REPORT

A toll for T cell costimulation

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Toll was originally found in *Drosophila* as a pattern recognition receptor associated with defence against fungal and bacterial infections.[1][2] Subsequently, toll was found in mammals and named toll-like receptor (TLR). At least 10 distinct TLRs have now been identified in humans.[3–9] TLRs are activated by pathogen associated molecular patterns (PAMPs) with target selectivity. PAMPs are integral structural components of pathogens that are thus thought to be essential for survival of infectious organisms and considered to be conserved among a range of pathogens including viruses, bacteria, and fungi.

TLRs act as primary sensors of microbial products and activate signalling pathways that lead to the induction of immune and inflammatory genes.[10] They belong to a broader family of proteins, which includes receptors for the pro-inflammatory cytokines interleukin (IL)-1, IL-18 and the orphan receptor T1/ST2.[11] All members of this superfamily signal inflammation in a very similar manner. This is due to the presence of a conserved protein sequence in the cytosolic domain called the Toll/IL-1 receptor domain, which activates common signalling pathways, most notably those leading to the activation of the transcription factor NF-κB and stress activated protein kinases.[11]

Most investigations of TLRs have focused on cells of the innate immune system. This is because TLRs are closely associated with the innate response. However, although innate immunity may constitute the primary function of TLRs, there is no a priori reason why TLRs may not have a direct role in adaptive immunity. We investigated the expression and functions of TLRs on T cells and found that TLR2 is expressed on the surface of activated and memory T cells. Furthermore, it functions as a costimulator receptor molecule for T cell activation and helps to maintain T cell memory. These data suggest a novel role for TLR2 and may explain how memory T cells are sustained in an immuno-competent host. The finding should have important implications for our understanding of the host response to infections.

EXPRESSION OF TLR2 AND TLR4 ON ACTIVATED HUMAN CD4+ T CELLS

To demonstrate the expression of TLRs on CD4+ T cells, we collected cord blood (containing mostly naive T cells) and purified CD4+ T cells by negative selection followed by positive selection on a cell sorter (>99.9% pure). The cells were cultured with medium alone, or immobilised anti-CD3 antibody, and the expression of TLR2 and TLR4 was determined by quantitative polymerase chain reaction (PCR) and flow cytometry. Naive CD4+ T cells expressed significant levels of mRNA and intracellular proteins of TLR2, TLR4, and MD2 but not CD14. This was not affected by anti-CD3+IFNα activation. In contrast, only activated T cells expressed appreciable levels of cell surface TLR2 or TLR4. Kinetic studies showed that cell surface TLR expression peaked between 12 and 72 hours following activation and was still at a substantial level by 96 hours. The cell surface expression of TLR2 and TLR4 on CD4+ T cells was also

visualised by immunofluorescence microscopy which clearly showed TLRs expressed in a polarised manner on CD3+ and CD4+ T cells. Similar results were obtained with cord blood CD8+ T cells. Since the T cell preparation was >99.9% pure and most (but not all) of the cells stained positive for TLR2 or TLR4, it is unlikely that the staining was due to antigen presenting cells (APCs), such as macrophages and dendritic cells. The polarised staining pattern is intriguing. It is tempting to speculate that TLRs tend to aggregate forming a lipid raft for effective function in a manner similar to T cell receptors (TCRs). We added interferon α (IFNα) to our culture medium as it enhanced the expression of the TLRs. The mechanism for this synergistic effect of TCR engagement and IFNα stimulation for TLR expression is currently under investigation. We elected to use plate-bound anti-CD3 antibody to activate the T cells in our culture system instead of the more conventional soluble anti-CD3 plus irradiated APCs to avoid the potential contribution of the APCs which carry TLRs and thus potentially indirectly affect the activation of T cells via cytokines.

BPL BUT NOT LPS ACTS AS A COSTIMULATORY MOLECULE FOR T CELL ACTIVATION

TLRs have been associated principally with the T helper (Th) 1 cell response.[12] To determine the functional significance of TLR expression on T cell differentiation, purified naive CD4+ T cells were cultured with anti-CD3 antibody and IFNα in the presence or absence of BLP (bacterial lipoprotein; Pam$_3$Cys-SK$_4$) or LPS (lipopolysaccharide), ligands for TLR2 and TLR4, respectively.[8][9] Proliferation of activated T cells was not affected by LPS, but it was modestly enhanced by BLP. However, BLP markedly enhanced the production of IFNγ, IL-2, and tumour necrosis factor α (TNFα) by activated CD4+ T cells. In contrast, LPS had little or no effect on cytokine production by the T cells. We therefore focused on the function of TLR2.

BLP ACTS DIRECTLY ON T CELLS AND NOT VIA APC

To further exclude the possibility that the observed effect of BLP on T cell activity may be due to an indirect effect of contaminating APCs, we performed similar experiments with T cells from TLR2 knockout (ko) mice. Purified (>95%) CD4+ T cells from TLR2$^{-/-}$ or wild-type mice were cultured in vitro with plate-bound anti-CD3 antibody and CD3+ T cells depleted wild-type APCs for three days. CD4+ T cells were then harvested, purified, and cultured with immobilised anti-CD3 for a further three days in the presence or absence of BLP. In such situations, T cells from both TLR2$^{-/-}$ and wild-type mice should contain similar levels of contaminating wild-type APCs. While T cells from wild-type mice showed enhanced proliferation and IFNγ production in response to BLP, T cells from the TLR2$^{-/-}$ mice failed to respond to BLP.

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Abbreviations: APC, antigen presenting cell; BLP, bacterial lipoprotein; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PAMP, pathogen associated molecular pattern; Th, T helper; TCR, T cell receptor; TLR, toll-like receptor

We then cultured purified CD4+ T cells from TLR2$^{-/-}$ or wild-type mice with immobilised anti-CD3 and deliberately added wild-type APCs in the presence or absence of BLP. While T cells from wild-type mice proliferated and showed increased IFNγ production in the presence of BLP, T cells from TLR2$^{-/-}$ failed to do so even in the presence of up to 10% deliberately added CD3 depleted APCs from wild-type mice. It should be noted that, unlike human T cells, murine T cells proliferated significantly in response to BLP. Furthermore, IFNα did not influence the activation of murine T cells by anti-CD3 antibody.

TLR2 IS CONSTITUTIVELY EXPRESSED ON MEMORY T CELLS

We then investigated the relative expression and function of TLR2 on memory and naive CD4+ T cells. CD4+ T cells were purified from peripheral blood of healthy donors and separated into CD45RA+ (naive) and CD45RO+ (memory) subsets. We analysed expression of cell surface TLR2 by flow cytometry. As expected, naive CD45RA+ cells did not express appreciable amounts of TLR2 in the absence of activation. In contrast, CD45RO+ memory T cells expressed significant levels of TLR2 ex vivo. The high level of TLR2 expression on CD45RO+ cells could not be further enhanced by stimulation with anti-CD3, or anti-CD3+BLP. In contrast, TLR2 expression by the naive CD45RA+ cells was significantly increased by stimulation with anti-CD3 and this was further enhanced by the presence of BLP. The expression of TLR2 paralleled the activation status of the cells as evident by their coexpression of human leucocyte antigen-DR (HLA-DR) antigen, an activation marker.

BLP ENHANCES IFNγ PRODUCTION AND PROLIFERATION OF MEMORY T CELLS

We then investigated the functional role of TLR2 expression in activated naive CD4+CD45RA+ T cells and memory CD4+CD45RO+ T cells in response to BLP in vitro. The cells were cultured with plate-bound anti-CD3 antibody ± BLP and cellular proliferation and cytokine production determined. Naive and memory CD4+ T cells did not show any detectable response when cultured with BLP alone. Both cell types underwent increased proliferation when cultured with anti-CD3 antibody which was not affected by BLP. Naive CD4+ T cells cultured with anti-CD3 produced low levels of IFNγ which was modestly enhanced by BLP. In contrast, memory CD4+CD45RO+ cells activated with anti-CD3 produced significant amounts of IFNγ which was markedly enhanced by BLP. IL-4 production was not detected. We further compared the role of BLP on naive and memory CD4+ T cells under a bystander activation condition with IL-2 and IL-15, two cytokines closely associated with memory cell expansion.[13–17] Naive CD4+CD45RA+ cells did not proliferate appreciably or show substantial levels of cytokines when cultured with IL-2 or IL-15 and this was not affected by BLP. In contrast, CD4+CD45RO+ memory T cells both proliferated significantly and showed significant increase in IFNγ levels when cultured with IL-2 or IL-15, and these functions were markedly enhanced by the presence of BLP. Neither IL-4 nor IL-10 were detected in the cultures.

DISCUSSION

TLR messages have been reported to be present in T cells.[18–24] We have now directly demonstrated the expression and function of TLRs on the surface of antigen specific T cells. The following lines of evidence support the functional expression of TLR2 on T cells: (a) the T cell population tested had no detectable CD14 message; (b) most of a 99.9% pure population of CD4+CD3+ cells stained positive for TLR2; (c) BLP alone did not induce detectable levels of cytokines or

proliferation; (d) in contrast with wild-type T cells, anti-CD3 activated T cells from TLR2$^{-/-}$ mice did not respond to BLP even in the presence of up to 10% wild-type APC.

Several features reported here are noteworthy.

- Surface expression (and hence function) of TLR2 was evident only after TCR activation and this was enhanced by the presence of the TLR2 ligand, BLP. Although the mechanism that induces TLR migration from the cytoplasm to the cell surface is currently unknown, the effect of BLP in this process may represent an important self-amplification loop for TLR functions.

- Although activated CD4+ T cells expressed similar levels of TLR4 and TLR2, the cells did not respond to LPS, a recognised ligand for TLR4.[25] This finding is consistent with a large number of reports in the literature.[26–28] LPS has recently been reported to activate CD4+CD25+ T cells in mice[24] but this remains to be confirmed. In the CD4+CD25+ T cell system, the potential involvement of cell types other than T cells could not be excluded. Using highly purified T cells, we were not able to activate murine CD4+CD25+ T cells to respond consistently to LPS. The reason for the failure of human T cells to respond to LPS, in contrast with cells of the monocytic lineage, is currently unclear but may be due to the lack of coreceptors (such as CD14) on these T cells essential for activation induced by LPS.

- Induction of TLR expression on T cells is dependent on TCR activation. Once induced, TLR2 signalling further enhances T cell function. This novel TCR–TLR interaction pathway suggests that TLR2 may be essential for optimal antigen specific T cell response.

- TLR expression depends on cell activation and is enhanced by the presence of IFNα, another innate cytokine. This demonstrates the role of TLR2 in networking with other innate mediators to enhance the adaptive immune response.

The present studies establish TLR2 as a costimulatory receptor for pathogen derived ligand, as opposed to conventional T cell costimulation ligands expressed on autologous cells. TLR2 may play an important role in adaptive immunity by directly enhancing antigen specific Th1 cell function, in addition to an indirect role through the activation of APCs.[9 12] Our results therefore demonstrate the existence of a T cell activation pathway via TLR2 which recognises the largest spectrum of PAMPs and stress proteins among the TLRs.[9 23 29] The constitutive and sustained expression of TLR2 on memory T cells may represent an important host mechanism for mounting an immediate strong response on encountering a reinfecting pathogen. Since BLP is highly conserved among bacterial strains, the results reported here may help to explain the often observed "flare up" of T cell mediated autoimmunity during microbial infection. Another important feature of the expression of TLR2 on memory T cells could be the contribution to the expansion and maintenance of memory T cells in the absence of specific antigen but in the presence of memory sustaining cytokines, including IL-2 and IL-15 produced by adaptive as well as innate immune cells. This mechanism could contribute to the perpetuation of long term memory by infectious agents with shared TLR2 ligand but is otherwise unrelated to the antigen specificity of the memory T cells. It should also be noted that in our studies BLP alone did not activate naive or memory T cells. It did so only in the presence of TCR activation or as a bystander effect of cytokines such as IL-2 or IL-15. This dual-signalling mechanism should avoid excessive T cell proliferation by BLP alone. In a recent report B cell receptor triggering led to upregulation of TLR9 that was constitutively expressed in

memory B cells.[30] Furthermore, human memory B cells proliferated and differentiated into plasma cells in response to CpG DNA.[31] The findings reported here suggest that there may be a parallel between T and B cells in that TLR signalling helps to maintain long term memory of these cells.

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REFERENCES

1 **Belvin MP**, Anderson KV. A conserved signaling pathway: the Drosophila toll-dorsal pathway. *Annu Rev Cell Dev Biol* 1996;**12**:393–416.
2 **Lemaitre B**, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 1996;**86**:973–83.
3 **Medzhitov R**, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997;**388**:394–7.
4 **Rock FL**, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A* 1998;**95**:588–93.
5 **Chaudhary P**, Ferguson C, Nguyen V, Nguyen O, Massa HF, Eby M, et al. Cloning and characterisation of two Toll/Interleukin-1 receptor-like genes TIL3 and TIL4: evidence for a multigene receptor family in humans. *Blood* 1998;**91**:4020–7.
6 **Takeuchi O**, Kawai T, Sanjo H, Copeland NG, Gilbert DJ, Jenkins NA, et al. TLR6: A novel member of an expanding toll-like receptor family. *Gene* 1999;**231**:59–65.
7 **Chuang T**, Ulevitch RJ. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. *Biochim Biophys Acta* 2001;**1518**:157–61.
8 **Aderem A**, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* 2000;**406**:782–7.
9 **Akira S**, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;**2**:675–80.
10 **Takeda K**, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003;**21**:335–76.
11 **Brint EK**, Fitzgerald KA, Smith P, Coyle AJ, Gutierrez-Ramos JC, Fallon PG, et al. Characterisation of signaling pathways activated by the interleukin-1 (IL-1) receptor homologue T1/ST2. A role for JUN N-terminal kinase in IL-4 induction. *J Biol Chem* 2002;**277**:49205–11.
12 **Schnare M**, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. *Nat Immunol* 2001;**2**:947–50.
13 **Geginat J**, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naïve, central memory, and effector memory CD4 (+) T cells. *J Exp Med* 2001;**194**:1711–19.
14 **Unutmaz D**, Pileri P, Abrignani S. Antigen-independent activation of naïve and memory resting T cells by a cytokine combination. *J Exp Med* 1994;**180**:1159–64.
15 **Zhang X**, Sun S, Hwang I, Tough DF, Sprent J. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* 1998;**8**:591–9.
16 **Marrack P**, Bender J, Hildeman D, Jordan M, Mitchell T, Murakami M, et al. Homeostasis of alpha beta TCR+ T cells. *Nat Immunol* 2000;**1**:107–11.
17 **Niedbala W**, Wei X, Liew FY. IL-15 induces type 1 and type 2 CD4+ and CD8+ T cells proliferation but is unable to drive cytokine production in the absence of TCR activation or IL-12/IL-4 stimulation in vitro. *Eur J Immunol* 2002;**32**:341–7.
18 **Muzio M**, Bosisio D, Polentarutti N, D'Amico G, Stoppacciaro A, Mancinelli R, et al. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol* 2000;**164**:5998–6004.
19 **Mokuno Y**, Matsuguchi T, Takano M, Nishimura H, Washizu J, Ogawa T, et al. Expression of toll-like receptor 2 on gamma delta T cells bearing invariant V gamma 6/V delta 1 induced by Escherichia coli infection in mice. *J Immunol* 2000;**165**:931–40.
20 **Zarember KA**, Godowsk PJ. Tissue expression of human toll-like receptors and differential regulation of toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* 2002;**168**:554–61.
21 **Bendigs S**, Sallzer U, Lipford GB, Wagner H, Heeg K. CpG-oligodeoxynucleotides co-stimulate primary T cells in the absence of antigen-presenting cells. *Eur J Immunol* 1999;**29**:1209–18.
22 **Matsuguchi T**, Takagi K, Musikacharoen T, Yoshikai Y. Tissue expression of human toll-like receptors and differential regulation of toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *Blood* 2000;**95**:1378–85.
23 **Zanin-Zhorov A**, Nussbaum G, Franitza S, Cohen I, Lider O. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. *FASEB J* 2003;**17**:1567–9.
24 **Caramalho I**, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med* 2003;**197**:403–11.
25 **Poltorak A**, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;**282**:2085–8.
26 **Vilanova M**, Tavares D, Ferreira P, Oliveira L, Nobrega A, Appelberg R, et al. Role of monocytes in the up-regulation of the early activation marker CD69 on B and T murine lymphocytes induced by microbial mitogens. *Scand J Immunol* 1996;**43**:155–63.
27 **Tough DF**, Sun S, Sprent J. T cell stimulation in vivo by lipopolysaccharide (LPS). *J Exp Med* 1997;**185**:2089–94.
28 **Castro A**, Bemer V, Nobrega A, Coutinho A, Truffa-Bachi P. Administration to mouse of endotoxin from Gram-negative bacteria leads to activation and apoptosis of T lymphocytes. *Eur J Immunol* 1998;**28**:488–95.
29 **Ohashi K**, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol* 2000;**164**:558–61.
30 **Bernasconi NL**, Onai N, Lanzavecchia A. A role for toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* 2003;**101**:4500–4.
31 **Bernasconi NL**, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 2002;**298**:2199–202.