Modulated rat dendritic cells in renal transplantation models : immune regulation and graft outcome

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CHAPTER 5

Generation and characterization of a novel anti-rat CD40L antibody with inhibitory activities in vitro and in vivo

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J. Immunol. Methods 2008 Jun 1;335(1-2):46-52.

Abstract

The CD40-CD40L interaction plays a critical role in cell mediated immune responses. Blocking this interaction has been shown to be beneficial in the treatment of various diseases studied in murine models. Although rats are widely used to test therapeutic strategies in several disease models, a monoclonal antibody (mAb) to block the CD40-CD40L interaction in rats is not broadly available. In the present study we generated Armenian hamster fibroblasts expressing rat CD40L and used these to generate a novel anti-rat CD40L mAb (AS1). In vitro studies showed that AS1 was able to block CD40L-induced DC maturation and B cell proliferation. Most importantly, in vivo, AS1 inhibited B cell responses in a dose-dependent fashion, as measured by the production of OVA specific antibodies after subcutaneous immunization with OVA. Taken together, AS1 was shown to be a powerful tool to modulate Ag presentation in vitro and in vivo. Elucidating the effect of AS1 in various rat models for human diseases will provide more insight into blocking CD40-CD40L interaction as a therapeutic strategy to prevent human diseases.

Introduction

CD40L (CD154) is a 33 kDa type II transmembrane protein and is a member of the TNF family of ligands. CD40L is expressed on activated T cells and platelets and interacts with CD40, a membrane molecule expressed on various cell types, including B cells, macrophages, dendritic cells, fibroblasts and endothelial cells [1].
Several signals are involved to induce full T cell activation. Resting naïve $CD4^+$ T cells are triggered by MHC/peptide complexes in cooperation with co-stimulatory molecules on the surface of dendritic cells (DC) and the presence of pro-inflammatory cytokines [2, 3]. As a consequence the T cells become activated and express several surface molecules, amongst which CD40L. Binding of CD40L to CD40 results in an enhanced expression of MHC class II and co-stimulatory molecules on DC, resulting in fully mature DC that is capable to induce strong activation of T cells [4, 5].
Studies in various in vivo models have shown that interference of the CD40-CD40L interaction abrogates cell mediated immune responses [6], implying its beneficial effect in therapeutic strategies of several disease models such as transplantation or autoimmunity. In agreement with these observations, studies in mouse models have shown that the anti-CD40L blocking antibody MR1 is able to prevent the induction of experimental autoimmune encephalomyelitis (EAE) [7], collagen induced arthritis [8] and lupus nephritis [9]. Furthermore, blocking the CD40-CD40L interaction in murine transplantation models has been shown to prolong transplant survival [10].
Besides mouse models, also rat models are frequently used to study the underlying mechanism of human diseases. However, relatively little information is available concerning the inhibition of CD40-CD40L interactions in rat disease models. A few studies have demonstrated that transplant survival could be prolonged when recipients were treated with an anti-rat CD40L mAb together with a co-treatment [11-13]. We believe that availability of a reagent that interferes with CD40-CD40L interactions in rats will provide more insight and help in the design of new therapeutic strategies.
The aim of the present study was therefore to generate a CD40L blocking mAb specific for rat. We generated a novel anti-rat CD40L antibody (AS1) and found that this antibody is able to block DC and B cell activation after CD40L stimulation. In vivo, AS1 was shown to be functional because it prevented an induced OVA response. The use of the novel anti-rat CD40L mAb provides the opportunity to obtain more insight into the effects of CD40-CD40L interactions in rat models for human diseases.

Materials and Methods

Animals

Seven to twelve week-old male Lewis (LEW RT-$1A^l$) rats purchased from Harlan (Horst, the Netherlands). Armenian hamsters (*Cricetulus migratorius*) were purchased from Cytogen (Boston, MA). The animals had free acces to water and standard chow. Experimental studies with animals were performed in accordance with local ethical committee guidelines of Leiden University Medical Center.

Generation of anti-rat CD40L mAb

Using electroporation Armenian hamster fibroblasts (ARHO 12, kindly provided by Dr. Hamann, AMC, Amsterdam, the Netherlands) were transfected with the expression plasmid pMErCD40L, which was generated by cloning the full length CD40L gene from cDNA of rat splenocytes using PCR based method. Transfected cells were selected with 400 ng/ml hygromycin B (Invitrogen, Breda, the Netherlands) and stably transfected clones were analyzed for rat CD40L expression by flow cytometry. ARHO-12 cells expressing high levels of rat CD40L (ARHO-rCD40L) were injected into Armenian hamsters. Four injections with $2x10^6$ irradiated cells (50 Gy) in PBS were given at weekly intervals. One day after the last injection, serum was tested for the presence of anti-rat CD40L antibodies by incubating serum on mouse fibroblasts expressing rat CD40L (L-rCD40L). The presence of hamster antibodies (Ab) against rat CD40L were detected by flow cytometry.
Two days later, animals with a strong anti-rat CD40L response were sacrificed and splenocytes were fused with mouse myeloma SP2.0 cells by standard hybridoma technology. Hybridoma supernatants were tested for binding to L-rCD40L cells by flow cytometry. Selected hybridomas were subcloned to obtain a stable clone (AS1). Monoclonal Ab produced by this clone were purfied on a protein A column (Amersham Biosciences AB, Uppsala, Sweden).

Flow cytometric analysis

Cells were harvested and washed in FACS-buffer containing 1% BSA, 0.02% NaN_3. Cells were stained with human CD40-Ig (12 μg/ml) followed by a mouse anti-human IgG (ATCC Hb43, 5 μg/ml, American Type Culture Collection, Halifax, GA, USA) or with purified AS1, hamster anti-mouse CD40L (MR1, kind gift from Dr. Flierman, LUMC, Leiden, the Netherlands), mouse anti-human CD40L (LL48) (10 μg/ml) or sera derived from AS1 injected rats. Binding was visualized with phycoerythrin (PE)-conjugated goat anti mouse Ig (Dako, Glostrum, Denmark) or PE-conjugated goat anti-hamster Ig (Southern Biotechnology, Birmingham, Alabama, USA). Fluorescence intensities were assessed with a FACS Calibur (BD, Mountain View, CA). Data analysis was performed using WinMDI 2.8 software.

Culture of rat dendritic cells

Bone marrow (BM) was isolated from tibias and femurs from LEW rats by flushing the bones with medium. Red blood cells were lysed with lysis buffer (Pharmacy, LUMC, Leiden, the Netherlands). The remaining BM cells were passed through an 80 μm sieve. Cells were cultured in 6 wells plates (Costar, Cambridge, MA) in 3 ml of RPMI+ medium containing RPMI 1640 (Invitrogen), 10% heat-inactivated FCS (BioWhittaker, Vervier, Belgium), penicillin/streptomycin (Gibco), Fungizone (Gibco), β-Mercapto-ethanol (50 μM, Merck, Darmstadt, Germany), L-Glutamine (2mM, Gibco). Rat GM-CSF (2 ng/ml, Invitrogen), rat IL-4 (5 ng/ml, Invitrogen) and human Flt3L (50 ng/ml, kindly provided by Amgen) was added and cells were cultured at a density of $1.5x10^6$ cells per well. All medium was removed and replaced by fresh medium containing cytokines at day 2 and 4. Non-adherent and semi-adherent cells were harvested at day 7 and stimulated with LPS (500 ng/ml, Salmonella Typhosa, Sigma, Zwijndrecht, the Netherlands) or

L-rCD40L in a 2:1 ratio. Non-transfected L-cells (L-orient) were used as control cells. Cells were plated in 6 well plates at a density of $1.5x10^6$ cells/well in the presence of GM-CSF (2ng/ml), IL-4 (5ng/ml) and Flt3L (50ng/ml) and stimulated for 24 hours in the presence of medium, AS1, MR1, LL48, CD40lg or hamster IgG (Biomeda, Foster city, CA, USA).

Generation of monocyte-derived human DC

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy blood donors by Ficoll density gradient centrifugation [14]. Cells were positively selected by CD14 MACS microbeads (Miltenyi Biotech, GmBH Berisch Gladbach, Germany), and cultured at a density of $1.5x10^6$ cells/well in a 6 well plate (Costar) in RPMI 1640 containing 10% heat inactivated FCS, penicillin/streptavidin, human GM-CSF (5 ng/ml, Leucomax®, Novartis Pharma BV, Arnhem, the Netherlands) and human IL-4 (10 ng/ml, Peprotech, RockyHill, USA).

Cytokine analysis or Ab detection by ELISA

ELISA's for the detection of rat IL-12p40, (Invitrogen) and human IL-12p70 (R&D, Abingdon, Oxon UK) were performed following the instructions provided by the supplier in supernatants of the stimulated cells. To detect a rat anti-hamster antibody response, 96 wells flat bottom plates (NUNC, Roskilde, Denmark) were coated with AS1 (10μg/ml). After 30 min. incubation with dilutions of rat sera, rat anti-hamster levels were detected using a horseradisch peroxidase conjugated rabbit anti-rat IgG (DAKO) and developed with 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid.

B cell proliferation

Splenocytes were derived from LEW spleen using a 70 μm cell strainer (BD, Breda, the Netherlands). $1x10^5$ splenocytes were cultured in RPMI+ together with irradiated (80Gy) L-orient or L-rCD40L cells in a 96-wells U-bottom plate (Costar) in a 10:1 ratio. On day 5, cells were pulsed with 0.5 μCi (37kBq) of [methyl-^{3}H] thymidine (NEN™ Life Science Products, Inc., Boston, MA,USA) for 16 hr to quantify cell proliferation. For inhibition studies AS1, MR1, LL48 or hamster IgG was added (5 μg/ml) at day 0. Results are presented as the mean counts per minute (cpm) ±SD obtained from triplicate cultures.

T cell stimulation

LEW splenocytes were incubating with anti-κ light chain (HIS8) and anti-MHC class II (OX6) in a buffer containing 2% heat inactivated FCS (BioWhittaker) and 2.5 mM EDTA. After incubation, cells were washed and subsequently incubated with goat anti-mouse beads (Polysciences Inc, Warrington, PA, USA). The negative population was isolated using a magnet (BD) and used as responder cells. Isolated T cells ($1x10^5$) were stimulated in 96-well flat-bottom plates coated with anti-CD3 (10 μg/ml, G4.18, BD) together with soluble anti-CD28 (1 μg/ml, JJ319, BD) for 24 hours. Cells were harvested and used for FACS analysis.

Blocking capacity of AS1 in vivo

LEW rats (n=5) were treated i.p. with 1.5 mg hamster IgG and 0.75 or 1.5 mg AS1 at day -1 and 3. All rats were immunized on day 0 with 50 μl of an emulsion of ovalbumine in PBS (OVA; 100 μg, Sigma) and an equal volume of complete Freunds adjuvant (Difco, Detroit, USA) subcutaneously in the tail. Serum was obtained at day 0 and at day 10 after immunization to measure IgG anti-OVA levels by ELISA. For this purpose OVA (10 μg/ml) was coated on a flat bottom ELISA plate (NUNC). Serum dilutions were incubated and IgG anti-OVA responses were detected by horseradish peroxidase conjugated rabbit anti-rat IgG (DAKO) and developed with 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid.

Statistical analysis

Statistical significant differences were determined using the Mann Whitney test. Differences were considered significant when $p<0.05$.

Results

Generation and characterization of an anti-rat CD40L specific monoclonal antibody

We recently cloned and expressed rat CD40L in murine L-cell fibroblasts and showed the potential of these cells for effective activation of CD40 expressing cells of rat origin. We used these cells to immunize mice but were unable to obtain a specific response recognizing rat CD40L. Alignment of the CD40L sequences showed a high homology between rat and mouse CD40L (95%), possibly explaining the lack of a specific response. We therefore tested the widely used hamster anti-mouse CD40L mAb, MR1, but although there was some recognition of rat CD40L, it was not able to inhibit CD40L induced responses (data not shown).

We thus decided to develop a hamster anti-rat CD40L mAb. Therefore we generated Armenian hamster fibroblasts expressing rat CD40L (ARHO-rCD40L) (**Fig. 1**). Subsequently, Armenian hamsters were immunized with these ARHO-rCD40L cells to generate AS1, a hamster mAb directed against rat CD40L (details described in materials and methods).

Next we examined the binding capacity of AS1, MR1 and LL48 (mouse anti-human CD40L) to various cell lines expressing rat or human CD40L. All cell lines transfected with either rat or human CD40L showed specific binding of human CD40-Ig, which was not observed with non-transfected cell lines. AS1 and MR1 showed binding to rat CD40L, whereas human CD40L was not recognized by either mAb. Monoclonal Ab LL48 exclusively bound to human CD40L (**Fig. 1**).

Functional characterization of AS1

To test the functional consequence of binding of AS1, rat DC were stimulated with rat CD40L in the presence or absence of various concentrations of AS1 for 24 hours. DC stimulated by rat CD40L showed strong production of IL-12. AS1 was able to

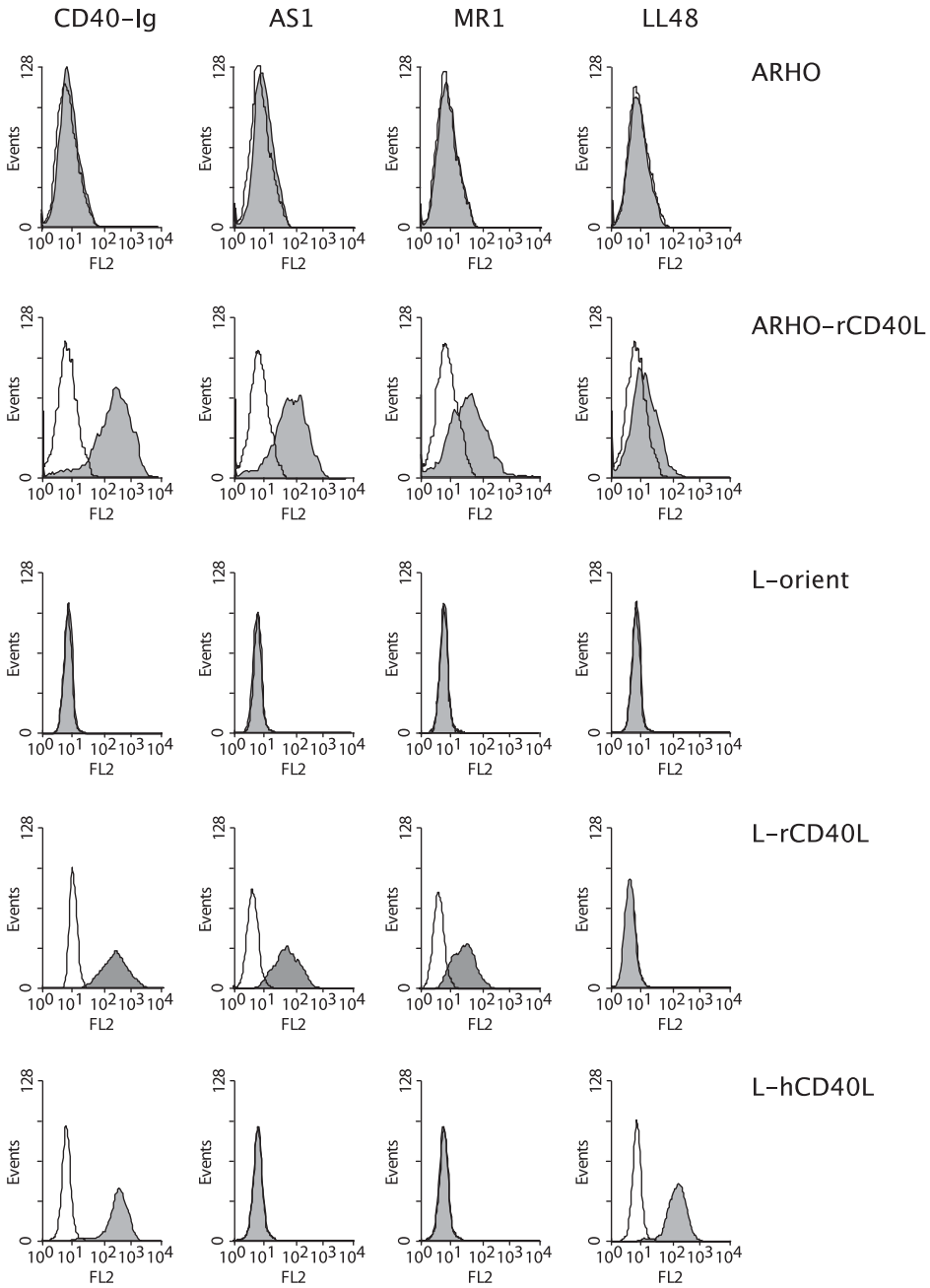

Figure 1. AS1 and MR1 bind to rat CD40L.
Binding of CD40-Ig, AS1, MR1 and LL48 was examined on ARHO-rCD40L, L-rCD40L expressing rat CD40L or L-hCD40L expressing human CD40L. As a control, non-transfected cell lines (ARHO or L-orient) were used. Binding capacity was detected by flow cytometry. Shown is a representative experiment out of 4.

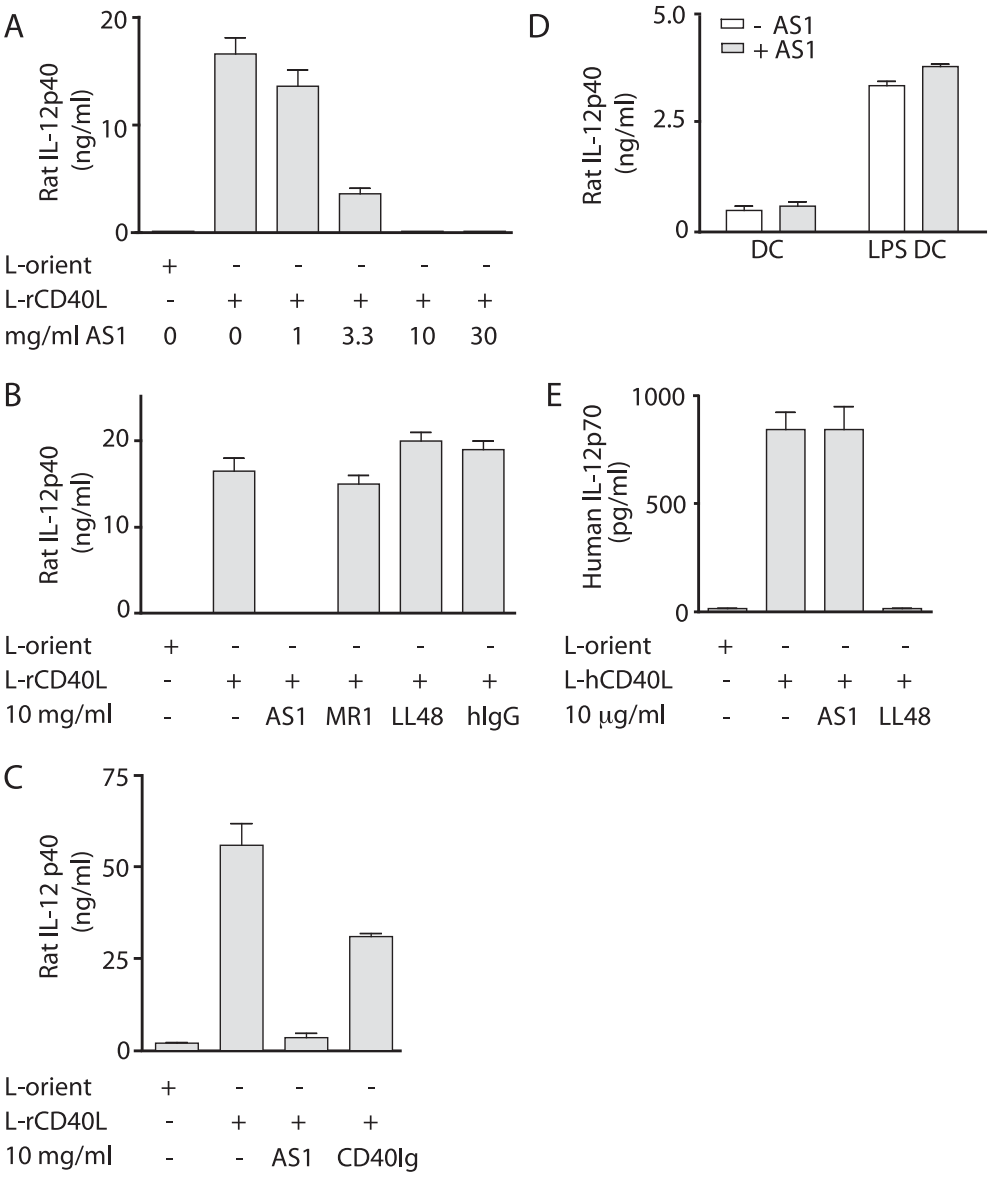

Figure 2. Only AS1 inhibits IL-12p40 production by CD40L stimulated DC.
A) LEW derived DC were stimulated by CD40L and cultured in the presence of various AS1 concentrations. L-orient was used as a negative control and L-rCD40L as a positive control B) LEW-derived DC were cultured in the presence of L-rCD40L and in the presence of 10 μg/ml AS1, MR1, LL48 or hamster IgG. C) LEW-derived DC were cultured in the presence of L-rCD40L in the presence or absence of 10 μg/ml AS1 or CD40Ig. D) LEW-derived DC matured with 500 ng/ml LPS were cultured in the absence (white bars) or presence (gray bars) of AS1. E) Human DC were stimulated by L-hCD40L in the presence or absence of AS1 or LL48 (10 μg/ml). Rat IL-12p40 or human IL-12p70 production was determined by ELISA. Results are mean ± SD of duplicate cultures representative of three independent experiments

completely inhibit this IL-12 production in a dose dependent fashion. Maximal inhibition of IL-12 production was observed with 10 μg/ml of AS1 (**Fig. 2A**). Similar concentrations of MR1, LL48 or hamster IgG were unable to inhibit the IL-12 production by CD40L-stimulated DC (**Fig. 2B**). In addition, human CD40-Ig partially inhibited the IL-12 production by DC when cocultured with L-rCD40L cells (**Fig. 2C**).

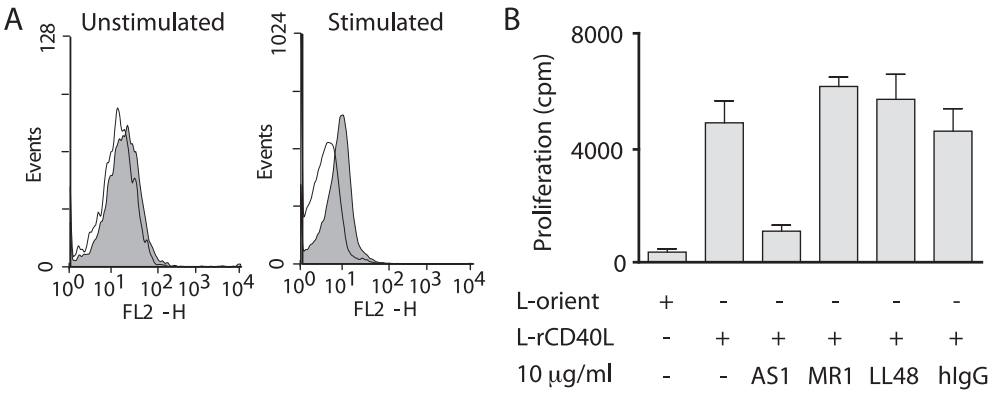

Figure 3. Inhibition of CD40L-induced B cell proliferation by AS1.
A) LEW T cells ($1x10^5$) were stimulated in 96-well flat-bottom plates using plate-bound anti-CD3 and soluble anti-CD28 for 24 hours. Stimulated T cells were analyzed for their binding of AS1 using flow-cytometry, as a control unstimulated T cells were used. Specific staining is represented by filled histograms, open histograms represent basal fluorescence level after staining with only PE-labelled goat anti-hamster secondary antibody. B) LEW-derived splenocytes ($1x10^5$) were cultured for 5 days in the presence of irradiated L-orient or L-rCD40L in a 10:1 ratio in the presence or absence of 10 μg/ml AS1, MR1, LL48 or hamster IgG. Proliferation was measured by incorporation of ^3H thymidine at day 5. Results shown are the mean ± SD of three experiments

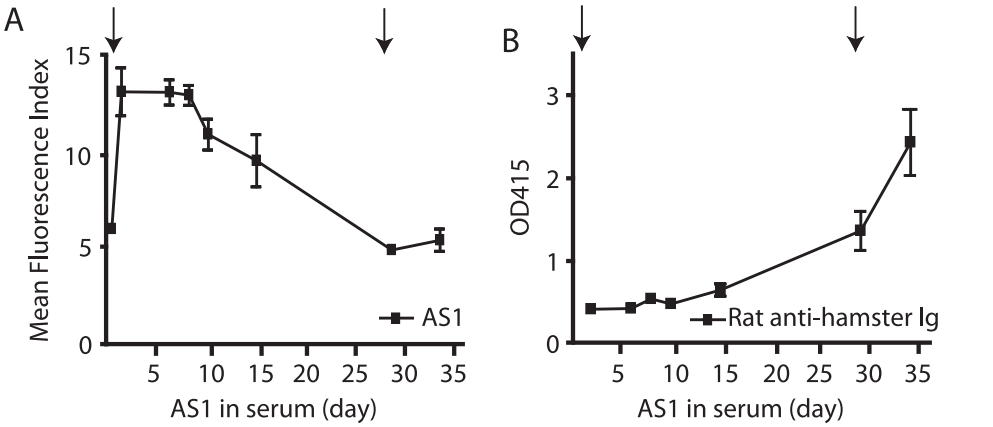

Figure 4. High levels of AS1 in serum during the first week after injection.
A) Sera derived from LEW rats (n=4) injected i.p. with AS1 (0.75 mg) on day 0 and 29 as indicated with an arrow, were collected at indicated time points and incubated with L-rCD40L cells. Day 29 serum was collected 6 hours after second AS1 injection. Binding of AS1 to rat CD40L was determined by flow cytometry. B) Presence of rat anti-hamster Ab were measured by ELISA. The same sera were used for the detection of AS1 and rat anti-hamster response.

To determine whether AS1 was specifically blocking the rat CD40-CD40L interaction, the effect of AS1 was examined on DC activated by another stimulus. DC stimulated by LPS showed production of IL-12, which was not inhibited in the presence of AS1 (**Fig. 2D**). In addition, CD40L-stimulated human monocyte-derived DC were not inhibited in their IL-12 production in the presence of AS1 whereas the anti-human CD40L Ab LL48 completely prevented IL-12 production (**Fig. 2E**). Thus AS1 specifically inhibits rat CD40-CD40L interaction.

Next we wanted to study whether AS1 exerted similar blocking capacities in vivo. It is known that in vivo interaction of CD40 on B cells with CD40L on T cells results in proliferation and production of Ab. We first explored whether AS1 could recognize native CD40L on stimulated T cells. Unstimulated and CD3/CD28 stimulated T cells were stained with AS1 and binding of AS1 was only detected on stimulated T cells (**Fig. 3A**). To investigate whether the CD40-CD40L interaction could be blocked, we measured B cell proliferation after CD40L stimulation in the presence or absence of AS1. Rat splenocytes were stimulated with rat CD40L and the effect of AS1, MR1, LL48 or hamster IgG was studied. CD40L induced strong B cell proliferation, which was inhibited by AS1. In contrast, similar concentrations of MR1, LL48 or hamster IgG showed no effect on the proliferation of CD40L-stimulated B cells (**Fig 3B**).

Blocking capacity of AS1 in vivo

To study the effect of AS1 in vivo we first investigated the time frame in which AS1 can be used in vivo. AS1 was measured in sera derived from rats injected i.p. with AS1 at day 0 followed by a second injection at day 29. L-rCD40L cells were incubated with sera obtained from various time points to measure the presence of AS1 by flow cytometry. AS1 levels were indeed detected in serum after the first injection, these levels were relatively stable until 8 days post injection. After 8 days AS1 levels started to decrease in serum (**Fig. 4A**). Interestingly, no detectable levels of AS1 were measured in sera taken 6 hours after a second injection on day 29.

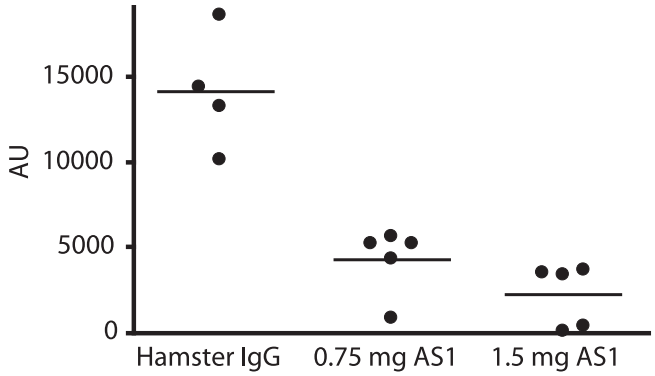

Figure 5. AS1 blocks OVA induced B cell response in vivo.
LEW rats were immunized s.c. with OVA in CFA. At day -1 and 3 various doses of AS1 or 1.5 mg hamster IgG were injected. Anti-OVA IgG levels were detected in day 10 serum using ELISA. Each dot represents one rat (* $p < 0.05$).

Since AS1 can be detected in serum 5 hours after injection, a rapid elimination of AS1 from the circulation after the second injection could have occurred. The induction of an anti-hamster response might explain these results. The same sera were used to measure the induction of a rat anti-hamster antibody response. We found a detectable increase in rat anti-hamster immunoglobulins from day 14 onwards (**Fig. 4B**).
To elucidate the ability of AS1 to inhibit the CD40L stimulatory capacity in vivo, rats were immunized with OVA in CFA on day 0. Groups (n=5) of rats were treated with 1.5 mg hamster IgG, 0.75 mg or 1.5 mg AS1 at day -1 and 3. At day 10 rats were sacrificed and IgG anti-OVA levels were determined in serum. Rats immunized with OVA and hamster IgG exhibited high levels of IgG anti-OVA in their serum. Treatment of rats with different doses of AS1 inhibited the IgG anti-OVA response dependent on the dose of AS1 given (**Fig. 5**). Similar results were obtained after immunization with keyhole limpet hemocyanin (KLH) (data not shown).

Discussion

In previous studies it has been shown that mAb against a number of murine molecules, including anti-mouse CD40L (MR1), are successfully generated using Armenian hamsters [6, 15, 16]. As the homology between mouse and rat CD40L is very high, we used Armenian hamsters for the generation of an anti-rat CD40L mAb. We identified a positive clone, AS1, which was expanded and characterized. In vitro and in vivo experiments showed that AS1 was able to specifically block CD40L induced activation.
We showed that both AS1 and MR1 were able to bind to rat and not to human CD40L. However, functional experiments demonstrated that although MR1 was able to bind to rat CD40L, it was not able to block the CD40-CD40L interaction in vitro, even not in high concentrations (data not shown). In contrast to MR1, AS1 completely blocked CD40L-induced maturation of DC and CD40L-induced B cell proliferation. These in vitro data imply that AS1 is able to block the CD40-CD40L interaction in rats. To determine the therapeutic window of AS1 in vivo, we monitored the presence of AS1 after injection and investigated the development of the rat anti-hamster response. We found that AS1 is present in serum at least until day 8. From day 14 an anti-hamster response was detectable, which lead to a rapid decrease of AS1 levels in serum. A second injection of AS1 at day 29 resulted in a rapid clearance of AS1 from circulation. Together, this indicates that AS1 can be used in vivo and will be most effective during the first week after injection.
To confirm the blocking effect of AS1 in vivo, we studied the effect of AS1 on the induction of OVA or KLH-specific Ab, which in mice were shown to be dependent on the interaction between CD40-CD40L [6]. We showed that OVA injected into rats resulted in high levels of anti-OVA IgG. Treatment of rats with AS1 inhibited the OVA response, indicating that the AS1 mAb is not only functionally blocking the CD40-CD40L interaction in vitro, but also in vivo.
In rat kidney transplantation models it has recently been shown that pretreatment of recipients with a hamster anti-rat CD40L mAb (AH.F5) prolonged survival [11-13, 17]. In addition, in more stringent primate transplantation models blockade of the CD40-

CD40L pathway was shown to be more promising than CTLA4-Ig, indicating that CD40L specific antibodies may play an important role in the induction of tolerance in humans [18, 19]. The mechanism of action of CD40L specific antibodies including AH.F5 and AS1 remains to be elucidated. It has been demonstrated that in mice binding of the hamster antibody MR1 to CD40L results in a selective depletion of activated T cells via a Fc-dependent mechanism rather than by co-stimulation blockade [20]. Since mice and rats are closely related, it is likely that a similar mechanism might be involved when AH.F5 or AS1 are applied in rats, although interaction of these hamster antibodies with rat Fc receptors still has to be demonstrated. The depletion of activated T cells could have a beneficial outcome for the immune response by creating a new balance in favor of regulatory versus effector T cells [20, 21].

In conclusion, we showed that the generated anti-rat CD40L mAb AS1 was able to block the CD40-CD40L interaction in vitro and in vivo. Studying this mAb in various rat models may therefore provide more insight into the role of CD40-CD40L interaction in these models and further support the identification of CD40-CD40L as therapeutic targets in human diseases.

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