptibility among inbred mouse strains with respect to survival time, mycobacterial burden in organs, and body weight loss following tuberculous challenge (22). Furthermore, F_1 mice display a hyperresistant phenotype (20, 23). Thus, this genetic model provides a useful tool for searching functional immunological correlates of the TB defense and/or severity. For obvious reasons our attention was concentrated primarily on T cell responses in infected lungs.

The following most notable interstrain differences were found. First, the mycobacteria-specific proliferative response of lung T cells was much higher in susceptible I/St mice (Fig. 2 and Table II). Paradoxically, this was accompanied by a diminished T cell accumulation in the lungs; significantly more bulk T cells and particularly $CD8^+$ T cells were recovered from resistant mice following challenge (Table I). Moreover, a higher proportion of A/Sn and F_1 $CD8^+$ lung T cells expressed activated $CD44^+/CD45RB^+$ phenotype compared with I/St (Fig. 4). Secondly, in I/St mice a higher proportion of lung $CD4^+$ cells expressed CD95 (Fas) receptor (Fig. 5) and underwent apoptosis (Fig. 6). This suggests that a more active lung T cell proliferation in these mice is counterbalanced with a more rapid apoptotic elimination. Thirdly, I/St lung cells produce more IL-5 and IL-10 and less IFN-γ and TNF-α in response to mycobacterial Ags (Table V), indicating a moderate bias toward a Th2-like response in susceptible mice.

Expansion of CD4 and CD8 T cells in murine lungs in response to mycobacterial infections was recently reported in the model of intratracheal infection with *M. bovis* Calmette-Guérin bacillus (37) and in the models of aerosol (38, 39), i.v. (25, 40), and i.p. (41) challenge with *M. tuberculosis*. In this study we have extended these observations by demonstrating that in the lungs of TB-resistant A/Sn and F_1 mice accumulation of $CD8^+$ cells reaches significantly higher levels than in susceptible I/St mice. These results are in line with numerous data indicating a protective role of $CD8^+$ T cells in mycobacterial infections (8, 10, 42, 43), particularly evident in the lungs (42, 44).

There was a clear interstrain difference in the capacity of T cells from infected mice to proliferate in response to mycobacterial Ags. I/St T cells demonstrated a significantly higher proliferative response both in vitro (Fig. 2 and Tables III and IV) and in vivo (Fig. 3) than their A/Sn counterparts, and F_1 T cells showed an intermediate response. This superior responsiveness of T cells from I/St mice was not due to a higher bacterial load in their lungs (Table II). Since the course of the disease is extremely severe in I/St mice, it

could be argued that the T cell proliferative response to mycobacteria is nonprotective. On the other hand, the fact that the most resistant F_1 mice show not the lowest, but an intermediate, level of T cell proliferation (Fig. 2) precludes ascribing a predominantly pathological, lung tissue-damaging role to T cell proliferation itself.

The capacity of I/St lung T cells to more readily proliferate in response to mycobacteria is in sharp contrast with their low accumulation in the infected organ. It is noteworthy that a higher proportion of I/St lung $CD4^+$ cells express CD95 apoptotic receptor (Fig. 5), ligation of which results in activation-induced cell death (45, 46), and are subjected to apoptosis (Fig. 6). One can speculate that an early onset and a high degree of T cell proliferation combined with their apoptotic elimination, i.e., high T cell turnover in lungs, lead to an unfavorable disease course. Since the expression of CD95L on lung T cells was virtually lacking (data not shown), elimination of Fas-positive T cells from the infected lung most likely involves non-T cells.

As infection progressed, both CD4 and CD8 lung T cells acquired activated $CD44^+CD28^+CD11\text{-}\alpha^+$ phenotype. This is in agreement with the observations of other authors. Thus, Serbina and Flynn (40) reported the expression of a high density CD44 molecule by >85% of both $CD4^+$ and $CD8^+$ lung T cells at 2 wk following TB onset. Feng et al. (39) have shown that the CD62L endothelium adhesion molecule is down-regulated, while the CD44 activation marker and CD11-α, CD49d integrins are up-regulated in the lung and lymph node $CD4^+$ and $CD8^+$ cells following aerosol *M. tuberculosis* challenge.

Earlier, Griffin and Orme (47) described the increase in CD44 expression along with the gradual decrease in CD45RB expression on $CD4^+$ splenocytes of infected mice. The shift in CD45RB expression following activation of $CD45RB^{high}$ naive/resting T lymphocytes occurs differently in CD4 and CD8 subsets. While this marker is uniformly down-regulated in activated $CD4^+$ cells (31, 47), several lines of evidence indicate that its expression is retained in the majority of activated $CD8^+$ cells (32, 33, 39, 48). In our study by week 5 postinfection the loss of CD45RB expression by CD4-positive cells, indicating their high activation, was more pronounced in I/St mice. In contrast, among their CD8-positive lung cells a significantly smaller population continued to express the $CD44^+/CD45RB^+$ double-positive phenotype by this time point compared with A/Sn and F_1 mice (Fig. 4). Although it is presently unclear what the functional difference is between $CD44^+/CD45RB^{-/low}$ and $CD44^+/CD45RB^+$ cells in the $CD8^+$ subset, some recent findings in humans indicate its possible prominence. Thus, it was reported that mature CD8-positive CTLs, possessing perforin and producing IFN-γ and TNF-α, reside within the $CD45RA^+$ population, whereas Ag-specific CTL precursors, which need to be restimulated to acquire cytotoxic function, belong to the $CD45RA^-$ subpopulation (49, 50). In our system it is possible that in I/St mice a stronger activation of $CD4^+$ (more $CD44^+/CD45RB^{low}$ cells) is accompanied by a weaker activation of effector $CD8^+$ (fewer $CD44^+/CD45RB^+$ cells) lung T lymphocytes.

Assessment of cytokine profiles has shown that lung cells from infected A/Sn and F_1 mice produced more type 1 cytokines. In contrast, in I/St cells there was a moderate bias toward the type 2 profile: somewhat lower IFN-γ and TNF-α and higher IL-10 and IL-5 production (Table V). An interesting feature of I/St lung cell response was that a lower IFN-γ was evident as early as 2 wk following challenge. The significance of the early IFN-γ production by CD4 T cells for TB control was recently shown by Caruso et al. (51). Thus, a bias toward a type 2 response in I/St lungs at the initial stages of the disease might contribute to its more severe course.

Among lung T cells, a high level of IL-10 synthesis was characteristic for I/St mice. This is in agreement with our previous finding that the majority of lung-derived T cell clones of I/St origin produce IL-10, even if IFN-γ is simultaneously produced (25). Interestingly, Gerosa et al. (52) have found that the majority of T cell clones derived from bronchoalveolar lavage of patients with active TB also produced both IFN-γ and IL-10. Thus, in mice and humans the activity of IFN-γ/IL-10-producing (Th0-like?) T cells in the lungs is prominent when the disease rapidly progresses. In view of the hypothesis that simultaneous synthesis of type 1 and type 2 cytokines during the TB course has a tissue-damaging effect (17, 18), conjunct production of these two, usually antagonist, cytokines in the lung may be considered an immunological correlate of the unfavorable development of pulmonary TB.

IL-10 is known to impair protective immune response to several infections by down-regulating Th1 function (53, 54). On the other hand, it probably regulates the balance between protective and pathologic immune responses during intracellular parasitic infections (55) and autoimmune disorders (56, 57). For example, IL-10 gene targeting results in systemic overproduction of proinflammatory cytokines and the development of lethal pathology (55). The role of IL-10 in tuberculosis is not completely understood. North (58) reported that IL-10 knockout mice and wild-type control mice display similar levels of TB protection. In our system IL-10 was produced in higher amounts by lung and lymph node T cells of susceptible mice. Another type 2 cytokine, IL-5, was produced almost exclusively by lung cells of I/St susceptible mice. Analogous results were recently obtained in other experimental infection models. Following infection with *Chlamydia*, a significantly greater amount of IL-10 was found in the lungs of susceptible compared with resistant mice (59). Huffnagle et al. (60) have reported IL-5 expression in the lungs of susceptible, but not of resistant, mice during pulmonary *Cryptococcus neoformans* infection. Thus, overproduction of IL-5 and IL-10 in the lung seems to be associated with susceptibility to pulmonary infections, although causality remains obscure.

Lung T cells from all three mouse strains exhibited a similar capacity to stimulate anti-mycobacterial activity of peritoneal syngenic macrophages in vitro, and there was a strong correlation between NO production and mycobacteria growth inhibition (Fig. 8). The lack of interstrain differences raises the question of the reason for the severity of *M. tuberculosis*-triggered disease in I/St mice, given that their lung T cells effectively promote macrophage-mediated inhibition of mycobacteria growth. At least two explanations are possible: 1) anti-mycobacterial activity of lung macrophages differs prominently from that of peritoneal ones and is selectively affected in I/St mice; and 2) the severe TB course in I/St mice is due to lung pathology rather than to impaired restriction of mycobacterial growth. Experiments are in progress to distinguish (or to combine) these two possibilities.

In conclusion, there is an agreement between the results on immune reactivity in the lungs of TB-susceptible and resistant mice presented here and our genetic mapping studies. A genome-wide scan in segregating backcross $(I/St \times A/Sn)F_1 \times I/St$ TB-infected mice demonstrated that the severity of tuberculosis is inherited as the oligogeneic quantitative trait and is controlled by more than two nonlinked quantitative trait loci (23). Analogously, interstrain differences in the lung T cell response to mycobacteria are quantitative, and the expression of major integrative phenotypes (survival, pathology, cachexia, etc.) depends, presumably, upon particular combinations of minor shifts in immune reactivity.

Acknowledgments

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