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**Deregulation of TNF expression can also cause heart valve disease**

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High levels of the pro-inflammatory cytokine tumour necrosis factor (TNF) have been associated with many diseases including rheumatoid arthritis (RA), ankylosing spondylitis (AS), inflammatory bowel disease (IBD) and psoriasis.

Although it has been clear for twenty-five years that TNF plays a major role in RA and AS, two major questions remain unanswered: 1) What mechanism underlies the loss of control of TNF levels in patients? 2) How does TNF exert its detrimental effects? Nonetheless, biological anti-TNF drugs have become the most successful treatment of these conditions with a third of patients entering remission, and the global market for biological TNF inhibitors is now estimated at around US$35 billions. However, their use is limited by their cost, the fact that they need to be injected, non-negligible side effects and the development of resistance due to the protein (thus antigenic) nature of these TNF inhibitors. It looks inevitable that new approaches to lower the amount of TNF should be considered. To do this, a better understanding of the regulation of TNF expression is necessary.

**High levels of TNF cause heart valve disease**

We have recently studied a spontaneous dominant mouse mutation that causes severe polyarthritis in BPSM1 (Bone Phenotype Spontaneous mutant 1) mice, and determined that it is due to the insertion of a retrotransposon in the 3' untranslated region (UTR) of TNF (1). This causes a strong overexpression of TNF, and a phenotype very similar to

that of the well-known $TNF^{\Delta ARE}$ mice (2). Surprisingly however, backcrossing the BPSM1 mice onto the BALB/c genetic background unveiled a fatal heart valve phenotype that was never observed before. Aortic root aneurism as well as thickening of the aortic and mitral valves were found in all BPSM1/BALB/c mice, and led to aortic regurgitation and cardiac failure around 100 days of age. BPSM1 mice on the C57BL/6 genetic background also showed valve thickening and aortic regurgitation, but only limited aneurism, and they didn't die of the disease, demonstrating that other genetic loci significantly influence the effect of excessive TNF levels. The BPSM1/BALB/c mouse is the first spontaneous model of aortic root aneurism and provides an excellent tool to dissect the causes and assay potential treatments for aneurisms.

In BPSM1 mice, bone erosion was most severe in joints with the highest load factor (ankles, wrists, T10-T13 vertebras, temporomandibular joint). In the heart, the aortic and mitral valves were affected, while the tricuspid and pulmonary valves, subjected to a much lower pressure, were unaffected. These observations indicate that mechanical stress is an important parameter in the disease triggered by excessive TNF, as observed in the $TNF^{\Delta ARE}$ mouse model (3), and may fuel the debate about the appropriateness or type of regular exercise in the management of rheumatoid arthritis.

**TNF 3'UTR contains a previously unidentified regulatory element**

TNF 3' UTR contains several AU-rich elements (ARE), and a constitutive decay element (CDE) that are the targets of CCCH-containing zing finger proteins (such as ZFP36/TTP1 or Rc3h1) responsible in part for the rapid turnover of its mRNA. To examine the potential role of all the CCCH-containing proteins on TNF expression, we engineered a range of reporter constructs based on GFP + various versions of TNF 3'UTR. As expected, ZFP36/TTP1 down regulated the expression of the reporter through the region containing the ARE. Two other CCCH-containing proteins, Zc3h12a and Zc3h12c, exerted a negative effect through a previously unidentified regulatory element close to the polyadenylation signal that we named NRE (new regulatory element). The negative role of Zc3H12a (also known as Regnase) on TNF expression needs to be further confirmed, as two previous studies reported contradictory results on this issue (4) (5). Nevertheless, we found that ARE and NRE acted synergistically: while removal of either of these

regulatory elements increased the expression of the reporter 3- to 4-fold, deletion of both elements increased expression of the reporter about 30-fold. This is particularly important when comparing our BPSM1 mice to the $TNF^{\Delta ARE}$ mice. In BPSM1 mice, insertion of the transposon at the start of the 3'UTR effectively suppresses the control by ARE, CDE and NRE and explains the very high levels of TNF observed in this model. Our results suggest that the deletion of the ARE alone should have a less drastic influence on the expression of TNF, and that as a consequence, $TNF^{\Delta ARE}$ mice should have a milder phenotype than BPSM1 mice. This is not the case, and we showed that $TNF^{\Delta ARE}$ mice even show the same heart valve phenotype as BPSM1 mice. However, examination of the cloning strategy used to generate $TNF^{\Delta ARE}$ mice showed that the NRE in these mice is disrupted by the introduction of a lox-neo-lox cassette, which leaves a 200 nucleotide sequence insertion in the NRE site after the removal of neo by cre-mediated recombination (2). It is therefore likely that the conclusions about the role of TNF 3'UTR ARE in the development of arthritis might have been overestimated and need reconsideration. In particular there has been intense investigation attempting to link ZFP36/TTP1 with inflammatory conditions such as RA. Our results however demonstrate that Zc3h12a and Zc3h12c warrant a similar level of investigation. Two CCCH-containing ZFP, Unkempt and Zc3h10, significantly increased the expression of the reporter. Whether these two proteins are responsible for high levels of TNF in inflammatory conditions is unknown at the moment. If this is the case, inhibiting them could provide an alternative strategy to lower TNF levels for therapeutic benefit.

In our animal facility, BPSM1 mice failed to develop obvious signs of inflammatory bowel disease (IBD). $TNF^{\Delta ARE}$ mice are prone to IBD, although it has been shown that they can develop arthritis in the absence of significant gut pathology (2). We surmise that this difference is due to a difference of diet or microbiota, and we are actively experimenting conditions that may favor the development of IBD in BPSM1 mice.

Collectively, our results show that excessive TNF not only causes polyarthritis, but also heart valve disease, the severity of which depends on additional genetic loci yet to be identified. TNF 3'UTR plays an important role in the regulation of the steady state levels of the cytokine, and contains several regulatory elements that cooperate to keep this expression within tight limits. It is obvious that mutations in any of the proteins that act

through these regulatory elements to maintain low levels of TNF may have inflammatory consequences.

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Figure legend

Figure 1: Diagrammatic representation of TNF 3'UTR in wild-type, $TNF^{\Delta ARE}$ and BPSM1 mice. The new regulatory element (NRE) is disrupted by a loxP site in $TNF^{\Delta ARE}$ mice, while all three regulatory elements are eliminated by the insertion of a SINE retrotransposon in BPSM1 mice.

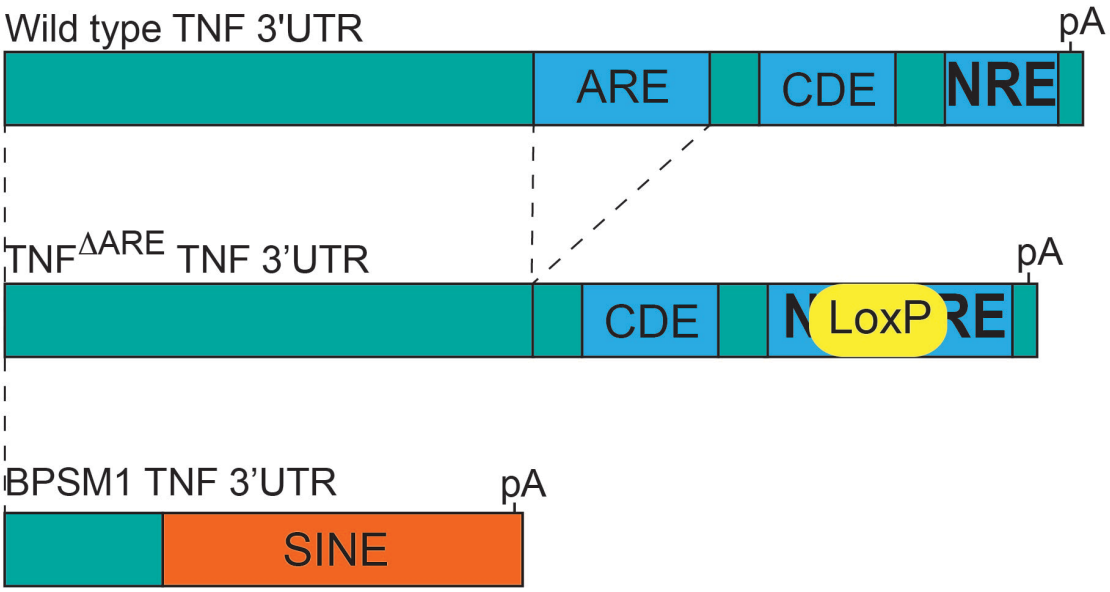
Wild type TNF 3'UTR
pA
ARE
CDE
NRE
TNF^ΔARE TNF 3'UTR
pA
CDE
LoxP
BPSM1 TNF 3'UTR
pA
SINE

Figure 1