Any value of podocyte B7-1 as a biomarker in human MCD and FSGS?

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1IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Centro Anna Maria Astori, Science and Technology Park Kilometro Rosso, Bergamo, Italy; 2Unit of Nephrology and Dialysis, Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy; and 3Department of Biomedical and Clinical Sciences, University of Milan, Milan, Italy

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Novelli R, Gagliardini E, Ruggiero B, Benigni A, Remuzzi G. Any value of podocyte B7-1 as a biomarker in human MCD and FSGS? Am J Physiol Renal Physiol 310: F335–F341, 2016. First published December 23, 2015; doi:10.1152/ajprenal.00510.2015.—Minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) are the most common causes of nephrotic syndrome in children and in young adults. Relapsing MCD carries the risk of severe complications and prolonged immunosuppression, whereas FSGS remains largely untreatable and urgently needs more effective treatments. Recently, induction of B7-1 (CD80), an immune-related protein expressed by antigen-presenting cells, was observed in podocytes of MCD and FSGS patients, suggesting that B7-1 plays a role in the pathogenesis of these diseases, and hence that abatacept, a B7-1 inhibitor, could be a possible treatment. Since previous studies raised serious concerns regarding the reliability of immunohistochemical assays for B7-1 detection and the efficacy of B7-1 inhibitory treatments, we investigated B7-1 podocyte expression in MCD and FSGS patients. Using different primary antibodies and immunohistochemical assays, no significant up-regulation of podocyte B7-1 was detected in patients’ biopsies compared with controls. To further confirm our findings, we analyzed mice with adriamycin-induced nephropathy, a model of human FSGS, and mice injected with LPS as additional control. Podocyte B7-1 was not observed in mice injected with adriamycin or LPS either. In conclusion, since B7-1 is not induced in podocyte of MCD and FSGS patients, the antiproteinuric action of abatacept, if confirmed, may not be the result of an effect on podocyte B7-1.

Minimal change disease; focal segmental glomerulosclerosis; podocytes; B7-1
proteinuria in one MCD patient, and unchanged proteinuria levels in one patient with primary FSGS and in two of three patients with recurrent FSGS after B7-1 blockade therapy, despite the presence of B7-1 glomerular staining. Finally, Grellier et al. (13) did not detect B7-1 staining in the glomeruli despite the presence of B7-1 glomerular staining. Finally, patients with recurrent FSGS after B7-1 blockade therapy, levels in one patient with primary FSGS and in two of three proteinuria in one MCD patient, and unchanged proteinuria levels in one patient with primary FSGS and in two of three patients with recurrent FSGS after B7-1 blockade therapy, despite the presence of B7-1 glomerular staining. Finally, Grellier et al. (13) did not detect B7-1 staining in the glomeruli of five patients with recurrent FSGS in whom belatacept treatment was unsuccessful.

Given the crucial importance of this highly controversial topic and the tireless interest of the scientific community in searching for breakthroughs for the treatment of devastating kidney diseases, here we investigated whether B7-1 was truly expressed in podocytes of our MCD and FSGS patient cohorts.

MATERIALS AND METHODS

Patient enrollment. Renal tissues were obtained from biopsy specimens from 15 patients affected by MCD and 16 patients with FSGS who were admitted for diagnostic reasons to the Nephrology Unit of the Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy. Demographic, clinical, and histological parameters at the time of the renal biopsy were retrieved from the hospital database. Renal biopsy specimens from the uninvolved portion of the kidney, collected for tumor nephrectomy from four age- and sex-matched nonproteinuric patients, were used as normal controls. Written, informed consent to undergo renal biopsy evaluations was obtained from all the patients enrolled in the study.

Experimental models. Male Balb/c mice (Charles River Laboratories Italia, Calco, Italy) were injected in the tail-vein with a single dose of adriamycin (ADR) (10.5 mg/kg; Pfizer Italia, Latina, Italy), as described. ADR-injected mice (n = 4) were euthanized 4 wk after disease induction. Healthy mice euthanized at 4 wk (n = 4) served as controls. Proteinuria was determined by the Coomassie method using a Cobas Mira auto-analyzer (Roche Diagnostic Systems, Basel, Switzerland). Male C57Bl/6 mice were obtained from Charles River Laboratories Italia (Lecco, Italy). Lipopolysaccharide (LPS; 400 μg/mouse; O111:B4; Sigma-Aldrich, St. Louis, MO) was intraperitoneally injected in mice (26–28 g body wt), which were euthanized 24 h after injection (n = 3). Mice injected with saline served as controls (n = 3). Renal function was measured by serum blood urea nitrogen (Reflotron test; Roche Diagnostics, Basel, Switzerland). Animal care and treatment were in accordance with institutional guidelines, in compliance with national (Decreto Legislativo n.116, Gazzetta Ufficiale suppl 40, 18 febbraio 1992, Circolare n.8, Gazzetta Ufficiale 14 luglio 1994) and international (EEC Council Directive 86/609, OJL358-1, December 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996) laws and policies. Animal studies were approved by the institutional animal care and use committees of Mario Negri Institute, Milan, Italy. Animals were

Table 1. Clinical parameters and immunohistological evaluations in MCD and FSGS patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age Onset, yr</th>
<th>Age Biopsy, yr</th>
<th>Creatinine Baseline, mg/dl</th>
<th>24-h Urinary Protein, g/24 h</th>
<th>B7-1 Immunofluorescence Analysis, %</th>
<th>B7-1 Immunohistochemical Analysis Score</th>
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<td>Primary MCD</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>M</td>
<td>1.7</td>
<td>34.9</td>
<td>0.85</td>
<td>0.15</td>
<td>0.77 ± 0.22</td>
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</tr>
<tr>
<td>2</td>
<td>M</td>
<td>2.8</td>
<td>43.6</td>
<td>0.83</td>
<td>0.07</td>
<td>0.65 ± 0.27</td>
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<td>3</td>
<td>M</td>
<td>3.5</td>
<td>19.3</td>
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<td>11.52</td>
<td>0.42 ± 0.12</td>
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<tr>
<td>4</td>
<td>M</td>
<td>5.7</td>
<td>26.4</td>
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<td>0.27</td>
<td>1.84 ± 0.55</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>21.5</td>
<td>32.8</td>
<td>0.86</td>
<td>6.78</td>
<td>0.79 ± 0.15</td>
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<tr>
<td>6</td>
<td>M</td>
<td>21.7</td>
<td>23.1</td>
<td>0.72</td>
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<td>0.94 ± 0.15</td>
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<td>9.43</td>
<td>0.29 ± 0.02</td>
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<td>9</td>
<td>F</td>
<td>34.3</td>
<td>34.6</td>
<td>0.86</td>
<td>8.50</td>
<td>0.84 ± 0.02</td>
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<td>1.04</td>
<td>6.23</td>
<td>0.77 ± 0.10</td>
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<tr>
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<td>0.74 ± 0.07</td>
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<td>1.49 ± 0.49</td>
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<td>71.2</td>
<td>1.01</td>
<td>12.48</td>
<td>1.03 ± 0.29</td>
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<tr>
<td>Secondary FSGS</td>
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<tr>
<td>14</td>
<td>M</td>
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<td>1.32</td>
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<td>16</td>
<td>M</td>
<td>64.4</td>
<td>65.4</td>
<td>6.61</td>
<td>9.28</td>
<td>1.05 ± 0.23</td>
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</tr>
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</table>

Immunofluorescence analysis values are means ± SE. MCD, minimal change disease; FSGS, focal segmental glomerulosclerosis; M, male; F, female. N/A, frozen sample not available.
housed in a constant-temperature room with a 12:12-h dark-light cycle and fed a standard diet.

**Immunofluorescence and immunoperoxidase analysis on renal tissue.** Snap-frozen renal specimens of MCD and FSGS patients and mice were used for immunofluorescence analysis. Snap-frozen kidney biopsies were cut in 3-μm sections and air-dried for 10 min at room temperature. Sections were fixed in cold acetone for 10 min and then washed in PBS twice for 5 min. After nonspecific sites were blocked with 1% bovine serum albumin (BSA), slides were incubated with polyclonal goat anti-human B7-1 (diluted 1:200; R&D Systems, McKinley Place, Minneapolis, MN; catalog number AF140), or polyclonal goat anti-mouse B7-1 (1:100; R&D Systems; catalog number AF740), or monoclonal hamster anti-mouse B7-1 (1:20; clone 16-10A1, BD Pharmingen, San Diego, CA; catalog number 553766), and mouse anti-human podocalyxin (PDX; 1:150, gift from Prof. Robert Atkins, Department of Nephrology, Monash Medical Centre, Clayton, VIC, Australia), or rabbit anti-mouse PDX (1:100, Novus Bio, Southpark Way, Littleton, CO), or mouse anti-human CD20 (1:100, Dako, Glostrup, Denmark), or hamster anti-mouse CD11c (1:20, BD Pharmingen), followed by the specific Cy3- and FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), previously exposed to either BSA or strain-specific murine serum. On human biopsies, we used a human IgG-adsorbed secondary antibody. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Milan, Italy), and the renal structure with lectin fluorescein wheat germ agglutinin (Vector Laboratories, Burlingame, CA). Negative controls were obtained by omitting the primary antibody on adjacent serial sections. Fluorescence was examined using an inverted confocal laser scanning microscope (LS 510 Meta, Carl Zeiss, Jena, Germany). Quantification of the B7-1-positive glomerular area was performed in all glomeruli found in human biopsies and expressed as a percentage of positive area on total glomerular tuft area. For B7-1 immunoperoxidase staining, Duboscq-Brazil-fixed paraffin-embedded human kidney sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling sections in a decloaking chamber (DC NxGen 220V, Biocare Medical) for 15 min at 110°C with DIVA decloaker buffer (Biocare Medical), then sections were incubated for 5 min with peroxidased 1 (Biocare Medical, Concorde, CA) to quench endogenous peroxidase. After blocking for 5 min with background punisher (Biocare Medical), sections were incubated with monoclonal mouse anti-human B7-1 (1:50; clone 37711, R&D Systems, catalog number MAB140), followed by MACH4 HRP-polymer kit (Biocare Medical) and diaminobenzidine (Biocare Medical) substrate solution.

**Immunofluorescence analysis of LPS-activated human splenocytes.** Human splenocytes (routinely collected from kidney donors at our Transplant Research Centre for immunological studies) were cultured with 20% male antibody human serum (resting condition) for 72 h in RPMI-1640 medium (Invitrogen, Gaithersburg, MA), supplemented with 20% male antibody human serum (resting condition) or with 50 μg/ml LPS (from E. coli D26:B6, Sigma Aldrich), as previously described (9). After incubation, cells were washed, cytocentrifuged on glass slides, and then fixed in 2% paraformaldehyde and 4% sucrose for 5 min. After blockade of nonspecific binding sites with 1% BSA, cells were incubated over night with goat anti-human B7-1 antibody (1:50, R&D Systems AF140), followed by rabbit anti-goat Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Nuclei were stained with DAPI (Sigma-Aldrich), and negative controls were obtained by omitting primary antibody. Fluorescence was examined by an inverted confocal laser scanning microscope (LS 510 Meta, Carl Zeiss).

**Statistical analysis.** Results are expressed as mean values ± SE. Data were analyzed using t-test for unpaired data. P < 0.05 was considered a statistically significant difference.

**RESULTS AND DISCUSSION**

We analyzed B7-1 protein expression in the kidney biopsies of 15 patients with diagnosed MCD and 16 with FSGS. Four uninvolved portions of kidneys collected from tumor nephrectomy samples served as controls. Clinical features of the analyzed patients are displayed in Table 1. In our cohort, 11 patients with MCD and 13 with FSGS had primary renal disease, while 4 MCD patients and 3 with FSGS had a secondary recognized cause of nephrotic syndrome, in most cases represented by chronic infections or hematological diseases.

Four subjects with MCD and two with FSGS experienced pediatric disease onset. Among MCD patients, five were in steroid-induced complete remission (urine proteins <0.3 g/day) at the time of the biopsy, while the remaining 10 patients had nephrotic syndrome, despite two of them apparently having nonnephrotic proteinuria levels (patient 10, 1.25 g/day, and patient 13, 2.7 g/day). Conversely, all FSGS patients were proteinuric at the time of biopsy: 14 patients had severe nephrotic syndrome, and only 2 had nonnephrotic proteinuria. As regards steroid sensitivity, all 11 primary MCD patients received steroids and had achieved remission; of these, 8 became steroid dependent over follow-up. FSGS patients were more clinically heterogeneous: 7 of the 13 patients who had received steroids were steroid resistant, and 4 of the 6 steroid-sensitive patients developed steroid dependency. Snap-frozen biopic renal specimens of subjects belonging to our patient cohort were stained using polyclonal goat anti-human B7-1 through indirect immunofluorescence, as previously described (2, 9). B7-1 was completely absent in glomeruli of all control subjects (Fig. 1A). In three MCD patients, no glomerular B7-1 expression was found (Fig. 1B), while few traces of B7-1 were observed in the glomeruli of the remaining subjects (Fig. 1D, inset). The quantification of B7-1 staining in MCD patients’ glomeruli revealed that only an average of 0.54 ± 0.12% (mean value ± SE) of the glomerular area was positive for B7-1 (Table 1). No differences in glomerular B7-1 expression were observed between patients in remission and those relapsing, or between those with pediatric or adult onset of the
disease. The same observations were made in FSGS patients’ biopsies. Two subjects were completely negative for glomerular B7-1, while in the remaining patients granular traces of B7-1 staining were occasionally detected along the periphery of the glomerular capillary loops (Fig. 1C). The quantification of B7-1 staining in all glomeruli found in FSGS biopsies revealed that it occupied a mean of 0.73 ± 0.11% of the tuft area, with a maximum of 1.49 ± 0.49% observed in one patient (Table 1). Primary and secondary FSGS patients displayed comparable glomerular B7-1 staining independent of steroid sensitivity. Glomerular staining that we quantified in frozen samples from both MCD and FSGS patients was often the result of an automatic subtraction (made using ImageJ software) of the signal obtained on the consecutive section treated by 10.220.33.6 on October 14, 2017 http://ajprenal.physiology.org/ Downloaded from

Fig. 2. Glomerular B7-1 is not induced in adriamycin (ADR)- and LPS-injected mice. A–C: representative images of B7-1 immunofluorescence staining using the pAb and mAb (red) in Balb/c mice injected or not with ADR. Nuclei were stained by DAPI (blue). A: B7-1 was not expressed in glomeruli of control (CTR) Balb/c mice. B and C: no B7-1 induction was observed in podocytes of ADR-injected Balb/c mice, even though B7-1-positive infiltrating inflammatory cells were found in the same specimens (C; arrowheads). Renal structures were stained with fluorescein WGA (green). D: co-staining of B7-1 staining with PDX (green) showed that B7-1 was not expressed by podocytes of ADR-injected mice. E: B7-1-positive (green) inflammatory cells also expressed CD11c (red) in ADR-treated murine renal samples. F–H: representative images of double staining of B7-1 with PDX or CD11c in C57BL/6 mice injected or not with LPS. B7-1 was not upregulated in podocytes of control (F) and LPS-injected mice (G). Some interstitial inflammatory cells were positive for B7-1 (G and H) and also expressed CD11c (H). Bar: 20 μm.
with the secondary antibody alone (negative control), and, in any case, it was so weak as to appear nonspecific. The co-
staining with PDX, a podocyte-specific marker, further con-
ﬁrmed that podocytes did not express B7-1 in either MCD or
FSGS patients (Fig. 1, D and E). The reliability of the primary
antibody used for human B7-1 detection was veriﬁed analyzing
human splenocytes that, when exposed to LPS, are a good
source of B7-1, as previously described (21). The positive
staining we observed in LPS-activated human splenocytes
unequivocally demonstrated that the primary antibody was
indeed speciﬁc for human B7-1 (Fig. 1F). Moreover, B7-1-
positive interstitial inﬂammatory cells (Fig. 1, B, C, G, and H,
arrowheads and insets) and injured epithelial tubular cells (Fig.
1, B and G) that could acquire antigen-presenting function (24)
found in patients’ biopsies attested that the B7-1 epitope was
not degraded in the analyzed samples. As expected, double
immunostaining revealed that B7-1-positive inﬁltrating inﬂam-
matory cells also expressed the B cell marker CD20 (Fig. 1, G
and H, arrowheads and insets) (6). These ﬁndings further
conﬁrmed the speciﬁcity of the employed antibodies and the
reliability of our immunostaining assays for B7-1 detection
when and where it is truly expressed.

To conﬁrm these observations, another assay for B7-1 de-
tection was performed. Dubosq-ﬁxed and parafﬁn-embedded
renal biopsy samples of all MCD and FSGS patients were
stained with a monoclonal mouse anti-human B7-1 antibody
using a previously described immunoperoxidase protocol with
antigen retrieval (9, 17). B7-1 was not expressed in glomeruli
of all analyzed patients (Fig. 1, J and K), even though clearly
detectable interstitial inﬂammatory cells and tubular epithelial
cells were strongly B7-1 positive (Fig. 1, J and K), providing
internal positive controls for the reliability of the B7-1 detec-
tion assay on parafﬁn-embedded sections.

Moving from patients to the experimental model, we ana-
alyzed B7-1 expression in kidneys from Balb/c mice injected
with ADR, a model that faithfully resembles human FSGS
histological lesions (19). Balb/c mice not injected with ADR
served as controls. Snap-frozen murine kidney specimens were
stained through indirect immunofluorescence using two differ-
ent anti-mouse B7-1 antibodies, one polyclonal and one mono-
clonal, followed by secondary antibodies formerly exposed to
the strain-speciﬁc murine serum, as previously described (9).
At 4 wk after disease induction, overt nephropathy and pro-
teinuria occurred (36.43 ± 5.83 mg/day vs. controls: 1.47 ±
0.28 mg/day, P value < 0.001). Glomerular B7-1 expression
was not detectable in the ADR-injected Balb/c renal speci-
mens, either with the polyclonal (Fig. 2B) or monoclonal
antibody (Fig. 2C), similar to controls (Fig. 2A). Co-immuno-
staining using the podocyte-speciﬁc marker PDX further doc-
umented that podocytes of ADR-injected mice did not express
B7-1 (Fig. 2D). By contrast, strong B7-1 positivity was ob-
served in interstitial inﬂammatory cells (Fig. 2, C and E,
arrowheads) that also expressed the dendritic cell marker
CD11c (Fig. 2E, arrowheads), revealing that B7-1-positive
interstitial cells were APCs. The presence of internal positive
controls ensures the reliability of our antibodies and experi-
mental protocols. Since B7-1 expression in murine podocytes
was ﬁrst described in LPS-injected mice (22), we also analyzed
frozen kidney specimens from C57BL/6 mice with renal in-
sufﬁciency induced by injection of LPS (blood urea nitrogen:
111.23 ± 1.87 mg/dl vs. controls: 17.53 ± 2.2 mg/dl, P value
< 0.0001). In these additional controls, double staining with
PDX showed that B7-1 was not upregulated in podocytes of
LPS mice (Fig. 2G), in contrast with previous ﬁndings (22).
B7-1 expression was conﬁned on interstitial inﬂammatory
cells, which were CD11c-positive APCs (Fig. 2, G and H).

Although the concept of biomarker-driven customized ne-
phrology using B7-1 podocyte expression as a discriminatory
feature for treatment with abatacept or belatacept gave new
hope, the present data cast further doubt on its reliability.
Although we analyzed a considerable number of patients with
heterogeneous clinical features, no signiﬁcant upregulation of
podocyte B7-1 emerged, and no glomerular B7-1 was found in
ADR and LPS-injected mice. Moreover, this study, with a
double investigation of podocyte B7-1 expression in both
frozen and parafﬁn-embedded patients’ biopsies, and irrefut-
able internal positive controls, helps us to understand that
parafﬁn-embedded samples are as reliable as frozen ones when
used for detecting B7-1.

Is it possible to generate a data-driven consensus on podo-
cyte B7-1 expression and its therapeutic targeting in renal
diseases? A fair answer to this question is threefold: 1) expres-
sion of B7-1 in podocytes of patients with MCD and FSGS is
not detectable by immunostaining of frozen or ﬁxed kidney
biopsy tissue, nor in kidney tissues of an animal model mim-
icking the human condition. Albeit highly unlikely, we cannot
exclude the possibility that B7-1 expression by podocytes is
present, but at levels that are too low for detection by immu-
nostaining techniques. By contrast, in these very same biop-
sies, B7-1 is clearly detectable in inﬁltrating inﬂammatory
cells, offering a strong argument for the robustness of negative
results; 2) B7-1 cannot be considered a biomarker of disease
activity and progression in the above-mentioned clinical con-
ditions; 3) the antiproteinuric action of abatacept in MCD and
FSGS is disputed. If conﬁrmed in rigorously designed studies,
it may be the consequence of inhibiting immune cell activation
rather than an action on podocytes.

With this in mind, we should not hasten to use B7-1 blockers
to treat MCD and FSGS patients before results from all the
ongoing studies on podocyte B7-1 expression are available.
Until that moment, even the rationale for embarking on clinical
trials with abatacept or belatacept is questionable.

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