The Transcription Factor PU.1 Is Involved in Macrophage Proliferation

By Antonio Celada,* Francesc E. Borràs,* Concepció Soler,* Jorge Lloberas,* Michael Klemsz,‡ Charles van Beveren,§ Scott McKercher,‖ and Richard A. Makill

From the *Departament de Fisiologia (Immunologia), Facultat de Biologia, and Fundació August Pi i Sunyer, Campus Bellvitge, Universitat de Barcelona, 08028 Barcelona, Spain; the ‡Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5120; §Sidney Kimmel Cancer Center, San Diego, California 92121; and ‖La Jolla Cancer Research Foundation, La Jolla, California 92037

Summary

PU.1 is a tissue-specific transcription factor that is expressed in cells of the hematopoietic lineage including macrophages, granulocytes, and B lymphocytes. Bone marrow–derived macrophages transfected with an antisense PU.1 expression construct or treated with antisense oligonucleotides showed a decrease in proliferation compared with controls. In contrast, bone marrow macrophages transfected with a sense PU.1 expression construct displayed enhanced macrophage colony-stimulating factor (M-CSF)–dependent proliferation. Interestingly, there was no effect of sense or antisense constructs of PU.1 on the proliferation of the M-CSF–independent cell line, suggesting that the response was M-CSF dependent. This was further supported by the finding that macrophages transfected with a sense or an antisense PU.1 construct showed, respectively, an increased or a reduced level of surface expression of receptors for M-CSF. The enhancement of proliferation seems to be selective for PU.1, since transfections with several other members of the ets family, including ets-2 and flt-1, had no effect. Various mutants of PU.1 were also tested for their ability to affect macrophage proliferation. A reduction in macrophage proliferation was found when cells were transfected with a construct in which the DNA-binding domain of PU.1 was expressed. The PEST (proline-, glutamic acid-, serine-, and threonine-rich region) sequence of the PU.1 protein, which is an important domain for protein-protein interactions in B cells, was found to have no influence on PU.1–enhanced macrophage proliferation when an expression construct containing PU.1 minus the PEST domain was transfected into bone marrow–derived macrophages. In vivo, PU.1 is phosphorylated on several serine residues. The transfection of plasmids containing PU.1 with mutations at each of five serines showed that only positions 41 and 45 are critical for enhanced macrophage proliferation. We conclude that PU.1 is necessary for the M-CSF–dependent proliferation of macrophages. One of the proliferation-relevant targets of this transcription factor could be the M-CSF receptor.

Mononuclear phagocytes represent a large family of cell types that includes tissue macrophages, Kupffer cells (liver), Langerhans cells (dermis), osteoclasts (bone), microglia (brain), and perhaps some of the interdigitating and follicular dendritic cells found in lymphoid organs (1). Macrophages originate from undifferentiated stem cells and require specific growth factors called colony-stimulating factors (IL-3, M-CSF, and GM-CSF) for their generation (2). The receptor for the growth factor M-CSF, the product of the c-fms gene, has been well characterized (3). The binding of M-CSF to its receptor induces receptor kinase activity and triggers a cascade of biochemical events that leads to the expression of M-CSF–responsive genes and subsequent cell proliferation.

Recently, we cloned a tissue-specific DNA-binding protein, called PU.1, that is an activator of transcription (4). This protein is expressed in macrophages, granulocytes, mast cells, osteoclasts, and B lymphocytes (5). PU.1 binds to a purine-rich sequence that contains a central core with the sequence 5′-GGAA-3′ (4). The DNA-binding domain, which is located near the COOH terminus, has significant sequence identity with the DNA-binding domain that is present in the Ets family of DNA-binding proteins (4). PU.1 is the product of the putative oncogene Spi-1 (6),

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which is activated in erythroleukemic cells induced by the insertion of spleen focus-forming virus (SFFV) (7).

Some of the genes thought to be regulated by PU.1 in lymphocytes include the Igκ light chain gene 3′ enhancer (8, 9), the Igλ 2-4 enhancer (10), the Ig heavy chain μ enhancer (11), the IgJ chain gene (12), and the mb-1 gene, which is expressed in early B cell differentiation (13, 14). PU.1 is believed to regulate the expression in macrophages of several receptors, which include the FcγRIIB gene (15), the FcγRIIA gene (16), the M-CSF receptor gene (17), the CD11b gene (18, 19), the scavenger receptor (20), the macrophage inflammatory protein 1α (MIP-1α) (21), IL-1β (22), and CD18 (23). Recently, it has been shown that PU.1 interacts with the B cell–specific factor NF-EM5 (Pip) and stimulates transcription from the Igκ 3′ enhancer (7, 24).

Using the technique of gene targeting, mice homozygous for the disruption of the PU.1 gene have been generated. Scott et al. (25) have reported the absence of homozygous mutant progeny, suggesting that disruption of PU.1 results in embryonic lethality. More recently, McKercher et al. (McKercher, S., B. Torbett, K. Anderson, D. Vestal, G. Henkel, C. Paige, and R.A. Mald, manuscript in preparation) have obtained mice homozygous for the disruption of the PU.1 gene that were born and survived for up to 2 wk when treated with the appropriate antibiotic. Neither macrophages nor B cells were present in either example, suggesting that PU.1 is involved in the development of these cells. Using PU.1+/− ES cells differentiated in vitro, Olson et al. (26) concluded that PU.1 is not essential for early myeloid gene expression, but is required for terminal myeloid differentiation.

In the present study we demonstrate that PU.1 is involved in macrophage proliferation. Transfection of a PU.1 expression construct into bone marrow macrophages was found to stimulate both M- and GM-CSF–dependent proliferation of these cells, whereas transfection of a PU.1 antisense construct or a construct that expressed DNA-binding domain of PU.1 into bone marrow macrophages was found to inhibit the proliferative effect of the growth factors. Thus, PU.1 seems to regulate the expression of genes involved within macrophage proliferation.

Materials and Methods

Mouse Bone Marrow–derived Macrophages. Macrophages derived from bone marrow cultures (BMDM) were obtained as described (27). 6-wk-old DBA/2 mice (The Jackson Laboratory, Bar Harbor, ME) were killed by cervical dislocation, and both femurs were dissected free of adherent tissue. The ends of the bones were cut off and the marrow tissue was eluted by irrigation with PBS. Cells were suspended by vigorous pipetting, washed once in PBS, and collected by centrifugation. We cultured 10^7 cells in a plastic, nontissue culture, 150-mm petri dish (Lab-Tek 4030; Miles Laboratories, Inc., Naperville, IL) in 50 ml of DME containing 2 mM L-glutamine, 1 mM Na pyruvate, 50 U/ml penicillin, 50 mg/ml streptomycin, 20% FCS, and 30% L cell–conditioned medium (LCM) as a source of M-CSF. The cell suspensions were incubated at 37°C in a humidified 5% CO_2 atmosphere. Macrophages were loosely adherent to the dishes and were harvested with cold PBS. In some experiments, serum-free media composed of CMRL-1066, insulin, glutamine, transferrin (GIBCO BRL, Gaithersburg, MD), and sodium bicarbonate, were used.

Cultured Cell Lines. The murine macrophage cell lines RAW264.7, IC-21, and BAC1 2F5-A were maintained in DME supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 1 mM Na pyruvate, and 5% FCS.

Proliferation Assay. Cell proliferation was measured as previously described (28) with minor modifications. After electroporation, cells (5 × 10^6) were incubated for 24 h in 24-well plates (3424 MARK II; Costar Corp., Cambridge, MA) in 1 ml of media with the indicated growth factor. For the experiments with oligodeoxynucleotides, 5 × 10^4 BMDM were incubated in 96-well plates with 100 ml of media containing M-CSF for 2 h. Media were aspirated and replaced by 0.2 ml of media containing [3H]thymidine (1.0 mCi/ml). After an additional 2 h of incubation at 37°C, media were removed and cells were fixed in methanol. After three washes in 10% TCA, cells were solubilized in 1% SDS and 0.3 M NaOH. Radioactivity was counted by liquid scintillation. All samples were prepared in triplicate and the results are expressed as the mean ± SD. Statistical analyses were performed using the Student’s paired t test, comparing the results of at least five independent experiments.

Transfections. Cells were removed from plates and washed twice in serum–free media. Cells were then incubated (12 × 10^6 in 1 ml of serum–free media) at 4°C in the cuvettes used for electroporation, with 2 µg of DNA for 15 min. Electroporation was carried out using a BTX electroporator (Transfactor 100; Bio-technologies & Exp. Research, San Diego, CA). Cells were incubated for a further 15 min on ice before they were distributed in 24-well plates. To determine the transfection efficiency in some experiments, we transfected an expression construct containing the green fluorescent protein gene (Clontech, Palo Alto, CA). After 2 d of culture, cells were fixed, stained, and counted (29).

In a series of preliminary experiments, we tested the appropriate conditions for transfection using the electroporation method (10 ms at 200 mV). We also found that increasing the amount of DNA transfected (any type) in BMDM resulted in a concentration–dependent decrease in proliferation. Therefore, we chose conditions that optimized the number of cells transfected, but also permitted proliferation. The amount of DNA used was 2 mg for 12 × 10^6 cells, which resulted in 7–12% of cells being transfected, as assessed by staining for green fluorescent protein.

Plasmids and Oligodeoxynucleotides. The PU.1 expression vector, PUPeCE, was constructed by ligating the full-length PU.1 cDNA into the EcoRI site of the expression vector, pECE (30). The retinoic acid receptor e/a3 pECE was a gift from M. Pfahl (La Jolla Cancer Research Foundation) (31). The PU.1 antisense construct was made by inserting the PU.1 cDNA into the HindIII – Sal sites of the pH2 APr-1-neo vector in the reverse orientation (32).

The pBluescript KS+ vector was purchased from Stratagene, Inc. (La Jolla, CA), and the pBL CAT vector was obtained from
Biosystems, Inc., Foster City, CA) and purified as described previously (34). The PU.1 antisense (5'-TTTGCACGCTTGATACTCCAGCTGACCTC-3') oligodeoxynucleotide synthesized for these studies straddled the predicted translation-initiation site of the PU.1 mRNA. A computer-assisted search of the GenBank database for mammalian sequences complementary to this antisense oligodeoxynucleotides revealed little homology with other genes.

Specific sites in the PU.1 gene were mutagenized using oligonucleotides spanning the desired site and a nearby unique restriction site, and generating the new fragment using the polymerase chain reaction. DNA fragments were isolated, the fragment was inserted into the PU.1 eukaryotic expression plasmid PUpECE previously described, and the DNA sequence was determined (8). The plasmids containing the DNA-binding domain (170-260) or the proline (P), glutamic acid (E), serine (S), and threonine (T) rich region (PEST) deletion (126-159) of PU.1 have been previously described (8). The plasmids containing e-S-2 and f-t-1 in the pECE vector have been previously described (35).

**Growth Factors and Interleukins.** Recombinant growth factors were a gift from DNAX (Palo Alto, CA). In some experiments we used LCM as a source of M-CSF. The amount of M-CSF present in LCM was determined using a M-CSF standard. The growth activity of LCM could be blocked by a specific mAb against M-CSF. The M-CSF blocking antibody was a gift from Dr. H.S. Lin (Washington University School of Medicine, St. Louis, MO) (36).

**Determination of M-CSF Receptors on the Cell Surface.** The determination of M-CSF receptors on the cell surface was performed using specific antibodies and cytometry analysis as previously described (37). Cells were washed three times in PBS and incubated with an excess of goat IgG (Sigma Chemical Co., St. Louis, MO) (250 mg/ml) to block Fc receptors. After 30 min at 4°C, cells were washed, the primary antibody (rabbit anti–mouse c-fes; Upstate Biotechnology Inc., Lake Placid, NY) was added at a 1/200 dilution, and cells were incubated for 45 min at 4°C. Finally, cells were incubated with fluorescein-conjugated goat affinity-purified F(ab')2 fragment to rabbit IgG (Cappel, Turhout, Belgium) at a dilution of 1/500, as recommended by the supplier, for 30 min at 4°C. Cytometry analysis was carried out using an Epics Elite (Coulter Corp., Hialeah, FL) apparatus.

**Results**

The transcription factor PU.1 has been linked to the immortalization of erythroblasts, and may be involved in the regulation of a number of genes in macrophages, some of which are involved in development or growth control. To examine its role in this latter process, we designed both antisense and sense expression constructs of PU.1 and transfected these constructs into BMDM. The cells were grown from bone marrow cells cultured in the presence of M-CSF for 5–7 d, which promotes the proliferation of macrophages but not other cell types. After 5–7 d, the BMDM were transfected with either the PU.1 antisense construct PU.1-pHb or the pHb vector alone and replated in media containing various concentrations of M-CSF. The transfection efficiency was estimated to be between 7 and 12% based on the transfection of a green fluorescent protein expression construct. BMDM transfected with the antisense PU.1 construct in the presence of M-CSF had a significantly reduced thymidine incorporation compared with the controls in which the vector alone was transfected into the cells (Fig. 1 A). The experiment was repeated using an antisense oligonucleotide made complementary to a region that included the initiation codon of PU.1. A sense oligonucleotide to the same region was made as a control. We observed that the antisense but not the sense oligonucleotide inhibited BMDM thymidine incorporation in a dose-dependent manner that was dependent on the concentration of M-CSF in the media (Fig. 1 B).

These results suggested that a block in the expression of PU.1 may inhibit macrophage proliferation. If this were the case, we reasoned that the overexpression of PU.1 may stimulate proliferation. To test this possibility, we generated a sense construct of PU.1, PU.1-pECE and proceeded to analyze the effect of the overexpression of PU.1 on the M-CSF-stimulated proliferation of BMDM. In the presence of M-CSF, the number of BMDM was significantly lower when the BMDM were transfected with PU.1 compared with the BMDM that were transfected with the vector.
Figure 2. PU.1 enhances M-CSF–dependent proliferation of macrophages. BMDM were transfected with PU.1 or the control vector (pECE) in the presence (striped bars) or absence (black bars) of M-CSF. After an overnight incubation, the number of cells was counted using a Coulter counter. In the presence of M-CSF, there was a significant difference between values of transfected cells with the control vector and the PU.1 vector (P < 0.01).

alone (Fig. 2). This result was also confirmed using thymidine incorporation as an index of macrophage proliferation. Thymidine incorporation was higher in BMDM treated with M-CSF and transfectd with the PU.1 expression construct when compared with BMDM treated with M-CSF and transfected with vector alone (Fig. 3).

To determine whether PU.1 acts as a general factor in macrophage proliferation, we incubated macrophages transfected with PU.1 or the control vector (pECE) in the presence of several recombinant growth factors or interleukins (Fig. 3). We observed an increase in thymidine incorporation to varying degrees in BMDM treated with M-CSF, GM-CSF, IL-3, IL-1, IL-4, and IL-6 compared with the control. No difference in thymidine incorporation compared with controls was found when cells were cultured in the presence of either G-CSF or IL-2. When macrophages were transfected with an expression construct containing PU.1, a significant increase in thymidine incorporation was observed only in cells treated with M-CSF or GM-CSF. These data suggest that PU.1 is involved in the regulation of macrophage proliferation that is M-CSF or GM-CSF dependent.

GM-CSF is known to induce the expression of M-CSF (38–41). To determine whether the increase in proliferation that we observed with the addition of GM-CSF to the cultures was due to the production of M-CSF, we added a mAb made against M-CSF to the culture media. The addition of anti-M-CSF antibodies caused a substantial decrease (30%) in BMDM proliferation in cells transfected with control vector alone. This may be due to the inhibition of the autocrine production of M-CSF induced by GM-CSF. We observed that in the presence of the mAb against M-CSF there was no stimulation of thymidine incorporation above background when GM-CSF was added to the cultures and the PU.1 expression construct transfected into the cells (Fig. 4). These data suggest that the enhancement of GM-CSF–dependent proliferation in PU.1-transfected macrophages may be related to the autocrine production of M-CSF (38–41).

The effect of PU.1 on proliferation appears to be linked to the growth factor–stimulated proliferation of M-CSF. PU.1 has been shown to bind to the promoter of the M-CSF receptor and therefore may be an important factor for the regulated expression of this receptor (17, 42). To address this possibility, we transfected BMDM with a PU.1 sense, antisense construct or vector alone as a control and measured the level of M-CSF receptor surface expression by flow cytometry. BMDM transfected with the vector alone

Figure 3. PU.1 enhances M-CSF– and GM-CSF–dependent proliferation. BMDM were transfected with PU.1 or the control vector and incubated for 24 h in the presence of different cytokines and growth factors. Thymidine incorporation was measured as described in Materials and Methods. Comparing cells transfected with the control vector and the PU.1 vector, only in the presence of M-CSF and GM-CSF, there was a significant difference (P < 0.01).
Transfection PU.1 Control PU.1 Control

Anti-M-CSF antibodies     +     +     +     +
GM-CSF                      +     +     +     +

Figure 4. PU.1 enhances GM-CSF-dependent proliferation and is abolished by mAbs against M-CSF. BMM were transfected with PU.1 or the control vector and incubated for 24 h in the presence of GM-CSF with or without mAbs to M-CSF, and thymidine incorporation was determined. There was a significant difference between the values of transfected cells with the control vector and the PU.1 vector in the absence (P < 0.01) but not in the presence of anti-M-CSF antibodies (P > 0.05).

were found to contain two populations of cells expressing the M-CSF receptor, one of which presented more receptors than the other (Fig. 5). Because these cells were proliferating, the heterogeneity in the number of M-CSF receptors may be related to the different stages of the cell cycle. The BMDM transfected with the sense PU.1 vector showed a marked increase in the population of cells with the higher number of receptors. In contrast, the BMDM transfected with the antisense PU.1 construct showed a reduction of the same cell population (Fig. 6). Similar results were obtained when antisense oligonucleotides were used (data not shown). Thus, PU.1, which has been shown to bind the M-CSF promoter and regulate expression from this promoter using a reporter construct, is also involved in the regulation of M-CSF receptor expression in macrophages in vivo.

Transfection of PU.1 into BMDM in the absence of M-CSF had no effect on the proliferation of the macrophages, suggesting that the effect of the antisense and sense PU.1 constructs was growth factor dependent. We therefore wanted to know what effect PU.1 may have on M-CSF-independent proliferation. For this purpose we transfected a series of cell lines that are growth factor independent with PU.1 in the sense or antisense orientation. The proliferation of three macrophage cell lines, IC-21, RAW264.7, and 2F5A, which express PU.1 endogenously, were tested using the sense and antisense constructs, and no effect on proliferation was observed (data not shown). These data suggest that PU.1 is involved in a proliferative mechanism that is only present in growth factor–dependent macrophages.

Since PU.1 belongs to a large family of related DNA-binding proteins (Ets family) (43, 44), we were interested in determining whether the effect of PU.1 on macrophage proliferation was specific for PU.1, or whether other Ets family members could have the same effect on macrophage proliferation. Expression constructs of ets-2 and fli-1 were generated to test this possibility. When expression constructs containing either ets-2 or fli-1 were transfected into BMDM, no increase in macrophage proliferation above that seen using the vector alone was detected (Fig. 6). Thus, we conclude that the effect of PU.1 on macrophage proliferation is specific for PU.1.

Previous work on the PU.1 protein has demonstrated that there are a number of functional domains. We were
been demonstrated to have functional importance are the proliferation. Two regions of the PU.1 protein that have been shown to be important for the interaction of PU.1 with the B cell-specific factor NF-EM5 (Pip) (8, 24). When a PU.1 expression construct lacking the PEST domain was transfected into BMDM, an enhancement of macrophage proliferation similar to that obtained using the wild-type protein in stimulating proliferation of BMDM (Fig. 7 B). In contrast, PU.1 protein carrying an alanine substitution at S41 or S45 not only failed to stimulate proliferation, but also lowered the basal level of proliferation. The effect of the S41/45A double mutation was about the same as either of the single mutations. It is therefore likely, at least in macrophages, that phosphorylation of amino acids 41 and/or 45 results in the activation of a domain important for the activity of PU.1.

Discussion

The data presented here indicate that PU.1 is involved in macrophage proliferation. The block in macrophage proliferation using either an antisense PU.1 expression construct or antisense oligonucleotides suggests that PU.1 plays a critical role in the proliferation of macrophages. The use of antisense oligonucleotides to inhibit gene expression and cell growth and development has provided new insight into the functional importance of a variety of genes (45). Antisense oligodeoxynucleotides of PU.1 inhibit the proliferation of erythroleukemia cells induced by the retrovirus SFFV (46). In this model, if the provirus integrates upstream of the PU.1 gene, PU.1 is overexpressed and the cell loses its commitment to terminally differentiate and becomes immortal. A reduction in the expression of PU.1 coincides with recommitment to the program of erythroid...
differentiation and the loss of immortality (47). The infection of erythroblasts with a retroviral vector expressing PU.1 was efficient for immortalization, suggesting that PU.1 perturbs the pathway that controls the potential for proliferation in these cells (48).

The enhancement of macrophage proliferation induced by the transfection of PU.1 appears to be related to the presence of M-CSF in the media. This may explain why PU.1 is not effective in cell lines whose proliferation is independent of growth factors.

The effect of PU.1 protein seems to be specific because other ets family members such as ets-2 and flI-1 were ineffective in stimulating proliferation. The ets family consists of a large number of proteins that have sequence identity within the DNA-binding domain, and all bind to a sequence containing the core sequence 5'-GGAA/T-3' (43). Whereas the core sequence appears to be necessary for binding it is known that flanking sequences also influence the binding of specific ets family members to DNA (44, and our unpublished results). This may be part of the explanation for why PU.1 has an effect on macrophage proliferation, but not ets-2 or flI-1.

PU.1 functions in association with other factors (8, 10, 19, 49, 50). In the mouse, κ 3' enhancer for example, PU.1 recruits a B cell–restricted factor, NF-EM5 (Pip) (8, 24). The protein–protein interaction is mediated through a 43–amino acid region with sequence homology to a PEST domain, i.e., one that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T), which is susceptible to protease degradation (51). The PEST sequence does not appear to have a role in the enhancement of macrophage proliferation by PU.1. However, expression of the PU.1 DNA-binding domain resulted in a reduced proliferation of BMDM. Dominant negative constructs of the PU.1 DNA-binding domain have proven useful in demonstrating the function of PU.1 in regulating gene expression. A dominant negative expression construct of PU.1 in which aminos acids 133 to the end were expressed was used to show that PU.1 was needed for expression from the J chain gene promoter (12). A dominant negative PU.1 expression construct (aminos acids 160–266) transfected into Ras-transformed NIH3T3 fibroblasts reverted the transformed phenotype (52). This latter result may be due to a general feature of the DNA-binding domains of ets family members since dominant negative constructs of ets-1 and ets-2 had the same effect. The finding that mutations at serine 41 and 41/45 caused a lowering of proliferation in BMDM suggests that this region plays an important role for this activity. The NH2-terminal half of PU.1 is known to be responsible for the transactivation properties of PU.1 (our unpublished results). This may provide part of the explanation for the results seen with the serine 41 and 41/45 mutations.

The number of genes regulated by PU.1 that could modulate macrophage proliferation in response to M-CSF is of present unknown. Because the enhancement of proliferation requires the presence of the growth factor M-CSF, one possible candidate is the M-CSF receptor (c-fms), which is specifically expressed in macrophages (3). Recently, it has been reported that PU.1 directs the expression of the M-CSF receptor (17, 42, 53). PU.1 binds to a specific site in the M-CSF receptor promoter just upstream from the major transcription initiation site. Mutations at this site have been shown to eliminate PU.1 binding and decrease promoter activity. PU.1 transactivates a reporter construct containing the M-CSF promoter. These experiments were performed by cotransfection in cells that normally do not express PU.1 or the M-CSF receptor, with PU.1 cDNA and a reporter construct containing the M-CSF promoter (17). It has also been reported that ets-2 transactivates the proximal promoter of the M-CSF receptor (42, 53). In our experiments, ets-2 had no effect on M-CSF–dependent macrophage proliferation. This may be due to an already high level of ets-2 expression in the cells (our unpublished results).

PU.1 is thought to regulate a number of other genes that could be involved in cell proliferation. For instance, PU.1 binds the tumor suppressor protein p53 which represses cell proliferation (49). The inhibition of an inhibitor could result in the activation of proliferation. In vitro, PU.1 was also shown to interact with the general transcription factor TFIID (49). PU.1 and PU.1–associated factor(s) could stabilize TFIID and allow assembly of the initiation complex of genes related to proliferation. However, the fact that enhancement of macrophage proliferation is M-CSF or GM-CSF dependent probably rules out this possibility.

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Address for correspondence: Dr. Antonio Celada, Departament de Fisiologia (Immunologia), Facultat de Biología, Universitat de Barcelona, Av. Diagonal, 645, 08028 Barcelona, Spain.

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