Tumor Necrosis Factor and Chemokine Interactions in the Formation and Maintenance of Granulomas in Tuberculosis

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Inflammatory cells migrate to the lungs in response to *Mycobacterium tuberculosis* infection. These infiltrating cells organize into a structure called a granuloma, which controls and contains infection. The signals that influence the formation of granulomas are largely unknown. Tumor necrosis factor (TNF) has been demonstrated to be required for formation of granulomas, in mouse models of tuberculosis, and for control of latent tuberculosis, in humans. We investigated the mechanisms by which TNF controls cell migration in response to *M. tuberculosis* infection, focusing on the effects of this cytokine on chemokine expression. Chemokines are small molecules that direct the migration of cells within the body. Our data support the notion that TNF is required for appropriate chemokine expression by *M. tuberculosis*–infected macrophages, both in vitro and in vivo.

Tuberculosis kills ∼2 million people every year. *Mycobacterium tuberculosis*, the bacterium responsible for this respiratory disease, has a unique ability to survive within the host for years or even decades. Initial infection leads to active disease in only a small subset of persons. Most infected people have an immune response to the bacillus that serves to keep it in check, resulting in an asymptomatic infection. However, clearance of *M. tuberculosis* from the body is difficult, and latent infection can persist for the lifetime of the host. Reactivation of this latent infection occurs in ∼10% of infected persons, often in response to an immunocompromising event (reviewed in [1]). For example, aging, steroid use, HIV infection, malnutrition, and substance abuse increase the risk of reactivation of tuberculosis. Recently, TNF-modulating treatments, such as TNF-neutralizing antibodies and receptor fusion proteins,

have been implicated in increased susceptibility to tuberculosis; in particular, reactivation of latent infection seems to be triggered by TNF modulation [2–4].

TNF IS REQUIRED FOR CONTROL OF *M. TUBERCULOSIS* INFECTION IN MICE

Our laboratory has had a long-standing interest in the role that TNF plays in the control of *M. tuberculosis* infection. Several years ago, we demonstrated that TNF was required for the control of acute infection in mice, with effects on macrophage activation and granuloma formation [5]. TNF receptor 1 (TNFR1; p55 receptor) knockout (TNFR1$^{-/-}$) mice or mice treated with neutralizing antibody against TNF were extremely susceptible to *M. tuberculosis* infection and died within 4 weeks.

The mouse model of tuberculosis allows one to study both acute and chronic infection [6]. Bacterial counts in the lungs increase for the first 3–4 weeks after infection via the aerosol route and then stabilize. The infection is controlled but not eliminated, and, eventually, the mice die of progressive infection. There is a long window of time in which to use this model to address the mechanisms responsible for maintaining a persistent *M. tuberculosis* infection. We used this model

to address the requirement for TNF in maintaining a persistent infection. Neutralization of TNF with antibody, beginning 4–6 months after infection, resulted in rapid reactivation of the infection [7]. This reactivation was characterized by a relatively small increase in bacterial numbers but a substantially worsened pathologic process, and mice died quickly. The most striking findings were the apparent loss of granuloma structure and the infiltration of cells throughout the lungs. This infiltration led to a destructive immunopathology that was not solely attributable to increased bacterial numbers. These results were in contrast to reactivation due to other immunocompromising events, including inhibition of nitric oxide synthase [8] or CD4 T cell depletion [9]. In association with these events, bacterial counts increased steadily, but granulomas did not lose their organization. The results of our studies implied that TNF plays a role in the maintenance of granuloma formation and the control of pathologic processes.

FORMATION OF GRANULOMAS

TNF is a pleiotropic cytokine that affects many pathways in the host response to *M. tuberculosis.* We sought to address how TNF controls the formation and maintenance of granulomas in both acute and persistent infection. We view the formation of granulomas in response to *M. tuberculosis* infection as follows: *M. tuberculosis* initially infects alveolar macrophages in the airways, resulting in cytokine production and the onset of an inflammatory response, with the influx of monocytes, neutrophils, and dendritic cells. At this point, the bacilli are likely taken up by dendritic cells, which then traffic to the lung-draining lymph nodes to prime naive T cells. The bacteria enter the lung parenchyma and infect tissue macrophages, inducing a series of inflammatory signals.

Effector T cells, B cells, and more monocytes migrate to the lung from the blood and then through the lung tissue to the local site of infection. The slow growth of the organism (doubling time, ~24 h in vivo) prevents an overwhelming bacterial infection from occurring prior to the onset of the granulomatous response. Once cells reach the site of infected macrophages, they form a structure that is meant to contain the infection (i.e., the granuloma). In granulomas, T cells can initially interact with infected macrophages and activate these cells to control infection. Because this activation is not completely successful, bacteria remain within the granuloma. However, in most cases, the remaining bacteria are controlled for an extended period. The granuloma serves 3 major purposes: it is a local environment in which immune cells can interact to kill bacteria, a focus of inflammatory cells that prevents inflammation from occurring throughout the lungs, and a barrier to dissemination of bacteria throughout the lungs and to other organs. Disruption of granuloma structure or function appears to be detrimental to the control of bacterial replication and the control of immunopathology in the lungs.

TNF AND CONTROL OF CHEMOKINE EXPRESSION IN VITRO

We were interested in the signals that induce cell migration within the lungs and result in formation of the granuloma, as well as the signals that prevent loss of granuloma structure during chronic infection. Our data pointed to a major role for TNF in both initial formation of the granuloma and maintenance of an established granuloma. TNF is likely to be a master regulator of the signals responsible for directing cells to the site of infection. Obvious candidates for these signals are chemokines. Chemokines are a large family of small molecules, similar to cytokines, that interact with G protein–coupled receptors on a variety of cells to direct cell movement. When this study was initiated, there were limited data on chemokines in tuberculosis (reviewed in [10]). We investigated how TNF controls chemokine expression in the mouse models of acute and chronic tuberculosis [11].

In vitro experiments demonstrated that *M. tuberculosis* infection of bone marrow–derived murine macrophages results in expression of TNF, as well as a number of chemokines, including ligands for the chemokine receptors CXCR3, CCR5, and CCR2 (figure 1). Expression of both proteins and mRNA was determined. The peak of the TNF and chemokine response occurred early, sometimes by 4 h after infection, and mRNA expression was greatly reduced by 24 h after infection. Neutralization of TNF by using antibody or TNFR1$^{-/-}$ macrophages demonstrated that expression of certain chemokines (CXCL9, CXCL10, CXCL11, CCL5, and CCL2) after *M. tuberculosis* infection was dependent, at least in part, on TNF. In particular, the early peak of CXCL9 and CCL5 production was greatly diminished when TNF was neutralized. However, the lack of TNF did not completely abrogate chemokine expression, indicating that there are other factors, induced as a result of infection, that stimulate chemokine production (figure 1).

EFFECTS OF TNF ON CHEMOKINE EXPRESSION IN THE LUNGS DURING *M. TUBERCULOSIS* INFECTION

We then turned to the in vivo mouse model of infection. Initially, we examined the dependence of chemokine expression on production of TNF in vivo by using real-time RT-PCR or RNase protection analysis of whole lung tissue obtained at various times after infection. However, in our model of low-dose infection (~50 cfu/lung, delivered via an aerosol route), chemokine expression was not detectable until at least 10 days after infection. Because we were interested in very early events whereby TNF might influence granuloma formation, this inability to detect chemokine expression at early time points was
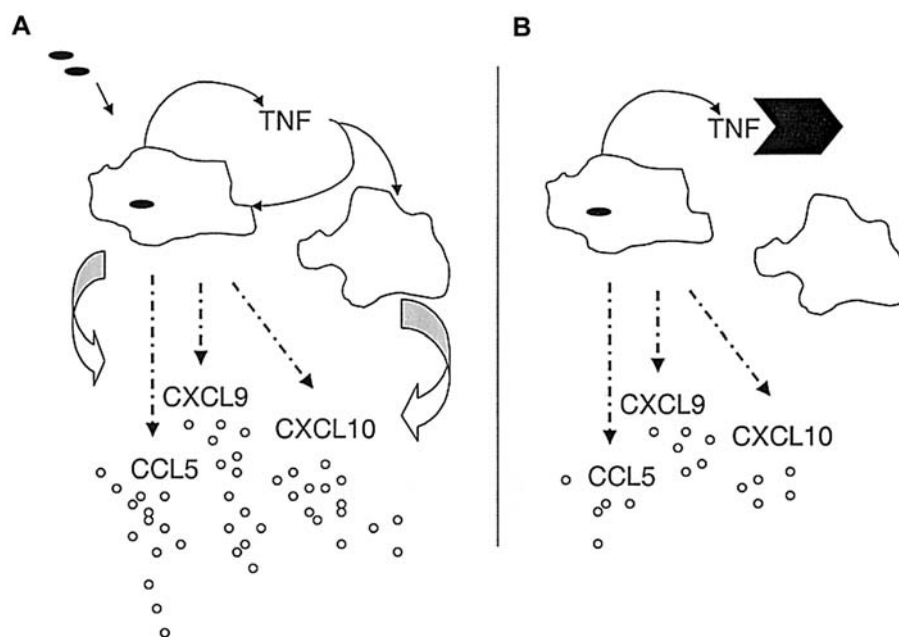
Figure 1. Influence of TNF on production of chemokines by macrophages during *Mycobacterium tuberculosis* infection. *A,* Initial interaction and infection with *M. tuberculosis* elicits production of TNF by macrophages within 4 h. TNF can then act on the infected macrophages or on other macrophages to induce chemokine production *(open arrows).* Chemokines (e.g., CXCL9, CXCL10, and CCL5) are also produced by macrophages following infection *(dotted arrows). B,* Blocking production of TNF (antibody or receptor fusion; *solid arrowhead*) reduces the quantity of chemokines produced by the macrophages, but a residual level of chemokine production that is not TNF dependent remains *(dotted arrows).*

problematic. In addition, once bacterial numbers began to diverge between wild-type *(wt)* and TNF-deficient mice, chemokine expression diverged as well. In fact, we found that chemokine expression in vivo increased rapidly in response to increased bacterial numbers, even in the absence of TNF. Therefore, it was essential to study the effects of TNF on chemokine expression before there were any changes in the bacterial numbers between *wt* and TNF-deficient mice.

To detect low-level chemokine expression in the lungs early during infection, we needed to select a population of cells that would be likely to produce chemokines. We reasoned that low-dose infection (∼50 cfu/lung) could, at most, result in infection of 50 pulmonary macrophages initially. This is a small percentage of the ∼10^5 macrophages in the lungs of uninfected mice. The replication rate of *M. tuberculosis* in vivo is estimated to be ∼24 h, depending on the strain. Therefore, even 5 days after infection, only a small percentage of macrophages would be infected and producing chemokines, which probably contributed to the difficulties in detecting chemokine expression early after infection. However, it is likely that bystander macrophages could be affected by the presence of the infection (including the production of TNF by infected macrophages) (figure 1). We decided to enrich for the macrophages from the lungs, to improve detection of chemokine expression.

CD11b$^+$ cells (which include both macrophages and neutrophils) were isolated from the lungs of mice at 4, 7, and 10 days after infection and were used for analysis of gene expression by using real-time RT-PCR. By 4 days after infection, in the macrophages from *wt* mice, we could detect increases in mRNA for the chemokines CXCL9, CXCL10, CCL3, CCL4, CCL5, and CCL12. However, the chemokine levels in the TNF-deficient mice remained at baseline levels at day 4 and still lagged behind the levels in *wt* mice at day 7. However, by day 10, mRNA levels for these chemokines increased to *wt* levels, likely in response to increased bacterial numbers in the TNF-deficient mice. Thus, the absence of TNF-mediated signaling through TNFR1 delayed production of chemokines by infected macrophages in the lungs and likely contributed to delayed or aberrant granuloma formation. These studies demonstrated that TNF affects chemokine expression by macrophages in vivo following infection and suggested that this is a mechanism by which TNF controls early cell migration and granuloma formation in the lungs.

We performed similar experiments in the lungs of mice chronically infected with *M. tuberculosis.* As discussed above, treatment of chronically infected mice with anti-TNF antibody leads to loss of granuloma structure, increased bacterial numbers, and destructive immunopathology [7]. Again, we were interested in early events (i.e., those that occurred prior to the onset of these phenotypic changes) to determine the key role that TNF plays in maintaining granuloma structure and function. Analysis of CD11b$^+$ cells isolated from the lungs of control and anti-TNF antibody–treated mice again revealed a transient decrease (up to

12 days of antibody treatment) in the expression of CXCL9, CXCL10, and CCL5 and shorter transient decreases in CCL3 and CCL4 in TNF-neutralized mice. These results suggested that, during chronic infection, TNF is affecting chemokine expression in the macrophages within the lungs. This may be a mechanism by which TNF participates in the maintenance of granuloma formation, because interference with chemokine gradients could cause cells to drift from the site of infection.

LOCALIZED REGULATION OF CHEMOKINE PRODUCTION BY TNF IN THE LUNGS OF INFECTED MICE

Localized production of chemokines is an important feature in the control of cell migration. We were interested in determining the expression of chemokines within newly forming granulomas, as well as the effects of TNF on this process. To examine local expression, we performed immunohistochemical analysis of lung samples obtained from infected mice. Although we could detect expression of certain chemokines in granulomas, particularly in chronically infected mice, when we used fixed tissue, the method was not sensitive enough to observe early expression of chemokines in the lungs. We next turned to in situ hybridization using 3 chemokine genes that we previously had demonstrated to be regulated by TNF as probes: CXCL9, CXCL10, and CCL5 [11]. We quantified expression of these chemokines by using an algorithm that relied on the numbers of cells in clusters of a certain size, as well as the numbers of cells in the cluster positive for the chemokine signal. Single cells and small (<100 cells) and large (>100 cells) clusters were analyzed, under the assumption that this represented the stages of granuloma formation within the first 12 days of infection. Unfortunately, the relatively high expression of CCL5 in cells other than macrophages in the lungs, even in uninfected lungs, precluded meaningful analysis of this chemokine. Our analysis revealed that CXCL9 and CXCL10 were regulated differently in terms of both the timing of expression and the size of the cluster of cells that expressed these chemokines. In addition, the effect of TNF could be observed on expression of these chemokines, but this effect was also dependent on the time and the stage of granuloma formation. CXCL10 expression in the newly forming granulomas (smaller infiltrates) was dependent on TNF, but this dependence was not observed in the larger infiltrates. Conversely, although there was a modest effect of TNF on CXCL9 expression in the early granulomas, this effect was most prominent in the larger infiltrates. These studies revealed a localized control of chemokine expression during granuloma formation by TNF and demonstrated that this regulation is affected by a number of factors. Although these 2 chemokines both bind to the same receptor, the differential expression may be involved in the complex organization of cells in a newly forming granuloma.

SUMMARY AND DISCUSSION

Chemokines function by setting up a gradient that cells with the correct receptors sense, which results in migration of cells toward the source of chemokine [10]. However, at a certain concentration in the gradient, cells no longer sense the gradient and either stop moving or orient toward a different signal. This feature is likely responsible for the multifactorial nature of the chemokine response. Cells do not simply sense one chemokine and migrate to that source. There are multiple simultaneous signals of different strengths, depending on the position of the cells, the sources of chemokines, and the receptors expressed by the migrating cell. The integration of this complex information by the cell is responsible for cell migration and, ultimately, is likely to affect granuloma formation. Granulomas are a complex environment, composed of different cell types, and a number of signals are likely to be produced, depending on a variety of factors. In terms of granuloma maintenance, production of chemokines at a certain level may be a factor in preventing cell movement out of the granuloma. If cells are not tightly controlled within the lungs, this could lead to excess inflammation. To reiterate, one of the major roles of the granuloma is to localize and contain not only the bacteria but, also, the inflammatory response to the bacteria. Thus, rigorous control of the organization of granulomas is likely necessary to prevent immunopathology. Our data support the notion that TNF plays a role in regulating chemokine expression in newly forming and established granulomas. TNF is also required to limit immunopathology in the lungs of chronically infected mice. Neutralization of this cytokine may wreak havoc on the sensitive control system necessary for preventing reactivation of infection and inflammation in the lungs.

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