Monoclonal Antibody Targeting the CD154 Cleavage Site Inhibits CD40-Dependent and -Independent Cleavage of CD154 from the Cell Surface

Suzanne Salti, Loubna Al-Zoobi, Youssef Darif, Ghada S. Hassan and Walid Mourad

ImmunoHorizons 2021, 5 (7) 590-601
doi: https://doi.org/10.4049/immunohorizons.2100062
http://www.immunohorizons.org/content/5/7/590

This information is current as of August 6, 2021.

References  This article cites 42 articles, 13 of which you can access for free at:
http://www.immunohorizons.org/content/5/7/590.full#ref-list-1

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://www.immunohorizons.org/alerts
Monoclonal Antibody Targeting the CD154 Cleavage Site Inhibits CD40-Dependent and -Independent Cleavage of CD154 from the Cell Surface

Suzanne Salti, Loubna Al-Zoobi, Youssef Darif, Ghada S. Hassan, and Walid Mourad
Laboratoire d’Immunologie Cellulaire et Moléculaire, Centre de Recherche du Centre Hospitalier de l’Université de Montreal, Montreal, Quebec, Canada

ABSTRACT
In addition to the membrane-bound molecule, soluble CD154 (sCD154) is also detected at high levels in the medium of activated T cells and platelets and in the serum of patients suffering from different inflammatory diseases. This sCD154 is the result of cleavage of the full-length molecule between the glutamic acid residue at position 112 (E112) and methionine at position 113 (M113) and can be derived from the intracellular milieu and from cleavage of cell surface molecules. We have recently reported that substitution of both E112 and M113 by alanine inhibits intracellular and CD40-induced membrane cleavage of CD154 and procures to CD154 an increased biological function as compared with cleavable CD154. Thus, in this study, and in the aim of developing tools inhibiting cleavage of CD154 from the cell surface, we generated a panel of anti-human CD154 mAbs. One of the derived mAbs that did not alter the binding of sCD154 to CD40, named in this study Clone 8 mAb, totally lost its binding activity against cells expressing CD154 mutated at its E112 and M113 residues. Treatment with Clone 8 mAb was shown to completely abolish CD40-dependent and -independent cleavage of CD154 from the cell surface. Our study is highlighting the development and characterization of an innovative therapeutic tool capable of inhibiting the release/cleavage of CD154 from cells and thus maintaining its availability on the cell surface and the high probably of increasing its potency as an activator of CD40-induced responses.

INTRODUCTION
CD154, also known as CD40L, is a type II transmembrane glycoprotein, with a molecular mass of 33–39 kDa, belonging to the family of TNFs (1, 2). Its coding gene is located on chromosome X (3) and was initially thought to be transiently expressed only on activated CD4+ T cells (2) but has been later identified on various cell types such as mast cells, basophils, and activated platelets (4). In addition to the membrane-bound molecule, CD154 can be found in a soluble form (sCD154) as an 18-kDa fragment without being expressed on the cell surface, resulting from an intracellular enzymatic cleavage between the glutamic acid residue at position 112 (E112) and methionine at position 113 (M113) (5, 6). Recently, we have demonstrated that membrane-bound CD154 can also become soluble upon its interaction with CD40 in a mechanism mediated by ADAM-10 and ADAM-17 metalloproteinases (7). Like other members of the TNF family, the two forms of CD154, soluble and membrane-bound, have a trimeric structure that is necessary for its biological activity (6, 8–10).

CD154 exerts a broad range of biological activities by interacting with five different receptors, CD40 (2), αIIbβ3 (11), α5β1 (12), αMβ2 (13), and αvβ3 (14). Its classical receptor, CD40, is a 45- to 50-kDa type I glycoprotein belonging to the TNFR family that is expressed constitutively on non-Hodgkin’s lymphoma and chronic lymphocytic leukemia cells (15), B cells,
dendritic cells, macrophages, basophils, platelets, epithelial and endothelial cells, fibroblasts, and vascular smooth muscle cells (16). The biological importance of the CD154/CD40 interaction in immune responses was initially described in patients suffering from the X-linked hyper IgM syndrome (17). These patients, with mutations in the gene encoding CD154, abrogating its interaction with CD40, present a defect in Ig class switching and a susceptibility to various infections (18). The CD154/CD40 dyad is at the basis of the interaction between T cells and APCs and is thus highly implicated in the development and/or initiation of several autoimmune diseases, cardiovascular complications, and cancer therapy (19). The interaction of sCD154 with the other receptors was also shown to be of high biological significance and to have been involved in various inflammatory responses and events (12, 13, 20). CD154 interacts with its various receptors via distinct residues, and such interaction, particularly that of the CD154/CD40 dyad, results in a bidirectional signaling in CD154- as well as in receptor-positive cells (21, 22). However, cleavage of CD154 from the cell membrane is a privileged property of CD40 (7).

Our group has recently demonstrated that cleavage-resistant CD154 mutant (mutated at the cleavage sites E112 and M113) exhibited an increased biological function as compared with the cleavable CD154 (23). These findings solicited our interest in developing tools to inhibit cleavage of CD154 from the cell surface. In this study, we developed a panel of mAbs directed against CD154, and one of them, named Clone 8 mAb, lost its binding activity against cells expressing the CD154 double-mutant described above and inhibits CD154 cleavage from the cell surface. This mAb will probably represent an exquisite therapeutic tool that could be used in various diseases for which upregulating CD154 and its immune functions are implicated in disease resorption and treatment.

**MATERIALS AND METHODS**

**Cell lines**

Mouse connective tissue fibroblasts (LTK), human embryonic kidney cells (HEK 293), Jurkat E6.1 (human lymphoblastic T cells), D1.1 [a subclone derived from Jurkat E6.1 that constitutively expresses human CE154 (hCD154) (24)], and the SP2 (mice myeloma B cells) were obtained from American Type Culture Collection (American Type Culture Collection, Manassas, VA), and maintained in RPMI 1640 supplemented with 5% heat-inactivated FBS (Wisent, Saint-Bruno, QC, Canada), 1% L-glutamine, penicillin, and streptomycin (PSG; Life Technologies, Burlington, ON, Canada).

**Plasmids and mutagenesis**

Human CD154 wild-type (WT) and its mutant CD154-EM113/AA were generated as we previously described (23) using the plasmid pCDNA4-TO-myc-HisA Zeocin (Invitrogen). HEK 293 cells stably transfected with the mutant were generated by the method of calcium phosphate and sorted using the anti-CD154 SC8 mAb, known to completely block the CD154/CD40 interaction (25) and according to the crystal structure of its Fab/CD154; it is directed against residue located far from the cleavage site (26). Sorted cells were then cultured in the presence of Zeocin (InvivoGen), a selective agent, at 100 μg/ml.

**Abs and reagents**

The SC8 hybridoma, obtained from American Type Culture Collection (Manassas, VA), was cultured in RPMI 1640 supplemented with 5% FCS. The mAb derived from this hybridoma was purified using G protein affinity columns and was biotin labeled according to the method provided by Pierce Biotechnology (Rockford, IL). The mouse anti-human CD3–Alexa Fluor 488 is from BD Biosciences (Mississauga, ON, Canada). Goat anti-mouse Alexa Fluor 488 (GAM-Alexa-488), streptavidin–Alexa Fluor 488, and streptavidin–PE come from Invitrogen/Life Technologies (Burlington, ON, Canada). Human sCD154 and soluble CD40 (sCD40) were generated in our laboratory, as described previously (22, 27). Alexa Fluor 488 labeling of sCD154 (sCD154-Alexa-488) and of sCD40 was performed according to the manufacturer’s instructions (12).

**mAb production**

The production of mouse mAbs against hCD154 was carried out as previously described (28). The method consists at immunizing BALB/c mice several times (days 0, 21, and weekly for three consequent weeks) with cell lysates of LTK-transfected cells. Spleen cells of sacrificed mouse were purified and fused with immortalized myeloma cells (SP2) by polyethylene glycol and maintained in 96-well plates in the presence of a selective agent, hypoxanthine, aminopterin, and thymidine. Two weeks later, supernatants were collected and tested for the production of IgGs by ELISA. Cells from positive wells were propagated in 24-well plates, and after 10 d, positive wells were tested by flow cytometry to verify their production of specific Abs against hCD154. The hybridomas producing mAbs directed against hCD154 were cloned twice, then the isotype of each clone was determined by a specific ELISA. Clones were then propagated in vitro, and mAb purification from cell supernatants was carried out using the G protein affinity columns, according to the manufacturer’s instructions (GE Healthcare Bio-sciences, Mississauga, ON, Canada).

**Cell stimulation for assessing cleavage**

Jurkat E6.1-hCD154 or HEK 293 cells transfected with CD154 were cultured alone or with Ramos CD40+ B cells for 1 h or 24 h, respectively, at 37°C in the presence of isotype controls Clone 8 or the 82102 mAb (an anti-human CD40 that blocks the CD40/CD154 interaction). CD154 cleavage was determined by measuring the residual cell surface expression of CD154 by flow cytometry analysis using SC8 biotinylated mAb and anti-human CD3–Alexa 488 mAb followed by streptavidin–PE. The concentration of sCD154 in the cell supernatant was measured.
by ELISA, as described in the datasheet (R&D Systems, Minneapolis, MN).

Evaluating CD154 expression on superantigen-stimulated PBMC

CD154 expression was assessed on human PBMCs. Briefly, blood was withdrawn in acid/citrate/dextrose-anticoagulated tubes from donors who gave their written consent according to the guidelines established by the Centre de Recherche du Centre Hospitalier de l’Université de Montréal ethical committee. PBMCs were purified using the Ficoll separation gradient (Wisent). PBMCs at a concentration of 1 x 10^6 cells/ml were then preincubated with Clone 8 or 82102 mAbs for 1 h at 37°C, then stimulated with a mix of superantigens (SAgs) (SEA-SEB and TSST-1) for 3, 6, and 24 h at 37°C. The concentration of sCD154 in the supernatant was measured by ELISA, as described in the datasheet (R&D Systems, Minneapolis, MN).

Western blotting

HEK 293 cells transfected with an empty vector (HEK 293–Vector) or cells expressing hCD154-WT were lysed for 1 h on ice in 40 μl of RIPA Lysis Buffer supplemented with protease inhibitors (Invitrogen). To test whether Clone 8 recognizes sCD154, 50 ng of sCD154 in 40 μl of RIPA Lysis Buffer was also used. Ten microliters of preheated sample buffer were added, and the mix was incubated at 95°C for 5 min. Proteins were separated by electrophoresis on 12% acrylamide gels and were transferred to PVDF membranes to be incubated with Clone 8 or 5C8 (mouse anti-hCD154, 1:1000) overnight at 4°C. After washing the membranes three times with TBS–TWEEN, we incubated them with HRP-conjugated goat anti-mouse Ab (1:20,000) for 1 h at room temperature. The membrane was washed with TBS–TWEEN, then incubated with an HRP substrate (Western Lightning Plus-ECL; PerkinElmer, Waltham, MA) in accordance with the manufacturer’s instructions to reveal the protein bands.

RESULTS

Generation of anti-hCD154 mAbs

We have generated a panel of mouse mAbs directed against hCD154 as previously described (28). These anti-hCD154 mAbs were first tested for their ability to recognize CD154 on LTK-transfected cells. The anti-hCD154 mAb 5C8 was used as a positive control. Ten hybridomas that specifically recognize CD154-transfected LTK cells but not vector-transfected ones were selected, cloned twice, and propagated in vitro. The ability of these selected clones to produce mAbs recognizing hCD154 expressed on the surface of HEK 293 or Jurkat E6.1 transfected with hCD154, the surface of D1.1 cells (clone derived from Jurkat E6.1 constitutively expressing CD154), and the surface of activated human T cells was then tested by flow cytometry. Table I summarizes the ability of these clones to recognize hCD154 expressed on all the tested cell types.

Selection of anti-hCD154 mAbs that do not interfere with CD154/CD40 interaction

Because our major goal is to generate mAbs directed against the cleavage site and capable of inhibiting CD40-induced CD154 cleavage from the cell surface, it was crucial to select those that do not interfere with the binding of CD154 to CD40. For this purpose, we first determined the isotype of our derived mAbs by specific ELISA (Table II) and purified them from cell supernatants using the G protein affinity columns. Clones producing mAbs that do not interfere with CD154/CD40 interaction were selected using three complementary approaches: 1) binding of sCD154-Alexa-488 to HEK 293 transfected with CD40, 2) binding of sCD40–Alex Fluor 488 to HEK 293 transfected with CD154, and 3) binding of sCD154 to sCD40-coated

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Binding to LTK-Vector</th>
<th>Binding to LTK-CD154</th>
<th>Binding to HEK 293-CD154</th>
<th>Binding to Jurkat D1.1</th>
<th>Binding to Jurkat E6.1-Vector</th>
<th>Binding to Jurkat E6.1-CD154</th>
<th>Binding to Activated T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5C8</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Clone 3</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Clone 4</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clone 5</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Clone 6</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clone 7</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Clone 8</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Clone 10</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Clone 11</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Clone 12</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Clone 14</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Specificities of the generated mAbs in recognizing hCD154 were expressed on the surface of different cell types are shown. LTK are mouse connective tissue fibroblasts (LTK-Vector are cells transfected with empty vector; LTK-CD154 are cells transfected with CD154). HEK 293 represents human embryonic kidney cells (HEK 293-CD154 are cells transfected with CD154). Jurkat E6.1 are human lymphoblastic T cells (Jurkat E6.1-Vector are cells transfected with empty vector; Jurkat E6.1-CD154 are cells transfected with CD154). Jurkat D1.1 is a subclone derived from Jurkat E6.1 constitutively expressing hCD154. These cells were stained for 30 min at 4°C with the various mAbs followed by 30 min at 4°C with GAM-Alexa-488, then analyzed by flow cytometry. The 5C8 mAb was taken as a control. Data presented are representative of five different experiments. Significant binding is represented by (+ + +) and no detectable binding is represented by (–).
96-well plates. In the first two approaches, CD40-transfected HEK 293 cells were incubated with sCD154-Alexa-488 and CD154-transfected HEK 293 cells were incubated with sCD40-labeled Alexa Fluor 488 in the presence or absence of the different anti-CD154 mAbs for 30 min at 4°C and analyzed by flow cytometry. In the third approach, sCD154 were added to CD40-coated 96-well plates in the presence of control mAb or anti-CD154 mAbs. The 5C8 mAb known to block the interaction of CD154 with CD40 was used as a control in all these experiments. Results in Table II show the effect of our mAbs on the CD154/CD40 interaction and clearly indicate that only Clone 6 mAb interferes with the CD154/CD40 interaction.

**Clone 8 mAb is directed against the residues required for CD154 cleavage**

To determine whether any of the derived mAbs is directed against the CD154 cleavage site, we first evaluated their recognition of our cleavage-resistant CD154 mutant (CD154-EM mutated at glutamic acid at position 112 and methionine at position 113) described previously (23). For this purpose, we transfected HEK 293 cells with CD154-WT and CD154-EM and assessed their binding to our above-generated mAbs using flow cytometry. In this study, we used the 5C8 mAb as a control because its recognition site is located far from the cleavage site and is capable of blocking the interaction of CD154 with CD40 (25, 26), making its ability to recognize the CD154 not impaired by the above mutation. Our data demonstrate that only Clone 8 mAb lost its ability to recognize the CD154-EM mutant (Table III, Fig. 1A), strongly suggesting that Clone 8 is directed against the major residues implicated in CD154 cleavage.

**Clone 8 mAb inhibits the spontaneous release of sCD154**

As Clone 8 is directed against the CD154 cleavage site, we hypothesize that this mAb would inhibit the cleavage of the molecule from the cell surface. In the first set of experiments and because it is well established that CD154 is expressed in a transient fashion on activated T cells, we studied the effect of Clone 8 mAb on the spontaneous release of CD154. For this

---

**TABLE II. Effect of the generated mAbs on the binding of CD154 to CD40**

<table>
<thead>
<tr>
<th>Anti-CD154 mAb</th>
<th>Subclass</th>
<th>Binding of sCD154 to HEK 293 CD40</th>
<th>Binding of sCD40 to HEK 293 CD154</th>
<th>Binding of sCD154 to CD40-Coated Wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>IgG</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>5C8</td>
<td>IgG2a</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Clone 3</td>
<td>IgG2b</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 4</td>
<td>IgG2b</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 5</td>
<td>IgG2a</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 6</td>
<td>IgG1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Clone 7</td>
<td>IgG2a</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clone 8</td>
<td>IgG2a</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 10</td>
<td>IgG1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 11</td>
<td>IgG1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 13</td>
<td>IgG1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 14</td>
<td>IgG2b</td>
<td>+++</td>
<td>+++</td>
<td>—</td>
</tr>
</tbody>
</table>

The ability of the generated mAbs to interfere with the binding of sCD154 to CD40 is outlined. HEK 293–CD154 and HEK 293–CD40 were incubated with sCD154 or with sCD40 in the presence of isotype control or specific Ab. High binding level is represented by (+++), medium level by (++), low level by (+), and complete inhibition of binding is represented by (−).

**TABLE III. Substitution of E112 and M113 at the CD154 cleavage site abolishes the ability of Clone 8 mAb to recognize the CD154 molecule**

<table>
<thead>
<tr>
<th>Anti-CD154 mAbs</th>
<th>Binding to HEK 293 CD154-WT</th>
<th>Binding to HEK 293 CD154-EM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5C8</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 3</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 4</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 5</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 6</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 7</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 8</td>
<td>+++</td>
<td>−</td>
</tr>
<tr>
<td>Clone 10</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 11</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clone 13</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Clone 14</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Recognition of hCD154-WT or hCD154 mutated at the cleavage site (CD154-EM) by the various mAbs using FACS analysis is outlined. Transfected HEK 293 were incubated for 30 min at 4°C with 1 μg/100 μl of the various mAbs followed by GAM-Alexa-488. The 5C8 mAb was taken as a control. Data presented are representative of five different experiments. High level of binding is represented by (+++), medium level by (++), low level by (+), and complete inhibition of binding is represented by (−).
purpose, hCD154-WT–transfected HEK 293 and Jurkat E6.1 cells were treated with isotype control or Clone 8 mAb for 24 h. Cell supernatants were then assessed for sCD154 by ELISA.

Treatment with Clone 8 mAb significantly inhibited the spontaneous release of sCD154 from both cell types (Fig. 1B), suggesting that the sCD154 usually detected in the medium of

FIGURE 1. Clone 8 mAb recognizes the glutamic acid residue at position 112 (E112) and the methionine at position 113 (M113) and inhibits the spontaneous release of sCD154.

(A) HEK 293 stably transfected with CD154-WT or the CD154-EM mutant were stained with anti-CD154 5C8 Clone 4 or Clone 8 mAbs followed by the GAM-Alexa-488 and analyzed by flow cytometry. (B) HEK 293 (0.5 × 10⁶ cells per well) and Jurkat E6.1 (10⁶ cells/well) stably transfected with vector or CD154-WT were cultured with Clone 8 mAb or an isotype control for 24 h at 37°C. Cell supernatants were collected and assessed by ELISA to quantify the concentration of sCD154. Results shown are mean values ± SD of five independent experiments. ***p < 0.001.
activated T cells or in the serum of patients with chronic inflammatory diseases can be also derived from a spontaneous cleavage of membrane-bound CD154.

**Clone 8 mAb inhibits membrane cleavage of CD154 induced upon polyclonal activation of T cells**

Induction and/or upregulation of CD154 can be triggered upon polyclonal stimulus or during cognate T/B cell interaction. To study the effect of Clone 8 mAb on the upregulation of CD154 and its release induced by the polyclonal activation of T cells, Jurkat E6.1 cells transfected with hCD154-WT were treated with isotype control or Clone 8 mAb and stimulated with PMA for 24 h. Membrane-bound CD154 was analyzed by flow cytometry, and sCD154 was analyzed by ELISA. Our results show that treatment with Clone 8 mAb significantly inhibited the release of sCD154 and maintained membrane-bound CD154 on the cell surface (Fig. 2A, 2B).

To rule out the possibility that the inhibiting effect of Clone 8 mAb toward the release of sCD154 is the result of a competition between the used mAbs and/or a steric hindrance, we performed the following experiments: 1) HEK 293 or Jurkat E6.1 cells stably transfected with CD154-WT were pretreated with mouse IgG (mIgG), Clone 8, or with 5C8 itself and incubated with biotinylated 5C8 and streptavidin–Alexa Fluor 488, then analyzed by FACS for the expression of CD154 on the cell surface (Fig. 3A shows that binding of Clone 8 mAb does not inhibit the binding of 5C8 to CD154); 2) sCD154 in the cell supernatant of PMA-stimulated Jurkat E6.1 transfected with CD154 was analyzed by ELISA after the addition of mIgG, Clone 8, or 5C8 mAb (Fig. 3B shows that adding Clone 8 mAb to the collected supernatants containing sCD154 did not alter the concentration of sCD154); 3) Western blot analysis (Fig. 3C) indicates that Clone 8 mAb is not capable of recognizing sCD154 involving residues 113–261.

**Clone 8 mAb inhibits CD40-induced cleavage of CD154 from the cell surface**

To verify the effect of Clone 8 mAb on CD40-induced cleavage of CD154 from the cell surface, coculture experiments using Jurkat E6.1 cells transfected with hCD154 and Ramos cells, a CD40-positive B cell line, were conducted. For this purpose, hCD154-transfected Jurkat E6.1 cells were treated with 2 µg of an isotype control or Clone 8 mAb for 20 min at 37°C, whereas Ramos cells (1 × 10⁶) were treated with isotype control or anti-CD40 mAb 82102, known to inhibit the CD154/CD40 interaction, prior to mixing the cells together for an additional 1 h. The expression level of CD154 on the cell surface was evaluated by two-color flow cytometry analysis, and the concentration of cleaved sCD154 was assessed by ELISA. Fig. 4A shows that treatment with Clone 8 significantly inhibited the cleavage of CD154 from the cell surface, induced by an interaction with CD40 as well as the spontaneous one (Fig. 4B). Similar results were obtained in the presence of the anti-CD40 82102 mAb used as a control (Fig. 4A, 4B).

**Clone 8 inhibits membrane cleavage of CD154 from SAg-stimulated cells**

It is well established that SAg mimics nominal Ag in initiating cognate T/B cell interaction and inducing CD154 expression on CD4-positive T cells in a β-chain V region-restricted fashion (29, 30). To further verify the inhibitory effect of Clone 8, PBMC were stimulated with SAg in the presence of isotype control, Clone 8 mAb, and anti-CD40 82102 mAb for 3, 6, and 24 h at 37°C (31). Next, we evaluated the level of CD154 expression on the surface of PBMCs by flow cytometry (Fig. 5A). Our results show that the surface expression of CD154 on PBMCs (on the T cell portion of these cells) is not decreased if PBMCs were treated with Clone 8 mAb. We also assessed the concentration of sCD154 in cell supernatants (Fig. 5B). Our ELISA data confirm those of the surface expression of the molecule obtained by flow cytometry, demonstrating that the Clone 8 mAb inhibits the spontaneous and the CD40-induced release/cleavage of CD154 from cells in a comparable way with the control used, the anti-CD40 82102 mAb.

Taken together, using three culture systems, we demonstrated that Clone 8 is capable of blocking the release/cleavage of CD154 from the surface of T cells.

**DISCUSSION**

CD154 plays a key role in the regulation of immune and inflammatory responses (4, 16, 32) and many autoimmune and inflammatory diseases that are characterized by increased levels of CD154 expression on the cell surface and elevated concentrations of sCD154 in patients sera (33). Like other members of the TNF family, CD154 is transiently expressed on activated T cells, and rapidly disappears from the cell surface (6). The sCD154 was initially thought to be mainly derived from intracellular cleavage (5); however, our recent study clearly demonstrated that it is also derived from a membrane-bound cleavage of CD154 (7). The biological role of sCD154 has not been well investigated, but it has been proposed that cleavage of CD154 attenuates the immune response. Our recent finding confirms this hypothesis (23), and most importantly, it indicates that cleavage of CD154 into sCD154 is a property required for its binding to the α5β1 integrin (34). An interest was created in defining how sCD154 is generated, with the aim of targeting this process ultimately for research or even therapeutic purposes. Thus, our work in this study outlines the generation of an anti-CD154 Clone 8 mAb directed against the CD154 cleavage site and demonstrates its ability to inhibit the spontaneous and the CD40-induced cleavage of CD154 from the cell surface without interfering with the interaction of CD154 with CD40.

The discovery of mAbs by Milstein and Köhler (28) in 1975 revolutionized the field of immunology. It has opened the door to interesting advances in the therapy of various autoimmune diseases and cancer immunotherapy. Taking
advantage of this approach, we generated a panel of mAbs directed against hCD154 that are capable of recognizing the molecule on the surface of different cell types in its conformational form. One of the generated mAbs, named Clone 8 mAb, completely lost its ability to recognize membrane-bound CD154 when the E residue at position 112 and the M at position 113 were substituted by A. Based on our recent study showing such substitution of these two residues at the cleavage site totally abolishes the spontaneous and the CD40-dependent cleavage of membrane-bound CD154 (23), it is therefore highly likely that binding of Clone 8 mAb to residues E112 and M113 completely abrogates the ability of ADAM-10 and ADAM-17 to recognize their substrate, the membrane-bound CD154 molecule.

The release and cleavage of CD154 has been previously assessed in T cells. It was initially reported that sCD154 encompassing residues M113–261, found in the supernatant of activated T cells and T cell clones, is an 18-kDa fragment derived from intracellular cleavage of CD154 without being expressed on the cell surface (6) and processed inside the microsomes (5). The same authors further reported that deletion of 12 aa (F111–A123) around the cleavage site abolished CD154 cleavage without altering CD40-induced responses (35). In contrast, Elmetwali et al. (36) have reported that deletion of 7 aa (S110–G116) abrogated CD154 cleavage and enhanced CD40-induced responses. In support of this latter observation, we have recently demonstrated that CD154 mutated at the cleavage site itself, namely the E112 and the M113 residues (CD154-EM), is resistant to being spontaneously released from cells or cleaved from their surface upon the interaction with CD40. Such cleavage-resistant membrane-bound CD154 was shown to trigger a significantly higher response in CD40-positive cells as compared with the cleavable form of the molecule (23). Indeed, the CD154 mutant resistant to cleavage upregulated the apoptotic response of susceptible B cell lymphoma cells as well as enhanced the proliferation and activation of immune cells (i.e., human B cells) (23). These observations strongly support a biological significance of our Clone 8 mAb, inhibiting CD154 cleavage from the cell surface and probably leading to a significant enhancement of CD40-mediated responses.

Generating an anti-CD154 mAb inhibiting CD154 cleavage will open the door for identifying the biological
The importance of this cleavage process. Thus, it was important to ensure that our anticleavage mAb did not interfere with the interaction of CD154 with CD40. The three complementary approaches used in the current study clearly show that Clone 8 mAb does not alter the interaction of CD154 with CD40. Indeed, our anti-CD154 mAb is aimed at enhancing the availability and thus the immune function of CD154 [as suggested by our previous work (23)] rather than at inhibiting its interaction with CD40. Not only that, the interaction of CD154 and CD40 is of particular importance at this point, as CD40 is the only one among CD154 receptors capable of inducing cleavage of the molecule, as previously shown by our group (7).

Our current study highlights the mere generation of an mAb that inhibits the cleavage of CD154 from the cell surface as well as its release from the intracellular milieu. Nevertheless, future studies (some of which are already ongoing) should characterize the biological importance of this mAb and its effect on various responses, including activation, proliferation, differentiation, and death of many types of immune as well as malignant cells.

FIGURE 3. Clone 8 mAb does not inhibit the binding of 5C8 to CD154 and fails to recognize sCD154-compensating residues 113–261.

(A) HEK 293 or Jurkat E6.1 stably transfected with CD154-WT were pretreated with mIgG, Clone 8 mAb, or with 5C8 mAb itself, followed by biotinylated 5C8 and streptavidin–Alexa488. Washed cells were analyzed by flow cytometry. (B) Clone 8 mAb was added for a period of 24 h to a supernatant derived from PMA (100 ng/ml)-stimulated Jurkat E6.1 cells stably transfected with CD154-WT prior to the sCD154 and quantified by ELISA. (C) Cell lysates of HEK 293-vector (HEK 293 Vect) or HEK 293 CD154 or recombinant of sCD154 compensating residues 113–261 were blotted using 5C8 mAb or Clone 8 mAb followed by HRP-conjugated goat anti-mouse Ab. Blots shown are a representative experiment of five independent ones.
Future studies will also be investigating the therapeutic efficiency of the Clone 8 mAb in animal tumor models, providing the proof-of-concept for its use in disease treatment. In fact, some disease conditions necessitate enhancing the function of CD154 for the disease to be alleviated, controlled, or cured. Indeed, the CD154/CD40 dyad has been implicated in antitumoral responses and is thus the target of several cancer immunotherapeutic approaches. Treatment with rCD154 or with agonistic Abs directed against its receptor CD40 has been assessed in animal models as well as in human clinical trials (37–39) and shown to have low feasibility (cells transfected ex vivo with CD154) or limited activation profile (anti-CD40 Abs...
Our study is offering an interesting and, probably, a highly efficient tool in this matter; mAbs that would inhibit the cleavage of CD154 from cells, maintaining the molecule on the cell surface and probably increasing its potency in inducing immune responses against cancer cells. It is important to mention, at this point, that the use of our mAb in the treatment of conditions benefiting from an enhanced immune/inflammatory function of CD154, such as antitumoral therapies of cancers, should be monitored for autoimmune and inflammatory complications, as is the case with most immunotherapeutic strategies (42).

In conclusion, based on research by several groups, including ours, showing that a CD154 mutated at its cleavage site and maintained on the cell surface exhibited more apoptotic functions against cancer cells, the cleavage site of CD154 was identified as a target for a better immune function against cancer cells. These interesting findings solicited us to develop our anticleavage mAb as a highly promising therapeutic tool in conditions requiring enhanced and more efficient CD154-mediated responses for their treatment. Cancers and malignancies figure on top of the list of these conditions and could highly benefit from our therapeutic agent in their treatment approaches.

DISCLOSURES

The authors declare no conflict of interest.
REFERENCES


