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Mammalian Host Responses to Proinflammatory Stimuli by Microbial Pathogens

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ABSTRACT OF THE DISSERTATION

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by

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The complex interplay between infectious agents and host defenses in both the innate and adaptive compartments of the immune system determines the outcome of host-pathogen interactions. Bacteria use virulence strategies to invade biological barriers, but active processes of host epithelial cells may also contribute to the endocytosis of microbial particles. To focus on the latter, the uptake of fixed bacterial particles by the inflamed intestinal epithelium was modeled *in vitro* by Caco-2BBe cells conditioned with TNF-α and an agonist antibody to the lymphotoxin-β receptor. *Staphylococcus* and *Yersinia* were readily endocytosed, compared with scant uptake of the enteric and pulmonary pathogens *Shigella , Salmonella,* and *Klebsiella*. Endocytosed *S. aureus*, but not *Yersinia*, was often associated with cytoplasmic claudin-4 vesicles, suggesting that cytokine treatment upregulated at least two distinct endocytic pathways. Treatment induced epithelial redistribution of β1 integrin; consistent with this effect, β1 integrin blockade reduced uptake of bacterial particles in epithelial layers. Together, these data indicate that cells of the inflamed mucosa selectively sample bacteria from the lumen,

perhaps as a mechanism for enhancing the antigen-specific repertoire of the mucosal lymphocyte population.

Other biological barriers to infection form a bulwark between immune cells in circulation and tissues at high risk for damage secondary to inflammation; these include the brain and eye. Infection of these tissues with the intracellular protozoan parasite *Toxoplasma gondii* induces a proinflammatory adaptive response. This is accompanied in the brain by the emergence of localized extracellular fiberlike networks along which parasite-specific T cells migrate. To identify molecules associated with tissue remodeling in the infected brain, matrix metalloprotease transcripts were measured. Substantial post-infection upregulation of MMP-8 and MMP-10 together with their endogenous inhibitor TIMP-1 was observed. Intracellular staining of brain mononuclear cells showed MMP-8 and -10 to be expressed by CD4+ and CD8+ cells; these populations, along with CNS-resident microglia and astrocytes, upregulated TIMP-1 during chronic infection. *T. gondii* burden was significantly reduced in the brains of TIMP-1 null animals. Together, these findings identify a role for specific MMPs and TIMP-1 in matrix remodeling and pathogen clearance associated with migration of brain-infiltrating leukocytes during *Toxoplasma* infection.

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INTRODUCTION

In the first century B.C., the Roman encyclopedist Celsus characterized inflammation by its hallmark signs, namely *calor, rubor, tumor* and *dolor*, or heat, redness, swelling and pain respectively. Inflammation is now understood to be an acute or chronic response to cellular injury that is marked by capillary dilation, leukocytic infiltration, and alterations to endothelial and epithelial integrity. It can result from various injurious stimuli including trauma, hypersensitivity, and infection. These processes serve to initiate cellular and chemotactic activity which are protective when they lead to wound healing and/or clearance of pathogenic agents from the affected local tissue environment.

Infection with microbial pathogens such as bacteria and intracellular parasites can initiate a spectrum of reactions in the host organism ranging from the rapid, generalized innate response to the specialization that characterizes the adaptive immune system of higher vertebrates. Both somatic cells and those of the innate compartment dedicated to antigen recognition are capable of recognizing molecular patterns associated with pathogens; binding of these pattern recognition receptors initiates a signaling cascade leading to production of soluble factors including cytokines and proteases [1]. These cytochemicals function variously to recruit effector cells to sites of insult, to mediate migration of leukocytes as they extravasate into inflamed tissue, and in some cases, to alter the functional activities of somatic cells in the local region of inflammation.

Infected cells respond to microbial signals with local production of general inflammatory cytokines including IL-1 and TNF-α, which in turn upregulate expression of cellular adhesion molecules on the luminal surfaces of nearby blood vessels [1]. The cascade of processes that then permits effector leukocytes to extravasate from vessels begins with rolling adhesion, which then slows to tight adhesion of leukocytes on high endothelial venule or capillary endothelial surfaces near sites of infection. This process of recruiting leukocytes from circulating blood is mediated by selectins and integrins expressed on the endothelial surface [2]. Once leukocytes have arrested, they can extend processes through pericellular spaces to migrate across the endothelium. Although early steps in diapedesis do not require protease activity, proteolysis is required for leukocyte transmigration of the vascular basement membrane. The identity of proteases involved in this process is not fully characterized, but is known for some populations to include the so-called 'matrix metalloproteases'. Furthermore, the effect of endogenous tissue inhibitors of metalloproteases on immune cell migration and the subsequent host capacity to clear pathogen are questions of importance to the understanding and treatment of chronic infection and autoimmune disease.

The work described here explores the function and effect of the inflammatory milieu at physically and functionally disparate physiological sites. In part one, the ability of proinflammatory cytokines to alter the endocytic capacity of cells of the intestinal and upper respiratory epithelium was investigated using *in vitro* models for the inflamed respiratory and intestinal epithelia. Part two elucidates the role of select host proteases

and an endogenous inhibitor in the controlled immune response to chronic brain infection with the apicomplexan parasite *Toxoplasma gondii*.

Chapter 1: Bacterial Particle Endocytosis by Epithelial Cells is Selective and Enhanced by Tumor Necrosis Factor Receptor Ligands

Abstract

Bacterial pathogens employ virulence strategies to invade epithelial barriers, but active processes of epithelial cells may also contribute to endocytosis. To focus on the latter, we studied uptake of fixed and fluorescently labeled bacterial particles in intestinal and bronchoepithelial cell cultures, and found it to be enhanced in NCI-H292 and Caco-2BBe cells after treatment with TNF-α and an agonist antibody against the lymphotoxin beta receptor. Confocal fluorescence microscopy, flow cytometry, and transmission electron microscopy revealed that *Staphylococcus aureus* and *Yersinia enterocolitica* were readily endocytosed, though there was scant uptake of *Shigella sonnei*, *Salmonella typhimurium* and *Klebsiella pneumoniae* particles. Endocytosed *Staphylococcus* was often associated with cytoplasmic claudin-4 vesicles; this was not found for *Yersinia*, suggesting that cytokine treatment upregulated two distinct endocytosis pathways. Interestingly, when both *Staphylococcus* and *Yersinia* were added to epithelial monolayers, only cells taking in the largest numbers of *Staphylococcus* particles also endocytosed *Yersinia*, though apparently in distinct compartments. Cytokine treatment induced an upregulation and redistribution of β1 integrin to the apical surface of NCI H292 cells; consistent with this effect, treatment with anti-β1 integrin antibody blocked uptake of both *Yersinia* and *Staphylococcus* in NCI H292 and Caco-2BBe cells. Our results suggest that capture of bacterial particles by mucosal epithelial cells is selective, and that different endocytic mechanisms are enhanced by pro-inflammatory cytokines.

Introduction

Chronic inflammation is associated with production of cytokines such as tumor necrosis factor alpha (TNFα), and lymphotoxin beta receptor ligands such as lymphotoxin (LT)α1β2 and LIGHT(16). In a seemingly disparate but equally critical role, TNFR ligands are also necessary for organogenesis and maturation of secondary lymphoid structures such as intestinal Peyer's patches and lymph nodes (5, 13, 14). The multiple effects of these cytokines on cells with essential roles in immune defense suggests that there are more complex aspects of the response to lymphotoxin/TNF ligands, including differences in the kinetics of cytokine production and the local tissue context of cytokine response. This may be especially true in the mucosal epithelium, where local cytokines may drastically affect barrier integrity (3).

Along with IFN-γ, TNF-α has been implicated in the compromise of epithelial barrier function which may result in entry and colonization by pathogenic bacteria ordinarily excluded from the subepithelial mucosa (7, 25). Among the mechanisms by which TNF-α may affect barrier function are loss of cells of the monolayer to apoptosis (8), internalization of tight junction proteins with consequent loss of tight junction integrity (1), and rearrangement of the peri-junctional cytoskeleton (26). While these studies point to important alterations of paracellular junctions by TNF-α, they do not take into consideration how epithelial cells themselves may actively

facilitate transmembrane entry of pathogenic bacteria under proinflammatory conditions.

Here, we compared the effects of incubating epithelial monolayers from both the bronchus and the intestine with a selection of fixed and fluorescently labeled bacterial pathogens, and examined how cells manage these particles after culture with LT/TNFR ligands. Intriguingly, we found that cytokine treatment induced a striking increase in endocytosis of select bacteria across epithelial cell surfaces. This effect was demonstrated in both an airway cell line (NCI-H292) and an intestinal epithelial cell line (C2BBe, a subclone of Caco2). Because pathogens including *Staphylococcus* and *Yersinia* have been shown to effect invasion by exploiting epithelial surface receptors via binding to the integrin β1 subunit (9, 21), we examined how TNFα and LT might alter β1 integrin expression in polarized epithelia. In the case of NCI-H292 cells, cytokine treatment correlated with an upregulation and redistribution of β1 integrin to the apical surface; accordingly, blockade of β1 integrin inhibited bacterial particle uptake. While the two cell lines showed some differences in their behavior, enhanced bacterial particle uptake under proinflammatory conditions suggests conserved mechanisms whereby epithelial cells may actively engage infectious organisms at the mucosal barrier.

Materials and methods

Cell culture. Cell lines NCI H292 (ATCC #CRL-1848) and a subclone of Caco-2, C2BBe (ATCC #CRL-2102), were obtained from the American Type Culture Collection and cultured using recommended media preparations. Freshly passaged cells were seeded onto 0.4 micron pore polycarbonate filter supports (Transwell filters; Corning Life Sciences) in standard media or with the added cytokines LTβR agonist antibody (5 μg/ml; R&D Systems) plus recombinant TNFα (100 ng/ml; Peprotech). Treatment of cells with cytokines was generally done over a period of 7 d. In endocytosis studies, cells in transwell cultures were cultured with or without medium including LTβR agonist and TNFα for 6 d (until stable transepithelial electrical resistance values were reached), and AlexaFluor 488-labeled *S. aureus* (Invitrogen) or AlexaFluor labeled bacterial strains cultured in our lab were then added during the final 12 h (C2BBe) or 2 h (NCI-H292) of culture. To block β1-integrin receptors on epithelial cells, 10 μg/mL mouse anti-β1 (Chemicon/Millipore) was added to culture medium during the last hour of incubation before bacterial particles were introduced.

Bacterial culture. *Y. enterocolitica* (ATCC 29913) and *K. pneumoniae* (ATCC 13883) were obtained from ATCC; *S. sonnei* and *S. typhimurium* were reconstituted from UC Riverside medical microbiology laboratory teaching stocks, and confirmation of genus and species was achieved by the appropriate API identification kit. All cultures were stored at -80°C and subcultured to trypticase soy broth (BD), shaken at 200 rpm at 37°C. Overnight broth cultures were diluted 1:20, grown to mid-log phase and harvested for

labeling. Bacteria were pelleted and washed 3x, fixed in PBS with 1% paraformaldehyde and 0.05% sodium azide for 30 minutes, washed 2x and labeled at RT with stirring overnight (21h±1h) with AlexaFluor-488 TFP ester, -568 succinimidyl ester, or -647 succinimidyl ester (Invitrogen) in 0.1M $NaHCO_3$. Labeled particles were washed 3x to remove free fluorophore and resuspended for storage at 4°C in PBS with 0.05% NaN_3.

Immunofluorescence staining. Cell cultures were fixed using PBS 1% paraformaldehyde/PBS, then permeabilized in 0.5% Triton-X 100/PBS, washed in 0.1% Triton-X 100/PBS and blocked in 0.1% Triton-X100/PBS containing 2% BSA. Cells were stained with antibodies to tight junction proteins claudin-4 (Abcam) and/or ZO-1 (Zymed), and/or to β1-integrin (Chemicon/Millipore) followed by secondary reagents Alexa Fluor 488-, Alexa Fluor 568- or Alexa Fluor 647-conjugated anti-rabbit or anti-mouse antibodies (Invitrogen). DAPI (Invitrogen) was used as nuclear counterstain. Slides were mounted using ProLong Gold antifade reagent (Invitrogen).

Confocal microscopy. Prepared slides were examined with a 63x water immersion objective using a Zeiss Observer Z1 inverted epifluorescence microscope equipped with a BD CARV II spinning disk confocal imager, and images collected with IPLab for Windows 4.0 software (BD Biosciences, Rockville, MD). Fluorescent images are either maximum image projections of z-stacks taken in multiple consecutive optical

planes, or 3D snapshots following image sequence deconvolution with Volocity imaging software (Improvision, Waltham, MA).

Transmission electron microscopy (TEM). Cell cultures were washed twice in PBS and fixed in PBS 2% paraformaldehyde/2% glutaraldehyde. Ultra-thin (80 nm) sections of cell-covered filters were prepared for TEM analysis by standard methods, as previously described (12). Observations were made using a Phillips Tecnai 12 microscope.

Flow Cytometry. Cells were plated on 24 well plates with or without inductive cytokines, and cultured for 48 hours. Then, during the last 12 h of incubation (C2BBe) or last two h of incubation (NCI-H292), fluorescent bacterial particles were added at a ratio of 200 bacteria per cell. Cells were detatched from plates using Versene (Gibco), washed x3, and data collected using a FACSCalibur (Beckton Dickinson) equipped with CellQuest Pro software (BD Bioscience). Data analysis was performed with FlowJo software (Tree Star).

Results

Preferential endocytosis of Staphylococcus aureus *and* Yersinia enterocolitica *by epithelial cells is enhanced in the presence of proinflammatory cytokines*

Pathogenic bacteria employ diverse virulence strategies such as endotoxin production, intracellular signaling and molecular mimicry to breach epithelial defenses (6). To examine how epithelial cells might actively mediate endocytosis of bacterial pathogens, we incubated intestinal epithelial cell monolayers with selected respiratory and enteric pathogens that had been fixed, killed and fluorescently labeled. We assessed particle uptake by use of confocal microscopy for qualitative studies; for more quantitative analysis, we also employed flow cytometry of monolayers that had been detached after ingesting fluorescent particles. This method enabled us to sample thousands of cells per experiment, and to generate quantitative estimates not only of the proportion of cells taking up particles, but of the number of particles per cell.

In a preliminary experiment, we found that NCI-H292T cells did not survive overnight exposure to bacterial particles; time course trials revealed that two hours of incubation with particles was sufficient to yield measurable ingestion. In the more orderly, tightly packed monolayers of columnar C2BBe intestinal cells, it was necessary to expose cell layers to particles for a minimum of twelve hours or overnight in order to observe measurable endocytosis.

Fluorescence microscopy revealed that in C2BBe cells the quantity and pattern of uptake varied per bacterial species. *Salmonella typhimurium*, *Shigella sonnei*, and *Klebsiella pneumoniae* were taken up sparsely, while *Staphylococcus aureus* and *Yersinia enterocolitica* appeared to be readily endocytosed. *Yersinia* was diffusely ingested, with many cells having taken up a few bacteria. The pattern of uptake for *Staphylococcus* was notably different in that, while fewer cells appeared to have taken up bacteria, many had ingested hundreds of particles (Fig 1.1). These observations were quantitatively confirmed by flow cytometry analysis, which showed by fluorescence shift that 53.4 % of cells had taken up some *Staphylococcus*, and that 27.8% had ingested *Yersinia*. *Shigella* were modestly taken up, while *Klebsiella* and *Salmonella* were largely excluded from endocytosis.

In 2004, Katakai *et al* reported that a combination of the proinflammatory cytokines TNFα and lymphotoxin β induced lymphoid stromal cell differentiation (10). As the epithelia overlying lymphoid tissue of the mucosa are enriched in cells specialized for antigen sampling (11, 17, 18), we wondered whether exposing epithelial cells to this particular cytokine combination could induce bacterial particle uptake in cell monolayers. We examined the effects of growing intestinal and airway epithelial cells in the presence of TNFα and LTβR on their propensity to ingest bacterial pathogens, and noted a modest but consistent increase in uptake by intestinal cells of all pathogens in the presence of cytokines (Fig 1.1), both by qualitative fluorescence microscopy and by flow cytometric quantitation. Notable endocytosis

of both *Staphylococcus* and *Yersinia* particles was observed in NCI-H292 (Fig 1.1B). While the distribution of *Yersinia* uptake appeared regular, with many cells ingesting 1-10 bacterial particles (TEM and immunofluorescence data not shown), many NCI-H292 cells had ingested large numbers (>100 particles, Fig 1.3B) of *S.aureus.*

To confirm that the observed association between fluorescently labeled bacteria and epithelial cells was not due solely to adherence, experiments were repeated by incubating cells at 4°C for one hour prior to the addition of bacterial particle suspension, and maintaining that temperature throughout the particle incubation. Uptake was nearly abolished (Fig 1.2), confirming that the fluorescence shift observed by flow cytometry was not due to mere adherence of fluorescent particles to cell surface moieties. To further confirm endocytosis, cell monolayers grown on permeable supports which had been incubated with fluorescent bacteria were fixed and immunostained with antibodies against the tight junction proteins claudin-4 and zonula occludens-1 (ZO-1). Confocal fluorescence imaging in the z-dimension showed both *Staphylococcus* and *Yersinia* located basal to apical cell surface markers (data not shown).

Bronchial epithelial cells ingest Staphylococcus aureus *in a dose-dependent fashion*

Our preliminary confocal fluorescence studies suggested that NCI-H292 cells exhibited an extraordinary capacity for endocytosis of *Staphylococcus aureus*. To explore the capacity of this uptake, we incubated NCI-H292 cells with *S. aureus* in tenfold dose increases (Fig 1.3A), and analyzed detached monolayers by flow cytometry. As the bacteria:cell ratio increased from 2:1 to 20:1, particle ingestion doubled in both treated and untreated cells. Another tenfold increase in bacterial dose resulted in a near-doubling of the number of untreated cells ingesting bacteria; in the cells which had been pretreated with TNF/LT, we observed a substantial and discrete population of cells which took up ≥ 100 particles. This was deduced as follows: we determined the mean fluorescence of the least 'positive' population of cells, presumed to have ingested at least one particle. We then measured mean fluorescence of the putative high-endocytosis population, and found it to have increased by more than two log cycles. This suggests a hundredfold or greater increase in the number of particles ingested. Transmission electron microscopy analysis of sections of cell monolayers incubated with *S. aureus* confirmed that many cells were capable of very high capacity endocytosis (Fig 1.3B). Our TEM sections revealed multiple cells which had ingested >100 bacterial particles without lysing; these high-uptake cells were also observed by confocal microscopy. It was this high-endocytosis population that increased most dramatically with cytokine treatment at a particle:cell exposure of 200:1.

S. aureus *and* Y. enterocolitica *are endocytosed via different pathways*

Immunofluorescence studies suggested that *Staphylococcus* and *Yersinia* were not endocytosed in the same compartments (Fig 1.4A). *Staphylococcus* was consistently taken up in cytoplasmic vesicles colocalized with claudin-4 (filled arrowheads), a protein normally confined to epithelial tight junctions which is also a receptor for *Clostridium perfringens* enterotoxin (15) . *Yersinia* particles rarely colocalized with claudin-4 (open arrowheads), nor with canonical bacterial PRRs TLR2 and TLR4 (data not shown). To measure co-ingestion of *Staphylococcus* and *Yersinia* particles in a more meaningful sample of cells, equivalent numbers (100:cell, each) of Alexa488-labeled *Staphylococcus* and Alexa647-labeled *Yersinia* particles were coincubated with either untreated or TNF/LT treated NCI-H292, and fluorescent cells detected by flow cytometry (Fig 1.4B). There were very few NCI-H292 cells which took up only *Yersinia*, and these cells ingesting *Yersinia* generally had also taken up many *Staphylococcus* particles. Pretreating the airway cells with TNF and LT cytokines increased the numbers of cells that ingested *Y. enterocolitica* along with large numbers of *S. aureus.* Thus, while uptake of the two species of bacteria appeared to be independent, the cells most capable of bacterial particle endocytosis readily took up both bacterial species.

Blockade of β1 integrin reduces endocytosis of* Yersinia enterocolitica *in both intestinal and bronchial epithelium, but* Staphylococcus aureus *uptake is blocked only in airway cells

Yersinia spp express invasin, a known opportunistic ligand for the epithelial α5β1 integrin receptor (2). Other pathogenic bacteria including *Staphylococcus* and *Streptococcus* have been shown to effect epithelial invasion by bridging to the endogenous β1 ligand fibronectin via fibronectin binding protein (FBP) (23). To date, invasin-integrin and FBP-integrin interactions have been well-studied in *in vitro* or *in vivo* experiments using live pathogens. To determine whether invasion and colonization exploiting β1 receptors can be strictly cell-mediated, we blocked receptors on cultured airway and intestinal epithelial monolayers with anti-β1 antibody prior to incubation with *Yersinia* and *Staphylococcus* particles, and examined by flow cytometry the effect on the ability of these cells to take up these particles. The effect of β1 blockade on ingestion of *S. aureus* in intestinal cells was unremarkable (Fig 1.5A); however, the proportion of C2BBe cells ingesting *Y. enterocolitica* was reduced in both untreated and cytokine-treated cells, by 13.2% and 16.7% respectively. Interestingly, ingestion of Y. *enterocolitica* by airway epithelial cells was also notably reduced (Fig 1.5B), though *Yersinia* is not typically described as a respiratory pathogen. The most dramatic β1 inhibition was of *S. aureus* endocytosis by NCI-H292 cells. In particular, the high uptake cell population (previously observed by increasing particle to cell ratios) was most abrogated by β1

inhibition; in untreated cells, endocytosis in the high uptake (>100 bacterial particles/cell) population was reduced 4.3-fold; a 2.4-fold reduction was observed in the high-uptake population treated with proinflammatory cytokines.

Redistribution of β1 *integrin to apical surfaces of cells treated with proinflammatory cytokines*

As integrins link extracellular matrix proteins such as laminin and fibronectin to the cytoskeleton, they are normally distributed to the basolateral surfaces of polarized epithelial cells (4). It has been previously reported that β1 integrin is distributed apically in M cells of the follicle-associated epithelium specialized for antigen sampling, though not in neighboring enterocytes (2). These observations were based on comparisons to cells staining positive for the lectin UEA-1 in mouse Peyer's patches. To test whether TNF family cytokines are involved in the redistribution of β1 integrin to the apical membrane of airway epithelial cells under proinflammatory conditions, we compared β1 distribution in untreated cells to cells treated with TNFα and LTβR agonist. Though some apical localization was observed in addition to the expected distribution at basolateral surfaces in untreated NCI-H292 cells (Fig 1.6A), there was a remarkable relocalization of β1 integrin towards the apical pole in those cells which were treated with TNF and LT cytokines (figure 1.6B). In addition to a notable redistribution of β1 in individual cells, samples treated with TNF and LT showed striking differences between cells expressing

abundant apical β1 (major population) alongside cells expressing little or no β1 at their surface.

Discussion

Studies on epithelial cells which rely on confocal fluorescence or electron microscopy imaging have only limited power to characterize the behavior of the majority of cells in a tissue sample or monolayer. Microscopy is of greatest value for close examination of specific subcellular processes, but for quantitative studies it can be limited by small sample size, even when multiple fields are sampled. In our hands, preparing adherent cultures for flow cytometry yields >90% viable cells for sampling, and cells which have ingested fluorescently labeled bacteria are detectable both by fluorescence shift and increased side scatter. Transmission EM confirmed that bacteria were ingested, and repeating incubations at 4°C abolished all but 8% of *S. aureus* and 4.08% of *Y. enterocolitica* uptake in NCI H292 cells, consistent with an endocytic process. We can therefore validate this tool as a quantitative assay for uptake of labeled bacteria by epithelial cells.

Flow cytometry also enabled us to discover that a subset of NCI-H292 cells was capable of endocytosis of very large numbers (100 or more) of *S. aureus*, and transmission EM provided corroborating evidence for such high capacity uptake. We discovered that this high-*Staphylococcus* endocytic population was enhanced by cytokine treatment, and that it was the most sensitive of all subpopulations to

inhibition of endocytosis by blocking β1 integrin. Similarly, flow cytometric analysis of the coincubation of cells with both *Staphylococcus* and *Yersinia* particles revealed that a very large subset of intestinal epithelia preferentially endocytosed *Yersinia.* By contrast in the airway epithelium, only those cells that had endocytosed many *Staphylococcus* also took up *Yersinia.*

Sinha et al (23) showed that *Staphylococcus* strains may employ 'zipper type' internalization to invade epithelial cells by binding to fibronectin, which in turn ligates surface α5β1 and other cellular adhesin receptors. Our results suggest the existence of additional epithelial mechanisms whereby microbes similar to *S. aureus* may invade epithelial monolayers, especially in the context of inflammation. It has been recently shown that certain 'carrier' strains of *S. aureus* , so named for their ability to delay host innate immune response and avoid clearance from the nasal epithelium, may utilize this strategy to persist in the nasal mucosa (19). This is intriguing in light of our identification of subpopulations of airway epithelial cells which internalize large numbers of *Staphylococcus* and *Yersinia,* and is suggestive of a potential "feed-forward" mechanism which may contribute to microbial invasion or penetration of epithelial barriers. Further investigation of how particles are managed *in vivo* will clarify whether this seemingly maladaptive internalization may in fact actually be beneficial in mucosal tissues. Starner (24) and Reibman et al (20) have shown that CCL20, which recruits dendritic cells via its ligand CCR6, is induced in human bronchial epithelial cells by TNF-α as well as by particulate matter. By

simultaneously increasing ingestion of pathogenic bacteria, epithelial cells may "focus" the invading pathogens to a specific site in the epithelium where innate immune responses (e.g., chemokine release) may more effectively trigger adaptive immune responses.

Endocytosis of *Y. enterocolitica* by intestinal epithelial cells has been shown to be enhanced in those cells upregulating β1 integrin at their apical surfaces (21). Whether invasion by this member of the enterobacteraciae is also enhanced in non-intestinal epithelia has not been demonstrated prior to this study. We found expression of β1 integrin to be enhanced by culturing bronchial epithelial cells with TNFR ligands, and that much of the protein was directed to the apical pole. This may be coordinated with increased expression of the endogenous α5β1 ligand, fibronectin, which occurs under proinflammatory conditions (22), though because of the distribution of the proteins, its significance is not clear. Apical β1 expression correlated with an increased tendency for airway epithelia to ingest both *S. aureus* and *Y. enterocolitica*; integrin binding may be directly responsible for capture of the bacteria, though the specific binding partner differs on each type of particle.

Interestingly, we observed *S. aureus*, but not *Y. enterocolitica*, colocalized in cytoplasmic vesicles with claudin-4, a protein that is ordinarily a key component of paracellular tight junctions. Previous studies on tight junction protein changes in response to inflammatory cytokines had not described any role in particle uptake;

however, given the presence of the tight junction at the apical pole of polarized epithelium, such a mechanism might be plausible, especially for uptake of such large particles. Though a specific mechanism remains to be identified, this suggests that *S. aureus* may be ingested by at least two distinct endocytosis mechanisms, which could help to explain its more abundant uptake in both C2BBe and NCI H292 cells.

In summary, these results suggest that epithelial cells of the intestine and airways are not passive victims during invasion of the mucosa by bacterial pathogens. Events such as redistribution of tight junction and cellular adhesion proteins may in fact be beneficial responses to enable a kind of immune surveillance function of epithelial cells.

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Figure 1.1. Selective uptake of bacterial particles by epithelial layers is enhanced under proinflammatory conditions. Caco2-Bbe cells seeded onto semipermeable filter supports to encourage polarization were cultured for 6 days to attain monolayers as determined by transepithelial resistance. Monolayers were treated (A, lower rows) or not (A, upper rows) with TNF-α and anti-LTβR . Green, bacterial particles; red, ZO-1; grey, DAPI. Flow cytometry plots are gated on cells positive for uptake of fluorescently labeled bacterial particles; gate values are percentages of cells positive for uptake. (B), intestinal and bronchoepithelial cell layers cultured in the presence of TNF-α and LTβR agonist ingest more *S. aureus* and *Y. enterocolitica* than do untreated monolayers.

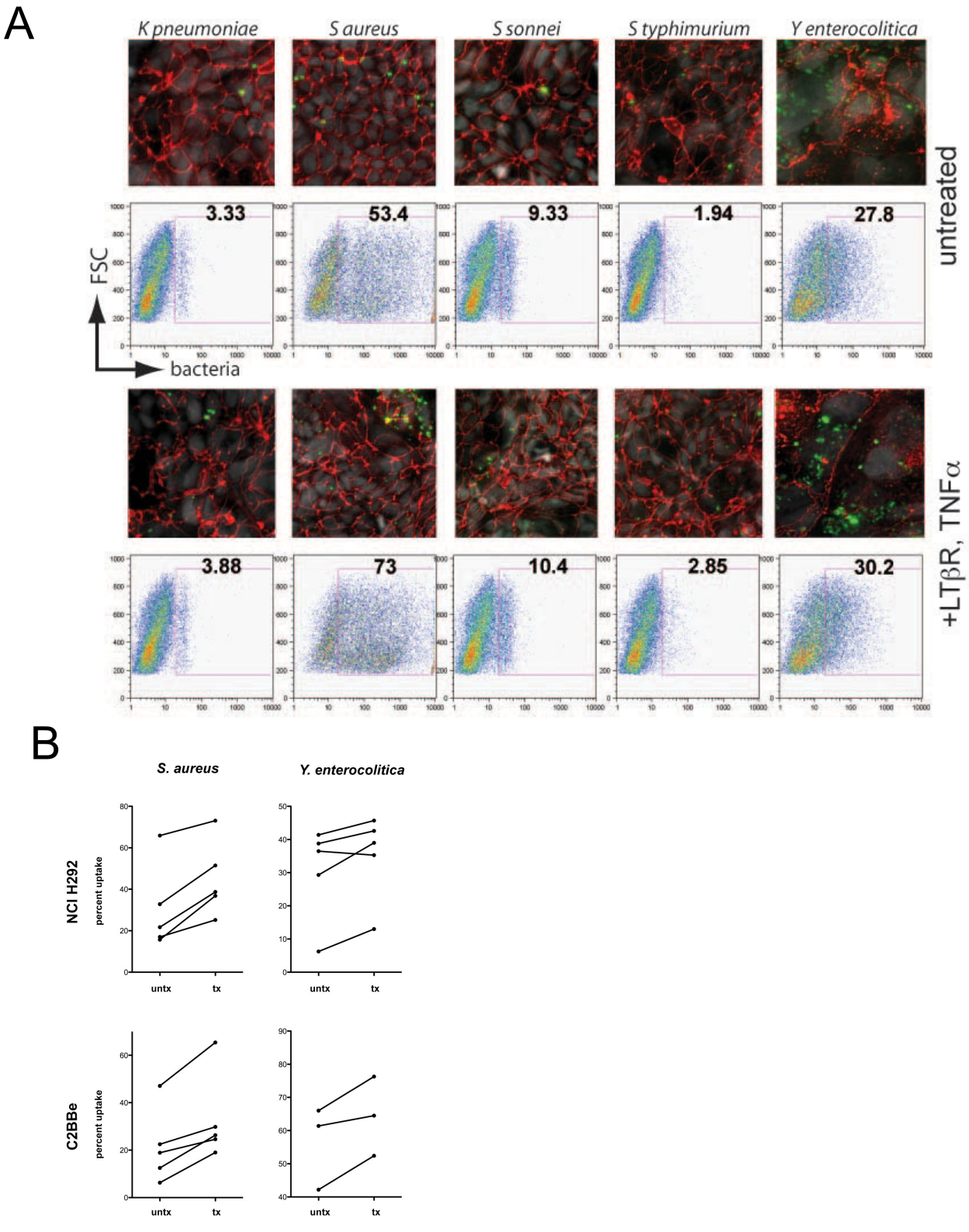

A
K pneumoniae
S aureus
S sonnei
S typhimurium
Y enterocolitica
untreated
FSC
bacteria
3.33
53.4
9.33
1.94
27.8
+LTβR, TNFα
3.88
73
10.4
2.85
30.2
B
S. aureus
Y. enterocolitica
NCI H292
percent uptake
C2BBe
percent uptake
untx
tx

Figure 1.2. Temperature reduction abrogates uptake of bacterial particles by epithelial layers. Cell layers incubated at 4°C for one hour prior to and throughout cytokine treatment period endocytosed few bacterial particles, excluding the possibility of non-specific adherence of particles to cell layers. Gates show percent cells positive for fluorescent particle uptake.

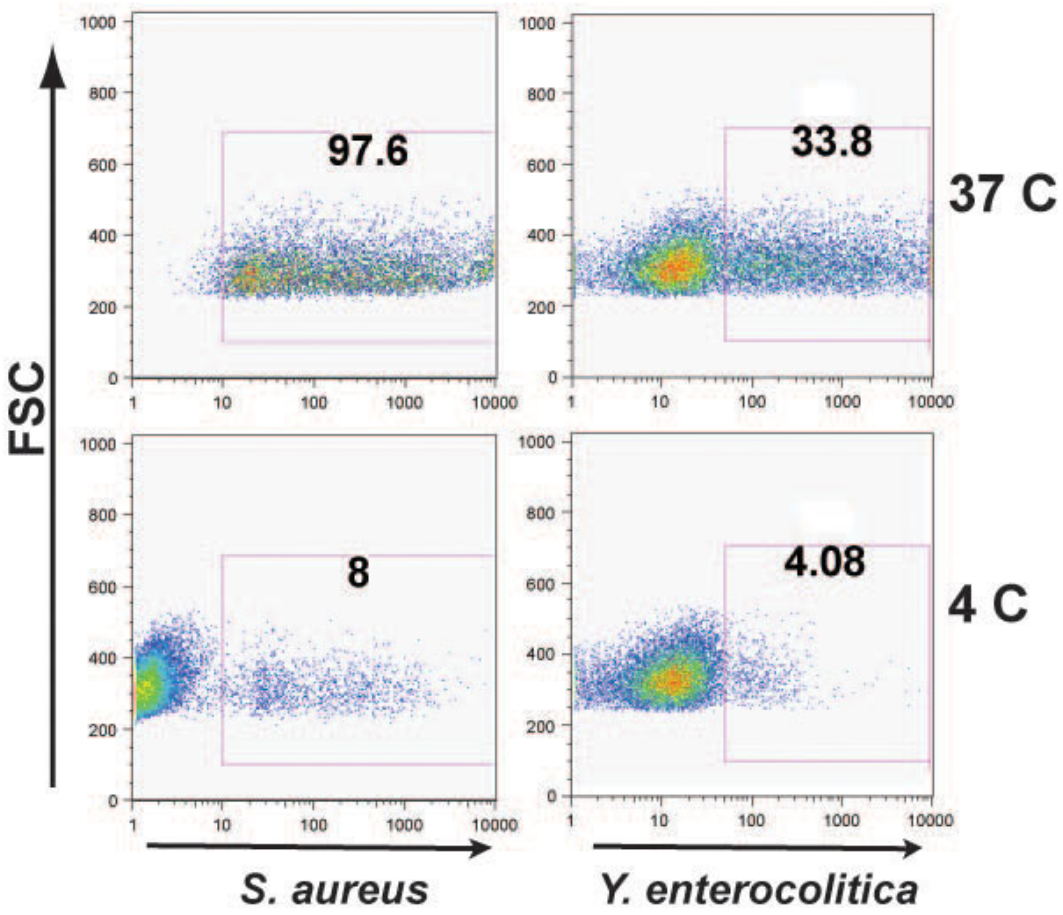
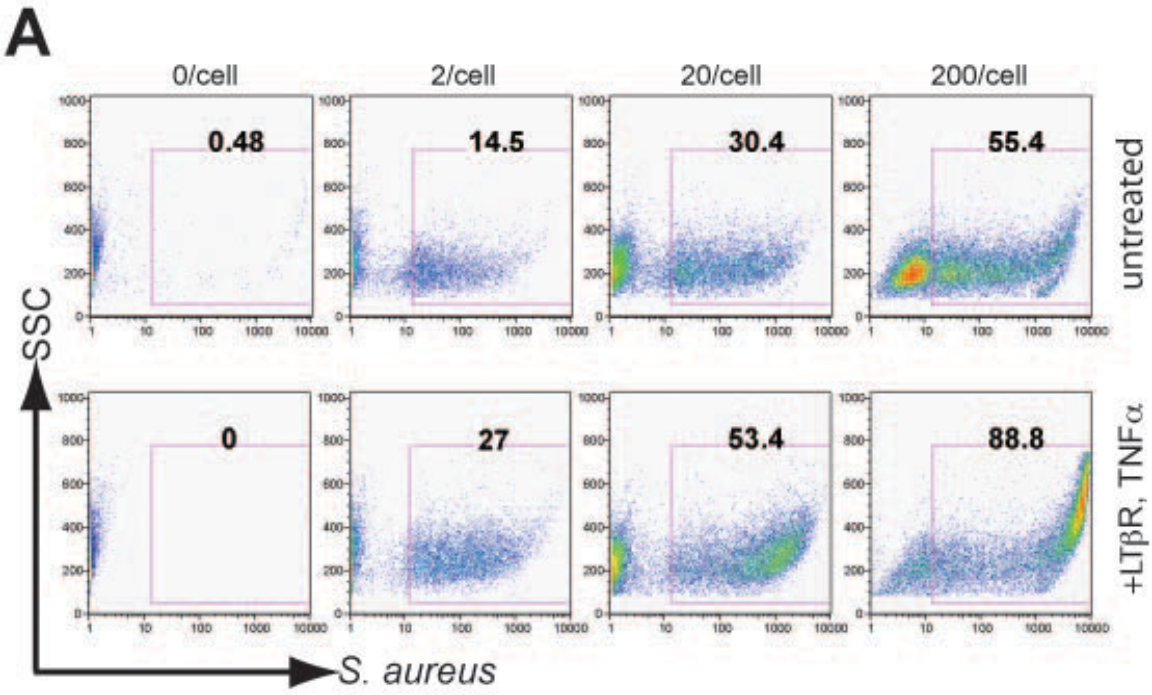

FSC
97.6
33.8
37 C
8
4.08
4 C
S. aureus
Y. enterocolitica

Figure 1.3 NCI H292 endocytosis of S. aureus at tenfold increases of particle/cell ratios in untreated and cytokine-treated cell layers. (A)at 200 particles/cell, a population of cells ingested very high (>100 particles/cell) (far right of fourth panel); this effect was enhanced in cytokine-treated epithelial layers. Load ratios of >200/cell induced lysis/cell death. Data are representative of three independent experiments. The cell depicted in panel (B) is representative of those observed by TEM in cytokine-treated cultures at a bacterial particle:cell ratio of 200:1. This cell has ingested >100 fixed *Staphylococcus*.


A
0/cell
2/cell
20/cell
200/cell
0.48
14.5
30.4
55.4
untreated
0
27
53.4
88.8
+LTβR, TNFα
SSC
S. aureus

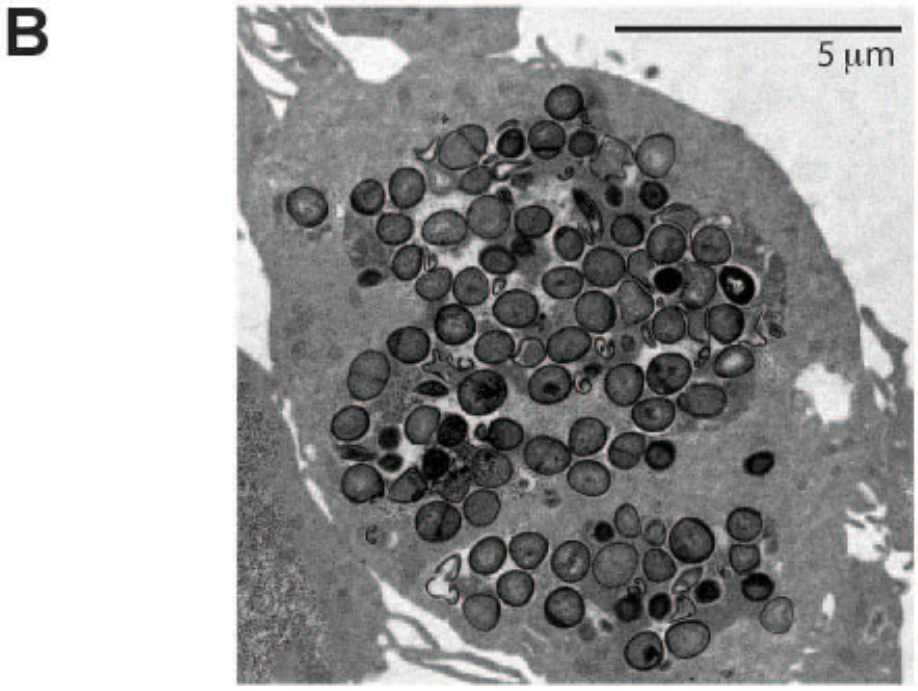

B
5 μm

Figure 1.4. (A), C2Bbe cell uptake of *S. aureus* (green) colocalized with Claudin-4 (red), but *Yersinia* (blue) rarely colocalized with Claudin-4. (B) Airway epithelial cells (NCI H292) coincubated with equivalent numbers of *S. aureus* and *Y. enterocolitica* particles. Cytokine treatment increased the numbers of cells that took up *Yersinia* alone, or that took up *Yersinia* + *Staphylococcus*, but not of those that endocytosed *Staphylococcus* only. There were few or no bronchoepithelial cells that took up *Yersinia* only; cytokine treatment enhanced the tendency of cells to ingest *Staphylococcus* and *Yersinia* together. The data shown are representative of seven (immunocyotchemistry) or three (flow cytometry) independent experiments.

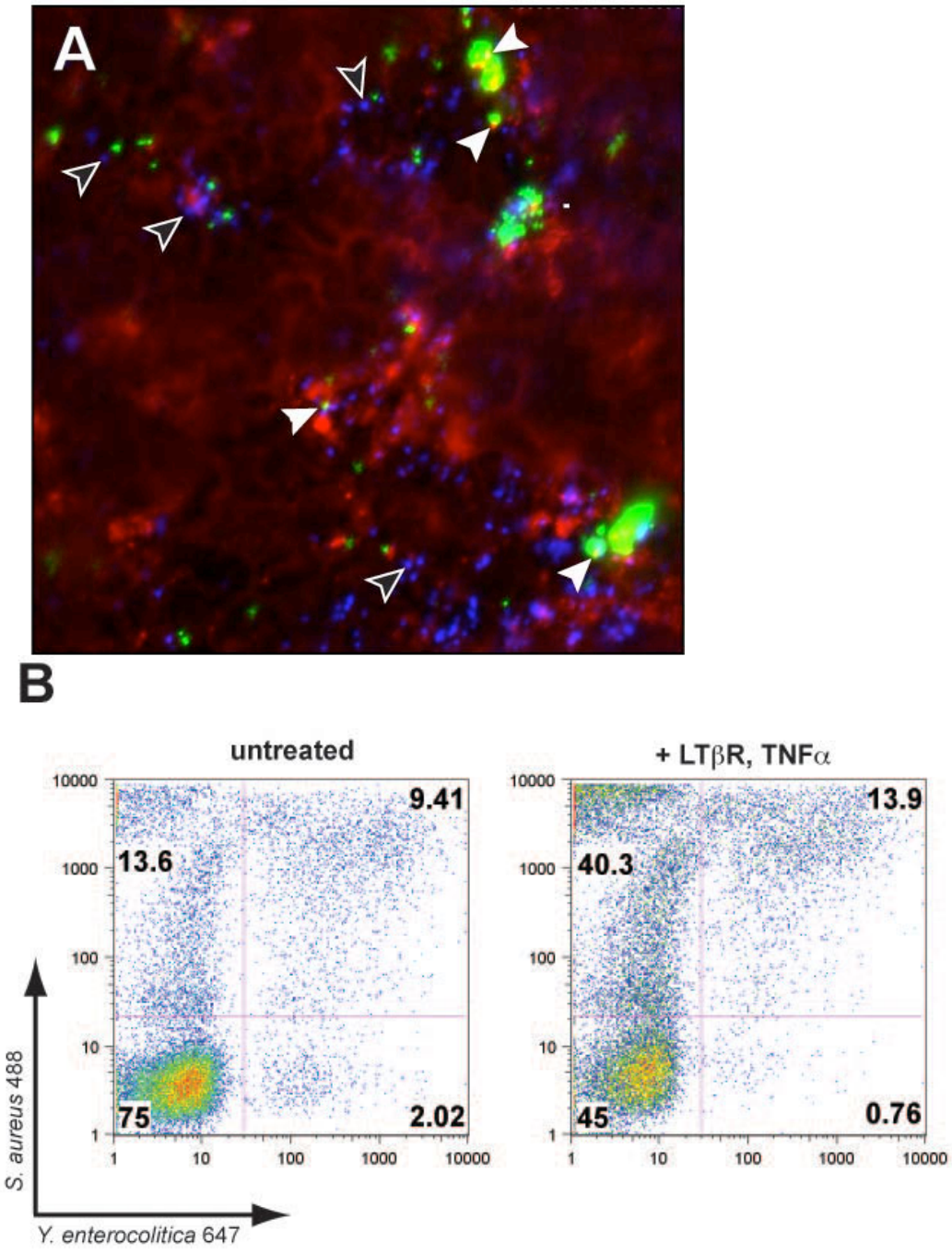

A
B
untreated
+ LTβR, TNFα
9.41
13.6
75
2.02
13.9
40.3
45
0.76
10000
1000
100
10
1
S. aureus 488
Y. enterocolitica 647

Figure 1.5. Effect of blocking β1 integrin receptors on bacterial particle uptake in intestinal (A) and airway (B) epithelial cells. β1 blockade reduced uptake of *S. aureus* in untreated and cytokine-treated H292 cells by an average of 5.0 and 18.9% respectively, with the greatest reduction in that population of cells which by fluorescence intensity and side scatter were determined to have endocytosed >100 particles. Uptake of *Yersinia* was similarly reduced, although the effect was more pronounced (12.5%) in airway cells treated with TNF-α and anti-LTβR. *S. aureus* uptake was not notably affected by blocking β1 in C2Bbe cells; uptake was reduced by 4.5 and 2.8% in untreated and treated cells, respectively. All data are means from at least three independent experiments.

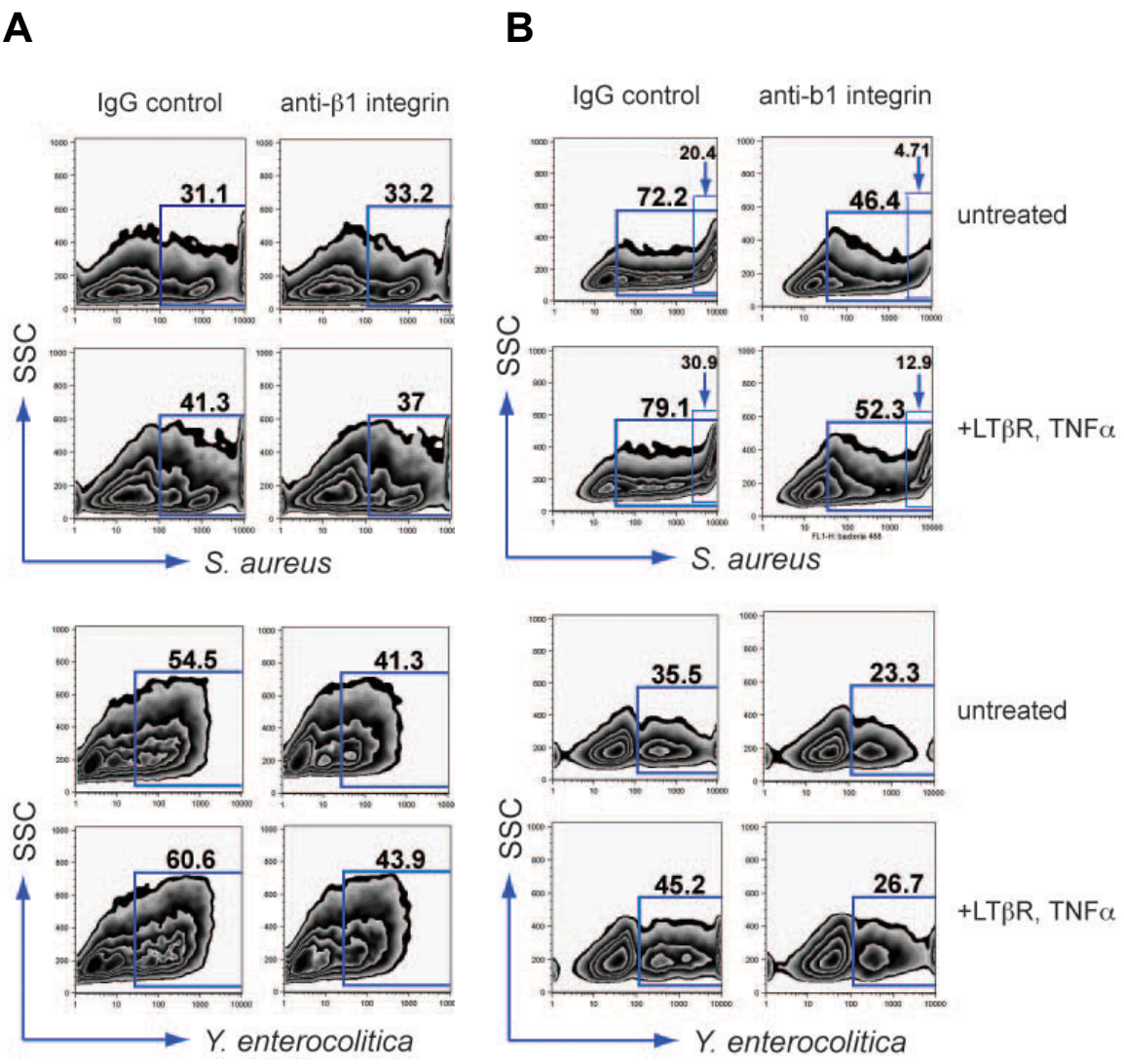
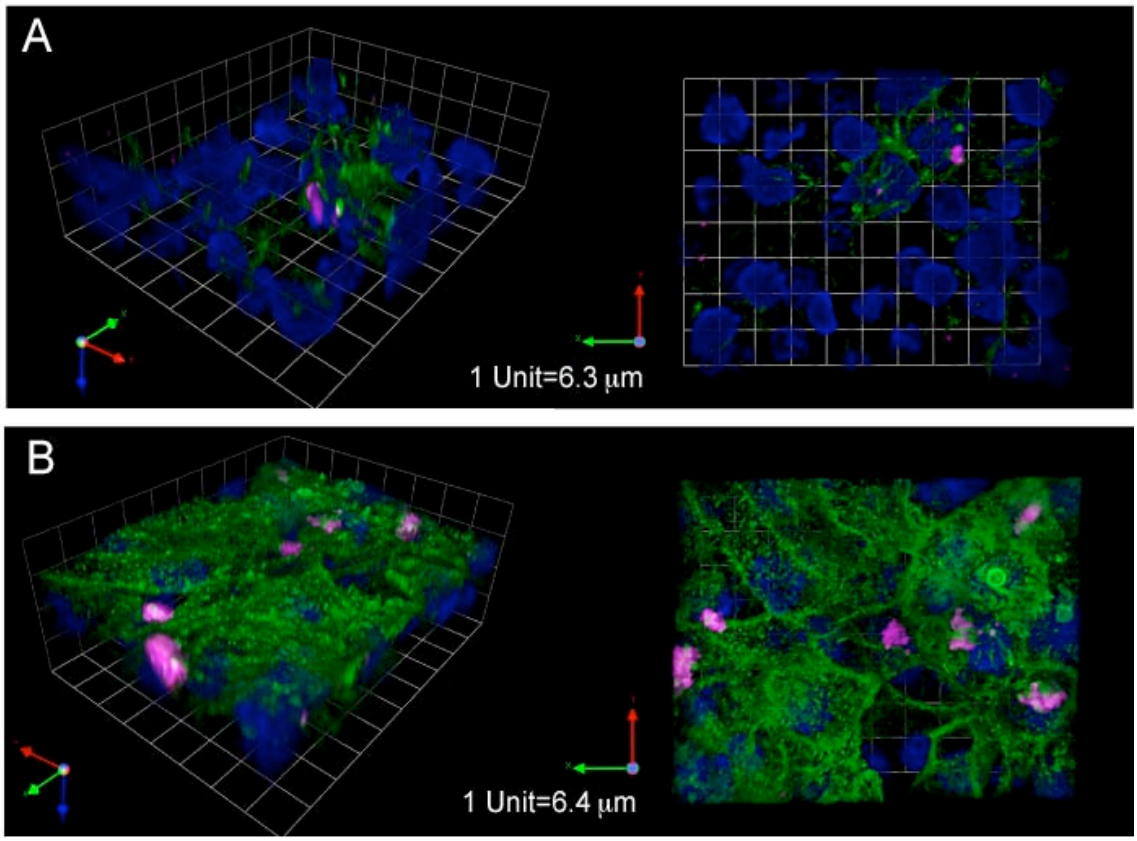

A
B
IgG control
anti-β1 integrin
anti-b1 integrin
31.1
33.2
41.3
37
20.4
72.2
4.71
46.4
30.9
79.1
12.9
52.3
untreated
+LTβR, TNFα
SSC
S. aureus
54.5
41.3
60.6
43.9
35.5
23.3
45.2
26.7
Y. enterocolitica

Figure 1.6. Increased uptake of *S. aureus* is accompanied by apical β1 integrin upregulation in inflamed epithelial cell layers. Spinning-disk confocal microscopy of immunostained airway epithelial cells either untreated (A) or treated with TNF-α/LTβR agonist (B). β1 integrin (green) is upregulated with cytokine treatment and blankets the apical surface of the majority of treated cells, although adjacent cells express no apical β1. Pink, *S. aureus*; blue, DAPI.


A
1 Unit=6.3 μm
B
1 Unit=6.4 μm

Chapter 2: Production of key matrix metalloproteases and TIMP-1 accompanies the immune response to CNS infection with *Toxoplasma gondii*

Abstract

Chronic infection with the intracellular protozoan parasite *Toxoplasma gondii* leads to tissue remodeling in the brain and a continuous requirement for peripheral leukocyte migration within the CNS. The mechanism and molecules involved in this cell migration in the brain are uncharacterized. In this study we investigated the role of matrix metalloproteases (MMPs) and their inhibitors. Increased expression of two key molecules, MMP-8 and MMP-10, along with their inhibitor, Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) was observed in the brain following infection. Analysis of infiltrating leukocytes demonstrated MMP-8 and -10 production by CD4+ and CD8+ T cells. In addition, infiltrating T cells and CNS resident cells increased their expression of TIMP-1 following infection. TIMP-1 deficient mice had a decrease in perivascular accumulation of lymphocyte populations accompanied by a reduction in parasite burden in the brain. Together, these findings demonstrate a role for MMPs and TIMP-1 in the migration of lymphocytes into the CNS during chronic infection in the brain.

Introduction

Infection can be described as invasion by and multiplication of pathogenic microorganisms in a body part or tissue, which may produce subsequent tissue injury and progress to overt disease through a medley of cellular or toxic mechanisms[1]. These sequelae may be due to direct effects of the infectious agent itself, but are frequently a consequence of the inflammatory response to infection. Inflammation is now understood to be a host response to cellular injury that is marked by capillary dilation, leukocytic infiltration, and alterations to endothelial and epithelial integrity. These processes serve

to initiate cellular and chemotactic activity that is protective when they lead to wound healing and clearance of pathogenic agents from the affected local tissue environment.

In 2002, 26% of deaths worldwide were attributed to infectious disease, and more than ten million people in the U.S. alone and an estimated 1-2% worldwide are affected by autoimmune disease and non-infectious immune pathologies [2]. The need to identify molecular agents of the immune response in both the infectious and other pathological contexts therefore remains an enduring medical challenge. Infection with microbial pathogens such as bacteria and intracellular parasites can initiate a spectrum of reactions in the host organism ranging from the rapid, generalized innate response to the specialization that characterizes the adaptive immune system of higher vertebrates. Both stromal cells and those specialized for immune response are capable of recognizing molecular patterns associated with pathogens; ligation of these pattern recognition receptors initiates a signaling cascade leading to production of soluble factors including cytokines, chemokines, and proteases [3]. These cytochemicals function variously to recruit effector cells to sites of insult, to mediate migration of leukocytes as they extravasate into inflamed tissue, and in some cases, to alter the functional activities of stromal cells in the local region of inflammation [4].

Other components of successful elimination of a microbial invader are pathogen killing, resolution of inflammation, and remodeling of affected tissue. Key in all of these processes are the matrix metalloproteases (MMPs). Although their typical

characterization as proteolytic shapers of the extracellular matrix (ECM) is critical to leukocyte migration and post-infection tissue repair, the ever-expanding repertoire of non-ECM substrates of MMPs has grown to include cytokines and chemokines, cell-surface receptors, adhesion molecules, and other proteases including MMPs themselves [4, 5]. First described nearly fifty years ago for their role in tissue development [6] and long a focus of study in cancer research for their involvement in tumorigenesis and metastasis [5], the role of MMPs is at the frontier of research in immunology and infectious disease .

The matrix metalloprotease family

The term *matrix metalloproteases* describes a family of proteases dependent upon zinc for their activity--hence the 'metallo-' prefix—and possessing the collective ability to degrade all components of the extracellular matrix. The MMPs are integral to developmental and homeostatic biological processes, including reproduction, embryonic development, wound repair, and diverse neurologic processes [7]. The first matrix metalloprotease was characterized by Gross and Lapiere, physician researchers using tadpole tail tissue at Harvard nearly five decades ago to identify agents of collagenolytic activity necessary for the extensive tissue remodeling necessary for normal growth and development[6]. Common names for this protease family often reflect the early tradition of establishing their nomenclature according to characteristic matrix substrates; well-studied subgroups include the collagenases (MMP-1, -8, and -13); gelatinases (MMP-2 and -9); and stromelysins (MMP-3, -10, and -11).

The 23 MMPs so far described in humans have minimal structural elements in common. These include the N-terminal pre-domain, the conserved pro-peptide domain, typically ~80 amino acids in length, which is cleaved following secretion of soluble forms, and the C-terminal hemopexin domain present in all but the simplest forms (MMP-7 and -26). MMPs -14-17, -24, and -25, also known as the 'MT-' or 'membrane-type' MMPs, -1 through -6, also possess a carboxyterminal domain anchored to the cell membrane; however, most metalloproteases are secreted in zymogen form, and become activated in the pericellular space by a 'cysteine switch' mechanism, when the association between the thiol group on a cysteine residue of the prodomain and the zinc ion at the site of activity becomes disrupted[3].

Powerful tools require careful control: MMP activation and inhibition

Following characterization of the first MMPs, subsequent observations in the Gross lab led to the hypothesis that the lack of enzymatic activity that could be extracted from the site of remodeling might be due to secretion of the enzyme in inactive zymogen form, a feature we now know to be characteristic of MMPs. In addition to being secreted in inactive form and consistent with their capacity for significant tissue proteolysis, MMPs are also tightly regulated at the transcriptional and post-translational levels. Additional regulatory controls include soluble inhibitors α2-macroglobulin and α1-antitrypsin secreted into the plasma from the liver, where they control serum MMP activity. Most relevant to infection of tissues, however, is local modulation of MMP activity achieved

by four tissue-inhibitor-of-metalloproteases, or TIMPs. Like the enzymes they inhibit, TIMPs are generally secreted proteins which suppress MMPs stoichiometrically in a 1:1 ratio by binding noncovalently at the enzyme activation site[8]. Although there is considerable redundancy amongst the TIMPs with respect to their ability to inhibit members of the MMP family, TIMP knockout animal studies have been useful in determining their relative ability to suppress specific MMP activity in vivo [8, 9]. TIMPs have been shown to be pleiotropic in that their function is not limited to MMP inhibition; however, this paper will address the consequences of dysregulation to the MMP/TIMP axis of expression during infectious and noninfectious inflammation.

The horse's mouth: sources of metalloprotease production during infection

During infection, pathogenic microbes may themselves be a source of metalloproteases whose activity is medically significant and/or analogous to that of mammalian MMPs. These can be important virulence factors, and often serve as useful target antigens for vaccination[10]. This review, however, will address upregulation, production, activity and disease sequelae of host MMPs as a consequence of infection and inflammation.

Clinically, MMPs and TIMPs are ubiquitous in the body fluids of infected patients. These include serum, cerebrospinal fluid (CSF), and bronchoalveolar lavage fluid (BALF). MMP-8 and -9, are both elevated in BALF during lung infection with *Pseudomonas aeruginosa.* Decreased MMP-9:TIMP-1 ratios were found in surviving patients; however, these and other clinical studies, though identifying potentially useful

prognosticators of outcome, do not identify cellular sources nor confirm function of MMPs in circulating fluids or those obtained from lavage. [11] Similarly, MMP-1, -2, -7, -9, and -10 have been shown to be increased in the peripheral blood or serum of patients infected with HIV [12-14], tuberculosis [15, 16], and Helicobacter [17]. Although these studies identify important pathogen-specific metalloprotease upregulation, detection in blood can supply little information regarding MMP sources or targets.

Skin and the epithelia constitute a vast surface area for effective but not impenetrable physical barriers to microbial infection. It is therefore common to detect MMP upregulation and activity at these bustling sites of host-pathogen interface. Metalloprotease transcripts and protein product have been detected during bacterial infection in tuberculous skin lesions [18], and in the respiratory, gastric, and intestinal epithelia [19-21]. Immunohistochemistry of the small intestine and MMP-$7^{-/-}$ mice have helped to identify paneth cells as sources of MMP-7 necessary to activate alpha- and beta-defensins by cleavage of the pro-form [22-24]. *In vitro* studies employing dermal and synovial fibroblast cultures have revealed them as the source of increased production of MMP-1, -2, -3, -7, -10, -11 during infection with *Staphylococcus aureus* [25]. ECM substrates of these MMPs include type I and II collagen, laminin, elastin, and e-cadherin, identifying MMPs as potential therapeutic targets in *S. aureus*-mediated septic arthritis and other infectious soft tissue conditions where overproduction of proteases results in tissue damage.

Trailblazers for migrating immune responders

The immune response is characterized by cell migration to and from sites of infection, and extravasation of leukocytes from the vasculature to infected stromal tissue. Despite the fact that the known substrates for the MMPs now include more non-matrix than ECM proteins, these processes require the activity of proteases capable of degrading components of the matrix for which these proteases were named. These include the collagens, gelatin, laminin, fibronectin, as well as tight junction proteins and adhesion molecules such as e-cadherin and zonula occludens-1, which are critical to maintaining basement membrane integrity [8]. Although broad substrate overlap amongst the MMPs has been demonstrated *in vitro*, their activity *in vivo* is determined by differential affinity for substrates present in the local tissue environment where they are secreted.

Metalloprotease production in the innate response to infection

As demonstrated by the activity of MMP-7 in the small intestine, metalloprotease production by stromal cells may be a nonspecific immune response that is evolutionarily conserved amongst vertebrates and less complex organisms. Knorr and others recently validated MMPs as immune effectors in invertebrates by showing significantly higher susceptibility to pathogenic fungus in the MMP-1 knockout *Tribolium* beetle [26]. The idea that vertebrate stromal cell production of MMPs may represent a rapid if primitive immune response to infection is further supported by recent studies showing that MMP upregulation proceeds via the transcription factor NF-κB [27-29], as this pathway is key in regulating the immune response to infection [1]. Although rapid and indiscriminate

protease production by stromal cells in the infection microenvironment may have evolved due to enhanced pathogen clearance, the unintended consequence of this activity may be tissue damage when broad substrate affinity results in cleavage of bystander host proteins. Chondrocytes, cartilage cells that produce proteins of the cartilaginous matrix, can also produce the proteases that degrade them. During infection with *Streptococcus pyogenes* and *Borrelia burgdorferi*, MMP-13, a potent collagenase, and MMP-19 respectively are produced by chondrocytes, implicating them in bacteria-induced cartilage damage [30, 31].

As the respiratory and gastrointestinal epithelia are an immense interface that is frequently the site of host-pathogen interactions, it is advantageous for epithelial cells to be capable of initiating antimicrobial responses such as MMP-7-mediated defensin production and activation. Gastric epithelial cells infected with *H. pylori* increase production of MMP-7, which facilitates shedding of heparin-binding epidermal growth factor (HB-EGF), soluble levels of which are associated with the development of gastric cancer [20]. Caruso *et al* used gastric epithelial cell lines to show that previously observed increases in *in vivo* expression of both MMP-2 and MMP-9 in *H. pylori*-infected gastric mucosa are mediated by IL-21 induced NF-κB upregulation [32]. To examine MMP activity in the infected respiratory tract, deBentzmann *et al* used cultured airway epithelial cells to identify the *P. aeruginosa* virulence factor PAO1 as causative in the overproduction of MMP-2 and MMP-9. These results suggest an important potential therapeutic target for preventing the lung tissue damage associated with *P aeruginosa*-

induced diseases such as cystic fibrosis and nosocomially-acquired pneumonia [19]. These responses are not restricted to prokaryotic pathogens, as the intracellular protozoan parasite *Toxoplasma gondii* induces a similar response in the ileal lamina propria, upregulating MMP-2 in an IL-23-dependent manner [33].

Fibroblasts are the most abundant cell type in connective tissue, and function to synthesize the extracellular matrix. They are also common sources of MMPs in health and frequent sources of their upregulation during infection. Induction of nearly all of the soluble MMPs by synovial fibroblasts has been reported in *S. aureus*-induced septic arthritis, suggesting metalloproteases-mediated immunopathology. Knockout of MMP-7 results in increased bacterial load despite decreased severity of arthritis symptoms [25, 34]. This serves to illustrate the 'Catch-22' of infection-induced MMP activity: the same proteolytic activity that effectively clears pathogen may exacerbate pathology.

Production by cells of the innate immune response

Also called 'macrophage elastase', MMP-12 was initially characterized in thioglycolate-stimulated macrophages more than 30 years ago [35]. Subsequently, macrophages have been shown to also produce MMP-1, -2, -7, -9, -10, MT1-, and MT6-MMP [13, 14, 36-38] and TIMPs -1, -2, and -3 during infection with bacteria, viruses, and parasites, with their monocyte precursors expressing a nearly identical MMP repertoire. This remarkable metalloprotease diversity may be explained by the variety of substrates mononuclear phagocytes must be competent to degrade in order to enter and traverse

infected tissue virtually anywhere in the host. Furthermore, macrophages are resident in lymphoid and non-lymphoid tissues including but not limited to subepithelial connective tissue, organ interstitial, and in the sinuses and sinusoid spaces of lymph nodes, liver, and spleen [1].

Like monocytes and macrophages, neutrophils arise from the myeloid lineage and are critical to the effective innate response. They are also potent producers of metalloproteases, although this is so far limited to MMP-8, or 'neutrophil collagenase,'and MMP-9, along with the serine protease neutrophils elastase. Despite their robust metalloprotease activity, TIMP production is rarely reported in neutrophils. This may be attributed to the short-lived nature of this population, as well as their rapid clearance from tissues following their migration to sites of infection in response to pathogen stimulus, abrogating the need for MMP inhibition.

Dendritic cells are resident in the subepithelia and throughout organs, where they are positioned to capture antigen and transport it to peripheral lymphoid organs, where it can be presented to lymphocytes. Immature dendritic cells in culture model *in vivo* populations such as Langerhans cells in the skin. Luplertop *et al* showed upregulation of the gelatinases MMP-9 and MMP-2 by infecting these cells with Dengue virus, one effect of which is to enhance endothelial permeability that is characteristic of Dengue hemorrhagic fever. This once again points to metalloproteases as attractive targets for therapeutic inhibition to restore endothelial integrity during Dengue infection [39].

Metazoan parasites can also upregulate metalloproteases, although infection with helminths, roundworms, flukes stimulates production of cytokines associated with a Th2 response [1]. In alternatively activated macrophages isolated from murine lungs infected with the hookworm *Nippostrongylus brasiliensis*, real time PCR showed upregulation of MMP-12 message [40], confirming its upregulation in this important macrophage subpopulation involved in tissue remodeling.

Mononuclear phagocytes infiltrating the infected and inflamed brain can also express the membrane type MMPs, a group whose substrates are frequently cell surface molecules. Bar-Or and others' comprehensive survey of MMP expression on leukocytes [41] from multiple sclerosis patients showed upregulation of MMP-2, along with MMP-14 (MT1-MMP) and TIMP-2, which activate and inhibit MMP-2, respectively. MT6-MMP on monocytes has been determined to generate immunogenic peptides by cleavage of myelin basic protein, identifying monocytes as key agents of autoimmune inflammation via the activity of this metalloprotease [38].

Although MMP production is typically increased in infected cells and tissues, the infectious conditions under which metalloprotease expression is reduced may be related to intracellular pathogen subversion of normal cellular pathways involved in response to inflammation. Of note are studies demonstrating that MMP-9 production is significantly diminished during viral infection. This is true of monocyte-derived macrophages

infected with HIV-1 [13], and of macrophages infected with human cytomegalovirus [42]. As previously noted, metalloprotease synthesis has been shown to be a downstream consequence of activation of the NF-κB pathway, which is known to be suppressed or abrogated by pathogens such as *T. gondii* which can impair host cell molecular processes including, but not limited to, MMP production [43, 44]. Future investigations may reveal that viral gene products interfere in a similar fashion with NF-κB-mediated synthesis of MMPs.

Metalloprotease production in the adaptive compartment

Nearly all of the secreted metalloproteases have been isolated from peripheral blood mononuclear cells under diverse infectious conditions [12, 15, 16], supporting the premise that leukocytes are important sources of MMPs both locally and systemically during infection. Compared with studies addressing pathogen-specific MMP production by cells of the innate immune system, there is little evidence to suggest which metalloproteases are produced by cells of the lymphoid lineage during the response to infection, despite their highly migratory phenotype and need to cross similar biological barriers. Our discussion of lymphocyte-associated MMPs therefore includes those produced in non-infectious pathologies where their activity may be analogous to the lymphocyte-mediated response to infectious insult.

Identification of agents responsible for degradation of the gut epithelia in ulcerative colitis and Crohn's disease is a focus of research in inflammatory bowel disease (IBD).

As a marked increase in gut IgG plasma cells (PCs) has been noted to be a feature of IBD, their potential contribution to the metalloprotease milieu was investigated by Gordon and others, who found both transcript and expression of MMP-3 to be increased in IgG PCs isolated from mononuclear cells of the lamina propria. This finding identifies MMP-3 as a potential target for therapeutic intervention in IBD, as this enzyme has high affinity for both e-cadherin and laminin, important cell adhesion and matrix molecules whose distribution is reduced during gut epithelium compromise [45].

Production of metalloproteases by T cells, especially during infection, is poorly characterized. The expression of MMPs by immunogenic T cells can, however, be informative in this context, as these populations display similar function and phenotype to those observed in the adaptive response to bacterial, viral, and some parasitic infections. Cultured T cells stimulated to express a Th1 phenotype show more active secretion of MMPs than do Th2 or Th0 groups, with marked increased in both transcript and expression of MMP-2 [46]. Of the metalloproteases significantly upregulated in spinal cords of mice with experimentally-induced encephalomyelitis (EAE), little significant MMP upregulation could be traced to T cells, which nonetheless increased expression of the closely related disintegrin-and-metalloprotease ADAM-12 [47] Data in preparation for publication from this laboratory show a dramatic upregulation of MMP-8 and -10 transcript in the brain following the establishment of chronic CNS infection with *Toxoplasma gondii.* Flow cytometry and fluorescence immunohistochemistry confirmed expression of these MMPs, along with their inhibitor TIMP-1, on CD4+ and CD8+ T cell

populations. This constitutes previously undescribed expression of the soluble MMPs likely to be functionally related to leukocyte migration on T cell populations isolated from infected tissue.

Granting immune privilege: metalloproteases in the infected and inflamed CNS

Because uncontrolled immune activity would be deleterious in tissues that cannot tolerate inflammation such as the bone-encased central nervous system, these are protected from both pathogens and immunogenic host cells by less permissive biological barriers. A canonical example of these is the blood-brain barrier (BBB). This barrier is continuous along virtually all capillaries of the CNS, and is comprised by selectivity of pericellular tight junction proteins between tightly packed vascular endothelial cells coupled with the glia limitans, or astrocyte endfeet that encircle and support vessels. Cells of the immune system responding to CNS infection signals rely on proteases to traverse not only the vascular but also the parenchymal basement membrane to egress to infected parenchymal tissue [48].

Cells of the brain vascular endothelium express MMP-1, -3, -7, -8, -9 during coinfection with *Anaplasma phagocytophilum* and *Borrelia burgdorferi*, which models concurrent transmission of these tick-borne pathogens [49]; whether this surprising array of proteases reflects the activation of multiple pathogen-associated pattern signaling pathways is unknown, but it is speculated that coinfection facilitates breach of the BBB by *Anaplasma* bacteria *in vivo* via this pattern of MMP activity. Production of MMP-2

by brain macrophages isolated from HIV-infected patients [13] and of MMP-12 in murine macrophages isolated from the brain during EAE [47] confirm that CNS-infiltrating macrophages, like their somatic counterparts, produce a variety of metalloproteases in response to diverse inflammatory stimuli.

Astrocytes are large stellate cells that constitute the most abundant glial population in the brain, where they perform diverse functions including biochemical support for neurons and propagation of calcium-dependent signaling. During CNS infection, they also function to protect neurons by performing various functions associated with innate immunity. In addition to the above-mentioned participation in formation of the BBB, astrocytes function as important antigen-presentation cells (APC) in an environment where major histocompatibility complex II (MHCII) + cells are largely absent, and can further interact with T cells to produce costimulatory activation signals via expression of CD40. It is therefore perhaps not surprising that this versatile cell population has been reported to express virtually every secreted MMP so far investigated in the context of diverse infectious and inflammatory conditions including CNS tuberculosis, Theiler's murine encephalomyelitis virus (TMEV), and EAE [50-52]. Given the need to limit unnecessary proteolysis in the brain, astrocytes can control MMP activity by production of TIMPs -1 and -2 under these same proinflammatory conditions. Data from our laboratory demonstrates similar careful regulation of the MMP/TIMP expression axis in cells infiltrating the brain during CNS infection with *T. gondii*, as MMP-8 and -10 + T cell populations also abundantly expressed TIMP-1.

The need for TIMP-1 in modulation of T-cell mediated CNS inflammation is demonstrated by increased severity of EAE symptoms in TIMP-1 null mice when compared with wild-type littermates [9]. However, its role in regulating inflammation during infection of the CNS has not been described.

Toxoplasma gondii is amongst the most successful of intracellular parasites, infecting virtually every warm-blooded animal including an estimated one third of the global human population [53]. Although symptoms of infection are largely subclinical in the immune competent individual, acquired or latent infection in the context of immunocompromise leads to focal intracerebral lesions caused by unchecked parasite reactivation and replication. Despite a robust proinflammatory response that effectively clears fast-replicating tachyzoites from the periphery, *Toxoplasma* converts to a slow-growing bradyzoite form which encysts in the brain parenchyma for the life of the host [54]. Throughout chronic infection, parasite reactivation is suppressed by a well-orchestrated immune response characterized by interferon-gamma (IFN-γ) producing CD4+ and CD8+ T lymphocytes. Recent *in vivo* observations of T cell behavior in *Toxoplasma*-infected brain tissue revealed that lymphocyte infiltration is accompanied by the appearance of a meshlike infrastructure along which parasite-specific T cells appear to migrate [55]. Although the composition of this reticular network is not yet known, its emergence is also observed in the context of EAE [55]. Together, these findings suggest inflammation-induced alterations to the brain extracellular matrix (ECM) involving T cell

migration, compelling investigation of the mechanisms and consequences of tissue remodeling in the *Toxoplasma*-infected brain.

To address the factors involved in this tissue remodeling and cell migration, we investigated the role of MMP production during infection. Here we demonstrate the upregulation of the metalloproteases MMP-8 and -10 in the brain that was accompanied by a striking increase in transcription of their inhibitor, TIMP-1. Using flow cytometry and immunohistochemistry to analyze the source of MMP production ex vivo we find that CD4+ and CD8+ T cells produce MMP-8 and MMP-10, and that these populations also contribute to the induction of TIMP-1 during early chronic brain infection. In addition, CNS resident astrocytes produce TIMP-1 in response to parasite antigens. Finally, parasite burden in TIMP-1 deficient mice is significantly reduced in association with efficient penetration of lymphocytes into the brain parenchyma. These data demonstrate the importance of the MMP/TIMP axis in migration of infiltrating populations to sites of infection, and to what factors may contribute to the significant tissue remodeling that has been observed in the context of *T. gondii* infection of the CNS. Furthermore, regulation of metalloproteases necessary for access of immune populations to infected CNS tissues may be key to the balanced, nonpathological yet persistent immune response that is the hallmark of chronic infection with *Toxoplasma*.

Materials and methods

Parasite culture and infections. *T. gondii* parasites (Type II, Prugniaud strain) were cultured in human foreskin fibroblast (HFF) cells at 37°C, 5% CO_2. For infection, parasites were purified by needle passage of infected HFFs , passed through a 5μm filter to remove cellular debris, washed, centrifuged and resuspended in sterile phosphate-buffered saline (PBS) at $5x10^4$ parasites/mL. C57Bl/6J, B6.129S4, and B6.129S4-$Timp1^{tm1Pds}$/J (TIMP-1 -/-) mice were obtained from the Jackson Laboratories and housed according to institutional protocol. Animals aged 8-12 weeks were infected by injecting 10^4 *T. gondii* tachyzoites in 200 μL sterile PBS intraperitoneally. Animals sham injected with 200 μL sterile PBS served as uninfected controls.

Cytokine measurement. Peripheral blood (100 μL) was collected from the tail vein of mice into capillary tubes at 7d and 14d post-infection. Samples were centrifuged at 13000 rpm at 4°C for five minutes to separate serum from cellular components. Serum was diluted tenfold for pro-inflammatory cytokine bead assay (CBA) (BD Pharmingen, San Diego CA) according to the manufacturer's protocol. Samples were collected on a FACSCanto II Flow Cytometer (BD Biosciences, San Jose CA), and concentrations of IFN-γ, IL-12p70, IL-6, MCP-1, TNF, and IL-10 were determined by comparison to a standard curve.

Quantitative RT-PCR. Immediately following euthanasia, naïve or *T. gondii*-infected animals were transcardially perfused and whole brains were collected. Total RNA from

three brains per post-infection time point (3, 7, 14, 21, 28, 35, 42, and 60 days post-infection or d.p.i.) was extracted using Trizol/$CHCl_3$ phase separation (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Pooled RNA was reverse transcribed using the oligo(dT) primer according to the First Strand cDNA Synthesis Kit protocol (Fermentas Life Sciences), and the resulting cDNA was used as template in an RT-PCR array of primers for extracellular matrix and ECM-associated molecules including metalloproteases and their inhibitors (SA Biosciences, Frederick, MD). To confirm upregulation of MMP-8, MMP-10 and TIMP-1 revealed by primer array, separate real-time PCR amplification was performed using primer sequences as previously described {Hasebe, 2007 #43} for MMP-10, forward, (5'-CCTGTGTTGTCTGTCTCTCCA- 3'): reverse, (5'-CGTGCTGACTGAATCAAAGGA-3'); and designed for MMP-8, forward, (5'-ACGGAGTGAGAGGTGTGGAT-3'); reverse, (5'-TCTGCCTGGGAACTTATTGG-3'); and TIMP-1, forward (5'-ATCTGGCATCCTCTTGTTGC- 3'): reverse (5'-CATTTCCCACAGCCTTGAAT-3'). DNA was amplified using a BioRad iCycler in the presence of SYBR-Green. Reaction conditions were as follows: denaturation at 95°C for 10 minutes, followed by 40 cycles consisting of 15s denaturation at 95°C, 30s annealing at 60°C , and 30s extension at 72°C. Melting curve analysis in 0.5°C increments from 95°C to 60°C was conducted to verify primer specificity. Threshold values were acquired and analyzed using the Bio-Rad iQ5 2.0 optical-system software. Fold induction was calculated using the comparative C_T method described by Livak and Schmittgen {Livak, 2001 #12}. Parasite burden was measured by amplifying the *T. gondii* B1 gene using the primer sequences:

forward, (5'-TCCCCTCTGCTGGCGAAAAGT-3'); reverse, (5'-AGCGTTCGTGGTCAACTATCGATTG-3'), followed by comparison to a DNA standard acquired from known numbers of purified parasites. Statistical differences between WT and TIMP-1$^{-/-}$ were performed by t test using Graphpad Prism v4.0 (Graphpad Software).

Astrocyte culture ELISA

Cell culture supernatants were prepared from astrocytes isolated from mixed glial cultures according to previously published methods [56]. Briefly, whole brains were collected from 1-3 days postnatal C57Bl/6 neonates, and regions caudal to the midbrain were discarded to exclude cerebellar tissue. The remaining forebrain tissues were strained, washed and plated in tissue-culture treated flasks for 12 days with medium change every 3 days. On the twelfth day, cultures were shaken at 37°C for 2h at 240 rpm, and the supernatant aspirated to remove less adherent cells. The cultures were then subjected to an additional 18h shaking at 37°C, after which the cells still adherent were enriched for astrocytes as confirmed by staining for glial fibriliiary acidic protein (GFAP). Astrocytes were trypsinized, counted in the presence of Trypan blue dye, and replated in multiwell tissue culture-treated plates at a density of $1x10^5$ viable cells/cm^2. Upon attaining confluency, cells were incubated with medium alone (DMEM, 10%FCS, 2 mM glutamine, 1% nonessential amino acids, 10 mM HEPES, 100 IU/mL penicillin, 100 μg/mL streptomycin) or 25 μg/mL soluble *Toxoplasma* antigen (sTAg) or 20

units/mL recombinant mouse IFN-γ (R&D Systems, Minneapolis MN), or with 100 ng/mL lipopolysaccharide (LPS), or were infected with the type I RH strain of *T. gondii* at a multiplicity of infection (MOI) of 5 parasites:cell. After 24 hours, culture supernatants were collected and centrifuged, and supernatants were diluted 1:100 for use in ELISA to measure TIMP-1 concentration. ELISA was performed using the Quantikine Immunoassay kit for mouse TIMP-1 (R&D Systems) according to the manufacturer's protocol. Statistical differences between untreated astrocytes and other groups were performed by t test using Graphpad Prism v4.0 (Graphpad Software).

Flow cytometry. Splenocytes were prepared from naïve and infected mice. Single cell suspensions were achieved by pressing tissue through a 40 μm filter mesh to dissociate cells from the matrix, and the resulting suspension was washed and incubated with 0.86% NH_4Cl in PBS to lyse erythrocytes. For isolation of brain mononuclear cells (BMNC), whole brain tissue was collected following sacrifice and transcardial perfusion with 20 mL ice cold PBS. Tissue was minced and then digested with collagenase/dispase (Roche Applied Science) at 37°C for 45 mins, followed by DNAse (Sigma-Aldrich, St. Louis, MO) at 37°C for 45 mins. A density gradient composed of 60% and 30% solutions of Percoll (GE Healthcare) in PBS or RPMI culture medium was used to separate cells from myelin and cell debris in the digestion product. Following washing, cells were counted and then resuspended in FACS buffer (1% BSA 0.1 mM EDTA in PBS) for incubation with antibodies against cell surface markers conjugated to fluorophores. For intracellular staining, cells were then fixed in 4% paraformaldehyde (EMS, Hatfield, PA) in PBS,

permeabilized with 0.3% saponin in PBS, and incubated with purified rabbit anti-mouse MMP-8, MMP-10, or TIMP-1 (Abcam). Secondary antibodies to rabbit IgG conjugated to Alexa 488 or Alexa 647 (Invitrogen, Carlsbad CA) were used for detection. A FACSCanto II (BD Biosciences) was used to collect fluorescence signal, and data were analyzed using FlowJo version 8.8.6 (Tree Star, Inc., Ashland OR).

Immunohistochemistry. Brain tissue was snap-frozen immediately following sacrifice by immersion in isopentane chilled on dry ice, and then mounted in OCT medium (Sakura Finetek). Samples were sectioned by Microm OMV cryostat to 6 μm (for hematoxylin and eosin staining) or 10-15 μm (for immunofluorescence or enzymatic immunohistochemical staining). For T cell receptor detection, sections were thawed and fixed in 75% EtOH, 25% acetone. For all other samples, sections were fixed in 2% paraformaldehyde and permeabilized in 0.5% Triton-X100, then blocked in 5% serum prior to incubation with purified antibodies. Primary antibodies against metalloproteases and TIMP-1 are as listed previously and were used at a concentration of 10 μg/mL. Purified rat-anti GFAP (Invitrogen) or goat-anti *Toxoplasma gondii* (Abcam) were used at 5 μg/mL and 20 μg/mL, respectively, incubated with tissue samples overnight at 4°C, and followed with appropriate secondary antibodies conjugated to Alexa 488, Alexa 568, or Alexa 647 at 2 μg/mL (Invitrogen). Samples were mounted in Prolong Gold with DAPI (Invitrogen) for nuclear counterstaining. Images were collected on a TCS/SP2 UV confocal microscope (Leica, Heidelberg GMBH), and analyzed using Improvision Volocity 5.0 (Perkin-Elmer, Waltham, MA).

For enzymatic immunohistochemistry using the avidin-biotin complex (ABC) method, sections were fixed in ice-cold acetone and blocked in 5% serum, 0.2% Triton-X before incubating overnight with anti-TIMP-1 as above. Secondary antibody was anti-rabbit IgG conjugated to horseradish peroxidase, followed by the addition of diaminobenzidine (DAB) substrate to form a brown precipitate. Slides were counterstained with hematoxylin and dehydrated in an ethanol gradient and toluene before mounting.

Results

MMPs and TIMPs are upregulated in the CNS following T. gondii infection

To discover which brain extracellular matrix (ECM)-related proteins are altered by *Toxoplasma gondii* infection of the CNS, whole brain mRNA was isolated from naïve and chronically infected mice at time points ranging from 3-60 days post-infection. Quantitative RT-PCR using an ECM-associated molecule array including primers for MMPs and TIMPs revealed selective infection-induced transcription. Fold induction over naïve transcript levels of all matrix metalloproteases and their inhibitors measured are listed in Table 2.2.

MMP-2 and MMP-9 are frequently upregulated in the CNS following traumatic or infectious insult [57, 58]. Although upregulation of MMP-2 (2.76-fold) and MMP-9 (4.02-fold) was observed in the *Toxoplasma*-infected brain, the most notable increase in transcription was of MMP-8 (~96-fold) and MMP-10 (~20-fold); this was accompanied

by a significant concomitant increase of their inhibitor TIMP-1 (~165-fold). This was notable also by comparison to upregulation of TIMP-2 and TIMP-3, which were increased just 3.56- and 3.44-fold, respectively. To focus on the post-infection kinetics of upregulation of these genes, brain tissue was collected for real time PCR at time points ranging from 3 days to 6 weeks post-infection (pi). An increase in MMP-8 transcript was detectable as early as 7 dpi, peaked at >14-fold over naïve at 28 dpi, and remained elevated twofold over naïve at 6 weeks post infection (Fig. 2.1A). In contrast, MMP-10 increase was intermittent, beginning at 3 dpi and peaking abruptly at 21 dpi, and decreasing rapidly to naïve levels by 6 weeks. The increase in TIMP-1 transcription displayed similar kinetics, but was of greater magnitude, beginning at day 7 pi and increasing to 192-fold over naïve at 4 weeks pi, with levels remaining >20-fold over naïve at 6 weeks. This suggests a continued need for MMP-8 and TIMP-1 during chronic infection, while the pattern of MMP-10 may be related to peak infiltration of T cells that occurs at approximately 21 dpi [59].

To assess the location and distribution of these molecules following infection, immunohistochemistry was performed on brain sections from chronically infected mice, and compared with uninfected tissue. In the naïve brain, MMP-8 protein signal was weak and diffuse; though not cellular in appearance, signal was frequently associated with the vasculature in infected tissue (Fig. 2.1B). MMP-10 protein was present on large parenchymal cells in the uninfected brain, and has been previously reported to be expressed by neurons in normal CNS tissue [58]. The pattern of expression is similar,

though signal is increased, in infected tissue (GFAP) (Fig 2.1B, center and right columns). TIMP-1 is detectable by enzymatic immunohistochemistry on glia limitans astrocytes in infected, but not naïve brain sections (Fig 2.1C, bottom panels), and was largely absent in naïve brains in fluorescence IHC sections.

MMPs and TIMP1 are expressed by infiltrating T lymphocytes in the infected brain, but only CD4+ cells are a significant source of MMP-10

Following *Toxoplasma* infection, infiltrating immune cells of both the innate and adaptive compartments can be detected in the brain of an infected animal by day 14 (unpublished data). Our observation that MMPs and TIMP1 peak at 21-28 dpi would coincide with the significant infection-induced presence of T lymphocytes infiltrating the CNS. We therefore examined the production of MMP-8, -10 and TIMP-1 by T cell populations isolated from the infected brain after chronic infection and immune surveillance has been established to assess whether their upregulation could be attributed to the adaptive immune response.

At four weeks post-infection, cells were collected from the spleen and brain and analyzed for surface markers and expression of MMP-8, -10 and TIMP-1. To verify the utility of intracellular immunostaining for identification of soluble MMP and TIMP production on BMNC and splenocytes, the capacity for this technique to detect MMP-8 signal on neutrophils was tested. SSC^{hi}, $Gr1^{hi}$ cells from the spleen and brain were significant producers of MMP8 consistent with its established production by neutrophils during

migration to sites of inflammation [60] (Fig. 2.2A). As this result confirmed the applicability of intracellular flow cytometry methods for detecting MMPs on leukocytes, MMP and TIMP production was then measured on BMNC and splenic populations gated on CD4+ or CD8+ (Fig 2.2B). MMP-8 was evident on naïve CD4+ splenocytes, and upregulated on CD4+ cells from infected spleens. The most profound increase in intensity of MMP-8 over isotype control, however, was on CD4+ from the infected brain. Naïve CD8+ splenocytes expressed very little MMP-8, while signal from the infected spleen and brain was more pronounced. Both populations strongly expressed TIMP-1; this expression was increased slightly on CD4+ and moderately on CD8+ in the infected spleen. Expression of TIMP-1 in the infected brain was fourfold over isotype on CD4+, and more than sixfold on CD8+ cells. This suggests a requirement for increased pericellular proteolysis by T lymphocytes responding to *Toxoplasma* infection , and a robust concurrent upregulation of endogenous MMP inhibition by these same populations.

Both CD4+ and CD8+ splenocytes expressed MMP-8 at 4 weeks post-infection. MMP-8 expression increased on infected CD8+ splenocytes when compared with those from uninfected spleens (Fig 2.2b). This suggests a general need for MMP-8 production by activated T lymphocytes, which must breach the blood-brain barrier to gain access to infected brain tissue; proteolytic activity is also needed to mediate migration through parenchymal tissue to maintain surveillance of the chronically infected brain. In contrast, MMP-10 expression was increased above isotype only on infected CD4+ populations

when compared with naïve CD4+ splenocytes (Fig. 2.2b and 2.2c). The same pattern of expression was observed on T cells infiltrating the *Toxoplasma*-infected brain. TIMP-1 was expressed by both CD4+ and CD8+ cells in the infected brain, and upregulated on splenocytes from infected animals at all time points (Fig. 2.2b and data not shown). Both T helper and cytotoxic T cell populations are therefore a source of MMP-8 and TIMP-1 during chronic *Toxoplasma* infection of the CNS, but only CD4+ T cells contribute significantly to MMP-10 production (Fig 2.2b).

Timp-1 is produced by CNS resident cells, and induced in astrocytes in response to infection

Control of chronic *Toxoplasma* infection in the CNS is achieved through mediation of proinflammatory factors such that reactivation is suppressed without deleterious effects of sustained activity by responding immune populations [61]. Similarly, uncontrolled MMP production, while potentiating access of infiltrating leukocytes, results in extensive tissue damage. In the brain, TIMP-1 production is conditionally altered in response to upregulation of MMP-2 and -9 [62, 63], and the present study shows that its upregulation in early chronic infection of the brain by *T. gondii* correlates to upregulation of MMP-8 and -10. Several studies have shown astrocyte production of TIMP-1 to be dysregulated in response to brain infection or inflammatory stimulus [64-66]. To investigate whether resident CNS populations can respond to *T. gondii* infection by producing TIMP-1, intracellular immunofluorescence and flow cytometry were used to compare its expression on microglia from naïve and *Toxoplasma*-infected mice. Comparison of

microglial populations isolated from naive and infected whole brain tissue shows modest production of TIMP-1 by microglia from uninfected brains, with no significant increase in production at four weeks post-infection (Fig 2.3a). Astrocyte TIMP-1 response to infection was assayed by comparing its production by primary astrocyte cultures treated as detailed in methods by infection with the RH strain of *T. gondii*, or stimulation with soluble *Toxoplasma* antigen (sTAg), or with recombinant mouse IFN-γ, with that from unstimulated astrocyte cultures. IFN-γ-stimulated astrocytes were included as a positive control for response to inflammatory stimulus. After 24h, culture supernatants were collected and analyzed by ELISA for the presence of TIMP-1. Unstimulated astrocytes demonstrated modest secretion of TIMP-1 (33.1±9.7 ng/mL), consistent with its role under normal physiological conditions [65] (Fig 2.3b). Astrocytes exposed to IFN-γ, a cytokine that is not specific to but nonetheless ubiquitous in *Toxoplasma* infection of the CNS, as well as those incubated with soluble parasite antigen, trended towards increased production of TIMP-1, although increase over untreated astrocytes was not significant. Significant increase in TIMP-1 production, however, was measured in astrocytes that had been directly infected with the RH strain of *Toxoplasma* (152.5±10.4 ng/mL), an increase of nearly fivefold over unstimulated ($p<.0001$). Fluorescence immunohistochemistry of tissue sectioned from the infected cortex supports the in vitro finding that astrocytes upregulate TIMP-1 during *Toxoplasma* infection, as images reveal abundant colocalization of TIMP-1 with the astrocyte cytoskeletal marker GFAP (Fig 2.3c). These data show that although astrocytes boost production of TIMP-1 in response to both nonspecific and parasite-specific inflammatory stimuli, upregulation is greatest

upon direct infection with *Toxoplasma*. These data further demonstrate that although this response may occur independent of the presence of responding immune populations, TIMP-1 expression on T cells and microglia as shown by flow cytometry and immunohistochemistry point to its collective production by both resident and infiltrating cells which may account for the magnitude of post-infection upregulation observed by quantitative PCR.

Parasite burden and perivascular cuffing in the brain are significantly diminished in the absence of TIMP-1 despite a normal systemic cytokine response to acute infection

Once established in the immunocompetent host, *T. gondii* maintains a subclinical presence through a balanced equilibrium involving immune evasion strategies, CD4+ and CD8+ production of proinflammatory cytokines, and constraint of inflammatory pathology by anti-inflammatory mediators [67-69]. To test how dysregulation of the endogenous MMP/TIMP response to infection might affect this balance, we infected animals deficient in TIMP-1 and compared pathology, immune function and parasite burden to that in wild type infected mice. No significant signs of murine systemic illness (hunching, fur ruffling) were observed in either group during the six week observation period following infection. Animals were weighed prior to and at regular intervals following infection. Both groups experienced a transient weight loss during acute infection, consistent with previous reports of systemic toxoplasmosis in mice [70] (Fig 2.4b). While the WT group recovered to pre-infection weight by day 15 p.i. (black triangles), the TIMP-1 null group failed to recover to naïve weight at four weeks p.i.

(filled black circles). Sham-infected animals had recovered pre-infection weight by 7 days (TIMP-$1^{-/-}$, grey filled circles) or 14 days (WT, grey triangles) post-injection, and both of these groups experienced a mean weight gain of 15% by the end of the measurement period. TIMP-1-/- animals displayed no alterations in cytokine production at 7 and 14 d.p.i (Fig 2.4a and data not shown, respectively). Together, these data point to an imbalance in the normal CNS immune control characteristic of chronic *Toxoplasma* infection, suggesting altered parasite burden or a dysregulated immune response.

Flow cytometry consistently shows that TIMP-1 is produced on infiltrating leukocyte populations at all post-infection time points. Because this data also shows MMP-8 and MMP-10 upregulated on CNS-infiltrating T cells post-infection, the absence of TIMP-1 could be expected to affect the ability of migrating populations to respond to chronic brain infection. We examined histological sections of brain tissue from WT and TIMP-1 null animals, and found perivascular cuffing characteristic of inflamed brain tissue in the infected WT (Fig 2.5a middle row and 2.5b, left column). In the infected TIMP-1 knockout, however, cuffing of brain vessels is reduced or absent (Fig 2.5a, bottom and 2.5b, right column). This decreased restriction of infiltrating cells to the perivascular space could be attributed to enhanced metalloprotease-mediated cleavage of basement membrane proteins due to reduced endogenous MMP inhibition . To measure the ability of CNS-responding immune cells to control parasite replication in the absence of TIMP-1, we compared parasites per milligram of tissue from the brains of infected WT and TIMP-$1^{-/-}$ mice. Parasite burden was reduced more than fourfold in the TIMP-1 null

brains (Fig. 2.5c), suggesting enhanced migration of parasite-controlling immune populations when metalloprotease suppression by TIMP-1 is not present.

Discussion

The inflammatory response is characterized by alterations to the motility of responding immune populations. For example, antigen-specific T cells demonstrate reduced velocity and arrest in response to their cognate antigen [55, 71]. Additionally, remodeling of the extracellular matrix is necessary to repair tissue damage sustained during infection and as a consequence of the subsequent host response [3]. The present study reveals that amongst the matrix remodeling metalloproteases induced in the CNS during chronic infection with *Toxoplasma* gondii, those most profoundly upregulated are MMP-8 and MMP-10. We further establish T lymphocytes as a source of these MMPs and TIMP-1, with MMP-10 expressed preferentially by CD4+ T cells. A nearly 200-fold increase in transcript of the endogenous MMP inhibitor TIMP-1 accompanies CNS toxoplasmosis; flow cytometry shows that TIMP-1 is also produced by T cells infiltrating the infected brain, while immunohistochemistry and western blot confirms TIMP-1 production by resident glial cells in response to infection. In animals lacking TIMP-1, perivascular cuffing and parasite burden are reduced during infection when compared with the brains of infected WT mice, perhaps due to reduced inhibition of metalloproteases enhancing T cell access to sites of infection, though the failure of the TIMP-1 null group to gain weight suggests a detrimental systemic effect of reduced MMP inhibition in the brain.

Certain MMPs are associated with, and therefore often known by, their expression on responding immune populations; examples include macrophage elastase (MMP-12) and neutrophil collagenase (MMP-8). Metalloproteases that are characteristically expressed by migrating lymphocytes, however, are less well-defined. Chronic brain infection with *Toxoplasma* gondii is accompanied by what appears to be significant tissue remodeling, and T cells that suppress parasite recrudescence must migrate deep within this tissue [55]. Due to the emergence of an infection-induced extracellular network in the brain associated with the parasite-specific T cell population, lymphocyte-associated protease activity may be responsible for matrix remodeling leading to the formation of a cell-trafficking network. The need to characterize the metalloprotease response during brain infection with *Toxoplasma* is therefore compelling. This study reveals significant upregulation of transcripts for MMP-8, MMP-10 and their tissue inhibitor TIMP-1 by both CD4+ and CD8+ T lymphocytes, populations required for controlling parasite reactivation in the CNS [67].

Leukocyte migration to sites of infection in the CNS involves non-proteolytic processes including chemokinesis and ligation of adhesion receptors [72]. However, the requirement for pericellular proteolysis to facilitate cell passage across the BBB and through parenchymal tissue is less well understood. In particular, it is unknown whether MMP activity is predictable in a given leukocyte population, or varies according to inflammatory context. MMP-14, a membrane-type metalloprotease also known as MT1-MMP, is expressed by T cells [73] and has been shown to cleave non-matrix substrates

such as the CD44 receptor [74] The role of soluble MMPs produced by T cells, however, is often to degrade tissue in the migratory pathway [75, 76]. Although specific non-matrix cleavage targets have been described for this protease family, MMPs are best characterized for their importance in degradation of ECM substrates. In EAE, for example, MMP-2 and -9 activity is required to permit egress of leukocytes from the perivascular compartment via degradation of dystroglycan at the perivascular basement membrane [48]. This is demonstrated by resistance to EAE in MMP-2/MMP-9 double knockout mice. Conversely, MMP-9 deficiency impairs host defense in a mouse model of bacterial meningitis induced by *Streptococcus pneumoniae* [77]. These studies demonstrate a requirement for MMP-2 and -9 in inflammatory brain pathologies.

A metalloprotease expression profile similar to that described here, along with an increase in MMP-12 and TIMP-1, has been described in association with T-cell mediated CNS inflammation in EAE [47, 52] and during viral encephalitis [78]. However, identification of the cells producing MMPs in the former studies has been attributed to nonspecific inflammatory infiltrates, or to lymphocytes isolated from the spinal cord. In the latter study, TIMP-1 was produced by CD4+ but not CD8+ cells during viral infection of brain tissue.

Unlike many other models of brain inflammation, the present investigation of metalloprotease expression in brain tissue following *Toxoplasma* infection revealed only modest upregulation in MMP-2 and MMP-9 transcripts compared with those of MMP-8

and -10. During cerebral malaria caused by the intracellular apicomplexan *Plasmodium,* transcripts of MMP-8 in the brain are increased, though the source of this upregulation is not known [79] The present study identifies brain-infiltrating T cells as a source of MMP-8, MMP-10 and TIMP-1, and further demonstrates TIMP-1 production by resident glial cells in response to protozoan infection. These results expand our existing knowledge of the role of MMP-8 and -10 and TIMP-1 in brain inflammation which has been best described to date in an autoimmune context.

Though originally characterized for their collective ability to degrade all proteins of the extracellular matrix, it has been subsequently demonstrated that the mammalian metalloproteases have diverse non-matrix substrates related to immunomodulatory function. MMPs have been shown to activate and degrade cytokines and chemokines [80], for proteolytic cleavage of proteins to generate autoimmunogenic peptides in EAE [38], and are frequently required for cleavage activation of the secreted proenzyme forms of other members of the metalloprotease family [5]. MMP-10, for example, converts MMP-8 to its active form by cleaving its zymogen precursor [81]. The implications for this study are that MMP-10 may be enhancing T cell surveillance in the infected brain by functioning to activate MMP-8 as well as to cleave matrix proteins. Measurement of MMP-8 activity following targeted MMP-10 suppression during chronic CNS infection with *Toxoplasma* would be useful to resolve which cleavage targets are most important in this context.

TIMP-1 is a pleiotropic glycoprotein shown to be involved in cell growth and proliferation in addition to being a broad endogenous inhibitor of the soluble metalloproteases [82]. It is frequently produced in autocrine fashion by cell populations expressing metalloproteases, though there is little evidence to date to suggest TIMP-1 production by T lymphocytes, and no study addressing production of this protein on T cells collected from infected tissue. The results presented here show a contribution by infiltrating CD4+ and CD8+ T cells to the striking upregulation in TIMP-1 transcript observed in chronically infected brain tissue. Although TIMP-1 production in the CNS is beneficial in that it has been shown to be neuroprotective [83], its MMP-inhibitory properties may contribute to chronic *Toxoplasma* infection by suppressing the metalloprotease activity, and therefore the surveillance efficiency, of parasite-patrolling T cells (Fig 2.6).

In the CNS, production of TIMP-1 is differentially dependent on inflammatory stimulus [52, 65, 84]. TIMP-1 production in the brain is inducible, and its dysregulation has been linked to multiple sclerosis, Parkinson's disease, and tumor progression [85]. We therefore examined its post-infection production in glial populations, and find that TIMP-1 is expressed during chronic infection with *T. gondii* by CNS-resident microglia, which have been shown to remain persistently activated in its absence during EAE [86].

Astrocyte production of TIMP-1 has been reported circumscribing inflammatory lesions [52], and to have a BBB protective effect during EAE [9]. Other groups have similarly

reported enhanced or prolonged EAE pathology in the TIMP-1-/- mouse [86]. In this study, astrocytes increased production of TIMP-1 in response to parasite-relevant stimuli, though response was most enhanced in parasite-infected cultures. This suggests that *Toxoplasma* infection may augment TIMP-1 production in astrocytes with or without the presence of metalloproteases in the microenvironment.

In the absence or inhibition of TIMP-1, infection with *Pseudomonas* increases metalloprotease-mediated destruction of the infected corneal epithelium[87], while resulting in a dramatically increased rate of pathogen clearance in the lung [84]. The current results show dramatic upregulation of two metalloproteases expressed on T cells isolated from parasite-infected brain tissue, both of which are inhibited by TIMP-1, which is also significantly increased during cerebral toxoplasmosis. Infecting animals deficient in TIMP-1 allows us to assess its role and importance in parasite-specific infection. Although the mechanism for the observed failure of infected TIMP-$1^{-/-}$ mice to gain weight as compared with uninfected or infected WT controls is not known, previous studies have demonstrated the influence of inflammatory mediators on similar patterns of weight loss in EAE, another T cell mediated CNS inflammatory disorder [88]. In particular, tumor necrosis factor alpha (TNF-α), known to cause wasting in cancer patients, is present during cerebral toxoplasmosis and required for parasite control and host survival [89].

Osiewicz et al (Ann NY Acad Sci 1999) found improved infection resistance in TIMP-1 null animals to be dependent on neutrophils known to secrete MMP-8 and MMP-9, both of which are inhibited by TIMP-1. Our finding that parasite burden is reduced in the absence of TIMP-1 during infection with *Toxoplasma* gondii demonstrates that this glycoprotein is a component of the balance between immune control and CNS homeostasis which is clinically characteristic of chronic infection in immunocompetence. Furthermore, histology shows a redistribution of infiltrating leukocytes from the perivascular aggregation typically seen during CNS infection in the WT mouse [90] to a more diffuse distribution in the parenchyma with little or no vessel cuffing. Given the requirement for the MMP-2 and -9 in mediating migration of leukocytes across the parenchymal basement membrane of the BBB [48], we propose that reduced parasite presence in TIMP-1 deficiency may be attributable to enhanced access of T lymphocytes to sites of parasite infection via deregulated production of MMPs. Taken together, these data describe a role for specific MMPs in CNS infection with *Toxoplasma* which may lead to targeted inhibition therapies in chronic brain infection as well as other T cell mediated inflammatory pathologies.

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Table 2.1. Matrix metalloproteases in infectious and immune-mediated disease.
Note: Since their original placement in the MMP nomenclature, MMP-4, -5, and -6 have been found to be 100% identical to previously identified MMPs.
MMP-18 is so far defined only in *Xenopus*.

MMP/ Inhibitor	Cell/tissue source	Infection/ Disease state	Target Substrate	Knockout phenotype	Reference
	brain vascular endothelial cells	*B. burgdorferi* + *A. phagocytophilum*			[49]
MMP-1	spleen	sepsis			[91]
	astrocytes	CNS TB			[50]
	dermal/synovial fibroblasts	*S.aureus*/ septic arthritis			[25]
	lung fibroblasts	TB			[29]
	monocytes, PBMC				[15]
	macrophages	EAE	dystroglycan		[48]
	monocytes	MS			[41]
	monocytes	*T. gondii*(↓)			[44]
	ileal lamina propria			enhanced survival	[33]
	gastric epithelial cells	*H. pylori*			[32]
	airway epithelial cells	*P. aeruginosa*			[92]
MMP-2	PBMC, macrophages	HIV			[12-14]

	h brain tissue macrophages		SDF-1		[93]
	dermal/synovial fibroblasts	*S.aureus*-septic arthritis			[25]
MMP-2	liver	schistosomiasis			[94]
	dendritic cells	Dengue virus			[39]
	serum	*H. pylori* gastritis			[17]
	skin lesions	*M. leprae*			[18]
	CSF	tuberculous meningitis			[95]
	LP plasma cells	IBD			[45]
	brain vascular endothelial cells	*B. burgdorferi*+ *A. phagocytophilum*			[49]
MMP-3	Peyer's patches, mesenteric LN	*S. typhimurium* *Y. enterocolitica*		↑ resistance	[96]
	astrocytes	CNS TB			[50]
	dermal/synovial fibroblasts	*S.aureus*/ septic arthritis			[25]
	astrocytes	TMEV			[51]

MMP-3	brain	*Trypanosoma. b. brucei*			[97]
	spleen	cerebral malaria			[79]
	monocytes, PBMC	TB			[15]
	astrocytes	CNS TB			[50]
	brain vascular endothelial cells	*B. burgdorferi* + *A. phagocytophilum*			[49]
MMP-7	macrophages	HIV			[14]
	dermal/synovial fibroblasts	*S.aureus*/ septic arthritis		↑ bacterial load ↓ arthritis severity	[25, 34]
	resp/intestinal epithelial cells	bacterial infection			[19, 21]
	Paneth cells	*Chlamydia*	defensins	↑ intestinal bacterial load ↓ antimicrobial peptides	[22-24] [22]
	gastric epithelium	*H. pylori*	HB-EGF		
	intestine	necrotizing enterocolitis			[98]
	bronchoalveolar lavage fluid	*P. aeruginosa* VAP			[11]
MMP-8	brain vascular endothelial cells	*B. burgdorferi*+ *A. phagocytophilum*			[49]

	gingiva	*P. gingivalis*-periodontitis		↑ bone degradation	[99]
	brain	*Trypanosoma. b. brucei*			[97]
		LPS+ hyperoxia	MIP-1α	↑PMNs, mac ↑ mortality	[100]
MMP-8	pt serum	*H. pylori* gastritis			[17]
	PMN	LPS	LIX/CXCL5/ CXCL8	↓PMN infiltration	[101]
	spleen, liver, brain	cerebral malaria			[79]
	granulocytes	EAE			[47]
	macrophages	EAE	dystroglycan	EAE resistance (w MMP-2 KO)	[48]
	monocytes	*T. gondii*			[44]
	macrophages				[36]
	gastric epithelium	*H. pylori*			[32]
	pt serum	*H. pylori* gastritis			[17]
MMP-9	cardiac tissue	CVB3 viral myocarditis		↑ viral titer, inflammation	[102]
	monocytes, PBMC	*M. tuberculosis*			[15] [16]

	lung tissue		↓macrophage recruitment to lungs	[103]
	BALF	*P. aeruginosa*		[11]
	airway epithelial cells			[92]
	cornea		↓corneal disease	[104]
	PBMC	EAE	↑resistance to EAE	[105]
MMP-9	PBMC	HIV		[12]
	macrophages*	HIV (↓)		[13]
	CSF	HIV-cryptococcosis		
	brain endothelium	*B. burgdorferi* *A. phagocytophilum*		[49]
	astrocytes	CNS TB		[50]
	pt serum	influenza virus		[106]
	astrocytes	TMEV		[51]
	CSF	meningitis		[107]
	CSF	tuberculous meningitis		[95]
	neutrophils	HSV	↓corneal angiogenesis	[108]
	pt serum	lethal sepsis (↓)		[109]

	dendritic cells	Dengue virus	[39]
	plasma, liver	CP-sepsis	[110]
MMP-9	hepatocytes	*T. cruzi*	[111]
	macrophages	HCMV (↓)	[42]
	airway epithelial cells	Human rhinovirus	[112]
	Skin lesions	*M. leprae*	[18]
	CSF	*A. cantonensis* - eosinophilic meningitis	[113]
	liver, brain, kidneys	cerebral malaria	[79]
	monocytes, PBMC	TB	[15]
MMP-10	dermal/synovial fibroblasts	*S.aureus*/ septic arthritis	[25]
	lung epithelium	*P. aeruginosa*	[19]
	astrocytes	TMEV	[51]
	T cells	EAE	[47]
	pt serum	sepsis	[109]

MMP-11	dermal/synovial fibroblasts	*S.aureus*- septic arthritis		[25]
	intestine	necrotizing enterocolitis		[98]
	macrophages	*M. tuberculosis*		[37]
	macrophages	EAE		[47]
	astrocytes	TMEV		[51]
MMP-12	A.A. macrophages	N. brasiliensis		[40]
	brain	*Trypanosoma. b. brucei*		[97]
	BALF	bacterial infection	secretory leukoprotease inhibitor	[114]
	liver	cerebral malaria		[79]
	astrocytes	TMEV		[51]
	chondrocytes	*S. pyogenes*	ECM	[30]
MMP-13	gingival tissue	bacterial periodontitis		[115]

	spleen, liver, brain	cerebral malaria		[79]
MMP-18	macrophages			[116]
MMP-19	chondrocytes	*Borrelia burgdorferi*		[31]
MMP-20	dentine	caries (↓)		[117]
MMP-21	macrophages	granulomatous skin lesions		[118]
MMP-23	tendon	inflammation		[119]
MMP-26	intestinal stroma	necrotizing enterocolitis		[98]
	monocytes	MS		[41]
	monocytes	*T. gondii*		[44]
	macrophages		CD44	[36]
MT1-MMP	corneal epithelium	*P. aeruginosa*		[120]
	diabetogenic T cells	type I diabetes	CD44	[74]
	spleen, liver	cerebral malaria		[79]
	neurons	HIV	pro-MMP-2	[93]
MT2-MMP	corneal epithelium	*P. aeruginosa*		[120]

	microglia	EAE (↓)			[47]
MT3-MMP	corneal epithelium	*P. aeruginosa*			[120]
MT4-MMP	corneal substantia propria	*P. aeruginosa*			[121]
MT5-MMP	corneal substantia propria	*P. aeruginosa*			[121]
MT6-MMP	cornea	*P. aeruginosa*			[121]
	macrophages	LPS	MBP		[38]
	myocardium	CVB3 myocarditis		attenuated myocarditis; ↑ survival	[122]
	pt BALF	*P. aeruginosa* pneumonia			[11]
	cornea, lung	*P. aeruginosa*		rapid pathogen clearance	[84, 123]
TIMP-1	spleen	splenic sepsis			[91]
	serum	viral encephalopathy			[106]
	astrocytes	TMEV			[51]
	liver	*S. mansoni*			[94]
	pt serum	sepsis			[109]

	circum-lesion astrocytes	EAE		[52]
	macrophages			[47]
	pt serum	*H. pylori* gastritis		[17]
	macrophages	HCMV		[42]
	CSF	viral meningitis		[107]
	monocytes	MS		[41]
		T. gondii (↓)		[44]
TIMP-2	Airway epithelial cells	*P. aeruginosa* (↓)		[92]
	astrocytes	CNS tuberculosis		[50]
TIMP-3	alveolar macrophages	lung sepsis	↑gelatinases, ↓collagen ↑lung compliance	[124]
TIMP-4	CSF	*A. cantonensis* meningitis		[113]

Table 2.2. Brain infection with *Toxoplasma gondii* induces upregulation of genes for matrix metalloproteases and their endogenous inhibitors. cDNA generated from whole brain mRNA collected at seven post-infection time points was used as template in qRT-PCR reactions with an array of 80+ ECM-associated gene primers. Fold induction shown is at day 21 post-infection as compared with transcript in uninfected brain tissue. mRNA were pooled from three animals per time point.

Gene product	Fold induction
TIMP-1	164.78
MMP-8	95.93
MMP-10	19.86
MMP-14	5.65
MMP-9	4.02
TIMP-2	3.56
TIMP-3	3.44
MMP-2	2.76
MMP-7	2.34
MMP-11	2.20
MMP-15	1.96
MMP-3	1.29
MMP-13	1.07

Figure 2.1 MMP-8, MMP-10, and their inhibitor, TIMP-1, are upregulated in the *T. gondii*-infected brain. a, quantitative real-time PCR of cDNA synthesized from mRNA harvested from forebrain tissue of C57BL/6 mice over a time course from 3-60 d post-infection. Induction is measured as fold change from naïve. b, fluorescence immunohistochemistry (FIHC) of cortical regions of infected mice collected four weeks post-infection shows MMP-8 absent in naïve tissue and associated primarily with the vasculature post-infection. MMP-10 expression on large parenchymal cells is increased post-infection. Green MMP-8 or MMP-10; red, GFAP; blue, DAPI. Bar, 50 μm. c, immunohistochemistry of naïve and infected brain sections for TIMP-1 counterstained with hematoxylin. TIMP-1 is absent in naïve tissue, and associated with astrocytes at the glia limitans.

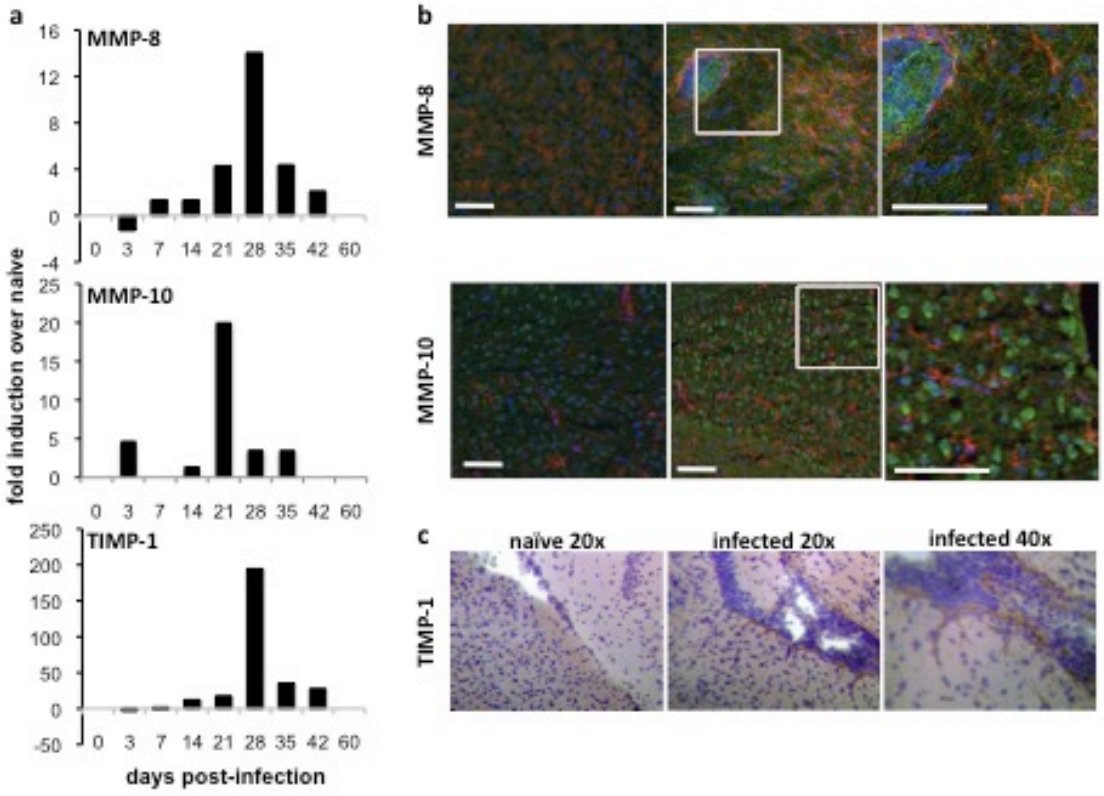

a
MMP-8
MMP-10
TIMP-1
fold induction over naive
days post-infection
b
MMP-8
MMP-10
c
naïve 20x
infected 20x
infected 40x
TIMP-1

Figure 2.2 CD4+ and CD8+ cells express MMPs and TIMP-1 in response to *Toxoplasma* infection. a. The majority of high SSC, GR-1hi cells from infected spleens are positive for MMP-8. Data represent 3 independent experiments; mean/SEM are from n=3 mice/group for each experiment. b. intracellular expression of MMP-8 and -10 and TIMP-1 is increased by CD4+ and CD8+ splenocytes at four weeks post-infection, and by CD4+ and CD8+ populations isolated from infected brain tissue. Shaded area, isotype control for MMPs and TIMP; fine line, uninfected; bold line, infected. Data are representative of two independent experiments, n=3-4 animals per treatment. C. T cell markers colocalize with MMPs in the infected brain cortex. FIHC shows MMP expression is frequently associated with perivascular T cells. Green, CD4; magenta, CD8; blue, DAPI; red, MMP-8 (left) and MMP-10 (right). Bar=50 μm.

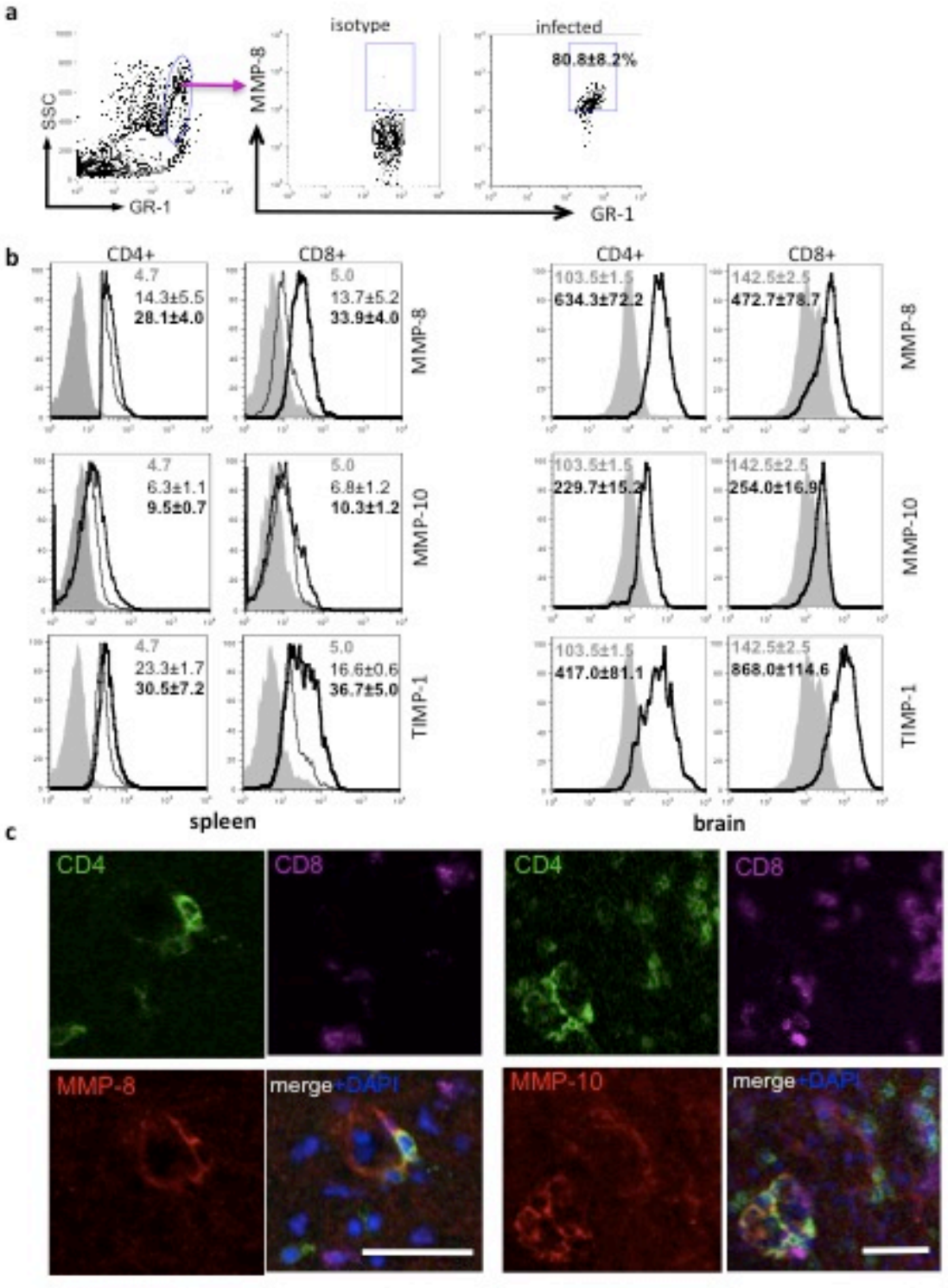

a
isotype
infected
80.8±8.2%
SSC
GR-1
MMP-8
GR-1
b
CD4+
CD8+
CD4+
CD8+
4.7
14.3±5.5
28.1±4.0
5.0
13.7±5.2
33.9±4.0
103.5±1.5
634.3±72.2
142.5±2.5
472.7±78.7
MMP-8
4.7
6.3±1.1
9.5±0.7
5.0
6.8±1.2
10.3±1.2
103.5±1.5
229.7±15.2
142.5±2.5
254.0±16.9
MMP-10
4.7
23.3±1.7
30.5±7.2
5.0
16.6±0.6
36.7±5.0
103.5±1.5
417.0±81.1
142.5±2.5
868.0±114.6
TIMP-1
spleen
brain
c
CD4
CD8
CD4
CD8
MMP-8
merge+DAPI
MMP-10
merge+DAPI

Figure 2.3 TIMP-1 is produced by microglia and upregulated by astrocytes in response to infection with *T. gondii*. A. Flow cytometry of microglia ($CD45^{int}$, CD11b+) isolated from brains of uninfected mice (fine line) compared with those from mice at 4 weeks post-infection (bold line), and isotype control (grey shaded area). Median fluorescence intensity of populations expressing TIMP-1 are compared. B. ELISA measurement of TIMP-1 production by primary astrocyte cultures incubated 24h in medium alone, infected with the RH strain of *T. gondii*, stimulated with sTAg, or with IFN-γ. LPS served as a positive control for proinflammatory stimulus. Data are from two replicates per sample, 3-5 bioreplicates per condition, and three independent experiments. *** $p<0.001$, ** $p<0.01$. C. Immunohistochemistry of chronically infected C57BL/6 cortical tissue section demonstrates colocalization (yellow) of TIMP-1 with astrocytes. Green, TIMP-1; red, GFAP; blue, DAPI. Bar, 50 μm.

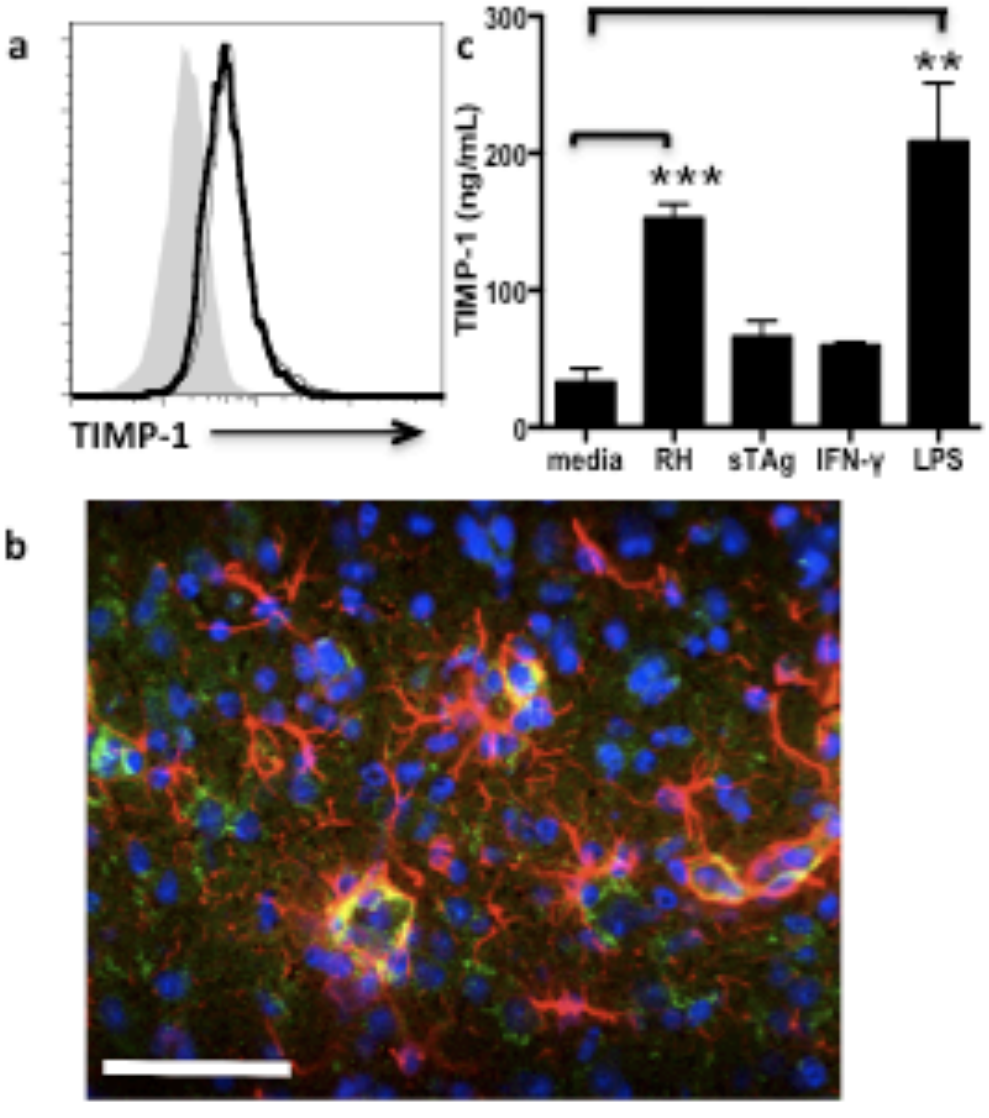

a
TIMP-1
c
TIMP-1 (ng/mL)
300
200
100
0
***
**
media
RH
sTAg
IFN-γ
LPS
b

Figure 2.4 The absence of TIMP-1 alters the kinetics of and immune response to chronic, but not acute, *T. gondii* infection. a. TIMP-1 null animals do not display dysregulated serum cytokine response to acute infection. Cytometric bead analysis of proinflammatory cytokines in serum from peripheral blood collected 7 days post-infection. Markers represent data from individual animals; bars represent mean levels per condition. b. Systemic recovery from acute parasite infection is delayed in the absence of TIMP-1 . Data points show mean percent change ± SEM from pre-infection weight within infected (n=7 or 8, black markers) and naïve (n=2, grey markers) groups of animals.

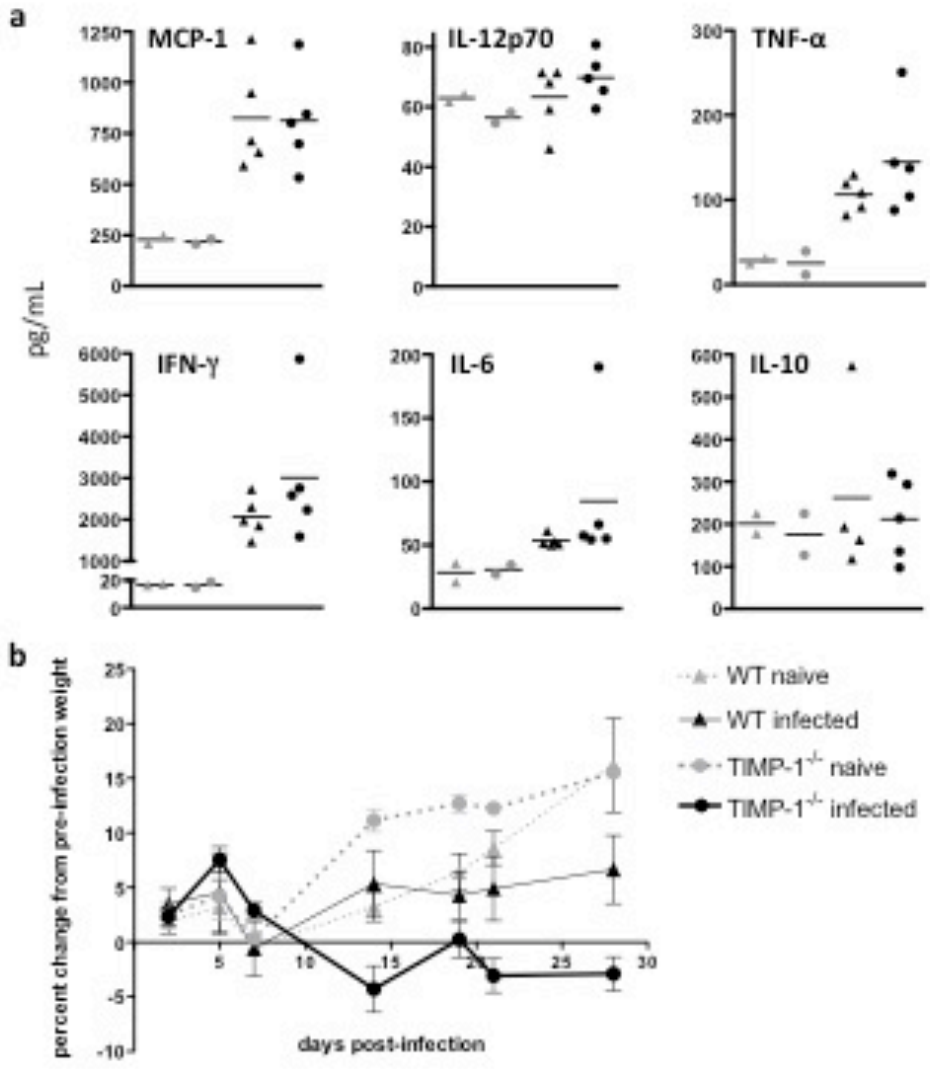

a
MCP-1
IL-12p70
TNF-α
IFN-γ
IL-6
IL-10
pg/mL
b
percent change from pre-infection weight
days post-infection
WT naive
WT infected
TIMP-1-/- naive
TIMP-1-/- infected

Figure 2.5 CNS parasite burden and perivascular cuffing are reduced in the absence of TIMP-1. a. hematoxylin and eosin histology at 100x magnification shows reduced perivascular cuffing and diffuse cellular redistribution in the forebrains of infected TIMP-$1^{-/-}$ mice (bottom row) as compared with infected WT B6.129S (middle row) at 4 wks p.i. b. immunohistochemistry of tissue in (a); glial fibrillliary acidic protein signal (magenta) demarcates glia limitans; nuclear counterstaining (blue) demonstrates perivascular aggregates in the WT (top row), but not in TIMP-$1^{-/-}$ infected brain cortex. Bar, 150 μm (left column); 47 μm (right column). c. real-time PCR of DNA isolated from whole brain tissue of WT and TIMP-$1^{-/-}$ at six weeks post-infection ampified with primers for *T. gondii.* Number of parasites per milligram of tissue were determined by comparison with a purified parasite DNA standard. Data are from two independent experiments, 3 animals per condition, 3 replicates per sample. **, $p<0.01$.

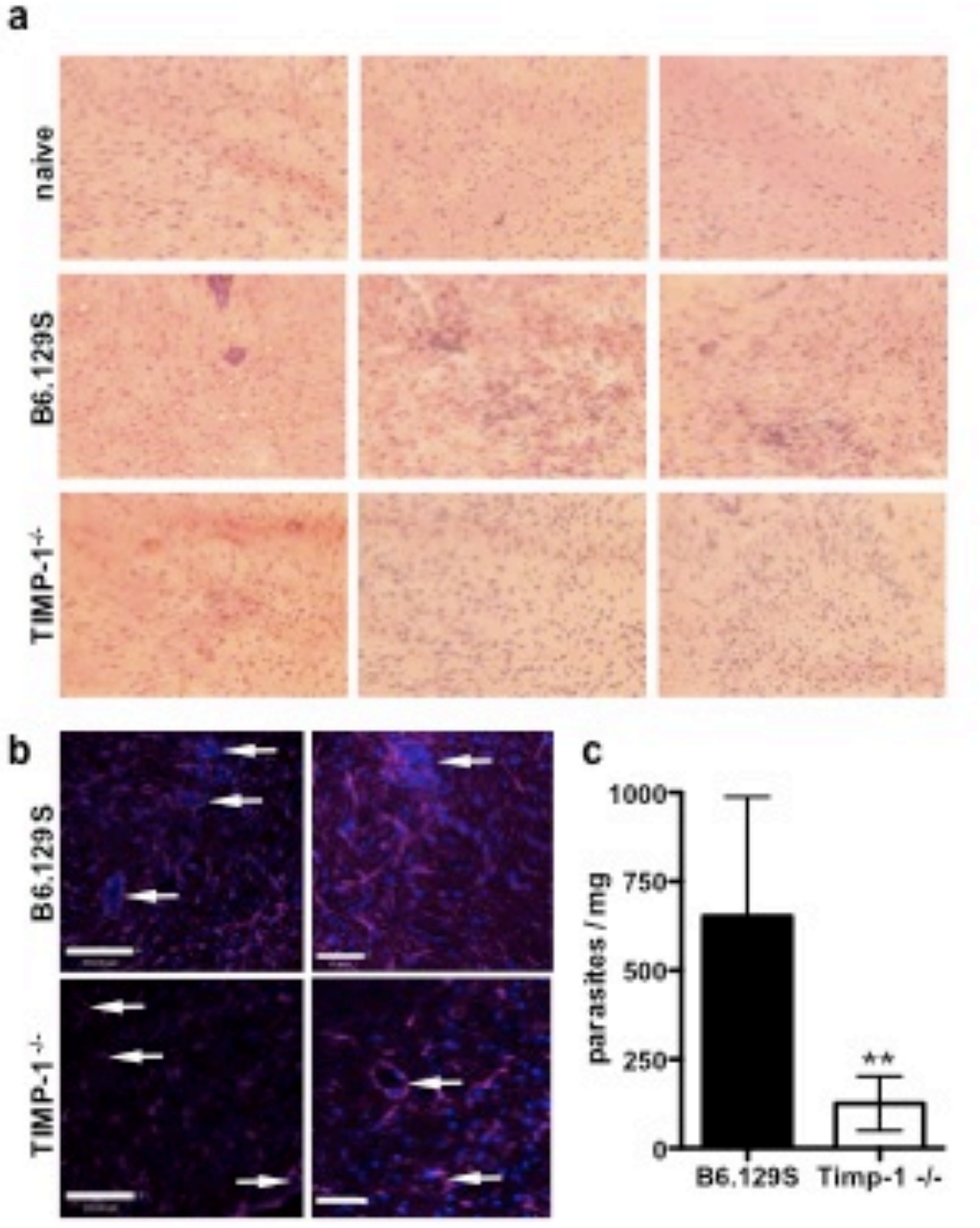
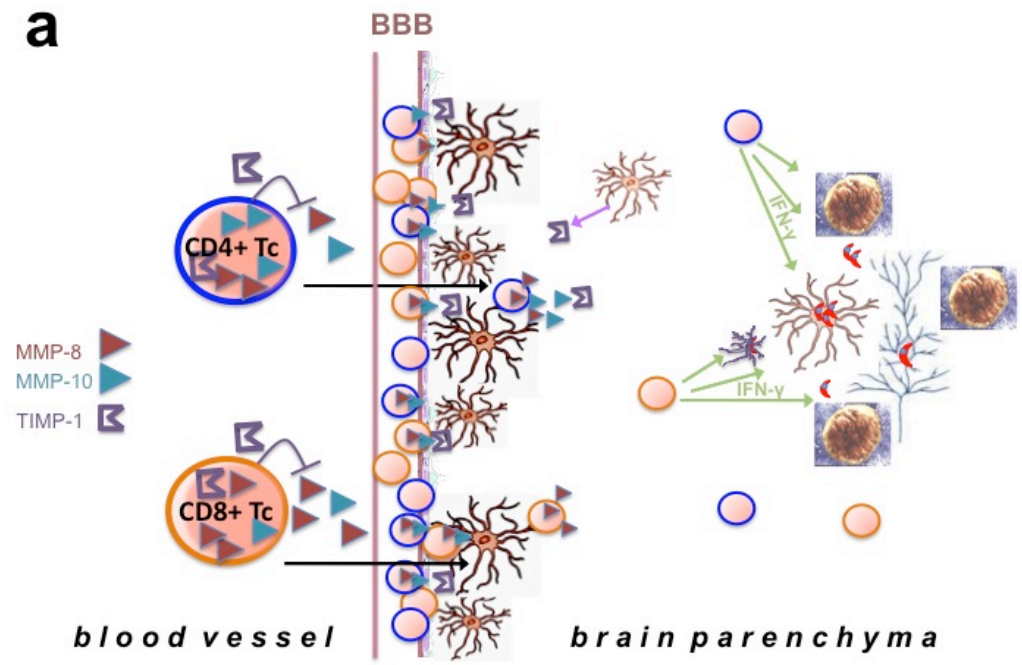
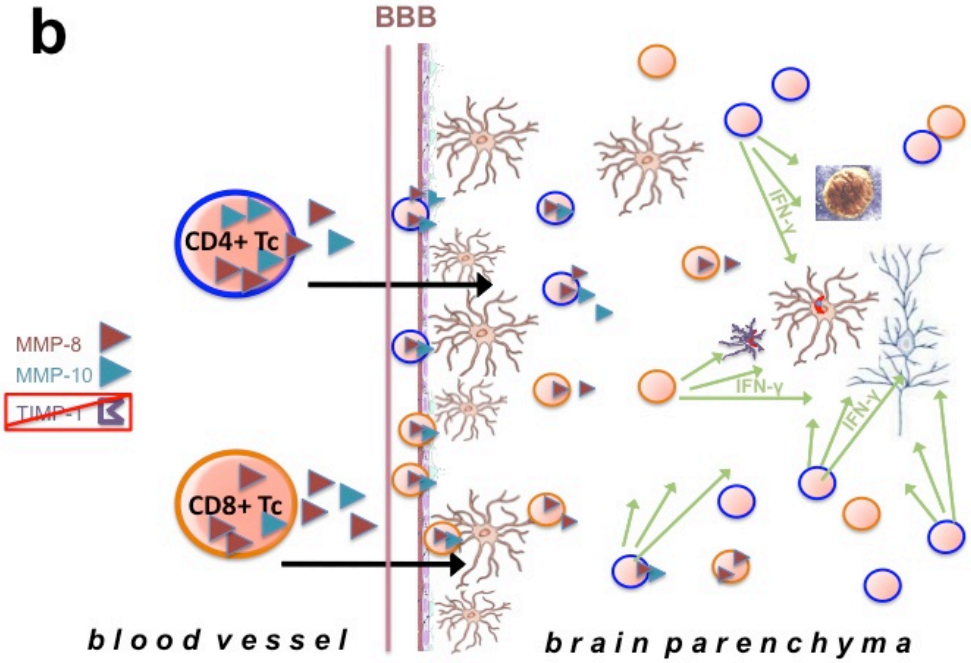

a
naive
B6.129S
TIMP-1-/-
b
B6.129S
TIMP-1 -/-
c
1000
750
500
250
0
parasites / mg
**
B6.129S
Timp-1 -/-

Figure 2.6. Timp-1 may interfere with clearance of *Toxoplasma* from the CNS.
a. T cell populations that produce MMP-8 and MMP-10 also produce TIMP-1 in response to infection. Astrocytes, the major resident glial population, upregulate production of TIMP-1 following infection. TIMP-1 production is particularly abundant by astrocytes functioning as the glia limitans at the blood brain barrier, perhaps in response to metalloprotease production by leukocytes accumulating in the perivascular space. Inhibiting T cell production of metalloproteases may limit their access to foci of parasite infection, where both CD4+ and CD8+ populations control parasite reactivation via effector mechanisms including production of the cytokine IFN-γ.
b. In the absence of TIMP-1, T cell production of MMPs required to transmigrate the parenchymal basement membrane of the blood-brain barrier is less inhibited. T cells responding to parasite infection of the brain are not restrained to the perivascular space, but may instead be distributed throughout the brain parenchyma. A consequence of this redistribution is increased and more diffuse delivery of parasite-inhibiting substances such as IFN-γ, resulting in decreased *Toxoplasma* burden during chronic CNS infection.


a
BBB
CD4+ Tc
CD8+ Tc
MMP-8
MMP-10
TIMP-1
IFN-γ
IFN-γ
blood vessel
brain parenchyma


b
BBB
CD4+ Tc
CD8+ Tc
MMP-8
MMP-10
TIMP-1
IFN-γ
IFN-γ
IFN-γ
blood vessel
brain parenchyma

CONCLUSIONS

In textbook descriptions of host defenses in higher vertebrates, biological surfaces such as the epithelia are often classified as 'mechanical' or 'physical' barriers to molecules and microbes that may pose a threat to the organism. A more nuanced characterization should acknowledge that these barriers are not impenetrable, but selective. More than just able to distinguish beneficial or innocuous microbes from pathogens, cells of the epithelia may even selectively admit disease-inducing bacteria for purposes of refining the immune repertoire. Increased uptake of bacteria has been observed during exposure of epithelia to proinflammatory cytokines, and has been previously thought to be attributable to bacterial invasion strategies and compromise of pericellular integrity. In chapter 1 of this dissertation, it was demonstrated that cells of both the intestinal and respiratory epithelia exposed to inflammatory cytokines may actively endocytose avirulent fixed bacterial pathogens, and that this phenomenon may be related to dramatic cytokine-induced upregulation of apical integrins. Identification of molecules associated with active uptake of bacteria at the epithelia may provide useful targets for development of mucosally-delivered vaccines, a primary goal of the Grand Challenges in Global Health and other organizations working to actualize needle-free vaccine delivery.

It is now understood that the process of inflammation is at the heart of diverse pathologies. Furthermore, it is now recognized that many chronic human diseases for which microbial triggers were never suspected are in fact sequelae of infection with pathogens and the inflammation that results [25]. Notable amongst these are peptic

ulcers which result from colonization of the gastric mucosa with *Helicobacter pylori*, and Human Papilloma Virus, which has been shown to trigger the development of cervical cancer. When we consider that host attempts to eradicate pathogenic invasion by infiltration of effector cells to infected tissue, killing of the pathogen, resolution of inflammation, and remodeling of the extracellular matrix, the importance of investigating matrix metalloproteases in the host immune response becomes clear.

Though metallprotease activity is required for many appropriate immune responses, it must be controlled in this context. Local suppression of MMPs by tissue inhibitors produced within local organ systems ultimately determines the degree of proteolysis. Whether the infected host benefits or is harmed by increased inhibition is multifactorial and infection-specific: it depends on whether proteolytic activity enhances or hinders access of responding immune populations; on whether increased immune activity results in pathogen clearance or immune pathology; and on the sensitivity of cells and tissues in the affected region.

Chapter 2 of this work shows that CNS-infiltrating T cells express previously unreported MMPs during murine infection with the globally significant parasite *Toxoplasma gondii*. Because of the pleiotropic activities of MMPs during both health and disease, their nonspecific inhibition as therapeutic intervention is likely to have unintended consequences. Identification of specific MMPs expressed by relevant populations during brain infection may have broader utility in targeting T cells that mediate autoimmune

diseases such as multiple sclerosis in the same tissue. We further demonstrate that suppressing metalloprotease inhibition can enhance clearance of *Toxoplasma gondii* from chronically infected brain tissue. There is currently no treatment for chronic brain infection with *Toxoplasma*. If it can be shown that this is achieved without CNS pathology to the host, targeting endogenous MMP inhibitors may prove an attractive therapeutic strategy for eradication of this tenacious parasite from infected brain tissue.