Identification of *Toxoplasma gondii* genes involved in the strain-specific modulation of IL-12 cytokine secretion.

by

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ABSTRACT

Toxoplasma gondii is an intracellular pathogen that causes life-threatening toxoplasmosis in developing fetuses and immune-compromised individuals. An immune response to a *Toxoplasma* infection is characterized by the stimulation of high levels of interleukin-12 (IL-12), followed by the production of interferon-γ (IFN-γ) by immune cells. Although IFN-γ is the main mediator of resistance to *Toxoplasma* infection, *Toxoplasma* is able to manipulate the immune response through the regulation of IL-12 production.

Toxoplasma has been shown to modulate the induction of IL-12 in a strain-specific manner. An infection with a type II strain, but not type I and III strains, induce high levels of IL-12 production by macrophages *in vitro*. Previous studies have implicated two *Toxoplasma* genes that play a role in this strain-specific difference in IL-12 production. A rhoptry protein kinase, ROP16, from type I and III strains, was found to be crucial in the suppression of IL-12. A type II dense granule protein, GRA15, induces IL-12 through NF-κB activation.

I have screened F1 progeny from a type III x type II cross for IL-12 induction and NF-κB activation. My preliminary experiments indicate that there are other *Toxoplasma* factors involved in the strain-specific inhibition of IL-12, and this inhibition has a genetic basis. To understand the role of IL-12 regulation by *Toxoplasma*, I intend to (i) identify novel *Toxoplasma* genes involved in the inhibition of IL-12 secretion (ii) test and characterize the effects of the Toxoplasma protein ROP38 on IL-12 signaling and (iii) determine the target/s of *Toxoplasma* inhibition of IL-12 production.

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Background and Significance

Toxoplasma gondii is an obligate intracellular protozoan parasite capable of infecting most warm-blooded animals including humans (reviewed in[1]). The sexual cycle of *Toxoplasma* occurs exclusively in felines, with two stages present in intermediate hosts: the rapidly replicating tachyzoites and the dormant encysted bradyzoites (reviewed in[2]). Intermediate hosts become infected with *Toxoplasma* by ingesting oocytes from cat feces or tissue cysts from the flesh of another intermediate host. The majority of *Toxoplasma* strains from Europe and North America belong to three distinct clonal lineages, referred to as types I, II and III[3]. Generally, immune-competent people infected with these strains do not suffer from any symptoms, although a life-long chronic infection is established. However, severe and life threatening disease in immunocompromised individuals and developing fetuses of pregnant women infected for the first time can develop.

Toxoplasma has three secretory organelles: micronemes, rhoptries and dense granules (reviewed in[4,5]). Active invasion of *Toxoplasma* into the host cell involves the sequential release of proteins from these organelles along with the formation of a parasitophorous vacoule (PVM) around the parasite (reviewed in[6]). Proteins released from the rhoptries and dense granules can traffic to the locations in the host cell, including the host nucleus and outside of the PVM where they are able to modulate host-signaling pathways.

A primary infection with a pathogen stimulates high levels of the proinflammatory cytokine interleukin-12 (IL-12) by phagocytic cells of the innate immune system. IL-12 then stimulates T cells and natural killer (NK) cells to produce the cytokine IFN-γ, which stimulates cells to induce anti-pathogen gene expression programs (reviewed in[7]). IL-12 is required for resistance to toxoplasmosis due to its essential role in stimulating the production of IFN- γ [8,9]. The biologically active form of IL-12 is comprised of two disulfide-linked subunits, p40 and p35 (reviewed in[7]). *In-vivo* studies have demonstrated the importance of both subunits in the resistance to *Toxoplasma* infection. Both IL-12p40-/- and IL-12p35-/- mice are highly

susceptible to toxoplasmosis, and neutralization of IL-12 in *Toxoplasma* infected mice results in reduced IFN-γ production, uncontrollable parasite load and increased susceptibility[10].

Toxoplasma has been shown to modulate the induction of IL-12 in a strain-specific manner. An infection with a type II strain, but not type I and III strains, induces high levels of IL-12 production by macrophages *in vitro*[11]. Initiation of signaling pathways that affect IL-12 production is one way this strain-specific difference arises. Previous studies have shown that interference with nuclear factor-κB (NF-κB) translocation, activation of signal transducer and activator or transcription 3 and 6(STAT3/6) and mitogen-activated protein kinases (MAPK) activation leads to the strain differences in IL-12 induction.

The *p40* promoter contains κB -binding sites and is transcriptionally regulated by NF-κB[12]. Infection of macrophages with type II strains, but not type I or III strains, induces the activation of NF-κB resulting in the induction of IL-12 production [11-13]. GRA15, a *Toxoplasma* dense granule protein, from type II strains activates NF-κB resulting in the induction of IL-12p40[14][table 1].

STAT proteins are transcription factors, which mediate the effects of many cytokines. Activated STAT3 and STAT6 negatively regulate IL-12 proinflammatory responses, thus preventing detrimental systemic inflammation as a result of uncontrolled proinflammatory cytokine production (reviewed in[15]). Activation of STAT3 inhibits *p40* gene expression by preventing NF-κB recruitment to its promoter[16]. Overexpression of STAT6 inhibits NF-κB nuclear translocation[17]. *Toxoplasma* has been shown to hijack these regulatory systems to suppress IL-12 production. A rhoptry protein kinase, ROP16, from types I and III strains, induces prolonged STAT3/6 phosphorylation leading to low levels of IL-12 production[18][table 1].

MAPK transduction cascades culminate in the induction of multiple proinflammatory genes that are important in the defense against pathogens, including
IL-12p40[19-21]. MAPK signaling is initiated through toll-like receptor (TLR) engagement of a pathogen associated molecular patterns (PAMPs) resulting in the recruitment of the adapter molecule myeloid differentiation factor 88(MyD88)(reviewed in [22]). This leads to the sequential association of intracellular protein molecules that activate MAPKs. Present in both cytosolic and nuclear compartments, activated

MAPKs include extracellular signal- regulated kinase (ERK), p38 MAPK, and stress-activated protein kinase/c-Jun N-terminal kinase(SAPK/JNK)(reviewed in[22]).Upon activation, they themselves activate numerous transcription factors including CCAAT-enhancer-binding protein β(C/EBPβ),which bind to the IL-12p40 promoter[23]. In response to soluble *Toxoplasma* antigen (STAg), macrophages activate the TRAF6-dependent phosphorylation of MAPK family members p38 and ERK1/2[24]. Using specific inhibitors of MAPK phosphorylation, *Toxoplasma*-induced phosphorylation of p38 promoted IL-12 production, while phosphorylation of ERK1/2 inhibited IL-12 [25]. Intestinal epithelial cells infected with strains from all three lineages, differed in their ability to induce phosphorylation of ERK1/2[26], but the genetic basis behind this difference is unknown.

Current data generated in the Saeij lab suggests that there are other *Toxoplasma* proteins involved in the strain-specific induction of IL-12.Expression of type I ROP16 ($ROP16_I$) in a type II strain (II: $ROP16_I$) decreases the levels of IL-12. Expression of $GRA15_{II}$ in a type I strain (I: $GRA15_{II}$) increases the levels of IL-12 secreted. However, a type I ROP16 knockout (I:ROP16KO) expressing $GRA15_{II}$(I:ROP16KO+ $GRA15_{II}$) still induces lower levels of IL-12 than infection with a type II strain [fig 1].

The goal of my thesis was to understand the role of IL-12 regulation by *Toxoplasma* through the following aims:

i. Identify novel *Toxoplasma* genes involved in the inhibition of IL-12 secretion.

ii. Test and characterize the effects of the *Toxoplasma* protein ROP38 on IL-12 signaling.

iii. Determine the target/s of *Toxoplasma* inhibition of IL-12 production.

RESULTS

Genetic analysis indicates more than two *Toxoplasma* genes regulate macrophage IL-12 secretion.

To further explore this possibility, I first screened F1 progeny from a type II x type III cross for IL-12 induction and NF-κB activation. Because *Toxoplasma* tachyzoites are haploid all alleles are expressed in the phenotype of interest because there is no masking of recessives by dominant alleles. Therefore, the F1

generation can be used to screen for genes without further crosses. These progeny contain the type II copy of both GRA15 and ROP16 (fig.2), thus eliminating the possibility that any detected difference between the progeny is due to these genes. These F1 progeny exhibited varied levels of IL-12 secretion and NF-κB activation [fig. 3]. This is consistent with our proposed hypothesis that, in addition to GRA15 and ROP16, there are other *Toxoplasma* molecules involved in the modulation of IL-12.

Coinfection indicates another *Toxoplasma* gene inhibits IL-12 induction.

Interestingly, a coinfection with F1 progeny strains that induce low levels of IL-12 and the F1 progeny that induces the highest level of IL-12, suggests that there may be an inhibitory type III *Toxoplasma* protein responsible for low levels of IL-12 production [fig. 4]. This inhibitory *Toxoplasma* factor responsible for low levels of IL-12 production is present in one or more of these progeny strains.

Future Directions

The experiments proposed below aim to determine the type III *Toxoplasma* gene(s) responsible for the inhibition of IL-12 induction. The genes discovered could then be further characterized to determine the molecular mechanisms governing their function. These experiments will further the understanding of how *Toxoplasma* is able to manipulate the host immune response through the selective regulation of IL-12, and may present new targets for treatment of *Toxoplasma*.

Experimental Design

Aim 1: Identify novel *Toxoplasma* genes involved in the inhibition of IL-12 secretion.

The overall goal of this aim is to determine new *Toxoplasma* factors that are responsible for inhibition of IL-12 induction. While enzyme linked-immunosorbent assay (ELISA) is most commonly used to detect cytokine levels, a reporter cell line would allow for quantification of IL-12 promoter activity. Also, one would gain some understanding of how IL-12 gene expression is regulated by *Toxoplasma* through the

specific and rapid analysis of its promoter activity. Therefore, a stable reporter cell line will be developed in RAW264.7 murine macrophages. Lentivirus will be used to deliver constructs containing the p35 and p40 promoter sequences driving the expression of reporter proteins, GFP and Firefly Luciferase. This will allow for the direct quantification of IL-12 mediated transcription in populations of *Toxoplasma*-infected cells.

Sub-aim 1.1: Identify genes necessary for IL-12 inhibition by mutagenesis

Toxoplasma has a high frequency of non-homologous end joining, thus allowing random insertional mutagenesis by transfection of a plasmid. Because of the haploid nature of *Toxoplasma* tachyzoites, insertional mutagenesis can lead to both loss of function and gain of function mutations without the requirement of further crosses.

F1 progeny (with type II copies of both ROP16 and GRA15) will be selected for mutagenesis based on two criteria. They should induce relatively low levels of IL-12 compared to the wild-type type II strain and be able to inhibit the IL-12 induction ability of the type II strain. After transfection of a plasmid, parasites containing insertions will be selected for and IL-12 reporter cell lines will be infected with pools of these mutants. Several rounds of flow cytometry (FACS) analysis will be done to obtain individual mutant parasites that induce higher levels of IL-12 than the unmutated F1 strain. Once mutants are found that do activate the reporter cell line, ELISA will be done to confirm this result. To identify the gene disrupted by plasmid insertion, plasmid rescue or inverse PCR will be done followed by sequencing.

An alternative approach would be to complement the F1 progeny strain that induces the highest level of IL-12 with a type III cosmid library. Cosmid clones capable of complementing the mutant phenotype will be determined by plasmid rescue or inverse PCR. Because many loci are covered by a single cosmid, the candidate loci of interest will be narrowed and confirmed using overlapping cosmids. Once type III genes are found, they will be cloned and knocked out or overexpressed in type II strains to confirm their inhibitory phenotype.

Sub-aim 1.2: Candidate gene approach

Given the F1 progeny data that I have generated [fig.2], an alternative approach would be to determine the genomic location common to all F1 progeny from the type II x type III cross that are able to inhibit IL-12. To achieve this, I will perform a coinfection experiment using all the F1 progeny with the type II copy of ROP16 and the F1 progeny strain that induces the highest level of IL-12. Importantly, the 40 F1 progeny from the type II x type III cross have been genotyped at 135 informative markers across the genome. This allows for the identification of candidate genes that are tightly linked to these genetic markers. The genomic locations common to all F1 strains that inhibit IL-12 will be examined and potential candidate genes will be chosen using a set of criteria. It is expected that candidate proteins will be secreted into the host cell and thus have a signal peptide. Candidate genes should also be polymorphic or differentially expressed between type II and III strains. Candidate type III genes that fit the above criteria and are present within the common genomic region will be cloned and expressed transgenically in type II strains and tested for IL-12 inhibition. Candidate gene knockouts or overexpression in the *Toxoplasma* strain from which it came from will be used verify its role in this phenotype.

Aim 2: Test and characterize the effects of the Toxoplasma protein ROP38 on IL-12 signaling.

A recently described rhoptry protein, ROP38, has been implicated in the modulation of host genes associated with ERK1/2 signaling[27]. Expression levels of ROP38 vary among strains and it is secreted into the host cell therefore presenting itself as a candidate gene for inhibition of IL-12 induction. Overexpression of ROP38 will be done to first determine if it is responsible for pERK1/2 levels in *Toxoplasma* infected cells. To investigate if ROP38 inhibits IL-12 secretion in a p-ERK1/2 dependent manner, the protein will be cloned and tagged from the strain in which it activates p-ERK1/2 and expressed transgenically in a strain that activates IL-12. I will use these transgenic parasites to infect bone-marrow derived macrophages (BMDMs) and ELISA will be performed to measure both IL-12p40 and IL-12p70 levels. Overexpression of ROP38

should replicate pERK1/2 inhibition of IL-12. To further characterize the role of ROP38 in IL-12 inhibition, microarrays will be done to assess the effect of ROP38 on global gene expression in infected BMDMs.

Aim 3: Determine the target of *Toxoplasma* inhibition in IL-12 induction.

The p40 and p35 genes are regulated at the level of transcription by a number of transcription factors (reviewed in[7]). In addition to NF-κB and C/EBPβ, the p40 gene is positively regulated by an ETS site upstream of the NF-κB binding sequence, which is made up of a large complex composed of ETS-2, PU.1, interferon regulatory factor-1 (IRF-1) and the NF-κB subunit, c-Rel [28]. Interferon consensus sequence binding protein (ICSBP) also interacts with the ETS-site[29] and ICSBP-/- mice are susceptible to *Toxoplasma* infection, which is associated with decreased IL-12p40 production[30]. ICSBP and IRF-1 also regulate human p35 transcription by binding to the ICSBP-response element *in-vivo*[31]. Inhibition has also been found to act at the level of transcription, with a repressor site located between the ETS and NF-κB sites. This site known as GA-12, (GATA sequence in the IL-12 promoter) is occupied by a GA-12-binding protein (GAP12) in unstimulated cells[32]. Interestingly, a past study showed that hyperactivation of the ERK pathway led to the activation of GAP-12, resulting in repression of IL-12p40 production in LPS-stimulated mouse macrophages[33].

Transcription of either the p40 or p35 genes could be inhibited by *Toxoplasma* through the competition by inhibitory proteins for DNA binding sites and the induction of inhibitory gene products. *Toxoplasma* may activate other pathways in the cell, for instance ERK1/2 signaling pathway, which can inhibit IL-12 production through any of the mechanisms mentioned above. The *Toxoplasma* protein identified from the mutagenesis experiment could play a role in the inhibition of *p40* or *p35* transcription.

To test if the candidate *Toxoplasma* protein competes for DNA binding sites, an electrophoretic mobility-shift assay will be done. Radiolabeled oligonucleotides encompassing the *p40* and *p35* promoter sequences will be incubated with the HA-tagged candidate *Toxoplasma* protein. The mixture will be resolved by SDS-PAGE and the presence of the protein will be assessed using anti-HA antibody. It is possible that the

Toxoplasma protein may not bind directly to the promoter, but may induce inhibitory proteins that are possible binding partners of the selected *Toxoplasma* protein. To identify possible binding partners, HA-tagged *Toxoplasma* protein will be ectopically expressed in the F1 strain from which it came from. Transgenic parasites will be infected into BMDMs, and cell extracts will be prepared post-infection. The proteins will be immunoprecipitated using anti-HA antibodies and interacting partners will be identified by mass spectrometry analysis. Confirmed interaction partners will prove useful in determining the mechanism of IL-12 inhibition.

Conclusion

The proposed experiments will help elucidate the mechanisms that *Toxoplasma* gondii uses to evade the host immune response and establish a chronic infection. Identification and characterization of the *Toxoplasma* genes involved in IL-12 inhibition will be useful in understanding how the pro-inflammatory response is manipulated by the parasite to ensure its survival and that of the host. The characterization of IL-12 inhibition may also present new targets for treatment of *Toxoplasma* in humans.

Material and Methods

Parasites and cells.

Parasites were maintained in vitro by serial passage on monolayers of HFFs at 37°C in 5% CO_2. Me49 and CTG were used as representative type II and type III strains respectively. The F1 progeny from type II x type III crosses were described previously [34]. BMDM were obtained from C57BL/6 mice and maintained in RPMI supplemented with 20% L929 cell-conditioned medium, 10% heat-inactivated FBS, 2mM 1-glutamine, 1mM sodium pyruvate, 1x MEM nonessential amino acids, and 50 μg/ml of streptomycin. A HEK293 stable cell line expressing four copies of the NF-κB consensus transcriptional response element driving the expression of GFP and luciferase (System Biosciences) were grown in DMEM supplemented

with 10% heat-inactivated FBS, 2mM 1-glutamine, 1-mM sodium pyruvate, 1 x MEM nonesstional amino acids, 10mM HEPES, 50 μg/ml of streptomycin and 20 μg/ml gentamycin.

In vitro cytokine ELISA.

C57BL/6 BMDMs were seeded (5 x 10^4 per well) in 96-well plates and left to adhere overnight at 37°C in 5% CO_2. Cells were infected with freshly lysed *Toxoplasma gondii* tachyzoites at MOI =10,5,2.5, and supernatants (200 μl) were collected 24hrs post-infection and stored at −20°C if necessary. IL-12p40/p70 levels were determined, for the cells infected with equal numbers of viable parasites as determined by plaque assay, using a commercially available ELISA kit (eBiosciences Mouse IL-12 (p40), IL-12 (p70) ELISA Set) according to the manufacturer's instructions.

Reporter assay

NF-κB/293/GFP-Luc cells were seeded (5 x 10^4 per well) in 96-well plates and left to adhere 4 hrs before infection at 37°C in 5% CO_2. Cells were infected with freshly lysed *Toxoplasma gondii* tachyzoites at MOI = 10,5,2.5. After 24hrs post-infection, cells were lysed and assayed with the Dual-Luciferase assay system (Promega). Firefly luciferase activity was measure in triplicate and normalized to uninfected cells.

Plaque Assay

For all infection assays, cells were infected with different MOIs and a plaque assay was done to determine the viability of each strain. 100 parasites per well were added to confluent HFFs in a 24-well plate and incubated for 5-7 d at 37°C. The number of plaques was counted using a microscope and the percent viability determined.

Table 1: Toxoplasma genes that modulate IL-12 production

	Type I	Type II	Type III
ROP16	+	-	+
GRA15	-	+	-
IL-12	-	+	-

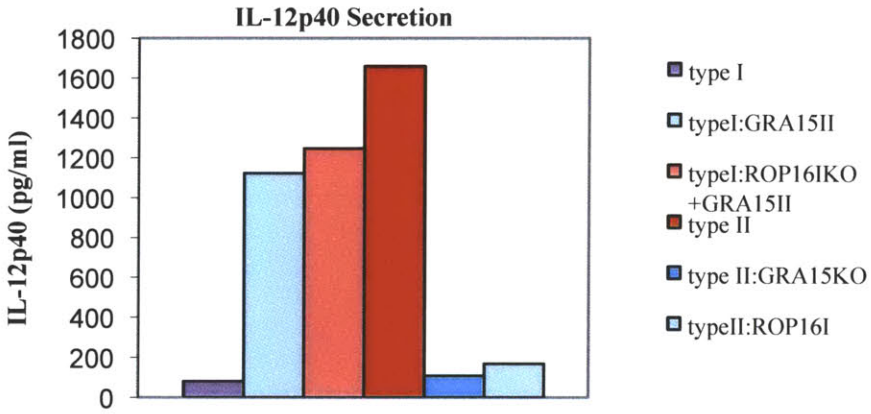


Figure 1: IL-12p40 secretion from *Toxoplasma* infected BMDMs 24hrs post-infection
Expression of $ROP16_I$ in a type II strain decreases the levels of IL-12 and expression of $GRA15_{II}$ in a type I strain increases the levels of IL-12 secreted. However, a type I ROP16KO expressing $GRA15_{II}$ still induces lower levels of IL-12 than infection with a type II strain, suggesting that other *Toxoplasma* proteins are involved in the strain-specific induction of IL-12

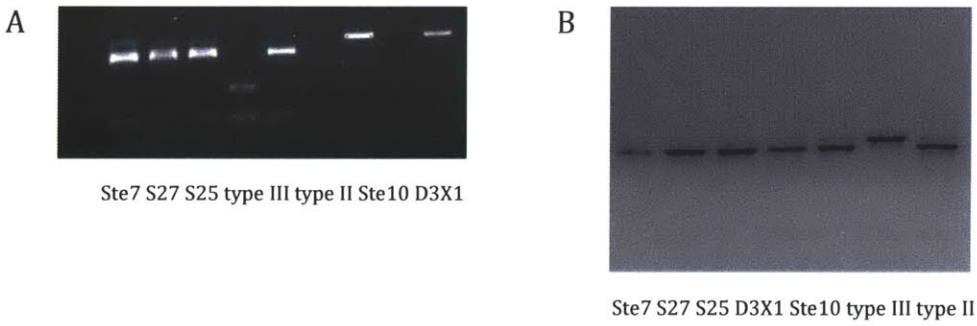
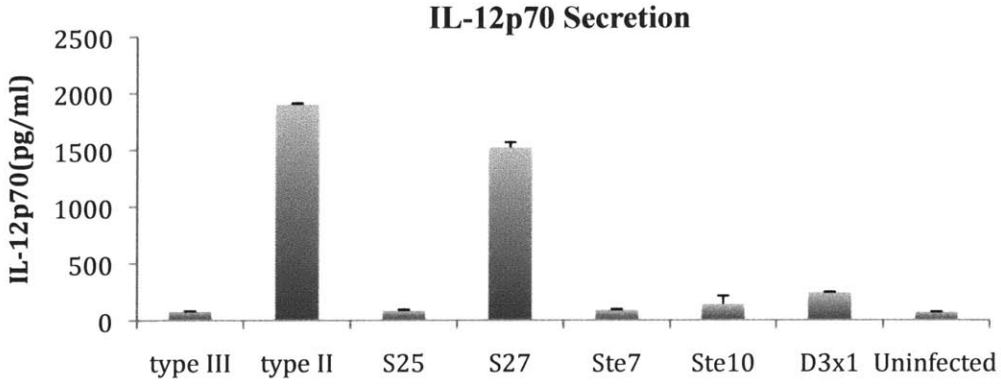
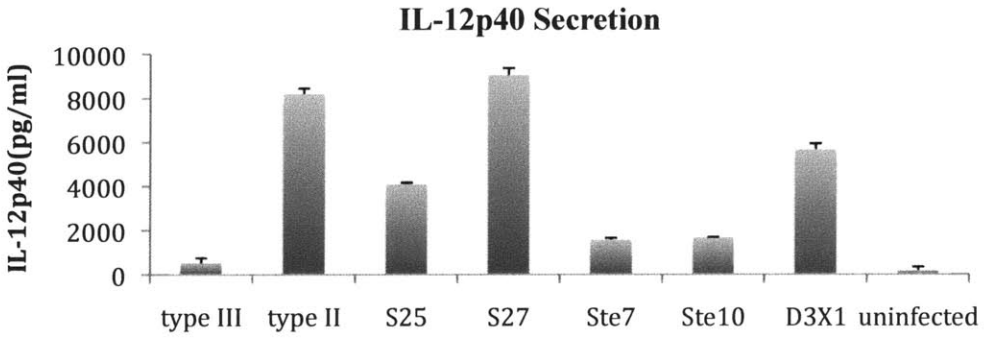


Figure 2: Genotyping of F1 progeny.
A, HindIII restriction digest pattern of *ROP16* fragment amplified from genomic DNA of *Toxplasma.* The restriction digest of the F1 progeny resulted in two fragments 1585bp and 539bp. This corresponds to type II *ROP16*. B, PCR amplified *GRA15* fragment from genomic DNA of *Toxoplasma*. The F1 progeny posses the type II *GRA15*(~1.7kb).





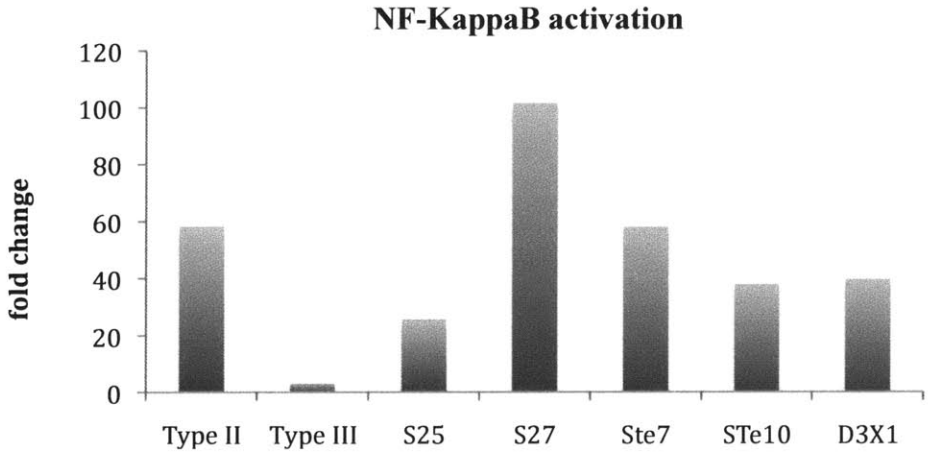


Figure 3: IL-12p40, IL-12p70 and NF-KappaB induction24 hrs post infection.
F1 progeny from a type II x type III cross exhibit varied levels of IL-12 secretion and NF-KappaB induction. These progeny contain the type II copy of both GRA15 and ROP16, thus eliminating the possibility that any detected difference between the progeny is due to these genes.

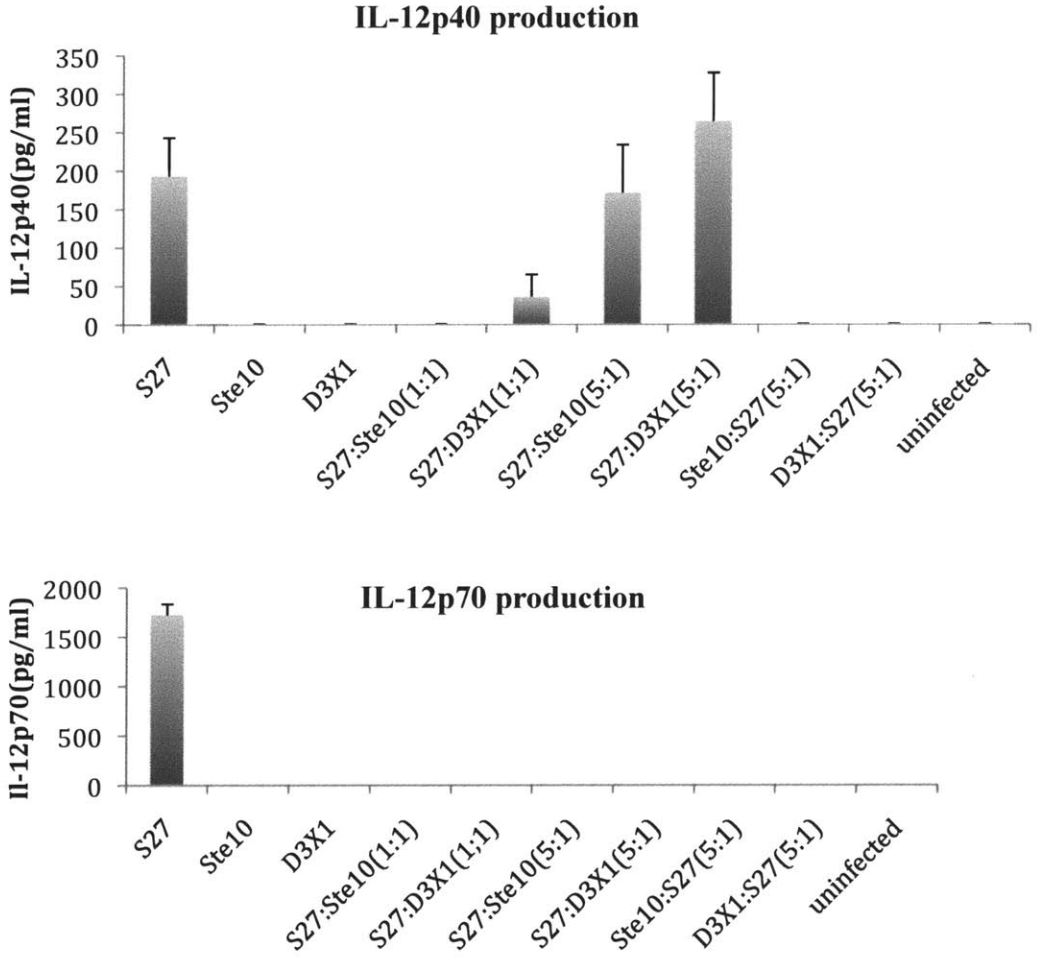


Figure 4: IL-12p40 and IL-12p70 secretion 24hrs post-infection.
A coinfection with F1 progeny strains that induce low levels of IL-12 (Ste10 and D3X1) and the F1 progeny that induces the highest level of IL-12 (S27), suggests that there may be an inhibitory type III *Toxoplasma* protein responsible for low levels of IL-12 production. The bracketed numbers represent the respective MOIs.

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