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Tuftsins signals through its receptor neuropilin-1 via the transforming growth factor beta pathway

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Abstract

Tuftsins (Thr-Lys-Pro-Arg) is a natural immunomodulating peptide found to stimulate phagocytosis in macrophages/microglia. Tuftsins binds to the receptor neuropilin-1 (Nrp1) on the surface of cells. Nrp1 is a single-pass transmembrane protein, but its intracellular C-terminal domain is too small to signal independently. Instead, it associates with a variety of co-receptors. Despite its long history, the pathway through which tuftsins signals has not been described. To investigate this question, we employed various inhibitors to Nrp1's co-receptors to determine which route is responsible for tuftsins signaling. We use the inhibitor EG00229, which prevents tuftsins binding to Nrp1 on the surface of microglia and reverses the anti-inflammatory M2 shift induced by tuftsins. Furthermore, we demonstrate that blockade of transforming growth factor beta (TGF β) signaling via TGF β R1 disrupts the M2 shift similarly to EG00229. We report that tuftsins promotes Smad3 phosphorylation and reduces Akt phosphorylation. Taken together, our data show that tuftsins signals through Nrp1 and the canonical TGF β signaling pathway.

INTRODUCTION

Tuftsins is a small, naturally occurring tetrapeptide with the sequence threonine-lysine-proline-arginine. It was originally described at its discovery in 1970 as a phagocytosis-stimulating factor derived from the proteolytic degradation of IgG (Nishioka *et al.* 1973, Najjar & Nishioka 1970). In cells of monocytic origin such as macrophages, microglia, and neutrophils, tuftsins promotes phagocytic activity. It can also stimulate migration and chemotaxis, as well as promote the antigen presentation of these cell types which ultimately aids in adaptive immune function through T cell populations (Siemion & Kluczyk 1999). In many animal disease models, such as sepsis (Wardowska *et al.* 2009) and multiple sclerosis (Bhasin *et al.* 2007), tuftsins treatment has been associated with anti-inflammatory effects, particularly among microglial cell populations (Wu *et al.* 2012).

Receptors for tuftsins have been described in the literature; using affinity chromatography, two binding activities were reported in peritoneal granulocytes corresponding to 250 and 500 kDa molecular masses consisting of two subunits of 62 and 52 kDa (Bump *et al.* 1986). More recently, neuropilin-1 (Nrp1) was identified as a receptor for tuftsins (von Wronski *et al.* 2006). Nrp1 is a single-pass transmembrane glycoprotein that plays important roles in development, immunity, and cancer. Nrp1 shares 44% homology with neuropilin-2, with which it shares many structural and biological properties; however, tuftsins has been shown

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to bind only to Nr1 (von Wronski et al. 2006). During angiogenesis, Nr1 binds vascular endothelial growth factor (VEGF) and promotes vessel formation (Gu *et al.* 2003, Soker *et al.* 1998), which is exploited by tumor cells to provide a blood supply to cancerous tissues (Klagsbrun *et al.* 2002). In neural development, Nr1 binds class 3 semaphorins (Sema3), which provide inhibitory signals in axonal guidance (Gu et al. 2003, Kitsukawa *et al.* 1995). Nr1 also plays an important role in the immune system by promoting long contacts between dendritic cells and immunosuppressive regulatory T cells (Treg) (Sarris *et al.* 2008, Tordjman *et al.* 2002), and can bind and activate transforming growth factor beta (TGF β), an anti-inflammatory cytokine (Glinka & Prud'homme 2008). Recently, Tregs have been shown to be recruited to tumors by Nr1 in a VEGF-dependent manner, which contributes to immune system evasion by tumor cells (Hansen 2013).

A notable feature of Nr1's structure is that it has a short cytoplasmic domain that is only about 44 amino acids long, and contains no known signaling motif (Vander Kooi *et al.* 2007). Although there have been several studies indicating that this domain is capable of signaling independently, the mechanism has not been well defined (Evans *et al.* 2011, Fantin *et al.* 2011). However, Nr1 can associate with various co-receptors via which downstream signaling can occur; PlexinA1 for Sema3, VEGFR (VEGF Receptor) for VEGF, T R1 (TGF β receptor 1) for TGF β , and c-Met for hepatocyte growth factor (HGF) (Prud'homme & Glinka 2012). Tuftsin competes with VEGF for binding on the Nr1 molecule, as it shares sequence homology with the C-terminus of VEGF (von Wronski et al. 2006). The structure of VEGF allows its N-terminus to interact with VEGFR while bound to Nr1 (Wiesmann *et al.* 1997), promoting co-receptor dimerization and signaling, which is not the case with tuftsin due to its small size. Although tuftsin has been used in various studies for over forty years, its mechanism of action and signaling pathway are still unknown.

In this study, we show that the Nr1 inhibitor EG00229 prevents tuftsin binding to the cell surface, and effectively blocks the anti-inflammatory shift induced by tuftsin in microglial cells. We also demonstrate that an inhibitor of T R1 function, but not of c-Met function, disrupts tuftsin's downstream effects similarly to EG00229. Moreover, we show that tuftsin signals through the canonical TGF β signaling pathway.

MATERIALS AND METHODS

Animals

C57BL/6 mice were bred in-house (SBU Animal Facility) under specific pathogen-free conditions set by the Division of Laboratory Animal Resources at Stony Brook University. The environment was controlled for temperature (21°C), and maintained under a 12-hour light/dark cycle. Access to food and water was ad libitum. All procedures were approved by the IACUC committee at Stony Brook University in compliance with the ARRIVE guidelines. Mice of both sexes were used.

Mixed cortical and primary microglia cultures

Microglia were isolated as previously described (Rogove & Tsirka 1998). In short, newborn (d0–d2) pups of wild-type mice were used to isolate cortical cells. The brains were removed, and cortices were freed from meninges, hippocampi and basal ganglia digested in 0.25% Trypsin/EDTA (Sigma) at 37 °C for 20 min. To obtain a single-cell suspension, the tissue was then triturated and filtered through a 40 μ m cell strainer, and plated in mixed cortical medium (DMEM, 10% FBS, 40 μ g/ml Gentamycin). Tissue culture plates used for plating mixed cortical cultures were coated overnight at 4°C with 5 μ g/ml poly-D-lysine (PDL, Sigma).

The medium was changed 3 days after plating. Microglial cells were harvested 10 days after plating. Briefly, lidocaine was added directly to the culture medium at a final concentration of 1mM and the culture left at room temperature for 15 min. The medium containing the floating microglia was collected and centrifuged at 500g for 5 min, following which the cell pellet resuspended in microglia medium (DMEM, 1% FBS) and counted on a hemocytometer. Cultures are >98% pure, as previously described (Rogove & Tsirka 1998, Siao & Tsirka 2002, Yao & Tsirka 2010).

Inhibitors used to treat microglia were diluted in DMSO, and were EG00229, a Nrp1 inhibitor (Jarvis et al. 2010); LY 364947, a T R1 inhibitor; and PF 04217903, a c-Met inhibitor (Tocris). The concentrations were chosen based on previous published work utilizing these compounds (Jarvis et al. 2010, Jia et al. 2010, Shiou et al. 2006, Zou et al. 2012). Microglial cells were plated at a density of 5×10^5 cells per 35mm plate, and NCM was utilized in a 1:1 ratio to microglial culture medium.

Primary Neuronal Cultures

Primary neuronal cultures were prepared from embryonic day 17–19 mice as previously described (Siddiq & Tsirka 2004). Briefly, mouse cortices were dissected and put in Hanks solution (HBSS), and triturated to form single cell suspensions. The cells were plated at a density of 100,000 cells/cm² in Neurobasal medium with B27 supplements, 25μM glutamate, 0.5 mM L-glutamine and 10 g/L gentamycin sulfate. Tissue culture plates used for plating neuronal cultures were coated overnight at 4 °C with 5 μg/ml poly-D-lysine (PDL, Sigma). Cells were utilized for experiments after seven days *in vitro*.

Neuronal conditioned media (NCM) was prepared from primary cortical neurons exposed overnight to 100 μM glutamate added directly to the culture medium to induce excitotoxic injury. Prior to treating microglia with NCM, the media was spun down to remove debris.

Tufts binding assay

To observe tufts binding to the cell surface in the presence or absence of Nrp1 inhibitor EG00229, primary microglia cells were exposed to varying concentrations of inhibitor at 37° C for 1 hour, and then treated with 100 μg/ml of biotinylated tufts (GeneScript) for 30 minutes at room temperature to minimize internalization of the peptide. After thorough washing, tufts's presence on the cell surface was observed by fixation of the cells and staining with Cy3-conjugated streptavidin.

Immunofluorescence

Cells used for immunofluorescence were fixed for 30 minutes at room temperature in 4% paraformaldehyde, but not permeabilized. After washing with PBS, they were blocked in serum of the host of the secondary antibody (5% serum in PBS with 0.5% TritonX-100), and then incubated overnight at 4°C with rabbit anti-mouse Nrp1 (Abcam) at a 1:500 dilution in PBS to detect tufts's receptor. After washing with PBS, cells were incubated with fluorescenceconjugated FITC goat anti-rabbit secondary antibody and Streptavidin-conjugated Cy3 (to detect surface bound biotinylated tufts) for 1 hour at room temperature to minimize potential internalization, washed 3 times with PBS, and mounted using Fluoromount-G with DAPI (Southern Biotech, USA). These were then visualized with a Nikon Eclipse E600 microscope.

Immunoblotting

Cells were lysed in 50 mM Tris-HCl (pH 7.4) containing 1% Nonidet P-40, 0.25% Nadeoxycholate, 150 mM NaCl, 1% SDS, and protease inhibitors cocktail (Sigma-Aldrich). After incubation on ice for 10 minutes, the lysates were centrifuged at 13,000 RPM for 10

minutes to remove debris, and the supernatant was collected. The extracts were separated on a reducing 10% SDS-PAGE gel, and blotted to PVDF membrane (Immobilon-P; Millipore). Membranes were then incubated overnight at 4° C with anti-phospho-Smad3 or anti-phospho-Akt (1:1000, cell signaling) primary antibodies. These were then incubated with Hrp-conjugated anti-rabbit secondary for 1 hour at room temperature, and visualized with ECL (Pierce). After stripping, membranes were reprobed with anti-Smad3 or anti-Akt antibodies (1:1000, cell signaling).

RNA isolation and quantitative real-time PCR

To prepare RNA, primary microglia that were treated for 10 hours were washed with PBS and lysed with RNA-Bee (Tel-Test), by the manufacturer's protocol. To obtain cDNA, one microgram of RNA was reverse transcribed on a Veriti Thermocycler (Applied Biosystems) using the High Capacity cDNA Reverse Transcription kit. Amplification was performed on a StepOnePlus real-time PCR machine using a SYBR Green kit (Applied Biosystems). Primer sequences are as follows: GAPDH forward, 5'-GCACAGTCAAGCCGAGAAT-3'; GAPDH reverse, 5'-GCCTTCTCCATGGTGGTGGA-3'; IL10 forward, 5'-TGGCCACACTTGAGAGCTGC-3'; IL10 reverse, 5'-TTCAGGGATGAAGCGGCTGG-3'; TNF forward, 5'-ATGAGCACAGAAAGCATGATC-3'; TNF reverse, 5'-TACAGGCTTGTCACCTCGAATT-3'. For gene expression analysis, the relative quantitation method was used (Ct) with GAPDH as an internal control.

Measurement of cytokine and NO levels

Primary microglial cells were treated with combinations of NCM, 100 µg/mL tuftsin, 10 µM T R1 inhibitor, 5 nM c-Met inhibitor, or 30 µM EG00229 for 10 hours.

To measure TGF levels, the Ready-Set-GO TGF cytokine ELISA kit from eBioscience was utilized. Briefly, 96-well plates were coated overnight with the appropriate dilution of capturing antibody in coating buffer (0.2 M sodium phosphate buffer pH 6.5). The plate was washed three times with PBS-T (PBS, 0.05% Tween-20) and blocked with Assay Diluent for 1 hour at RT. To activate latent TGF 1, samples were treated with 20 µl of 1N HCl per 100 µl of sample for 10 minutes, and then neutralized with 1N NaOH. After washing as before, 100 µl of sample or cytokines standard prepared in assay diluent were added followed by 2-hour incubation at RT. After 5 washes with PBS-T, 100 µl of assay diluent containing biotin-conjugated detection antibody and Avidin-HRP reagent at the appropriate dilutions were added and incubated for 1 h at RT. Following 7 washes with PBS-T, 100 µl of Substrate Solution was added to each well. After 30 min incubation in the dark, 50 µl of Stop Solution (2N H₂SO₄) were added and absorbance at 450 nm was read within 30 min on a SpectraMax microplate reader using the Softmax Pro software. Final readings were calculated with a dilution factor of 1.4 to account for acid activation/neutralization.

To observe nitric oxide levels, a nitrite assay was performed. Nitrite level measurement was based on the reaction of nitrites with 2,3-diaminonaphthalene (DAN) under acidic conditions, which results in 2,3-naphthyltriazole formation. Briefly, 100 µl of sample or standard (NaNO₂) was loaded in a black 96-well plate with a clear bottom. 20 µl of DAN (0.05 mg/ml in 0.62 M HCl) was added to each well. After proceeding at RT for 20 minutes, the reaction was terminated by the addition of 100 µl 0.28 M NaOH. After an additional 10-minute incubation at RT, fluorescence was measured on a Titertek Fluoroscan II fluorescence plate reader using a filter pair with excitation at 355 nm and emission at 460 nm.

Statistics

For multiple comparisons within a group, statistical analysis was performed using one-way ANOVA followed by a Bonferroni-Dunn test. For comparisons between groups, a two-tailed t-test was used, as indicated by the figure legends. For all figures, $p < 0.05$ was considered significant and is marked by *; $p < 0.01$ and $p < 0.001$ were considered very significant and are marked by ** and *** respectively. All results are represented as average with error bars indicating the standard error of the mean. In all experiments, n refers to the number of biological replicates used for each condition.

RESULTS

Neuropilin-1 inhibitor EG00229 prevents tuftsin binding to the cell surface

Tuftsin has been shown to bind to Nrp1 on endothelial cells (von Wronski et al. 2006). To examine if tuftsin is acting through Nrp1 on microglia, we used a small molecule inhibitor for VEGF binding to Nrp1 designated as EG00229. This inhibitor was designed around the final C-terminal residues of the VEGF molecule (DKPRR), as these were the residues that interacted with the Nrp1 binding pocket (Jarvis et al. 2010). Since tuftsin shares a strong homology with the C-terminus of VEGF (TKPR), EG0029 can function effectively as an inhibitor of tuftsin binding to Nrp1 as well.

Microglia readily express Nrp1, as shown in Figure 1. As there are no antibodies that can stain for tuftsin due to its small size, and conjugation with GFP could potentially disrupt its binding properties, we used biotinylated tuftsin that was then detected by streptavidin-conjugated Cy3 antibody. Primary microglia were treated with a combination of EG00229 and the biotinylated tuftsin. At all concentrations tested, the inhibitor seems not activate microglia as they remained in a ramified, resting state. Tuftsin binding to Nrp1 was significantly decreased by EG00229 (Fig. 1A), and appears to act in a dose-dependent manner (Fig. 1B). This result demonstrates that tuftsin's function on microglial cells is mediated by Nrp1 exclusively, and not by some alternative receptor (Bump et al. 1986), as its binding is prevented by the highly specific Nrp1 inhibitor.

EG00229 blocks the anti-inflammatory shift in microglia induced by tuftsin

When activated, microglia can be polarized to either a pro- or anti-inflammatory subset known as M1 or M2, respectively. M1 microglia, which are neurodegenerative in a model of spinal cord injury, produce TNF and nitric oxide, while neuroprotective M2 microglia release IL10 and TGF (Gordon & Martinez 2010, Michelucci *et al.* 2009, Kigerl *et al.* 2009). We previously reported that a 'two-hit' treatment with a combination of neuronal conditioned media (NCM), isolated from neurons treated overnight with 100 μ M glutamate to induce excitotoxic injury, and tuftsin reduced the release of TNF and promoted the release of IL10 in primary microglial cells, indicating an M2 shift in response to tuftsin treatment (Wu et al. 2012).

We wanted to examine whether EG00229 could prevent this tuftsin-mediated M2 microglial shift. We treated microglial cells for 10 hours with NCM in the presence or absence of tuftsin and increasing concentrations of EG00229, choosing our inhibitor concentrations based on previous studies (Jarvis et al. 2010, Jia et al. 2010). We then harvested RNA and performed quantitative real-time PCR to observe microglial phenotype based on TNF levels to indicate M1 polarization, and IL10 levels to indicate M2 polarization. While the combination of NCM and tuftsin reduced TNF levels and increased IL10, as we have previously shown (Wu et al. 2012), EG00229 reversed these effects (Fig. 2 A, B). While tuftsin and NCM alone significantly increase IL10 levels by about 3-fold, EG00229-treated cells at all concentrations showed no similar increase in IL10 levels, which remained

comparable to control levels (Fig 2B). Similarly, while cells treated with tuftsin and NCM resulted in a reduction in TNF α , the opposite was observed in groups treated with EG00229, which showed a slight increase in TNF α levels over control (Fig. 2A). Moreover, when the overall shift to an anti-inflammatory state in microglial cells was assessed, noted by the ratio of M2 to M1 gene expression, the EG00229 treatment resulted in reversion of these cells to a state similar to untreated controls (Fig. 2C). Thus, these experiments indicate that EG00229 can effectively prevent tuftsin's actions on microglial cells by blocking the M2 shift.

Blockade of T β R1 prevents the tuftsin-induced anti-inflammatory shift in microglia

Nrp1 employs different co-receptors which signal following ligand binding (Prud'homme & Glinka 2012). We investigated which one of these co-receptors is involved in mediating tuftsin signaling. A likely candidate is T β R1, since its classic ligand TGF β has been extensively associated with anti-inflammatory effects. Nrp1 can bind and activate the latent form of TGF β , which is associated with immunosuppressive regulatory T cell function (Wei *et al.* 2007, Karpanen *et al.* 2006). It is also essential in the development of alternatively activated M2 microglia (Zhou *et al.* 2012).

To test if T β R1 is the co-receptor involved in tuftsin signaling, we used an inhibitor capable of blocking the kinase activity of T β R1 at 10 μ M as previously described (Shiou *et al.* 2006). For comparison, we also used an inhibitor of c-Met kinase activity at 5 nM, in line with prior studies (Zou *et al.* 2012), which is an alternative co-receptor that Nrp1 could signal through (Prud'homme & Glinka 2012). Similarly to the experiments in Figure 2, microglia were treated for 10 hours with combinations of tuftsin and NCM, in the presence or absence of c-Met inhibitor or T β R1 inhibitor (Zou *et al.* 2012, Shiou *et al.* 2006). After harvesting RNA, qPCR was performed to quantify the expression of M1 and M2 markers. The ratio of M2/M1 in c-Met inhibitor-treated samples was comparable to controls, with a 3-fold decrease in TNF α and 3-fold increase in IL10 in tuftsin and NCM-treated samples. However in cells treated with T β R1 inhibitor a slight increase in TNF α levels and no change in IL10 levels were observed, as was the case for EG00229-treated microglia (Fig. 3A, B). Furthermore, while there was a significant anti-inflammatory switch in both control and c-Met inhibitor-treated cells when exposed to NCM and tuftsin, this was abolished in T β R1-treated samples (Fig. 3C). Taken together, these data indicate that tuftsin signals through the TGF β signaling pathway via T β R1.

Tuftsin signals through the canonical TGF β signaling pathway

We extended these observations by further investigating the downstream targets of T β R1. TGF β is characterized by signaling through two separate pathways, the canonical and non-canonical pathways, which have disparate effects. The canonical signaling pathway is associated with Smad2/3 phosphorylation, which then forms a complex with Smad4 that translocates to the nucleus, binds DNA, and regulates transcription. This series of events has been linked with inhibition of immune responses and cell proliferation (Rahimi & Leof 2007, Prud'homme & Glinka 2012). On the other hand, non-canonical TGF β signaling occurs through various molecules such as PI3k, Akt, or Erk (Zhang 2009). These two pathways have been shown to have complex cross talk, for the canonical and non-canonical pathways can antagonize each other. For example, hyperactivation of Akt can reduce canonical signaling, particularly through inhibition of Smad3 (Zhang 2009, Tian *et al.* 2011).

To investigate whether tuftsin signals through the canonical or non-canonical TGF β pathway, we performed immunoblots to examine Smad3 and Akt phosphorylation levels. Microglial cells treated with combinations of NCM and tuftsin for 10 hours in the presence

or absence of T_{R1} inhibitor or c-Met inhibitor were re-stimulated at hour 9 to allow for phosphorylation changes to be more readily observable. Control samples showed a significant increase of Smad3 phosphorylation in response to tuftsin and NCM, indicating that tuftsin signals through the canonical pathway. This increase was also observed in samples treated with c-Met inhibitor, but was abolished in those treated with T_{R1} inhibitor (Fig. 4A, B). Conversely, microglia treated with NCM and tuftsin revealed significantly decreased levels of Akt phosphorylation, indicating a decrease in non-canonical TGF β signaling similar to that in samples treated with c-Met inhibitor. However when the activity of T_{R1} was blocked, a decrease in Akt phosphorylation was not observed in the microglial samples (Figure 4C, D). Samples treated with 30 μ M EG00229 behaved similarly to those exposed to T_{R1} inhibitor, for both p-Smad3 and p-Akt (data not shown). These experiments suggest that tuftsin signals via the canonical TGF β signaling pathway.

Tuftsin promotes TGF β release from cells, which is prevented by T β R1 blockade

To further confirm polarization to an M2 subset as a result of tuftsin treatment, as well as the effect of c-Met, T_{R1}, and EG00229 inhibitors, we performed an ELISA assay for TGF β . We show that tuftsin and NCM promote a significant release of TGF β from microglial cells, similarly to those treated with 5 nM of c-Met inhibitor. However, this does not occur when cells are exposed to T_{R1} inhibitor, or EG00229 (Fig. 5A). To observe the release of reactive nitrogen species from cells as a means of determining a shift away from the M1 phenotype in cells, we quantified nitrite release by DAN reagent. However, there was no significant change between all treatments (Fig. 5B). Thus, blockade of tuftsin binding to Nrp1 by EG00229 or disruption of T_{R1} signaling prevents the tuftsin-mediated M2 shift in microglia.

DISCUSSION

In this study, we provide several pieces of evidence that support the hypothesis that tuftsin signals exclusively through its receptor Nrp1 via the canonical TGF β signaling pathway. Our previous work showed that tuftsin was capable of promoting an anti-inflammatory, M2 shift in microglia (Wu et al. 2012). In this report we show that blockade of tuftsin binding to Nrp1 prevents this shift, and that inhibition of T_{R1} similarly blocks microglial polarization to an anti-inflammatory phenotype. As the canonical TGF β pathway is associated with immunosuppression (Rahimi & Leof 2007), our current findings support our previously published work in the experimental allergic encephalomyelitis model of multiple sclerosis where tuftsin enhanced an M2 shift and promoted the expression of immunosuppressive Tregs (Wu et al. 2012).

It is notable that neither tuftsin treatment nor NCM treatment alone promotes an M2 shift in microglial cells (Wu et al. 2012). This is consistent with the idea that macrophages and microglia are often activated in a “two-hit” process. This model describes that a weaker, nonspecific initial signal produces a priming response in target cells, which, when faced with a second activation signal, are rapidly activated and produce a robust reaction (O’Leary *et al.* 2011, Hains *et al.* 2010). In our system, NCM would function as the initial signal, and tuftsin would then produce a strong anti-inflammatory response in “primed” microglial cells.

As EG00229 was originally generated as an inhibitor of VEGF binding to Nrp1 (Jarvis et al. 2010), it is possible that tuftsin is in fact signaling through the VEGF pathway. There are data both supporting and refuting this theory. It has been shown that treatment of cells with TGF β can promote the induction of VEGF (Shao *et al.* 2009), and that VEGF treatment promotes TGF β production in macrophages (Luo *et al.* 2012). However, VEGF binds to both Nrp1 and its co-receptor VEGFR concomitantly, which aids in the recruitment of VEGFR for signaling (Wiesmann et al. 1997). This would not occur for tuftsin, as it is comprised of

only 4 amino acids and binds deep within the VEGF pocket on the Nrp1 molecule (Vander Kooi et al. 2007), preventing its interaction with any other receptors. The fact that treatment of microglia with EG00229 resulted in small increase in TNF over control cells may possibly be due to the fact that EG00229 does not only block tuftsin binding, but prevents VEGF binding as well (Jarvis et al. 2010), so it can result in different behavior in microglia rather than just loss of tuftsin interaction alone.

Another immune cell type that readily expresses Nrp1 are regulatory T cells, Tregs (Weiss *et al.* 2012). Tregs cells play an essential role in suppressing ongoing inflammatory immune responses (Kohm *et al.* 2002, McGeachy *et al.* 2005). These cells differentiate in response to, as well as bind and activate, TGF β (Lohr *et al.* 2006, Glinka & Prud'homme 2008). It would thus be pertinent to investigate whether tuftsin also signals through the canonical TGF β signaling pathway in these cells as well.

As tuftsin has been used extensively over its 40-year history, the lack of knowledge of its mechanism of action has been a notable void in the information regarding this useful molecule. Our work is novel as although it has been shown that tuftsin binds to Nrp1 (von Wronski et al. 2006), Nrp1 utilizes a wide variety of co-receptors that function in disparate pathways for signaling (Glinka & Prud'homme 2008). Downstream signaling as a result of tuftsin binding to cells is an area that has not been investigated prior to our study. Here we present data that identify the canonical TGF β signaling pathway as the means by which tuftsin exerts its anti-inflammatory effects.

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Figure 1. EG00229 reduces tuftsin binding to the surface of microglia in a dose-dependent manner

Microglial cells were pre-blocked with the indicated concentrations of EG00229, and incubated with biotinylated tuftsin at room temperature as described in Methods. Cells were then fixed and stained for Nrp1 (FITC) and with streptavidin-conjugated Cy3 to visualize biotinylated tuftsin on the cell surface (A). Fluorescence intensity of tuftsin staining was quantified in (B). n= 3; ***, p<0.001.

Figure 2. The tuftsin-mediated M2 shift in microglia is disrupted by EG00229

Quantitative RT-PCR was performed to analyze changes in gene expression of the M1 marker TNF α (A) and the M2 marker IL10 (B). Primary microglia were treated 100 μ g/ml tuftsin or 100 μ g/ml tuftsin and NCM. Some groups were additionally treated with increasing concentrations of EG00229 as shown. (C) The ratio of the fold change of IL10 (M2) to the fold change of TNF α (M1). n= 3, *, p<0.05.

Figure 3. Blockade of T R1 prevents the tuftsin-mediated M2 shift in microglia

Quantitative RT-PCR was performed to analyze changes in gene expression of the M1 marker TNF α (A) and the M2 marker IL10 (B). Primary microglia were treated 100 μ g/ml tuftsin or 100 μ g/ml tuftsin and NCM. Some groups were additionally treated with 5nM c-Met inhibitor or 10 μ M T R1 inhibitor. (C) The ratio of the fold change of IL10 (M2) to the fold change of TNF α (M1). n= 3–5, *, p<0.05; **, p<0.01; ns, not significant.

Figure 4. Tuftsins signals through the canonical TGF β signaling pathway, which is prevented by T β R1 inhibition

Western blots were performed on primary microglia extracts that were exposed to NCM, 100 μ g/ml tuftsins, 5 nM c-Met inhibitor or 10 μ M T β R1 inhibitor for 10 hours. 1 hour prior to harvesting, cells were restimulated with their designated treatment to better observe phosphorylation changes. Blots were probed for Smad3 and phospho-Smad3 (A), and fold change relative to NCM treatment alone was quantified in (B), and adjusted to reflect changes in total Smad3 levels. Blots were probed for Akt and phospho-Akt in (C), and fold change relative to NCM alone is quantified in (D), and adjusted to reflect changes in total Akt levels. n= 3, *, p<0.05; **, p<0.01; ns, not significant.

Figure 5. Blockade of TR1 prevents tuftsin-mediated TGF upregulation

An ELISA assay probing for TGF levels (**A**) or a nitrite assay using DAN reagent (**B**) was performed on media isolated from primary microglial cells treated 100 μ g/ml tuftsin or 100 μ g/ml tuftsin and NCM for 10 hours. Some groups were additionally treated with 5 nM c-Met inhibitor, 10 μ M TR1 inhibitor, or 30 μ M EG00229, also for 10 hours. n= 3, **, p<0.01; ***, p<0.001, ns, not significant.