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Induction and expression of protective T cells during *Mycobacterium avium* infections in mice

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SUMMARY

Mycobacterium avium is an opportunistic pathogen that infects individuals suffering from chronic lung disease or immunocompromised patients such as AIDS patients. Here we show that a highly virulent isolate of *M. avium* proliferated as extensively in T cell deficient as in immunocompetent mice. T cell deficient mice allowed a progressive growth of a less virulent AIDS-derived isolate of *M. avium* while immunocompetent mice arrested the growth of this isolate. Adoptive transfer of T cell enriched spleen cells between congenic strains of mice differing at the *Bcg/Ity/Lsh* locus showed that only naturally resistant BALB/c.*Bcg*^{r} (C.D2) mice infected with the highly virulent strain of *M. avium* or the naturally susceptible BALB/c mice infected with the lower virulence isolate developed protective T cells and that these cells only mediated protection when transferred to naturally susceptible, but not to naturally resistant, mice. Both strains of *M. avium* proliferated in bone marrow-derived macrophages cultured *in vitro* and they were both susceptible to the bacteriostatic effects induced in the macrophages by crude lymphokines produced by concanavalin A-stimulated spleen cells.

Keywords mycobacteria *Mycobacterium avium* protective immunity T cells macrophages

INTRODUCTION

Mycobacterium avium is a rare human pathogen affecting individuals suffering from chronic lung disease and from immunodeficiencies such as AIDS [1–4]. The fact that a severe depletion of the CD4$^+$ subset of T cells in AIDS favours the establishment of infection by this microorganism suggests that T cells play a major role in the host defence against *M. avium* infection in humans. T cells are also central to the host defence against mycobacteria in mice [5–14]. It is thought that specifically sensitized T cells recognizing mycobacterial antigens activate the anti-microbial machinery of the macrophage [6]. In some cases, however, innate (natural) mechanisms in these phagocytes are able to establish some degree of resistance to infection [15]. In the mouse model it has been shown that different strains of animals differ in their resistance to *M. avium* infections [16–19]. This innate resistance is linked to the *Bcg/Ity/Lsh* gene [17] although other genes also control resistance to this pathogen [18]. Since the *Bcg/Ity/Lsh* gene is expressed in the resident macrophage [20] and because resident macrophages from the peritoneal cavity, liver and spleen in culture mimic the resistance pattern of the organisms from which they were collected [17,21–27], it has been postulated that mononuclear phagocytes are central cells in the resistance of mice to *M. avium* [17,27].

Here we analysed the participation of T cells in the host defense against *M. avium* looking at the effects of T cell depletion in mice as well as the protective ability of T cells from immune animals. Previous studies by others had shown that bacterial growth was necessary for the sensitization of protective T cells and that naturally susceptible (NS) strains of mice were able to mount a certain degree of resistance to infection [28]. However, naturally resistant (NR) mice were not dependent on T cells to resist infection [28]. Here we also show that in some cases, naturally susceptible mice can exhibit some T cell-mediated protection and that naturally resistant mice seem not to depend upon T cells to resist infection. In addition, we were able to distinguish between induction and expression of protective T cells since we used congenic mice and compared two different strains of *M. avium* with diverse virulence patterns.

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MATERIALS AND METHODS

Reagents and culture media

Media for the adoptive transfer of immune cells consisted of RPMI 1640 medium (GIBCO, Paisley, Scotland) supplemented with 2% fetal calf serum (FCS (GIBCO)). Media for the *in vitro* culture of macrophages consisted of Dulbecco's modified Eagle's medium (DMEM (GIBCO)) supplemented with 10 mM of

HEPES buffer (GIBCO) and 10% FCS. No antibiotics were used. Tween 80, concanavalin A, and saponin were from Sigma (St Louis, MO). Sephadex G50 was from Pharmacia (Uppsala, Sweden) and rabbit serum complement was from Serotec (Oxford, UK).

Bacteria

Mycobacterium avium ATCC 25291 and isolate 2447 were supplied by Dr F. Portaels (Antwerp, Belgium). The former comes from the ATCC collection and was originally isolated from an infected chicken. The latter was isolated from the bone marrow of an AIDS patient. From both strains, bacteria forming smooth transparent colonies were isolated and cultured in Middlebrook 7H9 medium (Difco) with 0·04% of Tween 80 (Sigma). At midlog phase the bacteria in the culture were centrifuged and resuspended in saline with 0·04% Tween 80, briefly sonicated, aliquoted, and frozen at −70°C until use as described previously [16,17].

Animals

BALB/c mice were purchased from the Gulbenkian Institute (Oeiras, Portugal). BALB/c. *Bcg*^{r} (C.D2) congenic mice were bred at our animal facilities from parent mice obtained from Dr E. Skamene (Montreal, Canada). DBA/2 mice were also bred at our animal facilities from parent mice purchased from Bantin & Kingman (Hull, UK). C57BL/6 nu/+ and nu/nu mice, and C.B-17 and SCID mice were purchased from Bommice (Ry, Denmark). T cell depletion of adult mice was performed in 4- to 6-week-old BALB/c and DBA/2 mice by surgically removing the thymus gland, irradiating the animals 2 weeks later with 750 cGy of X-rays, and reconstituting the haematopoietic system by transfusing 2×10^6 syngeneic bone marrow cells the next day (TXBM mice). Control mice were treated likewise except that thymectomy was omitted (XBM mice). The animals were infected after 4 weeks.

Study of the infection in vivo

Mice were infected intravenously with *M. avium* and, at different time intervals, groups of four mice were killed by cervical dislocation. Their organs (spleens and livers) were aseptically collected, homogeneized in a 0·04% Tween 80 solution, and serial dilutions (1 in 10) were plated onto Middlebrook 7H10 agar medium. After 2 weeks incubation at 37°C, colonies were counted and the total number of colony-forming units (CFU) per organ calculated. The geometric means of CFU and their standard deviations were calculated and plotted.

Adoptive transfer of protective immunity

Spleens from groups of four normal mice or mice infected for 4 weeks with *M. avium* were aseptically collected and spleen cells were isolated by pressing the spleens through a wire mesh and washing them with adoptive transfer medium. The cells were pelleted by centrifugation (100 ***g*** for 10 min) and resuspended in the same medium plus an equal volume of J11d.2 hybridoma supernatant (total volume of 30 ml) and incubated with rabbit complement (1:30 dilution) for 45 min at 37°C to lyse erythrocytes, B cells and granulocytes [29]. Clumps of cells and cell debris were discarded and the cell suspension centrifuged again. The pellet was washed once and added to a nylon wool column containing 1·2 g of wool in a 10 ml syringe barrel. The cells were incubated for 1 to 1·5 h at 37°C. Non-adherent cells were collected by washing the column with 20 ml of warm medium. This fraction was washed twice and intravenously administered in 0·5 ml of the same medium to recipient mice that had been irradiated with 500 cGy of ionizing radiation (X-ray sourse). One spleen equivalent was injected per recipient mouse. After a few hours, recipient mice were challenged intravenously with 2×10^8 CFU of *M. avium* and the bacterial proliferation assessed after 30 days as described above. The degree of protection was calculated by subtracting the geometric mean of CFU in the organs of mice receiving immune spleen cells to that in mice receiving normal spleen cells. Typical recovery of T cell-enriched spleen populations was about 30×10^6 cells from uninfected spleens (with 50–60% of Thy1.2$^+$ cells by immunostaining), 80×10^6 cells from spleens of infected resistant mice (40–50% Thy1.2$^+$ cells), and 120×10^6 cells from spleens of infected susceptible mice (30–50% Thy1.2$^+$ cells). The latter populations showed some contamination by mononuclear phagocytes.

Lymphokine-containing spleen cell supernatants and other conditioned media

To obtain a crude preparation of lymphokines with macrophage-activating properties the method described in ref. [17] was followed. Briefly, 50×10^6 spleen cells from normal C57BL/6 mice were cultured in 20 ml of DMEM with 10% FCS and 4 μg of concanavalin A/ml in a 100 mm^2 tissue culture Petri dish. After 48 h incubation in a humidified 7% CO_2 incubator, the supernatant was collected and cell debris was removed by centrifugation and filtration through a 0·2 μm membrane. This supernatant was then incubated for 2 h with Sephadex G50 equilibrated with the same medium in a 1:1 proportion to remove the concanavalin A remaining in solution. Aliquots were frozen at −70°C after being sterilized by filtration.

The J11d.2 B cell hybridoma and the L-929 fibroblastic cell line were obtained from Dr I. M. Orme. J11d.2 was cultured in DMEM with 10% FCS and the supernatant was removed from late exponential phase cultures and used as a source of antibody in cytolytic treatments. The fibroblast cell line L-929 was cultured in the same medium and the supernatants (LCCM) were used as a source of macrophage-colony stimulating factor in the culture of bone marrow cells.

Study of the infection in vitro

Bone marrow-derived macrophages were obtained from bone marrow cells cultured in the presence of L-929 cell conditioned medium (LCCM). Bone marrow cells were plated onto 24-well tissue culture plates ($0{\cdot}6 \times 10^6$ cells in 1 ml per well) in culture medium with 10% LCCM and incubated in a humidified 7% CO_2 incubator. At day 4 additional LCCM was added (100 μl per well) and the medium was substituted by fresh medium (with 10% LCCM) at day 7. After 10 days of culture, the medium was removed by aspiration and 200 μl of DMEM with 10% FCS and the appropriate number of bacteria was added to each well. The plates were incubated for 4 h and then washed extensively to remove unphagocytosed bacteria and 400 μl of fresh medium was added with or without 25% spleen cell supernatant. Every day, 100 μl of either fresh medium or spleen cell supernatant were added. At time zero (after the initial 4 h of infection and the extensive wash) and at different periods of infection, the volume of the wells was completed to 1 ml and saponin was added to a

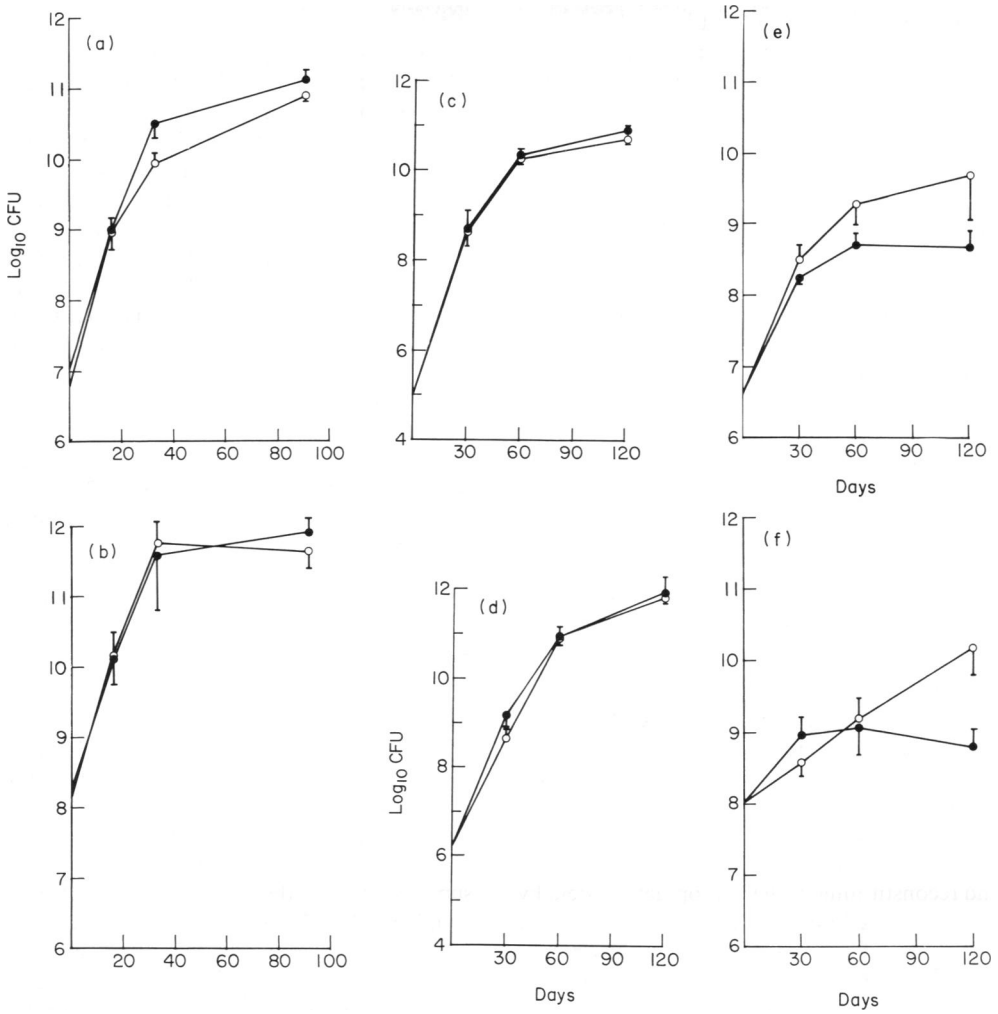

Fig. 1. Proliferation of *M. avium* in the spleens ((a), (c), (e)) and livers ((b), (d), (f)) of C57BL/6 nu/+ mice (closed symbols) and C57BL/6 nu/nu mice (open symbols). Mice were intravenously inoculated with (a, b) 2×10^8, (c, d) 2×10^6, or (e, f) 1×10^8 CFU of *M. avium* strain ATCC 25291 (a–d) and strain 2447 (e,f), and the number of CFU in the organs was determined at different time intervals. The implantation of the bacterial inocula at time 0 (18 h after the inoculation) was determined for the different mouse strains and showed similar results. To avoid overlapping of different symbols they were omitted at this point in this figure.

final concentration of 0·1% (w/v) to lyse the macrophages. The supernatant was vigorously pipetted to disrupt cell remnants, serially diluted in a 0·04% Tween 80 solution and plated onto 7H10 agar medium to determine the viable counts as described above. The mean number of CFU per well was calculated from triplicate wells for each time-point. The results are either shown as means of CFU or as growth index (GI) calculated as follows: GI = mean of log10 CFU at 7 days − mean of log10 CFU at time zero.

Statistical analysis

Student's *t*-test was used.

RESULTS

M. avium *ATCC 25291 grows as extensively in nude mice as in euthymic littermates*

The proliferation of *M. avium* ATCC 25291 in C57BL/6 nu/+ or C57BL/6 nu/nu mice is depicted in Fig. 1 (a–d). After infection of mice with 2×10^8 CFU, this virulent strain of

Table 1. Proliferation of *M. avium* (strains ATCC 25291 and 2447) in the spleen and liver after 90 days of IV inoculation of mice (results are expressed as growth indices (log10 CFU at day 90 − log10 CFU at day 0))

Strain		*M. avium* strain	Spleen	Liver
BALB/c	XBM	25291*	4·70	3·55
	TXBM		4·41	3·27
DBA/2	XBM	25291*	1·73	0·69
	TXBM		1·57	−0·14
C.B.-17	Control	25291†	5·89	5·18
	SCID		5·83	5·42
	Control	2447†	2·80	1·29
	SCID		3·95	2·67

* 10^8 CFU.
† 10^6 CFU.

Table 2. Induction of protective T cells in BALB/c and C.D2 mice infected with 2×10^8 CFU of *M. avium* strains ATCC 25291 (two experiments) and 2447 (one experiment)

Adoptive transfer		Strain ATCC 25291		Strain 2447	
Donor	Recipient	Spleen	Liver	Spleen	Liver
BALB/c	BALB/c	<0·01 <0·01	<0·01 <0·01	0·42*	0·69**
BALB/c	C.D2	<0·01 NT	<0·01 NT	0·09	0·24
C.D2	BALB/c	0·61** 0·28*	0·30** 0·49**	0·02	0·05
C.D2	C.D2	<0·01 NT	0·09 NT	0·20	0·08

One organ equivalent of T cell-enriched spleen cell preparations from donor mice infected for 4 weeks or control mice were adoptively transferred to sublethally irradiated syngeneic or congeneic mice. Recipient mice were challenged with the homologous strain of *M. avium* and bacterial growth determined 4 weeks later in the spleens and livers. Results are shown as log10 protection. Four mice were used per group.

Significantly different results are indicated for $P<0{\cdot}05$ (*) and $P<0{\cdot}01$ (**) according to Student's *t*-test.

NT = not tested.

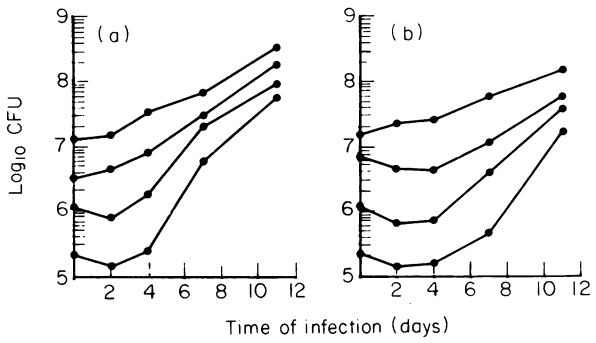

Fig. 2. Proliferation of *M. avium* strains 2447 (a) and ATCC 25291 (b) in bone marrow-derived macrophages from BALB/c mice. Macrophage monolayers were infected with different inoculum doses of bacteria for 4 h. After washing out the unphagocytosed bacteria, infection was monitored for 11 days.

M. avium grew progressively in the spleens and livers of the infected animals (Fig. 1a, b). After the first month of infection the bacterial number had increased by about 3 orders of magnitude. After that time, there was little or no further increase in bacterial counts. The bacterial growth in athymic nude mice was similar to that observed in euthymic mice in both organs. The growth pattern of this *M. avium* strain in mice given an inoculum dose 2 orders of magnitude lower (2×10^6 CFU) was similar to the one described above except that there was progressive proliferation for a longer period of time plateauing after 2 months of infection after having reached a similar bacterial load to that found with the higher inoculum dose

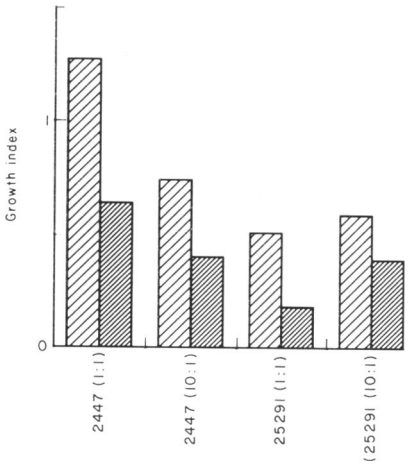

Fig. 3. Growth indexes for *M. avium* strains ATCC 25291 and 2447 infecting BALB/c bone marrow-derived macrophages for 7 days. Macrophages were infected at ratios of infection of 1:1 and 10:1 (bacteria:macrophage) and bacterial proliferation was studied in macrophages cultured in medium alone (light-hatched columns) or in medium supplemented with 25% lymphokine-containing (dark-hatched columns).

(Fig. 1 c,d). Again, there were no significant differences between euthymic and athymic mice.

M. avium *2447 grows more extensively in nude mice compared with euthymic littermates*

The proliferation of strain 2447 is shown in Fig. 1(e, f). The rate of proliferation of this strain during the first month of infection was smaller than that observed with the more virulent *M. avium* strain (an increase in viable counts of 1 to 1·5 orders of magnitude during the first 30 days). After this period of time, the bacterial growth was arrested in the euthymic mice after having reached a bacterial load about 3 orders of magnitude lower than the one found in the animals infected with the more virulent strain of *M. avium*. In the athymic animals, however, bacterial proliferation continued in both the spleens and the livers.

T cell-depleted mice of NR or NS background are not affected in their resistance or susceptibility to M. avium *ATCC 25291*

The effect of adult T cell-depletion on the growth of the virulent *M. avium* strain in BALB/c (NS) and in DBA/2 (NR) mice was studied (Table 1). The bacterial growth in the BALB/c mice was similar to the one found in C57BL/6 mice. Likewise, there was no significant difference in bacterial growth between TXBM and XBM mice. The naturally resistant DBA/2 mice restricted bacterial proliferation allowing only a small increase in viable counts in the spleens but no net increase in the livers. Again, there was no difference in mice depleted or not of T cells.

Proliferation of M. avium *in SCID mice*

The growth of the strains of *M. avium* in mice infected for 90 days was compared in SCID and C.B-17 mice. The results are shown in Table 1 and are similar to the results obtained for nude and control mice. There was no significant difference in the growth of the virulent strain ATCC 25291 between the immunocompetent and the immunodeficient mice. The strain 2447 multiplied more extensively in SCID mice than in control

C.B-17 mice. The sera for each SCID mouse was controlled for the level of antibodies revealing no leaky mutant (not shown).

Adoptive transfer of resistance

The ability of T-cell-enriched spleen cell suspensions from infected animals to confer protection to a challenge with the homologous microorganism in naive mice upon adoptive transfer was studied using a pair of congenic mouse strains that differ in the *Bcg/Ity/Lsh* allele and in their innate resistance to *M. avium* [17]. Since there were no differences in histocompatibility genes, both syngeneic and congeneic transfers were performed. Preliminary experiments did not find any effects of the transfer of congeneic cells from different immune compartments (spleen, thymus, bone marrow) on the resistance to *M. avium*. When immune spleen cells from mice infected with the more virulent strain of *M. avium* (ATCC 25291) were studied for their ability to protect irradiated recipient mice, the only cells that were shown to confer protection were those from C.D2 mice (NR) (Table 2). These cells were only able to transfer protection when transferred to susceptible BALB/c mice. When the less virulent 2447 strain was studied, only immune spleen cells from NS infected animals (BALB/c) were able to transfer any degree of protection (Table 2). Again, this protection was only detected in recipient mice of the susceptible strain.

In other experiments, different inoculum doses of *M. avium* ATCC 25291 were used to immunize BALB/c mice. There was no induction of protective T cells in these animals for inocula of 10^3, 10^4, 10^5, 10^6, or 10^7 CFU of *M. avium* when assessed in the susceptible BALB/c mice (data not shown). An inoculum of 10^6 CFU could also induce protective T cells in C.D2 mice (when assessed in the susceptible BALB/c mouse strain) (data not shown).

In vitro *growth of the two* M. avium *strains*

The proliferation of the two strains of *M. avium* in bone marrow-derived macrophages from BALB/c mice is shown in Fig. 2. Different numbers of bacteria were added to 10-day-old macrophages and after a 4 h infection period the cells were washed and bacterial growth monitored for 11 days by performing viable counts. It could be seen that both strains of *M. avium* proliferated in the macrophages after a 2 to 4 day lag period. The rate of growth of strain 2447 was slightly higher than that of strain 25291. In parallel experiments, the growth of the bacteria was studied in control macrophages and in macrophages that were activated by crude lymphokine supernatants (Fig. 3). Macrophages were infected for 4 h with different numbers of bacteria resulting in ratios of infection of 10:1 or 1:1 (bacteria: macrophages). Fresh medium or lymphokine-containing supernatants were added daily to the cultures. The growth index was calculated for untreated and treated cultures. Both strains of *M. avium* were sensitive to the bacteriostasis-inducing capacity of the lymphokines when proliferation was assessed at day 7.

DISCUSSION

We studied the role of T cells in the immunity to two strains of *M. avium* that differ in their *in vivo* growth behaviour. The strain ATCC 25291 proliferated progressively in both euthymic or athymic NS mice and eventually killed the animal from an overwhelming infection [16,17]. The plateauing of the bacterial proliferation before the death of the animals may be due to a limited supply of host cells to harbour the infectious organism rather than to an active immune-mediated anti-bacterial mechanism. This is supported by the findings that infections with smaller inoculum doses progress rapidly to the same bacterial load as the infections with higher inoculum doses and plateau at similar levels. The lack of protection by T cells in mice of the susceptible background against virulent *M. avium* is similar to what has been found with *M. lepraemurium* [30]. In contrast, the proliferation of strain 2447 was arrested by a T cell dependent mechanism since nude mice were not able to control the infection as well as the euthymic animals. The results with this latter strain mimic the results of Stokes & Collins [28] and Takashima & Collins [31] studying one strain of *M. avium* and one strain of *M. intracellulare*, respectively. The fact that the rate of proliferation of this bacterial strain is rather slow may explain the fact that only at rather late time points is there a clear difference in CFU between euthymic and athymic mice. Although athymic animals may still exhibit extrathymic T cell maturation [32] namely of $\gamma\delta$-positive T cells known to react with mycobacterial antigens [33,34], it is not known whether these cells are able to confer protection against mycobacteria. These aberrant T cell populations can, at least in theory, still be exerting some protection in nude animals. However, we found the same results when we studied SCID mice suggesting that the small and late differences in bacterial proliferation observed in nudes are most likely not related to either extrathymically matured T cells or $\gamma\delta^+$ T cells.

Since NR mice were able to abort *M. avium* proliferation from the start of the infection [16–19,27,28], we hypothesized that T cells were not involved in this type of resistance. In fact, T cell-depleted mice of NR phenotype (DBA/2) were still able to control the bacterial proliferation of the most virulent strain of *M. avium* used here. T cell-depleted mice of the NS phenotype were as susceptible as their controls as was found with the nude mice.

Looking at the mechanisms of induction of protective T cells, Stokes & Collins [28] showed for another strain of *M. avium* (TMC 702) similar to the strain 2447 that proliferation of the bacteria was necessary for the induction of protective T cells, a finding that applies to other mycobacteria [11,12,28,35,36]. We decided to compare the two *M. avium* strains not only with regard to induction but also to expression of protective immunity. We used for those studies a pair of congenic mice differing at the *Ity/Lsh/Bcg* locus that we had found to differ in their resistance to *M. avium* [17]. Since these mice do not differ at their histocompatibility loci, we performed adoptive transfer experiments in both syngeneic and congeneic combinations to allow us to discern the differences in induction and in expression of protective immunity. The most virulent *M. avium* strain did not induce any detectable population of protective T cells in NS mice but was able to induce protective T cells in the resistant mouse strain even though the level of protection transferred was low. This is different from the results of Stokes & Collins [28] who found no induction of protective T cells in resistant mice. This may be explained since these authors did not use congenic mouse strains to perform adoptive transfers between NR and NS mice and because the strain of *M. avium* used had a much more limited virulence for mice than the one studied by us which grew to some degree in the spleens of NR mice. This is compatible with the notion that bacterial proliferation is necessary for the induction of protective T cells [11,12,28,35,36].

The less virulent strain of *M. avium* that we studied behaved like the strain studied by Stokes & Collins [28] in that it induced protective T cells in the NS but not in the NR mice. None of the populations of protective T cells had any effect on NR mice. This may be due to the fact that these mice that are innately resistant to the infection cannot be further protected by T cells, i.e. that their macrophages are already maximally bacteriostatic.

We postulate that the lack of growth of *M. avium* in NR mice is dependent on a T-cell-independent constitutive function expressed in macrophages. In these mice T cells are not necessary for resistance to infection and any protective T cell generated during infection is unable to confer any further protection. The fact that additional *in vivo* activation of the macrophage is not able to induce killing would be explained by the high resistance of *M. avium* to the anti-microbial mechanisms of the activated macrophage [16,17,19]. Macrophages from NS mice lack such an intrinsic function and allow the growth of *M. avium*. According to the virulence of the bacterial strain, specifically sensitized T cells will or will not be generated. These protective T cells will be able to induce macrophages to acquire the ability to control the infection. In the case of the more virulent *M. avium* there might be a defect not only in the sensitization of T cells but also in other aspects of T cell–macrophage interaction so that the protective T cells would not be able to recognize bacterial antigens on the surface of infected macrophages and fail to activate their anti-mycobacterial machinery. This interpretation is supported by our data showing that soluble lymphokines were able to activate macrophages *in vitro* to restrict the growth of the two strains of *M. avium* that behave quite differently *in vivo*. This hypothesis is now under investigation using *in vitro*-cultured macrophages and immune T cells to try to mimic *in vitro* the *in vivo* situation. Finally we showed that there was a clear difference in the virulence of the two strains of *M. avium* when they were assessed *in vivo* or *in vitro*. Thus, strain 2447 was able to proliferate *in vitro* as well as or even more extensively as strain 25291 although it had a more limited capacity to proliferate *in vivo* even prior to the establishment of acquired immunity. We argue that much caution must be used in interpreting *in vitro* data as a measure of *in vivo* virulence. This is even more pertinent in the case of the human infection where we lack the *in vivo* growth data for the bacterial isolates.

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