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Nanoemulsion nasal adjuvant W_{80}5EC induces dendritic cell engulfment of antigen-primed epithelial cells

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Abstract

Nanoemulsions are adjuvants that enhance antigen penetration in the nasal mucosa, increase cellular uptake of antigens by both epithelial dendritic cells, and promote migration of antigen-loaded dendritic cells to regional lymph nodes within a day of vaccine administration. The objective of this study was to determine whether the W_{80}5EC nanoemulsion adjuvant enhances immune response not only by direct uptake of antigen by dendritic cells, but also indirectly, by phagocytosis of antigen-primed, apoptotic, epithelial cells. Consistent with this, we show that exposure of both epithelial cells (TC-1s) and dendritic cells (JAWS II or bone marrow derived dendritic cells (BMDCs)) to nanoemulsion exhibited augmented antigen uptake in cell culture. TC-1 cells subsequently underwent G_2/M cell cycle arrest and apoptosis, and when co-cultured with JAWS II or BMDCs were rapidly engulfed by the dendritic cells, which responded by up-regulating dendritic cell maturation marker CD86. Altogether these results suggest that the effectiveness of nanoemulsions as adjuvants stems, at least in part, from the engulfment of antigen-loaded epithelial cells, leading to enhanced antigen processing and a strong and balanced mucosal and systemic immune response.

Keywords

Immunization; antigens; cytokines; antibodies

Introduction

Despite many efforts aimed at developing improved adjuvants, very few have been approved for use in human vaccination [1–4]. Furthermore, most adjuvants are effective in stimulating humoral or cell-mediated immunity (CMI) but not both. For example, alum is pro-inflammatory compounds useful in augmenting Th2 humoral immune responses to bacterial toxoids and other antigens but not in consistently eliciting CMI [5, 6]. This deficiency is significant, since CMI play a crucial role in fighting intracellular pathogens. Other forms of particulate adjuvants (e.g., emulsions, microparticles, iscoms, and liposomes) have been

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proposed as alternatives to alum, but these adjuvants require additional pro-inflammatory immunostimulants to enhance the immune response [7]. Although liposomes and lipid formulations are capable of priming virus-specific CTLs [8], these formulations tend to be unstable, especially when mixed with antigen. Finally, the identification of innate immune receptors such as toll-like receptor (TLR) has led to the generation of a new class of adjuvants that stimulate CMI [9–12]. While promising, these compounds do not work for all antigens, and often require chemical coupling to antigens to be effective [13, 14]. Thus current adjuvant options fail to meet the need for stable formulations that elicit well-balanced strong humoral and CMI responses to a wide range of antigen types.

Adjuvants for mucosal vaccines are a novel class of compounds that offer unique advantages over both traditional and newer adjuvants. One of potential mucosal adjuvant is heat-labile enterotoxin. However, the enterotoxins raise serious safety issues [15] and therefore the use of enterotoxins as adjuvants in human vaccine formulations at this time is precluded [16].

NEs are oil-in-water emulsions (~400 nm droplet sizes) prepared using surfactants, solvent, soybean oil, and water and were developed as antimicrobial agents [17–21]. Recent studies have documented that NE can also be used as a mucosal adjuvant when mixed with soluble [22–25] or particulate [26–29] antigens. NE induces antigen-specific humoral and CMI responses in mucosal compartments, and protects against challenge with a pathogen [26–29]. The NE has also been shown to stimulate a Th17 response [30]. Finally, the W_{80}5EC nanoemulsion adjuvant is well tolerated in animals [24] and humans [31].

Despite these findings, the specific mechanisms by which NE stimulates a robust and balanced immune response are poorly understood. Here we describe a model system for the examination of NE-Ag action on epithelial cells (EC) and dendritic cells (DC). We report that the W_{80}5EC NE-Ag vaccine mixture induces antigen uptake and provide evidence that these antigen-primed ECs are engulfed by DCs. Furthermore, we demonstrate both antigen transfer to the DCs, as well as their subsequent maturation. Thus the uptake of antigen by ECs followed by their engulfment by DCs represents an indirect route for antigen acquisition by DCs that may contribute to the remarkable, broad-based adjuvant properties of the W_{80}5EC nanoemulsion.

Materials and Methods

Immunization and detection of humoral and CMI immune response

Female C57BL/6 mice 8–12 weeks were purchased from Harlan Sprague Dawley. Anesthetized mice were immunized with 20 μg of OVA mixed either with PBS or 20% W_{80}5EC and instilled i.n. at a volume of 10 μL per animal. Mice were immunized on day 0 and then three times at two-week intervals. OVA-specific immunoglobulins were evaluated using ELISA as described previously [29] with some modifications. Plates were coated with ovalbumin diluted in coating buffer (300 ng/well). Splenocytes from immunized mice were treated with OVA (20μg/mL) for48 hours. Supernatants were harvested and tested for the presence of cytokines using cytokine/chemokine LINCOplex kit (Millipore) as described previously [27]. Protocols for animal experiments were approved by the UCUCA at the University of Michigan, Ann Arbor, MI.

Cells

BMDCs were derived as described by Inaba et al. [32]. TC-1 (epithelial) and JAWS II DC lines, both of C57BL/6 origin were purchased from ATCC.

Reagents

The $W_{80}5EC$ was provided by NanoBio Corporation (Ann Arbor, MI). OVA was purchased from Hyglos GmbH (Regensburg, Germany).

Antigen uptake by TC-1 cells

The TC-1 cells were incubated overnight with R-PE (40 μg/mL) (AnaSpec, Inc.), or DQ-OVA (20 μg/mL) (Molecular Probes) either with or without 0.05% $W_{80}5EC$. After incubation cells were washed and analyzed using flow cytometry.

Engulfment of TC-1 cells by DCs and antigen transfer

PKH-26-stained TC-1 cells were treated with either 0.05% $W_{80}5EC$ or 1 μM staurosporine overnight. Then TC-1 cells were resuspended to a concentration of 3×10^5 cells/mL, and mixed with equal number of either JAWS II or BMDC cells and co-cultured for 4 hours at 37°C. Then cells were harvested and analyzed using flow cytometry. To exclude the potential for non-specific binding of fluorescent agents to the exterior of the cells, co-cultures were incubated at 4°C or DCs were either pretreated with 35mM NaN_3. In antigen transfer experiments, the TC-1 cells were loaded overnight with R-PE mixed with either $W_{80}5EC$ or staurosporine, then washed and incubated with CFSE-stained JAWS II cells. For confocal microscopy, PKH-26-TC-1 cells pretreated with NE were co-incubated with CFSE-JAWS II cells in chambered coverglass for 4 hours at 37°C. Then fixed with paraformaldehyde cells were imaged with a Leica inverted SP5X confocal microscope.

Expression of CD86 differentiation antigen on DCs

The PKH-26-TC-1 cells were incubated with 0.05% $W_{80}5EC$ for 6 hours. They were then washed and co-cultured overnight with CFSE-JAWS II cells. As a positive control, JAWS II cells were treated overnight with 10 μg/mL LPS (*Salmonella minnesota* from List Biological Laboratories, Inc.). The next day, the co-cultured cells were washed and stained with mouse anti-CD86 PE-Cy5-labeled antibody (eBioscience) and analyzed on flow cytometry. To analyze solely JAWS II cells, the red fluorescent TC-1 cells were gated out.

Statistical analysis

Results are presented as the mean ± SD. The data were analyzed by using Wilcoxon signed-rank test, with a significance level of $\alpha = 0.05$.

Results

Adjuvant activity of $W_{80}5EC$ in vivo

Intranasal immunization with $W_{80}5EC$ adjuvant produces a humoral immune response—The ability of $W_{80}5EC$ to function as a mucosal adjuvant was tested by immunizing mice i.n. with OVA+PBS or OVA+$W_{80}5EC$ four times at two-week intervals. Humoral immune response was assessed by measuring end-point titers of OVA-specific IgG (Fig. 1A). The first immunization resulted in an over 2-fold increase in IgG titer compared to control animals (Week 2), with second and third immunizations resulting in further increases of approximately one log each (Week 4 and Week 6). The endpoint titers of IgG1, IgG2b, and IgG2c subclasses of OVA-specific antibodies were evaluated (Fig. 1B). The IgG2a endpoint titer has not been evaluated due to deletion of the Igh-1^a gene in C57BL/6 mice which instead express a separate gene for the IgG2c (Igh-1^b) heavy chain isotype [33–35]. Both IgG1 and IgG2b subclasses increased between the first and second immunizations, with IgG1 reaching an endpoint titer of approximately $\log_2$ 16.5 and IgG2b an endpoint titer of $\log_2$ 15.4 at week 4. In contrast, IgG2c showed an insignificant increase in the endpoint titer.

Nasal immunization of W_{80}5EC adjuvant produces CMI (Th1, Th2 and Th17) response—To provide insight into the CMI, splenocytes from immunized mice were re-exposed to the OVA followed by assessment of cytokine response. The animals immunized with OVA+W_{80}5EC showed increased production of markers for Th1, Th2, and Th17 cellular response as compared to control animals (Fig. 2).

Adjuvant activity of W_{80}5EC in vitro

W_{80}5EC promotes antigen uptake by ECs and DCs—The broad based immune response to the W_{80}5EC adjuvant led us to consider possible mechanisms for this response. TC-1 cells were treated with either R-PE or DQ-OVA in the presence or absence of W_{80}5EC. Treatment of TC-1 cells with R-PE in the presence of W_{80}5EC increased the MFI 4 times as compared to cells uploaded with R-PE alone (Fig. 3A). Similar data were obtained when DQ-OVA was used as an antigen; treatment with DQ-OVA+W_{80}5EC increased the MFI 2.5 times over that of cells treated with DQ-OVA+PBS (Fig. 3B). Similar data were obtained when BMDCs were treated with OVA-AlexaFluor647+W_{80}5EC (Supplemental Figure 2).

Treatment of ECs with W_{80}5EC promotes engulfment by DCs which leads to indirect antigen uptake—We tested how DCs respond to ECs treated with W_{80}5EC using a co-culture system. PKH-25-stained (red fluorescence) TC-1 cells were incubated with W_{80}5EC, staurosporine, or vehicle control, and the next day were washed and mixed in equal number with CFSE-stained (green fluorescence) JAWS II cells. When untreated TC-1 cells were incubated with JAWS II cells, double-stained JAWS II cells occurred in 12% of the population (Fig. 4A). In contrast, when TC-1 cells were treated with W_{80}5EC, double-stained JAWS II cells increased to 42% (Fig. 4C), indicating a significant increase in the engulfment of ECs when pre-treated with W_{80}5EC. Staurosporine-pretreatment of TC-1 cells also increased the percentage of double-stained JAWS II cells, but to a lesser extent (28%) (Fig. 4E). When examined by confocal microscopy, fragments of W_{80}5EC-treated TC-1 cells (red) were clearly seen within the JAWS II cells (green) (Fig. 4H). Engulfment of the TC-1 cells did not take place at 4°C (Fig. 4B, D, F) and pretreatment of JAWS II cells with NaN_3 also inhibited the process (Fig. 4G). Moreover, non-phagocytic EL-4 cells did not engulf TC-1 cells pretreated with W_{80}5EC (data not shown). These studies indicate that engulfment of TC-1 is an active, cell-specific and energy-dependent process. We next investigated whether the engulfment of TC-1 cells may cause antigen transfer to DCs. CFSE-JAWS II cells were incubated with TC-1 cells loaded with R-PE in the presence or absence of W_{80}5EC overnight. TC-1 cells were then mixed in equal number with JAWS II cells, incubated for four hours, and analyzed using flow cytometry. When TC-1 cells loaded with R-PE antigen (red) were incubated with CFSE-JAWS II, 15% of CFSE-JAWS II cells were additionally stained with the R-PE (Fig. 5A). In contrast, when the TC-1 cells were also treated with W_{80}5EC, the double-stained CFSE-JAWS II cells increased to 26% (Fig. 5C). Staurosporine-treated TC-1 cells also increased the percentage of double-stained JAWS II DCs, to approximately 21% (Fig. 5B).

Co-culture of JAWS II cells and TC-1 cells treated with W_{80}5EC leads to up-regulation of CD86 on JAWS II cells—PKH-26-TC-1 cells were treated with 0.05% W_{80}5EC, washed and mixed with an equal number of CFSE-JAWS II cells. Co-cultured cells were incubated overnight and analyzed using flow cytometry. As shown in Figure 6B, JAWS II cells showed baseline CD86 antigen expression on approximately 27% of cells. LPS treatment (a positive control) up-regulates expression of CD86 on JAWS II cells to 79% (Fig. 6C). Co-culture of the JAWS II cells with untreated TC-1 cells had no effect on the expression of CD86 (Fig. 6D). In contrast, co-culture of the JAWS II with TC-1 cells pretreated with W_{80}5EC increased the expression of CD86 antigen in approximately 39% of

JAWS II cells (Fig. 6E). There was no difference in CD86 expression between JAWS II cells that had or had not engulfed TC-1 cells (upper right quadrant vs. upper left quadrant in Figure 6F).

NE causes cell-cycle arrest at G_2/M phase and apoptosis of epithelial cells— Since phagocytes engulf apoptotic and necrotic cells [36, 37], we next investigated whether increased engulfment of W_{80}5EC-treated TC-1 cells could be attributable to cell-cycle aberration and apoptosis. We indeed observed an increase of percentage of cells in the G_2/M phase after treatment with W_{80}5EC (Supplemental Figure 3).

To detect apoptosis W_{80}5EC-treated TC-1 cells were stained with annexin V (apoptosis) and PI (necrosis), and analyzed by flow cytometry. Treatment of TC-1 cells with W_{80}5EC resulted in approximately 8% early apoptotic cells at 48 hr (annexin V fluorescence alone) and 65% late apoptotic cells (annexin V and PI fluorescence). At 72 hr time-point the fraction of late apoptotic cells increased to 88% and no early apoptotic cells were recorded (Supplemental Figure 4C). In contrast, staurosporine-treated cells (Supplemental Figure 4B) showed only 16% late apoptotic/necrotic cells, while one-third of the cells were early apoptotic after 48-hour treatment. At 72 hour, the fraction of late apoptotic/necrotic cells increased up to 63%. RT-PCR analysis of genes involved in cell-cycle arrest and apoptosis showed that the pattern of gene expression in W_{80}5EC-treated and staurosporine-treated cells was different (Supplemental Table 1 and Figure 5).

Discussion

In this study potential nanoemulsion adjuvant for mucosal vaccine has been evaluated both *in vivo* and *in vitro*. The potential drawback of mucosal vaccine is how to avoid uptake by the olfactory nerve in the upper part of the nose and cause brain damage [38]. In our pilot unpublished study we addressed this issue. We examined whether one of the active components of NE – CPC can penetrate olfactory tissue and brain after intranasal instillation of 10 μl 20% W_{80}5EC. We did not detect any trace of CPC in brain using HPLC method (Paul Makidon – personal communication).

To evaluate the function of W_{80}5EC as a mucosal vaccine adjuvant we immunized C57BL/6 mice either with OVA+PBS or OVA+W_{80}5EC. A significant titer of specific IgG was observed two weeks after a single immunization with OVA+W_{80}5EC and further increased following subsequent immunizations (Fig. 1A). Since IgG2a isotype (Igh-1^a) is not expressed in the C57BL/6 strain due to deletion of the Igh-1^a gene [33] only IgG2b and IgG2c were evaluated. Evaluation of immunoglobulin subclasses showed that endpoint titers of both IgG1 and IgG2b increased after each additional immunization, while the endpoint titer of the IgG2c subclass did not increase after multiple immunizations, which may indicate a lack of Th1 response (Fig. 1B) [35].

Cytokine production in OVA+W_{80}5EC immunized mice was also evaluated. An increase in cytokine production as compared to the OVA+PBS group was observed for IFN-γ, IL-10, IL-17, IL-2, IL-4 and IL-5 (Fig. 2). This is interesting, because it demonstrates that the cells secrete Th1 markers (IL-2 and IFN-γ) despite the lack of elevated IgG2c antibodies. Furthermore, increasing levels of IL-4, IL-5 and IL-10 cytokines (Th2), and IL-17 cytokine (Th17) were also observed. Th17 response is implicated in clearing pathogens during host defense reactions and in inducing tissue inflammation in autoimmune disease [39]. The cytokine evaluation has been performed using splenocytes but similar pattern of cytokine production was noticed when lymphocytes isolated from cervical and inguinal lymph nodes were evaluated (data not shown). These results are consistent with previous studies that used HIV gp120 soluble antigen with W_{80}5EC for mucosal immunization in BALB/c mice [23].

To further define the function of the W_{80}5EC we examined mechanisms of NE adjuvant activity with respect to antigen uptake by ECs, antigen transfer to DCs and apoptosis. A study by our group has recently demonstrated that i.n. delivery of a W_{80}5EC-based vaccine results in the rapid uptake and internalization of antigen by both ECs and DCs present in the nasal mucosa and draining LNs [40] suggesting the possibility that the uptake of antigen not only by DCs, but also by ECs, may be an important aspect of the adjuvant activity of W_{80}5EC. In current study, we documented that W_{80}5EC promoted direct antigen uptake in both EC and DC cells. One possibility is that positively charged W_{80}5EC-Ag particles bind to cell membranes electrostatically and are delivered to the interior of the cell by endocytosis. Once inside the cell, the NE-Ag complex may then fuse with lysosomes to hydrolyze or to break down the complex. This hypothesis is supported by experiments using DQ-OVA, which only becomes fluorescent after hydrolysis inside lysosomes (Fig. 3B).

Whether antigen loaded into W_{80}5EC-treated ECs later gains access to DCs is an important question, since uptake of antigen by ECs followed by engulfment and secondary antigen uptake by DCs would represent indirect route for presentation of antigen to the immune system. To examine the effect of W_{80}5EC on antigen transfer from EC to DC cells, we applied a co-culture system. We demonstrate, first, that DCs engulf W_{80}5EC-treated TC-1 cells, but not untreated control cells (Fig. 4A, C). This suggested that W_{80}5EC may mediate antigen transfer from TC-1 cells to JAWS II cells. It was confirmed by the uptake of antigen by JAWS II cells from TC-1 cells that had been exposed to NE-Ag (Fig. 5).

Remarkably, W_{80}5EC-treated TC-1 cells induced maturation of the DCs. The W_{80}5EC-treated TC-1 cells enhance the expression of the CD86 antigen on the JAWS II cells (Fig. 6). This is consistent with a recent report documenting that exposure to emulsion-pretreated cells induced the maturation of DCs, resulting in enhanced surface expression of MHC class II molecules and the up-regulation of co-stimulatory molecules [41]. The CD86 receptor on the surface of DCs provides important co-stimulatory signals to augment and sustain a T-cell response *via* an interaction with CD28 [42–44]. Since NE-treated ECs affect rapid increase of $CD86^+$ DCs it is plausible that NE contributes directly or indirectly to both the maturation and activation of DCs. Since phagocytes readily engulf apoptotic cells [36, 37], we investigated whether increased engulfment of W_{80}5EC-treated TC-1 cells is associated with induction of cell-cycle arrest and apoptosis. We found that W_{80}5EC treatment does indeed lead to significant G_2/M arrest (Supplemental Figure 3), to a remarkable degree similar to staurosporine. However, the process blocking mitotic entry by NE and staurosporine appear different (Supplemental Figure 5).

The dead or dying cells generate danger signals that stimulate migration of APCs, facilitate antigen uptake, and induce the maturation of DCs [45–47]. APCs are thought to engulf apoptotic cells and subsequently load antigens on MHC class I and II and trigger downstream antigen-specific immune responses [48, 49]. Necrotic cells, on the other hand, serve as natural adjuvants to activate DCs by endogenous signals [50]. However, many recent studies have demonstrated that the mode of cell death does not impact uptake and presentation of cell-associated antigen by DCs or their maturation [51]. We used annexin V and PI to distinguish early- and late-apoptotic cells treated with NE (Supplemental Figure 4). We observed that NE-treated cells became increasingly double-stained (annexin V/PI) over time. This observation indicates that most of the cells either succumb to apoptotic necrosis, oncotic necrosis, or both. It is consistent with data obtained by Lecoeur, et al [52], who documented that the external PS exposure has not been exclusive to apoptotic cells, but also occurs in oncotic cells. Interestingly, RT-PCR analysis of genes involved in apoptosis showed that after treatment of TC-1 cells with NE three out of six genes down-regulated were genes coding caspases, directly involved in apoptosis (Supplemental Table 1). Altogether these results are consistent with data obtained by Yang, et al [53–55], who

documented that apoptotic and necrotic effects of emulsion-based adjuvants play a pivotal role in antigen delivery and presentation.

In conclusion, we showed that exposure of both epithelial cells and DCs to nanoemulsion exhibited augmented antigen uptake. Epithelial cells (TC-1) subsequently underwent apoptosis, and when co-cultured with DC cells were rapidly engulfed by latter cells, which responded by up-regulating DC maturation marker CD86. Altogether these results suggest that the effectiveness of nanoemulsions as adjuvants may stem, at least in part, from the engulfment of antigen-loaded epithelial cells, leading to enhanced antigen processing and a strong and balanced mucosal and systemic immune response. However, we have not documented yet whether the NE promotes their migration to lymph nodes, followed by the presentation of antigen to effector cells and thereby induces an unusually strong and balanced mucosal and systemic immune response. This is the focus of our ongoing studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

EC	epithelial cell
LDH	lactate dehydrogenase cytotoxicity assay kit
NE	nanoemulsion
PI	propidium iodide
PS	phosphatidylserine
XTT	cell proliferation assay kit

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Highlights

- Facilitates antigen uptake by epithelial and dendritic cells
- Promotes engulfment of epithelial by dendritic cells
- Augments antigen transfer from epithelial to dendritic cells
- Induces maturation of dendritic cells
- Contributes to cell cycle arrest and apoptosis

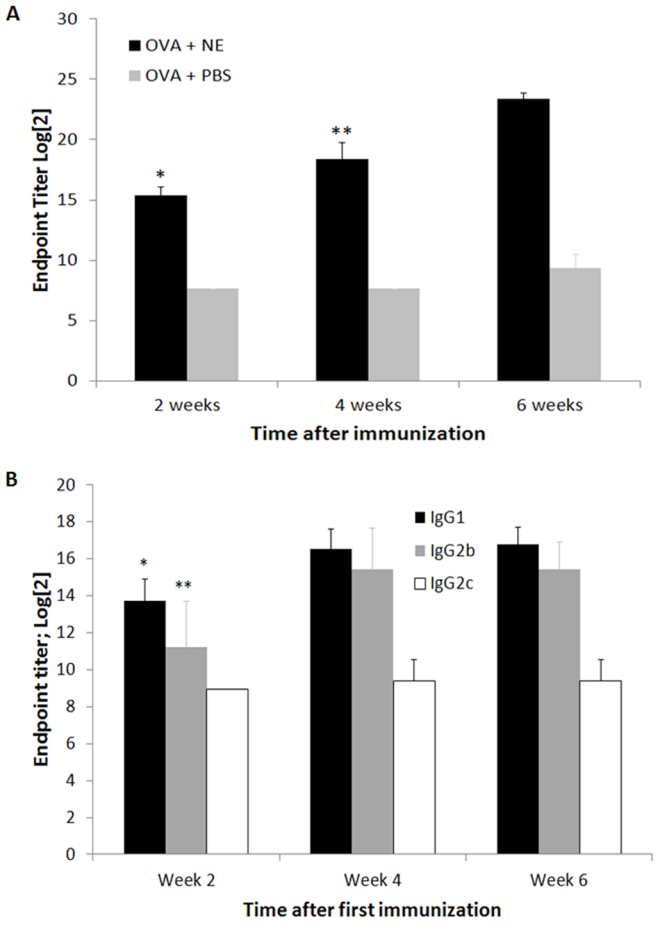

Figure 1.

Endpoint titer of total OVA specific IgG in sera (A). Mice (8 animals per group) were immunized on day 0 and then three times, two weeks apart. Sera were collected every two weeks. Each additional immunization increased the endpoint titer. Two weeks after the fourth immunization, there was no further increase in endpoint titer (data not shown). Data shown are representative of one of three independent experiments. * - significant difference ($p<0.005$) in endpoint titer of IgG between groups OVA+NE and OVA+PBS; ** - significant difference ($p<0.005$) in endpoint titer of IgG between week 2 and week 4 and week 4 and week 6. Endpoint titer of IgG1, IgG2b and IgG2c subclasses of OVA specific antibodies (B). Data shown are representative of one of three independent experiments. * - significant difference ($p<0.05$) in endpoint titer of IgG1 between week 2 and 4; ** - significant difference ($p<0.005$) in endpoint titer of IgG2b between week 2 and 4.

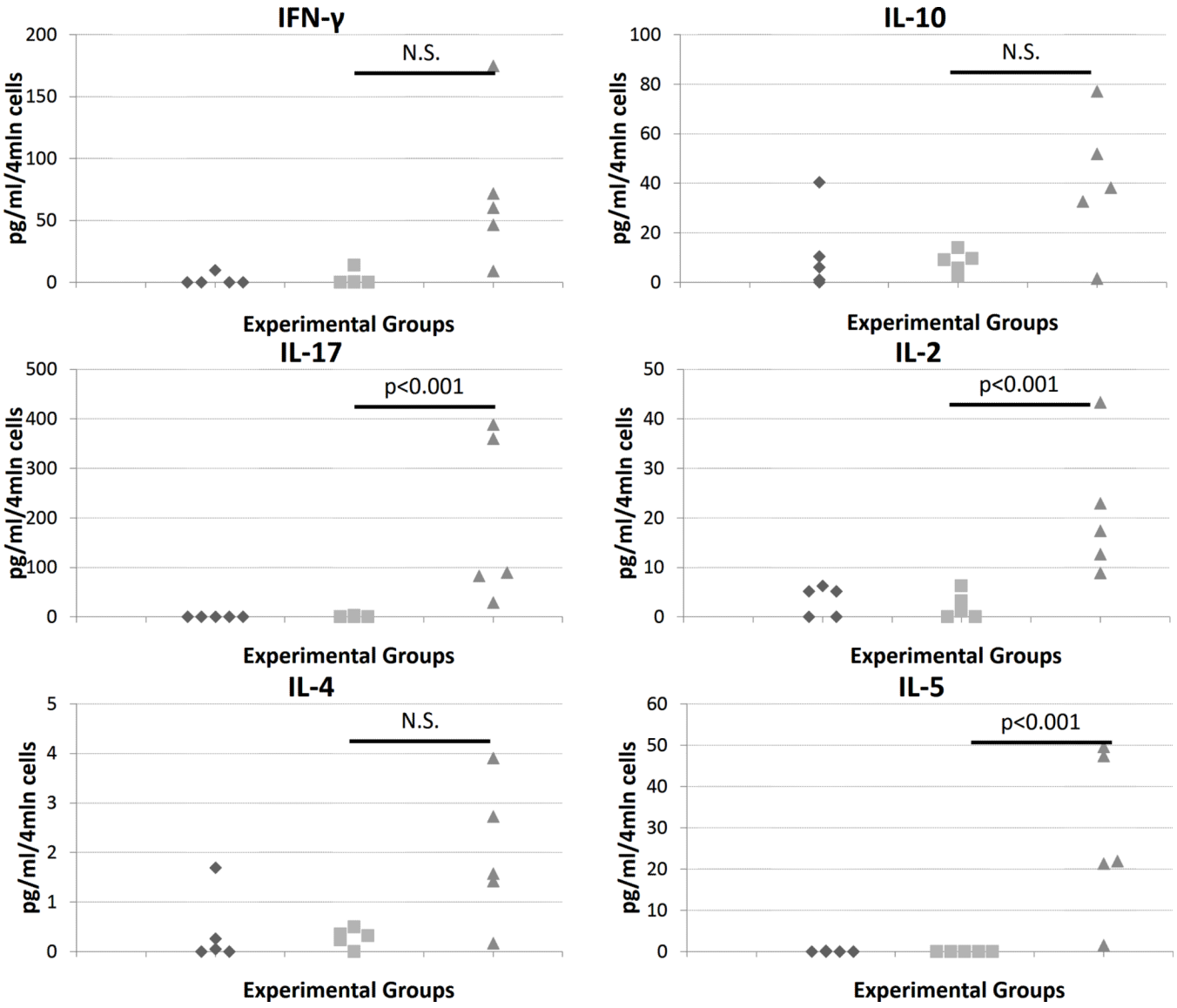

Figure 2.

Cytokine production by splenocytes obtained from mice immunized with PBS (◆), OVA and PBS (■), and OVA with NE (▲). Data shown are representative of one of three independent experiments. Statistical significance ($p<0.005$) has been observed for IL-2, IL-17 and IL-5 between groups OVA+PBS vs. OVA+NE.

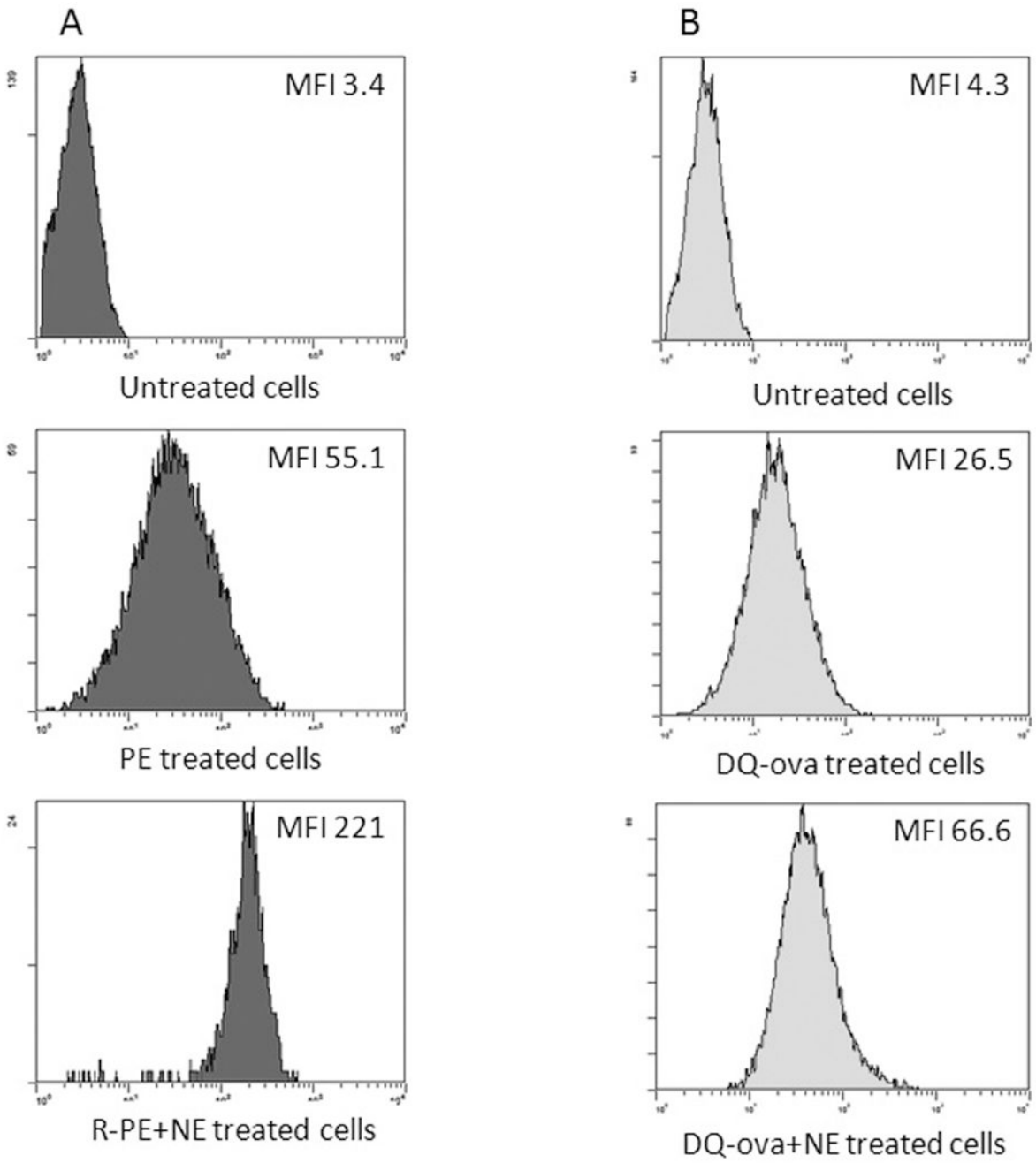

Figure 3.

The effect of NE on antigen uptake by TC-1 cells. The cells were incubated with either R-PE (A) or DQ-OVA (B) in the presence or absence of NE for 24 hours and then analyzed using flow cytometry. The experiments were repeated five times (rPE) and four times (DQ-OVA). Significant difference $p<0.01$ and $p<0.03$ in the MFI values between the uptake of rPE with and without $W_{80}5EC$ (N=5) and DQ-OVA with and without $W_{80}5EC$ (N=4), respectively have been observed as tested using Wilcoxon signed-rank test.

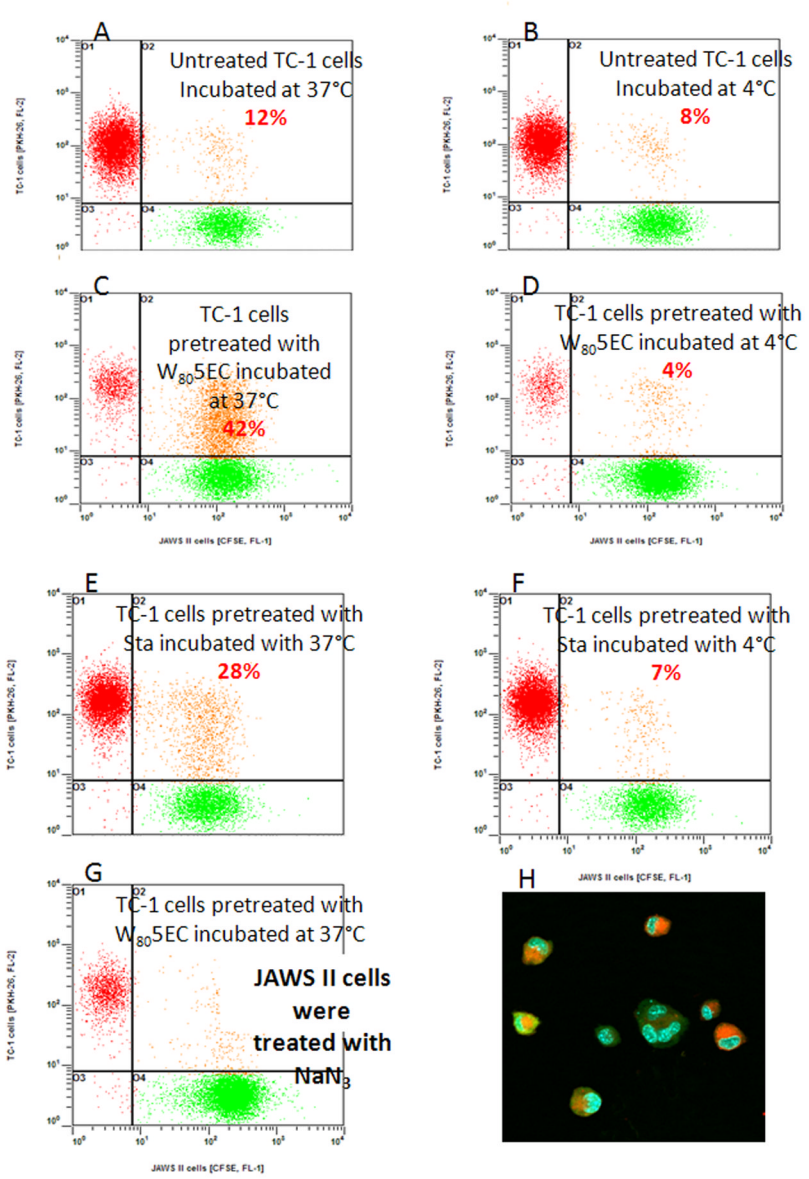

Figure 4.

NE facilitates engulfment of TC-1 cells by JAWS II cells. JAWS II cells were stained with CFSE (green fluorescence); TC-1 cells were stained with PKH-26 (red fluorescence). TC-1 cells were then incubated overnight with either NE (C, D) or staurosporine (E, F) or were left untreated (A, B). The next day, TC-1 ECs were mixed in equal number with CFSE-stained JAWS II DCs. The JAWS II cells were pretreated with 35 mM NaN_3 overnight (G). Mixed cultures were incubated for four hours either at 37°C or 4°C and analyzed using flow cytometry. Confocal microscopy microphotograph of TC-1 cells treated with NE and co-cultured with JAWS II cells (H). Data shown are representative of one of five independent experiments. Significant difference ($p<0.05$) in the percentage of engulfed TC-1 cells between untreated and treated with 0.05% of $W_{80}5EC$ (N=5) has been observed as tested using Wilcoxon signed-rank test.

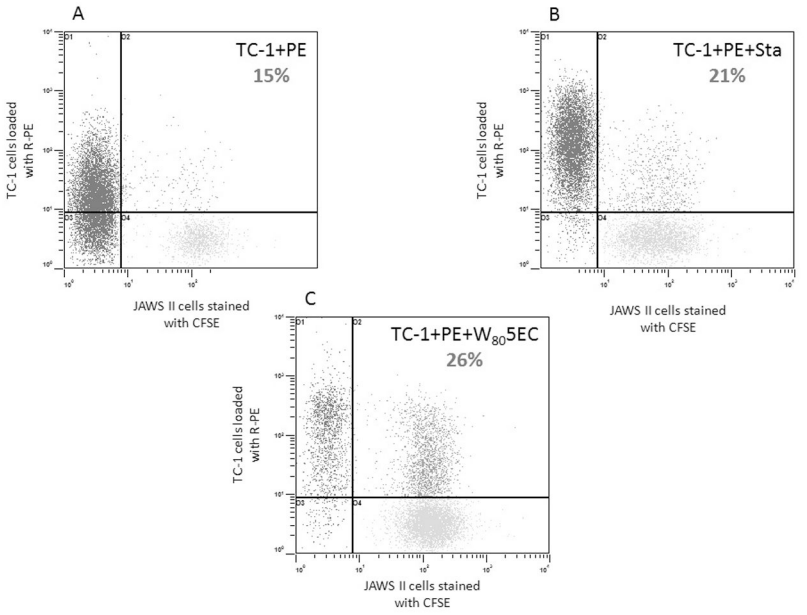

Figure 5.

NE augments antigen transfer from ECs to DCs. JAWS II cells were stained with CFSE (green fluorescence); TC-1 cells were uploaded overnight with 40 μg/mL R-PE (A), uploaded with R-PE and treated with 1μM staurosporine (B), uploaded with R-PE and treated with 0.05% W_{80}5EC (C). The next day TC-1 cells were mixed in equal number with CFSE-stained JAWS II cells and mixed cultures were incubated for four hours at 37°C and analyzed using flow cytometry. The experiments were repeated independently on three occasions. Data shown from a single experiment are representative for all tree experiments performed. Significant difference ($p<0.003$) in the percentage of JAWS II cells with R-PE antigen transferred from TC-1 cells untreated vs. treated with W_{80}5EC (N=6) has been observed as tested using Wilcoxon signed-rank test.

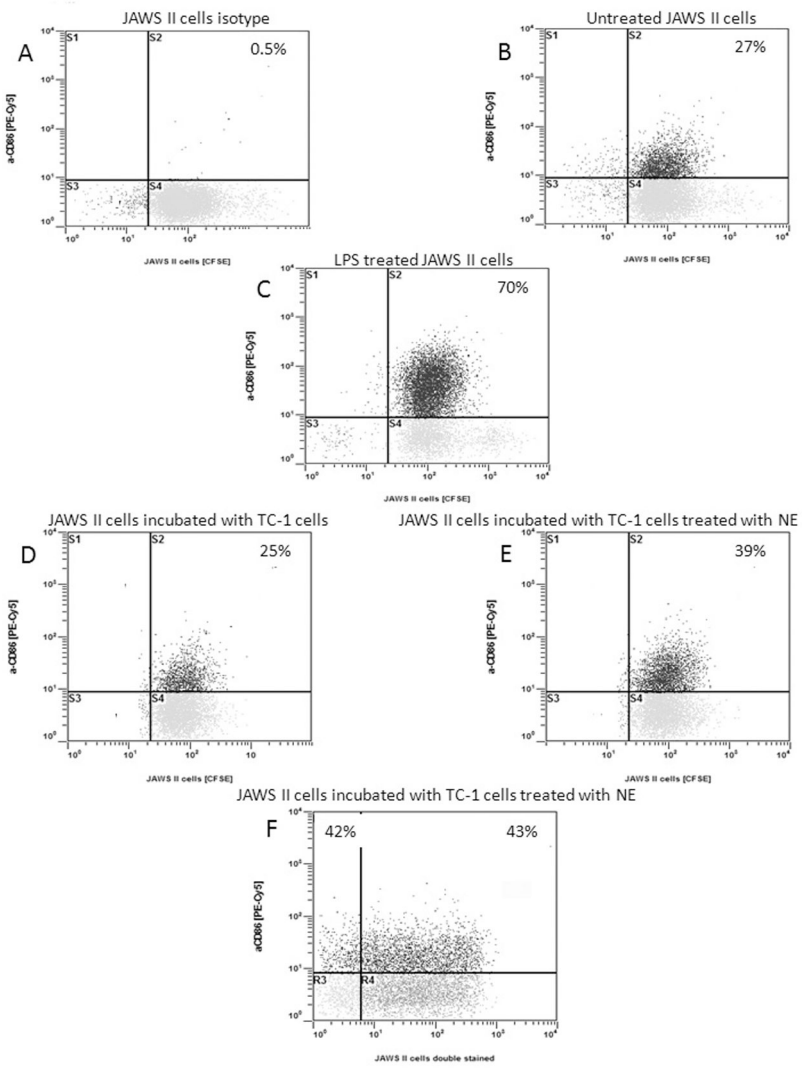

Figure 6.

Detection of expression of CD86 antigen on JAWS II cells. A) JAWS II cells stained with isotype control antibody. B) Untreated JAWS II cells stained with anti-CD86-PE-Cy5 antibody. C) JAWS II cells stimulated with 10 μg/mL LPS for 24 hours and stained with anti-CD86-PE-Cy5 antibody (positive control). D) JAWS II cells co-cultured with TC-1 cells and stained with anti-CD86-PE-Cy5 antibody. E) JAWS II cells co-cultured with TC-1 cells pretreated with $W_{80}5EC$ and stained with anti-CD86-PE-Cy5 antibody. F) Shows equal percentage of JAWS II cells expressing CD86 antigen between two subsets of JAWS II cells: those which phagocytosed TC-1 cells (the upper right quadrant) and those which did not (the upper left quadrant). To exclusively analyzed JAWS II cells, red fluorescent TC-1 cells were gated out. Cells were treated and stained with antibodies on two independent occasions. Significant difference ($p<0.01$) in the percentage of anti-CD86 expressed on JAWS II cells co-cultured with untreated TC-1 vs. treated with $W_{80}5EC$ (N=5) has been observed as tested using Wilcoxon signed-rank test.