Toll-like receptors: tools, assays, and implications for in vitro pyrogen tests
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Monoclonal antibody production against human toll-like receptors

Robert Kikkert, Els R. de Groot & Lucien A. Aarden
**Chapter 3**

**Monoclonal antibody production against human toll-like receptors**

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**Abstract**

Toll-like receptor (TLR) 2 and TLR4 are important receptors of the innate immune system that are activated by structural components of micro-organisms, and that play an important role in the generation of cytokine production and the development of antigen-specific acquired immunity. To study the role of TLR2 and TLR4 in the activation of the innate immune system, we sought to generate monoclonal antibodies to these TLRs by immunization of mice with human embryonic kidney (HEK) cells overexpressing CD14-TLR2 or CD14-TLR4 after a tolerization procedure with HEK cells expressing CD14. Using the intracellular dye CFSE a screening assay was developed that discriminated between hybridoma supernatants that bound to HEK-CD14 cells and HEK-CD14-TLR cells. Several immunizations led to the generation of three anti-TLR2 monoclonal antibodies, which were all of the IgM isotype subclass, whereas no mAbs recognizing TLR4 were produced.
Introduction

Toll-like receptors (TLRs) are germline-encoded pattern recognition receptors (PRRs) expressed on cells of the innate immune system, which recognize structural components conserved among different classes of microorganisms, also called pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs by TLRs initiates signal transduction pathways culminating in the generation of inflammatory cytokines and the expression of B7 costimulatory molecules as well as MHC II, which instruct the development of antigen-specific acquired immunity. At present, 11 mammalian TLRs have been discovered, each of which recognizes a particular PAMP, thereby enabling the host to mount a pathogen-specific immune response.

The first TLR member to be discovered was TLR4, which was demonstrated to be the long-sought receptor for bacterial lipopolysaccharide (LPS), the outer membrane component of Gram-negative bacteria. Subsequent generation of TLR-deficient mice has revealed that TLR2 is unusual compared to the other TLR members in that it is involved in the recognition of multiple PAMPs. It signals the presence of components of Gram-positive bacteria such as peptidoglycan, lipoteichoic acid and lipoproteins. In addition, lipoarabinomannan (LAM) from *Mycobacterium tuberculosis*, glycophosphatidylinositol anchors from *Trypanosoma cruzi*, porins from Neisseria meningitides, phosphatidylserine from Schistosoma mansoni and zymosan particles from the yeast *Saccharomyces cerevisiae* were demonstrated to signal through TLR2. The extent of the TLR2 ligand repertoire can be partially explained by the finding that TLR2 may form heterodimers with TLR1 and TLR6 to discriminate between the molecular structures of diacyl and triacyl lipopeptides, respectively.

For some TLRs (TLR3, TLR7-9) expression is limited to particular cell lineages. However, Northern blotting, RT-PCR, and immunostaining revealed that TLR2 is abundantly expressed on human monocytes, immature monocyte-derived dendritic cells and neutrophils. TLR4 is highly expressed also on monocytes, and moderately expressed on immature monocyte-derived dendritic cells and neutrophils. These studies also demonstrated that TLR2 and TLR4 expressed on these cells were functional. TLR2 and/or TLR4 stimulation may lead to cytokine production, the generation of superoxide, phagocytosis, chemotaxis, and the differentiation of naive T-cells to effector T-cells. TLR4 differs from TLR2 in that it, apart from recruiting MyD88 and Mal, is able to recruit additional adaptor proteins (TRIF, TRAM) following activation, which lead to qualitatively different immune responses. Interestingly, a body of evidence indicates that triggering of TLR4 on dendritic cells leads to the production of a Th1 type immune response, whereas TLR2 triggering promotes Th2 development.
At the time we initiated our experiments with the aim of producing TLR2- and TLR4-neutralizing antibodies, some TLR antibodies had already been described. The first mAb reported to neutralize TLR2 was TL2.1. This antibody (IgG1) raised against Chinese hamster ovary cells (CHO) stably transfected with human TLR2, partially (60%) inhibited Listeria monocytogenes (HKLM)-induced IL-6 release from CHO-TLR2 cells. Also in other studies, TL2.1 was used to inhibit TLR2-mediated effects on cells. However, in these studies, complete inhibition by TL2.1 of TLR2-mediated effects was not obtained. The same fusion by Espevik and co-workers also yielded TL2.3, a mAb which recognized TLR2 expressed on cells, but was not capable of inhibiting TLR2-mediated effects, and could be used for immunohistochemistry or Western blotting. TL2.1 and TL2.3 have been commercially available at several companies. Second, a mAb to human TLR2 was also generated at Genentech (San Francisco, USA). This mAb (mAb 2392; IgG2a) was raised to a TLR2-Fc fusion protein produced in a baculovirus expression system. Also this antibody partially inhibited TLR2-mediated effects. However, the method of generating this mAb was not accurately reported. Moreover, mAb 2392 had not been commercially available. Third, in 1999, Miyake and co-workers reported the generation of HTA125 (IgG2a), a mAb specific for TLR4 that was raised against murine Ba/F3 cells overexpressing TLR4. Again, the exact procedure of the generation of this mAb was, and still is unknown and was never published. HTA125 was used as an inhibitor of cytokine production by TLR4 agonists in several cell lines.

Thus, although three TLR-neutralizing mAbs had been described, information as to the generation of these mAbs, as well as to their precise specificity, was limited. In view of the aforementioned important role of TLR2 and TLR4 in the activation of monocytes, neutrophils, and dendritic cells, we sought to generate inhibitory and/or stimulatory monoclonal antibodies (mAbs) directed against TLR2 and TLR4 ourselves. Employing these mAbs would enable us to delineate in more detail the contribution of each individual TLR to the immune responses to particular pathogens.

Materials & Methods

Cell lines

Human Epithelial Kidney 293 (HEK) cells stably transfected with CD14, CD14/TLR2, or CD14/TLR4 were a kind gift from Drs. D. Golenbock and E. Latz, Worcester, MA, USA and have been described elsewhere. Transfected HEK cells, non-secreting mouse myeloma SP2/0-Ag14, and monocytoid THP-1 cells (ATCC TIB-202) were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM, Bio Whittaker, Verviers, Belgium) supplemented with 5% heat-inactivated fetal calf serum (FCS, Bodinco, Alkmaar, The
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Netherlands), penicillin 100 U/ml / streptomycin 100 µg/ml (Gibco, Merelbeke, Belgium), and 50 µM 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany). In medium for HEK cells and THP-1 cells, 5 µg/ml puromycin (Sigma-Aldrich) and 20 µg/ml human transferrin (Sigma-Aldrich) were supplemented, respectively. Cells were cultured at 37ºC in the presence of 5% CO₂, in a humidified incubator.

**Tolerization & immunization of mice**

To minimize generation of mAbs recognizing epitopes on HEK-CD14-TLR2 other than TLR2 itself, mice were tolerized for HEK-CD14 cells with cyclophosphamide (Sigma-Aldrich), using a well established tolerization protocol (see table 1, Dr. J. Wijdenes, Diaclone, Besançon, France, personal communication). Twelve-week-old male Balb/c mice received an intraperitoneal injection with HEK-CD14 cells (10x10⁶ cells/ 200µl PBS/ mouse), followed by an intraperitoneal injection with 1 mg cyclophosphamide/ 0.2 ml PBS/ mouse ten minutes later (day 1). Also the subsequent two days cyclophosphamide (1 mg/ 0.2 ml PBS/ mouse) was injected (day 2 + 3). This cycle (day 1-3) was repeated three times with two-week intervals. Following this tolerization procedure, the mice were immunized intraperitoneally three times, at two week intervals, with HEK-CD14-TLR2 or HEK-CD14-TLR4 cells (10x10⁶ cells/ 200µl PBS/ mouse, see table 1).

Before fusion, pre-immune sera, sera obtained after tolerization but before immunization, and immune sera were checked for reactivity with HEK-CD14 and HEK-CD14-TLR2/4 cells by FACS analysis using fluorescein isothyocyanate (FITC)-conjugated polyclonal goat-anti-mouse mAbs (CLB).

**Fusion of spleen and mouse myeloma cells**

Four days after the last booster injection, spleen cells and peripheral lymph nodes were fused with non-secreting mouse myeloma SP2/0-Ag14 cells at a 3:1 ratio (100,000 spleen cells/well) under standard conditions employing PEG 4000 (Sigma) as described before. Hybridomas were cultured in selection medium containing 100 µM hypoxanthine (Sigma) and 1 µg/ml azaserine (Sigma) in the presence of recombinant IL-6 (2 ng/ml, CLB) to maintain hybridoma proliferation, in 200-µl wells in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). After 8 and 10 days the medium was replaced with medium without azaserine. After another 2-3 days the supernatants were screened for mAb production. Clonality of hybridoma cells was achieved by limiting dilution.
Table 1: tolerization / immunization scheme

<table>
<thead>
<tr>
<th>day</th>
<th>procedure</th>
</tr>
</thead>
</table>
| 0   | -collection pre-immune serum  
     | -i.p. injection 10 x 10⁶ HEK-CD14  
     | -after 10 min. intraperitoneal cyclophosphamide injection (CP ip) |
| 1   | CP ip     |
| 2   | CP ip     |
| 14  | -i.p. injection 10 x 10⁶ HEK-CD14  
     | -after 10 min. CP ip               |
| 15  | CP ip     |
| 16  | CP ip     |
| 28  | -i.p. injection 10 x 10⁶ HEK-CD14  
     | -after 10 min. CP ip               |
| 29  | CP ip     |
| 30  | CP ip     |
| 42  | -i.p. injection 10 x 10⁶ HEK-CD14  
     | -after 10 min. CP ip               |
| 43  | CP ip     |
| 44  | CP ip     |
| 45  | -collection pre-immune serum  
     | - i.p injection 10 x 10⁶ HEK-CD14-TLR2 |
| 59  | i.p injection 10 x 10⁶ HEK-CD14-TLR2 |
| 66  | collection immune serum           |
| 73  | i.p/i.v. injection 10 x 10⁶ HEK-CD14-TLR2 |
| 77  | isolation of spleen cells + peripheral lymph nodes |

**Screening of hybridoma supernatants by FACS analysis**

To analyse the binding of hybridoma supernatants to both HEK-CD14-TLR2/4 and HEK-CD14 cells, hybridoma supernatants were added to a mixture of HEK-CD14 cells labelled with the intracellular fluorescent probe carboxyfluorescein-diacetate, succinimidyl ester (5(6)-CFDA, SE; CFSE, Molecular Probes, Eugene, OR, USA), and unlabeled HEK-CD14-TLR2/4 cells. Binding of hybridoma supernatant to HEK-CD14-TLR2/4 and/or HEK-CD14 cells was detected by polyclonal goat-anti-mouse immunoglobulin antibodies, as detailed out below.

HEK-CD14 cells were trypsinized at room temperature, washed in ice-cold IMDM containing 5% FCS, washed in ice-cold PBS, and resuspended in ice-cold PBS at a concentration of 20 x 10⁶ cells/ml. All incubations were performed at 4 ºC. One volume of HEK-CD14 cells was incubated with 1 volume of CFSE solution (0.33 μM) for 10 min. CFSE staining was terminated by addition of 2 volumes FCS. Following a 2 min...
incubation, HEK-CD14 cells were washed in IMDM containing 5% FCS, washed in PBA (PBS containing 0.5% (w/w) bovine serum albumin and 0.02% sodium azide) and resuspended in PBA at a final concentration of 4 x 10^6 cells/ml. HEK-CD14-TLR2/4 cells were trypsinized, washed with IMDM containing 5% FCS, washed in PBA and also resuspended in PBA at a concentration of 4 x 10^6 cells/ml. Equal volumes of HEK-CD14 and HEK-CD14-TLR2/4 cells were mixed and 50 µl aliquots containing 100,000 CFSE-labelled HEK-CD14 and 100,000 unlabeled HEK-CD14-TLR cells were added to 96-well round-bottom microtitre plates (Greiner Bio-One, Frickenhausen, Germany).

HEK-cell mixtures (50 µl) were incubated for 20 min with 50 µl of hybridoma supernatant, or with an anti-human TLR mAb (for TLR2 fusions mouse anti-human TLR2 mAb TL2.1, 20µg/ml, IgG2a, Cascade BioScience, Winchester, MA, USA and for TLR4 fusions mouse anti-human TLR4 mAb HTA125, 20 µg/ml, IgG2a, eBiosciences, San Diego, CA, USA) and an irrelevant mouse IgG2a isotype (20 µg/ml, CLB) as positive and negative controls, respectively. Incubations with antibodies were performed in PBA containing human gamma globulins (3 mg/ml, CLB) to prevent non-specific binding. HEK-cell mixtures were washed three times in PBA and incubated with a biotinylated polyclonal goat-anti-mouse antibody (1:1000, CLB) for 20 min. The cells were then washed three times in PBA and incubated with streptavidin-allophycocyanin (1:750; BD Pharmingen, San Diego, CA, USA) and measured on a FACS-calibur (Becton Dickinson, San Jose, Ca, USA).

Mouse anti-human HLA class I (W6/32), IgG2a was from Abcam, Cambridge, UK. Mouse anti-human CD14 (CD14.22) was from Dr. Van der Schoot, Sanquin, Amsterdam. An IgM raised against the allergen Corylus avellana 1 was kindly provided by Dr. R. van Ree, Sanquin, Amsterdam. The isotype of the mAbs produced was determined using a mouse monoclonal antibody isotyping kit (HyCult Biotechnology, Uden, The Netherlands).

Results

Screening of mice sera for reactivity with HEK-CD14 and HEK-CD14-TLR2/4 cells

To determine whether mice sera contained the desired antibodies, preimmune sera, sera collected after tolerization but before immunization, and immune sera, were compared for reactivity with HEK-CD14 and HEK-CD14-TLR2/4 cells. Compared to pre-immune sera, sera collected after tolerization did not show increased binding to HEK-CD14-cells (data not shown). After immunization with HEK-CD14-TLR2 or HEK-CD14-TLR4 cells, sera exhibited strong binding to the appropriate type of HEK-CD14-TLR cells (data not shown). However, these immune sera showed equally strong binding to HEK-CD14 cells.
### Table 2: Overview of 1st screening results.

<table>
<thead>
<tr>
<th>fusion</th>
<th>antigen</th>
<th>nr. of wells</th>
<th>1st screening: hybridoma supernatants positive for:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TLR mild</td>
<td>strong</td>
</tr>
<tr>
<td>1</td>
<td>TLR2</td>
<td>990</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.2%)</td>
<td>(0.3%)</td>
</tr>
<tr>
<td>2</td>
<td>TLR4</td>
<td>440</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.7%)</td>
<td>(0.2%)</td>
</tr>
<tr>
<td>3</td>
<td>TLR4</td>
<td>384</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.0%)</td>
<td>(0.3%)</td>
</tr>
<tr>
<td>4</td>
<td>TLR2</td>
<td>228[^V]</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3%)</td>
<td>(0%)</td>
</tr>
</tbody>
</table>

**I** TLR2/TLR4 indicates HEK cells expressing both CD14 and TLR2/TLR4

**II** Nr. of wells indicates the number of wells that were tested during the first (initial) screening

**III** A mild positive signal represents a binding to HEK-CD14-TLR cells that is less than three times the IgG isotype control mAb signal

**IV** A strong positive signal represents a binding to HEK-CD14-TLR cells that is more than three times the IgG isotype control mAb signal

**V** Fusion performed with a pool of spleen cells derived from mice tolerized/immunized at Diaclon, Besançon, France, using the same protocol.

without the appropriate TLR present (data not shown), indicating that tolerance had been broken during immunization. Of the eight mice immunized (1-4: TLR2, 5-8: TLR4), one mouse (nr. 4) did not produce antibodies reactive with HEK-CD14 or HEK-CD14-TLR2 cells (data not shown).

**Principle of FACS analysis screening.**

Technically, the fusions were successful. Out of 3000 wells tested, more than 96% of the wells showed growth of hybridomas. However, during the first screening (not shown) we noticed that a substantial part of the hybridomas bound to both HEK-CD14 and HEK-CD14-TLR cells. Therefore, we developed a screening method (as described in the
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materials & methods section) that enabled us to discriminate between supernatants only binding to HEK-CD14 cells and supernatants binding to HEK-CD14 cells expressing TLR. An example of this screening technique with HEK-CD14 and HEK-CD14-TLR2 cells using a mAb specific for TLR2 (TL2.1) is shown in figure 1. HEK-CD14 and HEK-CD14-TLR2 cells show equal (auto)fluorescence after incubation with PBA (fig 1A) or an irrelevant IgG2a isotype control (fig 1B). However, the TLR2 recognizing mAb TL2.1 specifically bound to HEK-CD14-TLR2, and not to HEK-CD14 cells (fig 1C). Comparable results were obtained when HEK-CD14-TLR4 and HEK-CD14 cells were stained with a mAb specific for TLR4 (HTA125, data not shown). As a positive control, a mAb specific for HLA class I (W6/32) was used, that bound equally well to HEK-CD14 and HEK-CD14-TLR cells (fig 1D). CD14 was found to be equally highly expressed on HEK-CD14-TLR2, HEK-CD14-TLR4, and HEK-CD14 cells (data not shown).

Screening of hybridoma supernatants by FACS analysis.

We performed four fusions with spleen cells from mice immunized with HEK-CD14-TLR cells (2x TLR2, 2x TLR4) and analysed the hybridoma supernatants by FACS analysis. An overview of the binding of these hybridoma supernatants is provided in table 2. The fusions of spleen cells derived from mice immunized with HEK-CD14-TLR2 yielded three mAbs (designated 1F8, 5C9, and 1B3). The binding of two hybridoma supernatants (1F8, 1B3) to HEK-CD14-TLR2 cells, as analysed by FACS, is shown in fig 2A. Despite the tolerization procedure, several hybridoma supernatants recognized HEK-CD14 and HEK-CD14-TLR2 cells equally well (fig 2C). Because the first fusion yielded one monoclonal antibody recognizing TLR2 (1F8) which was of the IgM subclass, an irrelevant murine IgM was included as a negative control in subsequent screenings (fig. 2D). All three monoclonal antibodies that bound to HEK-CD14-TLR2 cells were found to be of the IgM isotype. Two fusions performed with spleen cells from HEK-CD14-TLR4-immunized mice did not yield any mAb that was specific for TLR4.

Binding of IgM anti-HEK-CD14-TLR2 mAbs to THP-1 cells.

To ascertain whether anti-HEK-CD14-TLR2 mAbs also recognized endogenous TLR2 on human cells, THP-1 cells were stained with 3 IgM anti-HEK-CD14-TLR2 mAbs and TL2.1. None of the three IgM anti-HEK-CD14-TLR2 mAbs showed increased binding compared to the IgM isotype control, in contrast to control mAb TL2.1 (data not shown).
Chapter 3

Discussion

To study the role of TLR2 and TLR4 in the activation of cells of the immune system we sought to generate inhibitory and/or stimulatory monoclonal antibodies (mAbs) directed against TLR2 and TLR4. Since recombinant monomeric ectodomains of TLR2 and/or TLR4, with the correct folding and three-dimensional structure were not available for immunization (see chapter 2), HEK-cells overexpressing CD14 and the appropriate TLR were used for immunization. However, one of the disadvantages of immunizing mice with human cells is that antibodies which recognize abundant, immunodominant surface antigens other than the appropriate TLR, are likely to be generated. Therefore, a tolerization protocol with the alkylating agent cyclophosphamide was used. This strategy is based on the assumption that when the immune response is elicited after injection with HEK-CD14 cells, resulting in the clonal expansion of B and T lymphocytes, the dividing cells are preferentially killed by cyclophosphamide. This would result in the lack of an immune response after subsequent exposure to HEK-CD14 cells, which then would significantly increase the likelihood of an immune response directed against the appropriate TLR after immunization with HEK-CD14-TLR cells. This so-called subtractive immunization procedure has been proven to be useful for other antigens. In our experiments, the tolerization procedure was only partly successful: the binding to HEK-CD14 cells of immune sera after tolerization was comparable to that of pre-immune sera. However, after subsequent immunization with HEK-CD14-TLR cells, immune sera also bound to HEK-CD14 cells, which indicates that tolerization was, at least partly, lost.

We developed a screening assay that enabled us to compare the binding of hybridoma supernatant to both HEK-CD14 and HEK-CD14-TLR cells within one single FACS assay. To achieve this, HEK-CD14 cells were labelled with the fluorescent probe CFSE that has its peak excitation wavelength at 488 nm and peak emission wavelength at 530 nm, which can be recorded using the FL1 channel. However, CFSE has a more powerful and broader emission spectrum than other fluorescein dyes and is also detected in the FL2 and FL3 channels. Therefore, we used a combination of biotinylated polyclonal goat-anti-mouse IgG and streptavidin-allophycocyanin, which can be recorded at 660 nm using the FL4 channel, thus avoiding both the overlap of emission spectra that would require corrections, and autofluorescence.

Fusions of spleen cells derived from mice immunized with HEK-CD14-TLR4 cells did not yield any specific mAb for TLR4. TLR2 fusions yielded three mAbs that specifically recognized HEK-CD14-TLR2 and not HEK-CD14, which were all of the IgM subclass. As many screening assays, our assay may preferentially detect multivalent antibodies, such as IgM, because their avidity may be higher than that of bivalent IgG antibodies. However,
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Figure 1: Principle of FACS screening.
Mixtures of CFSE-stained HEK-CD14 cells with HEK-CD14-TLR2 cells were incubated with PBA (A), IgG2A isotype control (B), anti-TLR2 mAb TL2.1 (C), and anti-HLA class I mAb W6/32 (D).

titration of the control anti-TLR2 mAb TL2.1 indicated that the screening assay was able to detect as little as 1 µg/ml IgG2a-anti-TLR2 (data not shown), which is a concentration that is lower than that expected to be produced in the medium of hybridoma cells. In addition, the IgM anti-TLR2 mAbs did not recognize TLR2 on THP-1 cells, which may suggest that the density of TLR2 expressed on THP-1 cells was too low for a polyvalent interaction with the IgM ant-TLR2 mAbs. Antibody responses in mice against highly conserved mammalian proteins (such as TLRs) are often weak and mainly result in IgM antibodies, owing to the
lack of T-cell stimulation. Since the ectodomains of TLR proteins are comprised of leucine-rich repeats that are highly conserved in mammals and plants, this might account for the IgM response observed after HEK-CD14-TLR2 immunization.

After Beutler and co-workers discovered the TLR4 gene in 1998, most progress in the identification and characterization of new members of the TLR family has been obtained by the generation of TLR-deficient mice and the overexpression of human recombinant TLRs in mammalian cells. However, to study the role of TLRs in the activation of human blood cells it would be desirable to specifically block or activate a particular TLR with functional mAbs. Given this need for the use of functional anti-human TLR mAbs surprisingly few mAbs have been generated.

After its production the TLR2 neutralizing mAb TL2.1 antibody by Espevik and co-workers has been relatively little used, perhaps because only limited inhibition of TLR2 could be achieved. The mAb produced at Genentech has not been commercially available, but seems to be a better TLR2-neutralizing mAb. In a recent study, the inhibitory actions of TL2.1 and mAb 2392 on TLR2-ligand induced cytokine production in THP-1 cells were compared. The mAb 2392 blocked >99% of the TNF production induced by the TLR2 ligands lipoteicoic acid and Pam3Cys, and about 90% of the response induced by peptidoglycan, whereas TL2.1 was only capable of partially blocking Pam3Cys-induced TNF production.

The alleged TLR4-neutralizing mAb HTA 125 has been used as an inhibitor of cytokine production by TLR4 agonists in several cell lines. However, the usefulness of HTA125 was questioned when Wang and co-workers observed only weak binding of HTA125 to TLR4, which could be even further decreased by incubation with purified human IgG Fc, IgG1, and IgG4. Also, both our own as well as recent published experiments demonstrated that in whole blood, HTA125 was unable to block LPS-induced cytokine production. Perhaps because of these results obtained with these allegedly TLR-neutralizing mAbs, most research groups used TLR4-deficient C3H/HeJ mice to investigate the role of TLR4 in the immune response, rather than to use mAbs of questionable merit. Also Beutler mentioned that it is notoriously difficult to raise functionally antagonistic and/or agonistic mAbs against TLRs.

Nonetheless, recently, the generation of 15C1, a powerful mAb raised against TLR4, which inhibited LPS-induced cytokine production in a panel of primary cells and cell lines in vitro, was reported. This paper shed new light on the generation of TLR-neutralizing mAbs and reported some remarkable facts. First, the mice used in this study were immunized with CHO-cells co-expressing both TLR4 and MD-2. This was regarded essential for the generation of functional TLR4-neutralizing mAbs (G. Elson, NovoImmune SA, Switzerland, personal communication). Second, the final “hyper-booster” injection was
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Figure 2: Screening of hybridoma supernatants by FACS analysis.
Mixtures comprising CFSE-stained HEK-CD14 cells and HEK-CD14-TLR2 cells were incubated with hybridoma supernatants. A and B: Hybridoma supernatants (A, 1F8; B, 1B3) that specifically bound HEK-CD14-TLR2. C: an irrelevant IgM isotype control that bound to both HEK-CD14-TLR2 and HEK-CD14. D: Hybridoma supernatant that bound both to HEK-CD14-TLR2 and HEK-CD14.

not given with CHO-TLR4-MD2 cells, but with the extracellular soluble part of human TLR4. Third, the inhibition of TLR4 signalling was demonstrated to be caused not by the hindrance of the binding between LPS and TLR4-MD2, but by prevention of dimerization of TLR4 receptors. Finally, the TLR4 inhibitory effect was demonstrated to be dependent on the interaction of the Fc part of the TLR4-neutralizing mAb with FcγRIIa (CD32A).
At present, clinical trials are underway to test the efficacy of 15C1 against recurrent Gram-negative pulmonary infection (G. Elson, personal communication).

To summarize, we developed a new FACS assay which enabled us to compare efficiently the binding of hybridoma supernatants with HEK cells with and without TLR expressed. We attempted to generate mAbs against TLR2 and TLR4, but only produced three IgM anti-TLR2 mAbs. At present, we cannot account for the lack of a specific immune response after immunization with TLR-expressing cells. This, together with the fact that few mAbs against TLR2 and TLR4 have been developed by other research groups suggest that particular problems exist in the generation of a specific immune response to TLR structures, which remain to be elucidated. Recent reports suggest that, at least for TLR4, efficient generation of TLR-neutralizing mAbs depends on the presence of extracellular adaptor molecules, such as MD-2.

References

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