Details of the Results

Three-week-old chickens

Histopathology, immunohistochemistry for NDV, T cells, B cells
Results are presented in Table 1.

At the portal of entry, in the noninfected controls, there were no histologic abnormalities nor any signal for NDV. For T cells, a regular dense scattering was identified in the submucosa, and with approximately equal numbers of B cells directly below the T cells. At 1 dpi, there were rare clusters of epithelial cells with cytoplasmic NDV signal (Figure 1a), and overall lymphocyte numbers were increased to double the control levels as evidenced by immunohistochemistry for T and B cells. By 2dpi, rare epithelial ulceration was evident, and there were more epithelial cells with NDV signal than at 1dpi, including some positive cells in the submucosa. Signal for T cells and B cells was prominent, more than the controls, but less than that seen at 1 dpi. At 3 dpi, histopathologic changes were marked, with extensive ulceration, and depletion of all lymphoid zones. Correspondingly, the NDV signal was not present as filling cytoplasm of distinct cells, but rather consisted of fragments and spots of positivity distributed generously over a degenerating and necrotic background. Signal for both T and B cells at 3 dpi was minimal, with levels lower than those seen in the non-infected control birds.

Spleen was histologically unremarkable in the noninfected control birds and there was no signal for NDV on immunohistochemistry. Signal for B cells was evident in the outer regions of the splenic ellipsoids, and peripheral to that T cell signals were present. At 1 dpi, histologically, there was no difference from noninfected controls and no signal for NDV. Signal for T cells at this time point was in the same area as the noninfected controls, but signal was much stronger, approximately double that of controls. The same pattern was seen for B cells at this time point. At 2 dpi, there was general congestion of the spleen, and the ellipsoids had expanded, with marked hypertrophy of the inner zone and some degeneration of these cells, many of which contained signal for NDV. T-cell signal was decreased compared to 1 dpi, although still above control levels, but B-cell signal was markedly expanded, especially in the ellipsoids with the most NDV signal. By 3 dpi, many of the macrophages in the S-S sheaths were markedly vacuolated, or necrotic, with extensive fragments of NDV staining overlain. Some B-cell signal was evident, at levels more than in the noninfected controls, but T-cell signal was minimal, at levels less than half of those seen in the noninfected controls.

In the cecal tonsils, noninfected birds were histologically unremarkable, without any NDV signal. Both T- and B-cell signal was present in the lamina propria. At 1 dpi, histologically, lymphocytic infiltrates were present, and infrequent large cells in the lamina propria had NDV signal. Also, rarely, there was NDV signal filling the cytoplasm of scattered enterocytes. Both T- and B-cell signals were increased over noninfected levels, with T cells scattered throughout the lamina propria whereas B cells assumed a clustered format closer to the tunica muscularis. Histologically, increased lymphocytic cellularity of the lamina propria was evident at 2 dpi, with many large cells with cytoplasmic NDV signal. Both T- and B-cell signals was abundant at this time point, markedly more than noninfected controls. At 3 dpi, histologic changes were prominent, with extensive fragmentation and loss of cellularity evident throughout the lamina propria, small deposits of fibrin deep within the lamina propria, and NDV signal extensively present both within cells as well as dots and fragments overlying areas of depletion (Figure 3a). T-cell signal at this time was minimal, less than the noninfected controls, but B-cell signal remained slightly elevated over these levels, but much less than that at 2dpi.

Thymus of noninfected controls were histologically normal and there was no signal for NDV. Consistent signal for T cells was seen across all medullary areas. There was no signal for B cells in the noninfected control birds nor in any of the infected birds at any time point. At 1 dpi, histologically, there were no abnormalities, but there was signal for NDV in rare epithelial cells in the medulla. Signal for T cells was restricted to medulla but approximately 1.5X that of the noninfected controls. At 2 dpi, cortical zones were mildly to moderately depleted, and a starry sky appearance was evident in the medulla, but with prominent epithelial cells remaining. Signal for NDV was strong, in both epithelial cells of medulla as well as within numerous apoptotic cells throughout the cortex. Marked degenerative changes were more evident at 3 dpi, with extensive NDV positivity, but T-cell signal was less than that seen in controls.

Cloacal bursa in the noninfected control birds had no signal for NDV but extensive positive B-cell signal in all the central (medullary) portions of the follicles. There was no signal for T cells in any of the bursas at any of the time points, including the noninfected control birds. At 1 dpi, histologically, bursa was no different from the noninfected control bird. Rare single-cell signal for NDV was present at this time point, at the corticomedullary junction (Figure 2a), and there was a modest increase in B cell signal, still within the medullary zones. Histologically, medullary areas were decreased in size at 2 dpi, with infrequent positive NDV cells, but the same increase in B-cell signal as was seen at 1 dpi. By 3 dpi, many bursal follicles were markedly shrunken, with prominent depletion and apoptosis in cortical zones. Signal for NDV was prominent at

this time point, with extensive positivity in those cells at the corticomedullary junction, and present primarily in the follicles that were undergoing marked diminution. B-cell positivity was strong, more than double that seen in controls, and was evident in both cortex and medulla of many follicles (Figure 2c).

In situ hybridization for IL-1β, IL-6, TNF-α, and IFN-γ

Results are presented in Table 1. For all of the cytokine identifications in situ, the area where the cytokines were found most often corresponded to the zones that were positive by IHC for NDV.

IL-1β and IL-6 expressions had relatively similar tissue and quantity distributions. In the negative controls, both were present in low amounts. IL-1β signal was usually associated with cells morphologically consistent with macrophages, whereas IL-6 signal was found in a variety of cell types, often spindle-shaped cells indicating perhaps vascular smooth muscle cells or fibroblasts. At the portal of entry (Figure 1b), the presence of cells with these cytokines increased from 1-3 days. Similar increases were seen in the spleen. Modest increases were seen in the cloacal bursa (Figure 2b). In cecal tonsils, similar increases in IL-1β signals occurred and were sustained, but IL-6 expression had a modest increase and then decreased (Figure 3b). In thymus, medullary areas demonstrated increased amounts of cells expressing these cytokines through the period of infection. In all cases, the signal in the thymus was in the medulla.

TNF-α positivity was infrequently present. When present, it was in large cells. There were some small spots of positivity within the medulla of the thymus in uninfected control, but not in any of the other control tissues. In the thymus, the amount of TNF-α signal increased in infected birds up to 3 dpi, always in the medullary areas. At the portal of entry, TNF-α signal was absent until 2 dpi, when it was present in large amounts. Then the following day, 3 dpi, it was only minimally present. TNF-α signal was never identified in any of the spleen, cecal tonsil, or bursal tissues.

IFN-ɣ positivity was most reliably found in spleen and thymus. Its presence was always noted as just a small spot, within cells morphologically consistent with lymphocytes. In uninfected controls, IFN-ɣ signal was present only at the portal of entry, and in small amounts. Through the experimental period, IFN-ɣ expression was present sporadically but without any particular pattern. At the portal of entry, it was present only as few small spots within lymphoid follicles, at every time point (Figure 1c). It was present in increasing numbers in both spleen and thymus of infected birds. In thymus, it was present in the medulla early, but at later time points was in both cortex and medulla. In the spleen, it was predominantly in the ellipsoids. In cecal tonsils, IFN-ɣ expression was

absent in the negative control and 1 dpi infected birds, but then present as punctate areas in the lamina propria, but more extensively at 3 dpi (Figure 3c).

62-week-old birds

For the 62-week-old birds, the focus of investigation was on the portal of entry, spleen, cecal tonsils, and reproductive tract. For the latter, five areas were examined initially - ovary, infundibulum, magnum, isthmus, and shell gland. Only infundibulum and ovary are described here because signals were more pronounced in these tissues, with the other areas usually lacking any evidence of NDV, lymphocytes, or cytokines.

Histopathology, immunohistochemistry for NDV, T cells, B cells
Results for IHC are presented in Tables 3 and 4.

At the portal of entry, the noninfected controls were histologically normal and lacking any NDV positivity. These tissues had scattered infiltrates of T and B cells, in approximately equal numbers, as seen by immunohistochemistry. At 1 dpi and 2 dpi, there were infrequent focal ulcerations of the epithelium, with NDV signal in rare epithelial cells and even less frequently within submucosal cells. T and B cell signal was increased at 1 dpi, to approximately 1.5X that of control levels, and then even more so, to more than double control levels at 2 dpi. By 3 dpi, mucosal ulceration was marked, and these ulcerated foci had extensive amounts of NDV signal in the subjacent tissue. T- and B-cell signals throughout the submucosa were at the same levels at 3 dpi as was seen at 2 dpi.

Noninfected spleens were unremarkable and without any NDV signal. T- and B-cell signals were the same as seen in the noninfected spleens from the 3-week-old birds. Histologically, splenic ellipsoids were mildly expanded at 1 dpi but no NDV signal was present. Compared to non-infected controls, T and B cells were more prominent at 1 dpi, approximately 1.5X control levels, and then more at 2 dpi and 3 dpi, often to double the levels seen in the controls. The ellipsoids were very expanded at 2 dpi, occasionally to the point of confluence, with multifocal NDV signal throughout them. By 3 dpi, peri-arteriolar lymphoid sheaths were very decreased in size but NDV signal was extensive, within large cells in the ellipsoids.

Histology of cecal tonsils was unremarkable in the noninfected control birds and also at 1 dpi. No NDV signal was present in the noninfected control birds. Weak signal for both T and B cells within the lamina propria was present in the noninfected controls. At 1 dpi, a few epithelial cells were positive for NDV, T-cell positivity was no different from controls, but B-cell signal was increased, about 1.5X control levels. At 2 dpi,

histologically, there was a marked increase in lymphocytes within the lamina propria and numerous large cells with NDV cytoplasmic signal deep within the lamina propria. T-cell signal was strong at this time point, markedly more than the negative controls, and B-cell signal was slightly less than the signal for T cells. By 3 dpi, histologically the lamina propria of cecal tonsils was markedly depleted of cells, and NDV signal was present as dots and fragments distributed over the depleted background. Also, at 3 dpi, T-cell signal, often present as fragments, was extensive within cecal tonsils, and B-cell signal was present as small clusters deep within the lamina propria, both at more than double the level of the controls.

Infundibulum had mild changes at 2 dpi, consisting of some areas of focal epithelial necrosis that was accompanied by modest inflammatory infiltrates at 3 dpi. Similarly, ovary had some degeneration and necrosis evident at 2 dpi which progressed to mild to moderate inflammation through to 3 dpi. Both infundibulum and ovary had scattered but often limited NDV positivity at 2 and 3 dpi, in epithelium and submucosa of the infundibulum (Figure 4a), and in epithelial cells only in the ovary. Negative control tissues had infrequent T and B cells present. In the infected birds, the levels of T cells were similar to the control tissues, except for a mild increase in the ovary at 3 dpi and in the infundibulum at 2 dpi, with both always associated with inflammatory infiltrates. For B cells, numbers were increased at 2 and 3dpi in both tissues, and occurred in a more follicular arrangement that that seen in the negative control birds.

In situ hybridization for IL-1β, IL-6, TNF-α, and IFN-γ

Results are presented in Table 3. For all of the cytokine identifications in situ, the area where the cytokines were found always corresponded roughly to the zones that were positive by IHC for the NDV.

IL-1β signal was not present in any of the sections of nasal turbinate. In the spleen it was rarely present in the control bird, but then increased markedly through 1 and 2 dpi, with some decrease at 3 dpi, with signal consistently at the edges of the periarteriolar lymphoid sheaths (PALS), roughly the same areas as were positive for NDV. In cecal tonsil, IL-1β expression was present only at 2 dpi, in scattered areas within the lamina propria. In the infundibulum, IL-1β signal was evident at 1 and 2 dpi, often in abundance, and always in large cells associated with inflammation (Figure 4b). In ovary, pattern was similar but more intense and associated with necrosis and infiltration of inflammatory cells, predominantly lymphocytes.

IL-6 signal was not seen in any of the nasal turbinate sections, at any time points. For spleen, there was no positivity until 2 dpi, when the signal was extensive and often

clustered. In cecal tonsils, there was no signal until 3 dpi when the signal was scattered in the lamina propria and present in the same areas as IL-1β. In infundibulum and ovary, signal followed the same pattern as IL-1β, being prominent at 1 and 2 dpi.

In situ hybridization for TNF-α was negative in all tissues and at all time points, with the exception of infundibulum of infected birds at 2 dpi, where expression was present as a small cluster within a necrotic and inflammatory focus (Figure 4c).

IFN-ɣ positivity was not detected in any of the nasal turbinate tissues. In spleen, it was detected rarely, as single spot signals, at 1 and 3 dpi in infected birds. Similarly, in cecal tonsils, it was seen infrequently, as single points of positivity, at 2 dpi. It was absent in the infundibulum and ovary.