Diabetic Complications Consortium

Application Title: New diabetic wound healing model to functionally analyze human genetic variation.

Principal Investigator: Hesselson, Daniel

1. Project Accomplishments:

We have successfully established a zebrafish model to functionally analyze the role of human A20 variants on the in vivo inflammatory phenotype. Below we present some initial data with selected A20 variants and are currently using these models to test all reported coding variation at this locus. We anticipate that this approach will be useful for defining new inflammatory regulators that emerge from our ongoing whole-genome sequencing studies of patients with diabetic complications.

2. Specific Aims:

Specific Aim 1. Determine whether neutrophil responses and/or recruitment signals are defective under diabetic conditions.

Results: We did some pilot experiments using mRNA injections of the HyPer hydrogen peroxide sensor into 1-cell zebrafish embryos and found that the signal did not persist long enough for us to visualize the gradient at the wound site in diabetic embryos at the key stages where we had previously defined a neutrophil recruitment defect. We therefore generated a transgenic line expressing a more sensitive reporter (HyPer3) under a ubiquitious promoter. This reporter line now provides a robust readout of the hydrogen peroxide gradient. We are currently raising diabetic animals that express this reporter to address this aim.

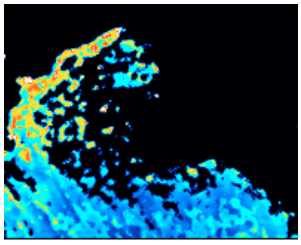

Figure 1. Hyrogen peroxide gradient in a single confocal section. Ratiometric image (488/405 nm excitation). High ratios are seen at the wound margin (white/red) compared to distal regions (black/blue). 6dpf zebrafish F1 transgenic larvae.

Specific Aim 2. Test whether normalizing glucose levels is sufficient to rescue neutrophil migration.

Results: We normalized glucose levels with a variety of small-molecules for 24h prior to performing the neutrophil recruitment assay. None of the treatments rescue the defect observed in diabetic larvae. Therefore, we suspect that it is hypoinsulinemia itself that is

important for this process. We are currently optimising dosing and delivery methods for recombinat human insulin to zebrafish larvae.

Specific Aim 3. Use zebrafish models to elucidate the function of human A20 variants.

Results: We have expended most of our effort on Aim 3. First we confirmed published data showing that zebrafish are sensitive to LPS added to their aqueous environment and that the effective lethal dose changes with develpmental age.

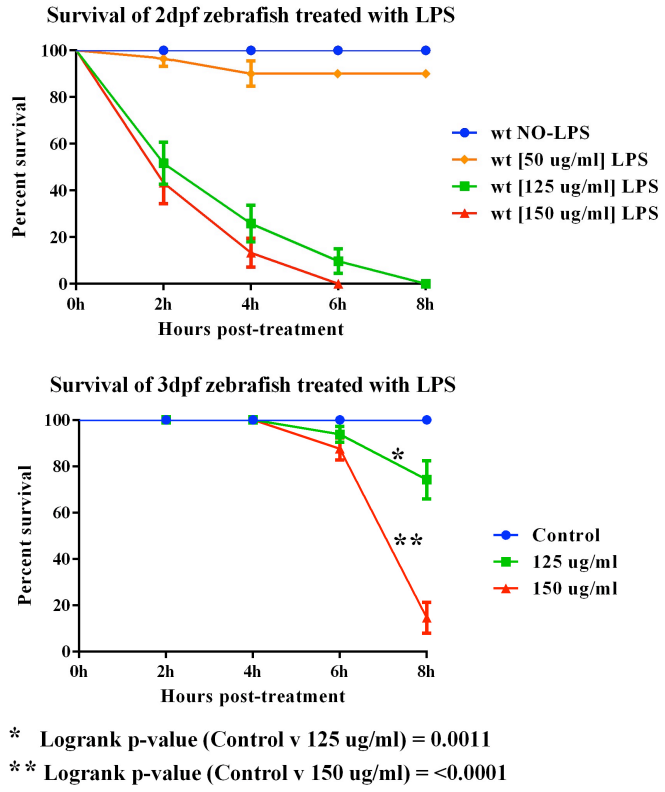

Figure 2. Survival analysis of 2 dpf and 3dpf zebrafish embryos with escalating doses of LPS.

* Logrank p-value (Control v 125 ug/ml) = 0.0011

** Logrank p-value (Control v 150 ug/ml) = <0.0001

To prepare to test the function of human A20 in this system we performed mock-injections to determine whether the physical stress of injecting the 1-cell embryo altered the LPS response.

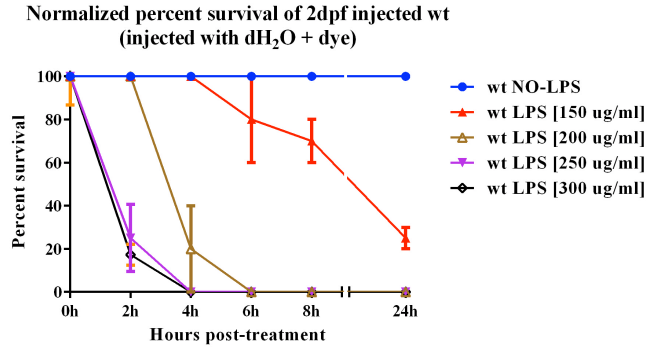

Figure 3. Mock injected embryos tolerate higher levels of LPS.

As shown in Figure 3 the presumed stress-response from the mechanical or osmotic disruption during injections reduced the sensitivity of zebrafish embryos to LPS. Using the optimised dose of 150 ug/mL at 2 dpf in a 24 h survival assay we tested the function of wild-type human A20 in

this system. It is important to note that we could not find any literature on the in vitro transcription of full-length human A20 mRNA. Therefore it required significant effort to find an appropriate expression system (Promega T7 Ribomax) that was capable of generating a full length mRNA.

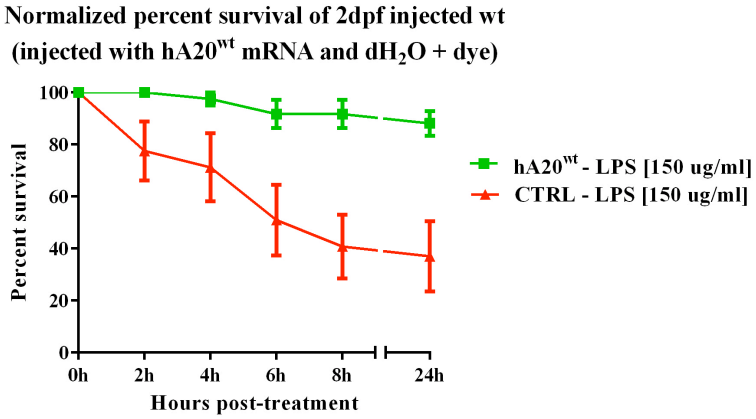

Figure 4. Wild-type human A20 protects zebrafish embryos from LPS

Expression of full-length human A20 increased survival of embryos compared to mock-injected controls. We proceeded to test two A20 variants that have been extensively characterized. C103A is a catalytically inactive variant and I325N is a partial loss-of-function that we identified in an mouse ENU screen for hyperinflammatroy phenotypes. Both mutations exhibited reduced ability to rescue survival in our assay (Figure 5).

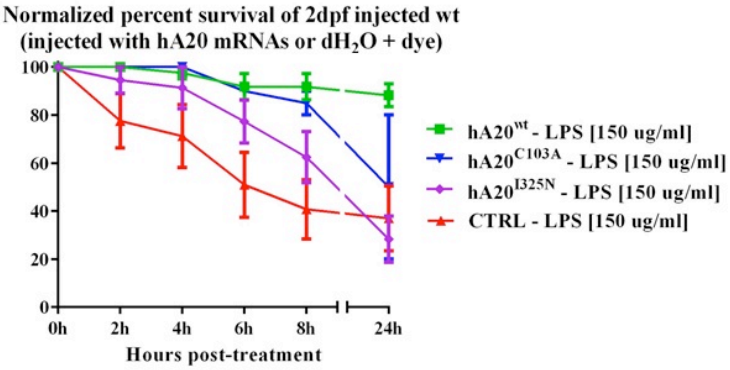

Figure 5. A20 variants reduce the rescue activity of human A20.

Data from

- **hA20^{wt} : 4 experiments (2 replicas of 10 embryos per group)**
- **hA20^{C103A} : 2 experiments (2 replicas of 10 embryos per group)**
- **hA20^{I325N} : 4 experiments (2 replicas of 10 embryos per group)**
- **CTRL : 11 experiments (2 replicas of 10 embryos per group)**

p-value (CTRL vs hA20^{wt}) : 0.0104

As pointed out by the original reviewers, we were concerned that endogenous zebrafish A20 might reduce the dynamic range of our assay. We have taken two approaches to address this issue. 1) Instead of relying on a binary readout (e.g. survival) we have time-courses a number of target genes that are downstream of A20/NFkB activation. Of these Il-1beta shows an 11-fold increase in mRNA expression at 1-hour post LPS exposure. We are currently testing whether this might be a more quantitative readout with greater dynamic range for A20 function. 2) We have generated a zebrafish A20 knockout using zebrafish. We recovered several frame-shift mutations

in the OTU domain that induce premature stop codons. We have generated F2 heterzyogous animals and it will be interesting to determine whether loss of zebrafish A20 further exacerbates the effects of LPS.

Finally, we have established a NFkB reporter strain (Figure 6) to provide rapid high-throughput readout of A20/NFkB pathway activity. This may prove particularly useful in the A20 knockout strain described above.

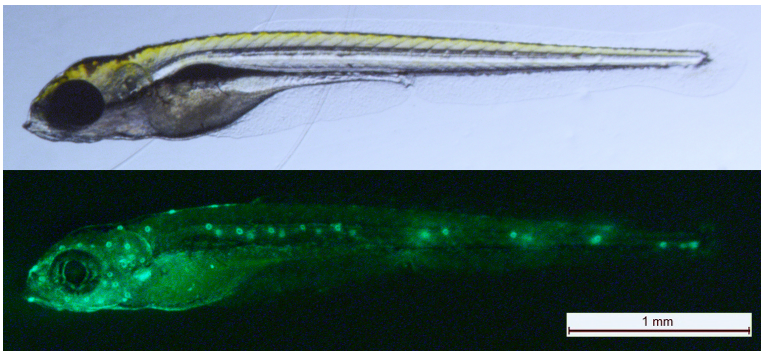

Figure 6. NFkB:GFP reporter transgenic strain. 6x NFkB response elements drive GFP expression in various tissues.

3. Publications:

Publications are still in preparation.