ELISPOT and intracellular cytokine staining: Novel assays for quantifying T cell responses in the chicken

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Summary
The measurement of T cell responses in chickens, not only for quantitative aspects but also for the qualitative nature of the responses, becomes increasingly important. However, there are very few assays available to measure T cell function. Therefore, we have developed enzyme-linked immunosorbent spot assay (ELISPOT) and an intracellular cytokine staining (ICCS) assay. ELISPOT assay for the detection of chicken interferon-gamma (ChIFN-γ) production was set up and shown to be reproducible for both polyclonal and antigen-specific stimuli such as Newcastle disease virus (NDV). However, the ELISPOT assay lacks the ability to identify individual cytokine-producing cells. Separation of CD4$^+$ and CD8$^+$ T cell populations gave additional information, but appeared to have the disadvantage of a loss of cell interactions during stimulation. In a further refinement, individual cells were identifiable by ICCS, which gives the possibility to characterize for multiple characteristics, such as cytokine production and phenotype of the cell. Using ICCS, ChIFN-γ production was evaluated. Although cells were detected at only low frequencies, polyclonal stimulation of peripheral blood mononuclear cell (PBMC) or spleen cells resulted in a significant increase in ChIFN-γ production by CD4$^+$ and CD8$^+$ cells.
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Introduction

Qualitative and quantitative methods to study T cell responses in mammals, such as intracellular staining of cytokines and enzyme-linked immunosorbent spot (ELI-SPOT), have not yet been properly established in avian research. These techniques provide highly sensitive and quantitative analysis of T cell activity and above all the ability to study the nature of T cell responses at the single-cell level. Intracellular staining of cytokines has the added advantage that qualitative analysis can be performed through labeling of cell surface markers, allowing identification of the specific cell subpopulations that contribute to cytokine production. By depletion of specific subsets of T cells using cell sorting methods additional information on the responding cells might also be found with the ELISPOT assay.

The purpose of this study was to develop an ELISPOT assay and intracellular cytokine staining (ICCS) for the detection of chicken interferon-gamma (ChIFN-γ) as novel tools to examine avian T cell responses, and to test their functionality in the study of host–pathogen interactions.

IFN-γ plays an important role in cell-mediated immune responses. ChIFN-γ production is used as an indicator for cell-mediated immune activity in various avian disease models [1,2]. In this study, birds immunized with a commercial Newcastle Disease Virus (NDV) vaccine were used to test whether anti-viral responses could be measured. A role for cell-mediated immunity has been shown in NDV infections, where proliferation of (mainly CD8^{+}) T cells was observed in response to NDV vaccination [3,4].

Materials and methods

Animals

Adult commercial White Leghorn and Silver Nick chickens were housed in groups and fed *ad libitum* on commercial feed. Chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

Virus

The NDV used for *in vitro* recall activation was obtained as commercial freeze-dried vials which contained at least 10^6 EID$_{50}$ per dose (Nobilis; ND clone 30; batch: 031046D, Intervet, The Netherlands).

The chickens were divided into two groups, a vaccine group and a control group. At 5 weeks of age, chickens were vaccinated with Nobilis Newcavac (Intervet) i.m. and live ND clone 30 by eye-drop, according to recommendation. The spleens were isolated at 17 days post-vaccination. All inocula were prepared in LPS-free phosphate-buffered saline (PBS) prior to use at a concentration of 10^6 EID$_{50}$/ml.

Preparation of splenocytes

Spleen tissue was squeezed through 70 μm mesh in RPMI1640 culture medium containing 2% FCS to prepare a single cell suspension. Splenocytes were isolated by density gradient centrifugation for 20 min at 850g using FICOLL-Hypaque 1.078, washed twice with PBS (Cambrex) and adjusted to 3×10^6 cells/ml in culture medium (RPMI1640 medium supplemented with 10% FCS, 2 mM glutamax-I, 50 mM β-mercaptoethanol (β-ME) and 100 U/ml penicillin/streptomycin).

ELISPOT assay

MAIPS4510 MultiScreen™-IP 96-well plates (Millipore, Billerica, USA) were coated with 5 μg/ml mouse-anti-ChIFN-γ (CAC1233; Biosource International, California, USA) in coating buffer (sodium bicarbonate, 50 mM, pH 9.6) for 2 h at 37 °C, 5% CO$_2$, or overnight at 4 °C. All incubation steps were performed with 100 μl/well. Plates were washed twice with blocking buffer (RPMI 1640 supplemented with 2% FCS, 2 mM glutamax-I, 100 U/ml P/S, 50 μM β-ME and incubated with blocking buffer for 1 h at 37 °C, 5% CO$_2$). The blocking buffer was discarded and splenocytes were seeded at 3×10^5 cells/well in triplicate in culture medium. Cells were incubated in the presence of either culture medium or medium supplemented with one of the following stimuli to a final volume of 200 μl per well: phorbol 12-myristate 13-acetate (PMA) and ionomycin, concanavalin A (ConA; Sigma), or NDV (ND clone 30). The cells were incubated for 24 or 48 h at 41 °C, 5% CO$_2$. Subsequently, the plates were washed twice with distilled water, and three times with washing fluid (PBS supplemented with 0.1% Tween-20). ChIFN-γ was detected by incubation with 1 μg/ml biotinylated mouse-anti-ChIFN-γ (CAC1233; Biosource International) in assay buffer (PBS supplemented with 0.1% Tween-20 and 1% BSA; 100 μl/well) for 1 h at room temperature. Plates were washed four times with washing fluid and incubated with 2 μg/ml streptavidin-alkaline phosphatase (Sigma; 100 μl/well) in assay buffer for 1 h at room temperature. Plates were washed five times with washing fluid and the assay was developed using 5-bromo-4-chloro-3-indolyl-phosphate/p-nitro blue tetrazolium chloride substrate (Roche, Basel, Switzerland). The plates were then washed with copious amounts of tap water, air dried and analyzed using the A·EL·VIS machine and the Eli.Analyse software (Version 4.0) that allows for automated counting of the number of spots based on size and intensity.

Magnetic bead depletion

Splenocytes were labeled with mouse anti-chicken CD4 (CT-4) or CD8α (CT-8; Southern Biotechnology Associates, Inc., Birmingham, USA) in MACS buffer (PBS supplemented with 1% FCS, 2 mM EDTA) for 20 min on ice. Cells were washed twice with MACS buffer, resuspended with goat anti-mouse IgG MicroBeads (Miltenyi Biotec, GmbH) and cell depletion was performed using a magnetic separation column according to the manufacturer's instructions. The cells were counted and seeded in duplicate in ELISPOT wells coated with mAb anti-ChIFN-γ (as described previously) at

3×10^5 cells/well. Cells were incubated for 48 h at 41 °C, 5% CO_2 in culture medium, or medium supplemented with ConA, or PMA and ionomycin. ChIFN-γ-producing cells were detected using the ELISPOT protocol as described above. Samples of each depleted cell population were taken to analyze bead depletion efficiency using flow cytometry.

ELISA for ChIFN-γ detection

A total of 2×10^6 splenocytes per chicken were incubated for 48 h in a 24-well plate in 500 μl of culture medium at 41 °C, 5% CO_2, without stimulation or in the presence of ConA. Afterwards, supernatants were tested for ChIFN-γ using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, California, USA) according to the manufacturer's instructions.

Intracellular staining of ChIFN-γ

Splenocytes were seeded in duplicate in 96-well round-bottomed plates (Corning Costar, NY, USA; 3799) at 10^6 cells/well in culture medium, and stimuli in culture medium were added to a final volume of 200 μl per well. The cells were incubated at 41 °C, 5% CO_2. After 2 h incubation Brefeldin A (Sigma; 5 μg/ml) was added and incubation continued for another 2 h. The cells were washed and labeled with fluorescein (FITC)-labeled or phycoerythrin-labeled mouse anti-chicken CD8α (CT-8), CD8β (EP42) or CD4 (CT-4; Southern Biotechnology Associates). Cells were washed twice with FACS buffer and incubated with Perm/Fix solution (Becton Dickinson, Franklin Lakes, USA) for 20 min on ice. Cells were washed by incubation with Perm/Wash solution (Becton Dickinson), supplemented with 0.25% normal mouse serum (NMS). Cells were intracellularly stained with mouse-IgG anti-ChIFN-γ antibody mAb80 [5] labeled with allophycocyanin. Cells were washed with Perm/Wash solution with 0.25% NMS followed by a wash step with FACS buffer, and the cell pellets were resuspended in FACS buffer. Cells were acquired on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) using Cell Quest software. Data analysis was done with FlowJo software (Treestar, Ashland, OR).

Statistical analysis

Statistical analysis was performed using SPSS version 12.0.1 for Windows. Analysis of data was performed using a paired *T*-test or one-way ANOVA. Results were considered statistically significant if $p \leqslant 0.05$.

Results

ChIFN-γ ELISPOT using mitogen stimulation

To set up the ELISPOT assay mitogen-stimulated splenocytes were used. ChIFN-γ production was measured at 48 h after stimulation with either ConA or PMA and ionomycin (Figure 1A). A highly significant ($p < 0.0001$) increase in the number of spots was found after mitogen stimulation when compared to unstimulated splenocytes.

To test the specificity of the spots, we compared ELISPOT plates coated with antibodies against either chicken IFN-γ or bovine IFN-γ. Chicken splenocytes and bovine peripheral blood mononuclear cells (PBMCs) were incubated for 24 h in culture medium with or without ConA or PMA and ionomycin. Production of IFN-γ was only detected with IFN-γ-specific antibodies of the same species-specificity as the coating antibodies (Figure 1B). High spot counts were found in wells with chicken splenocytes and anti-ChIFN-γ or bovine PBMC with anti-bovine IFN-γ antibodies (a gift from Dr. A. Koets). Wells with chicken splenocytes and anti-bovine IFN-γ antibodies were negative, confirming the absence of non-specific interactions between the culture supernatant and the well surface.

To test whether the ELISPOT results coincide with an existing assay to quantify ChIFN-γ at protein level, ChIFN-γ production was measured using both ELISPOT and ELISA (Figure 2). Though it must be taken into account that the correlation between number of cells that produce ChIFN-γ in the ELISPOT assay and the quantity of soluble ChIFN-γ as measured by ELISA is unknown, in both assays all birds showed a significant increase in ChIFN-γ after ConA stimulation.

To evaluate the contribution of different cell subpopulations to the ChIFN-γ response, CD4$^+$ and/or CD8α^+ splenocytes were depleted, ensuring depletion of both CD8$\alpha\alpha$ and CD8$\alpha\beta$ subpopulations, before performing the ELISPOT assay. Depletion with magnetic beads was between 93% and 96% efficient (Figure 3A).

After depletion of CD4$^+$ or CD8α^+ T cells, ChIFN-γ production decreased considerably ($p < 0.001$ for both depletions) in splenocyte cultures stimulated with PMA and ionomycin (Figure 3B). ChIFN-γ production was almost completely absent in ConA-stimulated splenocyte cultures after depletion ($p < 0.0001$).

ChIFN-γ production after vaccination

Splenocytes from NDV-vaccinated and unvaccinated chickens were stimulated with NDV for 24 h and tested for ChIFN-γ production using the ELISPOT assay (Figure 4). An increase in ChIFN-γ production was found after restimulation with NDV, and a significantly higher number of spots were found using splenocytes of NDV-vaccinated chickens compared to splenocytes of unvaccinated chickens ($p < 0.05$).

Intracellular staining of ChIFN-γ

Another powerful but demanding technique to study T cell responses is the intracellular staining of cytokines combined with surface staining of lymphocytes at single cell level. Splenocytes were stimulated for 4 h with PMA and ionomycin or ConA and double stained for T cell markers and ChIFN-γ. A representative example is shown in Figure 5A–C. The results show a significant ($p < 0.05$) increase in ChIFN-γ production by CD4$^+$ and CD8α^+ cells compared to cells stained with the isotype control (Figure 5D).

Based on the fluorescence intensity (FI) of CD8α, the CD8α^{int} positive cells were relatively more frequent ChIFN-γ^+ than the CD8α^{hi} positive cells (Figure. 5B plot 2). The cells were considered to be CD8α^{int} based on their FI ranging from
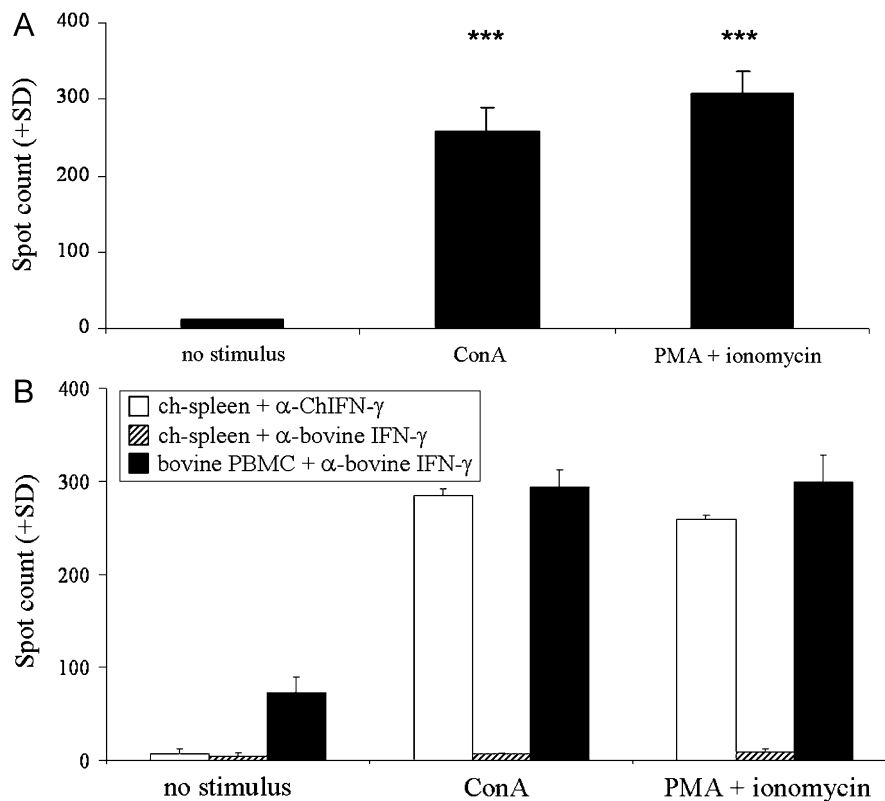
Figure 1 ELISPOT analysis of stimulated T cells. (A) Splenocytes were added in triplicate to ChIFN-γ-coated wells and stimulated for 48 h with medium, ConA (10 µg/ml), or PMA (100 ng/ml) and ionomycin (500 ng/ml). Spot counts represent the number of ChIFN-γ-secreting cells. *** Significantly different from unstimulated group ($p<0.0001$; $n=9$). (B) Chicken splenocytes were stimulated for 24 h with ConA (10 µg/ml), or PMA (100 ng/ml) and ionomycin (500 ng/ml), in wells coated with antibodies against chicken (white bars) or bovine (hatched bars) IFN-γ. Bovine PBMC were incubated under the same conditions for 24 h in wells coated with bovine IFN-γ antibodies (black bars).
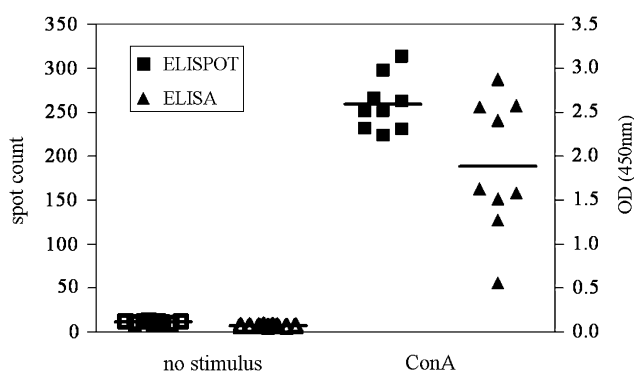

Figure 2 ChIFN-γ production measured using ELISA and ELISPOT. Splenocyte cultures ($n=9$) were stimulated for 48 h in culture medium with or without ConA (10 µg/ml). ELISPOT results for each bird (squares) are depicted as spot counts (left y-axis). ELISA results for each bird (triangles) are depicted as OD readings at 450 nm (right y-axis).

approximately 13 to 60 and CD8α^{hi} with an FI of 60–290 (gates based on FI are shown in Figure 5B and C plot 1). After triple staining for CD8α, CD8β and ChIFN-γ, the results indicated that both CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ cells produce ChIFN-γ (Figure 5C plot 3). Based on the percentage cells that were

CD8α^{int} or CD8α^{hi} and the percentage cells that were ChIFN-γ^+ CD8α^{int} and IFN-γ^+ CD8α^{hi}, we concluded that also in the triple stained cells the CD8^{int} positive cells produce ChIFN-γ relatively more frequently (Figure 5C plot 1 vs plot 3) than the CD8α^{hi}. A significant downregulation of CD4 was found after 4 h stimulation with ConA or PMA, whereas CD8α was only downregulated after 4 h stimulation with ConA (Figure 5E).

Discussion

In this paper we present a functional ELISPOT assay and an ICCS technique to measure ChIFN-γ production for analysis of both CD4 and CD8 T cell responses in chickens. IFN-γ production is routinely used as a marker for T cell activity in a wide range of species. The ELISPOT and ICCS assays are established methods for measuring IFN-γ production in other species such as mice, humans, cats and horses [6–9], but are not yet available in poultry research. They have several advantages over other methods that are currently used to measure T cell responses in poultry. ELISPOT and ICCS are more discriminative and antigen-specific, and do not require radioactivity or lengthy procedures. Furthermore, the ICCS provides additional qualitative information that is not available using techniques such as ELISA, quantitative
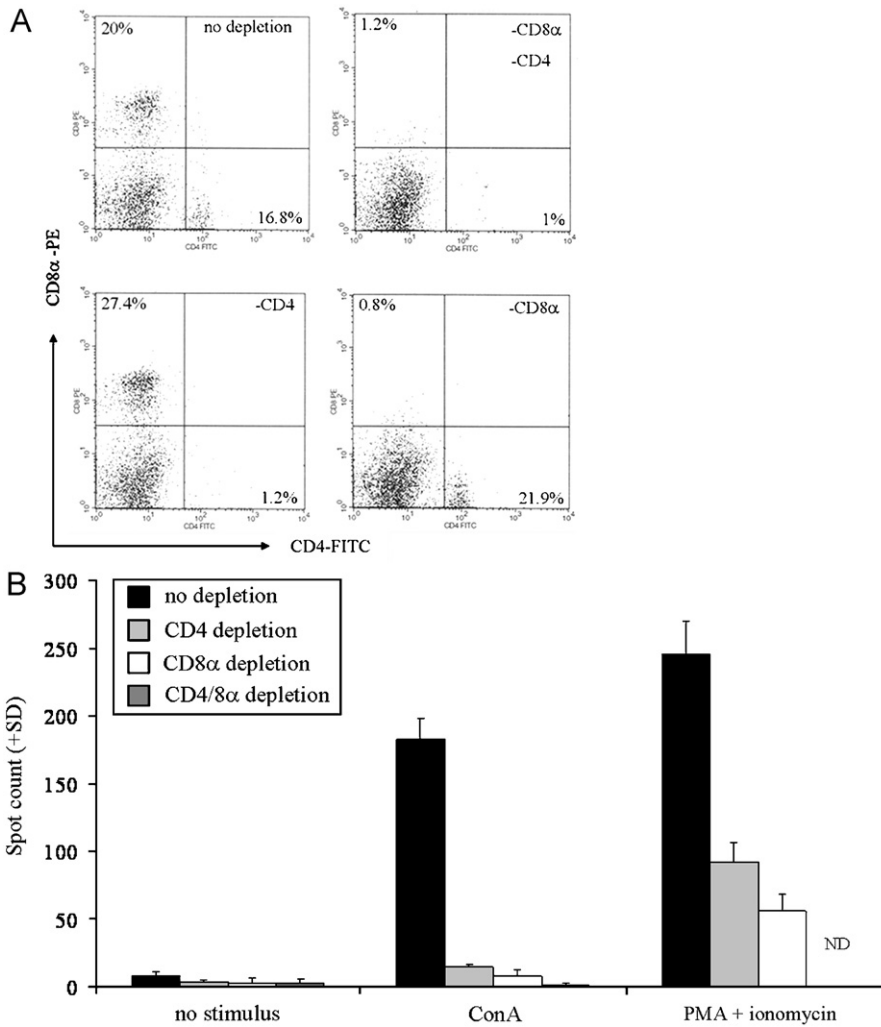
Figure 3 (A) Representative illustration of depletion of chicken splenocyte subpopulations using magnetic beads. Depletion efficiency is shown for CD4 (bottom left), CD8α (bottom right) or both (top right) cell subpopulations, as compared to an undepleted splenocyte sample (top left). (B) Splenocytes ($n = 2$) were incubated in triplicate for 48 h at 3×10^5 cells/well in ChIFN-γ-coated wells with medium, PMA (100 ng/ml) and ionomycin (500 ng/ml), or ConA (10 μg/ml). Spot counts represent ChIFN-γ-secreting cells from whole splenocyte cultures or depleted cultures as indicated. ND: not done.
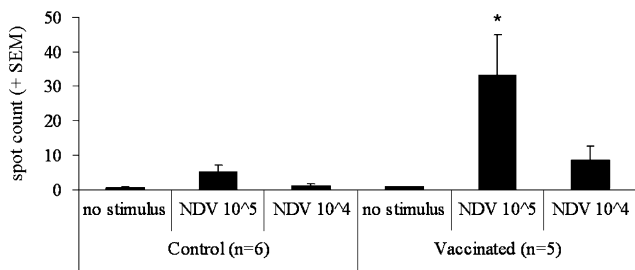

Figure 4 Splenocytes were incubated in ChIFN-γ-coated wells for 24 h in medium or medium containing NDV (10^5 or 10^4 EID$_{50}$). Spots represent ChIFN-γ-secreting cells.* Significantly different from control group ($p < 0.05$).

PCR (qPCR), chromium-51 (^{51}Cr)-release and proliferation assays.

When combined with magnetic bead depletion, the ELISPOT assay could not provide information about the contribution of specific subpopulations to the immune response. This was possibly due to loss of antigen presenting cells during the depletion procedure. ICCS has the advantage that qualitative information can be obtained without the need for depletion of certain cell types, a treatment that can influence the immune response due to loss of cell interactions.

To exclude non-specific binding of ChIFN-γ to the ELISPOT plate membrane or the capture antibodies, we incubated chicken splenocytes in wells coated with antibodies directed against bovine IFN-γ. Spots were found in wells coated with anti-chicken, but not with anti-bovine IFN-γ mAbs, confirming that the spots were specific for ChIFN-γ-producing cells.

Although the ELISPOT technique provides us with a way to quantify the immune response, it does not give qualitative information about the type of immune cells involved. After depletion of cell subpopulations using magnetic beads, qualitative information could not be obtained in addition to quantitative information when using ELISPOT. A possible explanation for the higher number of spots found in depleted cultures after PMA and ionomycin stimulation than
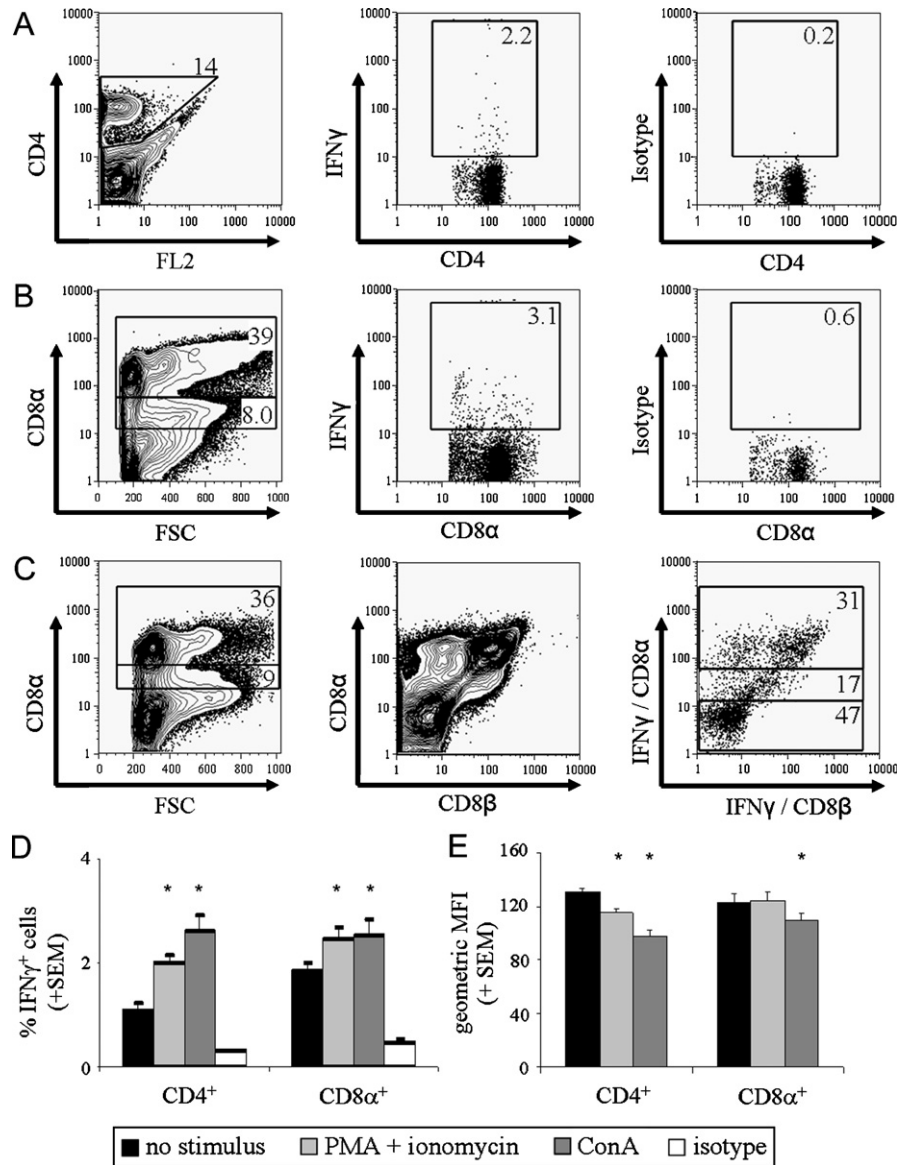
Figure 5 Intracellular staining for ChIFN-γ of splenocytes. Chicken splenocytes were stimulated for 4 h with PMA (50 ng/ml) and ionomycin (500 ng/ml), and live cells were gated for CD4 (A) or CD8α expression (B). Representative staining of ChIFN-γ produced by CD4$^+$ cells (A plot 2) and CD8α^+ cells (B plot 2), compared to the isotype control (A and B plot 3). Cells were triple stained for CD8α, CD8β and ChIFN-γ (C). Definition of CD8α^{hi} and CD8α^{int} positive cells as indicated by the gating is shown in the first plot. Identification of CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ cells is shown in the second plot. In the third plot, ChIFN-γ-producing cells expressing CD8β and/or CD8α are identified. The bottom left graph (D) shows the percentage of CD4$^+$ and CD8α^+ splenocytes that produce ChIFN-γ after stimulation with PMA and ionomycin, or with ConA (5 μg/ml) compared to an isotype control (+SEM; $n = 11$). Changes in geometric mean fluorescence intensity (MFI) of CD4$^+$ and CD8α^+ splenocytes after stimulation with PMA and ionomycin or ConA are shown in the bottom right graph (E;+SEM; $n = 11$). Asterisks indicate statistical significance compared to isotype control (D) or unstimulated cells (E) ($p < 0.05$).

after ConA stimulation is that PMA and ionomycin stimulation drives a more generalized activation of immune cells (including CD4$^-$ and CD8$^-$ cells) without the need for costimulation [10], whereas ConA-induced activation may require help from other cell types such as APCs [11] that are potentially lost in the depletion procedure.

In the ICCS assay, where depletion of cell subpopulations is not required for qualitative analysis, CD4$^+$, CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ cells were shown to produce ChIFN-γ. The percentage CD4$^+$ and CD8α^+ cells that produced ChIFN-γ increased significantly after stimulation with either ConA or PMA. Stimulation with ConA showed no difference between the percentage of ChIFN-γ-producing CD4$^+$ and CD8α^+ cells when using ICCS. CD8α^{int} positive cells produced ChIFN-γ relatively more frequently than CD8α^{hi} cells. This could be due to downregulation of CD8α on the cell surface upon activation with ConA. However, after 4 h stimulation with PMA we did not observe a downregulation of CD8α on the cell surface.

After stimulation with PMA, natural killer cells, which are CD8$\alpha\alpha^+$ [12], might be responsible for the relatively higher frequency of IFN-γ^+ cells. However, we do not yet know whether these cells are CD8α^{int}. Similar to human mononuclear cells, we saw downregulation of CD4 after 4 h stimulation with PMA and no downregulation of CD8α after 4 h stimulation with PMA [13], whereas both CD4 and CD8α were downregulated after 4 h stimulation with ConA.

To determine whether the ELISPOT assay is functionally applicable to the study of virus infections, we measured ChIFN-γ responses in splenocytes of chickens that had been previously vaccinated with NDV. After stimulation of splenocytes from NDV-vaccinated chickens with live NDV we measured a significant increase in the number of ChIFN-γ-producing cells, whereas no response was found in splenocyte cultures of unvaccinated chickens with the same dose of NDV.

We have developed convenient, sensitive and quantitative ELISPOT and ICCS assays for the chicken that can be used to measure ChIFN-γ responses induced by mitogens and additionally, in the case of the ELISPOT assay, for a selected pathogen. ICCS provides additional qualitative information on the cell subpopulations contributing to the response. These assays offer an alternative to assays such as ^{51}Cr-release, proliferation or qPCR assays in poultry. In the near future, we intend to adapt these assays for other cytokines as soon as antibodies become available.

Acknowledgments

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