FACULDADE DE ODONTOLOGIA

EXPRESSÃO IMUNOISTOQUÍMICA DOS RECEPTORES *TOLL-LIKE* 5 E 9 E DO FATOR DE TRANSCRIÇÃO FOXP3 EM LESÕES ORAIS DE LÍQUEN PLANO E LÚPUS ERITEMATOSO

VICTORIA MARTINA TRUCCI

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PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
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IMMUNOHISTOCHEMICAL EXPRESSION OF TOLL-LIKE RECEPTORS 5 AND 9 AND FOXP3 TRANSCRIPTION FACTOR IN ORAL LESIONS OF LICHEN PLANUS AND LUPUS ERYTHEMATOSUS

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Epígrafe

Si se es fiel al llamado de la vocación, los sacrificios y dificultades que uno pueda atravesar serán irrelevantes a la hora del balance final.

César Milstein (1927 - 2002)

Dedicatória

Aos meus pais, que também são merecedores,
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RESUMO

O presente estudo teve por objetivo avaliar o padrão de expressão dos receptores *toll-like* (TLR) 5 e 9 e do fator de transcrição forkhead box p3 (Foxp3) de células T regulatórias em lesões orais de líquen plano e lúpus eritematoso. Vinte e um espécimes de biópsias de líquen plano, 21 de lúpus eritematoso e 21 de hiperplasia fibrosa inflamatória (grupo-controle) foram submetidos a processamento imunoistoquímico com os marcadores anti-TLR5, anti-TLR9 e anti-Foxp3, e a quantificação da expressão desses marcadores foi realizada por meio do *software* Image Pro Plus 4.5.1 (Media Cybernetics). O padrão de expressão do Foxp3 diferiu significativamente entre os três grupos, sendo os maiores níveis observados no grupo líquen plano, e os menores, no grupo-controle. A expressão do TLR5 não diferiu significativamente entre os grupos lúpus eritematoso e controle, mas foi maior nesses grupos quando comparados ao grupo líquen plano. Ao discriminarem-se os subtipos de lúpus eritematoso sistêmico e discoide, não foi verificada diferença significativa na expressão do TLR5 entre líquen plano e lúpus sistêmico, mas foram verificados maiores níveis desse marcador no lúpus discoide e no grupo-controle quando comparados ao grupo líquen plano. O padrão de expressão do TLR9 não diferiu significativamente entre os grupos. Foxp3 e TLR5 apresentaram correlação negativa fraca ($r= -0.279$). As demais variáveis não apresentaram correlação, mesmo quando analisadas intragrupo. Os resultados do presente estudo permitem concluir que a expressão de Foxp3 está aumentada em lesões orais de líquen plano e lúpus eritematoso, enquanto TLR5 e TLR9 não exibem maior expressão nessas lesões; Foxp3 e TLR5 estão inversamente correlacionados. Novas investigações sobre células T regulatórias, receptores *toll-like* e citocinas pró-inflamatórias fazem-se necessárias para o melhor entendimento de doenças como líquen plano e lúpus eritematoso.

Palavras-chave: líquen plano, lúpus eritematoso, doenças do sistema imunológico, receptores toll-like, forkhead box p3, expressão imunoistoquímica

Summary

SUMMARY

The present study aimed at analyzing the expression of toll-like receptors (TLR) 5 and 9 and forkhead box p3 regulatory T cells transcription factor (Foxp3) in oral lesions of lichen planus and lupus erythematosus. Twenty-one biopsy specimens of lichen planus, 21 of lupus erythematosus and 21 of inflammatory fibrous hyperplasia (control group) were subjected to immunohistochemical staining with anti-TLR5, anti-TLR-9 and anti-Foxp3, whose expressions were quantified using Image Pro Plus 4.5.1 software (Media Cybernetics). Immunostaining for Foxp3 differed significantly between the three groups, where the highest levels were shown by lichen planus and the lowest ones by controls. TLR5 expression did not differ significantly between lupus erythematosus and control group, but it was significantly greater in these two groups than in lichen planus. When we considered the two types of lupus separately, there was no significant difference of TLR5 between lichen planus and systemic lupus erythematosus, but discoid lupus erythematosus as well as the control group showed significantly greater values than lichen planus. TLR9 immunostaining did not differ significantly between the groups analyzed. A weak negative correlation was observed between Foxp3 and TLR5 (r= -0.279), while there was no other correlation. When this analysis was performed within each group, no correlation was observed between the variables. In conclusion, Foxp3 expression is increased in oral lesions of lichen planus and lupus erythematosus, whereas TLR5 and TLR9 do not show increased expression in these lesions. Foxp3 and TLR5 are inversely correlated. Further investigations analyzing Treg cells and proinflammatory cytokines in these diseases are warranted.

Keywords: lichen planus, lupus erythematosus, immune system diseases, toll-like receptors, forkhead box p3, immunohistochemical expression

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

Doenças autoimunes podem desenvolver-se como resultado de falhas na tolerância imunológica, o que determina a ativação de células T autorreativas. Padrões moleculares exógenos microbianos ou endógenos ligam-se aos receptores *toll-like* (TLR) e ativam vias de sinalização em células do sistema imunológico inato, o que resulta na produção de citocinas pró-inflamatórias e ativação de células T que, até o momento, são consideradas o principal fator envolvido no desenvolvimento da autoimunidade (Mills, 2011). Os TLRs apresentam papel fundamental na resposta imunológica inata a agentes microbianos invasores. Tais receptores são expressos em monócitos, macrófagos, linfócitos, células dendríticas e granulócitos, bem como no epitélio das vias aéreas e na pele, importantes sítios de interação do organismo com patógenos (McInturff *et al*., 2005).

Além da ativação de células T efetoras, ligantes de TLRs podem modular direta e indiretamente a função das células T regulatórias (Tregs). Existem evidências de que a ativação de TLRs pode bloquear a resposta das células Treg, levando à quebra da tolerância a antígenos próprios (Pasare; Medzhitov, 2003; Yang *et al*., 2004; Peng *et al*., 2005; Mills, 2011). Diferentes estímulos extracelulares e diferentes moléculas sinalizadoras intracelulares controlam o desenvolvimento e a função das Tregs por meio da regulação da transcrição gênica do fator de transcrição forkhead box p3 (Foxp3) (Lal; Bromberg, 2009). Sob a influência de determinadas condições inflamatórias ou ligantes específicos, a ativação de certos TLRs resulta em reduzida expressão de Foxp3 com menor geração de Tregs capazes de suprimir a proliferação de células T autorreativas (Pasare; Medzhitov, 2003; Yang *et al*., 2004; Lal; Bromberg, 2009; Hackl *et al*., 2011).

Estudos recentes têm destacado que os TLRs estariam envolvidos na patogênese do lúpus eritematoso sistêmico (LES) (Nakano *et al*., 2008; Wong *et al*., 2010; Midgley *et al*., 2012). O LES representa o protótipo da doença autoimune, caracterizado por um amplo espectro de manifestações clínicas e produção de autoanticorpos (Midgley *et al*., 2012). As primeiras manifestações clínico-patológicas são consequência de uma inflamação tecidual local que tem início com a deposição de imunocomplexos nos diversos tecidos do corpo, uma vez que ocorre a produção de autoanticorpos contra componentes nucleares das células (Munoz *et al*., 2008). Uma falha na eliminação de células apoptóticas, principalmente neutrófilos, levaria ao acúmulo de autoantígenos nucleares (proteínas nucleares, DNA, RNA) que são modificados por clivagem enzimática durante o próprio processo de apoptose celular. Esse atraso na eliminação do material nuclear modificado permite que o mesmo seja reconhecido por TLRs em células dendríticas com consequente aumento da produção de interferon alfa (IFN-alfa), um potente mediador na patogênese do lúpus eritematoso sistêmico. Tal processo resulta na formação de autoanticorpos contra os autoantígenos nucleares (Munoz *et al*., 2008; Midgley *et al*., 2012). O IFN produzido medeia a maturação e a apresentação antigênica por células dendríticas convencionais e macrófagos. Além disso, ativa a geração de células B e T efetoras e de memória, enquanto Tregs são suprimidas, o que colabora para o estabelecimento do quadro de autoimunidade observado no lúpus (Rönnblom *et al*., 2011).

A etiologia do líquen plano ainda não foi totalmente esclarecida. No entanto, alguns fatores têm sido associados ao seu surgimento, entre os quais estão ansiedade, diabetes, doenças autoimunes, doenças intestinais, drogas, estresse, hipertensão, infecções bacterianas e virais, materiais dentários restauradores, neoplasias e predisposição genética (Canto *et al*., 2010; Roopashree *et al*., 2010). Ainda, acredita-se

na existência de uma possível susceptibilidade individual para o desenvolvimento da doença, que estaria na dependência da natureza do antígeno, da habilidade do indivíduo para apresentação de antígenos, da presença de células T capazes de reconhecer esse antígeno (receptores) e da possibilidade de herdar um perfil de citocinas e polimorfismos genéticos que estimulem resposta mediada por células ao antígeno (Thornhill, 2001). Há evidências de que uma desregulação imunológica mediada pelo componente celular do sistema imunológico estaria envolvida na patogênese do líquen plano oral, já que o infiltrado inflamatório nessa doença é composto predominantemente por células T e macrófagos, enquanto plasmócitos são observados raramente, e depósitos de imunocomplexos não são característicos (Lodi *et al*., 2005).

As lesões de líquen plano e lúpus eritematoso que se desenvolvem na mucosa oral podem apresentar aspecto clínico variado e semelhante, sendo necessária a realização de biópsia para o estabelecimento do diagnóstico diferencial (Neville *et al*., 2009). As lesões orais de líquen plano geralmente são bilaterais e simétricas, afetando a mucosa jugal, a gengiva e as porções lateral e dorsal da língua. São representadas tipicamente por estrias reticulares esbranquiçadas denominadas estrias de Wickham, que podem coalescer e formar pápulas e placas. As formas atrófica e erosiva são observadas frequentemente e, nos casos mais graves, a mucosa pode estar ulcerada. As lesões orais típicas do lúpus eritematoso ocorrem primeiramente nas mucosas jugal e labial, rebordo alveolar e vermelhão de lábio e são caracterizadas por uma área atrófico-ulcerada central, pequenas placas brancas ceratóticas com bordas elevadas e estrias brancas irradiadas. No entanto, o aspecto clínico é variado, o que pode dificultar o diagnóstico diferencial com o líquen plano (Farthing; Speight, 2006). Considerando-se que alterações na expressão da Foxp3, por meio da ativação de TLRs específicos, podem ter papel preponderante no desenvolvimento de doenças autoimunes, bem como a

semelhança clínica entre lesões orais de líquen plano e lúpus eritematoso, o presente estudo teve por objetivo avaliar, por meio de imunoistoquímica, o padrão de expressão dos *toll-like receptors* 5 e 9 e do fator de transcrição Foxp3 em lesões orais dessas duas doenças. O trabalho apresenta-se sob a forma de dois artigos científicos: o primeiro consiste em um artigo de revisão da literatura sobre o tema em questão, e o segundo compreende o experimento realizado.


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2 ARTIGO 1

O artigo a seguir intitula-se **Interrelationship of dendritic cells, system type 1 interferon, regulatory T cells and toll-like receptors in immune response and autoimmune diseases – a literature review** e foi formatado de acordo com as normas do periódico ***Archives of Oral Biology*** (Anexos A e B).

Interrelationship of dendritic cells, system type 1 interferon, regulatory T cells and toll-like receptors in immune response and autoimmune diseases – a literature review

Victoria Martina Trucci

Fernanda Gonçalves Salum

Maria Antonia Figueiredo

Karen Cherubini *

Postgraduate Program, Dental College, Pontifical Catholic University of Rio Grande do Sul - PUCRS

Running title: *Important factors in autoimmune diseases*

*** Corresponding author**

Karen Cherubini

Serviço de Estomatologia, Hospital São Lucas PUCRS

Av Ipiranga, 6690, sala 231

Porto Alegre RS Brazil

CEP 90610-000

Telephone/fax: 55(51)33203254

E-mail: karen.cherubini@pucrs.br

Abstract

The mechanisms involved in immune disturbances that lead to autoimmune diseases are not completely understood. Many studies focusing on this issue have been published, clearly indicating the great interest of the scientific community in improving the management and treatment of these diseases. There is evidence that the activation of some receptors of the toll-like family (TLRs) of the innate immune system, and also changes in expression levels of forkhead box p3 (Foxp3) protein, which is found in regulatory T cells (Tregs), could be involved in the development of autoimmunity. We present here a literature review focusing on the interrelationship of dendritic cells, TLRs, Tregs and type 1 interferon in the immune response and autoimmune diseases, with special interest in lupus erythematosus and lichen planus. Understanding the specific role of each of these factors would help elucidate the obscure etiology of such diseases and open new perspectives for their management and treatment.

Keywords: autoimmune diseases; TLRs; Foxp3; lichen planus; lupus erythematosus

Introduction

Autoimmune diseases are chronic conditions in which the immune system develops an inflammatory response to self cells and tissue components in the human body. This leads to inflammation and abnormal infiltration of lymphocytes and other leukocytes in the tissues, although they are free of infection.[1] Included among these conditions are some diseases such as lupus erythematosus,[2] which is a classic autoimmune disease where multiple autoantibodies are produced.[3] Nevertheless, there are some other immune disorders such as lichen planus, whose autoimmune nature has not yet been definitely established, as their initial triggers and pathogenesis are unknown.[4]

Therefore, lichen planus has been classified as either an autoimmune[5-9] or immune-mediated disease.[4,7,10-14]

Regardless of being a classic or a possible autoimmune disorder, the etiopathogenesis of these diseases needs to be elucidated, which has generated growing interest in investigating their relationship with some specific immune mechanisms.[15] Plasmacytoid dendritic cells, forkhead box $p3^+$ regulatory T cells ($Foxp3^+$ Tregs), toll-like receptors (TLRs) and type 1 interferon system (type 1 IFN), especially interferon-alpha (IFN-alpha), have been recognized as key elements involved in the process. An imbalance in the interrelationship between them could be the cause of the development of autoimmunity.[16-23]

Also, the interrelationship between environmental stimuli and the exacerbation of a self-reactive lymphocytic response, which culminates in lichen lesions, has been investigated. Studies have evidenced that plasmacytoid dendritic cells are present in skin lesions of patients with autoimmune diseases but absent in controls.[15] The constant presence of an endogenous or exogenous component recognized as an antigen in the body could persistently stimulate TLRs of plasmacytoid dendritic cells with consequent production of IFN and inhibition of $Foxp3^+$ Treg cells, which would establish a vicious circle and a consequent autoimmune state. We present here a literature review focusing on these important aspects of the immune system and their role in the immune response and autoimmune diseases, with special interest in lupus erythematosus and lichen planus.

Dendritic cells

Dendritic cells are immune cells originating from hematopoietic tissue, which, besides having the function of antigen-presentation, are also involved in the immune tolerance process.[24,25] These cells circulate in peripheral tissues capturing pathogens and/or

apoptotic cells and presenting them as antigens to T cells. T cells then proliferate and differentiate into Th1 (cellular immune response), Th2 (humoral immune response) and regulatory T cells, where the last are involved in the suppression mechanisms of immune response. Cytokines produced by dendritic cells contribute to the induction of T cell differentiation. Interleukin-6 (IL-6) induces $TCD4^+$ cells to differentiate into Th2 cells and suppresses Treg activity; interleukin-10 (IL-10) inhibits Th1 but induces Th2 responses; interleukin-18 (IL-18, IFN-gamma-inducing factor) interacts with IL-12 and induces naïve T cell proliferation and differentiation into IFN-gamma-producing Th1 cells.[19] Moreover, some subtypes of dendritic cells are highly specialized in the induction and differentiation of Treg cells, where a failure in this function can result in autoimmunity.[22]

Basically, dendritic cells are divided into myeloid and plasmacytoid. Plasmacytoid dendritic cells are found in blood and secondary lymphoid organs expressing blood dendritic cell antigen 2 (BDCA2), immunoglobulin-like transcript 7 (ILT-7) and interleukin-3 receptor (also known as CD123 antigen) on the cell membrane and are great producers of type 1 IFN.[21,24,26] Once activated by either endogenous or exogenous ligands, they secret considerable amounts of IFN-alpha[21,24] and IL-6, which stimulates plasma cells to produce antibodies.[24] Plasmacytoid dendritic cells express TLR7 and TLR9 in the endosomal membranes and can be activated by pathogens that invade them through endocytosis. In addition, these cells express interferon regulatory factors 5 (IRF 5) and 7 (IRF 7), which increase their capacity of producing IFN and other inflammatory cytokines.[21]

Type 1 interferon system

Type 1 interferon system (type 1 IFN), which includes interferon alpha (IFN-alpha) and interferon beta (IFN-beta), is produced against viral infections and can activate innate

and adaptive immune cells, thereby playing a role in cellular and humoral responses.[21] It has important immunomodulatory effects: maturation and migration of dendritic cells to secondary lymphoid organs; increase in antigen presentation and increase in its own production, especially in plasmacytoid dendritic cells. In natural killer (NK) cells, it increases cytotoxicity and cytokine production; in macrophages, it stimulates the intracellular destruction of pathogens; in T-lymphocytes, it increases IL-12 expression and generation of memory cells, as well as promoting suppression of Tregs.[21]

Regulatory T cells

Potentially pathogenic $CD4^+$ self-reactive T cells are found in peripheral tissues of normal individuals, and their activation/expansion is controlled by other $CD4^+$ cells called regulatory T cells (Tregs). These two populations of $CD4^+$ cells can be discriminated by the expression of some specific surface molecules. Treg cells, also called suppressor T cells, are a subtype of lymphocytes involved in the induction and maintenance of immune tolerance and in the control of the immune response as well. [27]

Tregs are induced by dendritic cells through several mechanisms that are dependent on indoleamine 2,3-dioxygenase, IL-10, IL-27, retinoic acid, vitamin D and transforming growth factor beta (TGF-beta). They comprise IL-10 secreting type 1 Tregs (Tr1), natural and adaptive $Foxp3^+$ Tregs, Th3 cells and double-negative Tregs.[22] Natural Tregs (*nTregs*) are induced in the thymus, show high CD25 (IL-2 alpha chain receptor) expression on the cell membrane, and are generally called $CD4^+CD25^+$ Tregs.[22,28,29] They suppress other T cells through cell-cell contact in a cytokine-independent mode, and it is believed that they play an important role in central tolerance to self-antigens.[28,30] Adaptive Tregs (*aTregs*), in turn, develop from mature $CD4^+CD25^-$ T cells in peripheral tissues under particular conditions or antigen-specific stimulation. *nTregs* and *aTregs* were recently defined also by the intracellular expression of Foxp3

($CD4^+CD25^+Foxp3^+$), which is a transcription factor needed for the differentiation and normal function of Tregs.[22,28,29]

Upon activation, *nTregs* predominantly express granzyme A, while *aTregs* express granzyme B. Both subtypes of Tregs exhibit perforin-dependent cytotoxicity against a variety of autologous target cells, including $CD4^+$ and $CD8^+$ T cells, $CD14^+$ monocytes, and dendritic cells.[31] Classic human Tregs ($CD4^+CD25^+Foxp3^+$) inhibit Th $CD4^+CD25^-$ cell proliferation through local action via cell-cell contact and induce them to assume an *aTreg* phenotype, which exerts its regulatory function by systemic action via TGF-beta. The secretion of this soluble suppressor mediator by *aTregs* is important for the systemic control of self-reactive effector T cells (Fig.1).[32]

To become suppressor cells, Tregs need to have their T-cell receptors (TCR) activated in the presence of IL-2, whose membrane receptor component is CD25 (de la Rosa *et al*., 2004). In naïve mice, CD25 protein is restricted to Tregs, whereas in humans just cells expressing large amounts of CD25 ($CD4^+CD25^{high}$ T cells) can be designated as Tregs. This is because activated T cells express intermediate levels of CD25, even though they do not show any suppressor function.[27,33,34]

$Foxp3^+$Treg cells play an important role in the maintenance of peripheral immune tolerance[17,22] and control of intense chronic inflammatory and allergic responses.[22] They are capable of blocking immune responses as well as inflammation and tissue destruction through functional suppression of many cells, including $TCD4^+$ and $TCD8^+$ lymphocytes, monocytes, NK cells,[34] and antigen-presenting cells, as well as antibody production by B lymphocytes.[17,34] Although it has not been confirmed, the site of Tregs action may be represented by draining lymph nodes or by the respective target organ or both.[35] Despite their importance, suppressor molecular mechanisms of

Tregs are still not completely known.[34] However, it is believed that their major function is to suppress the activation and expansion of naïve conventional T cells.[33]

It has been observed that proliferation and cytokine production of conventional $CD4^+CD25^-$ T cells, either from rats[36] or humans,[37] can be directly inhibited by Tregs, even in the absence of antigen-presenting cells. Different suppressor mechanisms may be used by Tregs depending on their state of activation, inflammatory site, and type and activation state of the suppressed target cell. Basically these mechanisms can be direct or indirect. Nevertheless, how the type of suppressor mechanism to be used is determined has not been identified.[34]

Direct suppression of effector T cells by Tregs can involve soluble immune suppressor factors or cell contact, whereas indirect suppression involves antigen-presenting cells.[34] The stimulation of effector T cells leads to the generation of a $Ca2^+$ signal and a cascade of protein phosphorylation and dephosphorylation events, which culminates in the activation of transcription factors that stimulate T cell proliferation and cytokine expression. The most important transcription factors for induction of cytokine expression are nuclear factor kappa B (NF-κB), nuclear factor of activated T-cells (NFAT) and activator protein 1 transcription factor (AP-1), which mediate gene activation of some cytokines such as IL-2 and IFN-gamma.[34,38,39] Tregs can rapidly suppress this cytokine expression in effector T cells through inhibition of $Ca2^+$ signals and consequently reduced NFAT and NF-κB activation.[34]

IL-10 and TGF-beta are inhibitory cytokines secreted by TCR-activated Tregs and are involved in *in vivo* and *in vitro* suppression of both proliferation and cytokine production of many cells such as T $CD8^+$, B, NK, and other innate immune cells.[35,37,40] Tregs can secret and express large amounts of soluble and membrane-bound TGF-beta, and the blockade of this protein production can partly nullify the suppression of T cell

proliferation, which suggests that TGF-beta produced by Tregs play an important role in autoimmunity control.[41,42]

The indirect pathway through which Tregs suppress the activation of conventional T cells occurs by means of impairing the stimulatory function of antigen-presenting cells.[43] Tregs induce an immunosuppressive pattern of cytokine production through reduced synthesis of IL-6 and increased levels of IL-10 by dendritic cells. In contact to $CD4^+$ T cells, dendritic cells would increase IL-6 and decrease IL-10 production; however, if in contact with $CD4^+CD25^+$ Tregs, the opposite would occur, with increase in IL-10 levels in detriment of IL-6 (Fig.1).[44] Also, Tregs can inhibit conventional T cell activation by reducing stable cell contacts between them and dendritic cells. Less time of contact between these cells has been observed in the presence of Tregs.[43]

Associated with the complex suppressor mechanism mediated by Tregs through IL-10, it was recently demonstrated that Tregs need IL-10 and not IL-35 and TGF-beta to control IFN-gamma production by T cells in inflamed skin, whereas in lymph nodes IL-10 is dispensable for control of IFN-gamma and T cell expansion. T cell cultures are capable of secreting IFN-gamma in the absence of Tregs, but incapable in their presence.[34,45]

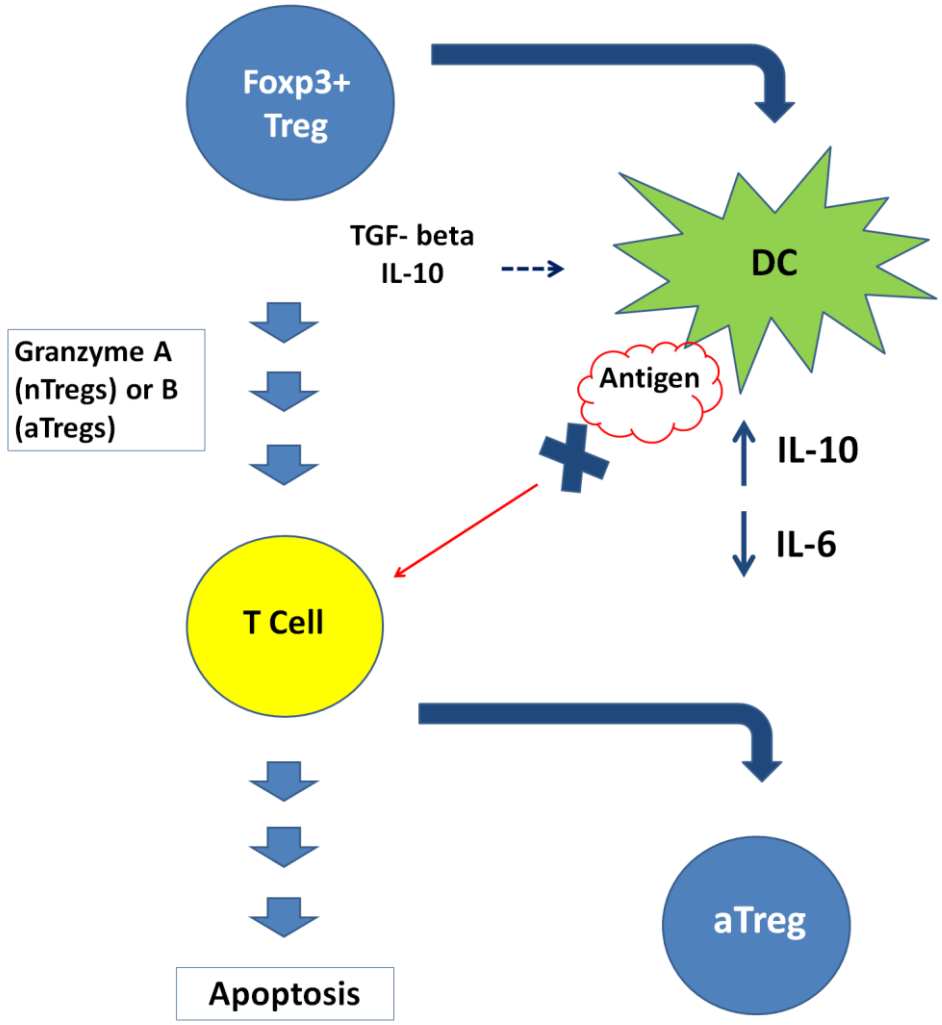

Figure 1 - Mechanisms of Foxp3^{+} Treg-mediated T cell supression: Regulatory T cell (Treg) express granzymes (nTreg = granzyme A; aTreg = granzyme B) which induce citotoxicity against autologous target cells in a perphorin-dependent manner. Through production of antiinflamatory cytokines [transforming growth factor beta (TGF-beta) and interleukin 10 (IL-10)], Tregs induce increased production of IL-10 and decresead production of interleukin 6 (IL-6) by dendritic cells. This would inhibit antigen presenting dendritic cell function.
nTreg= natural Treg; aTreg= adaptive Treg; DC= dendritic cell

Factors interfering with Foxp3 expression and Tregs stability

Foxp3 has unstable expression, and there are environmental or extrinsic factors that probably affect its stability. Therefore, depending on the type of stimulus and ligands, the natural history of inflammatory events can be marked by more or less Treg suppressor activity.[20] Different regulatory elements of the Foxp3 locus respond to

various signals. However, it is unknown how different extracellular signals affect the chromatin structure of these regulatory elements.[20,46]

IL-2 deficiency and the presence of IL-1, IL-4, IL-6, IL-23 and IFN-gamma can induce alterations in the chromatin of the gene locus of Foxp3, which impairs its transcription and thus promotes a decrease in its expression and suppressor effect.[17,20] Lal *et al*.[20] demonstrated that the two independent pathways of IL-6/IL-6 receptor/activator of transcription-3 (IL-6/IL-6R/STAT3) and peptidoglycan/TLR2/Myd88/IRF-1 resulted in inhibition of Foxp3 expression in *nTregs* and *aTregs*. These pathways lead to profoundly different changes in the structure of Foxp3 chromatin, with subsequent effects on T cell function. In the presence of TLR 7 and TLR9 ligands, cocultures of dendritic and T cells have shown greater production of IL-6, IL-12 and IL-17, with consequent reduced percentage of $Foxp3^+$ cells. That is, IL-6 as well as IFN-gamma and IL-4 produced in cocultures of dendritic and T cells in the presence of TLR7 ligands are critical factors for the decrease in Foxp3 expression.[47]

Toll-like receptors

Toll-like receptors (TLRs) are a group of glycoproteins that work as transmembrane receptors of the innate immune system, allowing antigen recognition and subsequent immune activation. They are located on the cell membrane surface (TLRs 1, 2, 4, 5, 6, 10) and in intracellular compartments such as the endoplasmic reticulum and endosomes (TLRs 3, 7, 8, 9), where they are more common in cells that initiate the primary immune response. Either immune cells such as monocytes, macrophages, dendritic cells, granulocytes, NK cells, and B and T lymphocytes, or non-immune cells such as keratinocytes and fibroblasts express TLRs,[23,48,49] where these receptors play different functions depending on the type of cell and tissue where they are expressed.[18] They stimulate an adaptive immune response through the activation of dendritic cells

and also increase the production of costimulating molecules and inflammatory cytokines such as TNF-alpha, IL-6 and IL-12.[50]

The ten human TLRs so far described are capable of recognizing a wide variety of molecules, ranging from those of bacterial origin such as lipoproteins (TLRs 1, 2 and 6), lipopolisaccharides (TLR4) and flagellin (TLR5) to viral RNA (TLRs 3, 7, and 8) and DNA (TLR9).[18,48] TLR 7 plays an important role in autoimmunity and antiviral defense,[47] and TLR9 contributes to the production of anti-DNA antibodies,[24] whereas TLR10 function is not completely known. TLR11 has been identified in mice and reported as capable of recognizing molecules of *Toxoplasma gondii*.[23] The function of recognizing infections and inducing signaling pathways that result in the expression of inflammatory mediators and immune response is common to different TLRs.[18] The presence of TLR ligands results in cytokine and chemokine secretion, which leads to immune activation against pathogens. TLRs 3, 7 and 9, for instance, respond to viral RNA and DNA with IFN-alpha production,[51] which exerts many immunomodulatory effects.[21]

The potent inflammatory response induced by TLRs is protective in most cases, since pathogens are destroyed before they can cause injury to the host. Exogenous ligands derived from viral or bacterial infections activate TLRs, thereby cause inflammation. This inflammation, in turn, induced by either endogenous or exogenous ligands, can cause cell necrosis and extracellular matrix damage, as well as proinflammatory cytokine production, which enables the release of new endogenous TLR ligands, making the process chronic.[18]

All TLRs share a conserved intracellular domain, the toll-interleukin-1 receptor (TIR) domain, which is responsible for signal transduction and adaptor recruitment. The extracellular domain, in turn, is called a leucine-rich repeat (LRR), which is responsible

for recognizing TLR ligands.[18] Until now, five TLR adaptor molecules have been described: myeloid differentiation factor-88 (MyD88),[52] TIR domain-containing adaptor protein (TIRAP),[53] TIR domain-containing adaptor inducing IFN-beta (TRIF or TICAM-1),[54] TRIF related adaptor molecule (TRAM or TICAM-2),[55] and sterile alpha and HEAT/Armadillo motif (SARM).[56] Once TLR is activated by an exogenous or endogenous ligand, its TIR domain interacts with the adaptor molecule, initiating a cascade of intracellular signaling pathways that lead to the activation of transcription factors.[23,57,58] The activation of these transcription factors results in the induction of gene expression of inflammatory cytokines such as TNF-alpha and type 1 IFN.[48,59,60] While most TLRs depend on the intracellular MyD88 signaling pathway, TLRs 3 and 4 use the TRIF pathway, which induces IFN-beta. The TNF receptor associated factor 6 (TRAF 6) pathway is considered an additional transducer for TLRs 7 and 9.[48,59,60]

The expression and function of TLR5 and TLR9 have been investigated in human keratinocytes. In normal epithelium, TLR5 is expressed in basal keratinocytes in the first and second layers. TLR9 is expressed in more external layers of the epidermis comprising the top two to three layers of cells. It is observed that transforming growth factor alpha (TGF-alpha), which is involved in epithelial healing, increases the expression of both these TLRs. Besides increasing the expression of TLRs, TGF-alpha stimulates their function by means of increasing IL-8 proinflammatory cytokine and human beta-defensin 2 (hBD-2) antimicrobial peptide production.[61]

Dendritic cells, type 1 IFN, TLRs and Tregs in autoimmunity

Jin *et al*.[19] observed that, in systemic lupus erythematosus (SLE) patients, plasmacytoid dendritic cells have a greater capacity of stimulating inflammatory T cells than in controls. These authors isolated dendritic cells from peripheral blood of 58 lupus patients and 62 controls and kept them in culture with or without apoptotic

polymorphonuclear cells and, afterwards, with T cells. In the presence of apoptotic polymorphonuclear cells, the plasmacytoid dendritic cells from controls induced the differentiation of T cells into $CD4^+CD25^+$ Tregs with high expression of Foxp3, reduced IL-6 production, and increased TLR9 mRNA transcription. On the contrary, plasmacytoid dendritic cells from lupus patients were functionally abnormal, since they induced inflammatory T cell proliferation even in the absence of apoptotic polymorphonuclear cells. They also lost TLR9 expression and were less efficacious in inducing Tregs differentiation after interaction with apoptotic polymorphonuclear cells. Therefore, in SLE patients, plasmacytoid dendritic cells are more effective in stimulating inflammatory T cells than in inducing Treg development. Such finding suggests that only healthy plasmacytoid dendritic cells can induce the development of suppressive Tregs.[19] In this way, these cells seem to be abnormal in lupus showing a lower capacity to induce Treg cell differentiation.[19,21]

Deficiency or dysfunction of Tregs may result in autoimmunity. Elimination of the peripheral population of $CD4^+CD25^+$ T cells in rats promotes a wide spectrum of organ-specific and systemic autoimmune diseases, as well as graft-versus-host disease,[27,33] whereas reconstitution of this cell population prevents the development of autoimmunity.[27] The alterations in number and function of $CD4^+CD25^+Foxp3^+$ Tregs were recently determined in autoimmune and inflammatory diseases such as psoriasis, multiple sclerosis, autoimmune polyglandular syndrome type II, rheumatoid arthritis, myasthenia gravis and type I diabetes.[62] High and stable expression of Foxp3 is needed for Treg suppressor activity, whereas its decrease favors loss of suppressor ability.[63] Also, similar alterations were observed in lichen planus. Using immunohistochemistry and real time RT-PCR, Tao *et al.*[62] found a greater number of $Foxp3^+$ cells in oral lesions of lichen planus patients than in controls. Reticular lesions of lichen in turn

showed a higher frequency of $Foxp3^+$ cells compared to erosive ones. In flow cytometry, they showed a significant increase in these cells in the peripheral blood of patients after treatment. Such findings indicate that $Foxp3^+$ Treg cells are involved in the etiopathogenesis of oral lichen planus, and they show an inverse correlation with disease activity.[62]

Disclosing global defects in Treg cells of autoimmune disease patients has been a challenging task because of the complexity of this kind of disturbance. There are many precipitating factors, genetic and environmental, contributing to the susceptibility to autoimmune diseases. Therefore, the failure of Tregs to function could be a consequence of a lack of Tregs with the specificity needed to suppress inflammation in a particular organ, cell-intrinsic defects, or extrinsic factors that inhibit the function of these cells.[29]

Excessive levels of tumor necrosis factor (TNF) and reduced IL-2 production commonly observed in SLE can contribute to a functional defect in $CD4^+CD25^+$ Treg cells, which start expressing lower levels of Foxp3 mRNA and lose their suppressive function.[64] Moreover, in SLE patients, the inability of plasmacytoid dendritic cells to induce Treg development contributes to immune tolerance breakdown and autoimmunity onset, with reduction of suppressor function of $CD4^+CD25^+$ Tregs and low expression of Foxp3 mRNA and protein.[19]

Valencia *et al.*[64] observed a significant reduction in suppressor function of $CD4^+CD25^{high}$ Tregs isolated from the peripheral blood of active SLE patients, which was confirmed by the expression of low levels of Foxp3 (mRNA and protein) and reduced inhibition of *in vitro* cytokine production and proliferation of $TCD4^+$. The same was not observed in Tregs isolated from patients with inactive disease, where Foxp3 expression and suppression of $TCD4^+$ proliferation were similar to that in controls. On

the other hand, *in vitro* activation of $CD4^{+}CD25^{high}$ Treg cells from active SLE patients, in culture with plate-bound anti-CD3 and high doses of IL-2, increased Foxp3 expression (mRNA and protein) and restored their suppressor function. To determine whether loss of suppressor function in active SLE would result from an intrinsic defect of $CD4^{+}CD25^{high}$ Tregs or from an increase in effector $CD4^{+}CD25^{-}$ T cell resistance, Valencia *et al.*[64] mixed cells from active SLE patients with cells of normal controls. They found that Tregs from active SLE patients failed to suppress the proliferation of both autologous and control effector T cells, whereas Tregs from controls were capable of suppressing the proliferation of effector T cells of active SLE patients.

TGF-beta is important to Treg development and function because it increases Foxp3 levels.[65] Nevertheless, if in the presence of IL-21 and especially IL-6, it can also evoke a Th17-type inflammatory response, where Foxp3 levels are reduced allowing Tregs to assume a Th17 phenotype,[65] which is associated with many autoimmune and inflammatory pathologies.[17,66] Noteworthy is the higher frequency of IL-17 producing Th cells (Th17 cells) in these pathologies.[19,21,66]

TLRs are involved in the pathogenesis of autoimmune diseases, where they can be activated by endogenous ligands.[18,23,24] TLR ligands can induce the secretion of cytokines capable of interfering with Treg plasticity and inhibiting their suppressor function.[20] Exogenous and endogenous TLR7 ligands, respectively found in viral infections and autoimmune diseases, reduce Foxp3 expression leading to lower numbers of Tregs and, therefore, lower capacity to suppress self-reactive T cell proliferation. The activation of dendritic cells through TLR7, for example, leads to the production of soluble factors that decrease the generation of Tregs, which probably favors their differentiation into proinflammatory effector Th cells and consequently promotes autoimmunity.[47]

Patients with rheumatologic autoimmune diseases, especially lupus erythematosus, have a higher production of type 1 IFN.[21] High serum levels of IFN-alpha are considered a heritable risk factor for SLE,[51] and are associated with higher disease activity, more severe clinical manifestations and immune activation signals.[21,67] IRFs can be activated by circulating immune complexes containing nucleic acids, which are recognized by endosomal TLRs. In addition, genetic variants of IRF-5 and IRF-7 have been associated with susceptibility to the disease, because they induce IFN-alpha production.[51] There is some evidence that activated plasmacytoid dendritic cells are responsible for the continuous production of IFN-alpha in lupus,[21,67] and the plasma of lupus patients has interferogenic immune complexes which are able to specifically activate these cells.[21,68]

In lupus, self-antigens derived from nucleic acids can be generated from apoptotic or necrotic cells, because of higher frequency of apoptosis or lack of clearance of apoptotic cells, and the immune complexes that activate TLR7 are potent inducers of IFN-alpha. Still, environmental factors such as ultraviolet light and demethylating agents increase the amount of autoantigens. This promotes greater production of interferogenic immune complexes and type 1 IFN, which promotes autoimmunity in a vicious circle.[21]

The effect of TLR2 stimulation on human Tregs has also been studied. It was observed that Treg suppressor activity is inhibited by TLR2 Pam3Cys ligand (synthetic bacterial lipoprotein), leading to a greater proliferation of effector T cells. TLR2 activation induces Tregs to secret IL-6, which in turn reduces Treg suppressor function and induces their differentiation into effector Th17 cells.[40] On the other hand, the activation of other TLRs such as TLR4 and TLR5 seems to have no effect on the generation of Tregs.[47] Nevertheless, in psoriasis, TLR5 and TLR9 are expressed in

larger amounts and show diffuse distribution in epithelium, with TLR5 expressed in three to eight of the deepest layers, and TLR9 occurring throughout all the epithelium.[61] Also, although evidence points to an important role of TLRs in lichen planus development, published studies are a little conflicting in this respect. Some have reported upregulation of TLR9[15,23,48] and TLR2[69] in lichen lesions, whereas another study has reported a significantly lower rate of TLR 1 and TLR2 expressions in lichen planus than in controls.[70]

The result of autoimmunity *versus* the immune tolerance process depends on the balance between stimulatory signals such as binding of ligands to TLRs, and antigen concentration, as well as on inhibitory signals mediated by Tregs.[35] This balance breaks down in SLE, where autoantibodies can bind to either native DNA or nuclear RNA-binding proteins such as Ro, La, Sm and RNP, and the immune complexes formed stimulate cells through endosomal TLRs, activating antiviral immunity and amplifying an autoimmune response. This abnormal process results in IFN-alpha production.[51] Moreover, persistent activation of TLRs involved in the induction of Th1/Th2 inflammatory cytokines such as IFN-gamma, type 1 IFN and other interleukins capable of downregulating Foxp3, can inhibit Treg activity.[19,20,29,47,71] Therefore, the release of TLR endogenous ligands during inflammatory processes and the consequent activation of their signaling pathways can represent a trigger mechanism of autoimmune diseases (Fig.2).[18]

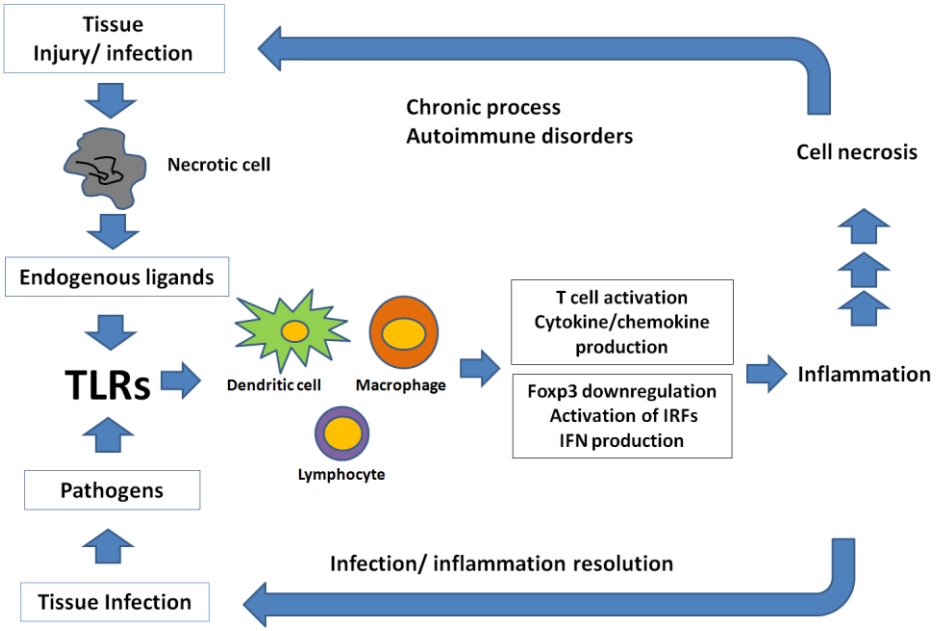

Figure 2 - Mechanism of immune activation by TLRs and their relationship with immune disorders. TLRs= toll-like receptors; Foxp3=forkhead box p3; IRFs= interferon regulatory factors; IFN= interferon

Final considerations

Despite the large number of published studies about autoimmunity and immune-mediated diseases, there is still no consensus on theories and concepts about how the dysregulation of the immune response actually occurs and which are the triggering factors. Although the Foxp3-mediated relationship between TLRs and Tregs has been extensively investigated,[20,35,47,50,72-78] it is known that many other factors and mechanisms are also involved in the process such as intracellular signaling pathways, gene polymorphisms and pro-inflammatory cytokine patterns, as well as different immune activation pathways.[3,20,46,54,76,79,80-86]

The involvement of some exogenous antigen such as viruses and bacteria invading host tissues has been pointed out in the etiology of autoimmune diseases.[87,88] Likewise, an association between hepatitis C virus (HCV) infection and oral lichen

planus has been reported. HCV is the infection most associated with immune manifestations.[88] There is evidence that HCV can subvert the immune system and induce autoimmunity through molecular mimicry. That is, T and B cells start recognizing self-antigens that share molecular similarities with HCV.[87] It is possible that the same process occurs with many other microorganisms in many other diseases, which would involve the mechanisms examined in this review. Therefore, considering the complexity of the immune response and the tremendous microbiota harbored by humans, it would be crucial in each specific case of autoimmune and immune-mediated disease to investigate and rule out first the involvement of exogenous agents, especially microorganisms, as the triggering factor.

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3 ARTIGO 2

O artigo a seguir intitula-se **Immunohistochemical expression of toll-like receptors 5 and 9 and Foxp3 transcription factor in oral lesions of lichen planus and lupus erythematosus** e foi formatado de acordo com as normas do periódico ***Journal of Oral Pathology and Medicine*** (Anexos C e D).

Immunohistochemical expression of forkhead box p3 transcription factor and toll-like receptors 5 and 9 in oral lesions of lichen planus and lupus erythematosus

Victoria Martina Trucci[1]

Márcia Fava[1]

Fernanda Gonçalves Salum[1]

Maria Antonia Figueiredo[1]

Vinicius Duval da Silva[2]

Karen Cherubini[1]

[1] Postgraduate Program, Dental College, Pontifical Catholic University of Rio Grande do Sul - PUCRS

[2] Department of Pathology, School of Medicine, Hospital São Lucas, Pontifical Catholic University of Rio Grande do Sul – PUCRS

Running title: *TLR5, TLR9 and Foxp3 in oral lesions*

Keywords: lichen planus, lupus erythematosus, toll-like receptors, forkhead box p3, immunohistochemical expression

Corresponding author

Karen Cherubini

Serviço de Estomatologia, Hospital São Lucas - PUCRS

Av. Ipiranga, 6690/231

Porto Alegre, RS, Brazil 90610-000

Telephone/Fax: 55 51 33203254

Email: karen.cherubini@pucrs.br

Abstract

Background: This study aimed at analyzing the expression of forkhead box p3 transcription factor (Foxp3) and toll-like receptors (TLR) 5 and 9 in oral lesions of lichen planus and lupus erythematosus.

Materials and methods: Biopsy specimens of lichen planus (n=21), lupus erythematosus (n=21) and inflammatory fibrous hyperplasia (n=21; control group) were subjected to immunohistochemical staining with anti-Foxp3, anti-TLR5 and anti-TLR-9, whose expressions were quantified.

Results: Immunostaining for Foxp3 differed significantly between lichen planus, lupus erythematosus and control group, where the highest expression levels were shown by lichen planus and the lowest ones by controls. TLR5 expression did not differ significantly between lupus erythematosus and control group, but it was significantly greater in these two groups than in lichen planus. When we considered the two types of lupus separately, there was no significant difference in TLR5 between lichen planus and systemic lupus erythematosus, but discoid lupus erythematosus as well as the control group showed significantly greater values than lichen planus. TLR9 immunostaining did not differ significantly between the groups analyzed. A weak negative correlation was observed between Foxp3 and TLR5 (r=-0.279), while there was no other correlation. When this analysis was performed within each group, no correlation was observed between the variables.

Conclusions: Foxp3 expression is increased in oral lesions of lichen planus and lupus erythematosus, whereas TLR5 and TLR9 do not show increased expression in these lesions. Foxp3 and TLR5 are inversely correlated. Further investigations analyzing Treg cells and proinflammatory cytokines in these diseases are warranted.

Introduction

Lupus erythematosus and lichen planus are immune-mediated disturbances whose specific etiologies are unknown (1-3). Both diseases determine the occurrence of oral lesions that are similar in appearance (4) and present as white striated lesions and white plaques, as well as erythematous and erosive areas with white striae (5). While lupus is considered the prototype of autoimmune disease, showing a variety of autoantibodies, in lichen planus no autoantibody has yet been identified. Nor has it been determined if an endogenous or exogenous antigen is the pivotal clue in the development of lichen planus (2,6,7). Nevertheless, the involvement of CD4 and CD8 lymphocytes and dendritic cells, which cause damage to the basal cell layer of the epithelium in oral mucosa is recognized in both diseases (3,8). Many other complex immune mechanisms are also involved in these processes (9), and recently, the role of regulatory T cells (Tregs), forkhead box p3 transcription factor (Foxp3) and toll-like receptors (TLRs) has been discussed in the field of autoimmune diseases (10-20).

Tregs are suppressor T lymphocytes that control peripheral self-reactive T lymphocytes and play an important role in the homeostasis of the immune response. Foxp3, in turn, is a transcription factor required for the normal development and function of Tregs, and it is also responsible for the phenotype definition of these cells (13,17,21-23). IL-2 production by activated effector T cells expands and sustains Treg cells, which in turn causes a feedback to suppress the effector T cell (Teff) response, maintaining normal immune homeostasis. Disruption of this cross-talk can lead to dysregulation of the Treg/Teff balance and contribute to the development of autoimmune diseases. It has been demonstrated that intra-islet Treg cell dysfunction secondary to defective IL-2 production is a major cause of progressive breakdown of self-tolerance (22).

TLRs are members of a family of signaling receptors of the innate immune system that recognize conserved pathogen-associated molecular patterns and play important functions in host defense in either innate or adaptive immunity. There are several known endogenous and exogenous TLR ligands that can be found in lesional tissues or infections, stimulating specific TLRs in T cells or in antigen-presenting cells and inducing them to secrete proinflammatory cytokines (14,17). Accordingly, TLR ligands can induce the secretion of cytokines capable of interfering with the plasticity of Tregs through epigenetic mechanisms regulating Foxp3 expression, and inhibiting their suppressive function (17,23,24).

Recent studies have reported that TLRs are capable of recognizing endogenous molecules released due to cell damage and necrosis, and they have also been found in numerous autoimmune diseases (14). For instance, DNA-immune complexes from systemic lupus erythematosus patients induce cytokine production by plasmacytoid dendritic cells in a pathway dependent on TLR9 (25), and TLR9 stimulation activates transitional B cells to differentiate into antibody-secreting cells (26). Thus, the constant presence of TLR9 endogenous ligands could continuously stimulate antibody-secreting B cells (26) and inhibit Tregs by inducing plasmacytoid dendritic cells to produce cytokines that block Foxp3 expression (24,27). This would establish a vicious circle, and a consequent autoimmune state. TLR9 is considered the major receptor related to autoimmune disturbances such as lupus erythematosus (12,14,28-30).

Like TLR9, TLR5 is expressed in epidermis (31), but it is also found on the surface of isolated T cells, and its mRNA is expressed at high levels by Tregs (32). This receptor is activated by flagellin, a bacterial protein (6,31,33), and it does not react with endogenous ligands such as DNA or RNA immune complexes (29). Therefore, TLR5 seems to be associated with exogenous ligands of microbial origin and has an important

role in maintaining homeostasis and regulating host defense against bacterial infection (31,33). TLR5 and its ligand flagellin modulate the suppressive activity of naturally occurring $CD4^{+}CD25^{high}$ Tregs by interfering with Foxp3 expression (32,34,35).

According to the type of the stimulus and ligands, the course of inflammatory events can be characterized by higher or lower suppressor Tregs activity, which interferes with the immune response. Therefore, knowing Tregs plasticity in different inflammatory conditions and their associated mechanisms is crucial to the development of immune therapies, in either the induction of tolerance of transplanted tissues or prevention of autoimmune diseases (17). Since changes in Foxp3 expression induced by the activation of specific TLRs can play a major role in the development of autoimmune diseases, this study aimed at analyzing the immunohistochemical expression of Foxp3, TLR5 and TLR9 in oral lesions of lichen planus and lupus erythematosus.

Materials and Methods

This study was approved by the Research Ethics Committee of Pontifical Catholic University of Rio Grande do Sul. The sample comprised biopsy specimens of oral lesions, which were allocated to 3 groups: group 1 (lichen planus) was composed of 21 specimens of oral lichen planus, where the diagnosis was confirmed through hematoxylin and eosin (H&E) and direct immunofluorescence staining; group 2 (lupus erythematosus) comprised 21 biopsy specimens of lupus erythematosus (12 discoid lupus erythematosus and 9 systemic lupus erythematosus), where the diagnosis was also confirmed by H&E and direct immunofluorescence; and group 3 (control group) was composed of 21 specimens of inflammatory fibrous hyperplasia of the oral mucosa from patients without any other disease. All the biopsied patients were female adults whose ages ranged from 26 to 85 years old.

Inclusion criteria

Inflammatory fibrous hyperplasias provided the control group, where specimens with minimum inflammatory infiltrate and no secondary infection were selected. Clinical criteria for oral lichen planus were the presence of white striated lesions in the buccal mucosa bilaterally and negative serology for antinuclear antibody (ANA), as well as for anti-DNA, anti-SSA, anti-SSB, anticardiolipins (IgG and IgM) and VDRL. For discoid lupus erythematosus, oral lesions were to have an erythematous or atrophic central area surrounded by delicate and irradiating white striae, with asymmetric distribution in the oral cavity (36) in patients who did not match the oral lichen planus criteria. The diagnosis of systemic lupus erythematosus was established when oral lesions occurred along with 4 or more criteria from the American Association of Rheumatology, simultaneously or periodically, in any time interval (37,38) and was confirmed by a rheumatologist's assessment. Patients whose lesions were in contact with amalgam dental fillings were excluded from the study.

On the H&E examination, the inclusion criteria for lichen planus were: (a) orthokeratosis and/or parakeratosis, (b) sharp epithelial crests, (c) basal cell layer degeneration, (d) band subepithelial infiltrate of lymphocytes. For systemic lupus erythematosus diagnosis, the criteria were (a) hyperkeratosis, (b) epithelial atrophy or atrophy alternating with hyperplasia, (c) basal cell layer degeneration, and (d) subepithelial, deep and perivascular lymphocytic infiltrate (5,39,40). Discoid lupus erythematosus was diagnosed by (a) basal cell layer degeneration, (b) pseudoepitheliomatous hyperplasia and/or epithelial atrophy, and (c) focal or diffuse and/or perivascular dense infiltrate of lymphocytes, with other chronic inflammatory cells (36,41,42). On direct immunofluorescence examination, lichen planus should show fibrinogen and C3 positivity in the basal membrane zone, whereas lupus erythematosus

should show negativity for fibrinogen and positivity for IgM or IgG or C3 also in the basal membrane zone. The sample size was calculated using WinPepi 5.6 software, based on Tao *et al.* (43), with a power of 80% and assuming a significance level of 5%.

Immunohistochemical processing

Three-micrometer-thick sections were obtained, deparaffinized and subjected to immunohistochemical processing. Antigen retrieval was performed in a 96^{o}C water bath for 20 min, using Tris-EDTA, pH 9. Endogenous peroxidase was blocked with 3% hydrogen in methanol. The antibodies used were anti-Foxp3 (clone 236A/E7, dilution 1:300, Abcam, Cambridge, UK), anti-TLR5 (clone 19D759.2, dilution 1:300, Abcam), and anti-TLR9 (clone 26C593, dilution 1:4000, Abcam). The sections were incubated with the specific antibody for 12 to 14 h at 4ºC. The detection system used was Picture Max, HRP Polymer Conjugate Broad Spectrum (Invitrogen, Carlsbad, CA, USA). Color development was with the chromogen 3,30-diaminoazobenzidine and phosphate buffer solution containing 0.002% hydrogen peroxide, and slides were then stained with hematoxylin, dehydrated, cleared, and coverslipped. Tonsil, prostate and pancreas specimens provided the positive controls respectively for Foxp3, TLR5 and TLR9, whereas the negative controls were performed by processing the specimens in the absence of the primary antibodies.

Histological analysis

The images were captured with a Zeiss Axioskop 40 light microscope (Zeiss, Goettingen, Germany), connected to a CoolSnap Pro videocamera (Media Cybernetics, Bethesda, MD, USA) linked to a computer with Image Pro Capture Kit Platform (Media Cybernetics). The images were captured using a 20x objective, and stored in TIFF (Tagged Image File Format). In each of the immunohistochemical

slides, 6 fields were captured in a standardized manner. The digital images were analyzed with Image Pro Plus 4.5.1 software (Media Cybernetics).

Histological analysis was performed by a calibrated observer blinded to the study. The calibration consisted of an evaluation of a series of 30 histological images for each marker at two different times. The agreement between these two evaluations was tested by using the intraclass correlation coefficient, which showed a strong correlation ($r > 0.8$).

Images were subjected to quantitative and qualitative analyses. The quantitative analysis of immunohistochemical expression of Foxp3, TLR5 and TLR9 was carried out using the semiautomated segmentation technique in Image Pro Plus 4.5.1 software (Media Cybernetics). In this technique, an immunostained point is selected by the observer and the software automatically selects and calculates all the stained area.

Statistical analysis

Immunohistochemical expression of Foxp3, TLR5 and TLR9 was compared between the lichen planus, lupus erythematosus and control groups by the Kruskal-Wallis and its multiple comparisons test. Correlation between these variables was analyzed using the Spearman correlation coefficient. Data were analyzed in SPSS 17.0 (Statistical Package for the Social Sciences Inc., Chicago, IL, USA) considering a significance level of 5%.

Results

Quantitative analysis

Foxp3

Immunostaining for Foxp3 differed significantly between the three groups evaluated, where the highest levels were shown by lichen planus followed by lupus erythematosus, and the lowest levels occurred in the control group (Table 1, Kruskal-Wallis, multiple

comparisons test, α=0.05). These results were also observed when systemic lupus erythematosus and discoid lupus erythematosus were considered separately (Fig. 1, Kruskal-Wallis, multiple comparisons test, α=0.05).

TLR5

Lupus erythematosus and the control group showed greater expression of TLR5 than did lichen planus, but the two former groups did not significantly differ between each other (Table 1, Kruskal-Wallis, multiple comparisons test, α=0.05). When we considered the two types of lupus separately, there was no significant difference in TLR5 between lichen planus and systemic lupus erythematosus, but discoid lupus erythematosus as well as the control group showed greater values compared to lichen planus (Fig. 1, Kruskal-Wallis, multiple comparisons test, α=0.05).

TLR9

The levels of TLR9 immunostaining did not significantly differ between the groups analyzed, even when systemic and discoid lupus erythematosus were considered separately (Table 1 and Fig.1, Kruskal-Wallis, α=0.05).

Table 1 – Immunostaining (μm^2) for forkhead box p3 (Foxp3), toll-like receptor 5 (TLR5) and toll-like receptor 9 (TLR9) in oral lesions of lichen planus, lupus erythematosus and control group

Marker	Lichen planus			Lupus erythematosus			Control		
	MD	P25	P75	MD	P25	P75	MD	P25	P75
Foxp3	1761.3^{A}	549.4	2140.2	296.8^{B}	119.2	1065.2	43.0^{C}	28.2	86.9
TLR 5	2431.8^{B}	1334.5	7129.1	7171.1^{A}	4270.4	10895.5	11898.0^{A}	4489.2	14527.1
TLR 9	8264.5^{A}	3539.5	16474.9	9627.6^{A}	4076.4	16749.8	8585.7^{A}	2473.7	12225.6

MD=median; P25=25th percentile; P75=75th percentile
Medians followed by different letters differed significantly
Kruskal-Wallis complemented by its multiple comparisons test, at a significance level of 5%

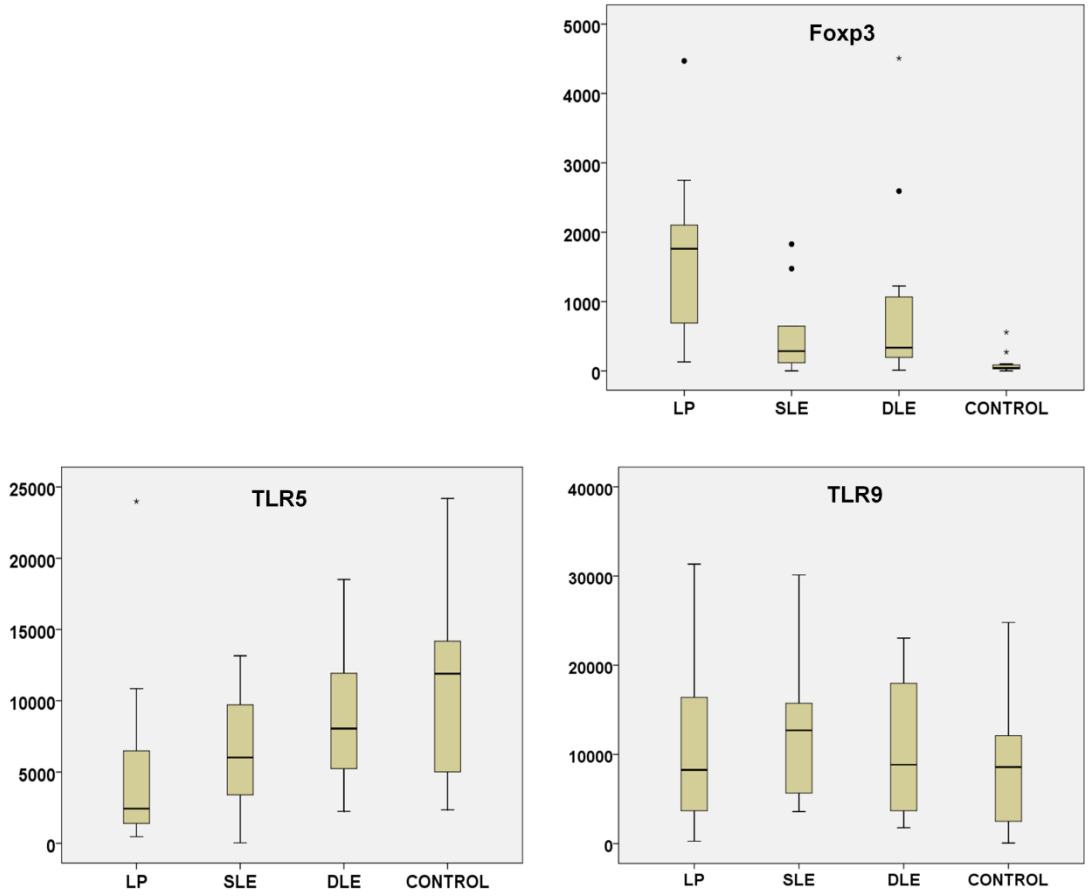

Figure 1 – Forkhead box p3 transcription factor (Foxp3), toll-like receptor 5 (TLR5) and toll-like receptor 9 (TLR9) immunostaining (μm^2) in oral lesions of lichen planus (LP), systemic lupus erythematosus (SLE), discoid lupus erythematosus (DLE) and control group

Correlation between Foxp3, TLR5 and TLR9

When Foxp3, TLR5 and TLR9 immunostaining levels were analyzed by the Spearman correlation coefficient, a weak negative correlation was observed between Foxp3 and TLR5 (r= -0.279), and there was no other correlation. When this analysis was performed within the groups, no correlation was observed between the variables (Table 2, Spearman correlation coefficient, significance level of 5%).

Table 2 - Correlation between forkhead box p3 (Foxp3), toll-like receptor 5 (TLR5) and toll-like receptor 9 (TLR9) analyzed by the Spearman correlation coefficient

	General	Groups		
		Lichen planus	Lupus erythematosus	Control
FOXP3 and TLR5	**-0.279***	0.100	0.197	-0.224
FOXP3 and TLR9	0.152	0.310	-0.088	0.077
TLR5 and TLR9	0.217	0.236	0.356	0.003

*Significant correlation at significance level of 5%

Qualitative analysis

The three groups evaluated (lichen planus, lupus erythematosus and control) showed a strong and focal immunostaining pattern for Foxp3 predominantly in connective tissue. TLR5 immunostaining occurred in either epithelial or connective tissue in all groups. Nevertheless, in connective tissue it showed a predominantly weak and focal pattern, whereas in epithelium it was strong and diffuse, with higher intensity in the zone of the epithelial basal layer. In the lichen planus and lupus erythematosus groups, TLR 9 immunostaining pattern ranged from intermediate to strong, with diffuse distribution in epithelium and focal in connective tissue. In the control group, TLR9 exhibited strong staining with diffuse distribution in the epithelium and focal distribution in the connective tissue. Figure 2 depicts the immunostaining for Foxp3, TLR5 and TLR9 in the groups analyzed.

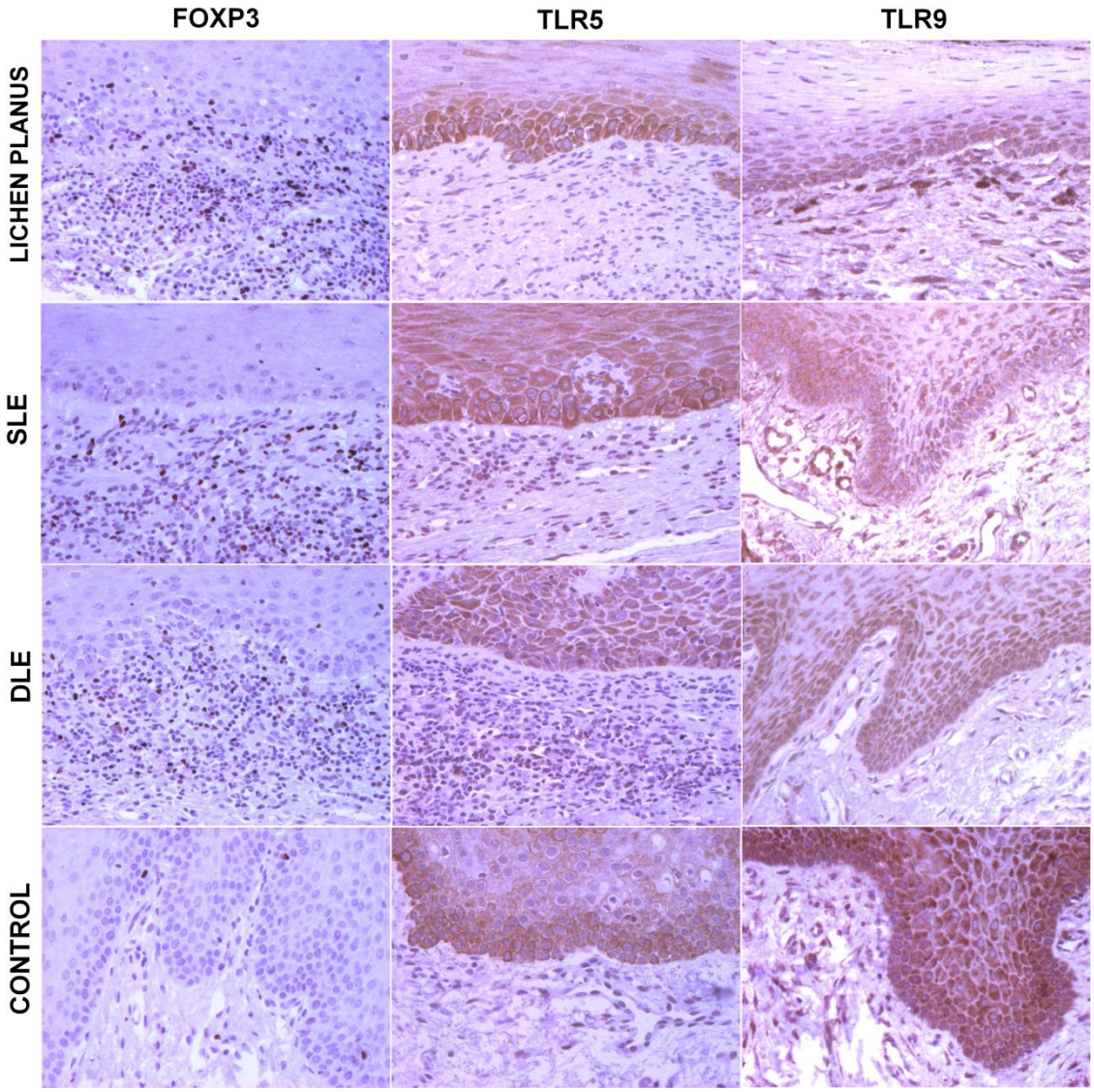

Figure 2 – Forkhead box p3 transcription factor (Foxp3), toll-like receptor 5 (TLR5) and toll-like receptor 9 (TLR9) immunostaining in oral lesions of lichen planus, systemic lupus erythematosus (SLE), discoid lupus erythematosus (DLE) and control group (x 200)

Discussion

In our results, immunostaining for Foxp3 and TLR5 differed significantly between the groups evaluated, whereas TLR9 did not show any significant difference between them. The highest Foxp3 expression occurred in the lichen planus group, followed by lupus erythematosus, and the lowest values occurred in the control group. The results for lichen planus and lupus erythematosus, showing expression of Foxp3 significantly

greater than the controls are in agreement with reports that Foxp3 is increased in inflammatory processes if compared to controls (11). Also, the lower expression of Foxp3 in lupus erythematosus if compared to lichen is corroborated by the autoimmune profile of lupus, where Tregs dysfunction seems to occur (44). It is important to recall here that the difference between lupus and the control group was smaller than that observed between lichen and lupus, which can indicate some failure in immune regulatory mechanisms mediated by Tregs occurring in lupus erythematosus (44,45). In this way, we could also infer that lichen planus would not be an autoimmune disturbance, or if considering it as autoimmune, its pathogenesis would be associated with factors other than Tregs failure (8,46,47). Another point in this respect is the different phases we can find in systemic lupus erythematosus, alternating with periods of higher and lower disease activity (48), which could also have interfered with our results. Because of the small size of our sample, we did not classify the cases of systemic lupus erythematosus according to SLEDAI (systemic lupus erythematosus disease activity index). Anyway, when we analyzed discoid and systemic lupus separately, no change was observed in these results. Maybe further studies with larger samples could clarify this issue. Also important is the high predominance of T CD4 lymphocytes, which express Foxp3, in lichen planus oral lesions, while in lupus lesions inflammatory infiltrate also comprises other cells besides lymphocytes, such as macrophages, mastocytes, plasma cells, and neutrophils in considerable amounts (5).

de Boer *et al*. (11) did not find any significant difference in Foxp3 expression between skin lesions of lichen planus and normal skin, which disagrees with our results. Nevertheless, these authors reported finding considerable variation in Foxp3 between the specimens within each group, including the control group, which was also observed in our study. On the other hand, one could point out that our control group was

composed of fibrous hyperplasias, which are also inflammatory conditions, and for this reason would show increased levels of Foxp3. However, besides using the inclusion criterion of minimal or no inflammatory infiltrate for control group, our results agree with those reported by Pereira *et al*. (49), who also demonstrated greater expression of Foxp3 in oral lesions of lichen planus than in fibrous hyperplasias.

Although Foxp3 is considered the best marker for Tregs (50,51), it is difficult to precisely evaluate Tregs number and function, as non-Treg CD4 or CD8 cells can sometimes express Foxp3 as well. The increased frequency of these cells with low levels of Foxp3 is associated with a defective suppressor function *in vitro* and with the development of specific anti-DNA antibodies in patients with active lupus (20). The possible presence of these non-Treg $Foxp3^+$ cells could have interfered with our results. Considering the existence of these distinct populations of $Foxp3^+$ cells, it would be interesting to find out which types of them actually occur in our sample and at what rates. Still, it is possible that in the lupus erythematosus group, the levels of Foxp3 are not sufficient to achieve the balance of immune response and therefore leading to the establishment of autoimmunity (44).

The expression of TLRs in epithelial cells confers protection against pathogens (31). In our results, TLR5 expression did not significantly differ between lupus erythematosus and the control group, but these two groups showed significantly greater values than did lichen planus. TLR5 is expressed in epithelial and dendritic cells, and it mediates the dynamic interaction between microbiota and the host immune system, showing positive correlation with microbiota density (33). Similar expression of TLR5 in lupus erythematosus and control group suggests that lupus etiopathogenesis is not related to flagellated bacterial agents. Anyway, the participation of some fungal or viral pathogen or even other bacterial types has not been ruled out, as TLR5 is not activated

by them. On the other hand, its lower expression in lichen could indicate some failure in this group to properly react against flagellated microbiota, even the commensals that invade the epithelium, with consequent loss of local homeostasis (33).

A former study reported greater expression of TLR4 and TLR9 in oral lichen planus lesions suggesting, respectively, a role of Gram-negative bacteria and human papillomavirus (HPV) infection in this disease (19). However, it is worth recalling that the sample of that study comprised either striated/hyperkeratotic or erosive-ulcerated lesions (19), where the latter are much more likely to harbor microorganisms (49). Our study, on the other hand, used only striated lesions, which apparently would diminish the possibility of a secondary infection of the specimens.

TLR9 immunostaining did not significantly differ between the groups analyzed, even when systemic and discoid lupus erythematosus were considered separately. Considering that TLR9 is activated mainly by viral agents (10,29), but also by unmethylated DNA sequences (CpG dinucleotides) found in bacteria (31), we could infer that this kind of pathogen would not be related to lichen planus or lupus erythematosus. Our results disagree with those of Li *et al*. (10) and Siponen *et al*. (19) who found greater immunohistochemical expression of TLR9 in lichen than in controls (normal mucosa and normal skin). Nevertheless, recent studies have reported that TLRs are also capable of recognizing endogenous molecules released due to cell damage and necrosis, and they have been found in many autoimmune diseases (14). TLR9 is considered the major receptor related to autoimmune disturbances such as lupus erythematosus (12,14,28-30). Accordingly, we expected higher levels of TLR9 in the lupus erythematosus group, which did not happen. A possibility to consider is that transforming growth factor-alpha (TGF-alpha), which regulates the expression and function of TLR5 and TLR9 in human keratinocytes (31) would also be increased in our

control group (fibrous hyperplasia). From this point of view, we would have increased levels of TLR5 and TLR9 also in controls. According to Miller *et al*. (31), the effect of TGF-alpha on the expression of TLR9 is more pronounced, which may be due to the growth and differentiation properties of TGF-alpha, which promotes the maturation of keratinocytes into the upper and most differentiated layers of the epidermis, where TLR9 is almost exclusively expressed. If we consider that all the groups of our study can show epithelial hyperplasia, especially the control group, and that TLR9 is more affected by TGF-alpha and also has a wider distribution in this tissue if compared to TLR5 (31), our lack of significant difference in TLR9 between the groups seems reasonable.

A weak negative correlation was observed between Foxp3 and TLR5, even though it was not found in the analysis within the groups. Accordingly, a former study reported that $TLR5^{-/-}$ rats showed higher levels of Foxp3 than did wild type rats (33). It seems that TLR5 activation promotes downregulation of Foxp3 expression as a pathway to defend the host against pathogens (33). This explains our result of negative correlation between these two markers.

When comparing our results for Foxp3, TLR5 and TLR9 with previously reported studies (10,11,19), some disagreements are observed. Regarding this, it is important to consider the rigorous criteria adopted in our study to define the diagnosis of lichen planus and lupus erythematosus, using strict clinical and histopathological criteria, including a direct immunofluorescence test. It is known how difficult the correct diagnosis of these lesions is, where misdiagnosis can result if rigorous criteria are not respected. Regarding the possible association of both lichen planus and lupus erythematosus with pathogens, including fungal and viral ones, not just other TLRs such as TLR2, TLR3 and TLR4 (19,52-56) should be tested in further studies, but also

the presence of microorganisms through specific techniques such as flow cytometry, PCR and RT-PCR (57-59). Such studies should especially focus on molecular mimicry/immunological cross-reactivity mechanisms by comparing cytometric features and protein sequences of known autoimmune immunogens with those of microorganisms commonly involved in human infections (60,61). Still, the profile and interaction of inflammatory cytokines such as IL2, IFN type I, IFN-gamma, IL6, and IL17 with Foxp3 and TLRs need to be investigated in these diseases.

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Discussão Geral

4 DISCUSSÃO GERAL

As doenças autoimunes e as desordens imunológicas que acometem o organismo humano são alvo de incessante investigação, haja vista a morbidade e a mortalidade que implicam. Muitos dos mecanismos etiopatogênicos envolvidos nessas enfermidades ainda não são completamente conhecidos. Entretanto, a compreensão efetiva dos fatores que contribuem para o desencadeamento das alterações imunológicas é imprescindível para a prevenção e para o melhor manejo dessas doenças.

O papel da imunidade inata, mais especificamente, dos receptores da família toll (*toll-like receptors*, TLRs) na ativação imunológica e em processos autoimunes, por meio do reconhecimento de antígenos próprios (Drexler; Foxwell, 2010; Lal *et al.*, 2011), tem sido alvo de destaque e investigação nos principais centros de Imunologia. TLRs têm como função primordial defender o organismo por meio do reconhecimento de patógenos e ativação da resposta imunológica adaptativa (Hari *et al*., 2010; Drexler; Foxwell, 2010; Valins *et al*., 2010; Ermertcan *et al*., 2011). Ainda nesse campo, tem sido investigada a relação desses receptores com a modulação da atividade supressora das células T regulatórias (Tregs) por meio de alterações da expressão de um fator de transcrição muito importante para o exercício de sua função, o forkhead box p3 (Foxp3). Falhas na expressão de Foxp3 por parte das Tregs estariam associadas a defeitos funcionais dessas células, com consequente desequilíbrio da resposta imunológica. Tal desequilíbrio, representado por uma disfunção do componente regulatório (supressão), permitiria o estabelecimento da autoimunidade (Lal; Bromberg, 2009; Lal *et al*., 2009; Lal *et al*., 2011; Long; Buckner, 2011).

Considerando-se que o líquen plano e o lúpus eritematoso são enfermidades autoimunes ou imunologicamente mediadas (Scully *et al*., 1998; Lodi *et al*., 2005; Perl, 2010) que exibem, frequentemente, manifestações orais (Farthing; Speight, 2006), o

presente estudo investigou, nessas lesões, o padrão de expressão imunoistoquímica dos TLRs 5 e 9 e do Foxp3. O TLR9, um receptor *toll-like* especializado no reconhecimento de partículas de DNA de origem bacteriana e viral, tem sido associado ao desenvolvimento de uma resposta autoimune por meio do reconhecimento de partículas de DNA do próprio organismo, além de ser capaz, mediante constante estimulação, de promover contínua baixa expressão de Foxp3 em células Treg com comprometimento de sua função na manutenção da homeostase do sistema imunológico (Pasare; Medzhitov, 2003; Means; Luster, 2005; Barrat *et al*., 2005; Marshak-Rothstein, 2006; Blanco *et al*., 2008; Drexler; Foxwell, 2010; Richez *et al*., 2011; Guerrier *et al*., 2012). O TLR5, por sua vez, tem como ligante principal a flagelina, que é expressa especificamente por bactérias portadoras de flagelo (Miller *et al*., 2005; Marshak-Rothstein, 2006; Demirci *et al*., 2007; Feng *et al*., 2012).

Os resultados da presente pesquisa evidenciaram diferença significativa do padrão de expressão imunoistoquímica do Foxp3 e do TLR5 entre os grupos avaliados, enquanto essa diferença não foi observada para o marcador TLR9. Além disso, foi observada correlação negativa entre Foxp3 e TLR5. O grupo líquen plano apresentou os maiores níveis de expressão do Foxp3, seguido do grupo lúpus eritematoso, e os menores níveis ocorreram no grupo-controle. A maior expressão de Foxp3 nos grupos líquen e lúpus em comparação ao grupo-controle estão de acordo com o estudo de De Boer *et al*. (2007) que demonstrou maiores níveis desse marcador em amostras de processos inflamatórios do que em controles. Por outro lado, a menor expressão de Foxp3 no grupo lúpus eritematoso, quando comparado ao grupo líquen plano, pode estar associada ao perfil autoimune da doença, que envolveria uma disfunção das células Treg (Mudd *et al*., 2006; Long; Buckner, 2011). Dessa forma, pode-se sugerir que o líquen plano não seja uma doença de natureza autoimune ou, se assim for, sua

patogênese estaria associada a outros fatores que não falhas na população de Tregs (Korn *et al*., 2007; Bettelli *et al*., 2008; Roopashree *et al*., 2010).

Além da análise da expressão de células Treg Foxp3^{+} nas lesões de líquen e de lúpus, o presente estudo avaliou a expressão de dois TLRs, o 5 e o 9. A princípio, informações sobre o envolvimento do TLR5 em tais enfermidades são escassas na literatura (Demirci *et al*., 2007). Por outro lado, tem-se especulado que o TLR9 participaria do desencadeamento de reações autoimunes que culminariam no desenvolvimento de lúpus eritematoso sistêmico (Marshak-Rothstein, 2006; Richez *et al*., 2011; Ghaly *et al*., 2012; Guerrier *et al*., 2012) e líquen plano (Li *et al*., 2007). Assim, investigou-se se o padrão de expressão desses TLRs em lesões orais de líquen plano teria alguma semelhança com o das lesões de lúpus, visto que isso forneceria alguma ideia quanto à etiopatogênese de ambas as doenças, bem como outras informações acerca dos TLRs 5 e 9. Nesse sentido, talvez fosse possível inferir que o líquen plano seria, de fato, uma enfermidade autoimune, o que ainda não está bem definido na literatura. A análise imunoistoquímica dos TLRs em lesões de hiperplasia fibroepitelial, por sua vez, possibilitaria o estudo do padrão de expressão dos mesmos em um processo sabidamente não autoimune, estabelecendo um meio de comparação com as lesões de líquen e lúpus. Sendo o TLR9 considerado o principal receptor *toll-like* associado a distúrbios autoimunes como o lúpus (Barrat *et al*., 2005; Marshak-Rothstein, 2006; Blanco *et al*., 2008; Drexler; Foxwell, 2010; Richez *et al*., 2011), esperava-se que seus níveis de expressão tivessem sido maiores no grupo lúpus eritematoso. No entanto, não foi observada diferença significativa em sua expressão entre os três grupos avaliados. Considerando-se que todos os grupos do presente estudo, em especial o grupo-controle, podem apresentar hiperplasia epitelial, que se trata de um processo proliferativo, tal resultado parece justificável. Processos proliferativos do

epitélio exibem maiores concentrações do fator de transformação do crescimento alfa (TGF-alfa), o qual regula a expressão e a função dos TLRs 5 e 9 elevando seus níveis nos queratinócitos humanos (Miller *et al.,* 2005). Isso justificaria a ausência de diferença significativa da expressão do TLR9 entre os grupos.

Também não foi observada diferença significativa da expressão do TLR5 entre o grupo lúpus eritematoso e o grupo-controle. Entretanto, tais grupos exibiram maior expressão desse marcador quando comparados ao grupo líquen plano. O TLR5 tem correlação positiva com a densidade da microbiota no tecido (Feng *et al*., 2012) e, portanto, sua menor expressão no grupo líquen plano poderia indicar uma deficiência da capacidade de reagir adequadamente à microbiota específica, com consequente desequilíbrio da homeostase local (Feng *et al*., 2012).

Ainda, foi observada correlação negativa, embora fraca, entre a expressão do Foxp3 e do TLR5. No estudo de Feng *et al*. (2012), ratos TLR5-/- apresentaram maiores níveis de Foxp3 do que os *wild type*, o que sugere que a ativação desse TLR promova a expressão de menores níveis de Foxp3 como um mecanismo de defesa do organismo contra patógenos. Isso explicaria a correlação negativa observada entre TLR5 e Foxp3 no presente estudo.

Tanto no líquen plano quanto no lúpus eritematoso, uma cascata de eventos inflamatórios ocorre nos sítios onde se desenvolvem as lesões. Todos esses eventos, marcados pela produção de uma gama de citocinas, quimiocinas e ativação de inúmeras vias de sinalização inter e intracelular, são, de certa forma, responsáveis pelo padrão de expressão dos componentes-alvo do presente estudo, bem como pelo próprio aspecto clínico das lesões. O resultado do processo "tolerância *versus* autoimunidade" pode culminar no aparecimento de tais lesões e depende do equilíbrio entre sinais estimulatórios, como a presença de ligantes de TLRs e a concentração de um dado

antígeno, seja ele exógeno ou endógeno, bem como de sinais inibitórios mediados por células Treg (Suri-Payer; Fritzsching, 2006). A quebra desse equilíbrio é observada no lúpus eritematoso sistêmico, em que autoanticorpos ligam-se ao DNA ou às proteínas conjugadas ao DNA/RNA provenientes de células apoptóticas (Munoz *et al*., 2008; Rönnblom *et al*., 2011), gerando imunocomplexos que podem estimular células por meio de TLRs, o que resulta na produção de citocinas inflamatórias como o IFN-alfa (Salloum; Niewold, 2011). A contínua ativação de TLRs envolvidos na indução de citocinas inflamatórias Th1/Th2, capazes de inibir a expressão de Foxp3, pode ser responsável pelo bloqueio da função de células Treg (Chang *et al*., 2009; Jin *et al*., 2010; Hackl *et al*., 2011; Lal *et al*., 2011; Long; Buckner, 2011). Assim, a presença de ligantes endógenos que, supostamente, poderiam estar sendo gerados em doenças como lúpus e líquen, teria papel preponderante no estabelecimento e na manutenção da autoimunidade (Drexler; Foxwell, 2010).

Nesse sentido, da mesma forma que ligantes endógenos poderiam estar persistentemente ativando todo esse círculo vicioso que resulta na cronicidade das doenças em questão, poder-se-ia especular que determinados agentes infecciosos constantemente presentes nos tecidos, como partículas virais, poderiam exercer esse mesmo papel. Além disso, é possível que tais agentes apresentem semelhanças moleculares com estruturas do próprio organismo, o que induziria a uma reação cruzada por permitir o reconhecimento de antígenos próprios pelo sistema imunológico e, com isso, o desencadeamento da autoimunidade (Westall, 2006; Sebastiani; Galeazzi, 2009).

A complexidade dos distúrbios autoimunes e imunologicamente mediados, em que tanto fatores genéticos/individuais quanto ambientais contribuem para a susceptibilidade à doença, exige que novas pesquisas continuem sendo conduzidas nessa área. Parece pertinente a investigação da relação de tais enfermidades com células

Treg, citocinas e quimiocinas pró-inflamatórias, bem como com alguns microrganismos possivelmente envolvidos no desencadeamento de reações autoimunes em função das semelhanças moleculares que guardam em relação a componentes do organismo humano. Até hoje, a maioria das doenças autoimunes e imunologicamente mediadas apresentam-se como enfermidades controláveis, porém incuráveis. Os pacientes resignam-se a uma vida inteira de tratamentos medicamentosos e controles clínicos. Tratamentos esses capazes apenas de inibir ou atenuar as manifestações da doença e, em geral, acompanhados de importantes efeitos colaterais. Somente a elucidação desses intrincados caminhos do sistema imunológico propiciará novas perspectivas de tratamento, cura e prevenção dessas enfermidades. Daí a necessidade constante de novas pesquisas nesse campo.

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Anexos

ANEXO A

Submission Confirmation for Interrelationship of dendritic cells, system type 1 interferon, regulatory T cells and toll-like receptors in immune response and autoimmune diseases - a literature review

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Authors: Victoria M Trucci, DDS; Fernanda G Salum, Ph.D.; Maria A Figueiredo, Ph.D.; Karen Cherubini, Ph.D.
Article Type: Review Article

Dear Karen,

Your submission entitled "Interrelationship of dendritic cells, system type 1 interferon, regulatory T cells and toll-like receptors in immune response and autoimmune diseases - a literature review" has been received by Archives of Oral Biology.

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ANEXO B

Normas para sumbissão de manuscritos ao periódico *Archives of Oral Biology*

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ANEXO C

Journal of Oral Pathology & Medicine

Submission Confirmation

Thank you for submitting your manuscript to *Journal of Oral Pathology and Medicine*.

Manuscript ID:	JOPM-01-13-OA-2459
Title:	Immunohistochemical expression of forkhead box p3 transcription factor and toll-like receptors 5 and 9 in oral lesions of lichen planus and lupus erythematosus
Authors:	Trucci, Victoria Fava, Márcia Salum, Fernanda Figueiredo, Maria Da Silva, Vinicius Cherubini, Karen
Date Submitted:	24-Jan-2013

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ANEXO D

Normas para sumbissão de manuscritos ao periódico *Journal of Oral Pathology and Medicine*

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ANEXO E

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 16 de Novembro de 2011

O Projeto de: Dissertação

Protocolado sob nº:	0069/11
Intitulado:	Expressão Imunoistoquímica dos Toll-like Receptors 5 e 9 e do Fator de Transcrição FOXP3 em Lesões Orais de Líquen Plano e Lúpus Eritematoso.
Pesquisador Responsável:	Profa. Dra. Karen Cherubini
Pesquisadores Associados:	Victoria Martina Trucci
Nível:	Dissertação / Mestrado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *16 de Novembro de 2011.*

Este projeto deverá ser imediatamente encaminhado ao CEP/PUCRS.

Profa. Dra. Ana Maria Spohr
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

Av. Ipiranga, 6681, Prédio 06 sala 210
Porto Alegre /RS – Brasil – Cx. Postal:1429
90619-900

Fone/Fax: (51) 3320-3538
e-mail: odontologia-pg@pucrs.br

ANEXO F

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

OF. CEP-1972/11

Porto Alegre, 26 de dezembro de 2011.

Senhora Pesquisadora,

O Comitê de Ética em Pesquisa da PUCRS apreciou e aprovou seu protocolo de pesquisa registro CEP 11/05676 intitulado **"Expressão imunoistoquímica dos toll-like receptors 5 e 9 e do fator de transcrição foxp3 em lesões orais de líquen plano e lúpus eritematoso"**.

Salientamos que seu estudo pode ser iniciado a partir desta data.

Os relatórios parciais e final deverão ser encaminhados a este CEP.

Atenciosamente,

Prof. Dr. Rodolfo Herberto Schneider
Coordenador do CEP-PUCRS

Ilma. Sra.
Profa. Karen Cherubini
FO
Nesta Universidade

PUCRS
Campus Central
Av. Ipiranga, 6690 – 3ºandar – CEP: 90610-000
Sala 314 – Fone Fax: (51) 3320-3345
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