Title:

Characterising regulatory T-cell subgroup phenotypes in human sepsis

Background:

Sepsis, although classically described as a hyper-inflammatory disease, can result in profound immunosupression (1, 2). The mechanisms underlying this immunosupression are poorly understood, although dysfunction has been found in neutrophils (3), monocytes (4), dendritic cells (5) and lymphocytes (6). Although lymphocyte anergy in sepsis has been described for some time (6), the recent finding of elevated numbers of immunosuppressive regulatory T-cells in sepsis (7) provides new insight into why this might happen and therefore opens up new therapeutic avenues.

Regulatory T-cells (Tregs) are a population of cells originally identified in murine models of auto-immune inflammation, with potent anti-inflammatory properties (8), and are characterised by the expression of the transcription factor Forkhead Box Protein 3 (FOXP3). Although FOXP3 expressing regulatory T-cells have been identified in humans (8), it is increasingly clear that human Treg biology is more complex than murine equivalents, and studies in healthy humans have identified 3 sub-types of Treg cell (9) these being 'resting/naive', 'cytokine secreting' and 'activated/highly suppressive' subsets which can be identified by staining for CD45RA and CD147 (9, 10).

Dr Conway Morris has recently identified elevated levels of CD4+CD25+FOXP3+ Treg cells during critical illness, and found they were strong predictors of subsequently developing ICU-acquired infection (11). These findings added predictive value to our previously observed associations with C5a-mediated neutrophil dysfunction (3, 12) and monocyte deactivation (4). In this secondary analysis of a cohort study 39% of critically ill patients had elevated levels of Tregs (defined as >10% of all CD4+ lymphocytes), and this was associated with a 2.8 (95% CI: 0.89-8.9, P=0.09) fold increased risk of death from sepsis. Levels of Tregs were found to peak between days 4 to 7, consistent with observations from other groups (7). These observations confirm the complex changes to immune function that occur during sepsis and other forms of critical illness, and suggest a potential key role of Treg populations in modulating alterations in immune function.

To date the characterisation of Tregs in human critical illness and sepsis has been basic and has not taken in to account the most recent insights into T-reg biology (9,10). These have shown three functional sub-sets of Treg cells (naive/resting, 'cytokine secreting' and activated/highly suppressive (9)), which cannot be detected by simple detection of CD25 and FOXP3 (10) Furthermore, although it is clear that Tregs inhibit lymphocyte proliferation, their potential synergies with other immune cells such as monocytes and neutrophils remain unexplored in sepsis.

Methods:

This study involves two major parts

1) Identification of Treg sub-sets in patients with sepsis, and linking this to outcomes (development of nosocomial infection and mortality)
2) Examination of the interactions between Tregs and neutrophils to determin any inhibitory effects and the mechanisms by which these may be mediated.

Progress to date:
Neutrophil-Treg interactions study. We have identified that Tregs are able to suppress two key antimicrobial functions of neutrophils, namely phagocytosis and reactive oxygen species production.
Phagocytosis was impaired by Tregs but not effector CD4+ cells (figure 1).

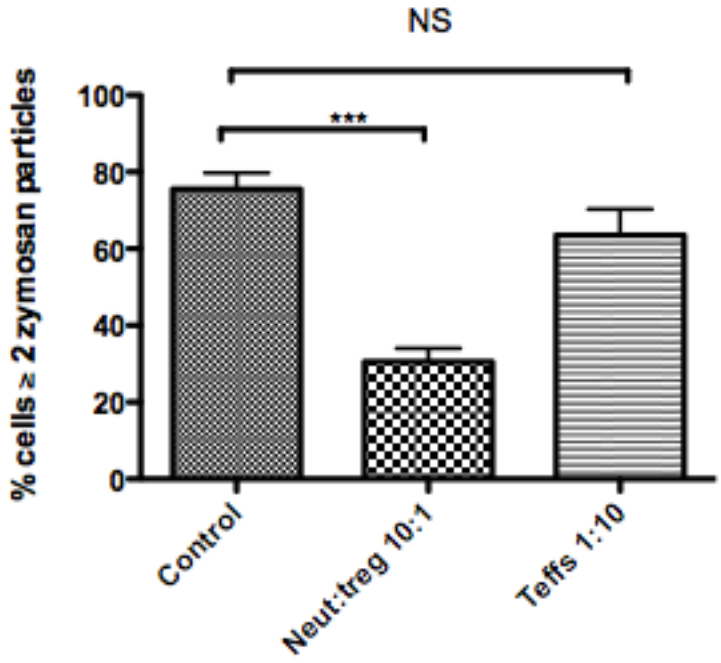

Figure 1: Inhibition of neutrophil phagocytosis by Tregs. P=0.0005 by Kruskal-Wallis, *** P<0.001 by Dunn's post-hoc test.

The effect is fairly potent, and even at a ratio of 1:200 Tregs to neutrophils, suppression remains present (figure 2)

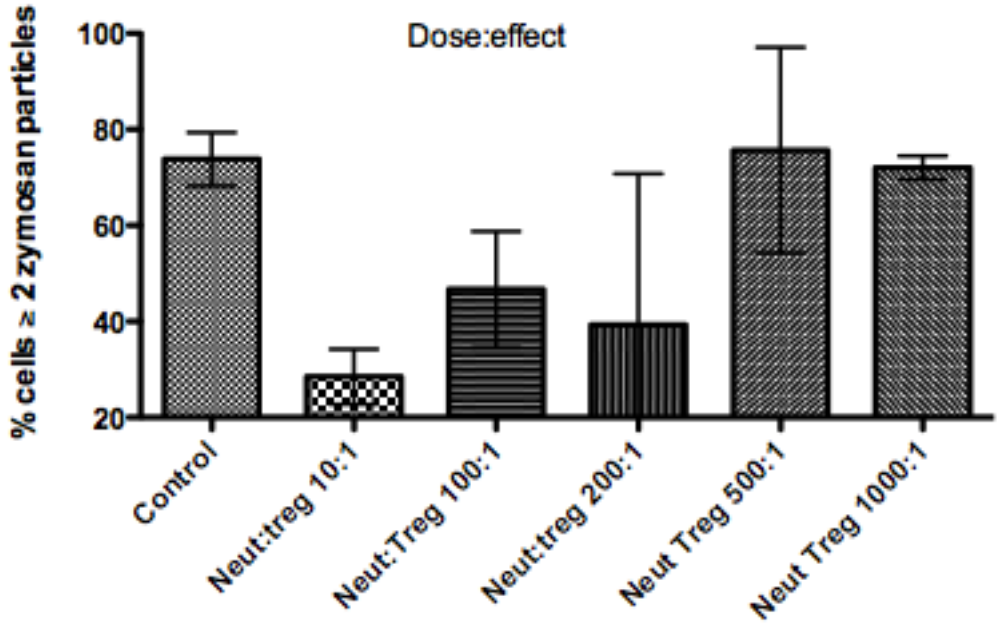

Figure 2: effect of decreasing ratios of Tregs:neutrophils on the ability to suppress phagocytosis.

We have identified that the suppressive effect is mediated by a soluble factor released by Tregs, as the effect can be reproduced by cell-free Treg conditioned media (figure 3) and is not abrogated by separating the Tregs from the neutrophils using a polycarbonate membrane (figure 4).

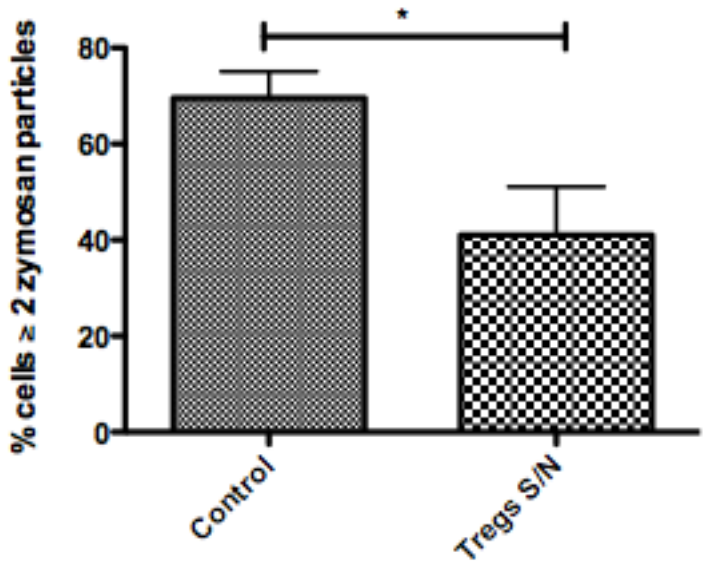

Figure 3. Treg conditioned media is able to impair neutrophil phagocytosis. *p=0.05 by Mann-Whitney U test.

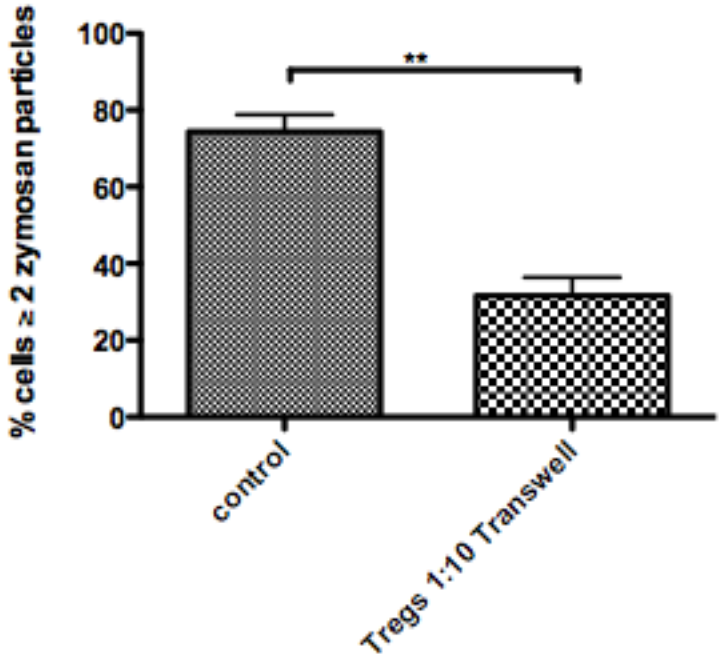

Figure 4: Tregs separated from neutrophils via a 0.4micron pore size polycarbonate transwell can impair phagocytosis. **P=0.008 by Mann-Whitney test.

The effect of Tregs on neutrophils can be prevented using selective inhibitors of the signalling molecules adenylate cyclase and PI3Kdelta (figure 5). We had previously shown that adenylate cyclase is a key mediator in beta-agonist induced defects in phagocytosis (12), whilst PI3Kdelta is a common downstream mediator of both C5a and beta-agonists(3,12).

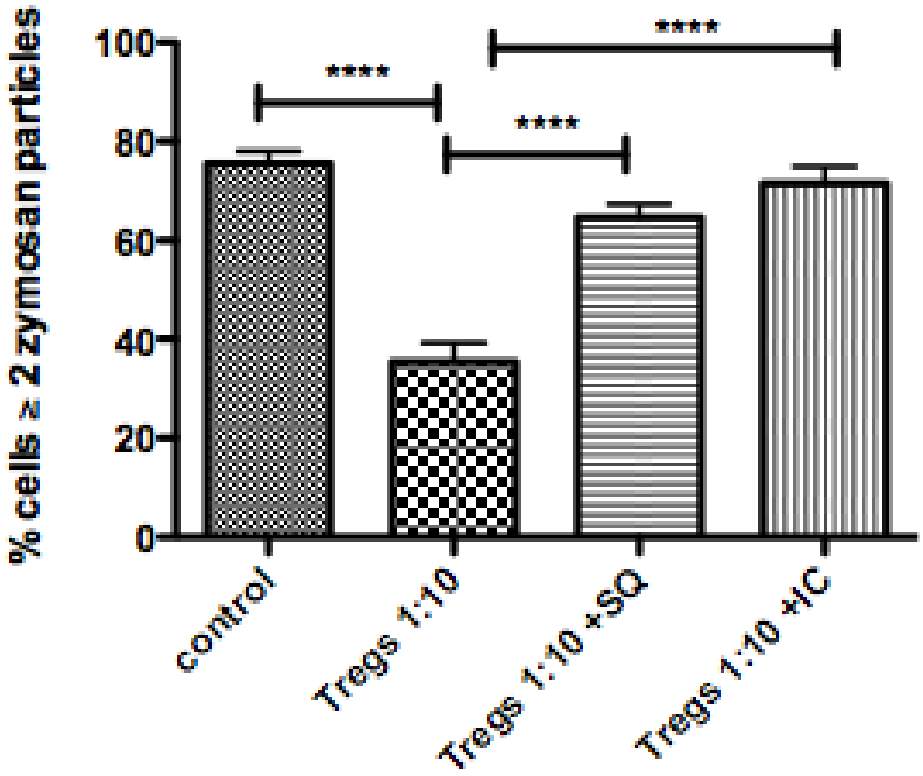

Figure 5: Treg-mediated impairment of phagocytosis can be prevented by inhibition of adenylate cyclase (SQ) or PI3Kdelta (IC). P=0.0004 by Kruskal-Wallis. **** P<0.001 by Dunn's post-hoc test.

Tregs are known to secrete prostaglandins (13), which have been previously shown to impair phagocytosis by macrophages (14) . To further identify the potential mediators we examined whether inhibition of cyclooxyganse could prevent the Treg effect. Both COX-2 selective inhibitor FK3311 and the non-selective indomethacin where able to prevent the phagocytosis inhibiting effect (figure 6)

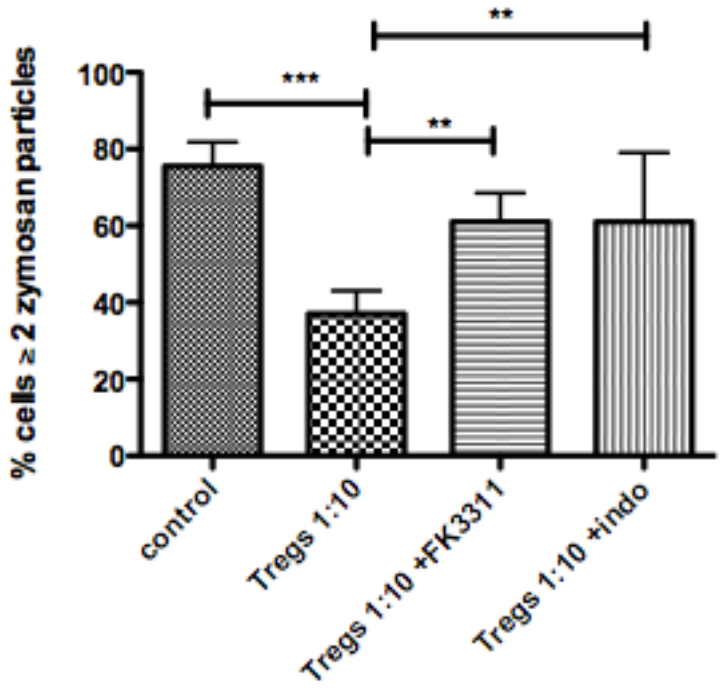

Figure 6 : The Treg mediated effect on neutrophil phagocytosis can be prevented by inhibiting cycoloxygenase. P=0.0001 by Kruskal-Wallis, ** P<0.01, ***P<0.001 by Dunn's post-hoc test.

Further evidence for the prostaglandin-dependent nature of this effect comes from the finding that blockade of the EP4 receptor, although interestingly not the EP2 receptor, was able to prevent the impairment of phagocytosis (figure 7).

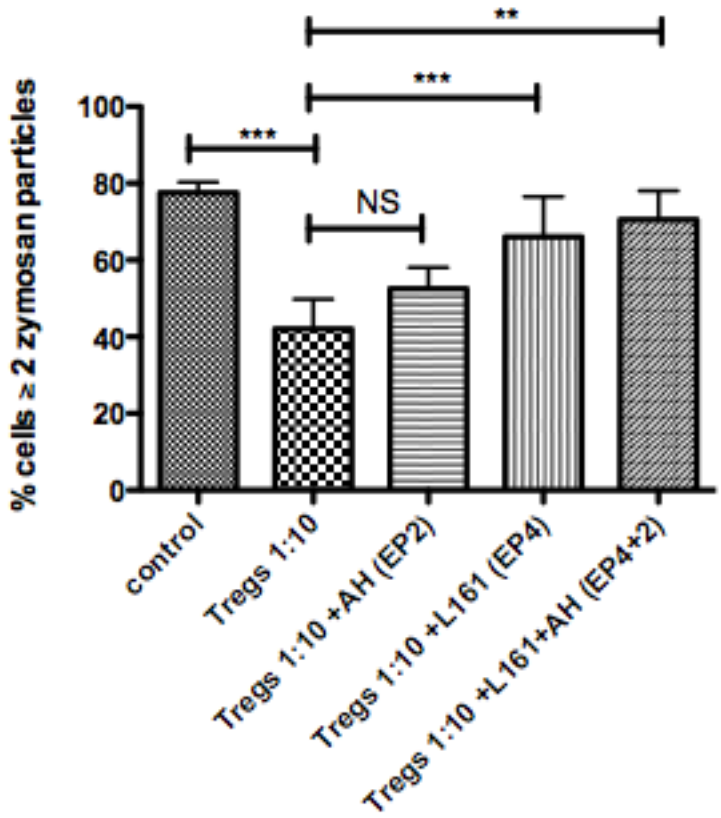

Figure 7. The effect of Tregs on neutrophil phagocytosis can be prevented by blockade of EP4 receptor (L161). EP2 receptor blockade (AH) alone does not reverse the effect and neither is it additive to EP4 receptor blockade. P=0.0001 by Kruskal-Wallis, ** P<0.01, ***P<0.001 by Dunn's post-hoc test.

To confirm the ability of prostaglandin E1 and 2 to impair phagocytosis, and that these molecules mediate their suppression via the similar pathways to Tregs we exposed healthy neutrophils to prostaglandin E1, E2 and 16,16 dimethyl PGE2 (a long acting analogue). These experiments demonstrate that all 3 molecules are able to suppress phagocytosis and that these effects are again dependent on adenylate cyclase, PI3Kdelta and the EP4 but not the EP2 receptor (figures 8-10).

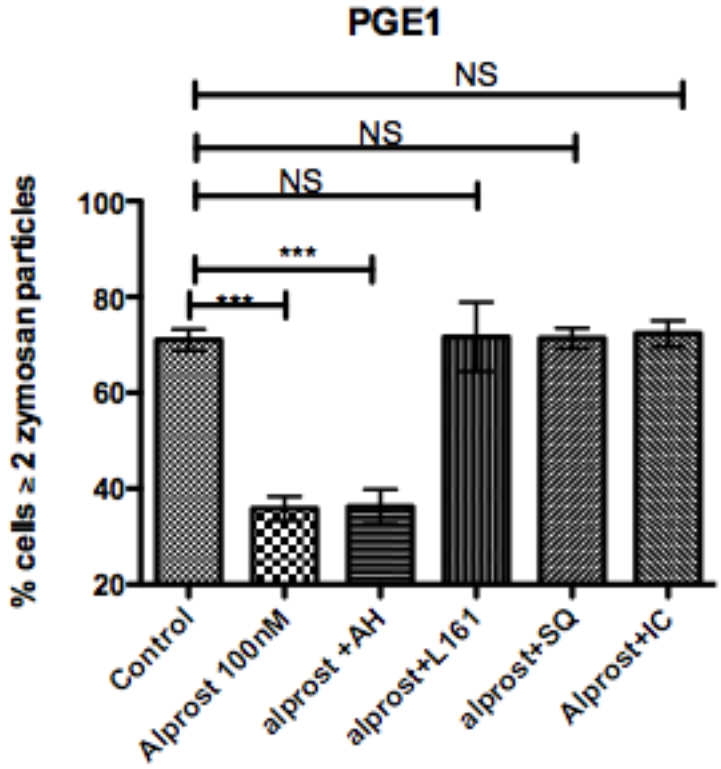

Figure 8. Effect of synthetic PGE1 (aplrostadil) on phagocytosis by neutrophils after 1 hour exposure. The effect can be blocked by inhibition of EP4 receptor (L161), adenylate cyclase (SQ) and PI3Kdelta (IC) but not by blockade of the EP2 receptor (AH). P=0.0001 by Kruskal-Wallis, ***P<0.001 by Dunn's post-hoc test.

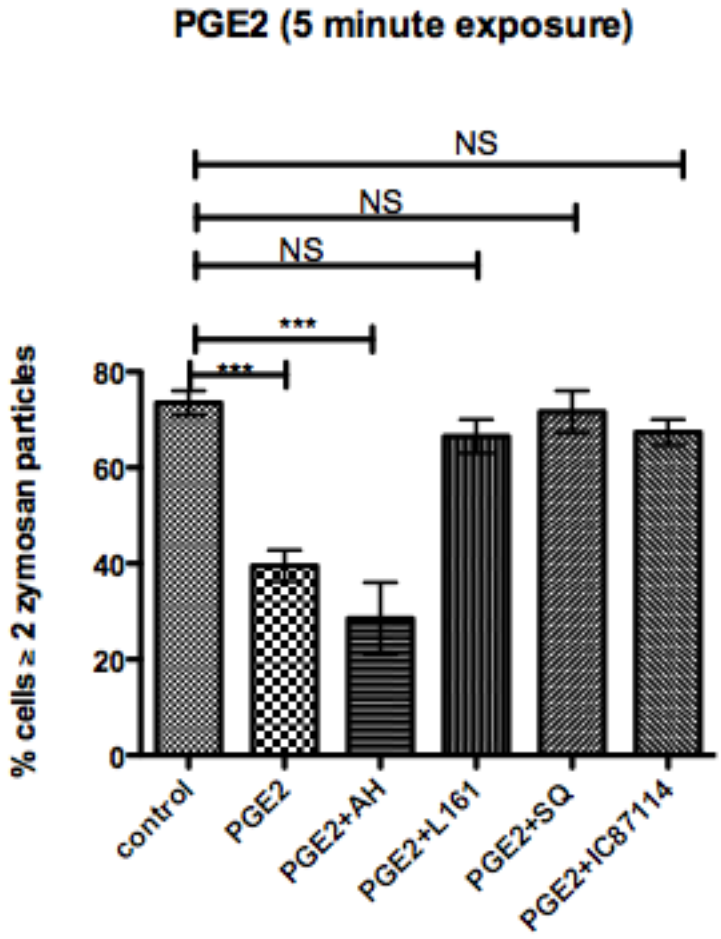

Figure 9. Effect of PGE2 on phagocytosis by neutrophils after 5 minutes exposure. The effect can be blocked by inhibition of EP4 receptor (L161), adenylate cyclase (SQ) and PI3Kdelta (IC) but not by blockade of the EP2 receptor (AH). P=0.0001 by Kruskal-Wallis, ***P<0.001 by Dunn's post-hoc test.

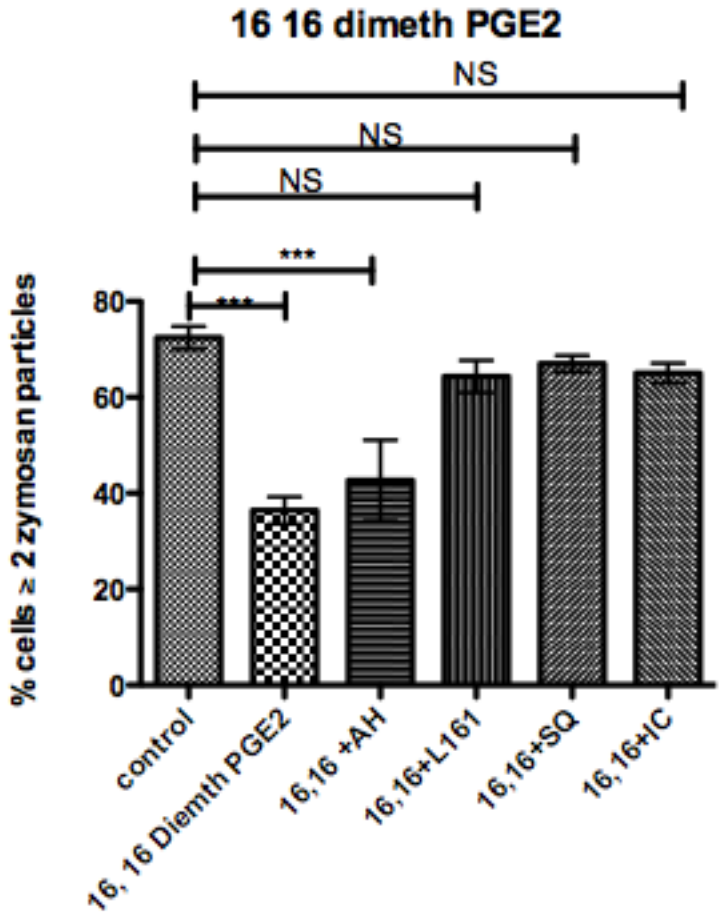

Figure 10. Effect of long-acting PGE2 analogue (16,16 dimethyl PGE2) on phagocytosis by neutrophils after 1 hour exposure. The effect can be blocked by inhibition of EP4 receptor (L161), adenylate cyclase (SQ) and PI3Kdelta (IC) but not by blockade of the EP2 receptor (AH). P=0.0001 by Kruskal-Wallis, ***P<0.001 by Dunn's post-hoc test.

Tregs are also able to suppress reactive oxygen species (ROS) production by neutrophils, in response to stimuli by both bacterial ligands (lipopolysaccharide) and direct activation of protein kinase C (using PMA). Unlike the effects on phagocytosis. the suppressive effects on ROS production were not mediated by COX-2 or adenylate cyclase dependent mechanisms (figures 11 and 12).

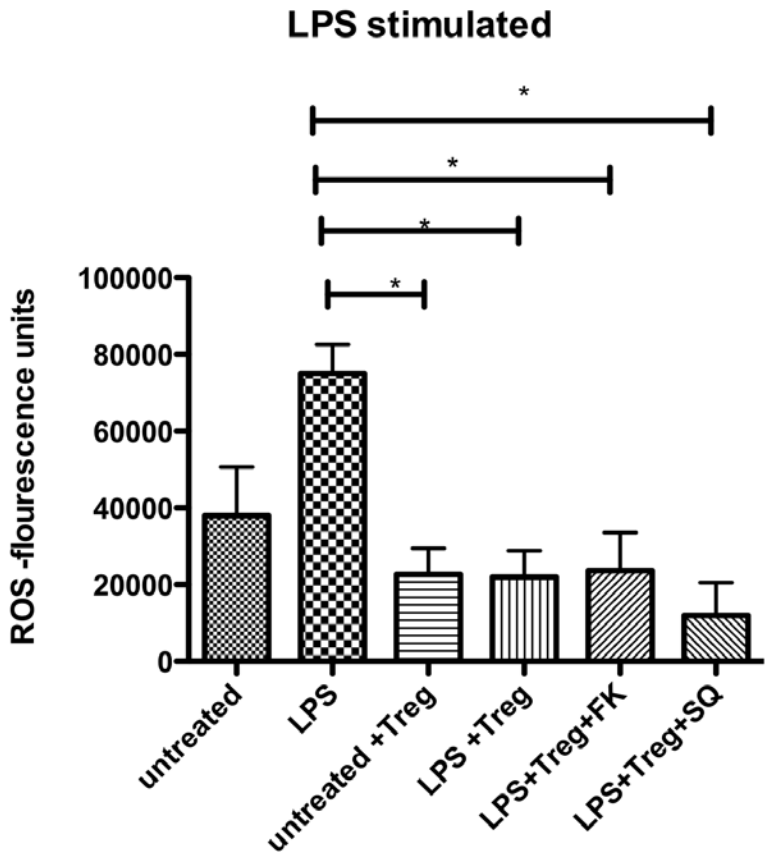

Figure 11. Reactive oxygen species generation by neutrophils stimulated with LPS, with and without Treg co-culture. Tregs were additionally co-cultured with inhibitors of COX-2 (FK) and Adenylate cyclase (SQ). P=0.02 by Kruskal-Wallis, *P<0.05 by Dunn's post-hoc test.

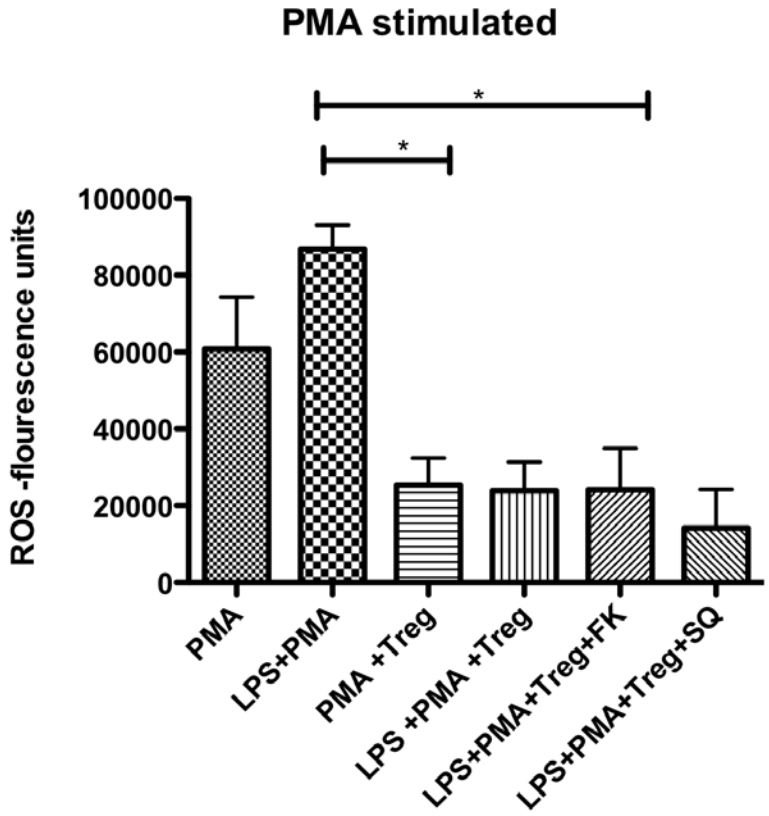

Figure 12. Reactive oxygen species generation by neutrophils stimulated with PMA and LPS, with and without Treg co-culture. Tregs were additionally co-cultured with inhibitors of COX-2 (FK) and Adenylate cyclase (SQ). P=0.01 by Kruskal-Wallis, *P<0.05 by Dunn's post-hoc test.

Further work is continuing to confirm the prostaglandin species which mediates the effects of Tregs on neutrophil phagocytosis, and also to identify the mechanism by which Tregs suppress reactive oxygen species production.

Patient study – In the initial phase of developing the Treg identification panel, we found that the originally planned marker CD147 was unable to reliably distinguish between subsets of Tregs. The revised panel of cell surface markers uses CD15S and CD45RA to distinguish between the three subsets (naive, activated and 'cytokine secreting'). The cytokine secreting cells are further identified by CD161 positivity. These panels have been tested in healthy volunteers and, following the securing of ethical and R&D approvals, in an initial sample of 20 patients with severe sepsis/septic shock. We are now recruiting the larger cohort of patients with sepsis, and will be undertaking functional assays of the Treg sub-sets to evaluate how their function compares to the same sub-sets in healthy patients, alongside enumerating the subsets and exploring how these relate to clinical outcomes for patients with sepsis.

References:

1 Boomer JS, To K, Chang KC, et al. Immunosuppression in patients who die of sepsis and multiple organ failure. *JAMA*. 2011;306(23):2594-605.

2 Wang, T. & Deng, J. Molecular and cellular aspects of sepsis-induced immunosuppression, *J Mol Med (Berl)*. 2008;86(5):495-506.

3 Conway Morris A, Brittan M, Wilkinson TS, et al. C5a-mediated neutrophil phagocytic dysfunction is RhoA-dependent and predicts nosocomial infection in critically ill patients. *Blood*. 2011;117:5178-88.

4 Volk, H.D., Reinke, P., Krausch, D et al. Monocyte deactivation--rationale for a new therapeutic strategy in sepsis. *Intensive Care Med. 1996;*22:S474-81.

5 Efron, P.A., Martins, A., Minnich, D., et al. Characterization of the systemic loss of dendritic cells in murine lymph nodes during polymicrobial sepsis. *J Immunol*. 2004; 173, (5): 3035-43.

6 Heidecke, C.D., Hensler, T., Weighardt, H., et al. Selective defects of T lymphocyte function in patients with lethal intraabdominal infection. *Am J Surg. 1999;* 178 (4):288-292.

7 Venet, F., Chung, C., Kherouf, H., et al. Increased circulating regulatory T cells (CD4+CD25+CD127-) contribute to lymphocyte anergy in septic shock patients. *Intensive care med. 2009;* 35 (4):678-686.

8 Langier, S., Sade, K. & Kivity, S. Regulatory T cells: the suppressor arm of the immune system. 2010. *Autoimmunity rev;* 10 (2):112-115.

9 Solstad T, Bains SJ, Landskron J et al. CD147 (Basigin/Emmprin) identifies FoxP3+CD45RO+CTLA4+-activated human regulatory T cells. *Blood*. 2011;118(19):5141-51.

10 Miyara M, Yoshioka Y, Kitoh A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*. 2009;30(6):899-911

11 Conway Morris A, Anderson N, Brittan M, et al. Combined dysfunctions of immune cells predict nosocomial infection in critically ill patients. *Br J Anaesth*, 2013 June 10 [Epub ahead of print]

12 Conway Morris A, Kefala K, Wilkinson TS et al. C5a mediates peripheral blood neutrophil dysfunction in critically ill patients. *Am J Respir Crit Care Med*. 2009;180:19-28.

13 Mahic M, Yaqub S, Johansson CC, Taskén K, Aandahl EM. FOXP3+CD4+CD25+ adaptive regulatory T cells express cyclooxygenase-2 and suppress effector T cells by a prostaglandin E2-dependent mechanism. *J Immunol*. 2006;177(1):246–254.

14 Ojielo CI, Cooke K, Mancuso P, et al. Defective phagocytosis and clearance of Pseudomonas aeruginosa in the lung following bone marrow transplantation. *J Immunol*. 2003;171(8):4416–4424.