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Mucosal Adjuvant Activity of Flagellin in Aged Mice

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Abstract

We evaluated the ability of flagellin, a highly effective mucosal adjuvant in mice and non-human primates, to promote mucosal innate and adaptive immunity in aged mice. We found that intratracheal instillation of flagellin induced a stronger respiratory innate response in aged mice than in young mice, and that intranasal instillation of flagellin was equally effective at triggering recruitment of T and B lymphocytes to the draining lymph nodes of young and aged mice. Intranasal immunization of aged mice with flagellin and the *Yersinia pestis* protein F1 promoted specific IgG and IgA production, but at lower levels and lower avidities than in young mice. Although intranasal instillation of flagellin and F1 antigen increased germinal center formation and size in young mice, it did not do so in aged mice. Our findings are consistent with the conclusion that flagellin can promote adaptive immune responses in aged mice, but at a less robust level than in young mice.

Keywords

vaccination; mucosa; memory

1. Introduction

Vaccination is much less effective in elderly populations than in young populations (Goodwin et al., 2006). In humans, aged individuals have reduced numbers of antigen reactive CD4+ T lymphocytes following immunization (Kang et al., 2004) as well as reduced levels of cytokine production on a per cell basis (Deng et al., 2004). The ability of immunization to generate cytotoxic T lymphocyte (CTL) activity and antibody responses in aged individuals is also significantly less than in young individuals (Powers and Belshe, 1993). Numerous mechanisms contribute to these deficits including decreased IFN-γ signaling in macrophages (Yoon et al., 2004), expansion of CD4+CD28− (Weyand et al., 1998) and CD8+CD28− cell populations (Goronzy et al., 2001; Saurwein-Teissl et al., 2002), decreased cytoskeletal rearrangement (Garcia and Miller, 2002) and impaired intracellular signaling following stimulation (Miller, 1989; Tamir et al., 2000). The B cell compartment is also subject to age-related impairments including decreased expansion of antigen-specific B cells (Dailey et al., 2001) and decreased hypermutation within responding populations of antigen-specific B cells (van Dijk- Hard et

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al., 1997; Troutaud et al., 1999). Attrition of the naïve T cell population (Lazuardi et al., 2005) and diminished TCR diversity in the total T cell population (Naylor et al., 2005) compound the detrimental effects of the loss of naïve B cells (Johnson et al., 2002), decreased B cell lymphopoeisis (Miller and Allman, 2003) and reduced antibody repertoire in aged humans (Kolibab et al., 2005; Smithson et al., 2005). The combination of loss of function by aged cells and reduction in the repertoire of cells capable of responding to a given antigen impose severe limitations on the aged immune system.

However, results from studies with humans reveal that an enhanced immunization regimen can compensate for some of the defects inherent in the aged immune system. Increasing the antigen dose at immunization (Roos-Van Eijndhoven et al., 2001; Keitel et al., 2006) and increasing the number of immunizations (Roos-Van Eijndhoven et al., 2001) results in higher antibody titers in aged humans compared to standard immunization schedules. While administering more antigen is biologically effective at the level of the individual, production constraints (Dennis, 2006) limit the practicality of immunizing entire populations with higher antigen doses. Other immunomodulatory approaches such as administering inflammatory cytokines at the time of immunization have proven effective at enhancing responses in aged mice (Haynes et al., 2004) but are not being considered for inclusion in human vaccines. Currently alum is the only adjuvant employed for widespread use in humans (Petrovsky and Aguilar, 2004). Alum is moderately effective in young and middle-aged humans, though other adjuvants in development have been shown to be more effective in these age groups (Lewis et al., 2003). The development of new adjuvants for use in humans may provide additional means of compensating for age-related defects of the immune system and increasing vaccine efficacy in the elderly.

Flagellin, the major structural component of bacterial flagella (Lowy and Hanson, 1964; Lowy and McDonough, 1964) and the ligand for Toll Like Receptor 5 (TLR5) (Hayashi et al., 2001), has long been known to possess in vivo adjuvant activity (Waldmann and Munro, 1975). Its adjuvant activity is believed to result from its ability to promote dendritic cell maturation (McSorley et al., 2002; Means et al., 2003; Cuadros et al., 2004; Pino, 2005) and consequently facilitate antigen specific T cell proliferation (McSorley et al., 2002). Flagellin has also been shown to be an effective systemic and mucosal adjuvant in mice (McEwen et al., 1992; Levi and Arnon, 1996; Ben-Yedidia et al., 1999; Strindelius et al., 2004; Honko et al., 2006) and non-human primates (Honko et al., 2006). Additionally, intratracheal (i.t.) instillation of young mice with flagellin triggers a robust respiratory innate response (Honko and Mizel, 2004). Flagellin has been shown to be effective at promoting anti-viral immunity in aged mice (Ben-Yedidia et al., 1998). In view of flagellin's efficacy as an adjuvant for immunization against bacterial (Newton et al., 1989; Honko et al., 2006), viral (McEwen et al., 1992; Levi and Arnon, 1996; Ben-Yedidia et al., 1998), and parasitic infections (Ben-Yedidia et al., 1999) in young mice, we have evaluated its ability to function as a *Y. pestis* vaccine adjuvant in aged mice.

Given the paucity of information on the adjuvant effect of flagellin in aged animals, we have examined key events in the development of the adaptive immune response in young and aged mice and determined how they differ. We have developed a *Y. pestis* vaccine (with flagellin as the adjuvant) which should soon enter Phase I clinical trials, and we anticipate that the results of our studies on aged mice will assist in shaping expectations of the ability of flagellin to function as an adjuvant in aged humans.

2. Materials and Methods

2.1 Mice

Eighteen months is a commonly accepted age for "aged" mice used for immunological studies (Tomer et al., 1991; Asanuma et al., 2001; Boehmer et al., 2004; Ehrchen et al., 2004; Mittler and Lee, 2004; Vannier et al., 2004; Alignani et al., 2005; El-Shaikh et al., 2006; Montes et al., 2006; Sen et al., 2006). For these studies, aged BALB/c mice from the Harlan colony were purchased through the National Institute on Aging, and 6 to 8 week-old BALB/c mice were purchased directly from Harlan (Indianapolis, IN). All mice were housed in the Wake Forest University Health Sciences animal facility in accordance with institutional and USDA regulations. All experiments were conducted in accordance with protocols approved by the Wake Forest University Health Sciences Animal Care and Use Committees.

2.2 Recombinant Proteins

The coding sequence for the F1 antigen of Yersinia pestis, caf1 (plasmid containing the entire caf operon kindly provided by J. B. Bliska, State University of New York, Stony Brook), was subcloned into the NdeI and XhoI sites of the pET29a expression vector from Novagen (EMD Biosciences, Inc., Madison, WI). Recombinant his-tagged Y. pestis F1 protein, Salmonella FliC flagellin, and the non-signaling FliC mutant 229 were produced as previously described (McDermott et al., 2000; Honko and Mizel, 2004; Honko et al., 2006). All recombinant proteins were purified using Acrodisc Mustang Q and E membranes (Pall Corporation, Ann Arbor, MI). Contaminating endotoxin levels were verified to be less than 10 pg LPS per μg protein by Limulus amebocyte lysate assay (Associates of Cape Cod, Inc., East Falmouth, MA).

2.3 Nonsurgical Intratracheal & Intranasal Instillation

Non-surgical i.t. instillation was conducted as previously described (Honko and Mizel, 2004). Forty μl was the standard volume used for all i.t. instillation. For intranasal (i.n.) instillations, 12 to 15 μl of solution were applied drop wise, alternating between nostrils. All immunizations with F1 protein were administered intranasally. For these experiments, flagellin and F1 were administered as separate proteins.

2.4 Bronchoalveolar Lavage & Nasal Wash

Mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation as a secondary method. Bronchoalveolar lavages (BAL) were conducted as previously described (Honko and Mizel, 2004), except that lungs were inflated only once with 1.5 to 2 ml of PBS. For nasal washes a similar procedure was followed except the tubing was inserted through an incision in the trachea toward the nasal passageway rather than toward the bronchi. Nasal passages were flushed with 0.35 ml of PBS, of which an average of 0.32 ml was recovered into a 1.5 ml tube held at the nose of the mouse. Bronchoalveolar lavage fluid (BALF) and nasal wash fluid were centrifuged to remove cells. Supernatant was stored at −70°C until analysis.

2.5 Cell counts and cytospins

Cells collected from BALF were counted in a hemacytometer. Two hundred microliters of cell suspension were dispersed onto slides using a cytospin machine set at 450 rpm for 5 min. Slides were stained using a Hema 3 stain set (Fisher Scientific Co.) according to the manufacturer's instruction.

2.6 Flow Cytometry

Lymph nodes were teased into a single cell suspension and washed. Red blood cells were lysed with ACK lysing buffer (Cambrex Bio Science, Walkersville, MD) and the remaining lymphocytes were washed again. Cells were incubated with anti-CD3 (145-2C11) and anti-

CD19 (1D3) antibodies from Becton, Dickinson and Company for 30 minutes at 4°C, washed, and fixed in 2% paraformaldehyde until analysis. Samples were acquired on a BD FacsCalibur flow cytometer and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

2.7 ELISA

TNF-α levels in BALF were measured using Becton Dickinson's OptiEIA ELISA kit according to manufacturer's instructions. Samples from at least six mice were analyzed for each group. For measurement of the anti-F1 antibody response, 96 well plates were coated overnight with 100μl F1 protein at a concentration of 10μg/ml. The plates were then washed 3x, blocked overnight with 250μl of 10% FBS, and then washed again. Samples were added to the wells and incubated overnight at 4°C before washing five times. Horse radish peroxidase conjugated anti-mouse IgG (Jackson ImmunoResearch) or IgA (Southern Biotech) was added to the wells and developed with tetramethlybenzidine (Sigma). After 30 min, development was stopped with 2N H_2SO_4 and absorbance at 450nm was measured. For all antibody experiments, ten mice were used per group. All samples were analyzed in triplicate.

ELISA to compare antibody avidity were conducted as described above with the addition of a 15 minute incubation of sodium thiocyanate (NaSCN) solution followed by 3 washes prior to incubation with horse radish peroxidase conjugated anti-mouse IgG. The avidity comparison is based on the concentration of NaSCN required to cause a fifty percent reduction in absorbance (Macdonald et al., 1988). In preliminary experiments we tested antibody dilutions ranging from $1:10^3$ to $1:10^5$ and determined $1:10^4$ to be the optimum sample dilution for comparison of avidity.

2.8 Histology and Immunofluorescence

Lungs were perfused for 15 min with 10% formalin following the same procedure used for collection of BAL before removal and transfer to 10% formalin for 24h. The tissue then was trimmed, embedded in paraffin, cut at 4μm, and stained with hematoxylin and eosin by routine methods. The nasal tissue was prepared by removing the skin from the head prior to preserving in 10% formalin overnight, followed by decalcification for 6.5h in Surgipath Decalcifier II solution. A coronal section immediately rostral to the eyes was embedded in paraffin, cut at 4μm and stained with hematoxylin and eosin by routine methods. For histological examination of the lungs and NALT, groups of four mice were used for each condition.

For immunofluorescence lymph nodes were harvested from mice and snap frozen in Optimal Cutting Temperature Compound (OCT) from Sakura (Torrance, CA). Six μm-thick frozen sections were cut from the blocks and dehydrated in acetone. Slides were then blocked with 2% BSA in PBS for 30 min at room temperature. For detection of germinal centers, slides were incubated with the rat anti-mouse IgD antibody 11–26c (eBioscience). After three rinses with 2% BSA in PBS, slides were incubated with AlexaFluor^{647} labeled goat anti-rat IgG and AlexaFluor^{568} conjugated peanut agglutinin (Invitrogen). The slides were again washed 3x with 2% BSA in PBS, and coverslips were applied using Prolong Gold antic-fade mounting reagent (Invitrogen). Slides were imaged using a Nikon Eclipse TE300 inverted microscope and a Retiga EX camera. Overlays were composed using Adobe PhotoShop 7.0.

Germinal centers were identified as areas of lymphoid tissue which bound peanut agglutinin (Rose et al., 1980). Tissue sections were cut to a thickness of 6 μm, and every twelfth section throughout the depth of the organ was collected for immunofluorescent analysis. The number of sections analyzed varied depending on the size of each individual lymph node. Generally, between 8 and 12 sections were analyzed per node. Germinal center area was measured in pixels using ImageJ 1.36b (NIH) and then converted to square millimeters. Germinal center volume was calculated by multiplying the average cross sectional area of each germinal center

by the depth of the germinal center. The depth of each germinal center was determined by multiplying the number of tissue sections in which the germinal center was detected by 72, which was the number of microns between analyzed sections. Germinal centers which appeared in only one section were assigned a depth of 72 μm.

2.10 Controls & Statistics

The non-signaling mutant flagellin, termed 229, was used as a control in all flagellin experiments. This protein consists of the hypervariable region of flagellin and does not bind TLR5. Recombinant 229 was produced using the same procedure as used for flagellin.

Statistical analysis of data was performed with SigmaStat 3.10 (Systat Software, Inc., Point Richmond, CA). For normally distributed data sets, significance was determined using the Student's T test. The significance of data sets which were not normally distributed or of unequal variance was determined using the Mann-Whitney rank sum test. P values of less than 0.05 were considered significant. Error bars represent the standard error of the mean.

3. Results

3.1 Flagellin triggers in vivo TNF-α production in aged mice

Toll-like receptor expression in cells from aged mice has been reported to be lower than in cells from young mice (Renshaw et al., 2002), and stimulation of aged macrophages with TLR ligands results in significantly lower levels of TNF-α production than stimulation of young macrophages (Renshaw et al., 2002; Boehmer et al., 2004). To evaluate the ability of flagellin to trigger TNF-α production in vivo in aged mice, we administered flagellin by i.t. instillation and collected BALF 4h later. Instillation of aged mice with flagellin resulted in TNF-α levels in BALF that peaked at 4h and returned to near baseline levels 24h after treatment. The difference in TNF-α levels in BALF from young and aged mice at 4h was not statistically significant (Fig 1A). This result conforms closely to previous findings in young mice (Honko and Mizel, 2004) and demonstrates that aged BALB/c mice are capable of mounting an initial flagellin induced innate immune response similar in magnitude to that of young mice.

In vivo studies examining cytokine production and lethality in aged mice following challenge with LPS have shown that aged mice are more susceptible to LPS challenge than young mice (Tateda et al., 1996). While these studies involved treatment of mice with extraordinarily high doses of LPS, they do raise the possibility that aged mice may exhibit an altered dose response to lower doses of TLR ligands. Previously published studies have shown that instillation of 5 μg of flagellin is required to achieve maximal TNF-α levels in the lungs of young mice (Honko and Mizel, 2004). We evaluated the dose response to flagellin in aged mice by i.t. instilling 5 μg 229 control protein, 1, 5, 15 μg flagellin, or 10μg CpGODN as a positive control and measuring TNF-α levels in BALF 4h later. Maximal TNF-α levels were achieved with of 1 μg of flagellin (Fig 1B). These levels were not significantly different from TNF-α levels following instillation of 5 or 15 μg of flagellin ($P>0.05$). Notably, the level of TNF-α present following instillation of 10 μg CpG-ODN was markedly less than the level achieved following instillation of 1 to 15 μg of flagellin (Fig 1B). Intratracheal instillation of flagellin in aged mice did not result in detectable levels of TNF-α in the plasma (data not shown). These results are consistent with the hypothesis that flagellin triggers an innate immune response in aged mice and that aged mice are more sensitive to flagellin than younger mice.

3.2 Flagellin triggers an acute innate response in the lungs of aged mice

To further characterize the innate immune response to flagellin in aged mice, 5 μg of flagellin or 229 control protein was instilled i.t., and 12h later lung tissues were collected for histological evaluation. Lungs from flagellin-treated aged mice exhibited acute inflammation. Diffuse

hyperemia and peribronchial neutrophil infiltrates were present to similar degrees in both age groups, while controls were within normal limits (Fig. 2). Bronchial associated lymphoid tissue was present in both flagellin treated and 229 control aged mice. Comparison of tissue sections from untreated aged and young mice revealed that aged mice consistently have more bronchus associated lymphoid tissue than young mice (N=3, data not shown).

Though acute inflammation in the lungs of young and aged mice following instillation of flagellin appeared similar, we also evaluated the numbers and types of cells recovered from BALF. To quantify the cellular infiltrate into the lungs of aged and young mice, we i.t. instilled aged mice with 5 μg of flagellin, and sacrificed mice at 4, 12, and 24h following treatment. Differential cell counts were performed on cell populations recovered from bronchoalveolar lavage fluid. Young mice were also instilled with 5 μg flagellin and sacrificed at 12h. As demonstrated for young mice (Honko and Mizel, 2004), inflammatory cell infiltration in the lungs of aged mice became maximal 12h after flagellin instillation and was largely resolved by 24h. However, slightly more than 3 times as many cells were recovered from the BALF of aged mice at the peak of the response as from young mice in the present study (Fig. 3A). Consistent with lung histology, neutrophils were overwhelmingly the dominant infiltrating cell type in both young and aged mice (Fig 3B).

3.3 Flagellin triggers cellular infiltrate in the upper respiratory tract of aged mice

Previous work has shown that i.n. immunization of young mice with flagellin is highly effective at promoting protective immunity (McEwen et al., 1992; Levi and Arnon, 1996; Ben-Yedidia et al., 1999; Honko et al., 2006). To compare the innate response to i.n. administered flagellin in aged and young mice, we instilled mice with 5 μg of flagellin or the control protein 229 and sacrificed mice 12h following treatment. Histological evaluation demonstrated similar tissue changes and acute inflammation in the nasal tissue of young and aged mice 12h after i.n. instillation of flagellin, compared to control animals. For example, neutrophil-rich exudates along the respiratory epithelium in the nasal cavity and loss of cilia, vacuolation and individual cell loss among the respiratory epithelial cells were evident (Fig. 4). None of these changes were observed in the control animals.

TNF-α was undetectable in nasal wash fluids or BALF 4h following i.n. instillation of flagellin (data not shown). Histological evaluation of lungs from mice 12h following treatment with flagellin was essentially normal, although slightly more cells were recovered in BALF of flagellin treated mice compared to controls (data not shown). Given the importance of the innate response in the development of an effective adaptive response (Pulendran and Ahmed, 2006), flagellin's ability to trigger a robust but transient innate response in the respiratory compartment of aged mice is highly significant.

3.4 Flagellin results in increased cellularity in draining lymph nodes

To test the hypothesis that flagellin promotes T and B lymphocyte recruitment into draining lymph nodes, we i.n. instilled groups of 5 young and aged BALB/c mice with 5 μg of flagellin or the control protein 229. Twenty-four hours following treatment, the numbers of total cells, T cells, and B cells in the cranial deep cervical, mandibular, and superficial parotid lymph nodes were determined (Fig. 5). (See (Van den Broeck et al., 2006) for a detailed description and standardized nomenclature of murine lymph nodes.) Instillation of flagellin had the greatest effect in the cranial deep cervical lymph nodes where instillation caused a 2.7-fold increase in total cell number in young mice (Fig 5A) and a 3.6-fold increase in aged mice (Fig 5B). Increases in the numbers of T and B cells accounted for nearly all of the increased cellularity in these nodes. Additionally, T and B cells were recruited at similar levels, thus treatment with flagellin does not preferentially recruit T rather than B cells to the draining lymph nodes. Flagellin-treated mice also had increased cell numbers in the mandibular lymph nodes in both

age groups, though this increase was not statistically significant (P>0.07). In contrast, the numbers of cells in the superficial parotid lymph nodes of young mice decreased somewhat following i.n. administration of flagellin, though treatment had no effect on numbers of cells present in these lymph nodes in aged mice (Fig 5C). The increases in cell numbers at this short time point after instillation of flagellin likely reflect a generic chemokine-mediated recruitment of lymphocytes from the blood rather than an increase in the number of flagellin-specific lymphocytes. These changes provide a mechanism that increases the likelihood of lymphocytes encountering their cognate antigen in an environment supportive of a productive immune response. Treatment of mice with flagellin did not result in increased CD69 expression by T cells in draining lymph nodes (data not shown).

3.5 Flagellin promotes antigen-specific antibody production in aged and young mice

In view of our finding that flagellin promoted recruitment or retention of large numbers of T and B cells into the draining lymph nodes of aged mice, we hypothesized that immunization with flagellin would also be effective at promoting antigen-specific antibody formation in aged mice. Groups of 10 mice were i.n. immunized with 1 μg of flagellin or the control protein 229 and 10 μg of the Y. pestis protein F1. Four weeks following primary immunization, mice were boosted with a repeat dose of the primary immunization, and sacrificed 7 days later. In aged mice, immunization with flagellin and F1 resulted in anti-F1 IgA titers of 38 in nasal wash and 13 in BALF. Young mice had anti-F1 IgA titers of 108 in nasal wash fluid and 56 in BALF. The difference in nasal wash anti-F1 IgA titers between the two age groups was not statistically significant (P=0.054). Immunization with the control protein 229 and F1 protein did not result in detectable IgA titers in either age group (Fig 6A). Immunization with flagellin and F1 also induced anti-F1 IgG titers in nasal wash fluid, BALF, and plasma of young and aged mice. Aged mice had anti-F1 IgG titers of 48 in nasal wash, 805 in BALF, and 4.1×10^5 in plasma. As with anti-F1 IgA, the response was somewhat less compared to that in young mice which had anti-F1 IgG titers of 530 in nasal wash, 4800 in BALF, and 2.8×10^6 in plasma. IgG levels in mice immunized with the control protein 229 and F1 protein were below the limit of detection in both mucosal compartments and less than 1000 in plasma (Fig 6B). These results clearly establish that flagellin is an effective adjuvant, promoting systemic and mucosal immune responses, in aged mice though not to levels as high as in young mice.

3.6 Flagellin promotes germinal center formation in young but not aged mice

Given the lower titer of F1-specific antibodies in aged mice, we considered the possibility that antigen-specific B cell expansion might be more limited in aged mice. To evaluate the ability of flagellin to promote germinal center formation in aged and young mice, we i.n. immunized groups of 5 to 6 mice with 1 μg of flagellin or the control protein 229 and 10 μg of the Y. pestis protein F1 and sacrificed mice ten days later. Germinal centers in the cranial deep cervical lymph nodes were discriminated on the basis PNA binding and IgD downregulation (Fig 7A). Immunization of young mice with flagellin and F1 protein resulted in significantly greater numbers of germinal centers per lymph node than in control mice (Fig 7B). These germinal centers were also significantly greater in volume. The numbers of germinal centers in lymph nodes from aged mice were highly variable in both flagellin and 229 immunized groups. Immunization of aged mice with flagellin did not produce a significant difference in number or volume of germinal centers compared to aged 229 immunized control mice. The lack of an effect of flagellin on germinal center formation in aged mice may be responsible in part for the reduced F1-specific antibody titer in aged mice.

3.7 Reduced Antibody Avidity in Aged Mice

In view of the more limited germinal center formation in aged mice following immunization, we considered the possibility that antibodies from aged mice may be of lower avidity than

antibodies from young mice. Plasma samples from groups of 10 young and aged BALB/c mice immunized with 1 μg of flagellin or 229 and 10 μg of F1 were analyzed by ELISA. Avidity was compared on the basis of antigen-bound antibodies' resistance to elution by sodium thiocyanate. The avidity index represents the concentration of sodium thiocyanate required to reduce absorbance as measured by ELISA by 50%. The average avidity index of plasma samples from young mice was 3M NaSCN while samples from aged mice had a significantly lower (P=0.002) average avidity index of 1M NaSCN (Fig 8A). Samples from aged mice showed more heterogeneity in their sensitivity to NaSCN than samples from young mice (Fig 8B & 8C). Incubation in 0.1 M NaSCN was sufficient to cause elution of some aged samples, while samples from young mice were unaffected by incubation with 1M NaSCN. These results establish that on average, antibodies from aged mice are less avid than antibodies from young mice. However, some aged mice are still capable of producing antibodies with avidities equal to antibodies from young mice.

4. Discussion

We have shown that mucosal administration of purified, endotoxin-depleted recombinant flagellin promotes a potent, but transient respiratory innate immune response in aged mice. As a mucosal adjuvant, flagellin also promoted a significant adaptive immune response in aged mice. In a related study (Ben-Yedidia et al., 1998), Ben-Yedidia and colleagues reported that intranasal immunization with flagellin-HA and flagellin-NP fusion proteins resulted in immunity to influenza in aged mice but that aged BALB/c mice had lower HA specific IgG and IgA titers following immunization than 3 month old mice.

The most notable finding in our study was that although flagellin evokes a stronger innate immune response in aged mice than in young mice, the increased innate response in aged mice fails to consistently promote a correspondingly strong adaptive response. Several other groups have reported increased response to TLR stimulation (Habicht, 1981; Esposito et al., 1989; Chorinchath et al., 1996; Gomez et al., 2007) or infection (Esposito et al., 1990) in aged mice. Gomez and colleagues reported that instillation of LPS in aged mice triggered increased levels of MIP-2, KC, and IL-1β in lung homogenates as well as increased infiltration of neutrophils compared to young mice (Gomez et al., 2007). However, TNF-α levels were not significantly different in young and aged mice. The reasons underlying the increased innate response in aged mice are unclear. Aoshiba and Nagai performed microarray analysis on cDNA from lungs of young and aged mice and found that several inflammation related genes, including IL-8RB and CXCR3 are more highly expressed in aged mice than in young mice (Aoshiba and Nagai, 2007). One likely explanation for these age-related differences is a diminished ability by aged mice to negatively regulate innate stimulatory pathways. Several lines of evidence have implicated phosphoinosotide-3-kinase (PI3K) in the altered regulation of innate immunity. For example, Ong and colleagues have shown that neutrophil recruitment into the lungs of PI3K gamma deficient mice is increased 100% compared to wild type mice in an *E. coli* sepsis model system (Ong et al., 2005). In another sepsis model system, inhibition of PI3K resulted in increased cytokine production as well as decreased survival time of septic mice (Williams et al., 2004). Importantly, inhibition of PI3K prior to stimulation of epithelial cells with flagellin resulted in increased expression of nitric oxide synthase, IL-6, and IL-8 (Yu et al., 2006). Thus our data showing increased neutrophil infiltration into the lungs of aged mice after treatment with flagellin compared to young mice are consistent with the hypothesis that aged mice have a reduced capacity to signal through PI3K that, in turn, results in an increased innate immune responsiveness to flagellin.

In addition to negatively regulating components of the innate immune response, the PI3K pathway upregulates phagocytosis (Cox et al., 2001), is necessary for DC migration in vivo (Del Prete et al., 2004), and may also negatively regulate TLR signaling (Guha and Mackman,

2002; Wrann et al., 2007). Agrawal and colleagues have reported that DC from aged humans exhibit a reduced ability to phagocytize antigens and migrate in response to chemokines. They also found that DC from aged humans produce higher levels of TNF-α and IL-6 in response to stimulation with LPS and ssRNA compared to DC from young humans (Agrawal et al., 2007). They attributed these changes in aged DC to reduced signaling through the PI3K pathway as measured by phosphorylation of AKT and found that expression of PTEN, a negative regulator of PI3K (Cantley and Neel, 1999), was increased in DC from aged humans. PTEN has also been shown to negatively regulate the CD4+ T cell (Suzuki et al., 2001) and B cell (Anzelon et al., 2003; Suzuki et al., 2003) responses. Thus decreased PI3K signaling resulting from increased PTEN expression in aged mice may account for the diminished adaptive response to immunization as well as the increased innate response. If reduced flux through the PI3K signaling pathway is responsible for the diminished adaptive response in aged mice, then it may be possible to enhance these responses by additional immunizations with flagellin and antigen or by pharmacological modulation of PTEN activity at the time of immunization.

Due to the recent National Institute of Aging mandated change in the availability of aged mice, we are currently unable to test the efficacy of our vaccine in aged mice or to proceed with studies on the optimization of the vaccination protocol (e.g., additional immunizations, increased dose, or alternate route of administration). Given the susceptibility of aged populations to respiratory pathogens and the reduced efficacy of vaccines among the elderly, our demonstration that flagellin promotes significant mucosal and systemic adaptive immune responses in aged mice is of major importance and thus clearly merits additional study. We believe that flagellin has significant potential for use as a vaccine adjuvant in humans. Indeed, we are very close to completing preclinical development of a flagellin/F1/V fusion protein for use human clinical trials. The flagellin/F1/V fusion protein vaccine has been produced and vialed under Good Manufacturing Practices conditions and has passed all of its quality control tests.

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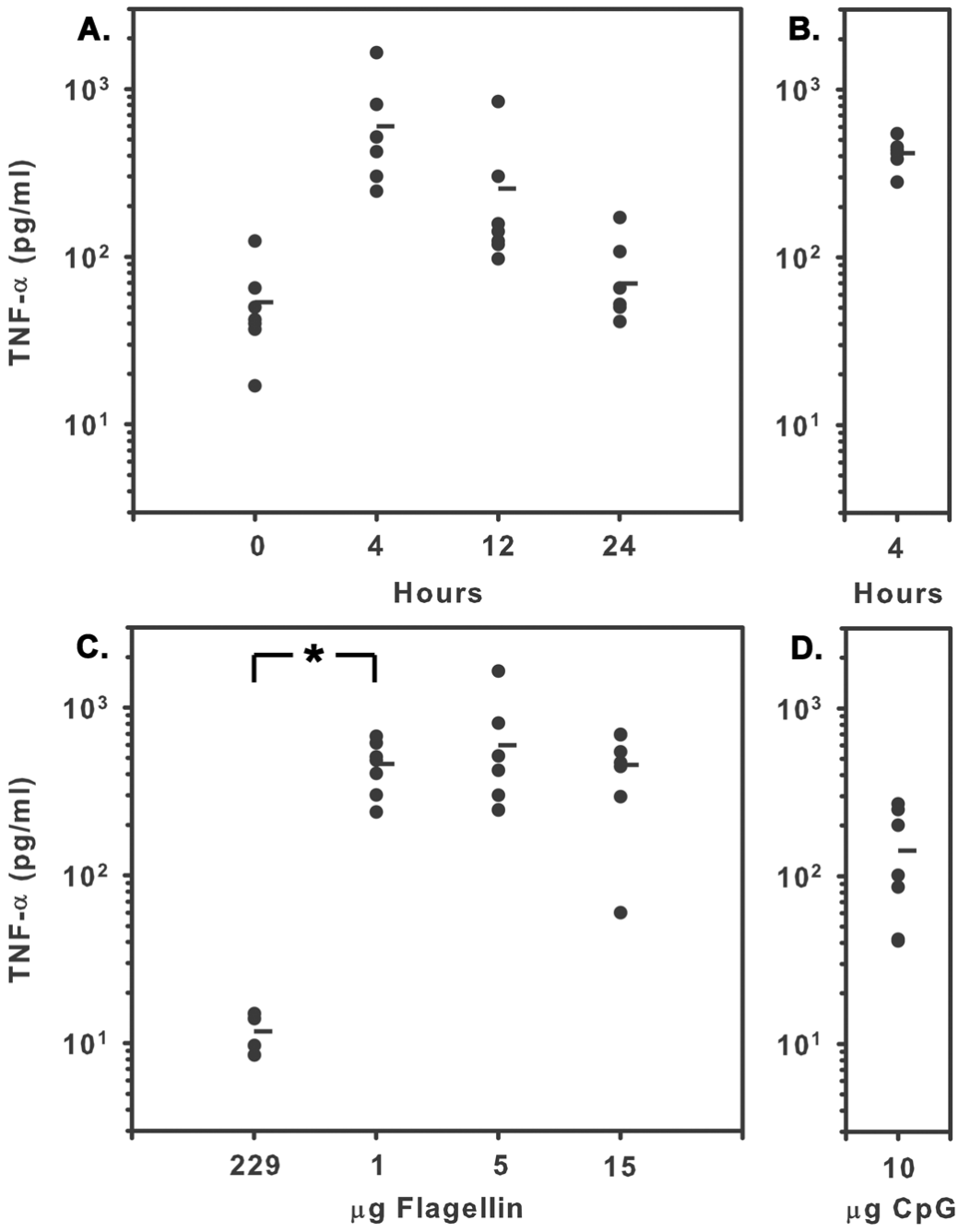

Fig. 1.

TNF-α levels in BALF from aged mice following intratracheal instillation of flagellin. (A) Aged mice were i.t. instilled with 5 μg of flagellin and sacrificed 4, 12, or 24 hours following treatment or at 0 hours without treatment. (B) Young mice were sacrificed four hours after receiving 5 μg of flagellin. TNF-α levels in aged mice peaked 4 hours after treatment and were not significantly different from levels in young mice at that time (t test, P=0.413). N≥6. (C) Aged mice were i.t. instilled with 5μg of the non-signaling flagellin mutant 229, 1, 5, or 15 μg flagellin. Instillation of 1 μg of flagellin resulted in significantly higher TNF-α levels than 229 (Mann-Whitney rank sum test, *P=0.006) or CpG (t test, P<0.001), however, instillation of 1 to 15 μg of flagellin did not result in significantly different levels of TNF-α (t test, P=0.961).

(D) I.t. Instillation of 10 μg CpG resulted in lower TNF-α levels than 1μg of flagellin. Horizantal bars represent the mean.

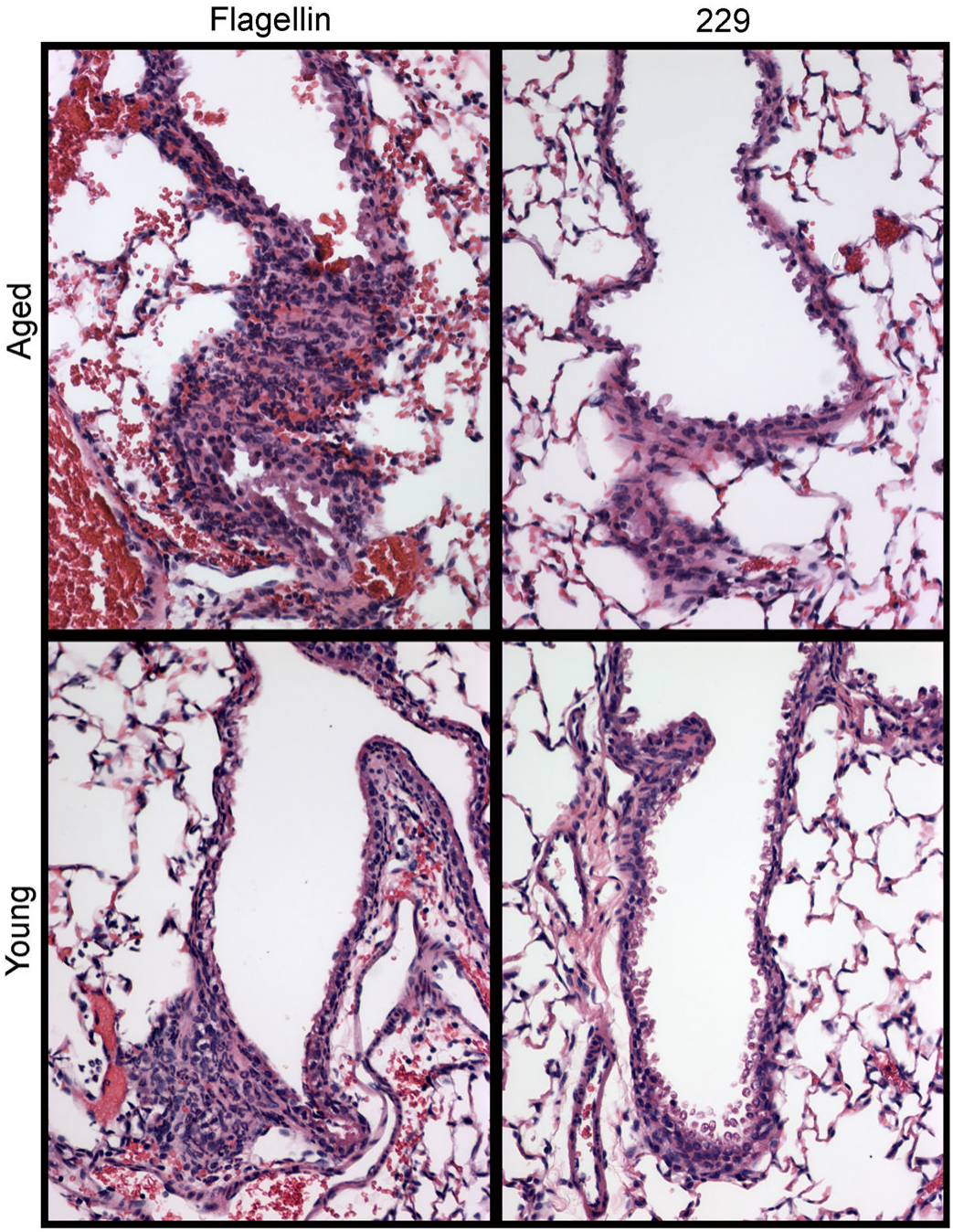

Fig. 2.

Innate response in the lungs of aged and young mice sacrificed 12 hours after i.t. instillation with 5 μg of flagellin or the control protein 229. The innate response in both age groups was characterized by diffuse hyperemia and peribronchial neutrophil infiltrates. Tissues from control mice were unaffected.

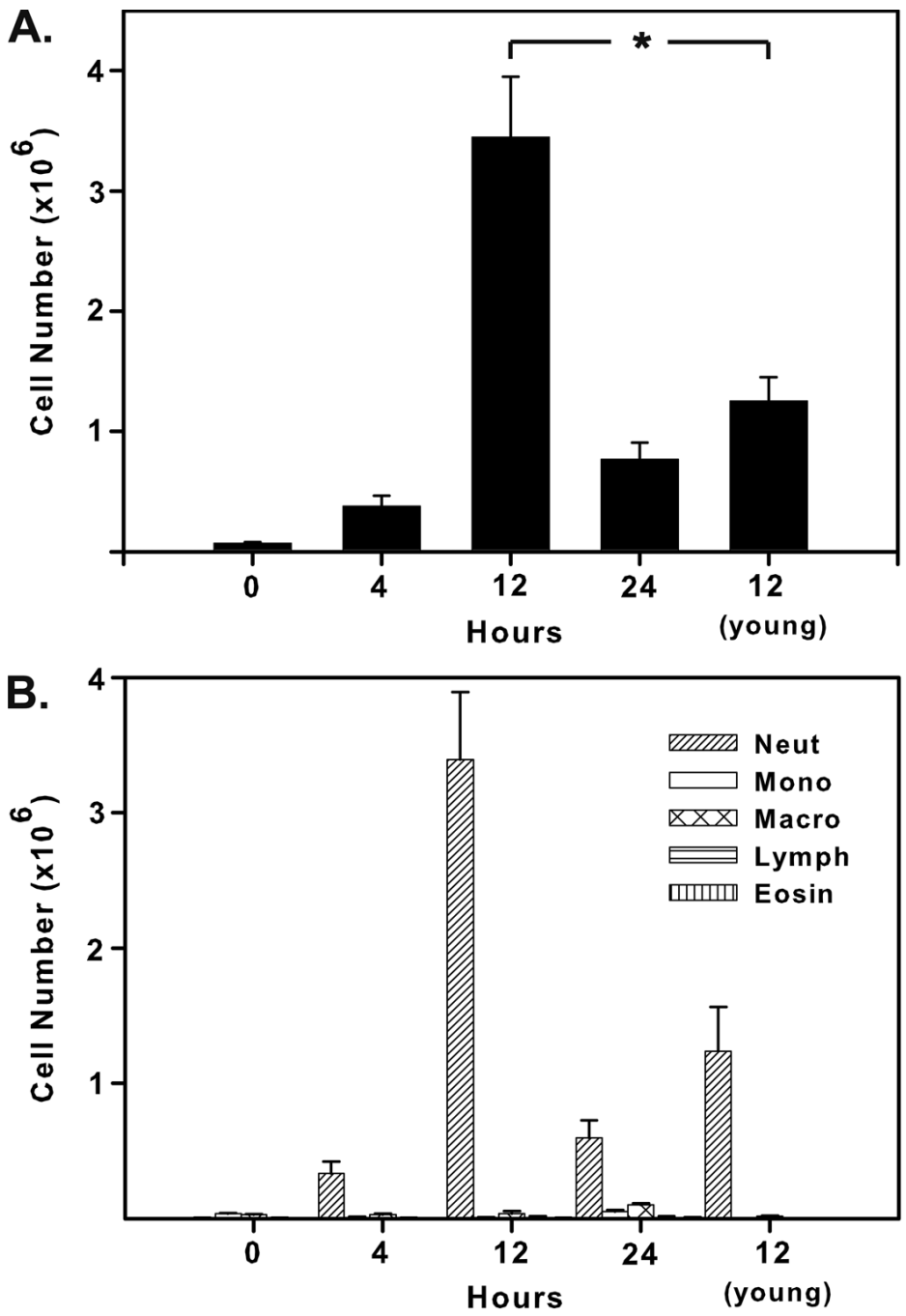

Fig. 3.

Cellular infiltrate in the lungs of aged mice following i.t. instillation of flagellin. Aged mice were instilled with 5 μg of flagellin and sacrificed 4, 12, or 24 hours following treatment or at 0 hours without treatment. Young mice were sacrificed 12 hours after receiving flagellin. (A) Total cellular infiltrate peaked in aged mice 12 hours after treatment and was 2.8 times greater than levels in young mice at that time point (t test, $^{*}P=0.002$). (B) Neutrophils were the dominant infiltrating cell type in young and aged mice at all observed times.

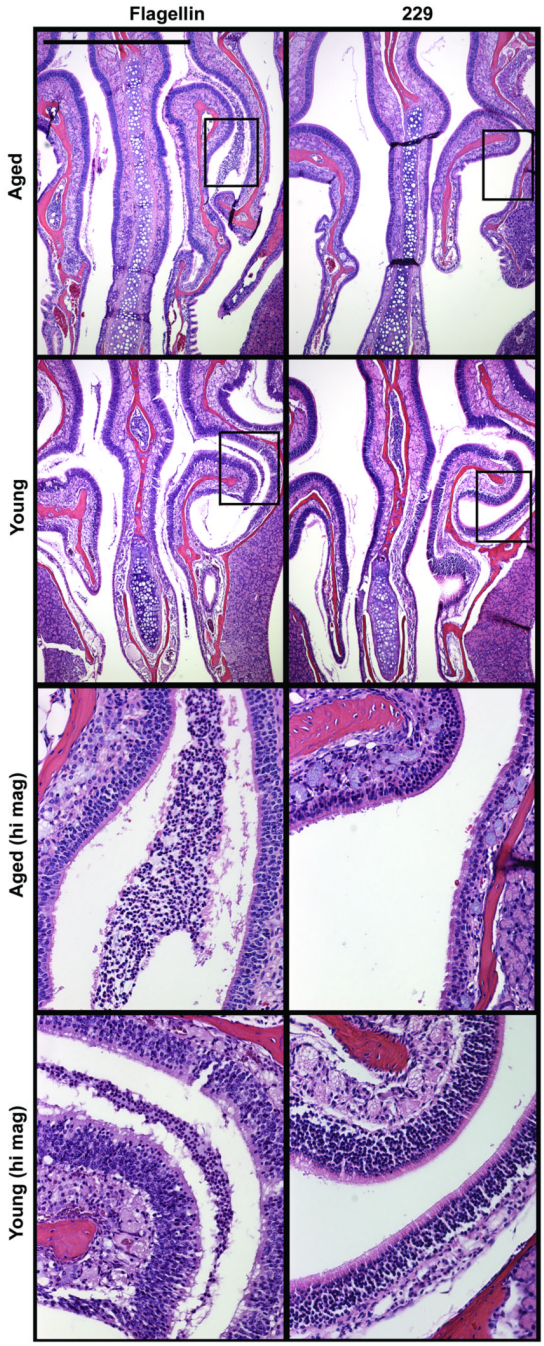

Fig. 4.

Cellular infiltrate in the nasal passageways of aged and young mice 12 hours following i.n. instillation of 5 μg of flagellin or the control protein 229. Neutrophil-rich exudates collected along the respiratory epithelium of aged and young mice. Loss of cilia, vacuolation, and individual cell loss among the respiratory epithelial cells were similar in aged and young mice. Control mice were unaffected. Scale bar represent 1 mm.

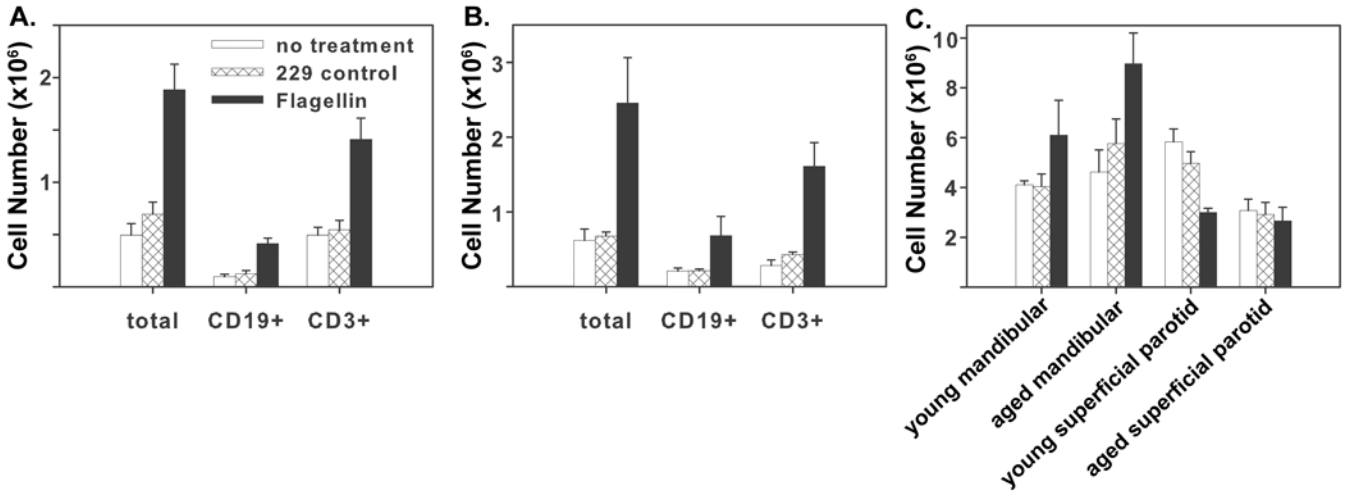

Fig. 5.

Increased cellularity in the cranial deep cervical lymph nodes of young and aged mice following i.n. administration of flagellin. Young and aged mice were i.n. instilled with 5 μg of flagellin and sacrificed 24 hours later. The number of total cells, CD19+, and CD3+ cells in the cranial deep cervical lymph nodes increased in (A) young and (B) aged mice following treatment with flagellin compared to 229 ($P<0.02$). (C) Increases in cell number in mandibular lymph nodes of young and aged mice following treatment with flagellin were not statistically significantly ($P>0.07$), and no increase in cell number occurred in the superficial parotid lymph nodes.

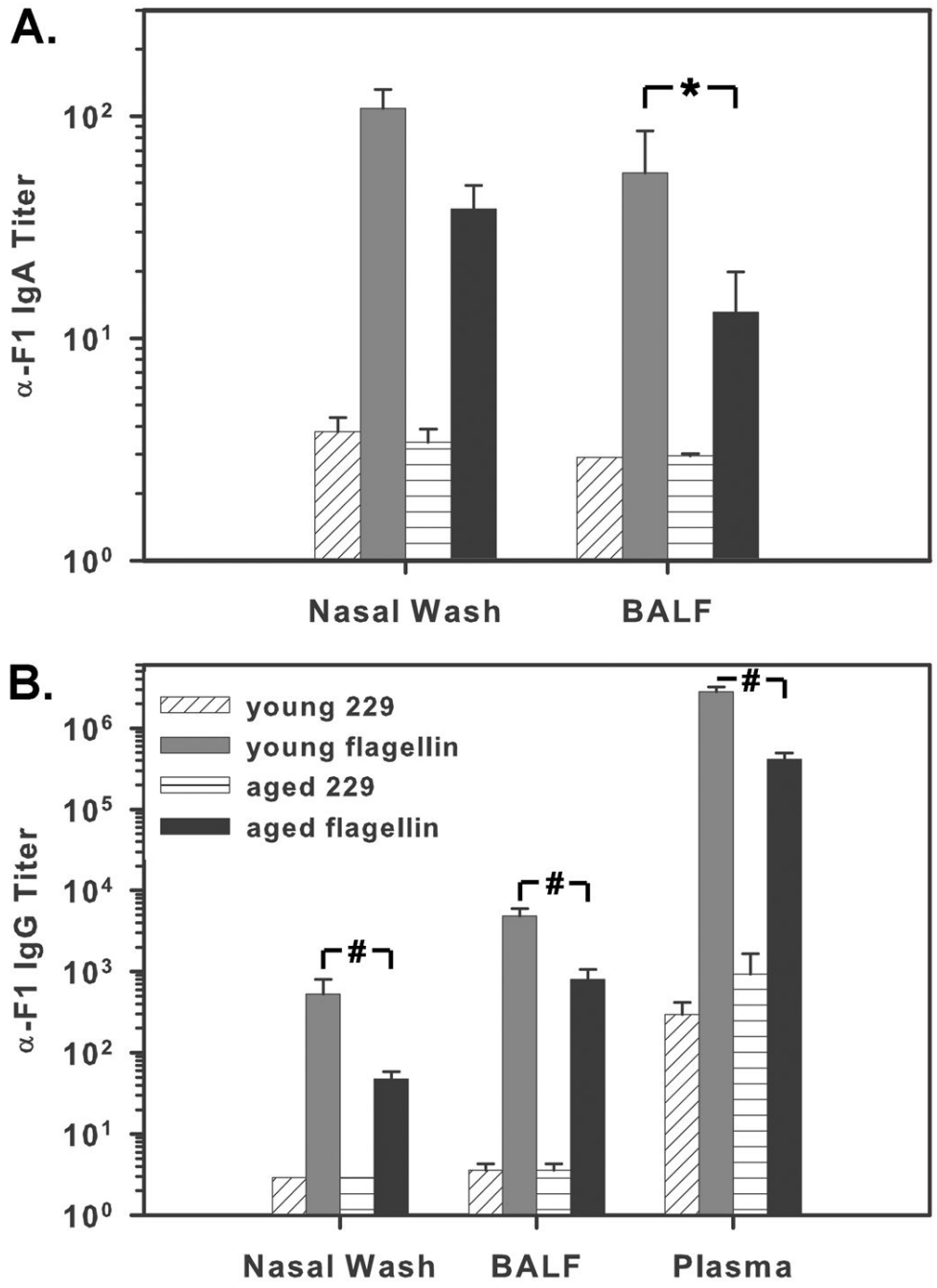

Fig. 6.

Antigen-specific antibody responses in young and aged mice. Mice were i.n. immunized with 1 μg of flagellin or the control protein 229 and 10 μg of F1. Mice were boosted on day 28 and sacrificed 7 days following boost. (A) Anti-F1 IgA titers were measured in nasal wash and BALF. Differences in NW IgA levels in flagellin-immunized young and aged mice were not statistically significant (P=0.054), though differences in BALF IgA levels were significant (*P=0.007). (B) Anti-F1 IgG titers were determined in nasal wash fluid, BALF, and plasma. Immunization with flagellin promotes F1-specific antibody formation in aged mice; however, IgG levels in flagellin-immunized aged mice are significantly lower than in flagellin-immunized young mice in all observed compartments ($^{\#}$P≤0.001).

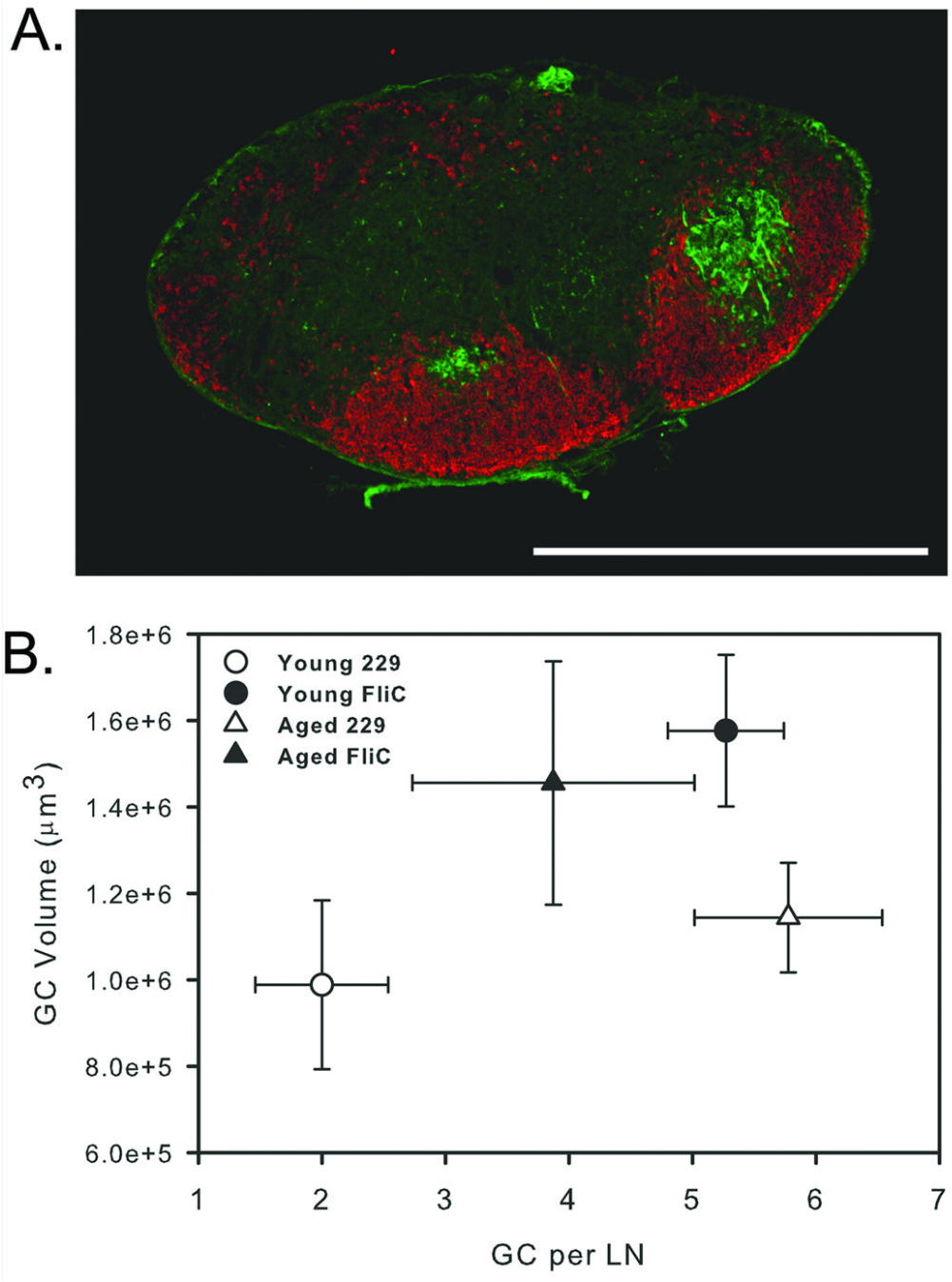

Fig. 7.

Number and volume of germinal centers in aged and young mice following immunization. Mice were i.n. instilled with 1 μg of flagellin or 229 control protein and 10 μg of F1. Mice were sacrificed 10 days later, and the number and volume of germinal centers in cranial deep cervical lymph nodes was analyzed by immunofluorescence. (A) Germinal centers were detected based on peanut agglutinin (green) binding ability, and B cell follicles were identified based on IgD expression (red). (B) Immunization of young mice with flagellin resulted in significantly larger and more germinal centers than with 229 ($P<0.05$). Germinal center number and volume was highly variable in aged mice and differences in number and volume of germinal

centers in flagellin and 229 control immunized mice was not significant (P>0.05). Scale bar represents 1 mm.

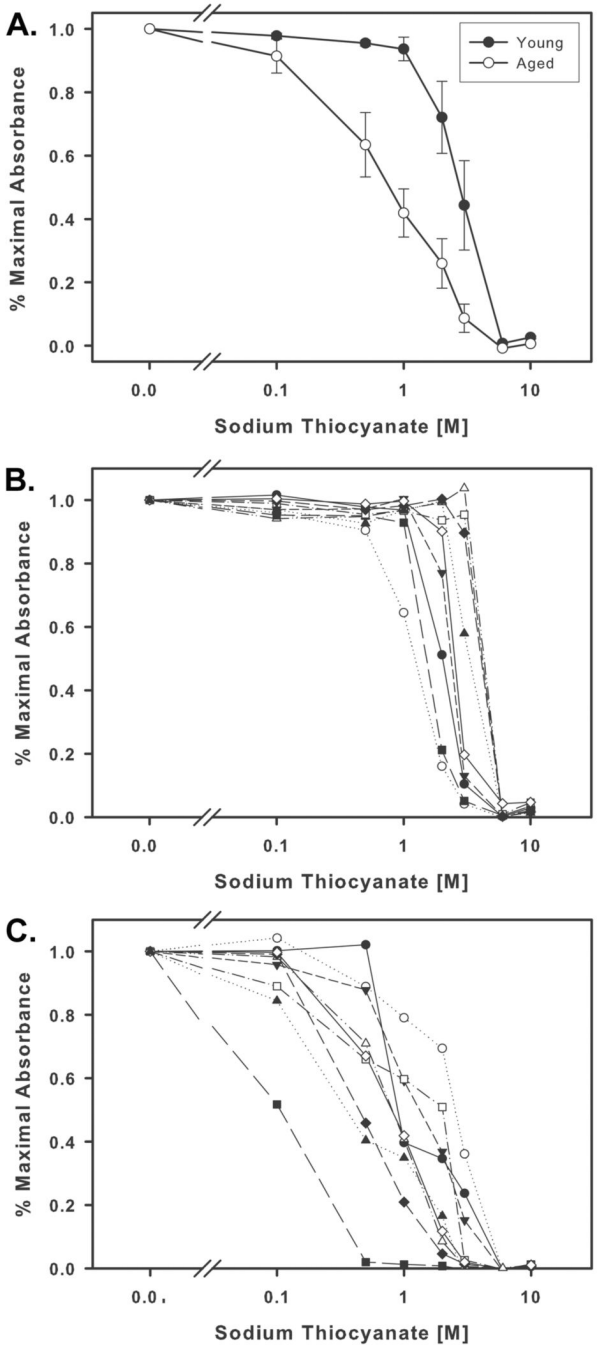

Fig. 8.

Avidity of anti-F1 plasma IgG in immunized young and aged mice. (A) Antibodies from aged mice exhibit lower avidity than antibodies from young mice. Incubation in approximately 1 M NaSCN was sufficient to reduce absorbance of aged plasma IgG by 50%, but young plasma IgG did not exhibit 50% reduction in absorbance until incubation with 3 M NaSCN (N=9, P=0.002). Variation of the concentration of NaSCN necessary to result in 50% reduction in absorbance was less in young mice (B) than in aged mice (C). Calculation of the standard deviation of the 50% reduction concentrations for young and aged mice yielded a standard deviation of 0.20 logarithmic units for the samples from young mice and 0.41 for the samples from aged mice.