Protease activated receptor-2 signalling by tissue factor on dendritic cells suppresses antigen-specific CD4+ T cell priming.

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Protease activated receptor-2 signalling by tissue factor on dendritic cells suppresses antigen-specific CD4\(^+\) T cell priming.

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Running title: Effects of TF/PAR-2 on DC

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Abstract

Background: The precise function of tissue factor (TF) expressed by dendritic cells (DC) is uncertain. As well as initiating thrombin generation it can signal through protease activated receptor (PAR)-2 when complexed with factor VIIa.

Methods: We investigated the expression and function of TF on mouse bone marrow (BM)-derived DC.

Results: 20% of BM-derived DC expressed TF, which did not vary after incubation with LPS or dexamethasone (DEX). However, the procoagulant activity of DEX-treated DC in recalcified plasma was 30 fold less than LPS-treated DC. In antigen-specific and allogeneic T cell culture experiments, the TF on DEX-DC provided a signal through PAR-2, which contributed to the reduced ability of these cells to stimulate CD4+ T cell proliferation and cytokine-production. In vivo, an inhibitory anti-TF Ab and a PAR-2 antagonist enhanced antigen-specific priming in two models where antigen was given without adjuvant, with an effect approximately 50% that seen with LPS, suggesting a similar mechanism was operational physiologically.

Conclusions: These data suggest a novel TF and PAR-2 dependent mechanism on DEX-DC in vitro and unprimed DC in vivo that contributes to the low immunogenicity of these cells. Targeting this pathway has the potential to influence antigen-specific CD4+ T cell activation.
Introduction

Initiation and propagation of the coagulation cascade in haemostasis and thrombosis is well characterised and results in the formation of a fibrin clot. Tissue factor (TF) is the physiological initiator of thrombin generation and is constitutively expressed on vascular adventitial cells, leading to the traditional concept that TF forms a ‘haemostatic envelope’ to prevent bleeding from damaged vessels.\(^1\)

TF can also be induced on inflammatory cells and has been implicated in the pathogenesis of a variety of diseases such as atherosclerosis, malignancy and Ab-mediated rejection\(^2\). Although some of these processes involve thrombosis, some of the effects of TF are fibrin-independent and involve signalling through protease activated receptors (PAR), which are cleaved by serine proteases to expose a neo-N-terminal activating tethered ligand\(^3\). Thrombin can cleave and activate PARs 1, 3 & 4, whereas TF bound to factor VIIa (FVIIa) in a binary complex is capable of activating PAR-2 directly. PAR-2 is also cleaved by a number of other proteases including FXa, trypsin, proteinase 3 and mast cell tryptase.

TF and PARs are expressed by a number of immune cells including monocytes, macrophages and DC\(^4\). On antigen-primed DC, thrombin, via PAR-1 has a profound influence on migration through lymph nodes and subsequent spread of systemic inflammation in murine models of endotoxaemia and infection\(^5\). Additionally, PAR-2 signalling has been shown to enhance uptake of antigens, trafficking and T cell activation by DC\(^6-10\).

In this paper, we investigated the function of TF on mouse bone marrow (BM)-derived DC, comparing DC outgrown under standard conditions with those incubated with either dexamethasone (DEX) or LPS. Our data suggests that, despite similar
expression levels of TF by all three, there is a hierarchy of procoagulant activity and
TF expressed by dexamethasone (DEX)-treated DC appears to provide signalling
through PAR-2 to sustain the low immunogenicity of these cells. Based on additional
data generated in vivo, we speculate that a similar mechanism operates
physiologically to limit CD4⁺ T cell priming to specific antigens encountered in the
absence of an adjuvant.
Methods

Animals

6-8 week old BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from Harlan Laboratories. Two strains expressing transgenic T cell receptors (TCR) were used to assess antigen-specific responses, chosen because they were available within the Immunology Department. The first, Marilyn, has a transgenic TCR specific for male HY antigen restricted by MHC class II and the second, DO11.10 has a TCR that recognises an OVA peptide (OVA323-339) restricted by H-2A^d. Both were kind gifts from Drs Jian Guo Chai and Andrew George (Imperial College London). All procedures were performed in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

Cell isolation and T cell assays.

BM-derived DC were cultured as described elsewhere 11. Briefly cells were flushed out from the femurs and tibiae of mice and passed through a nylon cell strainer. Red blood cells were lysed using ammonium-chloride-potassium buffer. After washing, 1x10^6/ml cells were seeded in RPMI growth medium plus 5% supernatant from a GM-CSF producing hybridoma cell line. On day 3, non adherent cells were discarded and fresh medium was added. In some cultures, either 1µM dexamethasone (DEX)(Sigma-Aldrich, UK) 12 or 1µg/ml LPS (Escherichia coli serotype 0128:B12)(Sigma-Aldrich, UK) was added on days 5 or 6. DCs were harvested on day 7.

T cells were isolated from the spleen and lymph nodes (mesenteric, inguinal and axillary). Organs were passed through a nylon cell strainer and red blood cells were lysed as above. Splenocytes were incubated with an Ab cocktail supplied by Invitrogen containing rat anti-mouse Gr, CD16/32, MHCII and CD8 antibodies for 20 min at 4°C before washing and incubation with sheep anti-rat magnetic beads for
negative selection according to manufacturer’s instructions. The resulting CD4+ T cells were 90-95% pure.

To assess T cell proliferation against alloantigens, 2x10^5 BALB/c T cells were stimulated with 1x10^4 irradiated C57BL/6 DCs in 200µl complete medium unless otherwise stated. To assess antigen-specific proliferation, 2x10^5 female Marilyn CD4+ T cells were stimulated with 1x10^4 male C57BL/6 DCs in 200µl complete medium. In some assays, rabbit polyclonal anti-TF Ab (American Diagnostica) or control rabbit Ig were added at beginning. Proliferation was measured by adding ³H-thymidine on day four of culture and harvesting 16 to 18 hours later to determine T cell proliferation as assessed by incorporated radioactivity.

**Flow cytometric analysis**

All flow cytometry was performed on a FACSCalibur flow cytometer and analysed using Cellquest (BD BioSciences, UK) or Flojo (Treestar, USA) software. For cell surface analysis, the following antibodies were used; rat anti-mouse CD4, CD8, (e-Bioscience USA) FITC-CD80 (Serotec UK), FITC-CD86 (Becton Dickinson UK); hamster anti-mouse FITC-CD3, FITC-CD11c, FITC-MHC II (e-Bioscience USA); rabbit polyclonal anti-TF, anti-TFPI (both American Diagnostica), PAR-3, PAR-4 (Santa Cruz USA); mouse anti-PAR-1 (Becton Dickinson), PAR-2 (Santa Cruz).

Where appropriate, the following second layers were used; Swine anti-rabbit FITC-Ig (Dako, Denmark); Goat anti-rabbit FITC-Ig, anti-rabbit PE-Ig (Sigma-Aldrich UK), anti-mouse FITC IgG (Dako); mouse anti-rat FITC-Ig (e-Bioscience). 2x10^5 cells were analysed immediately or fixed in 2% paraformaldehyde in PBS and analysed within three days.

Intracellular cytokine staining as performed as previously described¹³. Briefly, cells were stimulated with 50ng/ml PMA (Sigma-Aldrich, UK) plus 500ng/ml ionomycin
(Calbiochem, Germany) for 4 hours, with 10µg/ml brefeldin A (Sigma-Aldrich) for the final 2 hours. All washes and incubations were done in buffer containing 0.5% Saponin (Sigma-Aldrich). Cells were stained with rat anti-IFNγ, IL-4 or IL-10 (all from BD Pharmingen USA)

**RNA extraction and RT-PCR**

Between 5x10⁶ and 1x10⁷ cells were washed thoroughly with PBS before RNA was extracted using phenol and chloroform and re-suspended in RNAse-free water (Sigma-Aldrich). RNA was assessed using agarose gel analysis and Quanti-iT Ribogreen RNA reagent and kit (Invitrogen, UK). RT-PCR was performed using reagents from Applied Biosystems, USA, including primers for PARs 1-4 and beta-actin. All PCR products were run on 1% agarose gel.

**Clotting Assay**

Mouse acetone brain extract (Sigma, UK), used as a standardized source of TF and all other reagents were suspended in 50mM Tris-HCL, 150 mM NaCl and 1 mg/ml human albumin pH 7.4. For test samples, cells were suspended at a concentration of 1x10⁷/ml. Serial dilutions of brain extract (in 80µl) or 1x10⁷ cells /ml (80µl) were mixed in a glass tube with 80µl phospholipid and 80µl pooled normal mouse plasma at 37°C for one minute. To start the clotting assay 80µl 65mM CaCl₂ was added, and, whilst being continuously agitated, the time for a clot to form in the tube was measured. In some assays, rabbit polyclonal anti-TF Ab or control rabbit Ig were added at beginning. All samples performed in triplicate. A standard curve generated from the TF in mouse brain extract was used to measure relative TF function in the test samples.

**In vivo antigen specific T cell sensitisation**
Cells from lymph nodes and spleen of naive DO11.10 mice were labelled with 2.5µM CFSE (Cambridge Biosciences, UK) using standard protocols. 1x10^7 cells were injected into the tail vein of BALB/c mice. 24 hours later, the mice were given an i.p. injection of either saline, 250µg anti-TF Ab 14, control rabbit Ig, 25µg LPS (Escherichia coli serotype 0127:B8) (Sigma-Aldrich, UK) or LPS alone followed by 5mg whole OVA. 72 hours later, lymph node (cervical, axillary, brachial and inguinal) cells were harvested for analysis of proliferation and IFN-γ, IL-4 and IL-10 production by flow cytometry.

**Contact sensitivity responses**

On day 0, C57BL/6 mice were sensitised by application of 5% oxazalone in ethanol and acetone (4:1, 50ul) to the shaved abdomen. At the same time mice were injected i.p. with either 100µl saline control, 1uM/kg PAR 2 agonist or 100uM/kg PAR-2 antagonist. Mice were re-challenged on day 5 by applying 1% oxazolone in olive oil and acetone (4:1, 10ul) to the right ear, whereas the left ear was painted with vehicle alone. Ear thickness was measured using a digital micrometer. Measurements were done on day 5 as a baseline and then 24 and 48 hours following ear painting.
Results

TF expression and procoagulant function on in-vitro cultured DC

C57BL/6 BM-derived DC (>85% CD11c+) were examined by flow cytometry. Figure 1 illustrates the expression of MHC class II, CD80 and CD86 by cells grown under three different conditions. As expected, DEX-treated DC expressed low levels of MHC II and co-stimulatory molecules whereas LPS-treated DC had higher expression. DC cultured without either DEX or LPS (‘untreated’) had an intermediate phenotype. Subpopulations of DC from all three conditions expressed detectable TF antigen on their cell surface, representing 15-20% of the CD11c+ cells, with similar population MFI (figure 1B-D), suggesting similar levels of TF expression. Similar results were obtained with DC grown from BALB/c mice (data not shown).

In clotting assays using re-calcified mouse plasma, addition of all three DC populations promoted shorter clotting times compared to the ‘no cells’ control, with a hierarchy of clotting times (LPS<untreated<DEX) (figure 2A), each of which was prolonged by an inhibitory anti-TF Ab (figure 2B) suggesting that there were significantly different levels of procoagulant TF present on each type of DC. This was confirmed when the relative TF procoagulant activity on each was calculated (Figure 2C). In these experiments, the time to clot spontaneously in glass tubes (‘no cells’ in Figure 2A) represents the clotting induced by the (TF-independent) contact activated pathway. These data indicated that a greater proportion of TF on DEX-treated DC was in the non-procoagulant or ‘encrypted’ form. There were no detectable differences in annexin V binding, protein disulphide isomerase or murine tissue
factor pathway inhibitor expression by any of the DC used in these assays (data not shown).

**Biological activity of TF on DEX-treated DC**

To investigate whether TF on DEX-treated DC had a biological function, male DCs were co-cultured with naïve CD4⁺ T cells from female Marilyn mice, which possess a transgenic TCR that recognises the male HY antigen restricted by MHC class II. Compared to DEX-treated DC incubated with isotype control Ig, DC incubated with inhibitory anti-TF Ab stimulated significantly greater proliferation (figure 2D). These experiments were repeated with allogeneic DC and WT CD4⁺ T cells, with similar results (figure 2E), suggesting that the effect was not due to antigen processing. The same Ab did not enhance the near-maximal proliferation induced by untreated DC (figure 2F) and had no impact on proliferation of CD8⁺ T cells induced by DEX-treated DC (figure 2G). These results indicated that inhibiting TF on DEX-DC specifically enhanced proliferation by CD4⁺ T cells and suggested that the TF on DEX-DC was acting to limit the capacity of these cells to induce CD4⁺ T cell proliferation.

The TF-FVIIa complex can cleave and activate PAR-2 signalling. Analysis of PAR expression by DEX-DC revealed transcripts for all PARs (figure 3A), but only PAR-2 and PAR-4 were detectable on the cell surface by flow cytometry (figure 3B), which may reflect known differences in the importance of mRNA stabilisation for expression of different PARs. To address whether the effect of TF was due to signalling through PAR, agonist or antagonist peptides were added to DEX-DCs, prior to coculture with CD4⁺ T cells. Figures 3C&D show that, in contrast to when PAR-1 or -4 agonists were used, a selective PAR-2 agonist inhibited the effect of the anti-TF Ab,
indicating that PAR-2 activation was able to compensate for the inhibition of TF when Ab was present. In contrast, a PAR-2 antagonist enhanced the proliferative capacity of DEX-treated DC to the same extent as the anti-TF Ab (figure 3E), suggesting that it had the same functional effect as the Ab. These data are consistent with the hypothesis that the (mostly) encrypted TF on DEX-treated DC influences the capacity to stimulate T cells through activation of PAR-2.

Inhibition of TF/PAR-2 signaling enhances T cell priming in vivo

To assess whether any of this was relevant in vivo, lymph node and spleen cells from naïve DO11.10 mice were isolated and CFSE labelled. T cells from these mice possess a transgenic T cell receptor that recognises an OVA peptide (OVA323-339) restricted by H-2A^d [372- Murphy KM Science 1990 “Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo”]. The labelled cells were injected intravenously into naïve BALB/c mice 24 hours before IP sensitisation with whole OVA. 72 hours later lymph nodes from the mice were harvested and examined by flow cytometry. As shown in figure 4, compared to mice receiving control IgG, there were increased numbers of DO11.10 cells producing IFNγ in mice given an anti-TF Ab, the frequency of which approached that seen in mice when LPS was used as adjuvant. Proliferation of these IFNγ-producing CD4+ cells was also increased compared to when OVA was administered with the control, but these data just failed to reach statistical significance. No differences in IL-4 production were noticed. No TF was found on CD4^+ or CD8^+ T cells isolated from spleen and lymph nodes of these mice as described (data not shown), indicating that the anti-TF Ab
was unlikely to be acting directly on the T cells and consistent with it having an
influence on T cell priming via DC.

To further investigate, a model of DC-dependent delayed type hypersensitivity was
used. For these experiments, a specific PAR-2 antagonist was used instead of the
anti-TF Ab, so that only the signalling via PAR-2 was inhibited. C57BL/6 mice were
injected with a PAR-2 antagonist peptide immediately prior to oxazolone
sensitisation on the abdominal skin. Five days later mice were re-challenged with
oxazolone and antigen-specific swelling calculated. As shown in figure 4C-D, mice
given a PAR-2 antagonist at the time of sensitisation developed significantly greater
antigen-specific swelling compared to control mice, consistent with the hypothesis
that constitutive PAR-2 signalling modulates T cell priming.
Discussion

TF expression has been previously defined on human DC in lymphoid follicles\textsuperscript{17}, DC derived from monocytes\textsuperscript{4,18} and on murine DC\textsuperscript{5}. In this report, we confirm TF expression by 10-15\% of mouse BM-derived DC and show that this proportion and the level of TF expression was not influenced by addition of DEX or LPS in the final 2 days of culture. However, the procoagulant activity was significantly different on these cell populations, with DEX-treated DC having approximately 5-fold less functional TF activity compared to untreated DC and 30-fold less than that of LPS-treated DC (figure 2). These data suggest that DC have a mechanism to regulate the functional activity of TF, in line with their state of maturity, such that TF on immature DC exists predominantly in an encrypted or non-procoagulant state.

The molecular basis of TF encryption/decryption remains enigmatic. Potential mechanisms for decryption include an increase in the amount of procoagulant phosphatidylserine expressed on the cell membrane, and the conversion of Cys186-Cys209 disulphide bonds by protein disulphide isomerase\textsuperscript{19}. We assessed the levels of both these on the surface of our DC and found no differences (data not shown). Tissue factor pathway inhibitor can also regulate the differential functions of TF\textsuperscript{20}, but there were no differences in the levels of murine tissue factor pathway inhibitor on all three types of our DC (data not shown), so the mechanism by which TF was regulated on these DC remains unclear.

Encrypted TF/FVIIa is known to signal through PAR-2\textsuperscript{15,21}. Using an inhibitory anti-TF Ab, and a combination of PAR-2 agonists and antagonists, our data indicates that the encrypted TF on DEX-treated DC provides a signal through PAR-2 that suppresses CD4+ T cell proliferative responses, so that the anti-TF Ab or PAR-2
antagonist significantly enhanced CD4+ T cell proliferation after both allogeneic and antigen-specific stimulation. In the presence of the anti-TF, these responses could be re-suppressed by pre-incubation of DC with the PAR-2 agonist. This mechanism was not apparent when the anti-TF Ab was used with untreated or LPS-treated DC, suggesting either that it was not operational on these cells, or that the effect was masked by the near maximal proliferative responses induced by these cells.

In vivo, the anti-TF Ab enhanced the priming of naïve antigen-specific transgenic T cells to whole OVA (administered without an adjuvant), as shown by increased production of IFNγ and a trend towards increased proliferation. The magnitude of the effect of anti-TF Ab was approximately 50% of control LPS adjuvant, indicating the potency of the TF-mediated suppression of priming in this system. Additionally, the PAR-2 antagonist given to mice at the time of dermal priming to oxazolone enhanced the subsequent recall response, evident by a significant increase in the ear swelling on secondary challenge with the antigen. In these experiments, a PAR-2 agonist given during the priming phase had no significant impact on recall responses. These data suggest that the mechanism we have defined in vitro has a functional relevance in vivo.

The effects of PAR-2 signalling on DEX-treated DC we report here are very different to those previously reported by others. Fields et al, described that PAR-2 signals were required for DC maturation during GM-CSF-induced outgrowth from BM cells, although these data have since been challenged. A number of studies have concluded that PAR-2 signalling enhances antigen uptake and trafficking by murine DC, and increases T cell activation in response to specific antigens. In trying to reconcile our data with these, one obvious difference is that we have focussed on DEX-treated immature DC; the mechanism we describe appears irrelevant in mature
DC. It may also be relevant that others have compared responses in WT and PAR-2-deficient mice and have addressed the effect of proteases other than TF/FVIIa.

In summary, we report that the activity of TF, expressed on a subset of murine DC, varies with maturation state, such that TF on LPS-treated DC is procoagulant whereas on DEX-treated DC it appears to provide a tonic signal through PAR-2 that maintains an aspect of the low-immunogenic phenotype characteristic of these cells. This mechanism appears relevant in vivo, partially suppressing priming and subsequent recall responses. This novel role for TF/PAR signalling augments our understanding of DC function and highlights that manipulating this pathway might influence antigen-specific responses.
Acknowledgements

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The authors declare no conflict of interest
References.


Figure legends

Figure 1: Flow cytometric analysis of murine BM-derived DC to assess TF expression on DC at different states of functional maturity.

A: C57BL/6 BM cells were grown in GMCSF and DC harvested on day seven of culture before analysis by flow cytometry with the antibodies as indicated. Cells that were otherwise untreated are compared to those in which dexamethasone (DEX - 1μM) or LPS (1μg/ml) were added into the culture for the final 48 or 24 hours respectively. Representative of 4 experiments.

DEX-treated (B), untreated (C) and LPS-treated (D) DCs were harvested on day seven of culture and analysed for co-expression of CD11c and TF. Profiles show isotype control and specific binding. Numbers in quadrants represent percentage of positively stained cells within each quadrant. Representative profiles from one of four experiments.

Figure 2: Analysis of TF function murine DC.

A-B – Clotting assays with recalcified mouse plasma in presence of dexamethasone-treated (DEX), untreated (UNTX) or LPS-treated (LPS) DCs or B16F10 tumour cells known to express high levels of surface TF (positive control in A). The ordinate shows the time to clot in seconds (±SEM). Error bars in some columns are too small to see. In (B) respective DCs were incubated with increasing doses of anti-TF (closed triangles)) or rabbit control (open triangles) Ig.
C: Relative TF activity on dexamethasone (DEX)- and LPS-treated DC compared to untreated (UNTX) DC (=1) calculated from the clotting times from standard curves generated by titrating fixed concentrations of TF from mouse brain extract into the clotting assay. *P<0.01 compared to untreated.

D: C57BL/6 male DEX-DCs were incubated with either medium, polyclonal rabbit anti-TF antibody or control rabbit Ig as indicated and co-cultured with purified Marilyn female CD4⁺ T cells. T cell response was measured by ³H incorporation on day three of culture. Graph shows mean +/- SEM of triplicate well. Compared to control Ig *p=0.05.

E: Experiment similar to that in D, except DEX-treated BL/6 DC incubated with BALB/c CD4⁺ T cells. Compared to control Ig, *p<0.002.

F: As E, except untreated DC used as stimulators. P=NS

G: As E, except CD8⁺ T cell used as responders. P=NS

All data representative of three experiments.

Figure 3: Experiments to determine PAR expression on mouse DC and interaction of TF with PAR-2

A: RT-PCR analysis of RNA extracted from dexamethasone-treated (D), untreated (U) or LPS-treated (L) DC for expression of PAR-1-4. Control lane (N) contains RNA from DEX-treated cells without reverse transcriptase. Beta actin expression used as positive control for RNA integrity.


C&D: dexamethasone (DEX)-treated DC were incubated with medium alone (medium), anti-TF Ab, or control rabbit Ig as indicated with or without additional
agonist peptides to activate PARs-1 and -4 (C) or PAR-2 (D), before co-culture with purified BALB/c CD4\(^+\) T cells. Graphs show mean \( ^3 \text{H} \)-thymidine incorporation (±SEM) of triplicate wells on day five of culture. Representative of three experiments. p=NS all comparisons.

E: As D but with additional control of dexamethasone (DEX)-treated DC incubated with a selective PAR-2 antagonist. Representative of three additional experiments. *p<0.05

**Figure 4: In vivo experiments demonstrating potential physiological role for TF/PAR-2 during sensitisation.**

A: 1x10\(^7\) CFSE stained lymph node and spleen cells from naïve DO11.10 mice were injected intravenously into naïve BALB/c mice. 24 hours later mice were immunised with 5mg whole OVA given with either an anti-TF Ab (250\( \mu \)g) or control Ig or 25\( \mu \)l LPS (positive control). Negative control mice received no OVA or LPS only. 72 hours later the lymph node cells were harvested from individual mice and analysed for proliferation and cytokine production (IFN-\( \gamma \) and IL-4). Representative profiles from 1 of 9 mice from each group are shown.

B: Experiment performed exactly as in A. The number of IFN-\( \gamma \) producing CFSE+ CD4\(^+\) T cells were enumerated from each mouse given OVA plus polyclonal Ig control (open bar), anti-TF (striped bar) or LPS (filled bar). Fold increase was calculated as the ratio of this number for each mouse to the equivalent number of
CD4⁺ T cells enumerated for mice given LPS alone. Mean and standard error was calculated for each group and is plotted as shown.

C-E: Contact hypersensitivity was induced by sensitisation with 5% oxazolone on the shaved abdomen of C57BL/6 mice. 5 days later the mice were re-challenged with vehicle alone on the left ear and oxazolone on the right. Graphs show difference in ear thickness between right and left ears 24 (C) and 48 (D) hours after re-challenge (n=12). Prior to initial sensitisation, mice received either a PAR-2 antagonist, PAR-2 agonist or normal saline control IP. Data are expressed as an increase in ear thickness. Statistical analyses performed using two-way ANOVA with Bonferroni's post test (* p<0.05 at both time points compared to saline)
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Figure 1 – Shrivastava et al

A

CD11c

DEX

UNTREATED

LPS

Counts

0

10^6

10^7

10^8

FL1-H

Anti-CD11c

B: DEX-TREATED

MFI=425

2.5%

2.5%

4%

4%

1%

15%

2%

50%

C: UNTREATED

MFI=425

4%

1.9%

1%

20%

2.5%

55%

D: LPS-TREATED

MFI=390

2.5%

2%

22%

45%

MFI=425

Anti-CD11c

Anti-CD11c

Anti-TF

Anti-TF

Anti-TF

Anti-TF
Figure 2 – Shrivastava et al

A

B

C

D

E

F

G

Ab concentration (µg/ml)

Relative TF activity (normalised to untreated DC)

Male DEX-DC, Female Marylin CD4+ T cells

BL/6 DEX-DC, BALB/c CD4+ T cells

BL/6 untreated DC, BALB/c CD4+ T cells

BL/6 DEX-DC, BALB/c CD8+ T cells

Medium Control anti-TF
Figure 3 – Shrivastava et al

A

PAR1 PAR2 PAR3 PAR4 β actin
D U L N

B

Pre-incubation of DEX treated DC with:

PAR-1
PAR-2
PAR-3
PAR-4

Counts

C

Medium
Control Ig
α-TF

PAR-1 & 4 agonist

D

Medium
Control Ig
α-TF

PAR-2 agonist

E

Medium
α-TF
α-TF + PAR-2 agonist
PAR-2 antagonists

Pre-incubation of DEX treated DC with:
Mice immunized with OVA plus:

- Control
- Ig
- α-TF
- LPS alone
- OVA + LPS
- OVA + control
- Saline

Fold increase in IFNγ producing CFSE+ CD4+ T cells

Antigen-specific ear swelling (mm)

IP treatment accompanying initial sensitisation

Figure 4 – Shrivastava et al
Protease activated receptor-2 signalling by tissue factor on dendritic cells suppresses antigen-specific CD4+ T cell priming.

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Keywords: tissue factor, protease activated receptor-2, dendritic cell, dexamethasone, CD4+ T cell

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Abstract

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Results: 20% of BM-derived DC expressed TF, which did not vary after incubation with LPS or dexamethasone (DEX). However, the procoagulant activity of DEX-treated DC in recalcified plasma was 30 fold less than LPS-treated DC. In antigen-specific and allogeneic T cell culture experiments, the TF on DEX-DC provided a signal through PAR-2, which contributed to the reduced ability of these cells to stimulate CD4\(^+\) T cell proliferation and cytokine production. In vivo, an inhibitory anti-TF Ab and a PAR-2 antagonist enhanced antigen-specific priming in two models where antigen was given without adjuvant, with an effect approximately 50% that seen with LPS, suggesting a similar mechanism was operational physiologically.

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Introduction

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TF can also be induced on inflammatory cells and has been implicated in the pathogenesis of a variety of diseases such as atherosclerosis, malignancy and Ab-mediated rejection. Although some of these processes involve thrombosis, some of the effects of TF are fibrin-independent and involve signalling through protease activated receptors (PAR), which are cleaved by serine proteases to expose a neo-N-terminal activating tethered ligand. Thrombin can cleave and activate PARs 1, 3 & 4, whereas TF bound to factor VIIa (FVIIa) in a binary complex is capable of activating PAR-2 directly. PAR-2 is also cleaved by a number of other proteases including FXa, trypsin, proteinase 3 and mast cell tryptase.

TF and PARs are expressed by a number of immune cells including monocytes, macrophages and DC. On antigen-primed DC, thrombin, via PAR-1 has a profound influence on migration through lymph nodes and subsequent spread of systemic inflammation in murine models of endotoxaemia and infection. Additionally, PAR-2 signalling has been shown to enhance antigen uptake by DC and T cell activation by DC.

In this paper, we have investigated the function of TF on mouse bone marrow (BM)-derived DC, comparing DC outgrown under standard conditions with those incubated with either dexamethasone (DEX) or LPS. Our data suggests that, despite similar
expression levels of TF by all three, there is a hierarchy of procoagulant activity and TF in particular expressed by dexamethasone (DEX)-treated DCs, on which TF appears to provide signalling through PAR-2 that sustains the low immunogenicity of these cells. Based on additional data generated in vivo, we speculate that a similar mechanism operates physiologically to limit CD4+ T cell priming to specific antigens encountered in the absence of an adjuvant.
Methods

Animals

6-8 week old BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased from Harlan Laboratories. Two strains expressing transgenic T cell receptors (TCR) were used to assess antigen-specific responses, chosen because they were available within the Immunology Department. The first, Marilyn, has a transgenic TCR specific for male HY antigen restricted by MHC class II and the second, DO11.10 has a TCR that recognises an OVA peptide (OVA323-339) restricted by H-2A^d. Both mice were kind gifts from Drs Jian Guo Chai and Andrew George (Imperial College London). All procedures were performed in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

Cell isolation and T cell assays.

BM-derived DC were cultured as described elsewhere \textsuperscript{11}. Briefly cells were flushed out from the femurs and tibiae of mice and passed through a nylon cell strainer. Red blood cells were lysed using ammonium-chloride-potassium buffer. After washing, 1x10^6/ml cells were seeded in RPMI growth medium plus 5% supernatant from a GM-CSF producing hybridoma cell line. On day 3, non adherent cells were discarded and fresh medium was added. In some cultures, either 1µM dexamethasone (DEX) (Sigma-Aldrich, UK) \textsuperscript{12} or 1µg/ml LPS (Escherichia coli serotype 0128:B12) (Sigma-Aldrich, UK) was added on days 5 or 6. DCs were harvested on day 7.

T cells were isolated from the spleen and lymph nodes (mesenteric, inguinal and axillary). Organs were passed through a nylon cell strainer and red blood cells were lysed as above. Splenocytes were incubated with an Ab cocktail supplied by Invitrogen containing rat anti-mouse Gr, CD16/32, MHCII and CD8 antibodies for 20 min at 4°C before washing and incubation with sheep anti-rat magnetic beads for
negative selection according to manufacturer’s instructions. The resulting CD4+ T cells were 90-95% pure.

To assess T cell proliferation against alloantigens, 2x10^6 BALB/c T cells were stimulated with 1x10^4 irradiated C57BL/6 DCs in 200µl complete medium unless otherwise stated. To assess antigen-specific proliferation, 2x10^5 female Marilyn CD4+ T cells were stimulated with 1x10^4 male C57BL/6 DCs in 200µl complete medium. In some assays, rabbit polyclonal anti-TF Ab (American Diagnostica) or control rabbit Ig were added at beginning. Proliferation was measured by adding ^3H-thymidine on day four of culture and harvesting 16 to 18 hours later to determine T cell proliferation as assessed by incorporated radioactivity.

**Flow cytometric analysis**

All flow cytometry was performed on a FACSCalibur flow cytometer and analysed using Cellquest (BD BioSciences, UK) or Flojo (Treestar, USA) software. For cell surface analysis, the following antibodies were used; rat anti-mouse CD4, CD8, (e-Bioscience USA) FITC-CD80 (Serotec UK), FITC-CD86 (Becton Dickinson UK); hamster anti-mouse FITC-CD3, FITC-CD11c, FITC-MHC II (e-Bioscience USA); rabbit polyclonal anti-TF, anti-TFPI (both American Diagnostica), PAR-3, PAR-4 (Santa Cruz USA); mouse anti-PAR-1 (Becton Dickinson), PAR-2 (Santa Cruz).

Where appropriate, the following second layers were used; Swine anti-rabbit FITC-Ig (Dako, Denmark); Goat anti-rabbit FITC-Ig, anti-rabbit PE-Ig (Sigma-Aldrich UK), anti-mouse FITC IgG (Dako); mouse anti-rat FITC-Ig (e-Bioscience). 2x10^5 cells were analysed immediately or fixed in 2% paraformaldehyde in PBS and analysed within three days.

Intracellular cytokine staining as performed as previously described. Briefly, cells were stimulated with 50ng/ml PMA (Sigma-Aldrich, UK) plus 500ng/ml ionomycin
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(Calbiochem, Germany) for 4 hours, with 10µg/ml brefeldin A (Sigma-Aldrich) for the final 2 hours. All washes and incubations were done in buffer containing 0.5% Saponin (Sigma-Aldrich). Cells were stained with rat anti-IFNγ, IL-4 or IL-10 (all from BD Pharmingen USA).

**RNA extraction and RT-PCR**

Between 5x10⁶ and 1x10⁷ cells were washed thoroughly with PBS before RNA was extracted using phenol and chloroform and re-suspended in RNAse-free water (Sigma-Aldrich). RNA was assessed using agarose gel analysis and QuantiT Ribogreen RNA reagent and kit (Invitrogen, UK). RT-PCR was performed using reagents from Applied Biosystems, USA, including primers for PARs 1-4 and beta-actin. All PCR products were run on 1% agarose gel.

**Clotting Assay**

Mouse acetone brain extract (Sigma, UK), used as a standardized source of TF and all other reagents were suspended in 50mM Tris-HCl, 150 mM NaCl and 1 mg/ml human albumin pH 7.4. For test samples, cells were suspended at a concentration of 1x10⁷/ml. Serial dilutions of brain extract (in 80µl) or 1x10⁷ cells /ml (80µl) were mixed in a glass tube with 80µl phospholipid and 80µl pooled normal mouse plasma at 37°C for one minute. To start the clotting assay 80µl 65mM CaCl₂ was added, and, whilst being continuously agitated, the time for a clot to form in the tube was measured. In some assays, rabbit polyclonal anti-TF Ab or control rabbit Ig were added at beginning. All samples performed in triplicate. A standard curve generated from the TF in mouse brain extract was used to measure relative TF function in the test samples.

**In vivo antigen specific T cell sensitisation**
Cells from lymph nodes and spleen of naive DO11.10 mice were labelled with 2.5µM CFSE (Cambridge Biosciences, UK) using standard protocols. 1x10^7 cells were injected into the tail vein of BALB/c mice. 24 hours later, the mice were given an i.p. injection of either saline, 250µg anti-TF Ab, control rabbit Ig, 25µg LPS (Escherichia coli serotype 0127:B8) (Sigma-Aldrich, UK) or LPS alone followed by 5mg whole OVA. 72 hours later, lymph node (cervical, axillary, brachial and inguinal) cells were harvested for analysis of proliferation and IFN-γ, IL-4 and IL-10 production by flow cytometry.

**Contact sensitivity responses**

On day 0, C57BL/6 mice were sensitised by application of 5% oxazalone in ethanol and acetone (4:1, 50ul) to the shaved abdomen. At the same time mice were injected i.p. with either 100µl saline control, 1uM/kg PAR 2 agonist or 100µM/kg PAR-2 antagonist. Mice were re-challenged on day 5 by applying 1% oxazolone in olive oil and acetone (4:1, 10ul) to the right ear, whereas the left ear was painted with vehicle alone. Ear thickness was measured using a digital micrometer. Measurements were done on day 5 as a baseline and then 24 and 48 hours following ear painting.
Results

TF expression and procoagulant function on in-vitro cultured DC

C57BL/6 BM-derived DC (>85% CD11c⁺) were examined by flow cytometry. Figure 1 illustrates the expression of MHC class II, CD80 and CD86 by cells grown under three different conditions. As expected, DEX-treated DC expressed low levels of MHC II and co-stimulatory molecules whereas LPS-treated DC had higher expression. DC cultured without either DEX or LPS ('untreated') had an intermediate phenotype. Subpopulations of DC from all three conditions expressed detectable TF antigen on their cell surface, representing 15-20% of the CD11c⁺ cells, with similar population MFI (figure 1B-D), suggesting similar levels of TF expression. Similar results were obtained with DC grown from BALB/c mice (data not shown).

In clotting assays using re-calculated mouse plasma, addition of all three DC populations promoted shorter clotting times compared to the ‘no cells’ control, with a hierarchy of clotting times (LPS<untreated<DEX) (figure 2A), each of which was prolonged by an inhibitory anti-TF Ab (figure 2B) suggesting that there were significantly different levels of procoagulant TF present on each type of DC. This was confirmed when the relative TF procoagulant activity on each was calculated (Figure 2C). In these experiments, the time to clot spontaneously in glass tubes (‘no cells’ in Figure 2A) represents the clotting induced by the (TF-independent) contact activated pathway. These data indicated that a greater proportion of TF on DEX-treated DC was in the non-procoagulant or ‘encrypted’ form. There were no detectable differences in annexin V binding, protein disulphide isomerase or murine tissue
factor pathway inhibitor expression by any of the DC used in these assays (data not shown).

**Biological activity of TF on DEX-treated DC**

To investigate whether TF on DEX-treated DC had a biological function, male DCs were co-cultured with naïve CD4^+ T cells from female Marilyn mice, which possess a transgenic TCR that recognises the male HY antigen restricted by MHC class II. Compared to DEX-treated DC incubated with isotype control Ig, DC incubated with inhibitory anti-TF Ab stimulated significantly greater proliferation (figure 2D). These experiments were repeated with allogeneic DC and WT CD4^+ T cells, with similar results (figure 2E), suggesting that the effect was not due to antigen processing. The same Ab did not enhance the near-maximal proliferation induced by untreated DC (figure 2F) and had no impact on proliferation of CD8^+ T cells induced by DEX-treated DC (figure 2G). These results indicated that inhibiting TF on DEX-DC specifically enhanced proliferation by CD4^+ T cells and suggested that the TF on DEX-DC was acting to limit the capacity of these cells to induce CD4^+ T cell proliferation.

The TF-FVIIa complex can cleave and activate PAR-2 signalling. Analysis of PAR expression by DEX-DC revealed transcripts for all PARs (figure 3A), but only PAR-2 and PAR-4 were detectable on the cell surface by flow cytometry (figure 3B), which may reflect known differences in the importance of mRNA stabilisation for expression of different PARs. To address whether the effect of TF was due to signalling through PAR, agonist or antagonist peptides were added to DEX-DCs, prior to co-culture with CD4^+ T cells. Figures 3C&D show that, in contrast to when PAR-1 or -4 agonists were used, a selective PAR-2 agonist inhibited the effect of the anti-TF Ab,
indicating that PAR-2 activation was able to compensate for the inhibition of TF when Ab was present. In contrast, a PAR-2 antagonist enhanced the proliferative capacity of DEX-treated DC to the same extent as the anti-TF Ab (figure 3E), suggesting that it had the same functional effect as the Ab. These data are consistent with the hypothesis that the (mostly) encrypted TF on DEX-treated DC influences the capacity to stimulate T cells through activation of PAR-2.

Inhibition of TF/PAR-2 signaling enhances T cell priming in vivo

To assess whether any of this was relevant in vivo, lymph node and spleen cells from naïve DO11.10 mice were isolated and CFSE labelled. T cells from these mice possess a transgenic T cell receptor that recognises an OVA peptide (OVA323-339) restricted by H-2A^d [3729 Murphy KM Science 1990 "Induction by antigen of intrathymic apoptosis of CD4^+CD8^+TCR^lo thymocytes in vivo"]. The labelled cells were injected intravenously into naïve BALB/c mice 24 hours before IP sensitisation with whole OVA. 72 hours later lymph nodes from the mice were harvested and examined by flow cytometry. As shown in figure 4, compared to mice receiving control IgG, there were increased numbers of DO11.10 cells producing IFNγ in mice given an anti-TF Ab, the frequency of which approached that seen in mice when LPS was used as adjuvant. Proliferation of these IFNγ-producing CD4^+ cells was also increased compared to when OVA was administered with the control, but these data just failed to reach statistical significance. No differences in IL-4 production were noticed. No TF was found on CD4^+ or CD8^+ T cells isolated from spleen and lymph nodes of these mice as described (data not shown), indicating that the anti-TF Ab
was unlikely to be acting directly on the T cells and consistent with it having an influence on T cell priming via DC.

To further investigate, a model of DC-dependent delayed type hypersensitivity was used. For these experiments, a specific PAR-2 antagonist was used instead of the anti-TF Ab, so that only the signalling via PAR-2 was inhibited. C57BL/6 mice were injected with a PAR-2 antagonist peptide immediately prior to oxazolone sensitisation on the abdominal skin. Five days later mice were re-challenged with oxazolone and antigen-specific swelling calculated. As shown in figure 4C-D, mice given a PAR-2 antagonist at the time of sensitisation developed significantly greater antigen-specific swelling compared to control mice, consistent with the hypothesis that constitutive PAR-2 signalling modulates T cell priming.
Discussion

TF expression has been previously defined on human DC in lymphoid follicles, DC derived from monocytes and on murine DC. In this report, we confirm TF expression by 10-15% of mouse BM-derived DC and show that this proportion and the level of TF expression was not influenced by addition of DEX or LPS in the final 2 days of culture. However, the procoagulant activity was significantly different on these cell populations, with DEX-treated DC having approximately 5-fold less functional TF activity compared to untreated DC and 30-fold less than that of LPS-treated DC (figure 2). These data suggest that DC have a mechanism to regulate the functional activity of TF, in line with their state of maturity, such that TF on immature DC exists predominantly in an encrypted or non-procoagulant state.

The molecular basis of TF encryption/decryption remains enigmatic. Potential mechanisms for decryption include an increase in the amount of procoagulant phosphatidylserine expressed on the cell membrane, and the conversion of Cys186-Cys209 disulphide bonds by protein disulphide isomerase. We assessed the levels of both these on the surface of our DC and found no differences (data not shown). Tissue factor pathway inhibitor can also the regulate the differential functions of TF, but there were no differences in the levels of murine tissue factor pathway inhibitor on all three types of our DC (data not shown), so the mechanism by which TF was regulated on these DC remains unclear.

Encrypted TF/FVIIa is known to signal through PAR-2. Using an inhibitory anti-TF Ab, and a combination of PAR-2 agonists and antagonists, our data indicates that the encrypted TF on DEX-treated DC provides a signal through PAR-2 that suppresses CD4+ T cell proliferative responses, so that the anti-TF Ab or PAR-2
antagonist significantly enhanced CD4+ T cell proliferation after both allogeneic and antigen-specific stimulation. In the presence of the anti-TF, these responses could be re-suppressed by pre-incubation of DC with the PAR-2 agonist. This mechanism was not apparent when the anti-TF Ab was used with untreated or LPS-treated DC, suggesting either that it was not operational on these cells, or that the effect was masked by the near maximal proliferative responses induced by these cells.

In vivo, the anti-TF Ab enhanced the priming of naïve antigen-specific transgenic T cells to whole OVA (administered without an adjuvant), as shown by increased production of IFNγ and a trend towards increased proliferation. The magnitude of the effect of anti-TF Ab was approximately 50% of control LPS adjuvant, indicating the potency of the TF-mediated suppression of priming in this system. Additionally, the PAR-2 antagonist given to mice at the time of dermal priming to oxazolone enhanced the subsequent recall response, evident by a significant increase in the ear swelling on secondary challenge with the antigen. In these experiments, a PAR-2 agonist given during the priming phase had no significant impact on recall responses. These data suggest that the mechanism we have defined in vitro has a functional relevance in vivo.

The effects of PAR-2 signalling on DEX-treated DC we report here are very different to those previously reported by others. Fields et al, described that PAR-2 signals were required for DC maturation during GM-CSF-induced outgrowth from BM cells, although these data have since been challenged. A number of studies have concluded that PAR-2 signalling enhances murine DC antigen uptake and trafficking by murine DC, and increases T cell activation in response to specific antigens. In trying to reconcile our data with these, one obvious difference is that we have focussed on DEX-treated immature DC; the mechanism we describe appears
irrelevant in mature DC. It may also be relevant that others have compared
responses in WT and PAR-2-deficient mice and have addressed the effect of
proteases other than TF/FVIIa.

In summary, we report that the activity of TF, expressed on a subset of murine DC,
varies with maturation state, such that TF on LPS-treated DC is procoagulant
whereas on DEX-treated DC it appears to provide a tonic signal through PAR-2 that
maintains an aspect of the low-immunogenic phenotype characteristic of these cells.
This mechanism appears relevant in vivo, partially suppressing priming and
subsequent recall responses. This novel role for TF/PAR signalling augments our
understanding of DC function and highlights that manipulating this pathway might
influence antigen-specific responses.
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The authors declare no conflict of interest
References.


Figure legends

Figure 1: Flow cytometric analysis of murine BM-derived DC to assess TF expression on DC at different states of functional maturity.

A: C57BL/6 BM cells were grown in GMCSF and DC harvested on day seven of culture before analysis by flow cytometry with the antibodies as indicated. Cells that were otherwise untreated (UNTX) are compared to those in which dexamethasone (DEX -1μM) or LPS (1μg/ml) were added into the culture for the final 48 or 24 hours respectively. Representative of 4 experiments.

Figure 2: Functional analysis of TF function murine DC.

A-B – Clotting assays with recalcified mouse plasma in presence of dexamethasone-treated (DEX), untreated (UNTX) or LPS-treated (LPS) DCs or B16F10 tumour cells known to express high levels of surface TF (positive control in A). The ordinate shows the time to clot in seconds (±SEM). Error bars in some columns are too small to see. In (B) respective DCs were incubated with increasing doses of anti-TF (closed triangles)) or rabbit control (open triangles) Ig.
C: Relative TF activity on dexamethasone (DEX)- and LPS-treated DC compared to untreated (UNTX) DC (=1) calculated from the clotting times from standard curves generated by titrating fixed concentrations of TF from mouse brain extract into the clotting assay. *P<0.01 compared to untreated.

D: C57BL/6 male DEX-DCs were incubated with either medium, polyclonal rabbit anti-TF antibody or control rabbit Ig as indicated and co-cultured with purified Marilyn female CD4+ T cells. T cell response was measured by 3H incorporation on day three of culture. Graph shows mean +/- SEM of triplicate well. Compared to control Ig *p=0.05.

E: Experiment similar to that as in AD, except DEX-treated BL/6 DC incubated with BALB/c CD4+ T cells. Compared to control Ig, *p<0.002.

F: As E, except untreated DC used as stimulators. P=NS

G: As E, except CD8+ T cell used as responders. P=NS

All data representative of three experiments.

Figure 3: TF/PAR-2 Experiments to determine PAR expression on mouse DC and interaction of TF with PAR-2

A: RT-PCR analysis of RNA extracted from dexamethasone-treated (D), untreated (U) or LPS-treated (L) DC for expression of PAR-1-4. Control lane (N) contains RNA from DEX-treated cells without reverse transcriptase. Beta actin expression used as positive control for RNA integrity.


C&D: dexamethasone (DEX)-treated DC were incubated with medium alone (medium), anti-TF Ab, or control rabbit Ig as indicated with or without additional
agonist peptides to activate PARs-1 and -4 (C) or PAR-2 (D), before co-culture with purified BALB/c CD4⁺ T cells. Graphs show mean ³H-thymidine incorporation (±SEM) of triplicate wells on day five of culture. Representative of three experiments. 

p=NS all comparisons.

E: As D but with additional control of dexamethasone (DEX)-treated DC incubated with a selective PAR-2 antagonist. Representative of three additional experiments. 

*p<0.05

**Figure 4: In vivo experiments demonstrating potential physiological role for TF/PAR-2 during sensitisation.**

A: 1x10⁷ CFSE stained lymph node and spleen cells from naïve DO11.10 mice were injected intravenously into naïve BALB/c mice. 24 hours later mice were immunised with 5mg whole OVA given with either an anti-TF Ab (250µg) or control Ig or 25µl LPS (positive control). Negative control mice received no OVA or LPS only. 72 hours later the lymph node cells were harvested from individual mice and analysed for proliferation and cytokine production (IFN-γ and IL-4). Representative profiles from 1 of 9 mice from each group are shown.

B: Experiment performed exactly as in A. The number of IFN-γ producing CFSE⁺ CD4⁺ T cells were enumerated from each mouse given OVA plus polyclonal Ig control (open bar), anti-TF (striped bar) or LPS (filled bar). Fold increase was calculated as the ratio of this number for each mouse to the equivalent number of
CD4⁺ T cells enumerated for mice given LPS alone. Mean and standard error was calculated for each group and is plotted as shown.

C-E: Contact hypersensitivity was induced by sensitisation with 5% oxazolone on the shaved abdomen of C57BL/6 mice. 5 days later the mice were re-challenged with vehicle alone on the left ear and oxazolone on the right. Graphs show difference in ear thickness between right and left ears 24 (C) and 48 (D) hours after re-challenge (n=12). Prior to initial sensitisation, mice received either a PAR-2 antagonist, PAR-2 agonist or normal saline control IP. Data are expressed as an increase in ear thickness. Statistical analyses performed using two-way ANOVA with Bonferroni’s post test (* p<0.05 at both time points compared to saline)