



# Distinct regulatory roles of NF- $\kappa$ B in CpGA and CpGB- induced IFN- $\alpha$ production by plasmacytoid dendritic cells

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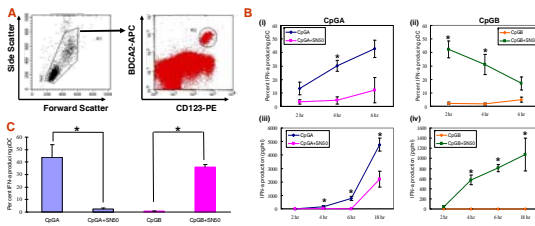
## ABSTRACT:

Two distinct types of CpG oligodeoxynucleotides have different functions in stimulating plasmacytoid dendritic cells (pDC): CpGA induces high amounts of IFN- $\alpha$ , but CpGB induces pDC maturation. Here, we demonstrate that the inhibition of NF- $\kappa$ B activation by SN50 or NBD conferred CpGB to induce IFN- $\alpha$ , while CpGA-induced IFN- $\alpha$  was decreased. Using ImageStream imaging flow cytometer, we found that both CpGA and CpGB-induced NF- $\kappa$ B translocation was inhibited by SN50. Although CpGA-induced IRF7 translocation was inhibited by SN50, SN50 conferred the ability of CpGB to induce IRF7 translocation as early as 1 hr. CpG endosomal localization has been shown to be important in the IFN- $\alpha$  production. We found that both CpGA and CpGB moved from early to late endosomes from 30 min to 2 hr. However, neither CpGA nor CpGB was found in either early or late endosomes in large quantities upon NF- $\kappa$ B inhibition. We also found that TLR9 was constitutively localized in the early endosomes and recruited from ER upon stimulation with CpGA in 15 min. These results suggest that NF- $\kappa$ B plays an inverse role in the regulation of IFN- $\alpha$  production by pDC in response to CpGA or CpGB stimulation.

## MATERIALS AND METHODS:

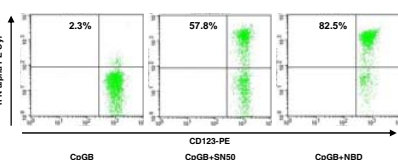
**Preparation of pBMC:** pBMC were isolated by Ficoll-Hypaque gradients from fresh heparinized peripheral blood obtained with informed consent from healthy donors.  
**pDC enrichment:** pDC were negatively selected from pBMC using the Miltenyi pDC isolation kit.  
**IFN- $\alpha$  ELISA:** pBMC were pre-incubated with or without SN50 (50  $\mu$ g/ml) or NBD peptide (50  $\mu$ g/ml) for 1 hr and stimulated with CpGA (5  $\mu$ g/ml) or CpGB (5  $\mu$ g/ml) for different time points. Supernatants were collected and analyzed by ELISA using BenderMed Systems IFN- $\alpha$  module set.  
**Flow cytometry:** Stimulated pBMC or pDC were surface stained with anti-CD123 PE and BDCA-2 APC Abs, or anti-BDCA-2 and BDCA-4 PE Abs, respectively. For IFN- $\alpha$  intracellular flow, pBMC were treated with the indicated stimuli and incubated for different time points. 6-hr time point was treated with or without Brefeldin A (BFA, 5  $\mu$ g/ml). Cells were stained and fixed overnight. On day 2, cells were permeabilized and stained with biotinylated anti-IFN- $\alpha$  (PE<sub>1</sub> clone MHF42) followed by Streptavidin-PE-Cy7, and acquired using a FACScan and analyzed by Cell Quest software (BD). For pIRF7 intracellular flow, after stimulation, cells were fixed in 1% paraformaldehyde for 10 min, followed by surface staining with anti-CD123 PE and HLA-DR APC Abs. On day 2, cells were intracellularly stained with anti-pIRF7 AF488 Ab.  
**ImageStream analysis:** Surface-stained enriched pDC were intracellularly stained with anti-IRF7 Ab or anti-p65 Ab (Santa Cruz) followed by goat anti rabbit IgG-FITC, or anti-CD71 Ab, anti-CD107a Ab and anti-EEA1 Ab (BD). For nuclear translocation analysis, nuclei were stained with DRAQ5 before acquiring. Samples then were acquired using ImageStream multispectral imaging flow cytometer and analyzed with Amnis IDEAS software (Amnis Corporation, Seattle, WA).

## The effect of NF- $\kappa$ B inhibition on the IFN- $\alpha$ production induced by CpGA and CpGB in pDC



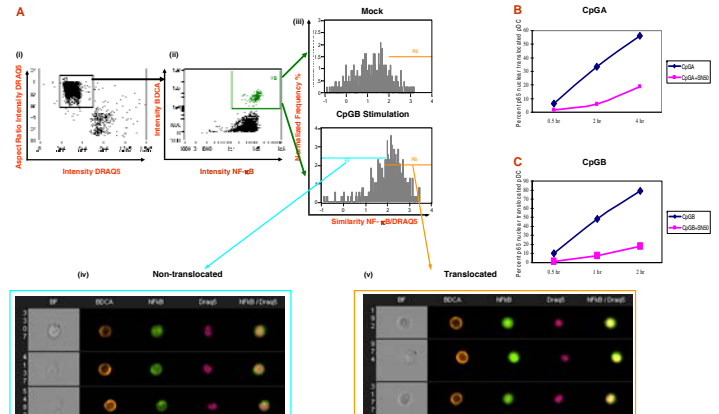
**Figure 1. CpGA-induced IFN- $\alpha$  is inhibited by the NF- $\kappa$ B inhibitor, SN50, while SN50 allows CpGB to induce a large amount of IFN- $\alpha$  in pDC.** A, pDC were gated from R1 by their CD123 and BDC2A positive phenotype (R2). B, pBMC were stimulated with CpGA or CpGB, with or without SN50 pretreatment for one hour, for different time points. Following stimulation, supernatants were collected for ELISA analysis (iii and iv). Cells were surface stained for pDC, fixed overnight and intracellularly stained for IFN- $\alpha$  (i and ii). C, BFA was added to the extra 6-hr incubation cell groups 2 hrs before staining. Data show mean  $\pm$  SE of three independent experiments. \*, p < 0.05, paired t-test.

## NF- $\kappa$ B inhibitors, NBD peptide and SN50, have similar effects on CpGB treated pDC



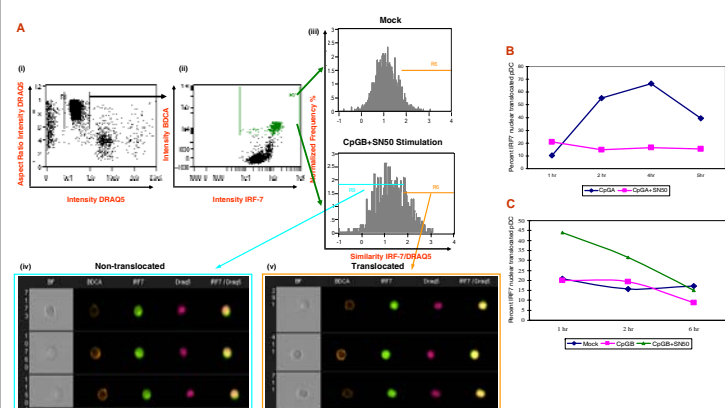
**Figure 2. The NF- $\kappa$ B inhibitor, NBD peptide, has a similar effect as SN50 on the induction of IFN- $\alpha$  by CpGB.** pBMC were treated with CpGB alone, or preincubated with SN50 or NBD peptide for 1 hr followed by 6-hr stimulation with CpGB. IFN- $\alpha$  production was analyzed by flow cytometry.

## NF- $\kappa$ B nuclear translocation induced by CpGA and CpGB is inhibited by SN50



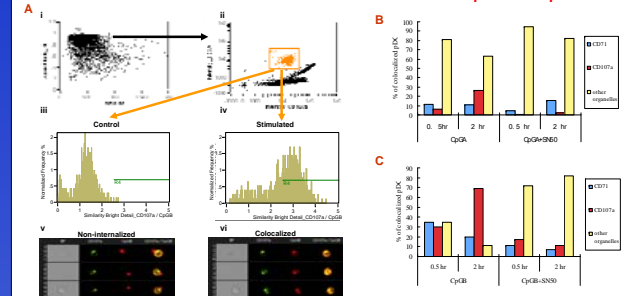
**Figure 3. CpGA- and CpGB-induced NF- $\kappa$ B nuclear translocation is inhibited by SN50.** A, pDC-enriched pBMC were preincubated with or without SN50 for 1 hr, and stimulated with CpGA or CpGB for different time points. NF- $\kappa$ B nuclear translocation was analyzed by Amnis ImageStream. Single cell population (R1) was gated by DRAQ5-positive events and high DRAQ5 aspect ratios (i). pDC (R2) were identified from R1 by gating on their NF- $\kappa$ B bright staining and BDC2A/4-positive phenotype (ii). Nuclear translocation of NF- $\kappa$ B from either mock or CpGB-treated pBMC was measured with the similarity score (iii). (iv) and (v) represent the images of non-translocated and translocated pDC, respectively. B, C, Time course of NF- $\kappa$ B nuclear translocation induced by CpGA  $\pm$  SN50 and CpGB  $\pm$  SN50 in pDC.

## Distinct effect of NF- $\kappa$ B inhibition on IRF7 nuclear translocation induced by CpGA or CpGB



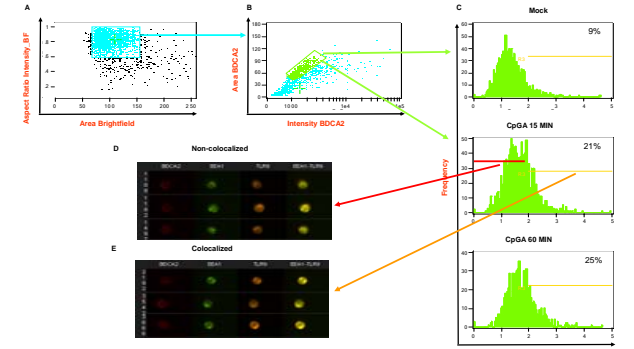
**Figure 4. CpGA-induced IRF7 nuclear translocation is inhibited by SN50, while SN50 confers the ability of CpGB to induce IRF7 nuclear translocation in pDC.** A, pDC-enriched pBMC were preincubated with or without SN50 for 1 hr, and stimulated with CpGA or CpGB for different time points. IRF7 nuclear translocation was analyzed by Amnis ImageStream. Single cell population (R1) was gated by DRAQ5-positive events and high DRAQ5 aspect ratios (i). pDC (R2) were identified from R1 by gating on their IRF7 bright staining and BDC2A/4-positive phenotype (ii). Nuclear translocation of IRF7 from either mock or CpGB+SN50-treated pBMC was measured with the similarity score (iii). (iv) and (v) represent the images of non-translocated and translocated pDC, respectively. B, C, Time course of IRF7 nuclear translocation induced by CpGA  $\pm$  SN50 and CpGB  $\pm$  SN50 in pDC.

## The effect of SN50 on endosomal localization of CpGA and CpGB



**Figure 5. The endosomal localization of CpGA and CpGB is inhibited by SN50.** A, pDC-enriched pBMC were preincubated with or without SN50 for 1 hr, and stimulated with CpGA-Cy5 or CpGB-Cy5 for different time points. Early endosome (CD171) or late endosome / lysosome (CD107a) co-localization with CpGA or CpGB was analyzed by Amnis ImageStream. Single cell population (R1) was gated by Area<sub>BF</sub> and high aspect ratio. BF (i). pDC (R2) were identified from R1 by gating on their BDC2A/4-positive phenotype (ii). Co-localization of CD107a with CpGB from either mock or CpGB-treated pBMC was measured with the similarity score (iii, iv). (v) and (vi) represent the images of non-internalized and co-localized pDC, respectively. B, C, Time course of endosomal localization induced by CpGA  $\pm$  SN50 and CpGB  $\pm$  SN50 in pDC.

## TLR9 early endosome localization



**Figure 6. TLR9 is constitutively localized in early endosomes and the early endosomal localization is enhanced by CpGA.** pDC-enriched pBMC were stimulated with CpGA for different time points. Early endosome (EEA1) and TLR9 co-localization was analyzed by Amnis ImageStream. Single cell population (R1) was gated by Area<sub>BF</sub> and high aspect ratio. BF (i). pDC (R2) were identified from R1 by gating on their BDC2A-positive phenotype (ii). Co-localization of EEA1 with TLR9 from either mock or CpGA-treated pBMC was measured with the similarity score (C). (D) and (E) represent the images of non-colocalized and co-localized pDC, respectively.

## CONCLUSIONS:

- CpGA-induced IFN- $\alpha$  is inhibited by the specific NF- $\kappa$ B translocation inhibitor, SN50.
- CpGB can not induce detectable IFN- $\alpha$  production in pDC, but with the NF- $\kappa$ B inhibition by SN50 or NBD peptide, a large amount of IFN- $\alpha$  is induced by CpGB.
- CpGB- and CpGA- induced NF- $\kappa$ B translocation is inhibited by SN50.
- CpGA-induced IRF7 nuclear translocation is inhibited by SN50.
- Although CpGB itself can not induce IRF7 nuclear translocation, SN50 confers the ability of CpGB to induce high levels of IRF7 translocation and this happens as early as 1 hr.
- CpGB transfers from early endosome to late endosome within 2 hrs. But the SN50 treatment inhibits the localization of CpGB to both early endosome and late endosome. They are clustered in other organelles in the cytoplasm.
- TLR9 is constitutively localized in early endosomes and can be enhanced upon CpGA stimulation.