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Existence of Conventional Dendritic Cells in Gallus gallus Revealed by Comparative Gene Expression Profiling

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The existence of conventional dendritic cells (cDCs) has not yet been demonstrated outside mammals. In this article, we identified bona fide cDCs in chicken spleen. Comparative profiling of global and of immune response gene expression, morphology, and T cell activation properties show that cDCs and macrophages (MPs) exist as distinct mononuclear phagocytes in the chicken, resembling their human and mouse cell counterparts. With computational analysis, core gene expression signatures for cDCs, MPs, and T and B cells across the chicken, human, and mouse were established, which will facilitate the identification of these subsets in other vertebrates. Overall, this study, by extending the newly uncovered cDC and MP paradigm to the chicken, suggests that these two phagocyte lineages were already in place in the common ancestor of reptiles (including birds) and mammals in evolution. It opens avenues for the design of new vaccines and nutraceuticals that are mandatory for the sustained supply of poultry products in the expanding human population. The Journal of Immunology, 2014, 192: 000–000.

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onventional dendritic cells (cDCs) are functionally characterized by their exquisite capacities to present Ags to naïve T cells, having a key role in maintenance of tolerance and induction of immune effectors against invading pathogens (1). cDCs constitute a unique immune cell lineage, as recently revealed in mice by genetic cell fate mapping (2) and the precursor–progeny relationship at the single-cell level (3) and in humans by comparative gene expression profiling (4).

The online version of this article contains supplemental material.

Abbreviations used in this article: cDC, conventional dendritic cell; GSEA, gene set enrichment analyses; LC, Langerhans cell; MGG, May–Grünwald–Giemsa (stain); MP, macrophage; pDC, plasmacytoid DC; qPCR, qualitative PCR.

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phagocyte subsets of mice, sheep, and humans (4, 24, 25). In this article, a comparative gene expression analysis between chicken (*Gallus gallus*), human and mouse immune cell subsets shows the existence in chicken of MPs and of bona fide cDCs that are capable of T cell activation. These data suggest that the establishment of the cDC lineage occurred before the common bird/mammal ancestor, and is likely to apply to amniotes in general.

### Materials and Methods

#### Animals

Highly inbred white Leghorn chickens that originate from the GB1 Athens line and are homozygous for the B13 histocompatibility B complex were used for the cDCs, MPs, and T and B cell characterization. The PA12 outbred line was used to isolate CD4+ T cells in the MLR experiments; the PA12 chickens present mainly the B21 MHC haplotype (97%), and a small proportion of them present the B19 MHC haplotype (3%) (B. Bed’hom, personal). Chickens were 6.5–8.5 wk of age and were raised under specific pathogen-free conditions (Plate-Forme d’Infectiologie Experimentale-Institut National de la Recherche Agronomique, Nouzilly, France). In compliance with French law, the chickens were euthanized according to protocols approved by the Animal Ethics Committee (Région Centre, France).

#### Spleen cell subset isolation

Spleen from four different exsanguinated chickens, indifferently male or female, were used for cell isolation. After an incubation of 30 min at 37°C in RPMI 1640 plus collagenase D (400 U/ml; Roche Applied Science), and DNase (15 μg/ml; Sigma-Aldrich), spleen pieces were crushed on a steel sieve. Isolated splenocytes were washed and centrifuged on a 1.077 density Histopaque gradient (Sigma-Aldrich) to remove the nucleated erythrocytes. For B and T cell sorting, splenocytes were labeled with 2 μg/ml PE-conjugated anti–B220 mAb (AbD Serotec), and they were gassed above background in at least one subset, and 3) were differentially expressed (≥2-fold) across all cell subsets (cDC, MP, and T and B cells). Of these 116 chicken genes, 94 were found to have an orthologous number GSE55642 (www.ncbi.nlm.nih.gov/geo/), and they are publicly available. To statistically test whether mouse and human cell transcriptional fingerprints were enriched in specific chicken cell types, we performed pairwise comparisons between cell types, using the gene set enrichment analysis (GSEA) method from the Massachusetts Institute of Technology (www.broad.mit.edu/gsea).

The mouse and human cell-specific transcriptional fingerprints (Supplemental Dataset 2) correspond to genes that were found more highly expressed (>1.5 fold) in the cell population of interest compared with all other cell populations considered in the preselected compendium of arrays [see list of public database array IDs taken into account in the human and the mouse compendia (Supplemental Dataset 1)]. The cut-off of 1.5-fold has been used by the stringent “min/max” procedure calculating fold change as (minimum expression among all replicates for all other cell types / selected/maximum expression among all other replicates for all other cell types), as previously published (29). To perform GSEA on chicken expression data, using fingerprints composed of human or mouse genes, we used annotations provided by the Sigenea pipeline on the orthologs in human or mouse of the chicken genes.

### RNA extraction and hybridization on microarrays

Total RNA from subsets was extracted using the PicoPure RNA Isolation Kit (Arcturus Life Technologies) and checked for quality with an Agilent 2100 Bioanalyzer using RNA 6000 Nano or Pico Kits (Agilent Technologies). All RNA samples had an RNA integrity number ≥9. Hybridizations were performed at the Platform Biopuces et Séroquenç (Institut de Génétique et de Biologie Moléculaire et Cellulaire, 67400 Illkirch, France, www.igbmc.fr/grandesstructures/cbi/). When insufficient total RNA amounts were available (<50 ng), the RNA from the sorted subsets of distinct spleen pools was mixed. RNA labeling was performed using the one-color Low Input Quick Amp Labeling Kit (Agilent Technologies) and following the manufacturer’s recommendations. Each RNA sample (50 ng) was amplified and cyanin 3 labeled, and subsequently the cRNA was checked for quality on Nanodrop and the Agilent 2100 Bioanalyzer. Subsequently, the cRNA (600 ng) was fragmented and used for hybridization on custom-designed Agilent chicken arrays. Our custom-designed array is based on 248 immunologically related genes (sensors, cytokine, cytokine receptors, signaling receptors) from which 116 genes 1) were present on the chicken array, 2) gave signals above background in at least one subset, and 3) were differentially expressed (≥2-fold) across all cell subsets (cDC, MP, and T and B cells). Of these 116 chicken genes, 94 were found to have an orthologous gene on both human and mouse gene chips (except CLEC2B, for which an orthologous gene was present only on the human chip) as well as having a differential expression of ≥2-fold across all murine and human cell subsets. Whenever several probes were found for a given gene, the most highly expressed probe across all cell subsets in each species was selected. Gene expression values of the three species were then cross-normalized: Each of the three batches of arrays, corresponding to cell subsets from the three species, were scaled by setting the mean expression value to 0 and the variance to 1.

### Real-time PCR

For relative quantitation of gene expression in cellular subsets, RNA was reverse transcribed using random primers and SuperScript III Reverse Transcriptase (Invitrogen). Real-time qualitative PCR (qPCR) was carried out with 10 μM primers in a final reaction volume of 15 μl of 2 iQ SYBR Green Supermix (Bio-Rad). The primers used to amplify the chicken cDNA were designed with the eArray software from Agilent Technologies. The new microarray data have been assigned the Gene Expression Omnibus number GSE55642 (www.ncbi.nlm.nih.gov/geo/info/s), and they are publicly available.
Results

Identification of MP and cDC candidates in chicken spleen

We previously identified DC-like cells in chicken splenocytes that were morphologically distinct from MPs and that were not labeled by KUL01, a mAb usually considered to mark MPs (18, 28). To gain further insight into the existence of distinct MP and cDC phagocytes in chicken, we followed a flow cytometry sorting strategy to isolate candidates from suspensions of inbred SPF Leghorn chicken splenocytes, using a combination of pertinent markers among the relatively few number of mAbs available in this species. We based our reasoning on the fact that in mammals, most cDCs are “lineage”-negative cells that coexpress CD11c and MHC-II molecules. We used the well-characterized mAbs directed to the CD3 and Bu-1 Ags to exclude T and B cells, respectively, and the KUL01 to exclude MPs. After electronic exclusion gating of cells positive for CD3, Bu-1, or KUL01, we sorted the remaining cells in four populations based on their staining pattern with an anti–MHC-II mAb and the 8F2 mAb thought to target chicken CD11c, which was previously shown to label bone marrow–derived DCs (Fig. 1A). We sorted, in parallel, MP candidates as KUL01+ MHC-II+ cells (designated as KUL01+), as well as T (CD3+) and B (Bu-1+) cells (Fig. 1B–D). In an exploratory approach, we tested these cell subset candidates for the expression of three murine/human cDC-associated genes (FLT3, ZBTB46 alias BTBD4, ARGHAP22) (4), and two murine/human MP-associated genes (MAFB, CD14) (4, 31), using qPCR. MAFB and CD14 mRNA were found to be expressed at the highest level in KUL01+ cells.
(Fig. 1G). FLT3 and ZBTB46 mRNA was found expressed at the highest level in MHC-II⁺ CD11c⁺ splenocytes, and at relatively low levels in MHC-II⁺ CD11c⁻ and MHC-II⁻ CD11c⁺ splenocytes. As expected, comparatively low expression of these cDC- and MP-associated genes was detected in T and B cells. In May–Grunwald–Giemsa (MGG) staining (32), the KUL01⁺ cells resemble typical MPs with a high cytoplasmic/nuclear ratio and vacuolated cytoplasm, whereas MHC-II⁺ CD11c⁺ cells presented large dendrites (Fig. 1E, 1F). The KUL01⁺ MHC-II⁺ cells appeared to often include KUL01hi MHC-IIint and KUL01int MHC-II⁺ populations (Fig. 1D) that appeared indistinguishable in the exploratory cDC and MP gene expression analysis and that presented a homogeneous morphology in the MGG staining (data not shown). Thus, the exploratory qPCR and morphological analyses reassured us in selecting for the next steps the whole KUL01⁺ and the MHC-II⁺ CD11c⁺ populations as the MP⁺ and cDC⁺ candidates, respectively (*marks the candidate status in this article).

Transcriptome mapping of chicken cDC* and MP*

To align the chicken MP⁺ and cDC⁺ with mammalian counterparts, we devised a scale-up of a global gene expression analytic method that was previously demonstrated to be relevant for distinguishing new myeloid human subsets (33, 34) and for characterizing sheep cDC subsets (24). The transcriptome of each chicken subset—that is, B, T, MP⁺, and cDC⁺ (independent triplicates)—was obtained from mRNA hybridization on customized chicken gene chips (see Materials and Methods). From publicly available expression data (Supplemental Dataset 1), we identified mouse and human fingerprints for T cells, B cells, plasmacytoid DCs (pDCs), and CD8α⁺/BDCA3⁺ cDCs, which were established as the list of genes expressed at least 1.5-fold higher in the index cell population than in a large number of other immune cell types (Supplemental Dataset 2). We also identified mouse and human “cDC” and “myeloid” fingerprints as genes overexpressed in all cDCs compared with other myeloid cells (i.e., monocytes and/or MPs from many different tissues), and reciprocally (Supplemental Dataset 2). We next tested whether the selected mouse and human fingerprints were enriched between the four chicken subsets by performing pairwise comparisons using GSEA (35). In all comparisons, the chicken cDCs⁺ were found enriched for the mouse and human cDCs and CD8α⁺/BDCA3⁺ Genesets (q values < 0.05 in most cases), whereas the chicken MPs⁺ were found enriched for the mouse and human myeloid Genesets (q values < 0.05) (Fig. 2). In the cDC⁺ versus MP⁺ comparison, the chicken and CD8α⁺/BDCA3⁺ signatures were the only ones to be significantly enriched in a conserved manner. Conversely, in the MP⁺ versus cDC⁺ comparison, the dominant signature was the myeloid one. As positive controls, we confirmed that chicken T and B cells were enriched for the respective mouse/ human T and B cell Genesets in all comparisons (q values < 0.05). The human and mouse pDC Genesets were not found together enriched in most comparisons using our selected populations of chicken splenocytes, consistent with the fact that our strategy was not designed to identify pDCs in chicken that would have required use of different cell surface marker combinations.

Thus the gene expression profiles show that cDCs⁺ and MPs⁺ are distinct cell types in the chicken and that they strongly resemble mouse and human cDCs and myeloid cells, respectively. Furthermore, chicken cDCs⁺ appear to include a significant proportion of cells of the CD8α⁺/BDCA3⁺ cDC types.

Establishment of core gene expression signatures for T, B, cDC, and MP subsets across mice, humans, and birds

We sought to identify core gene expression signatures for T, B, cDCs, and MPs that would allow distinguishing these subsets from other immune cell types across chickens, humans, and mice, and that would likely apply to amniotes.

We selected the mRNA transcripts that contribute most to the enrichment of the mouse T, B, cDC, CD8α⁺, and MP fingerprints in all the chicken subset pairwise comparisons and that are provided as “leading edge” lists by the GSEA. We repeated the same procedure with the human fingerprints. We identified as core signatures the genes found in common in the mouse against chicken and in the human against chicken leading edges lists (Fig. 3). The MP core signature across chicken, human, and mouse encompasses the TLR4, CTSB, and CTSD genes. The transspecies cDC core signature encompasses CHITA, FAM46C, KIT, ZBTB46, FLT3, PLEKHA5 (cDC fingerprint) as well as the XCR1 and

**FIGURE 2.** Murine and human cDC and myeloid fingerprints are enriched in the candidate chicken cDC⁺ and MP⁺, respectively. GSEA was performed using sets of genes corresponding to the transcriptional fingerprints of specific murine (left) and human (right) cell types generated from compendia of expression data from many leukocytes (see Supplemental Dataset 2). Pairwise comparisons between chicken cDCs⁺, MPs⁺, and B and T cells were performed to assess enrichments of the mouse and human fingerprints. Results are represented as dots: The red and blue colors of the dots correspond to the color attributed to the subset in which the Geneset was enriched (see boxed legend within the figure); the circle surface area is proportional to the absolute value of the normalized enrichment score (NES), which varies between -1 (no enrichment) and 5 (best enrichment possible). The color intensity of circles is indicative of the false-discovery rate (FDR) statistical q value.

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**Table 1.** Summary of chicken and rodent cDC and MP core gene expression signatures.

<table>
<thead>
<tr>
<th>Species</th>
<th>Core Gene Expression Signatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>CHITA, FAM46C, KIT, ZBTB46, FLT3, PLEKHA5</td>
</tr>
<tr>
<td>Mouse</td>
<td>TLR4, CTSB, CTSD</td>
</tr>
<tr>
<td>Human</td>
<td>TLR4, CTSB, CTSD</td>
</tr>
</tbody>
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**Table 2.** Comparison of chicken cDC and MP fingerprints with rodent counterparts.

<table>
<thead>
<tr>
<th>Chicken</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDC</td>
<td>cDC</td>
<td>cDC</td>
</tr>
<tr>
<td>MP</td>
<td>MP</td>
<td>MP</td>
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</tbody>
</table>

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**Table 3.** Comparison of chicken and rodent MP fingerprints.

<table>
<thead>
<tr>
<th>Chicken</th>
<th>Mouse</th>
<th>Human</th>
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<td>T</td>
<td>T</td>
<td>T</td>
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<tr>
<td>cDC</td>
<td>cDC</td>
<td>cDC</td>
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<tr>
<td>MP</td>
<td>MP</td>
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**Table 4.** Comparison of chicken and rodent cDC fingerprints.

<table>
<thead>
<tr>
<th>Chicken</th>
<th>Mouse</th>
<th>Human</th>
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<td>cDC</td>
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<td>cDC</td>
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<td>MP</td>
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In humans and mice, DC subsets and MPs express specific and relatively conserved profiles of immune response genes, although partially overlapping (30). To identify immune response genes that are differentially regulated in cDCs and monocytes/MPs across humans, mice, and chickens, we analyzed the expression of a selection of immune response genes across the arrays used in this study (Supplemental Dataset 1; see Materials and Methods). A group of genes clustered together that appeared to be expressed at higher levels in monocytes/MPs and cDCs than in lymphoid cells in several comparisons (Supplemental Fig. 2, pink dendogram). Among these genes, some showed a higher expression in cDCs than in monocytes/MPs in all three species, that is, FLT3, XCR1, and TLR3 (Fig. 4, right panel). Several immunity molecule mRNAs were overexpressed in chicken cDCs compared with MPs, for example, for CD86, ICOSLG, and CSFR2A, whereas it was only the case in one of the two mammals. Conversely, other genes showed a higher expression in monocytes/MPs than in cDCs in all three species: CD14, TLR2, TLR4, P2RY13, SIRPα, and CSFR1 (Fig. 4, left panel). Finally, inflammatory cytokine genes (IL6, IL1B) were markedly overexpressed in chicken MPs compared with cDCs and far less clearly so in the corresponding mammalian subsets. The different array data originated from cells that were isolated from distinct tissue types with different markers for subsetting, which may hinder similar regulation of gene expression in homologous immune subsets across species. The expression of CD14, TLR2, and TLR4 was higher in monocytes/MPs than in cDCs and lymphoid cells, demonstrating a conserved specialization of MPs in the recognition of bacteria in all three species.

**Chicken cDCs are efficient at CD4+ T cell activation**

In mammals, cDCs are potent APCs that can activate naive T cells with optimal efficacy. We thus tested whether chicken cDCs presented the capacity to activate allogeneic CD4+ T cells. In five independent experiments, we observed that chicken spleen cDCs, without exogenous activation, were efficient at stimulating allogeneic CD4+ cells with optimal efficacy. We thus tested whether chicken cDCs isolated in parallel were not (Fig. 5). Thus, as in mammals, cDCs are more potent in activating allogeneic T cells than are MPs.

**Discussion**

In this article, we show that MP and cDC subsets exist in the chicken as two distinct immune cell lineages that display gene expression profiles sharing substantial homology with their mammalian counterparts. To our knowledge, this is the first time that cDCs have been identified outside mammals, and it shows that the cDC/MP paradigm extended beyond mammals, to vertebrates that diverged ∼300 million years ago from the common ancestor of reptiles, birds, and mammals. Birds also present other phagocytic cells that mammals do not—including heterophils (the chicken functional equivalent of mammalian neutrophils) and thrombocytes (homologous in function to mammalian platelets, which are absent in the chicken)—for which their role as APCs is not established.

Comparative gene expression profiling allowed us to determine similarities between immune cell subsets across the chicken, human, and mouse. Whereas this approach avoids biases of a priori hypotheses, it unravels similarities between subsets, but not exact equivalences, for different reasons. First, the MHC-II+ CD11c+ subset that was sorted with our strategy, corresponding to ~1% of splenocytes, was clearly enriched for bona fide cDCs, but it is possible that non-c-DCs, such as NK and innate lymphoid cells, were included. In addition, our selection process of human and murine cell subset fingerprints was stringent and eliminated many genes that were not found in common in the whole range of the datasets corresponding to a population type (MP, DC), owing to tissue or species exception, absence of functional probes, or mis-
annotated probes. An example is the absence of CD14 and MAFB from the mouse myeloid signature because of their low expression in MPs of some tissues. Consequently, CD14 and MAFB, which are highly expressed in chicken MPs, do not fall in the myeloid core signature across the chicken, human, and mouse. To avoid this problem, the human and mouse fingerprints should have been

**FIGURE 4.** Comparative expression of immune response–related genes in cDCs, MPs, and T and B cells across chicken, human, and mouse species. Bar chart representing the relative expression (mean ± SD) of a selection of monocyte/MP-specific (top) or cDC-specific (bottom) immune response–related genes in chicken (left), mouse (middle), and human (right) cDC and monocyte/MP populations (cDC in red/orange and MP in black/gray in the legend, respectively). These populations include, for chicken: MPs and cDCs; for human: monocyte (mono)–derived MPs, PBMC-derived MPs, nonclassical blood monocytes, classical monocytes, BDCA3+ cDCs, and BDCA1+ cDCs; for mouse: peritoneal cavity MPs, lung MPs, nonclassical monocytes, classical monocytes, splenic CD8α+ cDCs, cutaneous lymph node CD8α+ cDCs, splenic CD11b+ cDCs, and cutaneous lymph node CD11b+ cDCs. Values were computed from two to five independent replicates for each cell population, except for human mono-derived MP (n = 1). Normalized gene expression values were scaled for each gene and species to the highest expression value across all cell types.
and B lymphocytes, with no difference between cDCs and MPs. In agreement with this finding, a newly generated anti-chicken CD205 mAb was found to label both putative DCs (CD8α+) and MPs (KUL01+) in chicken spleen (22). Thus, in the chicken, CD205 may not discriminate cDCs from MPs.

The core gene expression profiles we identified in this study allow proposal of a unifying molecular definition of T, B, MP, and cDC subsets across the chicken, human, and mouse, which should be valid across amniotes. These core signatures can be used to search for homologous subsets in other model and application species, including distant vertebrates such as fishes. Most genes of the transspecies MP and cDC core signatures are included in the core gene lists of MP and cDCs across mouse tissues identified in an independent study by the Immgen group (31). Several of the CD8α+ cDC-type genes, such as CADM1 and XCR1, were found in the transspecies cDC core signature, strongly indicating that chicken are equipped in CD8α+-type cDCs and that their cDC lineage is likely composed of the two subsets previously identified in mammals, CD8α+-type and CD11b+-type cDCs. Whereas several of the cDC signature have already been studied for their biological implication in cDC biology, nothing is known regarding PLEKHA5 and FAM46C. Similarly, the molecules of the T and B cell core signature are well known for their function, except for KHLH14. As these genes are conserved in the gene expression program of specific immune cell subsets across evolution, they should be studied for their role in the specific function or development of these subsets.

Our results underline some degree of conservation of functions for cDCs and MPs across amniotes. Several immunity-related genes were similarly regulated between cDCs and MPs across the three species under study. The conserved selective expression of sensors that are considered more viral (TLR3) and bacterial oriented (TLR2, TLR4, CD14) in cDCs and MPs, respectively, suggest that these two lineages are specialized in distinct pathogen sensing in amniotes. Furthermore, CTSB and CTSD are members of the MP core signature, further pointing to the strong lysosomal activity in MPs for degradation purposes. In addition, the stronger capacity for CD4 T cell activation, as well as the higher expression of the CD86 and ICOSLG genes in chicken cDCs compared with MPs, shows that in all species, cDCs developed as specialized in T cell activation.

Efficient strategies to prevent disease outbreak in chicken farms is mandatory to supply the growing world demand for poultry products that is expected to increase at a 2.5% rate per year until 2030. The poultry sector strongly relies on vaccination for maintenance of health and production status, with birds receiving >20 vaccines during their short life (16). Our identification and molecular characterization of cDCs in chickens will help in designing better vaccine strategies and in developing new immunomodulators as alternatives to antibiotics.

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Disclosures
The authors have no financial conflicts of interest.
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