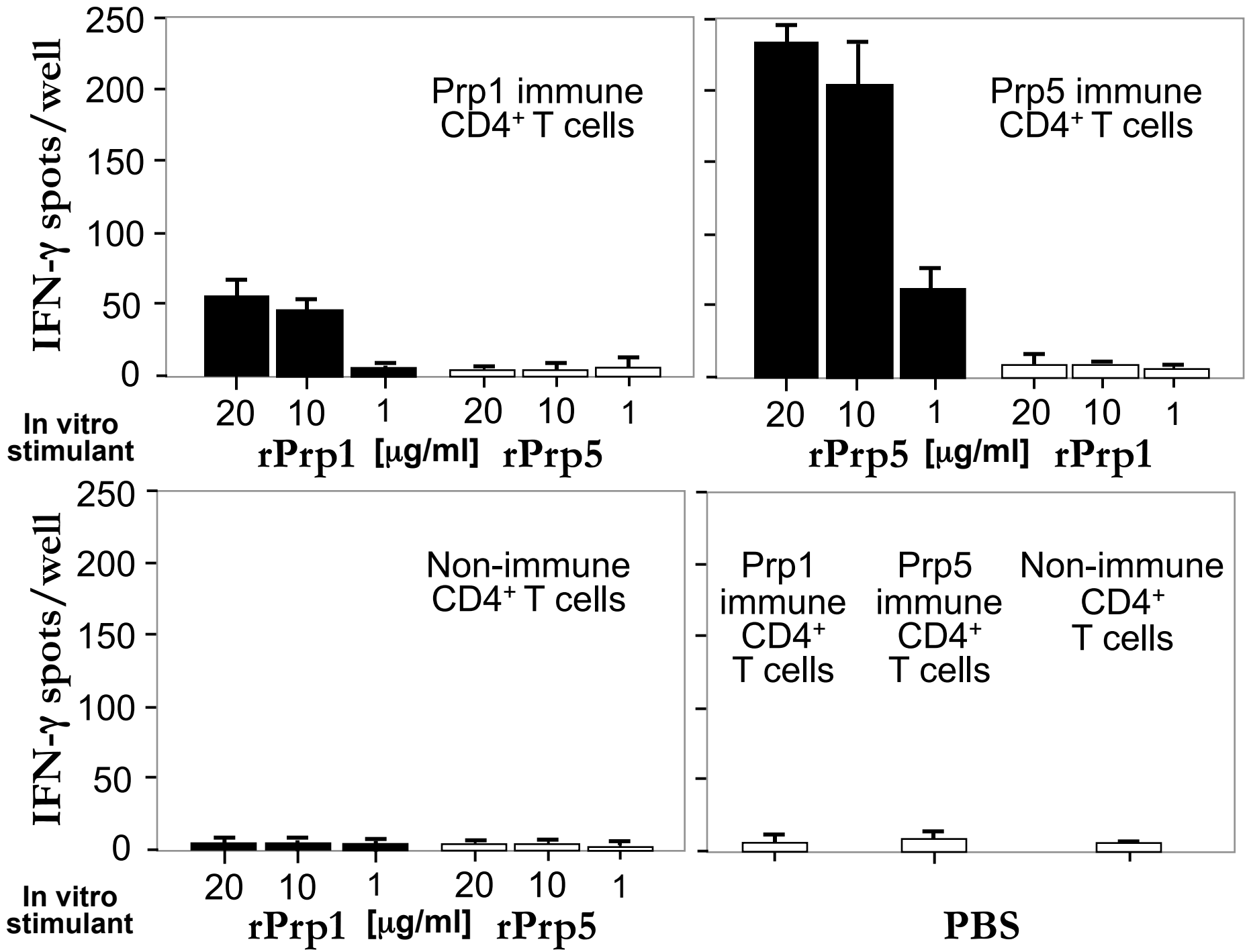
IFN-γ spots/well
250
200
150
100
50
0
Prp1 immune
CD4+ T cells
20 10 1 20 10 1
In vitro stimulant
rPrp1 [μg/ml] rPrp5
Prp5 immune
CD4+ T cells
20 10 1 20 10 1
rPrp5 [μg/ml] rPrp1
Non-immune
CD4+ T cells
Prp1 immune CD4+ T cells
Prp5 immune CD4+ T cells
Non-immune CD4+ T cells
PBS

Assessment of IFN-γ production by immune and non-immune $CD90^+$ T cells derived from C57BL/6 mice conducted by IFN-γ ELISPOT assays

Purification of the *E. coli*-expressed recombinant proline-rich protein (rPrp1) was conducted as reported (Herr. et al., 2007). The pET32b vector was used for expression of rPrp5. This vector encodes a N-terminal thioredoxin fusion peptide (Trx-Tag™; Novagen; San Diego, CA), which enhanced solubility of the recombinant protein during the chromatographic isolation procedure. The thioredoxin fusion peptide was removed from the Ni-affinity-purified recombinant protein by proteolysis with biotinylated thrombin (Novagen). The thrombin was then removed with small aliquots of streptavidin-agarose as recommended by the supplier. The endotoxin content of stock solutions of the two recombinant proteins was determined by use of a *Limulus* ameboycte lyase kit (QCL-1000; BioWhittaker, Walkersville, MD) as reported (Herr. et al., 2007). Purified rPrp1 or rPep5 (1 μg), plus an adjuvant that contained unmethylated CpG dinucleotides in a synthetic oligodeoxynucleotide preparation as reported (Li, et al., 2001), were used to immunize four 8-week-old female C57BL/6 mice. The immunization protocol was the same as previously described (Herr et al, 2007). Two weeks after the second immunization, the spleens of the two groups of mice were separately harvested, pooled and macerated. Isolation of $CD90^+$ T cells from the total splenocytes, collection of the antigen-presenting cells (APCs), and performance of the enzyme-linked immunospot (ELISPOT) assays were the same as reported (Tarcha et al., 2006). In brief, 96-well microplates containing a PVDF membrane disc in each well (Multiscreen HTS™ IP; Millipore; Billerica, MA) were coated with monoclonal anti-murine IFN-γ antibody (Clone# AN-18; eBioscience; San Diego, CA), washed with PBS and blocked with HL-1 growth medium (BioWhittaker) containing 2% fetal calf serum. $CD90^+$ immune T cells (4×10^5) plus APCs (2.5×10^5) were then added to each well and incubated with or without the recombinant protein (rPrp1, rPrp5, or medium alone) as described (Tarcha et al.,

2006). After incubation, the cells were removed from the filtration membrane of each well, the plates were washed, and biotin-labeled anti-IFN-γ monoclonal detection antibody (Clone# R4-6A2-biotin; eBioscience) was added to the wells followed by streptavidin-alkaline phosphatase. The plates then were washed and the phosphatase substrate BCIP/NBT (KPL, Gaithersburg, MA), was added for color development. Each membrane was analyzed using an automated ELISPOT reader system (ZellNet Consulting Inc. Fort Lee, NJ). The frequency of IFN-γ secreting antigen-specific $CD90^+$ T-cells was calculated as the number of spots per 4×10^5 $CD90^+$ T-cells seeded in the presence of antigen minus the number of spots per equal number of $CD90^+$ T-cells in medium alone.

References

1. Cox, R. A., and D. M. Magee. 2004. Coccidioidomycosis: host response and vaccine development. Clin. Microbiol. Rev. **17:**804-839.

2. Herr, R. A., C.-Y.Hung, and G. T. Cole. 2007. Evaluation of two homologous proline-rich proteins of *Coccidioides posadasii* as candidate vaccines against coccidioidomycosis. Infect Immun. **75**: 5777–5787.

3. Kulkarni, R. D., H. S. Kelkar, and R. A. Dean. 2003. An eight-cysteine-containing CFEM domain unique to a group of fungal membrane proteins. Trends Biochem. Sci. **28:**118-121.

4. Li, K., J.-J. Yu, C.-Y. Hung, P. F. Lehmann, and G. T. Cole. 2001. Recombinant urease and urease DNA of *Coccidioides immitis* elicit an immunoprotective response against coccidioidomycosis in mice. Infect. Immun. **69:**2878-2887.

5. Tarcha, E. J., V. Basrur, C.-Y. Hung, M. J. Gardner, and G. T. Cole. 2006. A recombinant aspartyl protease of *Coccidioides posadasii* induces protection against pulmonary coccidioidomycosis in mice. Infect. Immun. **74:**516-527.

6. Zhu Y., V. Tryon, D.M. Magee, and R.A. Cox. 1997. Identification of a *Coccidioides immitis* antigen 2 domain that expresses B-cell-reactive epitopes. Infect. Immun. **65**: 3376-3380.

Assessment of IFN-γ production by immune and non-immune $CD90^+$ T cells derived from C57BL/6 mice conducted by IFN-γ ELISPOT assays

Purification of the *E. coli*-expressed recombinant proline-rich protein (rPrp1) was conducted as reported (Herr. et al., 2007). The pET32b vector was used for expression of rPrp5. This vector encodes a N-terminal thioredoxin fusion peptide (Trx-Tag™; Novagen; San Diego, CA), which enhanced solubility of the recombinant protein during the chromatographic isolation procedure. The thioredoxin fusion peptide was removed from the Ni-affinity-purified recombinant protein by proteolysis with biotinylated thrombin (Novagen). The thrombin was then removed with small aliquots of streptavidin-agarose as recommended by the supplier. The endotoxin content of stock solutions of the two recombinant proteins was determined by use of a *Limulus* ameboycte lyase kit (QCL-1000; BioWhittaker, Walkersville, MD) as reported (Herr. et al., 2007). Purified rPrp1 or rPep5 (1 μg), plus an adjuvant that contained unmethylated CpG dinucleotides in a synthetic oligodeoxynucleotide preparation as reported (Li, et al., 2001), were used to immunize four 8-week-old female C57BL/6 mice. The immunization protocol was the same as previously described (Herr et al, 2007). Two weeks after the second immunization, the spleens of the two groups of mice were separately harvested, pooled and macerated. Isolation of $CD90^+$ T cells from the total splenocytes, collection of the antigen-presenting cells (APCs), and performance of the enzyme-linked immunospot (ELISPOT) assays were the same as reported (Tarcha et al., 2006). In brief, 96-well microplates containing a PVDF membrane disc in each well (Multiscreen HTS™ IP; Millipore; Billerica, MA) were coated with monoclonal anti-murine IFN-γ antibody (Clone# AN-18; eBioscience; San Diego, CA), washed with PBS and blocked with HL-1 growth medium (BioWhittaker) containing 2% fetal calf serum. $CD90^+$ immune T cells (4×10^5) plus APCs (2.5×10^5) were then added to each well and incubated with or without the recombinant protein (rPrp1, rPrp5, or medium alone) as described (Tarcha et al.,

2006). After incubation, the cells were removed from the filtration membrane of each well, the plates were washed, and biotin-labeled anti-IFN-γ monoclonal detection antibody (Clone# R4-6A2-biotin; eBioscience) was added to the wells followed by streptavidin-alkaline phosphatase. The plates then were washed and the phosphatase substrate BCIP/NBT (KPL, Gaithersburg, MA), was added for color development. Each membrane was analyzed using an automated ELISPOT reader system (ZellNet Consulting Inc. Fort Lee, NJ). The frequency of IFN-γ secreting antigen-specific $CD90^+$ T-cells was calculated as the number of spots per 4×10^5 $CD90^+$ T-cells seeded in the presence of antigen minus the number of spots per equal number of $CD90^+$ T-cells in medium alone.

References

1. Cox, R. A., and D. M. Magee. 2004. Coccidioidomycosis: host response and vaccine development. Clin. Microbiol. Rev. **17:**804-839.

2. Herr, R. A., C.-Y.Hung, and G. T. Cole. 2007. Evaluation of two homologous proline-rich proteins of *Coccidioides posadasii* as candidate vaccines against coccidioidomycosis. Infect Immun. **75**: 5777–5787.

3. Kulkarni, R. D., H. S. Kelkar, and R. A. Dean. 2003. An eight-cysteine-containing CFEM domain unique to a group of fungal membrane proteins. Trends Biochem. Sci. **28:**118-121.

4. Li, K., J.-J. Yu, C.-Y. Hung, P. F. Lehmann, and G. T. Cole. 2001. Recombinant urease and urease DNA of *Coccidioides immitis* elicit an immunoprotective response against coccidioidomycosis in mice. Infect. Immun. **69:**2878-2887.

5. Tarcha, E. J., V. Basrur, C.-Y. Hung, M. J. Gardner, and G. T. Cole. 2006. A recombinant aspartyl protease of *Coccidioides posadasii* induces protection against pulmonary coccidioidomycosis in mice. Infect. Immun. **74:**516-527.

6. Zhu Y., V. Tryon, D.M. Magee, and R.A. Cox. 1997. Identification of a *Coccidioides immitis* antigen 2 domain that expresses B-cell-reactive epitopes. Infect. Immun. **65**: 3376-3380.