Fc Chimeric Protein Containing the Cysteine-rich Domain of the Murine Mannose Receptor Binds to Macrophages from Splenic Marginal Zone and Lymph Node Subcapsular Sinus and to Germinal Centers

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Summary

Ligands for the cysteine-rich (CR) domain of the mannose receptor (MR) were detected by incubating murine tissues with a chimeric protein containing CR fused to the Fc region of human IgG1 (CR-Fc). In naive mice, CR-Fc bound to sialoadhesin+, F4/80low+, macrophilic macrophages (Mo) in spleen marginal zone and lymph node subcapsular sinus. Labeling was also observed in B cell areas of splenic white pulp. Western blotting analysis of spleen and lymph nodes lysates revealed a restricted number of molecules that interacted specifically with CR-Fc. In immunized mice, labeling was upregulated on germinal centers in splenic white pulp and follicular areas of lymph nodes. Kinetic analysis of the pattern of CR-Fc labeling in lymph nodes during a secondary immune response to ovalbumin showed that CR ligand expression migrated towards B cell areas, associated with cells displaying distinctive dendritic morphology, and accumulated in developing germinal centers. These studies suggest that MR+ cells or MR-carbohydrate-containing antigen complexes could be directed towards areas where humoral immune responses take place, through the interaction of the MR CR domain with molecules expressed in specialized macrophage populations and antigen transporting cells.

The mannose receptor (MR) is a 175-kD type I transmembrane protein expressed in macrophages (Mo), dendritic cells (1), and hepatic endothelial cells, that mediates phagocytosis of saccharide-coated particles as well as pinocytosis of soluble glycoconjugates (2). The expression of the MR is highly regulated; it appears only during terminal differentiation of Mo and is modulated by steroids and cytokines. In biogel polyacrylamide bead-elicited peritoneal Mo MR mRNA and endocytic activity are downregulated by IFN-γ and upregulated by IL-4 and IL-13 (3–5). Three types of domain have been described in the extracellular region of this molecule: a cysteine-rich (CR) domain that contains a module that resembles a domain of the B chain of the plant lectin Ricin (6), a domain with fibronectin type II repeats (FNII), and eight C-type carbohydrate recognition domains (CRD) that mediate ligand binding (7–10). Of these, only CRDs 4–8 show affinity for carbohydrate and only CRD4 exhibits multiple specificities (9, 10). This structure is conserved among the members of the "MR family" that includes the dendritic cell and thymic epithelium marker DEC-205 (11), which contains 10 CRDs, and phospholipase A2 receptors (12, 13).

No function has been found for the CR and FNII domains of the MR, but amino acid sequence comparison between the human and murine forms of the receptor shows 86% identity in the case of the CR and 93% in the case of the FNII domain (4). Such a degree of conservation is suggestive of an important function for these regions. In this study we have used chimeric proteins containing both CR and FNII domains or only the CR domain of the murine MR fused to the Fc region of human IgG1 (14), to detect ligands in mouse tissue. In non-immunized mice, both proteins bound equally to the marginal zone of spleen, the subcapsular sinus of lymph node and B cell areas of splenic white pulp. Phenotypic characterization of the cells that express the ligand(s) will be presented. Several molecules that interact specifically with CR-Fc in Western blot analysis, have been detected in spleen and lymph node protein lysates.
After antigen stimulation ligand activity was also detected in germinal centers in spleen and lymph nodes. Kinetic studies showed that cells with ligand activity appeared to migrate towards B cell areas and accumulate in developing germinal centers of draining lymph nodes. These studies indicate that MR (i.e., MR$^+$ cells) could be directed, through its interaction with specific molecules expressed in spleen and lymph nodes via domain(s) not involved in carbohydrate recognition, to areas where affinity maturation of B cell responses occur (i.e., the germinal center).

Materials and Methods

Animals. C57Bl/6, Balb/c and op/op mice were bred at the Dunn School of Pathology and used at 7-8 wk of age. Balb/c SCID mice were obtained from Harlan (Bicester, UK) and tissues from rat/tag mice were a kind gift from Dr. D. Koussou (NIMR, Mill Hill, London).

Antibodies. The following rat mAb were prepared in our laboratory: Sc-4 and 3D6, used together to detect sia!oadhesin (Sn) (15, 16), F4/80, specific for a Mo plasma membrane differentiation antigen (17), and PAI1 that recognizes macroslasin (18). Other antibodies used were: FDC-M1 (19), MS/114 (specific for MHCI11), obtained from the American Tissue Type Collection (Rockville, MD), 8C12 (specific for complement receptor 1, CR1) (20), and B220, obtained from PharMingen (San Diego, CA). Purified human IgG1 was obtained from Sigma (Poole, Dorset, UK).

Immunization. Mice were primed subcutaneously in the back of the neck with OVA precipitated in alum (100 µg) and 2 wk later challenged intraperitoneally (100 µg) or subcutaneously (50 µg per limb) with the same antigen, as described elsewhere (21).

Fc Receptors. Plasmid MR1:5'GGGATAATGACCGGTTGAATCTGAGCCAAAGGGGCAACCTTG3' and MR5:5'GGGATTCTCATTACCTGCTTCGTCCCCTCCCAAGTGGCAATGGACAAAA3' were used to amplify the leader sequence, CR domain and FNII domain of the MR by RT-PCR using as template cDNA from digested-elicited peritoneal Mo that had been induced to express high levels of CR by IL-4 treatment (25 ng/ml), as described (3, 5). To amplify only the leader sequence and CR domain a new primer was designed: MR1:5'GGGATAATGACCGGTTGAATCTGAGCCAAAGGGGCAACCTTG3' and MR5:5'GGGATTCTCATTACCTGCTTCGTCCCCTCCCAAGTGGCAATGGACAAAA3' were used to amplify the leader sequence, CR domain and FNII domain of the MR by RT-PCR using as template cDNA from digested-elicited peritoneal Mo that had been induced to express high levels of MR by IL-4 treatment (5 ng/ml), as described (3, 5). To amplify only the leader sequence and CR domain a new primer was designed: MR1:5'GGGATAATGACCGGTTGAATCTGAGCCAAAGGGGCAACCTTG3' and MR5:5'GGGATTCTCATTACCTGCTTCGTCCCCTCCCAAGTGGCAATGGACAAAA3' were used to amplify the leader sequence, CR domain and FNII domain of the MR by RT-PCR using as template cDNA from digested-elicited peritoneal Mo that had been induced to express high levels of MR by IL-4 treatment (5 ng/ml), as described (3, 5).

Immunohistochemistry. Organs were collected and frozen in O.C.T. compound (BDH-Merck, Poole, Dorset, UK) cooled in isopentane over dry ice. Frozen sections were cut at 5 µm, air-dried for 1 h, and stored at -20°C. Before staining, sections were thawed at room temperature for 1 h and fixed for 10 min in 2% paraformaldehyde in Hepes-buffered isotonic saline at 4°C or in acetone at room temperature. In some cases, animals were perfusion-fixed with 2% periodate-lysine-paraformaldehyde. After postfixing in the same solution for 2 h, tissues were rehydrated in 20% (wt/vol) sucrose in 0.1 M phosphate buffer overnight, before freezing and sectioning.

Fixed sections were washed in PBS containing 0.1% (vol/vol) Triton X-100 and endogenous peroxidase activity blocked by incubating sections with 10% (vol/vol) glucose, 10-3 M NaN3, 40 U glucose oxidase (Sigma) in 100 ml phosphate buffer for 15 min at 37°C. When chimeric proteins were used, sections were incubated with 5% (vol/vol) normal goat or mouse serum (blocking buffer) for 30 min and with hlgGl or Sn1-Fc, MAG1-5-Fc, CR-FNII-Fc, or CR-Fc proteins diluted at 10 µg/ml in blocking buffer for 60 min. Binding was detected by incubation of sections with a biotinylated goat anti-human IgG (Vector, Peterborough, UK) or biotinylated mouse anti-human F(ab)2 (Jackson Immunoresearch Labs., West Grove, PA) diluted in blocking buffer, followed by avidin-biotin-peroxidase complex (ABC elite; Vector) and 0.5 mg/ml diaminobenzidine (Polysciences Inc., Northampton, UK) with 0.024% H2O2 in 10 mM Imidazole in PBS, pH 7.4. In the case of rat mAb, the blocking buffer was 5% (vol/vol) normal rabbit or mouse serum and a biotinylated rabbit anti-rat IgG (Vector) or mouse anti-rat IgG (Fab')2 (Jackson) was used to detect binding. Sections were counterstained with crystal violet or methyl green. In the case of double immunocytochemistry with CR-Fc and rat Ab, the first staining step with the above protocol was followed by blocking with avidin-biotin (Vector). For the second step, avidin-biotin-alkaline phosphatase complex (ABC-AP) and Vector blue detection system (Vector) were used. Cytospin preparations were air dried and kept at -20°C. Before use, cells were fixed, permeabilized, and endogenous peroxidase quenched as above. In this case binding was performed in 5% (vol/vol) normal mouse serum in PBS. Cells were incubated with MAG1-5-Fc or CR-Fc (10 µg/ml) diluted in 5% (vol/vol) normal mouse serum for 1 h, and CR-Fc binding detected as above. Cells were counterstained with hematoxylin. For double immunohistochemistry on isolated cell preparations, fixed and permeabilized cells were incubated in 5% (vol/vol) normal mouse serum for 30 min, followed by CR-Fc (10 µg/ml) and rat mAb diluted in 5% (vol/vol) normal mouse serum for 60 min. Binding was detected by incubation with biotinylated mouse anti-human F(ab)2, and FITC-mouse anti-rat IgG (Fab')2 (Jackson) for 30 min and Texas red-avidin (Vector) for 30 min.

Enrichment of CR-Fc+ Cells. Spleens and peripheral lymph nodes from normal mice were digested using 2.5% (wt/vol) collagenase D and 0.1% DNase (Boehringer Mannheim, Lewes, East Sussex, UK) in Iscove's modified Dulbecco's medium (IMDM). After digestion, erythrocytes in spleen were lysed following Gey's procedure (24). Cell suspensions were washed and loaded on a performed continuous percoll gradient (Pharmacia) (1-2 × 108 cells/10 ml) and centrifuged at 400 g for 30 min. The two major bands (low and high density cells) were collected, washed in IMDM and prepared for analysis by immunocytochemistry. 105 cells were plated on poly L-lysine treated slides by centrifugation at 400 rpm for 5 min using a cytocentrifuge (Life Sciences International, Basingstoke, UK).
Results

Localization of Ligands for the CR Domain of the MR in Mouse Tissue Sections by Immunocytochemistry in Normal Mice. The CR and FNII domains of the murine MR or the CR domain alone were fuses to the Fc portion of human IgG1 (CR-FNII-Fc and CR-Fc) (Fig. 1) and used to label various mouse tissues using human IgG1 or other chimeric proteins or human IgG1 and CR-Fc binding was not influenced by the presence of the Fc receptor blocking reagent 2.4G2 (PharMingen; data not shown). No specific binding was observed in lung, liver, muscle, skin, epiphysis or dermis, brain or bone marrow (data not shown). In spleen, CR-FNII-Fc and CR-Fc labeling included metallophilic MZ Mo which express high levels of sialoadhesin (Sn) (Fig. 2, compare 1C, 1D, 1G). Double immunolabeling using CR-Fc and 3D6/SER-4 (specific for Sn) confirmed this observation (Fig. 3 C). CR ligand activity was absent in Sn+ Mo in the outer MZ and in the red pulp (Fig. 3, compare A with B). Double immunolabeling using CR-Fc and F4/80 antibody, specific for red pulp Mo in the spleen, established that neither probe bound to cells in the outer MZ (Fig. 3 D). CR-Fc+Sn+ metallophilic Mo also expressed the pan-Mo and dendritic cell marker macrophage in addition was found in red pulp, white pulp and outer MZ Mo (Fig. 3 E). Weak CR-Fc labeling was detected in B cell areas in the white pulp of normal spleen that are strongly positive for complement receptor 1 (CR1) (Fig. 2, 1B) and weakly positive for the follicle dendritic cell marker M1 (FDC-M1, Fig. 2, 1F). No labeling in T cell areas was found.

In unstimulated peripheral lymph nodes, CR-Fc binding was detected in a subpopulation of Sn+ macrophage Mo located in the subcapsular sinus (Fig. 4, compare B, C, D). Again no co-localization of CR-Fc ligand(s) (Fig. 4 B) and F4/80 (Fig. 4 F) was observed.

The characterization of MZ CR-Fc+ cells and subcapsular sinus CR-Fc+ cells as Sn+ macrophage Mo was confirmed by labeling spleens from op/op mice which lack MZ and MZ metallophilic Mo, M-CSF-dependent Mo populations (25) (Fig. 2, column 3). In these animals no CR-Fc binding to the MZ of spleen (Fig. 2, 3C and 3D) and no 3D6/SER-4 labeling (Fig. 2, 3G) were detected. Similar results were obtained in the case of the subcapsular sinus of LN (data not shown). Work with op/op mice also showed that the labeling observed in follicular areas was independent of the presence of MZ, because CR-FNII-Fc and CR-Fc still bound to B cell areas (CR1+, FDC-M1+) (Fig. 2, compare 3C, 3D, 3E, 3F).

CR-Fc binding was Ca2+- and Mg2+-independent, because no changes in the labeling pattern were noticed in the presence of EDTA (data not shown). CR binding activity was also T and B cell-independent; CR-Fc bound to similar areas in spleen and lymph nodes from SCID and Rag-/- mice though the abnormal morphology of the MZ in spleen makes any precise comparison difficult (data not shown).

Ex Vivo Phenotypic Characterization of CR-Fc+ Cells. The procedure used to enrich CR-Fc+ cells was originally developed to isolate follicular dendritic cells (FDCs) from immune lymphoid tissue (26). In the present case we used tissue from naive animals and compared the cells obtained from collagenase digested spleen and lymph nodes. Cell suspensions were fractionated by percoll gradient and high and low density fractions collected as described in Materials and Methods.
Immunocytochemical analysis of cytospin preparations from both cell populations was performed with CR-Fc to estimate enrichment of CR-Fc+ cells. These cells were found in the low density fraction where they represented less than 0.1% of total cells. No significant number of CR-Fc+ cells was found in the high density fraction. No FDC-M1+ cells were obtained from non-immunized mice under these conditions. CR-Fc+ cells appeared large with cytoplasmic processes and were isolated or part of cellular clusters. They contained a granular cytoplasm and CR-Fc staining was localized strongly but not exclusively to the plasma membrane (Fig. 5). Double immunofluorescence showed that most CR-Fc+ cells isolated from naive animals are macrosialin+, Sn+, MHCIIlow/−, F4/80low/−, CR1 negative, and B220 negative (data not shown). Therefore the populations of CR binding cells isolated following this method are likely to represent mainly MZ metallophilic Mø from spleen and subcapsular sinus Mø from LN.

Detection of Molecules with CR Binding Activity. Blotting of normal tissue lysates with CR-Fc allowed the characterization of molecules that interact with CR. As shown in Fig. 6, several proteins ranging in size from 80 to more than 300 kD were specifically recognized by CR-Fc in spleen and LN lysates. No proteins were detected by CR-Fc in liver lysates (which totally lack reactivity with CR-Fc by immunocytochemistry) or by other control chimeric proteins in any of the lysates tested.

This complex pattern was highly reproducible, was obtained with CR-FNII-Fc and CR-Fc (data not shown) and was independent of the detergent used to prepare the lysate (β-octylglucoside, CHAPS, or NP-40). In an attempt to increase sensitivity, lysates were immunoprecipitated with CR-Fc, electrophoresed under non-reducing conditions, blotted, and incubated with CR-Fc conjugated to digoxigenin followed by an anti-digoxigenin antibody. No differences were observed (data not shown).
Localization of Ligands for CR-Fc in Immunized Mice. After induction of germinal center formation in normal mouse spleen by immunization with OVA (Fig. 2, column 2), CR-FNII-Fc and CR-Fc also labeled germinal centers and co-localized with CR1 and FDC-M1 which are both upregulated under these conditions (Fig. 2, compare 2C and 2D with 2E and 2F). Similar results were observed in germinal centers generated in draining LN after subcutaneous immunization (data not shown).

These findings were suggestive of a role for the CR
ligand or CR ligand bearing cells during immune responses. To investigate this possibility the time course of localization of CR-Fc binding in LN during a secondary immune response was studied. As shown in Fig. 7, changes in CR-Fc labeling were observed at different times during a secondary response to ovalbumin. Background labeling using a control chimeric protein was minimal (Fig. 7 A). Before injection (Fig. 7 B, Day 0) the CR-Fc protein bound to cells restricted to the subcapsular sinus and trabeculae entering the tissue. Within 24 h of antigenic challenge (Fig. 7 C), labeling appeared to have begun to move into the first layers of lymphocytes. CR binding activity continued to move deeper by 48 h (Fig. 7 D), with CR-Fc+ cells displaying distinctive dendritic processes that could be especially seen by day 3 (Fig. 7 E). At this point the label appeared to have began to accumulate in the B cell follicles as well as to move towards the paracortex. Interestingly, as seen by day 4 (Fig. 7 F), cells generating the FDC network (arrows) in the developing germinal center (PNA labeling; serial section, Fig. 7 K) also bound the CR-Fc protein; labeling diminished as the other positive cells approached the T cell zone. This appearance may indicate that the ligand was internalized and degraded. From day 5 (Fig. 7 G) onward (Fig. 7 H, I, and J, respectively, days 6, 10, and 12), labeling became more restricted to the subcapsular sinus and the FDC network (Fig. 7, G-J, arrows) within germinal centers (PNA labeling serial sections, Fig. 8, L-O, days 5, 6, 10, and 12, respectively). By day 12, the distribution resembled that of day 0 with the additional labeling of the antigen-retaining FDCs. These results show that during an immune response CR-Fc binding activity moved towards the follicles and became concentrated in the germinal centers.

Discussion

MR plays an important role in endocytosis and phagocytosis of sugar bearing molecules and particles. As such, the focus of studies to date has been on the domains involved in carbohydrate recognition (CRDs), defining receptor specificity, binding properties (9, 10), and its intracellular trafficking (2). The most conserved regions between the human and murine forms of the MR are the CR and FNII domains which are located at the amino terminus of the molecule (4). This degree of conservation as well as their localization at the same region in other members of the MR superfamily, i.e., DEC205 and phospholipase A2 receptors, suggest an important role for these domains that might be common to all these proteins. Our results show that there is/are ligand(s) for the CR domain of the murine form of the MR in mouse spleen and lymph nodes. Our strategy involved the use of Fc chimeric proteins containing either both CR and FNII domains of the MR, or the CR domain alone (Fig. 1 A), to search for ligands in murine tissues. The observation that CR-FNII-Fc and CR-Fc showed identical labeling patterns (Fig. 2, compare row C with D) suggested that their binding was due to the presence of the CR domain alone and that this region has a similar conformation in both proteins. Further characterization of the molecule(s) that interact with CR will allow us to test if the CR domain as part of the whole MR molecule, behaves in the same way as in CR-FNII-Fc and CR-Fc. The possible binding of CR-Fc through Fc receptors in the tissue labeling experiments was excluded by the addition of normal mouse serum and the Fc receptor blocking antibody 2.4G2 (data not shown) and the use of other Fc chimeric proteins (MAG1-5-Fc and Sn1-Fc) or human IgG1 as controls. All the results indicated that the interaction of CR-Fc with its ligand(s) was not mediated by its Fc region.

In tissue sections from normal mice, CR-FNII-Fc and CR-Fc mostly bound to two subpopulations of F4/80+/Sn- M-CSF-dependent (Fig. 2, 3C and 3D) Mo which express macrosialin (FA11+) and the adhesion molecule Sn (SER-4/3D6+); MZ metallophilic Mo in spleen (Figs. 2 and 3) and Mo in the subcapsular sinus of LN (Fig. 4). Not all Sn+ cells are recognized by CR-Fc; red pulp and outer MZ Mo (Sn+low) in the spleen and medullary Mo (Sn+high) in the LN, as well as other Sn+ cells located in other organs (data not shown), do not interact with this protein. Detection of CR-Fc+ cells after collagenase digestion and selection of low density cells of spleen and LN, confirmed that ligands for CR are cell associated and that only a fraction of Sn+ cells bound CR-Fc. Therefore CR-Fc recognized specialized Mo populations that express the Mo restricted marker Sn and lack the Mo differentiation antigen F4/80.

Areas where most CR-Fc+ cells are located in naive animals are thought to be involved in cell and antigen trafficking. Previous studies have described binding of lymphocytes (27-30), macrophages (31), dendritic cells (32), as well as of exogenous antigens (33, 34) to splenic MZ (35) or to the subcapsular sinus of LN (30, 31).

Additional weaker staining with CR-Fc was observed in follicular areas (CR1+, Fig. 2 1E, Fig. 3 F) in normal splenic white pulp, which we have not yet assigned to a specific cell type; no FA11+, Sn- cells that bound CR-Fc were found after spleen or LN digestion suggesting that CR-Fc+ follicular cells were not isolated by this procedure. Specific follicular labeling correlated with the degree of antigen stimulation, was more commonly found in spleen and mesenteric lymph nodes than in the peripheral lymph nodes.
of naive animals and varied among mice independent of the constant presence of CR-Fc binding activity in the subcapsular sinus of LN and MZ of spleen. In particular, labeling of op/op mouse spleens (Fig. 2, column 3) showed that this follicular label was present in the absence of MZ Mo. After immunization with ovalbumin, follicular labeling increased markedly in spleen and LN; it was located in germinal centers and co-localized with CR1, the FDC marker FDC-M1 (36) (Fig. 2, compare 2C and 2D with 2E and 2F) and PNA labeling (data not shown).

To identify the origin of the follicular labeling observed after antigen stimulation, peripheral lymph nodes collected at different times during a secondary immune response were labeled with CR-Fc. The results from this experiment, shown in Fig. 7, suggested that the origin of the follicular staining could be the concentration of CR-binding cells in this area. Early after immunization CR binding activity, associated with cells that were dendritic in appearance, seemed to migrate towards the B and T cell areas and then became localized in germinal centers. This could be due to the movement of CR-Fc* cells or the transfer of CR ligand(s) from one cell type to another. According to our results, the time course of CR-Fc* cells moving into these regions paralleled the kinetics for the movement of antigen into germinal centers reported by Szakal et al. (37), and these cells may correspond to the antigen transporting cells characterized by these authors.

Five species of CR binding proteins have been detected in protein lysates from spleen and lymph nodes (Fig. 6) which raised concern about the specificity of CR-Fc in this assay. Because the patterns obtained were highly reproducible and specific for the organs where CR binding was detected, and because no other chimeric proteins recognized any of these species, we consider these proteins to be putative ligands for the CR domain of the MR. Their natures is undefined; they could represent multimers, different spliced or cleaved forms of the same molecule or distinct molecules that share the same CR binding motif. Nor is it known whether particular cells that bind CR-Fc by immunocytochemistry express all of these molecular species detected in tissue lysates. Experiments are in progress to purify the putative ligand(s) from spleen lysates, and to characterize the nature of their interaction with the CR domain of the MR. At the same time comparison between the patterns obtained with tissues from naive and immunized mice is under way to characterize molecules that could be upregulated after antigen stimulation.

The data presented in this study indicate that MR is able to interact through its CR domain with ligands expressed by cells located in areas involved in cell and antigen transport and regulated by antigen stimulation. The binding of the CR domain to ligands could mediate the trapping of MR* cells (Mo and dendritic cells) and their delivery to follicular areas. The relevance of the interaction between MZ metallophilic and subcapsular sinus Mo with MR* cells through the MR, a receptor that mediates phagocytosis and endocytosis, is not clear. A possibility we considered is the existence of a soluble form of the MR, as has been described for the human 180-kD receptor for secretory phospholipases A2 (12), that could be trapped by cells bearing CR ligands and transported to follicular areas. Such a form has recently been found in supernatants from biogel-elicted peritoneal Mo (Martinez-Pomares, L., J. Mahoney, and S. Gordon, manuscript in preparation), but no in vivo data are yet available. We postulate that carbohydrates or carbohydrate-bearing molecules could be directed, through their binding to the CRDs of the mannose receptor and subsequent interaction of the complex with the CR-ligand(s), to B cell areas in a way similar to complement and antibody-bound antigens. This would be an alternative mechanism for the transport of native antigens to sites where high affinity B cells responses are occurring.

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Figure 7. Expression of CR ligand during an immune response. Mice were primed behind the neck with ovalbumin precipitated in alum and 4 wk later, challenged with the same antigen in each limb. The draining lymph nodes were taken at 24-h intervals until day 12 (C, day 1; D, day 2; E, day 3; F and K, day 4; G and L, day 5; H and M, day 8; I and N, day 10; J and O, day 12) as well as before the secondary immunisation (A and B). Sections were stained as described in Materials and Methods with control chimeric protein (A), CR-Fc (B-F), and PNA (K-O).
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