CD11d is a novel antigen on chicken leukocytes

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ABSTRACT

In life sciences, antibodies are among the most commonly used tools for identifying, tracking, quantifying and isolating molecules, mainly proteins. However, it has recently become clear that antibodies often fall short with respect to specificity and selectivity and in many cases target proteins are not even known. When commercial availability of antibodies is scarce, e.g. for targeting proteins from farm animals, researchers face additional challenges: they often have to rely on cross-reactive antibodies, which are poorly characterized for their exact target, their actual cross-reactivity and the desired application.

In this study, we aimed at identifying the true target of mouse monoclonal antibody 8F2, which was generated against chicken PBMC and used for decades in research, while it´s actual target molecule remained unknown. We used 8F2 antibody for immunoprecipitation in chicken PBMC and subsequently identified its true target as CD11d, which was never described in chicken lymphocytes before, by quantitative LC-MSMS. The most abundant interactor of CD11d was identified as integrin beta 2.

The existence of this alpha integrin was therefore clearly proven on protein level and provides a first basis to further assess the role of CD11d in chickens in future studies.

Data are available via ProteomeXchange with identifier PXD017248.

KEYWORDS

CD11d; integrin alpha D; CD18; chicken; identifying target of mAb; immunoprecipitation

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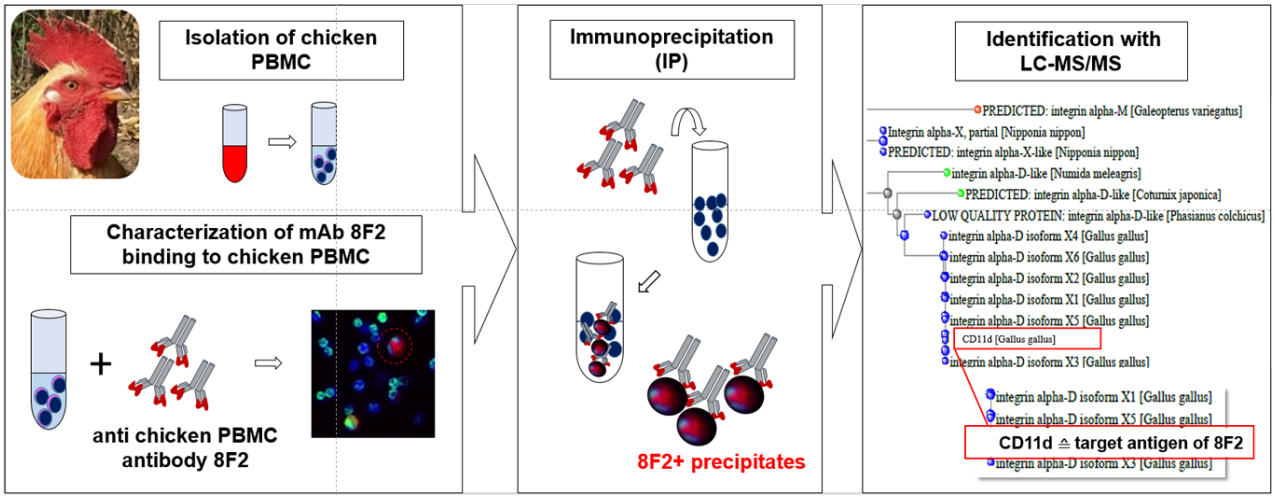
Isolation of chicken PBMC
Characterization of mAb 8F2 binding to chicken PBMC
anti chicken PBMC antibody 8F2
Immunoprecipitation (IP)
8F2+ precipitates
Identification with LC-MS/MS
PREDICTED: integrin alpha-M [Galeopterus variegatus]
Integrin alpha-X, partial [Nipponia nippon]
PREDICTED: integrin alpha-X-like [Nipponia nippon]
integrin alpha-D-like [Numida meleagris]
PREDICTED: integrin alpha-D-like [Coturnix japonica]
LOW QUALITY PROTEIN: integrin alpha-D-like [Phasianus colchicus]
integrin alpha-D isoform X4 [Gallus gallus]
integrin alpha-D isoform X6 [Gallus gallus]
integrin alpha-D isoform X2 [Gallus gallus]
integrin alpha-D isoform X1 [Gallus gallus]
integrin alpha-D isoform X5 [Gallus gallus]
CD11d [Gallus gallus]
integrin alpha-D isoform X3 [Gallus gallus]
integrin alpha-D isoform X1 [Gallus gallus]
integrin alpha-D isoform X5 [Gallus gallus]
CD11d ≙ target antigen of 8F2
integrin alpha-D isoform X3 [Gallus gallus]

INTRODUCTION

Antibodies are important to establish a basis in research of the immune system of farm animals and other species. They are used for the enrichment, detection, and quantitation of proteins and their modifications in cells involved in immune response. Antibodies are applied in a variety of methods, including western blots, immunohistochemistry, immunoprecipitation (IP), flow cytometry and cell sorting as well as enzyme-linked immunoassays (ELISA) for detection of respective targets or for ligation of the molecule and hence interference with its function. These antibodies may be monoclonal, polyclonal, or recombinant from different hosts and they may be used to get information about biological systems and signaling pathways in general or for diagnosis and therapy of disease [1, 2].

For some animal species or model systems the commercially available amount and variety of antibodies is limited, making research more difficult and challenging. Often, assumedly cross-reactive antibodies have to be used that are poorly characterized for their exact target, their actual cross-reactivity and for the desired application [3]. It is important to verify that antibodies recognize their proposed targets in the intended species as well as in additional organisms, because they often do not recognize the target or they additionally react with other proteins [4, 5]. Specific isolation of these unknown or poorly characterized target molecules from complex sample mixtures can be effectively achieved by immunoprecipitation, and subsequent mass spectrometry analysis can then be used to uncover the actual antibody target [6]. Mass spectrometry has the unique ability to identify the actual antibody target but additionally also interacting proteins in a sample [7-9]. Therefore, mass spectrometry currently beats all other existing methods of antibody-antigen identification and characterization. Unlike other methods such as western blots or ELISAs, mass spectrometry also directly provides unbiased information on target abundance and non-specific activities of the antibody tested.

In the study conducted here, we aimed at specifically identifying the target of a mouse monoclonal antibody generated against chicken peripheral blood derived mononuclear cells (PBMC). The antibody clone 8F2 has been widely used to

identify populations and subpopulations of leukocytes in the chicken [10-18], despite the lack of protein identity. 8F2 was reported to react with lymphocyte subsets, dendritic cells, NK cells, heterophils, monocytes and macrophages [10-18]. From immune precipitation studies and western blots, it was deduced that the target could be CD11c and so the specificity was given as “putative” CD11c [10-16]. To clarify the target of respective 8F2 antibody, we decided to perform an IP with a traditional method for efficient target antigen recovery and identify it´s respective antigen by quantitative label-free mass spectrometry.

MATERIAL AND METHODS

Sample preparation

Peripheral blood derived leukocytes from a total of 25 chickens (Ross 308 broilers from Aviagen and Lohmann selected Leghorn from Lohmann Tierzucht) were used in this study. In detail, we used PBMC from three different chickens for immune precipitation (and subsequent mass spectrometric analysis), six for western blots, eight for FACS analyses, three for MACS experiments and five for cytological characterizations. Animals were vaccinated for infectious bronchitis virus (IBV) at day 0 and 18 and for infectious bursal disease virus (IBDV) at day 12. Slaughtering of broilers aged 39–42 days was performed at a local abattoir. Chicken venous whole blood was collected immediately upon slaughtering process in lithium-heparin coated tubes. Blood was then diluted 1:2 in PBS (NaCl 136,9 mM, Na_2HPO_4 x $2H_2O$ 8,1 mM, KH_2PO_4 1,4 mM and KCl 2,6 mM) and PBMC were subsequently isolated by density gradient centrifugation (RT, 650 x g, 12 min, brake off) using Pancoll separating solution (PanBiotech, Aidenbach, Germany). PBMC were withdrawn from intermediate phase. Cells were washed twice in PBS (6 °C, 225 x g, 10 min) and immediately used for further experiments. No experimental animals were used in this study, blood was received fresh from a local abattoir.

Immunocytological characterization of 8F2 target in chicken PBMC

For these experiments, chicken PBMC were stained in 96 well round bottom plates with 1×10^6 cells per well. Primary antibodies were diluted in staining buffer (1% BSA + 0.001% NaN_3 in PBS) and incubated with cells for 30 min at 4 °C (8F2

monoclonal antibody from in-house invention and production; neat or anti-GST1 as isotype control). After washing with staining buffer, secondary antibody goat anti-mouse IgG:Alexa546 (Invitrogen, Karlsruhe, Germany, 1:500) was added and incubated for 30 min at 4 °C. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Karlsruhe, Germany, 1:1000). After staining, cells were transferred to microscope slides and mounted with coverslips using fluorescence mounting medium. Binding pattern of $8F2^+$ PBMC was monitored using using a Leica Dmi8 microscope with associated LAS-X-software (both Leica, Wetzlar, Germany).

Magnetic activated cell sorting (MACS) sort for morphological characterization of CD11d+ and CD11d- PBMC

4×10^7 chicken PBMC were washed in sorting buffer (PBS pH 7.2 containing 0.5% BSA and 2 mM EDTA) and incubated with 4 ml anti-chicken PBMC antibody solution (clone 8F2, monoclonal antibody from in-house invention and production, purified, 4µg/ml for 25 min at 4°C. After washing, 80 µl anti-mouse IgG1 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to 1.6 ml of cell suspension, mixed thoroughly and incubated for 15 min at 4°C. Mixture was washed, resuspended in 500 µl sorting buffer and loaded onto rinsed LS Columns (Miltenyi Biotec, Bergisch Gladbach, Germany) for magnetic separation. Eluted positive and negative fractions were separately transferred to microscope slides by cytocentrifugation and stained with hematoxylin and eosin (H&E) for microscopical assessment of cell morphology, using a Leica Dmi8 microscope with associated LAS-X-software.

Characterization of CD11d expression by flow cytometry

PBMC of five chickens were stained in 96 well round bottom plates with 1×10^6 cells per well. Mouse anti-chicken PBMC antibody (clone 8F2, from in-house invention and production) was diluted 1:250 in PBS and incubated with cells for 30 min at 4 °C. As isotype control, mouse IgG1 anti-GST1-antibody was used. After washing with PBS, goat anti-mouse F(ab')2 IgG Alexa Fluor 647 secondary antibody (Jackson, Dianova, Hamburg, Germany, 1:400) was added and incubated for 30 min at 4 °C.

As positive controls and for double stainings, mouse monoclonal IgG1 antibodies to Bu-1 (clone 21-1A4, B-cells), TCR1 (γδ T cells), TCR2 (VαVβ1 T cells), TCR3 (VαVβ2 T cells), CT-4 (CD4) and CT-3 (CD3) as well as mouse monoclonal IgG2b antibody 3-298 (CD8α) were used (all Biozol, Eching, Germany). Determination of cell viability was done by staining with propidium iodide (PI, 1:100; Miltenyi Biotec, Bergisch Gladbach, Germany). Only viable cells were included in further analyses. Measurement was performed on MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach Germany) and results were analyzed with Flowlogic software V7 (inivai, Miltenyi Biotec, Bergisch Gladbach Germany).

Immunoprecipitation (IP) of chicken PBMC

5 x 10^8 PBMC derived from whole blood were lysed in IP-lysis-buffer (TBS containing 50 mM Tris and 150 mM NaCl, 2% CHAPS, Roche complete EDTA-free protease inhibitor) and protein concentration was determined by use of Bradford protein assay (Sigma-Aldrich, Taufkirchen, Germany). Protein G Sepharose Beads (GE Healthcare, Freiburg, Germany; 40 µl per 1 mg PBMC protein) were washed with IP-buffer (50 mM Tris and 150 mM NaCl), diluted with TBS and incubated separately with mouse anti-chicken PBMC antibody (clone 8F2, monoclonal antibody from in-house production, isotype IgG1; neat) diluted in IP-buffer or respective isotype control (clone 2C8, to irrelevant target M-GST; isotype mouse IgG1; neat). Beads were added to the lysates and incubated on a tube rotator overnight at 4°C and subsequently washed with IP-lysis-buffer. Immuno-precipitates were eluted with Laemmli-buffer (stored at -20°C).

Analysis of antibody target by western blot

From each immunoprecipitation sample, 12 µl eluate were separated by SDS-PAGE on 8-10% gels and blotted semidry onto PVDF membranes (GE Healthcare, Freiburg, Germany). To prevent unspecific binding, membranes were blocked with 4% BSA. After washing, blots were incubated with monoclonal anti chicken PBMC clone 8F2 (neat, from in-house production) at 4°C overnight. HRP-conjugated anti-mouse IgG antibody (Sigma-Aldrich, Taufkirchen, Germany; 1:5000) was used for incubation at RT for one hour. After washing steps, signals were detected by

enhanced chemoluminescence (Amersham Imager 680, Analysis2.0, GE Healthcare, Freiburg, Germany).

Sample preparation for LC-MS/MS mass spectrometry

Laemmli eluates from IP were digested by a modified filter-aided sample preparation (FASP) protocol and described [1]. Briefly, eluates were diluted 1:10 with 0.1 M Tris/HCl pH 8.5 and 50 µl 100 mM dithiothreitol was added for 30 min at 60°C. After cooling down, 500 µl UA buffer (8 M urea and 1 M Tris-HCl pH 8.5 diluted in HPLC-grade water) and 100 µl 300 mM iodoacetamide were added and incubated for 30min at RT in the dark. Eluates were transferred to 30 kDa cut-off centrifuge filters (Sartorius, Göttingen, Germany) and washed 5 times with 200 µl UA-buffer and 2 times with 100 µl ABC buffer (50 mM NH_3HCO_3 diluted in HPLC-grade water). After washing, proteins were subjected to proteolysis at RT for 2 h with 0.5 µg Lys C in 40 µl ABC-buffer followed by addition of 1 µg trypsin and incubation at 37°C overnight. Peptides were collected by centrifugation and acidified with 0.5% trifluoroacetic acid.

Mass spectrometric analysis and label-free quantification

Acidified eluted peptides were analyzed in the data-dependent mode on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) online coupled to a UItimate 3000 RSLC nano-HPLC (Dionex). Samples were automatically injected and loaded onto the C18 trap column, eluted after 5 min and separated on the C18 analytical column (Acquity UPLC M-Class HSS T3 column, 1.8 µm, 75 µm x 250 mm; Waters) by a 90 min non-linear acetonitrile gradient at a flow rate of 250 nl/min. MS spectra were recorded at a resolution of 60,000 and after each MS1 cycle, the 10 most abundant peptide ions were selected for fragmentation. Acquired raw data was loaded into Progenesis QI software for proteomics for MS1 intensity based label-free quantification (v3.0, Nonlinear Dynamics, Waters), and analyzed as described [19]. MSMS spectra were exported and searched against the UniProt chicken database (35,128 sequences) using the Mascot search engine (version 2.6.2). Search settings were: enzyme trypsin, 10 ppm peptide mass tolerance and 0.02 Da fragment mass tolerance, one missed cleavage allowed, carbamidomethylation was set as fixed modification, methionine

oxidation and asparagine and glutamine de-amidation were allowed as variable modifications. A Mascot-integrated decoy database search was performed with an average false discovery rate of <1%. Peptide assignments were re-imported into the Progenesis QI software. The abundances of all unique peptides allocated to each protein were summed up. The resulting normalized abundances of the individual proteins were used for calculation of fold-changes of protein ratios between 8F2- and isotype control IP samples. Only proteins quantified by at least two unique peptides were taken for further analysis. Sequence coverage was illustrated in Scaffold (version_4.8.2, Proteome Software Inc., Portland, OR).

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [20] partner repository with the dataset identifier PXD017248.

RESULTS

The target of 8F2 antibody is found on different chicken blood cells

We evaluated cellular localization of the 8F2 antigen with immunofluorescence stainings of chicken PBMC (Fig. 1a-d). We could locate 8F2 primarily on the larger cells, but also on some lymphocytes (Fig. 1c/d). Among the few 8F2-positive lymphocytes, expression was less abundant than in the larger cells (Fig. 1c/d).

Magnetic activated cell sorting using 8F2 antibody enriches heterophils and monocytes from chicken leukocytes

Applying MACS sorting as a gentle cell isolation method, that enables the separation of 8F2+ and 8F2 negative cells under preservation of cell integrity and characteristics, we were able to further analyze the respective cell populations. There was a clear difference between the unbound cells (Fig. 2a) and the cells sorted through binding to 8F2 (Fig. 2b). We could confirm with this experiment the broad abundance of 8F2 antigen on heterophils and monocytes [15, 21], that were all found in the positive fraction (Fig. 2a/b, inserts depict respective cell types found in the two fractions). Additionally, basophils were enriched in the 8F2+ cell fraction and a further lymphocyte-like cell type with more cytoplasm, probably NK cells (Fig. 2b). Since only a minor proportion of lymphocytes was 8F2+ as shown in Figure 1,

more lymphocytes remained in the 8F2 negative fraction after MACS sort (Fig. 2a/b).

In lymphocytes, 8F2 is primarily associated with a subpopulation of CD8+ cells

Next, we wanted to characterize which subpopulations of lymphocytes (Fig. 3a) harbour the target of 8F2 (Fig. 3b). Therefore, we further characterized 8F2+ PBMC with double stainings for lymphocyte subsets in flow cytometry (Fig. 3, representative images). The 8F2 target was found on 5.7% (± 13.5%) of B cells (Fig. 3c; Bu-1) and 3.0% (± 3.3% of CD3+ lymphocytes (3d: CT-3) in average. Further characterisation of subpopulations revealed, that only 2.4% (± 7.2%) of CD4+ T cells (Fig. 3e; CT-4), but 45.6% (± 9.6%) of CD8α+ T cells (Fig. 3f; 3-298) were double positive for 8F2. Moreover, the target of 8F2 was expressed on 5.3% of γδ T cells (3g, TCR1), 11.8% of $V\alpha V\beta_1$ T cells (3h; TCR-2) and 12.8% of $V\alpha V\beta_2$ T cells (3i; TCR-3).

8F2 antibody precipitates a target at around 125 kD

For further investigation of the target protein of 8F2, eluates from immunoprecipitation were separated by SDS-PAGE, blotted onto PVDF membranes and stained with 8F2 followed by HRP-coupled secondary antibody. Samples from immunoprecipitation of PBMC with 8F2 showed enrichment of a band around 125 kDa (Fig. 4, IP mAb 8F2), suggesting a selective binding of the antibody to this protein in near native conformation in protein lysates.

CD11d was identified as target candidate by mass spectrometry

With mass spectrometry, a total of 62 proteins were detected in the precipitates of 8F2 and the isotype control antibody from chicken PBMC (data are available via ProteomeXchange with identifier PXD017248). Of these, only seven proteins were nominally enriched (> two fold) with immunoprecipitation in the three chicken PBMC lysates tested (table 1). Five of these proteins were only minimally enriched by 8F2 and with a low number of unique peptides (between 2 and 5 per protein), namely vimentin, lysozyme, tropomyosin beta, tubulin beta and Myl12, a so far uncharacterized protein in chickens (table 1). In contrast, CD11d (ITGAD) was 37fold enriched and covered by 32 unique peptides, and integrin beta 2 (ITGB2;

88kD) was 22fold enriched, covered by 28 unique peptides (supplemental table 1 and data available in Pride). Sequence coverage as calculated by Scaffold was 27% for CD11d (Fig. 5a) and 35% for integrin beta 2 (Fig. 5b).

DISCUSSION

Well-characterized antibodies are valuable tools for research, diagnostics and therapy. For farm animals, there is a lack of such antibodies against many molecules, which, on the contrary, can be readily studied in species like mice or man. We used a classical immunoprecipitation protocol to enrich the target of the widely used chicken monoclonal antibody 8F2. Subsequent mass spectrometry analysis of the enriched protein fraction unraveled CD11d and integrin beta 2 (=CD18) as highly enhanced candidates. Analyzes with western blot confirmed the mass spectrometry results, and further suggests that the primary target of 8F2 is the molecule CD11d. While both methods can not directly distinguish between primary targets and interacting proteins in immunoprecipitations, the fact that the only band revealed by Western Blot after immunoprecipitation is ~125 kDa suggests that rather CD11d (113 kDa), than ITGB2 (88 kDa) is the direct target of the antibody. Additionally, CD11d was identified with especially high relative abundance compared to ITGB2 and the other identifications (Abundances in supplemental table 1; ratio CD11d (ITGAD)/isotype control = 36.9) and the purification of the target antigen via IP was achieved from the cells´ native environment (we used primary, untreated cells immediately after isolation from blood for all our analyzes). These commonly suggested criteria for reliability of specific antigen recognition by antibodies [22] support our finding of CD11d as primary target of 8F2.

Our conclusion, that we unambiguously identified CD11d as target of the antibody 8F2 is based on a very high sequence coverage of 27% when applying high stringency for positive identification allowing only unique peptides and applying strict rules of parsimony using the Uniprot chicken database containing 27542 proteins. The UniProt knowledgebase (UniProtKB; https://www.uniprot.org/) is the central hub for the collection of functional information on proteins, with accurate, consistent and rich annotation. In addition to capturing the core data mandatory

for each UniProtKB entry (mainly, the amino acid sequence, protein name or description, taxonomic data and citation information), as much annotation information as possible is added. UniProtKB consists of two sections: a reviewed (Swiss-Prot) one, containing manually annotated records with information extracted from literature and curator-evaluated computational analysis and an unreviewed (TrEMBL) one, consisting of computationally analyzed records that await full manual annotation. To date, there are several alpha integrins from chicken in the Uniprot database (27,542 proteins from January 15, 2020), namely Cd11d (ITGAD), alpha-2 (ITGA2), alpha-3 (ITGA3), alpha-IIb (ITGA2B), alpha-6 (ITGA6), alpha-7 (ITGA7), alpha-8 (ITGA8), alpha-10 (ITGA10) and alpha-11 (ITGA11); as well as entries for alpha-1 (ITGA1) and alpha-V (ITGAV) and a fragment of CD11b (ITGAM).

Therefore, the scientifically sound conclusion of our analyses is, that the target is CD11d or an as yet unidentified gene translating into a protein with extremely high sequence homology, explaining all the identified peptides. This would be most probably ITGAX, but currently there is no such entry in the database, which either means it still has to be found and added or it does not exist in the chicken. A NCBI BLASTp of the identified peptides to all species shows the highest homology for CD11d of other species and then ITGAX of Lynx canadensis (query cover of 74%, supplemental table 2).

Since the four members of the CD11 leukocyte integrin family (CD11a, CD11b, CD11c, CD11d) are in other species non-covalently associated with the common beta subunit CD18 [23], it is of interest, that we could additionally confirm this interaction for chicken CD11d with CD18 through our experiments. A lowercase letter following the CD number indicates in the case of CD11 several molecules that share a common chain, since all CD11`s share CD18 as a common chain to form different dimers, but they are not different members of the same gene family like e.g. in CD66a-f [24].

Besides determination of target proteins, immunoprecipitation also enables identification of interactors of a protein of interest, because these will attach to the candidate protein and will consequently also be enriched when present in the input

sample [9]. Whether the five proteins that were also slightly enriched through the immunoprecipitation procedure in our experiment, are true interactors of CD11d in chicken PBMC, or if they are present due to unspecific background binding needs to be clarified in further experiments in our opinion. Tubulin beta, like vimentin, is a cytoskeletal protein [25], and both support the functional capacity, biogenesis, morphology and energy flux regulation of various cells [26]. Tropomyosin beta, on the other hand, generally binds to actin filaments and may have a role in receptor internalization in immune cells [27]. Further, we detected the expression of myosin light chain 12 (Myl12) protein, which is also needed to maintain cellular integrity [28] and lysozyme on chicken PBMC in this study. Lysozyme is an enzyme with important functions in innate immune system [29]. Through hydrolysis of cell wall peptidoglycan they possess an all-purpose weapon to kill bacteria and it has further anti-bacterial mechanisms that are hydrolase-independent [29]. Lysozyme additionally modulates the host immune response of infections [29].

So far, there was no information about interaction of these candidates with CD11d in chickens. A potential cooperation between lysozyme and CD11d in the chicken immune system is an especially interesting finding from this experiment and merits further investigations in our opinion. CD11d is known as myeloid-specific leukocyte receptor and forms the α_D subunit of the $\alpha_D ß_2$ receptor [30]. In this study, we confirmed earlier findings, that the 8F2 antigen is highly abundant on chicken heterophils [15]. Further, we showed its expression on heterophils, basophils and a minor subpopulation of chicken lymphocytes.

CD11d is the latest member of this integrin family and information about its function is scarce in all species to date [30]. To our knowledge, no functions of CD11d in chickens were described so far and this first identification of an alpha integrin on protein level is interesting for avian immunology in our opinion. Since the 8F2 antibody was already used in many different studies and cellular subsets of chickens, these data build a first basis of information about CD11d in this species [10-16]. From other species it is known that the $\alpha_D ß_2$ receptor is a cell surface adhesion receptor that mediates adhesion to extracellular matrix or other cells, through heterodimerization and connecting to the cytoskeleton [31]. This could

also be the case in chickens, since we demonstrated the concomitant enrichment of the beta integrin family member CD18 and several other proteins that are linked to the cytoskeleton. Further interesting functions of CD11d are phagocytosis of blood-borne pathogens from blood and various signaling molecules. Additionally, CD11d was found upregulated on M1 macrophages at inflammatory sites in a mouse model of atherosclerosis [32]. Upregulation of CD11d contributed to macrophage retention at inflammatory sites in these mice was additionally shown in several dysregulated inflammatory diseases, e.g. lethal systemic infections [31]. Deletion of α_D in animals infected with the malaria pathogen *Plasmodium berghei* led to a survival advantage in this otherwise lethal disease model [33]. This advantage could be attributed to an altered pattern of inflammatory cytokines after knock out of α_D [33]. In contrast, genetic deficiency of integrin αDβ2 in mice enhanced lethality of animals infected with *Salmonella serovar typhimurium* [34], through impaired accumulation of leukocytes in response to peritoneal infection by *S. typhimurium*, impaired pathogen clearance *in vivo*, defective bacterial elimination by cultured peritoneal macrophages and enhanced cell death process triggered by Salmonella [34]. These observations indicate important contributions of αDβ2 in clearing this bacterial infection [34]. Since Salmonella also play a major role in chickens, the role of CD11d and the αDβ2 integrin can now be studied with the 8F2 antibody [34]. The discovery of the first member of the alpha integrin family on protein level enables further investigations in the function of respective integrin in birds. This will clarify, if the protein behaves like CD11d in other species or more like another, closely related member of CD11.

CONCLUSIONS

In this study, we achieved to determine the previously unknown target of an antibody against chicken PBMC with immunoprecipitation and mass spectrometry. We identified the primary target of the antibody as CD11d, a novel antigen on chicken peripheral blood derived mononuclear cells, which interacts with integrin beta 2 (CD18). The technique we used proved valuable to identify the selectivity of the antibody analyzed and is the first direct evidence for a member of the alpha integrin family on protein level in the chicken.

AUTHOR CONTRIBUTIONS

C.D. conceived, designed, performed and analyzed the experiments and supervised the project; R.D., I.G., M.W., S.H., B.A., S.M.H. performed the experiments; and analyzed the data; I.G. and C.W. contributed reagents, materials and analysis tools; C.D. wrote the manuscript. All authors critically read the manuscript and approved the final version to be published.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURE AND TABLE LEGENDS

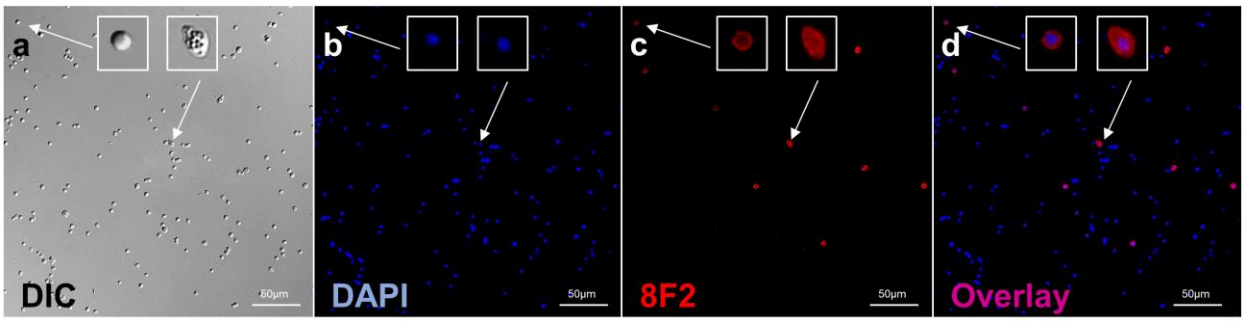

Figure 1: Immunocytological characterization of 8F2 binding to chicken PBMC. a) Differential interference contrast image (DIC) image of chicken PBMC, b) Cell nuclei were stained with 4′,6-Diamidin-2-phenylindol DAPI (blue), c) 8F2 + cells (red fluorescence) were primarily larger cells, but also some lymphocytes (Fig. 1c/d). Among the few 8F2-positive lymphocytes, expression was less abundant than in the larger cells (Fig. 1c/d), d) Overlay image of b and c; magnification: 400x.

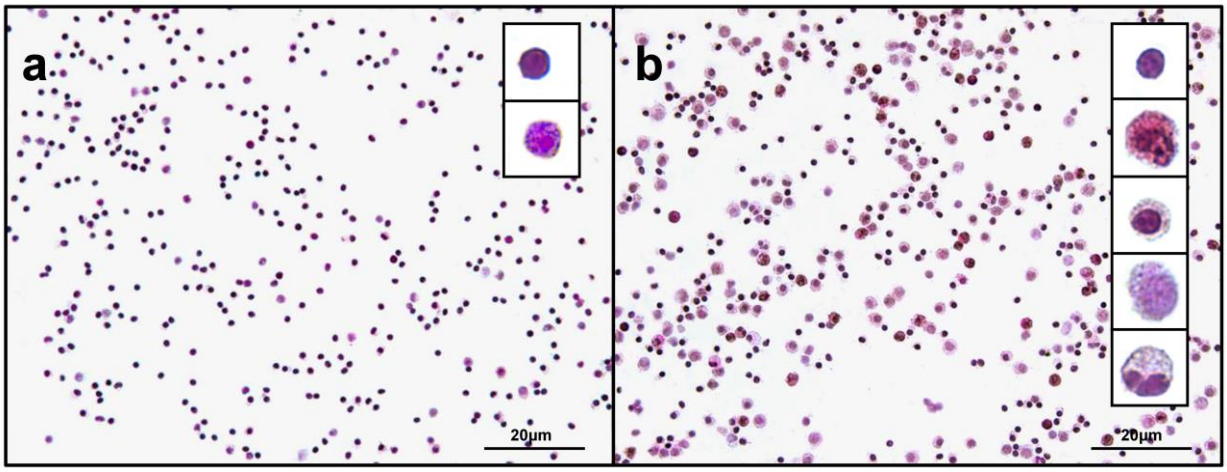

Figure 2: Magnetic activated cell sorting enriches 8F2+ cells from chicken PBMC

a) Cells that were not bound by 8F2 (negative sort), b) 8F2+ PBMC sorted by MACS, stained with hematoxylin and eosin; magnification: 1000x. Insets: representative images of celly types detected per fraction.

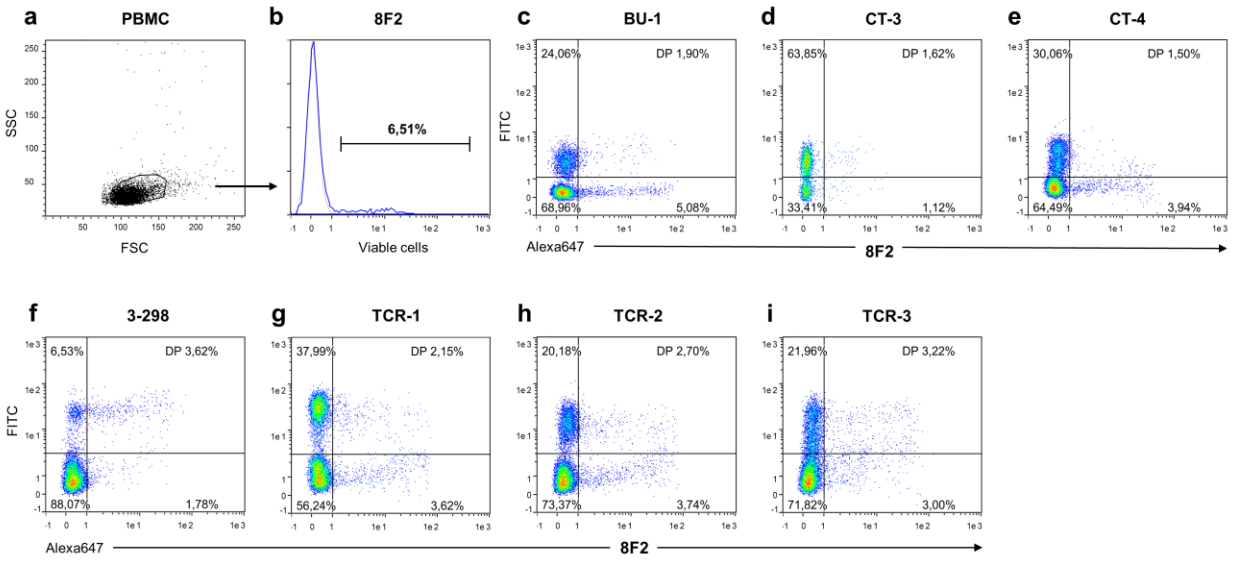

Figure 3: Characterization of 8F2 target in chicken PBMC with flow cytometry. Representative images of flow cytometry results in different leukocyte subsets. The target of 8F2 was expressed in subpopulations of chicken PBMC, a) FSC/SSC image; only viable PBMC were used for further analysis in double stainings), b) 8F2+ viable cells from gate, c) Double stainings reveal percentages of viable 8F2 positive cells (x-axis) per subpopulation (y-axis) of c) B-cells (BU-1) , d) CD3+ T cells (CT-3), e) CD4+ T cells (CT-4), f) CD8α+ T cells (3-298), g) γδ T cells (TCR-1), h) VαVβ1 T cells (TCR-2), i) VαVβ2 T cells (TCR-3).

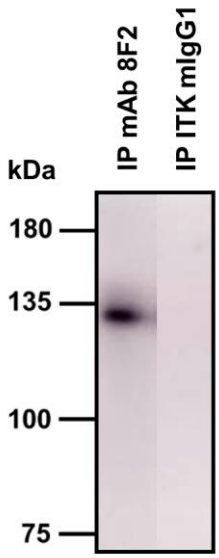

Figure 4: Antibody 8F2 precipitates antigen in chicken PBMC. Representative image of western blot analysis of eluates from 8F2 immunoprecipitation with chicken PBMC lysate. In 8F2 eluate, target protein was enriched and detected by monoclonal 8F2 antibody at around 125kD (left section, IP mAb 8F2), whereas mIgG1 isotype control showed no enrichment (right section, IP ITK mIgG1).

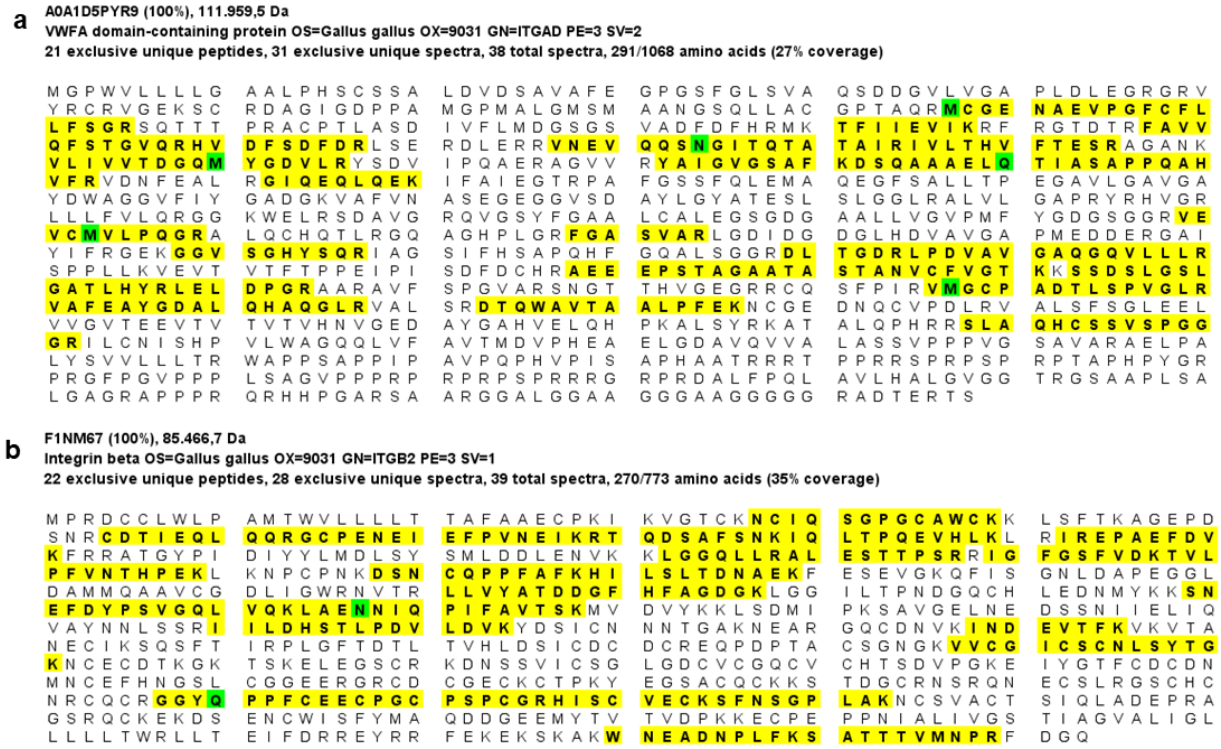

Figure 5: Sequence coverage of the two major enriched proteins CD11d and beta integrin 2. a) Identified peptides from integrin CD11d (yellow) and sequence coverage given by scaffold, b) Identified peptides from integrin beta 2 (CD18, yellow) and sequence coverage given by scaffold.

Table 1. Proteins enriched at least 2fold from chicken PBMC with monoclonal antibody 8F2. a) protein accession numbers from UniProt; b) gene name, c) protein name, d) amount of peptides used for the identification of each protein, e) amount of peptides used for the quantification of the proteins; f) confidence score, g) mass in Dalton, h) ratio 8F2/isotype control calculated with normalized protein abundances from mass spectrometry output.

Accession	**Unique peptides**	**Confidence score**	**Mass**	**ratio 8F2/ ITK**	**Gene symbol**	**Description**
A0A1D5PYR9	32	2336	112918	36,9	**ITGAD**	**VWFA domain-containing protein OS=Gallus gallus OX=9031 GN=ITGAD PE=3 SV=2**
F1NM67	28	1616	88717	22,4	**ITGB2**	**Integrin beta OS=Gallus gallus OX=9031 GN=ITGB2 PE=3 SV=1**
A0A1L1RXL9	2	141	52759	3,1	VIM	Vimentin OS=Gallus gallus OX=9031 GN=VIM PE=3 SV=2
B8YK79	2	81	16741	2,7	LYZ	Lysozyme OS=Gallus gallus OX=9031 GN=LYZ PE=3 SV=1
A0A452J805	2	192	38117	2,5	TPM2	Tropomyosin beta chain OS=Gallus gallus OX=9031 GN=TPM2 PE=3 SV=1
A0A1D5PEI5	2	244	55780	2,3	TUBB3	Tubulin beta chain OS=Gallus gallus OX=9031 GN=TUBB3 PE=3 SV=2
A0A1L1RM08	5	406	22868	2,0	MYL12A	Uncharacterized protein OS=Gallus gallus OX=9031 GN=MYL12A PE=4 SV=1

Supplemental figure 1: Additional information about the identified proteins with immunoprecipitation.

Supplemental figure 2: Results of protein blast of identified CD11d peptides in NCBI (searched against all species).