*Brief Definitive Report*

# Evidence for an $\alpha/\beta$ T Cell–independent Mechanism of Resistance to Mycobacteria. Bacillus-Calmette-Guerin Causes Progressive Infection in Severe Combined Immunodeficient Mice, but Not in Nude Mice or in Mice Depleted of $CD4^+$ and $CD8^+$ T Cells

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## Summary

Depleting thymectomized mice of $CD4^+$ T cells, or $CD4^+$ plus $CD8^+$ T cells, rendered them incapable of resolving Bacillus-Calmette-Guerin (BCG) infection in their livers, spleens, kidneys, and lungs. However, it did not render them incapable of stabilizing infection in the latter three organs after an initial period of BCG growth. Athymic nude mice showed a similar capacity to control BCG growth in these organs after a certain stage of infection. In contrast, congenitally severe combined immunodeficient (SCID) mice appeared to offer no resistance to BCG infection, in that the organism grew progressively in all organs of these mice and was lethal for them beginning on day 55 of infection. The results suggest that, although $CD4^+$ T cells are important for resolving BCG infection, an $\alpha/\beta$ T cell–independent mechanism of resistance can be acquired at 2–3 wk of infection that is capable of inhibiting further BCG growth in all organs except the lungs. Because this mechanism is absent from SCID mice, it is likely that it depends on the functions of $\gamma/\delta$ T cells, B cells, or both types of cells. In keeping with this possibility is the additional finding that SCID mice engrafted with lymph node cells depleted of $CD4^+$ or $CD8^+$ T cells were capable of expressing an appreciable level of resistance against BCG infection.

It is generally believed, on the basis of results obtained from adoptive immunization and T cell depletion studies, that immunity to infection with mycobacteria is mediated by T cells. It has been known for some time, for example, that mice in the process of resolving BCG infection acquire T cells that are capable, on passive transfer, of conferring increased antimycobacterial resistance on naive recipient mice (1, 2). More recently, experiments with mice depleted of T cell subsets have shown that depletion of $CD4^+$ T cells, but not of $CD8^+$ T cells, results in a reduced capacity to resolve BCG infection (3) and to defend against infection with *Mycobacterium tuberculosis* (4). However, in the case of BCG infection, the same T cell depletion studies (3) revealed that the absence of $CD4^+$, or both T cell subsets, did not leave mice completely defenseless against infection. On the contrary, mice so depleted showed a surprising capacity to halt progressive growth of BCG after a certain stage of infection. Therefore, the interpretation that $CD4^+$ T cells are pivotal for resistance to BCG infection is not supported by the evidence.

This paper will show that mice depleted of $CD4^+$ plus $CD8^+$ T cells, and athymic nude mice, can acquire the ability to stabilize BCG infection after 2–3 wk of BCG growth. It will show, in addition, that SCID mice permit progressive BCG growth in all organs and begin dying of infection from day 55 on, but not if they are engrafted with coisogenic lymph node cells depleted of $CD4^+$ or $CD8^+$ T cells. Therefore, acquired resistance to BCG infection is partly dependent on a mechanism that is independent of $CD4^+$ and $CD8^+$ T cells.

## Materials and Methods

*Mice.* $B6D2F_1$ (C57BL/6 × DBA/2) mice, BALB/c *nu/nu*, BALB/c *nu/+* mice, CB-17 *scid/scid* (SCID) mice, and their coisogenic CB-17 ($H\text{-}2^d$) counterparts were used. All mice were between 8 and 9 wk of age when they were used in experiments. They were purchased from the Trudeau Institute Animal Breeding Facility and were known to be free of common and viral pathogens according to routine screening performed by the Research Diagnostic Laboratory, University of Missouri. The SCID mice were


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pneumocystis free as a result of having been bred from B cell- and T cell-engrafted parents (5). They were maintained in isolator cages supplied with filtered air, and were provided with sterilized food and water. Nude mice were maintained under similar conditions.

*BCG. Mycobacterium bovis*, strain BCG Pasteur (TMC no. 1011), was supplied by the Trudeau Institute Culture Collection as a frozen mid-log-phase culture. Before each experiment a vial was thawed, subjected to brief ultrasound, and diluted in PBS containing 0.01% Tween. It was inoculated in a dose of $2 \times 10^6$ CFU in 0.2 ml PBS via a lateral tail vein. BCG was enumerated in lungs, livers, spleens, and kidneys by plating 10-fold serial dilutions of homogenates of these organs on 7H11 agar (Difco Laboratories, Detroit, MI) and counting 2–3 wk later.

*T Cell Depletion and Cytofluorometry.* $B6D2F_1$ mice were thymectomized at 4 wk of age and used in experiments 4 wk later. 2 d before inoculating BCG the mice were injected with 0.5 mg of anti-CD4, anti-CD8, or both mAbs intravenously. On days 7, 14, 21, and 32 after BCG inoculation, mice were again injected intravenously with 0.25 mg of these mAbs. The anti-CD4 mAb was produced by clone GK1.5 (provided by Frank Fitch, University of Chicago, Chicago, IL) and the anti-CD8 mAb by clone TIB 210 (American Type Culture Collection, Rockville, MD) growing as ascites in immunodepressed mice. Anti-Thy-1.2 mAb was produced by clone 30.H.12 (American Type Culture Collection). The mAbs were purified as described previously (6). The extent of T cell depletion was determined at progressive stages of infection by subjecting spleen cells to cytofluorometric analysis on a FACScan® cytofluorometer (Becton Dickinson & Co., Mountain View, CA) after incubating them in FITC-conjugated $F(ab')_2$ fragments of anti-Thy-1.2, anti-CD4 , or anti-CD8 mAb. Preparation of $F(ab')_2$ fragments and their conjugation to FITC was as described previously (6). Dead cells were stained with propidium iodide and excluded from calculations done on 3,000 events.

*Engrafting of SCID Mice with Lymphoid Cells.* In one experiment, SCID mice were engrafted with $10^7$ lymph node cells from CB-17 mice, or from CB-17 mice depleted of $CD4^+$ T cells or $CD8^+$ T cells by treatment with anti-T cell subset mAbs as described above. The mAbs were given to the CB-17 donors 2 d before harvesting their popliteal, axillary, brachial superior, and mesenteric node cells. 2 d after infusing SCID recipient mice with CB-17 lymph node cells, the recipients were themselves infused with 0.5 mg of the appropriate anti-T cell subset mAb to eliminate any T cells that survived mAb treatment in the donors. After a further 3 d, they were inoculated intravenously with $2 \times 10^6$ BCG, as were appropriate SCID and CB-17 controls.

## Results

*Failure of BCG to Grow Progressively in Mice Depleted of T Cells with mAbs.* Fig. 1 shows the growth of BCG in the livers, spleens, kidneys, and lungs of immunocompetent control mice, thymectomized control mice, and thymectomized mice depleted of $CD4^+$ T cells alone, or $CD4^+$ plus $CD8^+$ T cells. It can be seen that after ~20 d of BCG growth, immunocompetent mice, but not T cell–depleted mice, began progressively resolving infection in their livers, spleens, and kidneys. However, although incapable of resolving infection in these organs, mice depleted of $CD4^+$ or both T cell subsets were nevertheless capable of preventing further BCG growth after day 20 of infection. Consequently, the infection was stabilized until the experiment was terminated on day 80. This was not the case in the lungs, however, in that

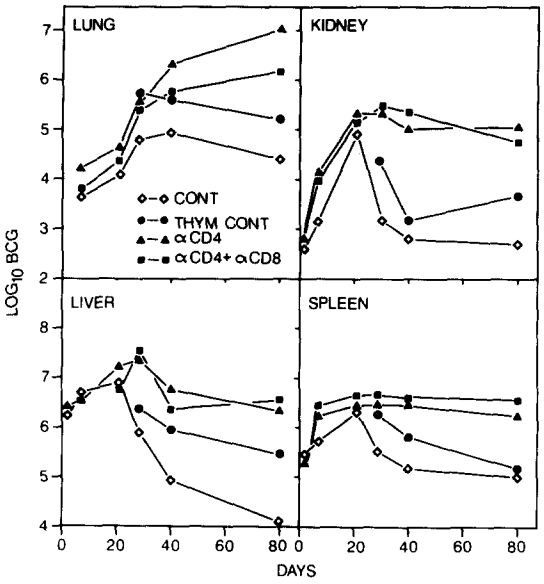


**Figure 1.** Evidence that BCG infection was progressive in the lungs, but not in the kidneys, livers, and spleens of thymectomized mice depleted of $CD4^+$ ($\alpha$CD4), or $CD4^+$ plus $CD8^+$ ($\alpha$CD4 + $\alpha$CD8) T cells, in that BCG growth plateaued in the last-mentioned organs after about day 20 of infection. In contrast, infection underwent resolution after about day 20 in all organs of immunocompetent control (*CONT*) and thymectomized control (*THYM CONT*) mice. However, thymectomized controls showed a reduced ability to resolve infection, which was not reduced further by depleting them of $CD8^+$ T cells alone (data not shown). Data are means of five mice per group per time point.

BCG grew progressively in this organ in mice depleted of $CD4^+$ or both subsets of T cells while it was being inactivated in control mice. Fig. 1 also shows that thymectomized control mice were deficient in their capacity to deal with infection in all organs, in that the rate of resolution of infection was slower than in controls. It was found, in addition (result not presented), that treating thymectomized mice with anti-CD8 did not significantly reduce their ability to resist BCG infection in any organ. Therefore, $CD4^+$ T cells, rather than $CD8^+$ T cells, are involved in resolution of BCG infection.

*Efficiency of T Cell Depletion by mAbs.* The foregoing results showing that mice depleted of $CD4^+$ T cells, or both subsets of T cells, retain an appreciable capacity to stabilize infection after ~20 d of progressive BCG growth could mean that T cell subset depletion was not complete. This was investigated by subjecting spleen cells of mAb-treated mice to cytofluorometric analysis. The results in Table 1 show that in infected immunocompetent mice and in infected thymectomized mice, 9.4% and 6.3%, respectively, of spleen cells on day 40 of infection were $CD4^+$ T cells. In contrast, only 0.3–0.4% of spleen cells were $CD4^+$ in thymectomized mice repeatedly treated with anti-CD4 mAb or with both mAbs. Treatment with both mAbs reduced the number of $CD8^+$ T cells to a level that was not detectable by cytofluorometry. Thus, it is apparent that depletion of $CD4^+$ and $CD8^+$ T cells was essentially complete in terms of the per-

**Table 1.** *Cytofluorometric Assessment of T Cell Subset Depletion in B6D2F$_1$ Control and Day 40 BCG-infected Mice*

| Treatment | No. of cells in spleen (%) | | | |
|---|---|---|---|---|
| | Thy-1$^{+}$* | CD4$^{+}$ | CD8$^{+}$ | All cells |
| | | $\times 10^6$ | | |
| Uninfected control | 16.7 (15.2) | 10.3 (9.38) | 5.8 (5.29) | 118 |
| Uninfected thym. control | 16.8 (11.2) | 14.4 (9.6) | 7.8 (5.2) | 150 |
| Infected control | 70.8 (13.3) | 50.0 (9.4) | 17.0 (3.2) | 532 |
| Infected thym. control‡ | 37.4 (10.0) | 23.6 (6.3) | 9.3 (2.5) | 374 |
| Infected thym. given αCD4§ | 8.3 (4.2) | 0.6 (0.3) | 7.1 (3.6) | 198 |
| Infected thym. given αCD4 + αCD8$^{b}$ | 8.5 (2.3) | 1.5 (0.4) | - (0)‖ | 368 |

* F(ab')$_2$-FITC reagents were used for cytofluorometry.
‡ Thymectomy was performed when mice were 4 wk old, 4 wk before infection.
§ 0.5 mg of mAb was given 2 d before infection with 2 × $10^6$ BCG cells, and this was followed by injection of 0.25 mg mAb on days 7, 14, 21, and 32.
‖ Level was below that of detection using the FACScan® cytofluorometer.

cent that survived, relative to the percent present in controls. However, when these results are considered in terms of total numbers of cells, the spleens of anti-CD4-mAb–treated mice contained 6 × $10^5$ CD4$^+$ T cells, compared with 5 × $10^7$ in the spleens of infected immunocompetent controls, and 2.3 × $10^7$ in the spleens of infected thymectomized controls. Therefore, it could be argued that 6 × $10^5$ CD4$^+$ T cells are enough to inhibit BCG growth as shown in Fig. 1, assuming that a proportion of these cells was, in fact, specific for BCG. It can also be seen in Table 1 that the spleens of infected thymectomized mice depleted of CD4$^+$ and CD8$^+$ T cells contained an appreciable number of Thy-1$^+$CD4$^-$ CD8$^-$ cells.

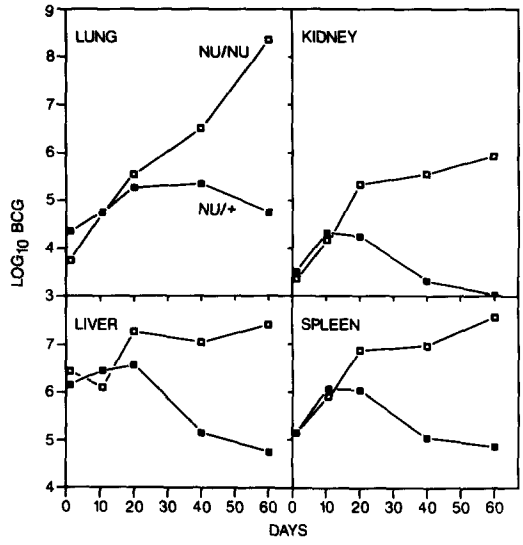


**Figure 2.** Evidence that BCG infection was progressive in the lungs, but not in the kidneys, livers, and spleens of athymic nude (*NU/NU*) mice. In euthymic (*NU/+*) control mice, infection underwent progressive resolution in all organs beginning between days 10 and 20 postinoculation. Data are means of five mice per group per time point.

*Ability of Athymic Nude Mice to Acquire Resistance to BCG Infection.* As shown above, thymectomized mice repeatedly treated with anti-CD4 and anti-CD8 mAbs, although ~99% depleted of CD4$^+$ and CD8$^+$ T cells on day 40 of infection, were nevertheless capable of preventing further growth of BCG in their livers, spleens, and kidneys after day 20 of infection. It was anticipated, therefore, that the same result would be obtained with 8-wk-old athymic nude mice, which possess a very small number of α/β T cells (7, 8), apparently few of which are functionally normal in vivo and in vitro (9–11). As shown in Fig. 2, athymic nude mice were capable of greatly reducing any further growth of BCG in their livers, spleens, and kidneys, although not in their lungs, after about day 20 of infection. This resulted in an approximate plateauing of BCG growth in all organs except the lungs from day 20 to day 60 of infection when the experiment was terminated. In contrast, syngeneic euthymic mice were able to resolve infection progressively in all organs starting between days 10 and 20.

*Progressive Lethal Growth of BCG in SCID Mice but Not in SCID Mice Engrafted with Lymph Node Cells Depleted of CD4$^+$ and CD8$^+$ T Cells.* The foregoing results show that mice depleted of CD4$^+$ and CD8$^+$ T cells, and nude mice, can acquire a mechanism of resistance capable of restricting the growth of BCG after a certain stage of infection. It could be suggested as an argument against this interpretation, however, that in the absence of T cell–mediated immunity BCG growth is eventually self limiting in liver, spleen, and kidney, but not in lung. This possibility was investigated by following the growth of BCG in SCID mice that are devoid of functional T cells (including γ/δ T cells) and consequently possess

no capacity to resist infection with a range of pathogenic and nonpathogenic organisms. The results in Fig. 3 show unequivocally that BCG grew progressively with little interruption in all organs of SCID mice until the experiment was terminated on day 60. These mice began dying of infection after day 55. Therefore, the growth of BCG in the liver, spleen, and kidney of mice is not self limiting in vivo.

On the other hand, SCID mice engrafted with lymph node cells from CB-17 donors were capable of expressing an appreciable level of resistance against BCG infection. This was the case, moreover, even with lymph node cells from CB-17 mice that had been essentially depleted of $CD4^+$ or $CD8^+$ T cells. This can be seen in Fig. 4, which shows the results of an attempt to provide SCID mice with a capacity to resist infection with $2 \times 10^6$ BCG by infusing them with $10^7$ lymph node cells from normal CB-17 mice or from CB-17 mice depleted of $CD4^+$ or $CD8^+$ T cells by treatment with anti-CD4 or anti-CD8 mAb 2 d before transfer. At the time of transfer, lymph node cells of anti-CD4-treated or anti-CD8-treated CB-17 donors contained <0.1% $CD4^+$ T cells and an undetectable number of $CD8^+$ T cells, respectively. Moreover, in this experiment, recipient SCID mice were themselves treated with anti-CD4 or anti-CD8 mAbs in order to destroy any $CD4^+$ or $CD8^+$ T cells that escaped destruction in the donors. It is obvious from Fig. 4 that lymph node cells depleted of $CD4^+$ T cells, although less protective at day 40 of infection than total lymph node cells, nevertheless provided SCID recipients with a substantial capacity to resist infection in all organs except the lungs. Indeed, the numbers of BCG in the organs of engrafted SCID recipients on day 40 is in keeping with the interpretation that recipients that had received $CD4^+$ T cell–depleted lymph node cells were able to stabilize infection, whereas recipients that received total lymph node cells were as capable as CD-17 donor mice at resolving infection (see Fig. 3).

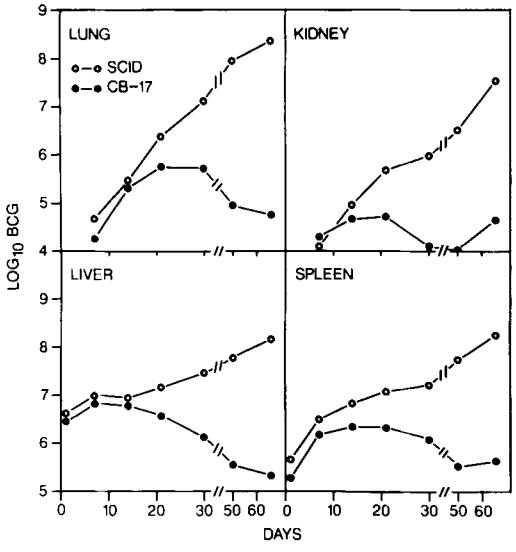


**Figure 3.** Evidence that BCG infection was progressive in all organs of SCID mice. In CB-17 coisogenic mice, in contrast, infection underwent progressive resolution in all organs after 2–3 wk of BCG growth.

## Discussion

This study shows that $CD4^+$ T cells, rather than $CD8^+$ T cells, are needed by mice to resolve intravenous infection with BCG. However, it shows in addition, that at a certain stage of infection, mice depleted of $CD4^+$ T cells alone, or of both subsets of T cells, acquired the ability to prevent further growth of BCG in their livers, spleen, and kidneys, although not in their lungs, for a protracted period of time. A similar ability to stabilize infection was acquired by athymic nude mice. Stabilization of infection was not the result of self-limiting growth of BCG in vivo, because the organism grew progressively without interruption in all organs of SCID mice, and was lethal for these mice beginning on about day 55 of infection. Therefore, cells exist in mice depleted of $CD4^+$ and $CD8^+$ T cells, and in nude mice, but not in SCID mice, that can stabilize BCG infection after 2–3 wk of progressive BCG growth. Because $\gamma/\delta$ T cells and B cells are possessed by the former mice, but not the latter, it seems reasonable to suggest that either or both of these cell types was involved in the resistance observed. A suggested role for $\gamma/\delta$ T cells is supported by published evidence showing that

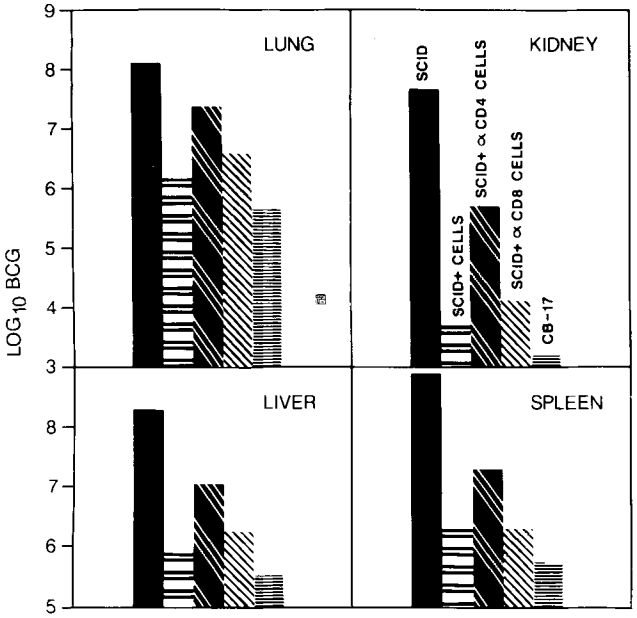


**Figure 4.** Result of attempt to provide SCID mice with the capacity to control or resolve BCG infection by infusing them intravenously with $10^7$ lymph node cells from CB-17 mice, or from CB-17 mice depleted of $CD4^+$ or $CD8^+$ T cells by treatment with anti-CD4 or anti-CD8 mAbs (0.5 mg), respectively, 2 d before death. In this experiment SCID recipients of lymph node cells depleted of $CD4^+$, or $CD8^+$ T cells, were themselves treated with anti-CD4 or anti-CD8 mAb (0.5 mg), respectively, 2 d after cell transfer, and were inoculated intravenously with $2 \times 10^6$ BCG cells 3 d later. Whole lymph node cells provided SCID mice with an impressive capacity to resist BCG infection, in that by day 40 they showed about the same ability as CB-17 donors to inactivate BCG in their lungs, kidneys, livers, and spleens. Lymph node cells depleted of $CD4^+$ T cells showed a reduced, although appreciable, capacity to protect SCID mice against infection in their kidneys, livers, and spleens, although not in their lungs. Lymph node cells depleted of $CD8^+$ T cells were almost as protective as control lymph node cells. Data are means of five mice per group.

a proportion of $\gamma/\delta$ T cells are specific for mycobacterial antigens, including epitopes of the highly conserved 60-kD heat-shock protein (12–15). However, a role for B cells also needs to be considered, because in spite of past failures to transfer antimycobacterial resistance with serum antibodies (16, 17), it is possible that these cells serve to secrete relevant lymphokines and to act as APC at sites of infection. Studies designed to investigate the roles of $\gamma/\delta$ T cells and B cells based on lymphocyte engraftment experiments with SCID mice are currently in progress in this laboratory. The initial results of these studies presented here clearly show that SCID mice engrafted with $\sim 10^7$ lymph node cells from naive coisogenic CB-17 donors displayed an almost normal capacity to resolve BCG infection. These results show, in addition, that an appreciable level of resistance was expressed in SCID mice engrafted with lymph node cells from CB-17 donors depleted of $CD4^+$ or $CD8^+$ T cells. Indeed, when examined at day 21 of infection, SCID mice so engrafted contained no detectable $CD4^+$ or $CD8^+$ T cells. Thus, it seems likely that this model will allow successful analysis of the cellular basis of antimycobacterial immunity.

None of the evidence presented here contradicts evidence published by others (3, 4) showing that $CD4^+$ T cells play an important role in defense against BCG infection. On the contrary, our results support an important role for this subset of $\alpha/\beta$ T cells in resistance to BCG. However, their main purpose is to show that a $CD4^+$ T cell–independent mechanism of defense can be called on by the host to combat infection with this organism. Indeed, an examination of the results of T cell depletion studies published by others (3) shows quite clearly that thymectomized mice treated with anti-CD4 mAb, although deficient in their capacity to resolve BCG infection, retained a capacity to stabilize infection and to even begin resolving infections after a delay. This is entirely in keeping with the data presented here. Unfortunately, it is not possible to know in the case of this published study whether the onset of resolution of infection was the result of incomplete depletion of targeted $CD4^+$ T cells, because the results of cytofluorometric analysis at the time of resolution of infection were not presented. In the case of the study presented here, repeated treatment with anti-CD4 mAb was shown to result in essentially complete depletion of $CD4^+$ T cells, in that the treated mice possessed only 1.2% of the splenic $CD4^+$ T cells possessed by control mice at day 40 of infection. However, because infection caused a large increase in the cellularity of the spleen, the 99% depletion of $CD4^+$ T cells still left mice with $6 \times 10^5$ of these T cells on day 40, a number that cannot be disregarded. It should be brought to mind in this connection, however, that, unlike the repertoire of B cells, which is based to an appreciable extent on somatic hypermutation in Ig V region genes, the repertoire of T cells is relatively fixed after immunologic maturation (18). Therefore, it is likely that few of the 0.3–0.4% $CD4^+$ T cells that existed in anti-CD4-treated thymectomized mice at the beginning of infection were specific for BCG. Indeed, because anti-CD4 mAb was given repeatedly during infection it should have resulted in the repeated destruction of any $CD4^+$ T cells that multiplied in response to infection. Moreover, nude mice that are known to possess only very small absolute numbers of $CD8^+$ and $CD4^+$ T cells, presumably few of which are capable of expressing $\alpha/\beta$ TCRs (7, 8) and of functioning normally in vivo (9, 10) or in vitro (11), gave the same results as T cell–depleted mice. Therefore, the results support the interpretation that $CD4^+$ T cells are not needed for stabilization of BCG infection in the liver, spleen, and kidneys after 2–3 wk of progressive BCG growth. These results with nude mice are in agreement with growth curves published some years ago by others, which on examination show that nude mice (19, 20) and neonatally thymectomized mice (17), although incapable of resolving infection, were capable of restricting BCG growth to a considerable extent in their livers and spleen after a certain stage of infection. The results presented here showing that SCID mice engrafted with lymph node cells depleted of $CD4^+$ T cells are capable of expressing a substantial level of anti-BCG resistance support this conclusion.

It needs to be emphasized that although mice depleted of $CD4^+$ T cells, nude mice, and SCID mice engrafted with $CD4^+$ T cell–depleted lymphoid cells were capable of gaining control of BCG infection in their livers, spleens, and kidneys, they were much less capable of controlling infection in their lungs. Indeed, the results suggest that $CD4^+$ T cells are essential for the control, as well as the resolution, of BCG infection in the lungs. This indicates that the $\alpha/\beta$ T cell–independent mechanism of resistance that exists in other organs may not exist in the lungs. In this regard it has been noted repeatedly over the years that the lung is the most susceptible organ to infection with BCG and *M. tuberculosis* (22).

In conclusion, this study draws attention to the antimycobacterial resistance that remains in mice in the absence of $CD4^+$ and $CD8^+$ T cells, rather than to the resistance that is lost. Given that the resistance that remains is relatively powerful, and the possibility that it is mediated by $\gamma/\delta$ T cells specific for mycobacterial antigens, it cannot be ignored in any analysis of the cellular basis of antimycobacterial immunity.

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