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Tumor Necrosis Factor Receptor I blockade shows that TNF-dependent and independent mechanisms synergise in TNF Receptor Associated Periodic Syndrome

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Complete List of Authors:	Fairclough, Lucy; The University of Nottingham, School of Life Science Stoop, A.; GlaxoSmithKine, Negm, Ola; The University of Nottingham, School of Life Sciences Radford, Paul; The University of Nottingham, School of Life Sciences Tighe, Patrick; The University of Nottingham, School of Life Sciences Todd, Ian; The University of Nottingham, School of Life Sciences
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Tumor Necrosis Factor Receptor I blockade shows that TNF-dependent and independent mechanisms synergise in TNF Receptor Associated Periodic Syndrome

Lucy C. Fairclough[1], A. Allart Stoop[2], Ola H. Negm[1,3], Paul M. Radford[1], Patrick J. Tighe[1], Ian Todd[1]

[1]School of Life Sciences, University of Nottingham, Nottingham, UK

[2]GlaxoSmithKline, Stevenage, UK

[3]Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

Keywords: Autoinflammatory disease; domain antibody; TNF; TNFR1; TRAPS

Correspondence: Dr Lucy C. Fairclough, School of Life Sciences, The University of Nottingham, A Floor West Block, Queen's Medical Centre, Nottingham NG7 2UH, UK. Tel: +44 115 8230729. Fax: +44 115 8230759.
E-mail: lucy.fairclough@nottingham.ac.uk.

Non-standard abbreviations: dAb – single domain antibody; DD – death domain; FL – full-length; PTX3 – pentraxin-3; TNFR1 – TNF receptor-1; sTNFR1 – soluble TNF receptor-1; *TNFRSF1A* – TNF receptor superfamily 1A gene; TRAPS – TNF receptor associated periodic syndrome.

Abstract

TNF Receptor Associated Periodic Syndrome (TRAPS) is an autoinflammatory disease involving recurrent episodes of fever and inflammation. It is associated with autosomal dominant mutations in *TNFRSF1A* localised to exons encoding the ectodomain of the p55 TNF receptor, TNFR1. The aim of this study was to investigate the role of cell-surface TNFR1 in TRAPS, and the contribution of TNF dependent and independent mechanisms to the production of cytokines. HEK-293 and SK-HEP-1 cell-lines were stably transfected with WT or TRAPS-associated variants of human *TNFRSF1A*. An anti-TNFR1 single domain antibody (dAb), and an anti-TNFR1 mAb, bound to cell-surface WT and variant TNFR1s. In HEK-293 cells transfected with death domain-inactivated (R347A) TNFR1, and in SK-HEP-1 cells transfected with normal (full-length) TNFR1, cytokine production stimulated in the absence of exogenous TNF by the presence of certain TNFR1 variants was not inhibited by the anti-TNFR1 dAb. In SK-Hep-1 cells, specific TRAPS mutations increased the level of cytokine response to TNF, compared to WT, and this augmented cytokine production was suppressed by the anti-TNFR1 dAb. Thus, TRAPS-associated variants of TNFR1 enhance cytokine production by a TNF-independent mechanism and by sensitising cells to a TNF-dependent stimulation. The TNF-dependent mechanism requires cell-surface expression of TNFR1, as this is blocked by TNFR1-specific dAb.

Introduction

TNF Receptor Associated Periodic Syndrome (TRAPS) is an autoinflammatory disease involving mutations localised to the ectodomain of the p55 TNF receptor, TNFR1 [1]. TRAPS involves stimulation of inflammation by misfolding of mutated TNFR1 causing TNF-independent signalling by intra-cellular, aggregated mutant TNFR1 and/or an unfolded protein response and stimulation of ROS production [2-10]. However, studies in mutant TNFR1 knock-in mice showed the TRAPS-like phenotype to be associated with the mutant/WT TNFR1 heterozygous state (as is also the case in TRAPS patients) and not with mutant/mutant homozygosity [7]. This indicates that synergy between TNF-independent effects of intra-cellular mutant TNFR1 and TNF-dependent signalling by cell-surface WT receptor are necessary to generate the full TRAPS phenotype.

TRAPS is associated with both 'structural' and 'non-structural' TNFR1 variants [7]. The former inevitably generate TRAPS and involve amino acid substitutions with significant structural consequences; these include cysteine mutations that disrupt disulphide bond formation (e.g. C33Y), and non-cysteine mutations (e.g. T50M); these mutations result in intra-cellular retention and reduced TNF-binding capacity, which is most profound in the cysteine mutants. Non-structural variants are R92Q and P46L, which occur in 1-10% of the general population as well as in TRAPS patients; these variants show cell-surface expression and TNF binding capacity similar to WT-TNFR1 and generate a relatively mild clinical phenotype.

In order to investigate the contribution of cell-surface TNFR1 to the TRAPS phenotype, we have employed an anti-human TNFR1 single domain antibody (dAb; DMS5541) that neutralises activation of TNFR1 by TNF without inhibiting TNF-TNFR1 interaction [11]; a similar anti-mouse TNFR1 dAb has also been reported [12]. This demonstrates both TNF-dependent and independent aspects of cytokine production associated with TRAPS, but with different contributions dependent on cell type, TNFR1 mutation and cytokine produced.

Results and discussion

The anti-TNFR1 dAb binds to HEK-293 cell surface WT and variant TNFR1. HEK-293 cells transfected with full-length (FL) WT or TRAPS-associated variants of *TNFRSF1A* under doxycycline control [2, 4, 13] were cultured with or without doxycycline to induce expression of the recombinant TNFR1. Figure 1a and b show cell-surface binding of anti-TNFR1 mAb (mAb225) and dAb (DMS5541), with non-specific staining subtracted in each case. As reported previously [2], surface expression of TNFR1 was very low in the HEK-293 transfectants not treated with doxycycline (i.e. 'non-induced') (Fig. 1a). Stimulation with doxycycline induced significantly higher cell-surface expression of recombinant protein in cells transfected with WT or non-structural variants of TNFR1 (R92Q or P46L) (Fig.1b). As expected, only a minor increase in cell-surface expression of the T50M structural mutant was observed and there was nearly no surface expression of the C33Y mutant. The binding of anti-TNFR1 dAb to HEK-293 cell-surface WT or variant TNFR1 was very similar to the binding of the anti-TNFR1 mAb. (The slightly higher staining by the dAb relative to the mAb at low levels of expression of TNFR1 is explained by the signal amplification given by the biotinylated-dAb/ Streptavidin-Alexa Fluor 647 compared to the directly phycoerythrin-labelled mAb (Fig. 1a).)

HEK-293 cells transfected with the R347A mutant of WT or TRAPS-associated variants of *TNFRSF1A* under doxycycline control were employed, as in our previous studies [4]. The R347A substitution inactivates the death domain (DD) in the cytoplasmic region of TNFR1. This inhibits signalling via the DD (including DD-dependent induction of apoptosis); it also reduces the normal intra-cellular accumulation of TNFR1 that is DD-dependent [4]. Thus, as expected, doxycycline induction of the R347A transfectants led to very much higher cell-surface expression of WT, R92Q and P46L TNFR1 (Fig. 1d); cell-surface expression of the T50M structural mutant was also detectable, and cell-surface C33Y increased only slightly, which is consistent with our previous report that structural mutants of TNFR1 with the R347A substitution are largely retained in the cytoplasm of HEK-293 cells, even following doxycyclin induction [4]. In all cases, both the anti-TNFR1 dAb and mAb bound to a similar extent, indicating that the dAb binds to structural and non-structural variants of TNFR1, as well as to the WT. The binding of dAb and mAb to

the non-induced R347A-TNFR1 transfectants was similar to that seen with the FL-TNFR1 transfectants (Fig. 1c).

The anti-TNFR1 dAb inhibits TNF synergy with TNFR1 variants in inducing IL-8 in HEK-293 cells.

The tetracyclin-suppressed strong CMV-promoter associated with the *TNFRSF1A* transgene in the pcDNA4TO plasmid allows low (near physiological) expression of the recombinant TNFR1 in the absence of induction with doxycycline (i.e. 'non-induced' cells). We showed previously that the HEK-293 transfectants produce high levels of IL-8 when over-expression of recombinant WT or variant TNFR1 is induced with doxycycline [2]. However, in order to investigate synergy between exogenous TNF and the recombinant TNFR1, non-induced HEK-293 cells transfected with FL or R347A *TNFRSF1A* variants were analysed for production of IL-8 (Figs. 1e-h).

In the absence of TNF, the non-induced R92Q and T50M FL-TNFR1 transfectants showed slight, but significant, elevation of IL-8 production compared to WT transfectants; this appeared to be TNF-independent as there was no significant inhibition by the anti-TNFR1 dAb (DMS5541) relative to the non-specific control dAb (DMS5556) (Fig. 1e). Very similar results were obtained for IL-8 production by the non-induced R347A-TNFR1 transfectants in the absence of TNF, with the R92Q and T50M variants enhancing IL-8 production significantly and without significant inhibition of this by the anti-TNFR1 dAb (Fig. 1g).

When the non-induced HEK-293 FL TNFR1 transfectants were treated with 3ng/ml TNF, IL-8 production was greatly augmented (> 10-fold) for all TNFR1 variants, although the highest IL-8 production was given by the WT transfectant (Fig. 1f). In all cases, the anti-TNFR1 dAb caused very significant inhibition of IL-8 production, indicating that it was highly dependent on TNF signalling via TNFR1 (Fig. 1f). Interestingly, the non-induced R347A-TNFR1 transfectants behaved differently from the FL-TNFR1 transfectants when treated with 3ng/ml TNF (Fig. 1h) – lower levels of IL-8 were produced by the WT and non-structural TNFR1 variants (R92Q and P46L) whereas significantly higher levels were produced by the C33Y and T50M structural variants (Fig. 1h). In all cases, the anti-TNFR1 dAb again inhibited

IL-8 production very significantly (Fig.1h). This indicates that TNF stimulation via endogenously-expressed TNFR1 synergises with transgenically-expressed TNFR1 structural variants (C33Y and T50M) to enhance IL-8 production by the HEK-293 cells via pathways not dependent on the DD of the structural variants; this might involve other signalling domains of the cytoplasmic region of the TNFR1 structural mutants or, possibly, an unfolded-protein response triggered by the misfolding of these mutants.

The anti-TNFR1 dAb binds to SK-HEP-1 cell surface TNFR1.

The SK-Hep-1 cell-line naturally expresses higher levels of TNFR1 than do HEK-293 cells. We have demonstrated previously that SK-Hep-1 cells transfected with FL structural, or non-structural variants of *TNFRSF1A* produce significantly higher levels of G-CSF, GM-CSF, CCL-2, CCL-5 and pentraxin-3 (PTX3) than do WT TNFR1 transfected SK-Hep-1 cells when induced with doxycycline [6]. Furthermore, the non-induced transfectants (i.e. not treated with doxycycline) express near physiological levels of recombinant TNFR1 and, under these conditions, we have found that the C33Y and R92Q variants induce activation of multiple inflammatory signalling pathways relative to WT TNFR1 ([10] and Abduljabbar et al., unpublished observations).

Figure 2 (a-d) shows expression of cell-surface WT TNFR1 in the SK-Hep-1 transfectants in the absence of doxycycline as detected by binding of anti-TNFR1 mAb (minus non-specific staining) or binding of anti-TNFR1 dAb (minus binding of the non-specific dAb). The WT, non-structural and structural TNFR1 variant transfectants all show cell-surface TNFR1 expression. (Fig. 2e). As mentioned previously, the higher staining by the dAb relative to the mAb is explained by the signal amplification given by the biotinylated-dAb/ Streptavidin-Alexa Fluor 647 compared to the directly phycoerythrin-labelled mAb (Fig. 2e).

The anti-TNFR1 dAb inhibits TNF synergy with TNFR1 variants in inducing cytokines in SK-Hep-1 cells.

Figure 3 shows the production of IL-8 (a, b), CCL-2 (c, d), G-CSF (e, f), PTX-3 (g, h), CCL-5 (i, j) and IL-6 (k, l) by the non-induced SK-Hep-1 transfectants in the absence or presence of TNF. Without stimulation with exogenous TNF, only the R92Q

transfectant gave significantly enhanced production of IL-8 (Fig. 3a) and CCL2 (Fig. 3c) relative to the WT transfectant; T50M gave significantly enhanced production of G-CSF (Fig. 3e) and both R92Q and T50M significantly enhanced PTX-3 production (Fig. 3g) relative to WT; only P46L gave enhanced CCL-5 (Fig. 3i); more IL-6 was produced by the WT and non-structural variants than the structural variants (Fig. 3k). In none of these cases did anti-TNFR1 dAb significantly inhibit the cytokine production, indicating this to be independent of stimulation, or clustering, of cell-surface TNFR1 in the absence of TNF.

Stimulation of the SK-Hep-1 transfectants with 1ng/ml TNF induced moderately higher production of all cytokines. In particular, both R92Q and T50M transfectants produced significantly higher levels than WT of IL-8 (Fig. 3b) and G-CSF (Fig. 3f); P46L also enhanced production of G-CSF (Fig. 3f), and only P46L induced higher levels of CCL-5 (Fig. 3j); the spread of replicate values for CCL-2 (Fig. 3d) and PTX3 (Fig. 3h) meant that no differences were significant, and no TNFR1 variant transfectants produced higher levels of IL-6 than did the WT transfectant (Fig. 3l). In many instances, the production of cytokines was significantly inhibited by the anti-TNFR1 dAb, indicating its dependency on cell-surface TNFR1 activation by TNF. Further increases in cytokine production were observed when the cells were treated with 3ng/ml TNF (which was also significantly inhibited by anti-TNFR1 dAb), indicating a TNF dosage effect (data not shown). Thus, different TNFR1 variants differentially enhanced the production of particular cytokines in both TNF-independent and TNF-dependent ways.

Concluding remarks

Both structural and non-structural TRAPS-associated TNFR1 variants enhance cytokine production by both TNF-independent and TNF-dependent mechanisms. The TNF-dependent mechanism requires cell-surface expression of TNFR1, as this can be blocked by the TNFR1-specific dAb. The observation that the anti-TNFR1 dAb does not affect the stimulation of cytokine production by TNFR1 variants in the absence of exogenously added TNF (although the dAb can bind to these variants) is consistent with a TNF-independent component of stimulation that involves intra-cellular TNFR1.

We observed different behaviour of the WT and variant TNFR1s in the HEK-293 and SK-Hep-1 cell lines, which is not surprising, given their very different natures. Differences in behaviour have also been observed between, for example, myeloid cells and fibroblasts in relation to TRAPS [7, 13].

In the SK-Hep-1 transfectants, different TNFR1 variants affected the production of different cytokines. No enhancement of cytokine production was induced by the C33Y FL-TNFR1 mutant, although we showed previously that doxycycline-induced C33Y FL-TNFR1 SK-Hep-1 transfectants produce higher levels of G-CSF, GM-CSF, CCL-2 and PTX3 than both the WT and the R92Q and T50M transfectants [6]. Thus, the effect of this cysteine mutation on cytokine production may be much more dependent on its level of intra-cellular expression than upon synergy with exogenous TNF. Interestingly, TNF stimulation led to significantly increased IL-8 and G-CSF, for the R92Q and T50M mutants, and G-CSF and CCL-5 for P46L, when compared to WT. This would suggest an increased sensitivity to extra-cellular TNF in these mutants, as this augmented response is inhibited by the anti-TNFR1 dAb.

The dAb inhibited TNF-stimulated cytokine production only to the level observed in the absence of TNF stimulation; this suggests that the ligand-independent component of cytokine production induced by e.g. the R92Q TNFR1 variant is not driven solely by changes to extracellular receptor clustering [14].

Our findings are consistent with Simon et al. [7], indicating synergy between variant and WT TNFR1 in generating the full TRAPS phenotype, and their proposal that expression of TRAPS-associated TNFR1 variants confers hypersensitivity to external inflammatory stimuli [7]. This might explain why TRAPS patients experience recurrent flares rather than unremitting inflammation – the baseline level of ligand-independent signalling by variant TNFR1 alone might not generate clinically overt symptoms, but a very small external inflammatory stimulus, which would normally be below the threshold to generate an obvious effect, leads to exaggerated signalling that induces the flare.

This study highlights the complex of pathogenesis of TRAPS, and the need for more therapeutic options in addition to steroids and cytokine-blocking biologics. For

example, directly targeting inflammatory signalling pathways, as well as targeting TNFR1 itself, as demonstrated here with a TNFR1-specific dAb.

Materials and methods

Production of recombinant variants of *TNFRSF1A* and transfected cell lines. The production of recombinant DNA clones of the WT and variant (R92Q, P46L, T50M, C33Y) *TNFRSF1A*, encoding either the full-length (FL) receptor or receptor with an R347A substitution, has been described [2, 4, 6, 13]. The production of HEK-293 and SK-Hep-1 cell-lines stably transfected with these *TNFRSF1A* constructs have also been described [2, 4, 6, 13].

Detection of cell-surface TNFR1 by transfected cell-lines. This was performed on a Beckman-Coulter FC500 flow cytometer following incubation of the cells with anti-human TNFR1-phycoerythrin mAb (mAb225) or IgG1-phycoerythrin non-specific control (R&D Systems) as described [2, 4, 6, 13]. Anti-human TNFR1 dAb (DMS5541) and the negative control dAb (DMS5556) were produced and purified by GlaxoSmithKline [11]; theywere biotinylated with EZ-Link Sulfo-NHS-LS-Biotin (Thermo Scientific). Binding of biotinylated dAb (10µg/ml) to cell-surface TNFR1 was assessed by flow cytometry using 1µg/ml Streptavidin-Alexa Fluor 647 (Life Technologies). 10nM Sytox Green (Invitrogen) was used for live/dead cell discrimination.

Production of cytokines by transfected cell-lines. The transfected cell-lines were cultured at 1.7 x 10^4 cells/well in flat-bottomed 96-well plates (Nunc, Δ-surface), with or without recombinant human tumor necrosis factor-alpha (R&D Systems), and with 10µg/ml anti-human TNFR1 dAb (DMS5541) or the negative control dAb (DMS5556), for 72 hours. The caspase inhibitor ZVAD.FMK (10µM, Calbiochem) was added to the cultures containing TNF. The culture supernatants were harvested and assayed for the presence of cytokines by ELISA (R&D Systems Duoset) to detect IL-8 production by the HEK-293 transfectants, or by protein micro-array, as described [15], to detect production of IL-8, CCL-2, G-CSF, PTX-3, CCL-5 and IL-6 by the

SK-Hep-1 transfectants. Statistical differences in cytokine production were determined by t-test using the Sidak-Bonferroni correction for multiple comparisons in Prism 6 (GraphPad Software).

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Conflict of Interest Disclosure

Dr. Stoop was an employee of GlaxoSmithKline. Dr. Stoop is inventor on patents assigned to GlaxoSmithKline concerning TNFR1 antagonist.

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FIGURE LEGENDS

Figure 1. Binding of anti-TNFR1 dAb to HEK-293 *TNFRSF1A* transfectants, and effects on TNF-dependent and independent IL-8 production. (A-D)) Flow cytometric analysis of the cell-surface binding of anti-TNFR1 mAb225 (white circles) and anti-TNFR1 dAb DMS5541 (black squares) to the following HEK-293 cells transfected with WT or variant forms of TNFR1: (a) uninduced FL-TNFR1 transfectants; (b) doxycyclin-induced FL-TNFR1 transfectants; (c) uninduced R347A-TNFR1 transfectants; (d) doxycyclin-induced R347A-TNFR1 transfectants. (A-D) Each point represents the 'delta median fluorescence intensity' (ΔMFI) for an individual experiment and bars show the means of the triplicate experiments. For mAb binding, ΔMFI = mAb225 MFI – IgG MFI. For dAb binding, ΔMFI = DMS5541 MFI – DMS5556 MFI. (E-H) ELISA determination of IL-8 (pg/ml) secreted over 72h by the following uninduced HEK-293 cells transfected with WT or variant forms of TNFR1: (e) FL-TNFR1 transfectants without TNF; (f) FL-TNFR1 transfectants with 3ng/ml TNF; (g) R347A-TNFR1 transfectants without TNF; (h) R347A-TNFR1 transfectants with 3ng/ml TNF. The cultures contained either anti-TNFR1 dAb DMS5541 (black circles) or negative control dAb DMS5556 (white squares). Each point represents an individual experiment and bars show the means of the triplicate experiments. * Significantly higher cytokine concentrations in the indicated variant TNFR1 transfectant cultures relative to the equivalent WT-TNFR1 transfectant cultures (both in the presence of the control dAb DMS5556). § Significantly lower cytokine concentrations in the indicated cultures containing the anti-TNFR1 dAb DMS5541 relative to the equivalent cultures containing the control dAb DMS5556, $p \leq 0.008$ for * and $p < 0.002$ §, determined by t-test using the Sidak-Bonferroni correction for multiple comparisons.

Figure 2. (A-D) Binding of anti-TNFR1 dAb to SK-Hep-1 WT TNFR1 transfectants: (a) Binding of anti-TNFR1 dAb to SK-Hep-1 transfectants was determined by flow cytometry. Example of a dot plot of forward-scatter versus side-scatter showing gating on the live cells. (b) example of Sytox Green staining (FL1) – the gated viable cells (R3) show low fluorescence. (c) example of a histogram showing binding of anti-TNFR1-PE mAb (solid line) and IgG-PE (dotted line). (D) Example of a histogram showing binding of biotinylated anti-TNFR1 dAb DMS5541 (solid line), or

negative control dAb (dashed line), plus Streptavidin-Alexa Fluor 647, or Streptavidin-Alexa Fluor 647 only (dotted line). (A-D) One representative out of three independent experiments is shown. (E) Cell-surface binding of anti-TNFR1 mAb225 (white circles) and anti-TNFR1 dAb DMS5541 (black squares) to uninduced SK-Hep-1 cells transfected with WT or variant forms of FL-TNFR1. Each point represents the 'delta median fluorescence intensity' (ΔMFI) for an individual experiment and bars show the means of the triplicate experiments. For mAb binding, ΔMFI = mAb225 MFI – IgG MFI. For dAb binding, ΔMFI = DMS5541 MFI – DMS5556 MFI.

Figure 3. Cytokines secreted by uninduced SK-Hep-1 cells. (A-L) Cytokines secreted over 72h by uninduced SK-Hep-1 cells transfected with WT or variant forms of FL-TNFR1 were determined by protein microarray (pg/ml) [15]. The cells were cultured without (a, c, e, g, i, k) or with (b, d, f, h, j, l) 1ng/ml TNF. The cultures contained either anti-TNFR1 dAb DMS5541 (black circles) or negative control dAb DMS5556 (white squares). Each point represents an individual experiment and bars show the means of the triplicate experiments. The cytokines and other inflammatory mediators assayed were: (a, b) IL-8; (c, d) CCL-2; (e, f) G-CSF; (g, h) PTX-3; (i, j) CCL-5; (k, l) IL-6. * Significantly higher cytokine concentrations in the indicated variant TNFR1 transfectant cultures relative to the equivalent WT-TNFR1 transfectant cultures (both in the presence of the control dAb DMS5556). § Significantly lower cytokine concentrations in the indicated cultures containing the anti-TNFR1 dAb DMS5541 relative to the equivalent cultures containing the control dAb DMS5556. † Data unavailable. , $p<0.015$ for * and $p<0.01$ §, determined by t-test using the Sidak-Bonferroni correction for multiple comparisons.

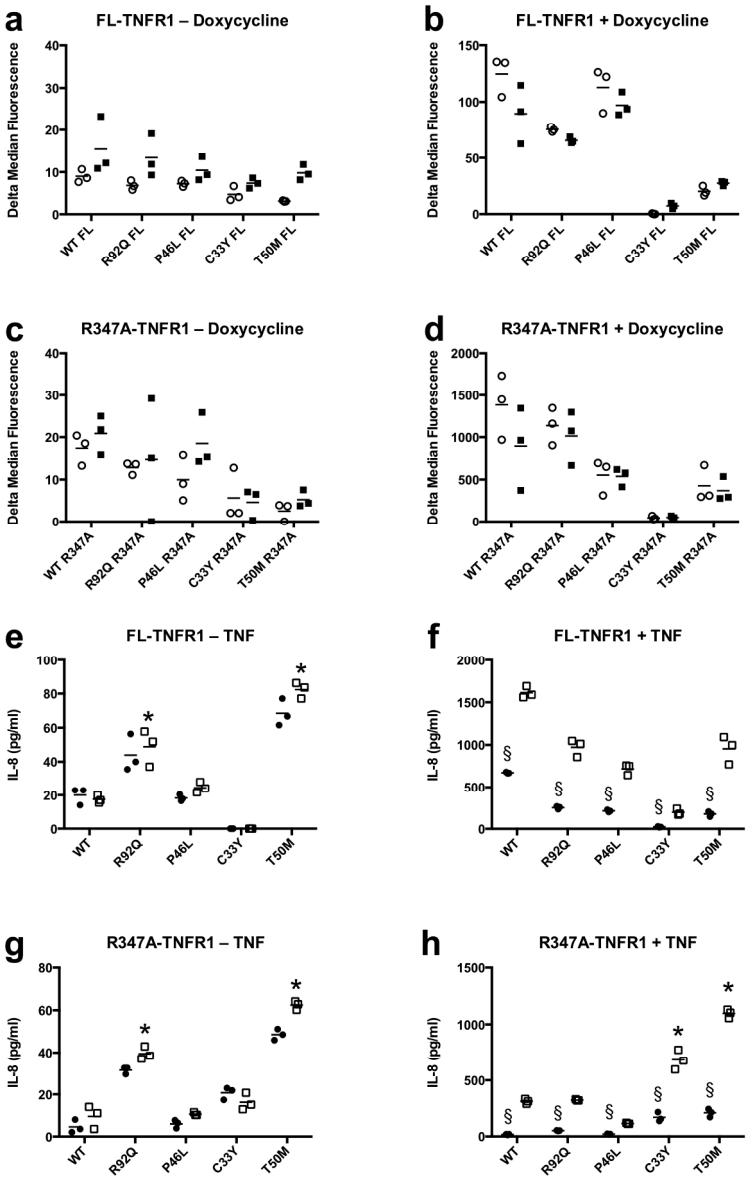

Figure 1. Binding of anti-TNFR1 dAb to HEK-293 TNFRSF1A transfectants, and effects on TNF-dependent and independent IL-8 production. (a-d) flow cytometric analysis of the cell-surface binding of anti-TNFR1 mAb225 (white circles) and anti-TNFR1 dAb DMS5541 (black squares) to the following HEK-293 cells transfected with WT or variant forms of TNFR1: (a) uninduced FL-TNFR1 transfectants; (b) doxycyclin-induced FL-TNFR1 transfectants; (c) uninduced R347A-TNFR1 transfectants; (d) doxycyclin-induced R347A-TNFR1 transfectants. Each point represents the 'delta median fluorescence intensity' (ΔMFI) for an individual experiment, and bars show the means of the triplicates. For mAb binding, ΔMFI = mAb225 MFI – IgG MFI. For dAb binding, ΔMFI = DMS5541 MFI – DMS5556 MFI. (e-h) ELISA determination of IL-8 (pg/ml) secreted over 72h by the following uninduced HEK-293 cells transfected with WT or variant forms of TNFR1: (e) FL-TNFR1 transfectants without TNF; (f) FL-TNFR1 transfectants with 3ng/ml TNF; (g) R347A-TNFR1 transfectants without TNF; (h) R347A-TNFR1 transfectants with 3ng/ml TNF. The cultures contained either anti-TNFR1 dAb DMS5541 (black circles) or negative control dAb DMS5556 (white squares). Each point represents an individual experiment and bars show the means of the triplicates. * Significantly higher

cytokine concentrations in the indicated variant TNFR1 transfectant cultures relative to the equivalent WT-TNFR1 transfectant cultures (both in the presence of the control dAb DMS5556). § Significantly lower cytokine concentrations in the indicated cultures containing the anti-TNFR1 dAb DMS5541 relative to the equivalent cultures containing the control dAb DMS5556.
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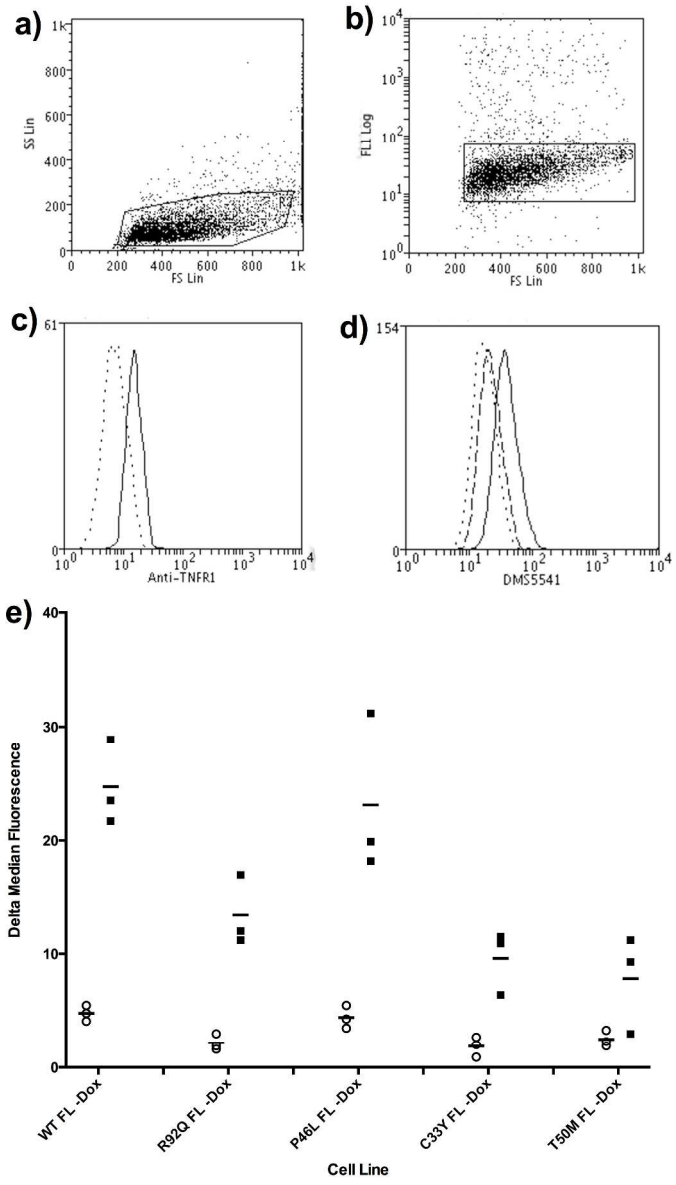

Figure 2. Binding of anti-TNFR1 dAb to SK-Hep-1 transfectants determined by flow cytometry. (a) example of a dot plot of forward-scatter versus side-scatter showing gating on the live cells. (b) example of Sytox Green staining (FL1) – the gated viable cells (R3) show low fluorescence. (c) example of a histogram showing binding of anti-TNFR1-PE mAb (solid line) and IgG-PE (dotted line). (d) example of a histogram showing binding of biotinylated anti-TNFR1 dAb DMS5541 (solid line), or negative control dAb (dashed line), plus Streptavidin-Alexa Fluor 647, or Streptavidin-Alexa Fluor 647 only (dotted line). (e) cell-surface binding of anti-TNFR1 mAb225 (white circles) and anti-TNFR1 dAb DMS5541 (black squares) to uninduced SK-Hep-1 cells transfected with WT or variant forms of FL-TNFR1. Each point represents the 'delta median fluorescence intensity' (ΔMFI) for an individual experiment, and bars show the means of the triplicates. For mAb binding, ΔMFI = mAb225 MFI – IgG MFI. For dAb binding, ΔMFI = DMS5541 MFI – DMS5556 MFI.
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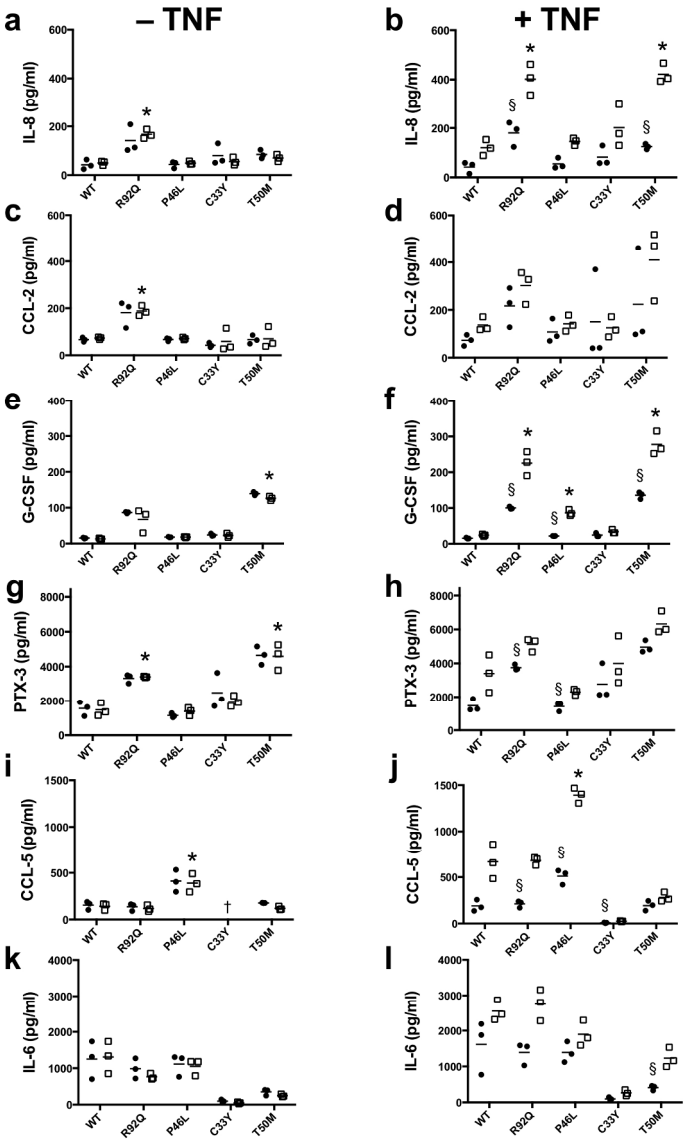

Figure 3. Cytokines secreted (pg/ml) over 72h by uninduced SK-Hep-1 cells transfected with WT or variant forms of FL-TNFR1 as determined by protein microarray [15]. The cells were cultured without (a, c, e, g, i, k) or with (b, d, f, h, j, l) 1ng/ml TNF. The cultures contained either anti-TNFR1 dAb DMS5541 (black circles) or negative control dAb DMS5556 (white squares). Each point represents an individual experiment and bars show the means of the triplicates. The cytokines and other inflammatory mediators assayed were: (a, b) IL-8; (c, d) CCL-2; (e, f) G-CSF; (g, h) PTX-3; (i, j) CCL-5; (k, l) IL-6. * Significantly higher cytokine concentrations in the indicated variant TNFR1 transfectant cultures relative to the equivalent WT-TNFR1 transfectant cultures (both in the presence of the control dAb DMS5556). § Significantly lower cytokine concentrations in the indicated cultures containing the anti-TNFR1 dAb DMS5541 relative to the equivalent cultures containing the control dAb DMS5556. † Data unavailable.
154x267mm (300 x 300 DPI)