View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Charitable Trust (to J.E.C.), an American Cancer Society Research Scholar Award (to J.E.C.), and an Irma T. Hirschl/Monique Weill-Caulier Trust Research Award (to J.E.C.). This work was also supported in part by two research grants (5-FY11-74 and 1-FY13-416) from the March of Dimes Foundation (to J.E.C.), an Einstein Research Fellowship (to S.Y.W.), an American Skin Association Medical Students Grant (to S.Y.W.), an American Federation for Aging Research MSTAR Grant (to J.C.S.), and the Developmental Research Pilot Project Program within the Department of Oncological Sciences at Mount Sinai (to E.B., J.T.C., and J.E.C.). We would also like to thank Joanna Dong for assistance.

Shira Y. Wieder[1,2,3,4,8], Madhavika N. Serasinghe[1,2,3,8], Julie C. Sung[1,2,3,8], Daniel C. Choi[1,2,3], Miriam B. Birge[2,3,5], Jonathan L. Yao[2,3,5], Emily Bernstein[1,2,3,6], Julide T. Celebi[2,3,5,6] and Jerry E. Chipuk[1,2,3,6,7]

[1]Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA; [2]Department of Dermatology, Icahn School of Medicine at Mount Sinai, New York, New York, USA; [3]The Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, New York, USA; [4]Albert Einstein College of Medicine, Bronx, New York, USA; [5]The Department of Pathology, Icahn School of Medicine at Mount Sinai, New York, New York, USA; [6]The Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA and [7]The Diabetes, Obesity, and Metabolism Institute, Icahn School of Medicine at Mount Sinai, New York, New York, USA
E-mail: jerry.chipuk@mssm.edu
[8]These authors contributed equally to this work.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Bollag G, Hirth P, Tsai J *et al.* (2010) Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* 467:596–9

Cassidy-Stone A, Chipuk JE, Ingerman E *et al.* (2008) Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. *Dev Cell* 14: 193–204

Cerami E, Gao J, Dogrusoz U *et al.* (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2:401–4

Gao J, Aksoy BA, Dogrusoz U *et al.* (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Science Signal* 6:pl1

Goldstein AM, Tucker MA (2013) Dysplastic nevi and melanoma. *Cancer Epidemiol Biomarkers Prev* 22:528–32

Holderfield M, Deuker MM, McCormick F *et al.* (2014) Targeting RAK kinases for cancer therapy: BRAF-mutated melanoma and beyond. *Nat Rev Cancer* 14:455–67

Mishra P, Chan DC (2014) Mitochondrial dynamics and inheritance during cell division, development and disease. *Nat Rev Mol Cell Biol* 15: 634–46

Nasrallah CM, Horvath TL (2014) Mitochondrial dynamics in the central regulation of metabolism. *Nat Rev Endocrinol* 10:650–8

Pearlstein MV, Zedek DC, Ollila DW *et al.* (2014) Validation of the VE1 immunostain for the BRAF V600E mutation in melanoma. *J Cutan Pathol* 41:723–32

Renault TT, Floros KV, Elkholi R *et al.* (2015) Mitochondrial shape governs BAX-induced membrane permeabilization and apoptosis. *Mol Cell* 57:69–82

Serasinghe MN, Wieder SY, Renault TT *et al.* (2015) Mitochondrial division is requisite to RAS-induced transformation and is targeted by oncogenic MAPK pathway inhibitors. *Mol Cell* 57:521–36

Smirnova E, Griparic L, Shurland DL *et al.* (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell* 12:2245–56

Yoon Y, Pitts KR, McNiven MA (2001) Mammalian dynamin-like protein DLP1 tubulates membranes. *Mol Biol Cell* 12:2894–905

The Possible Interaction between Receptor Activator of Nuclear Factor Kappa-B Ligand Expressed by Extramammary Paget Cells and its Ligand on Dermal Macrophages

Journal of Investigative Dermatology (2015) **135,** 2547–2550; doi:10.1038/jid.2015.199; published online 18 June 2015

TO THE EDITOR

A mammary gland is a specific type of apocrine gland located in the subcutaneous fat of the breast (Ackerman *et al.*, 2007). The interaction between receptor of activator nuclear factor kappa-B (RANK) and its ligand RANKL is the main mediator of progesterone-induced proliferation of mammary epithelial cells, and the activation of this pathway promotes mammary tumorigenesis (Bhatia *et al.*, 2005; Gonzalez-Suarez, 2011). Extramammary Paget's disease (EMPD) is an uncommon adenocarcinoma of apocrine origin. It usually affects older patients and is highly metastatic to other organs, including bone (Shiomi *et al.*, 2013). On the basis of these findings, we hypothesized that the interaction between RANK and RANKL has a role in the tumorigenesis of EMPD.

To test this hypothesis, we collected archival formalin-fixed paraffin-embedded skin specimens and cryosections from EMPD patients (Supplementary Table S1 and S2 online) for immunohistochemical and immunofluorescence analysis. This study was approved by the ethics committee of Tohoku University Graduate School of

Abbreviations: Arg1, arginase 1; DC, dendritic cell; EMPD, extramammary Paget's disease; LC, Langerhans cell; MMP-7, matrix metalloprotease-7; RANK, receptor of activator nuclear factor kappa-B; Treg, regulatory T cell

Accepted article preview online 27 May 2015; published online 18 June 2015

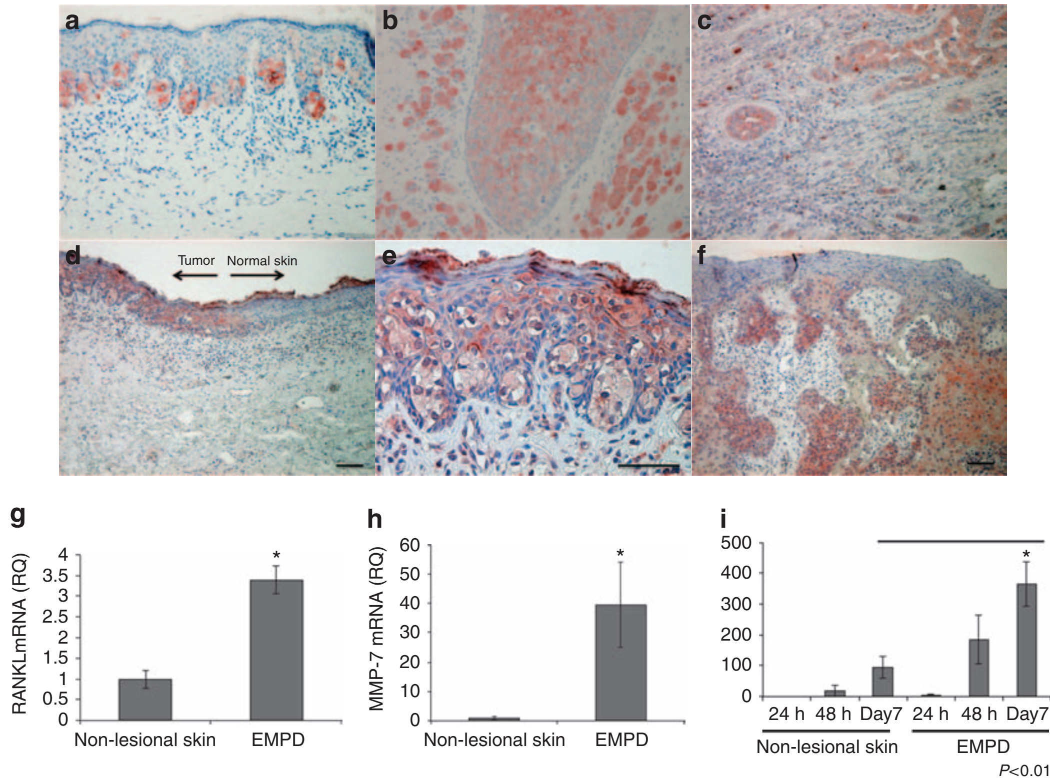

Figure 1. Extramammary Paget's disease lesions, both noninvasive and invasive, express receptor of activator nuclear factor kappa-B ligand and matrix metalloprotease-7. Immunohistochemical staining for RANKL (**a**–**c**) and MMP-7 (**d**–**f**). Scale bar = 100 μm. The expression of RANKL mRNA (**g**) and MMP-7 mRNA (**h**) in laser-captured epidermis of EMPD was analyzed by quantitative RT-PCR, using the ΔΔCt method. The mean ± SEM of data from three cases of EMPD and non-lesional skin is presented. The production of sRANKL from EMPD tissue culture was measured using ELISA (**i**). $^*P < 0.05$ by Student's *t*-test (**g**–**i**). RT-PCR, reverse transcriptase–PCR.

Medicine, Sendai, Japan (2013-1-521), and all patients gave written informed consent. The antibodies and antigen retrieval methods used are described in Supplementary Table S3 online. For laser capture microdissection, three samples of EMPD tissue and surrounding normal skin tissue were collected at the time of resection. For the preparation of samples for tissue culture, three samples of EMPD tissue and surrounding normal skin tissue were obtained and cultured in complete medium for 7 days. Full details are available in the Supplementary Methods online.

Figure 1 shows that Paget cells strongly expressed RANKL (Figure 1a–c and Supplementary Table S1 online) and the matrix metalloprotease (MMP)-7, which cleaves RANKL to release a soluble form (sRANKL), (Figure 1d–f and Supplementary Table S1 online) on their surface (Lynch *et al.*, 2005). Notably, keratinocytes of either lesional or normal epidermis did not express RANKL or MMP-7 (Figure 1a and d, and Supplementary Figure S1A online), whereas there were several RANKL-expressing stromal cells scattered in the lesional dermis (Figure 1c). In addition, apocrine glands in the lesional skin of EMPD expressed RANKL and MMP-7 (Supplementary Figure S1B and S1C online), whereas apocrine glands in normal skin did not express MMP-7 (Supplementary Figure S1D online).

To analyze the mRNA expression of RANKL and MMP-7 by EMPD, we performed quantitative real-time reverse transcriptase–PCR using mRNA recovered from the laser-captured epidermis from the lesional skin containing Paget cells and that from the uninvolved skin of three cases of EMPD (Figure 1g and h and Supplementary Figure S1E online). The expression of RANKL mRNA (Figure 1g) and MMP-7 mRNA (Figure 1h) was significantly more abundant in the lesional epidermis of EMPD, suggesting the expression of RANKL and MMP-7 mRNA by Paget cells. As MMP-7 expression by Paget cells suggested that they might release sRANKL into the extracellular space of the lesional skin of EMPD, we cultured the lesional and non-lesional skin of EMPD and measured the concentration of sRANKL in the culture supernatants using ELISA. The results clearly demonstrated that the culture supernatants of the lesional skin contained significantly more sRANKL compared with those of the non-lesional skin (Figure 1i). These results indicate that the extracellular space surrounding EMPD tissue is rich in sRANKL.

To clarify the significance of RANKL expression by Paget cells, we immunohistochemically stained cryosections of EMPD tissue for RANK. RANK was not expressed by Paget cells or surrounding keratinocytes but was instead expressed on scattered dermal cells (Figure 2a), as well as on epidermal dendritic cells (DCs; Figure 2a insert). In addition, RANK mRNA expression in the lesional epidermis of EMPD was lower than that in the non-lesional epidermis, which

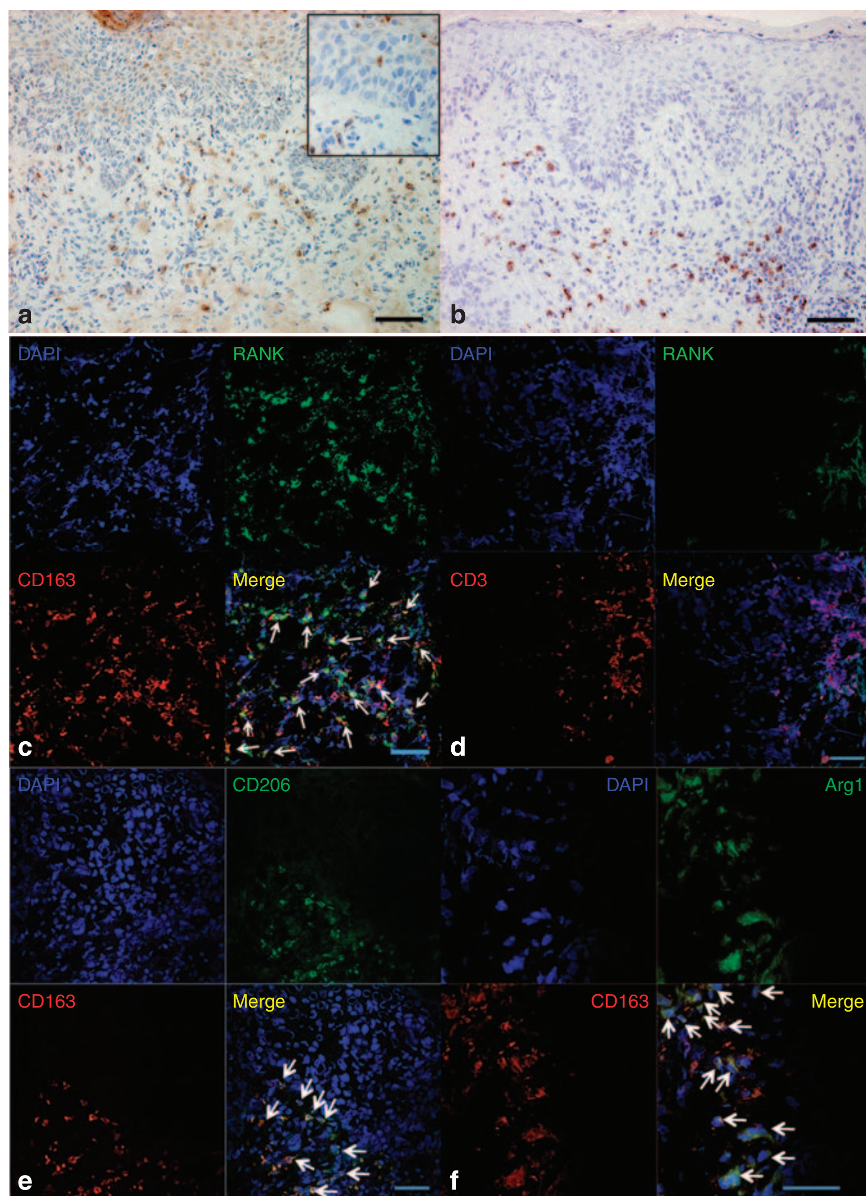

Figure 2. CD163$^+$ CD206$^+$ Arg1$^+$ M2 macrophages express RANK in the lesional skin of extramammary Paget's disease. Immunohistochemical staining of EMPD for RANK (**a**) and CD163 (**b**). Double immunofluorescence staining of EMPD for CD163 and RANK (**c**) (red: CD163, green: RANK, blue: DAPI, yellow: merge), for CD3 and RANK (**d**) (red: CD3, green: RANK, blue: DAPI, yellow: merge), for CD163 and CD206 (**e**) (red: CD163, green: CD206, blue: DAPI, yellow: merge), and for CD163 and Arg1 (**f**) (red: CD163, green: Arg1, blue: DAPI, yellow: merge). Scale bar = 100 µm (**a** and **b**) and 40 µm (**c**–**f**). Arg1, arginase 1; RANKL, receptor of activator nuclear factor kappa-B ligand.

supported the immunohistochemical demonstration of the lack of RANK expression by Paget cells (Supplementary Figure S1F online). Although it is well known that epidermal Langerhans cell (LC)s express RANK (Loser *et al.*, 2006), the origin of the RANK$^+$ cells scattered in the dermis has not yet been clarified. When we immunohistochemically stained the lesional skin of EMPD with several antibodies against macrophages and DCs such as anti-CD163 Ab (Figure 2b), anti-CD1a Ab, anti-CD205 Ab, or anti-CD208 Ab (data not shown), the distribution of the cells expressing CD163 appeared to overlap with that of dermal RANK$^+$ cells (Figure 2b).

Therefore, to clarify the origin of the RANK$^+$ dermal cells, we conducted double immunofluorescence staining of cryosections with combinations of antibodies against RANK and CD163 (Figure 2c), against RANK and CD3 (Figure 2d), against CD163 and CD206 (Figure 2e), and against CD163 and arginase 1 (Arg1; Figure 2f). The average expression of RANK on CD163$^+$ macrophages and that of CD163 on RANK$^+$ cells was 48% and 62%, respectively (Figure 2c, Supplementary Table S2 online). There were a few CD3$^+$ cells that co-expressed RANK (Figure 2d). Moreover, CD163$^+$ macrophages surrounding EMPD expressed both CD206 (Figure 2e) and Arg1 (Figure 2f), which suggests that these CD163$^+$ macrophages including RANK-expressing cells are M2 macrophages (Hao *et al.*, 2012).

We have demonstrated here that RANK is mainly expressed by CD163$^+$ Arg1$^+$ CD206$^+$ macrophages, suggesting that the sRANKL released from Paget cells stimulates these macrophages via RANK. The role of these macrophages in EMPD remains to be clarified. Accumulating evidence has suggested that RANKL-mediated modulation of surface barrier DCs including LCs results in the suppression of autoimmune responses and of detrimental immune responses toward self-antigen, as well as innocuous foreign antigen. As an explanation of this phenomenon, it was reported that RANKL-stimulated LCs induce the expansion of regulatory T cells (Tregs) in UV-irradiated skin (Loser *et al.*, 2006). We previously reported that a significant number of both M2 macrophages and Tregs infiltrate EMPD lesions (Fujimura *et al.*, 2012; Fujimura *et al.*, 2013). However, the interaction between Tregs and these macrophages in EMPD was unclear. Press *et al.* (2011) suggested that increased Tregs are associated with more extensive cases of vulvar EMPD and disease recurrence. Similarly, breast cancers with a high risk for recurrence are accompanied by the recruitment of greater numbers of Tregs to the tumor microenvironment (Bates *et al.*, 2006). Moreover, a high expression level of CD163 on tumor-associated macrophages in breast cancer is associated with reduced overall survival and reduced recurrence-free survival (Medrek *et al.*, 2012). These studies suggested that both Tregs and macrophages have a crucial role in

constituting the immunosuppressive microenvironment that causes poor prognosis in EMPD, as well as in breast cancer. In the accompanying paper, we demonstrate a role for RANKL-stimulated macrophages in recruiting Tregs into the skin.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (23791249 and 25461682).

AUTHOR CONTRIBUTIONS

TF and SA involved in conception and design; YK, TF, and SF involved in development of methodology; YK, TF, SF, MA, and AK involved in acquisition of data; YK, TF, MA, and SA involved in analysis and interpretation of data; TF and SA involved in writing, review, and/or revision of the manuscript; TF involved in administrative, technical, or material support; TF and SA involved in study supervision.

Yumi Kambayashi[1,2], Taku Fujimura[1,2], Sadanori Furudate[1], Masayuki Asano[1], Aya Kakizaki[1] and Setsuya Aiba[1]

[1]Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan.
E-mail: tfujimura1@mac.com
[2]The first two authors contributed equally to this work.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Ackerman AB, Kessler G, Gyorfi T *et al.* (2007) Contrary view: the breast is not an organ per se, but a distinctive region of skin and subcutaneous tissue. *Am J Dermatopathol* 29: 211–8

Bates GJ, Fox SB, Han C *et al.* (2006) Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 24:5373–80

Bhatia P, Sanders MM, Hansen MF (2005) Expression of receptor activator of nuclear factor-kappaB is inversely correlated with metastatic phenotype in breast carcinoma. *Clin Cancer Res* 11:162–5

Fujimura T, Furudate S, Kambayashi Y *et al.* (2013) Potential use of bisphosphonates in invasive extramammary Paget's disease: an immunohistochemical Investigation. *Clin Dev Immunol* 2013:164982

Fujimura T, Kambayashi Y, Hidaka T *et al.* (2012) Comparison of Foxp3+ regulatory T cells and CD163+ macrophages in invasive and non-invasive extramammary Paget's disease. *Acta Derm Venereol* 92:625–8

Gonzalez-Suarez E (2011) RANKL inhibition: a promising novel strategy for breast cancer treatment. *Clin Transl Oncol* 13:222–8

Hao NB, Lu MH, Fan YH *et al.* (2012) Macrophages in tumor microenvironments and the progression of tumors. *Clin Dev Immunol* 2012: 948098

Loser K, Mehling A, Loeser S *et al.* (2006) Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells. *Nat Med* 12: 1372–9

Lynch CC, Hikosaka A, Acuff HB *et al.* (2005) MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. *Cancer Cell* 7:485–96

Medrek C, Ponten F, Jirstrom K *et al.* (2012) The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC Cancer* 12:306

Press JZ, Allison KH, Garcia R *et al.* (2011) FOXP3+ regulatory T-cells are abundant in vulvar Paget's disease and are associated with recurrence. *Gynecol Oncol* 120:296–9

Shiomi T, Noguchi T, Nakayama H *et al.* (2013) Clinicopathological study of invasive extramammary Paget's disease: subgroup comparison according to invasion depth. *J Eur Acad Dermatol Venereol* 27:589–92

Th2 Cytokines Suppress Lipoteichoic Acid–Induced Matrix Metalloproteinase Expression and Keratinocyte Migration in Response to Wounding

Journal of Investigative Dermatology (2015) **135,** 2550–2553; doi:10.1038/jid.2015.181; published online 28 May 2015

TO THE EDITOR

Recent clinical trials have established a central role for the T helper type 2 (Th2) cytokines IL-4 and IL-13 in the pathology of atopic dermatitis (AD) (Beck *et al.*, 2014). Although blockade of the IL-4 and IL-13 receptors causes significant clearing of skin lesions, a direct effect of Th2 cytokines on wound healing processes has not yet been demonstrated. Furthermore, AD patients are frequently affected by infection with the pathogen, *Staphylococcus aureus* (Boguniewicz and Leung, 2011). Elevated levels of staphylococcal products are frequently found on the skin of affected patients (Travers *et al.*, 2010), and these products may affect the wound healing process as well.

A primary event in skin healing is induction of matrix metalloproteinases (MMPs) (Inoue *et al.*, 1995). MMPs-1, -9, and -10 are expressed at the leading edge of the wound (Inoue *et al.*, 1995; Rechardt *et al.*, 2000; Turchi *et al.*, 2003) and are required for keratinocyte migration into the damaged area (Pilcher *et al.*, 1997; Agren, 1999). Inhibition of MMP function effectively blocks keratinocyte migration and wound healing (Mirastschijski *et al.*, 2002b). However, overexpression of MMPs has been reported in skin diseases and can inhibit wound closure as well (Saarialho-Kere *et al.*, 1994; Mirastschijski *et al.*, 2002a). In this study, we determined the effects of staphylococcal products and AD-associated Th2 cytokines on MMP expression and on keratinocyte migration.

We first examined the effect of bacterial products on MMP levels.

Abbreviations: AD, atopic dermatitis; LTA, lipoteichoic acid; MMP, matrix metalloproteinase; siRNA, small interfering RNA; STAT6, signal transducer and activator of transcription 6; Th2, T helper type 2; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumor necrosis factor-α

Accepted article preview online 7 May 2015; published online 28 May 2015