Part I

Progress Report: The role of G-protein coupled chemokine signaling in a murine model of ulcerative colitis

Since being awarded funds from the Moran Foundation, significant progress has been made on multiple fronts in this project. Briefly, the questions this project seeks to answer include:

1. What are the kinetics and cellular sources of elevated IL-12 production in the gut and/or lymphoid tissue of Giα2 -/- mice observed even before the onset of colitis?

2. Are defects in the immune system limited to T-cells in these animals, or are they 'innocent bystanders' just responding to abnormal innate immune cells, such as macrophages?

3. How does the lack of Gia2 affect signaling through chemokine receptors on leukocytes, and are there alterations in the utilization of other G-protein coupled pathways?

4. Can the reintroduction of Giα2 into T-cells or colonic epithelium reverse the effects on cytokine production, T-cell activation, and/or the colitis phenotype?

5. Which of the Gia2- dependent downstream signaling pathways coupled to chemokine receptors are involved in one or more of these cell types that lead to increased expression of IL-12 and IFNγ?

Preliminary data, summarized here, pose insight into some of these questions.

Question 1:
Sources of Elevated IL-12 in splenocytes, and macrophages in vitro

1: WT vs Giα2 -/- Splenocyte results

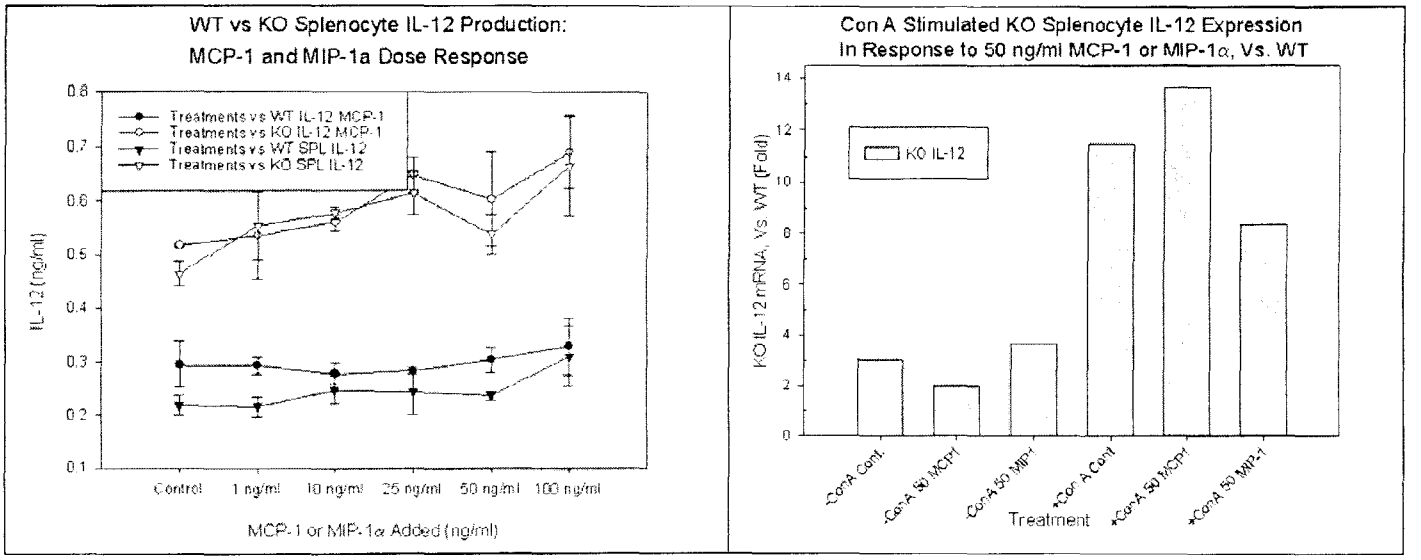


Figure 1: Splenocytes were isolated from colitis-free WT and KO mice and cultured at 3 million/ml in the presence of 3 μg/ml Con A and MCP-1 or MIP-1α as shown. At 48 hours, culture sups were taken for IL-12 ELISA (left panel), or cellular RNA was isolated and used for quantitative RT-PCR for IL-12 (right panel).

RESULT: Compared to WT, Giα2 -/- splenocytes produce significantly more IL-12 for all concentrations of MCP-1 or MIP-1α tested (left panel). While neither chemokine appears to affect WT splenocyte IL-12 production, KO splenocytes appear to produce more IL-12 in the presence of higher concentrations of MCP-1 or MIP-1α. At the mRNA level, Con-A activated KO splenocytes produce 8 to 13-fold more IL-12 than WT cultures. MIP-1α appears to downregulate IL-12 compared to MCP-1, which is the opposite of what would be expected given MCP-1's established role in suppressing IL-12 production.

Correlation of altered IL-12 levels with Dysregulation of Th1 cytokines and Chemokine levels.

Additional experiments with KO splenocytes have revealed additional alterations in cytokine and chemokine production. They produce higher levels of Th1 (IFNγ: 1.4 to 8 fold; IL-12: 2 to 13.6 fold) and lower levels of Th2 cytokines (IL-4: 0.3 to 0.8 fold; IL-13: 0.4 to 0.84 fold) compared to WT splenocytes.

In addition, KO splenocytes overproduce TNFα compared to WT, with a similar trend towards increased TNFα production at higher concentrations (50 and 100 ng/ml) of MCP-1 or MIP-1α. In contrast, the chemokine C10 is produced at considerably lower levels than in knockout splenocytes, averaging about 0.3 ng/ml across the range of MCP-1 or MIP-1α tested, compared to roughly 1 ng/ml for similarly treated WT splenocytes (data not shown).

WT vs KO Macrophage Experiments:

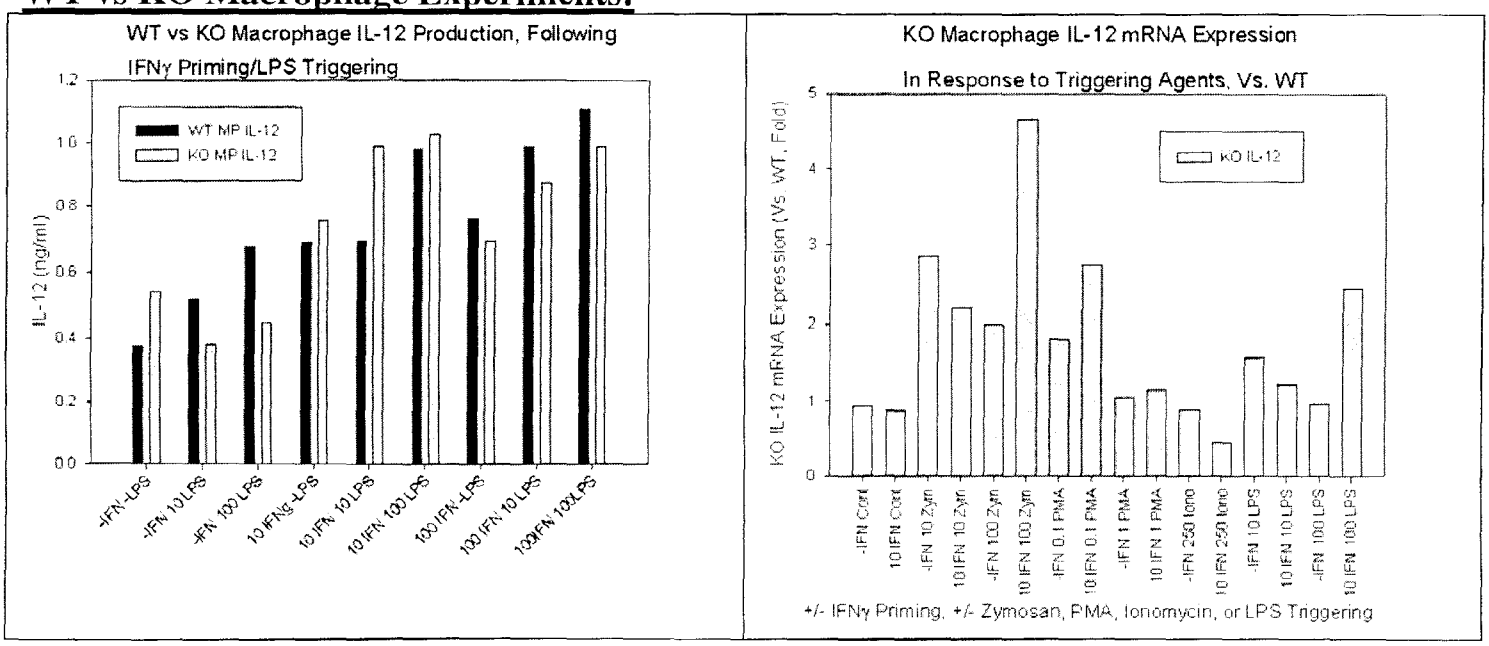


Figure 2: 5 days after IP injection of 4% thioglycollate, peritoneal macrophages were isolated, plated at a density of 250,000 cells/ml, enriched to ~90% purity by plastic adherence, and primed for 4 hours with 0, 10, or 100 ng/ml IFNγ. Cells were washed, then activated with various activators as shown for 20 hours. Culture sups

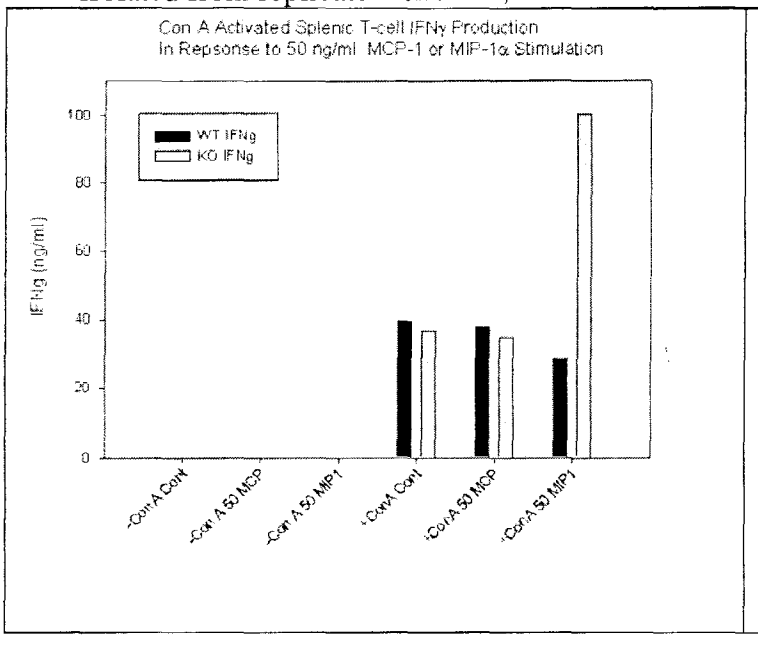
were taken for IL-12 ELISA (left panel), or cellular RNA was isolated and used for quantitative RT-PCR for IL-12 (right panel).

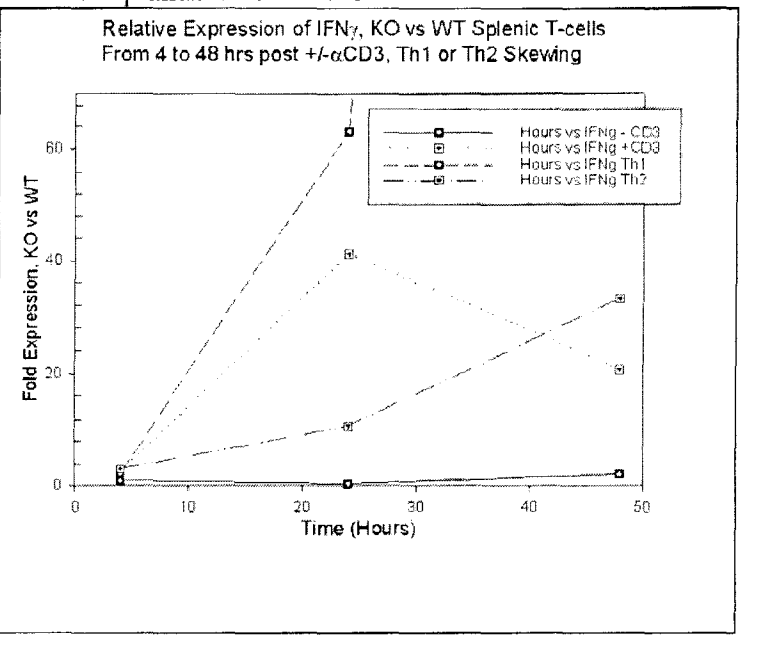
RESULT: Initial experiments with LPS-activated KO macrophages failed to demonstrate increased levels of IL-12 mRNA or protein when activated with IFNγ and LPS (left and right panels). Subsequent work shows that the macrophage activator Zymosan induces IL-12 mRNA expression 2 to 4 fold higher in KO macrophages compared to WT cells. This finding suggests that induction of KO macrophage IL-12 expression depends on stimulating macrophage phagocytic activity.

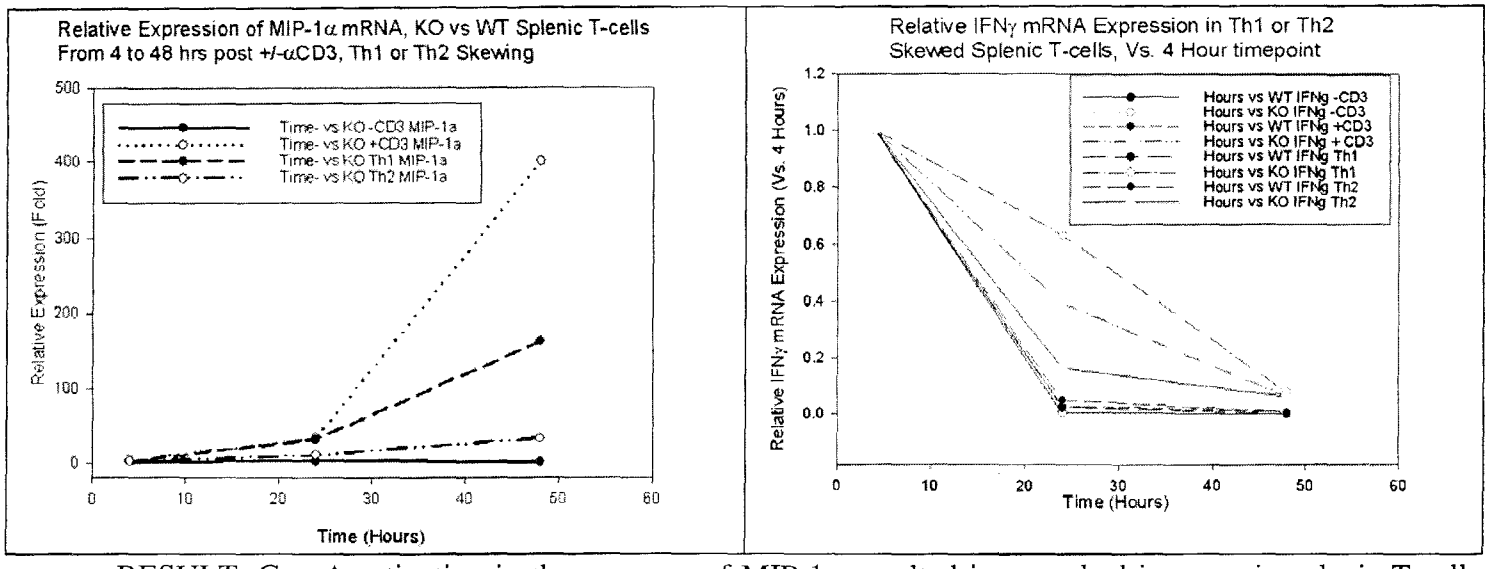
In other experiments, KO macrophages clearly produce higher levels of the chemokines IP10 (WT: 0.05 to 0.4 ng/ml; KO 1.4 to 1.6 ng/ml) and RANTES (WT 0-1 ng/ml; KO 0.5 to 4.5 ng/ml) when primed and triggered with the IFNγ/LPS priming/triggering regimen illustrated in Figure 2. In contrast, macrophage production of MIP-1α is significantly inhibited both in WT and KO macrophages when primed with IFNγ; this inhibition is much more pronounced in KO (0.2-0.4 ng/ml) compared to WT (0.7-2 ng/ml) macrophages.

Question 2: WT vs KO Splenic T-cell Experiments

Figure 3 (next page): Splenic T-cells were isolated from whole splenocytes by positive selection using Thy 1.2 magnetic beads on a Miltenyi Biotech LS column. Purified T-cells were cultured at 3 million/ml under 2 skewing regimens as described. Briefly, Regimen 1 involves Anti-CD3 activation under Th1 (IL-12 and anti-IL4) or Th2 (IL-4 and anti-IFNγ) conditions; regimen 2 involves 3 μg/ml Con A activation with 50 ng/ml MCP-1 or 50 ng.ml MIP-1α. At 48 hours, supernatants were harvested for ELISA (upper left). Alternatively, RNA was isolated from replicate wells at 4, 24 and 48 hours for use in quantitative RT-PCR.







RESULT: Con A activation in the presence of MIP-1α resulted in a marked increase in splenic T-cell IFNγ production at 48 hours compared to WT T-cells (upper left). Using TaqMan analysis, the same T-cells activated with anti-CD3 produce markedly more MIP-1α mRNA at 24 and 48 hours post-activation than WT cells (lower left). Th1 skewing induces more robust MIP-1α expression than under Th2 conditions.

IFNγ mRNA levels are also markedly elevated in KO T-cells compared to WT cells, especially under Th1 conditions (upper right). The 48 hour Th1 timepoint for KO cells is greater than 1000 fold higher than WT cells and is thus off the chart. When IFNγ mRNA levels are compared as a fraction of their expression at 4 hours post-activation (lower right), it is clear that KO T-cells under Th1 conditions are still making ~65% as much IFNγ message at 24 hours, compared to 2% for WT T-cells. Therefore, KO T-cells make more IFN message for much longer periods of time following activation.

Conclusions, questions 1 and 2:

In conclusion, this supplemental data supports the ideas that activated KO macrophages and T-cells produce increased levels of IL-12 and IFNγ, respectively, compared to their WT counterparts. Furthermore, KO macrophages and T-cells respond to and produce MIP-1α in a manner that is distinct from WT cells. The mechanisms underlying these findings will be the subject of further experimentation.

Progress and Future Directions, Questions 3-5:

Question 3:

The issue of how other G-protein coupled pathways are affected by the loss of particular G-protein alpha subunits is beginning to be investigated. First, I have obtained mice that are deficient in two other pertussis toxin-sensitive G-proteins, Gia1 and Gia3. These animals do not develop ulcerative colitis. The use of cells from these three lines of knockout mice will enable a molecular dissection of specific vs. redundant usage of G-protein alpha subunits by chemokine receptors in the immune system.

Using a complementary methodology, I have designed and am testing oligonucleotise probes specific for these alpha subunits in an effort to understand how the absence of one affects the expression and usage of the

other alpha subunits. Insight into this issue may help explain the intricacy of how certain G-protein coupled receptors activate their downstream effector pathways.

Question 4:

Addressing this question involves the production of two new lines of transgenic mice who produce the Gia2 protein only in T-cells or in colonic enterocytes. To date, both lines of mice have been established, and both transgene constructs have been shown to be transmissible in the germline. Currently, there are approximately 30 mice positive for one of the transgene constructs. The next step with these animals is to demonstrate that each transgene is being expressed in the tissues to which they have been targeted. With this completed, breeding the transgenic mice onto a background similar to the original Gia2 -/- mice will be done, at which point the effect of the transgene on the development of ulcerative colitis can be investigated.

Question 5:
Work on the question of downstream effectors involved in the transduction of G-protein coupled signals to affect IL-12 production has not yet begun. However, identifying alterations in downstream effector pathways will tie our knowledge about what cells, G-protein coupled cell surface receptors, and downstream pathways into a more global understanding of the disease phenotype in Gia2 -/- mice.

Part II:

Portions of the work shown in this progress report have been presented in Pathology Dept. Grand Rounds presentations made at the University of Kentucky, Lexington, KY, Indiana University, Indianapolis, IN, and The University of California at Irvine, Irvine, CA. An abstract for poster presentation has been scheduled for the FASEB meeting to held in New Orleans, LA on April 20-24, 2002.

Part III:

This project is still being actively pursued.