Supplementary Material

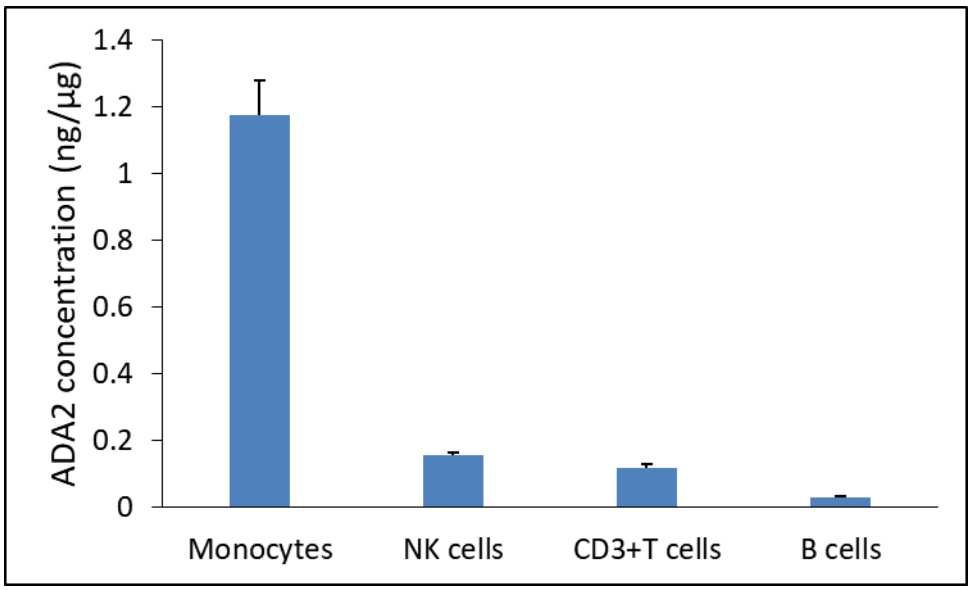
Supplementary Methods

mTLR9 binding to CpG ODN 2006 PTO

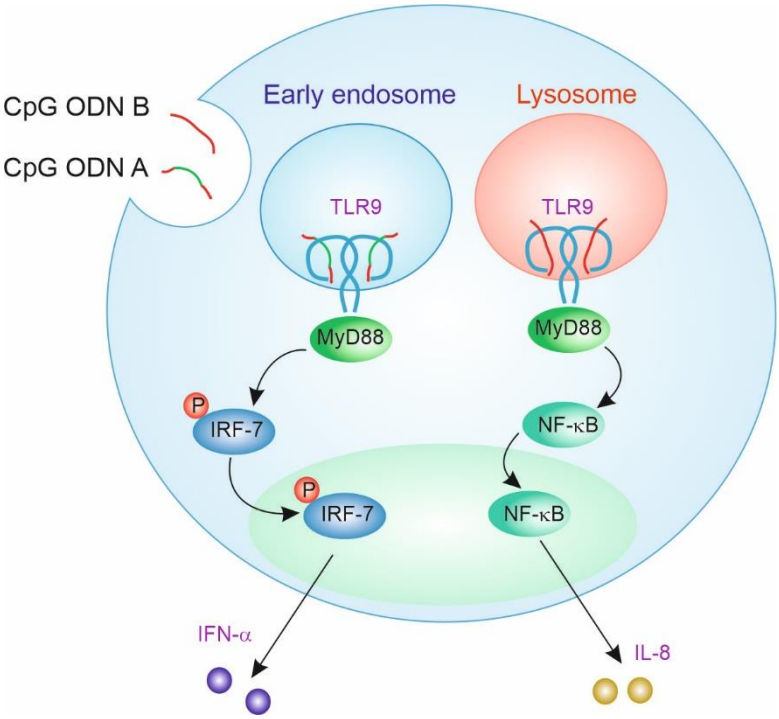
ELISA plates (Greiner Bio-One) were coated overnight at 4°C with 100 µl of 5 µg/ml goat anti-mouse antibodies (Sinobiological) in PBS with 0.02% NaN_3. After washing the plates 3 times with 200 µl PBS-Tween 20 buffer and blocking with 200 µl of 2% BSA in PBS for 1 hour, 100 µl of 100 ng/ml Fc-mTLR9 (R&D Systems) in PBS containing 10% FBS and 0.02% NaN_3 were added to the wells. The plates were incubated for 1 hour at RT on a shaker. Subsequently, the plates were washed 3 times with 200 µl PBS-Tween 20, and a 100 µl mix of 4 nM CpG ODN 2006 biotin in buffer A with different concentrations of inhibitory CpG ODNs was added to the wells. In the experiment in Supplementary Fig. 2B, 50 µl of ADA2 with indicated concentrations was added to the Fc-mTLR9 plate following the addition of 50 µl of increasing concentrations of CpG ODN 2006 biotin. The plate was incubated for 30 min at room temperature on a shaker and washed 3 times with buffer A. After 100 µl 1:1000 Streptavidin-HRP (Abbkine) in 20 mM Tris HCl pH 6.8, 50 mM NaCl, 10 µM $ZnCl_2$ containing 10% FBS (buffer B) was added to the wells, and the plates were incubated for 30 min at room temperature on a shaker following 4 washes with the same buffer. The color reaction was initiated by adding 100ml of TMB substrate (BioLegend). The reaction was stopped by adding 100 ml of 2 M HCl, and the plates were read at 450 nM in a plate reader (Thermofisher).

Supplementary Table 1. The oligonucleotide sequence and IC_{50} of ODNs PTO were used in the experiment described in Fig. 2. Bases shown in capital letters are phosphodiester, and in lowercase are phosphorothioate (nuclease resistant). A palindrome is underlined.

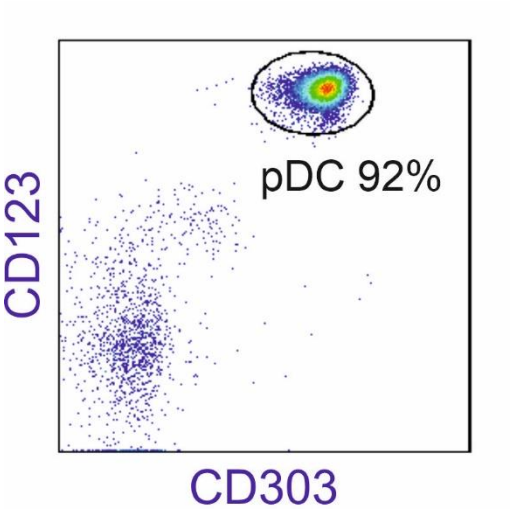
ODN	Oligonucleotide sequence	IC_{50} (nM)
	Class A	
ODN 2216	ggGGGACGATCGTCGggggg	1326
ODN 2336	gggGACGACGTCGTGgggggg	2525
	Class B	
ODN 2006	tcgtcgttttgtcgttttgtcgtt	0.6
ODN 2006 GC	tgctgcttttgtgcttttgtgctt	3.2
ODN BW006	tcgacgttcgtcgttcgtcgttc	98.3
ODN D-SL01	tcgcgacgttcgcccgacgttcggta	6.9
	Class C	
ODN 2395	tcgtcgttttcggcgcgcgccg	7.6
ODN M362	tcgtcgtcgttcgaacgacgttgat	45.3
ODN D-SL03	tcgcgaacgttcgccgcgttcgaacgcgg	17.6



Supplementary Fig. 1. The concentration of ADA2 in the main types of immune cells expressing the ADA2 gene, according to proteinatlas.org. The cells were isolated from PBMCs using magnetic beads, and ADA2 concentration in the cell lysates was analyzed by ELISA (11). The concentration of ADA2 is normalized to 1 µg of total protein determined by the Pierce BCA Protein Assay. The error bars represent the standard deviation, and the results were obtained from four independent replicates.

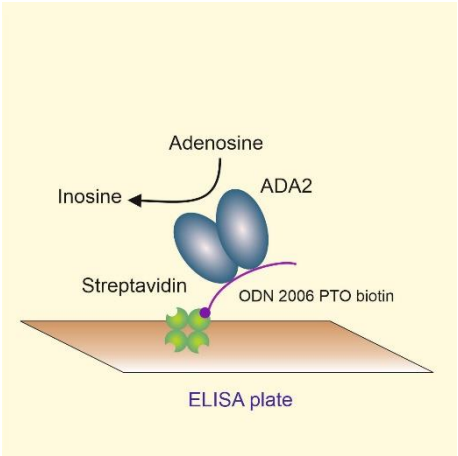


Supplementary Fig. 2. A scheme shows the release of IFN-α and IL-8 from human pDC in response to TLR9 activation with class A and B ODNs PTO.



Supplementary Fig. 3. The purity of pDC cells was assessed by flow cytometry after purification using the Miltenyi kit (130-092-207). The purified cells were stained with anti-CD303 FITC and anti-CD123 PE antibodies from BD Biosciences. Analysis of the flow cytometry data showed a cell purity of 92% for the pDC population.

A



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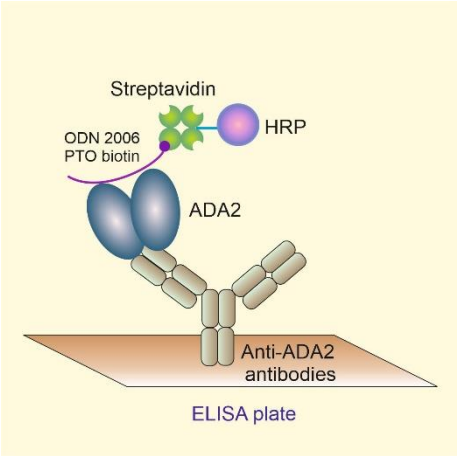
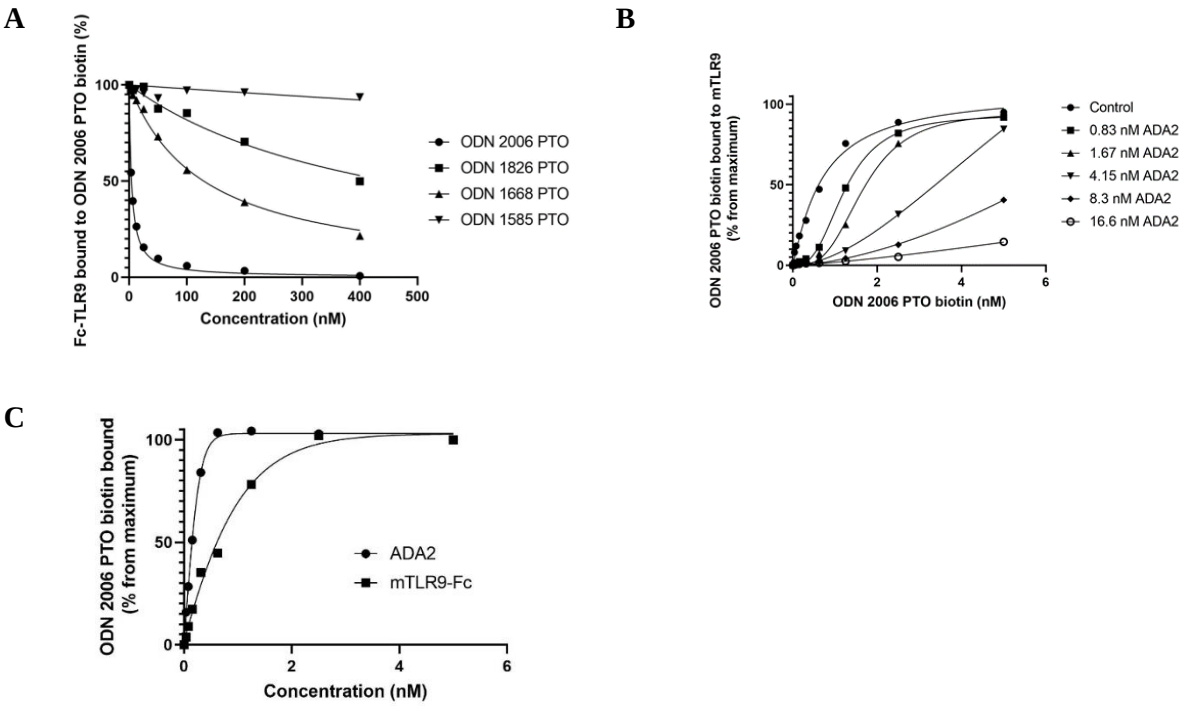
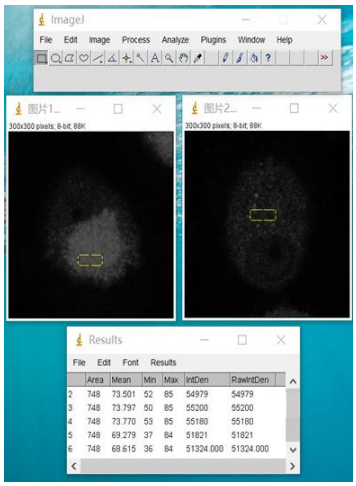


Supplementary Fig. 4. This scheme outlines the process for studying the binding of ADA2 to ODNs. In A, ODN 2006 PTO is bound to streptavidin to capture ADA2, followed by the addition of adenosine to detect ADA2 bound to ODN. In B, anti-ADA2 rabbit polyclonal antibodies capture ADA2, and ODN 2006 PTO Biotin is detected with HRP-Streptavidin.



Supplementary Fig. 5. Mouse Fc-TLR9 binding to ODN PTO. (**A**) Inhibition of mouse Fc-TLR9 binding to ODN 2006 PTO by class A (ODN 1585 PTO) and class B (ODN 2006 PTO, ODN 1826 PTO, ODN 1668). (**B**) Binding of mouse Fc-TLR9 to ODN 2006 PTO in the presence of increasing concentrations of ADA2. (**C**) Binding of mouse Fc-TLR9 and ADA2 to ODN 2006 PTO. Each dot represents a mean of three replicates.

A



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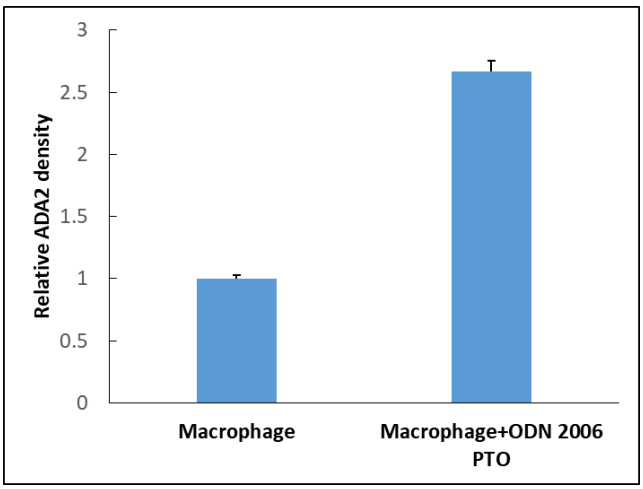
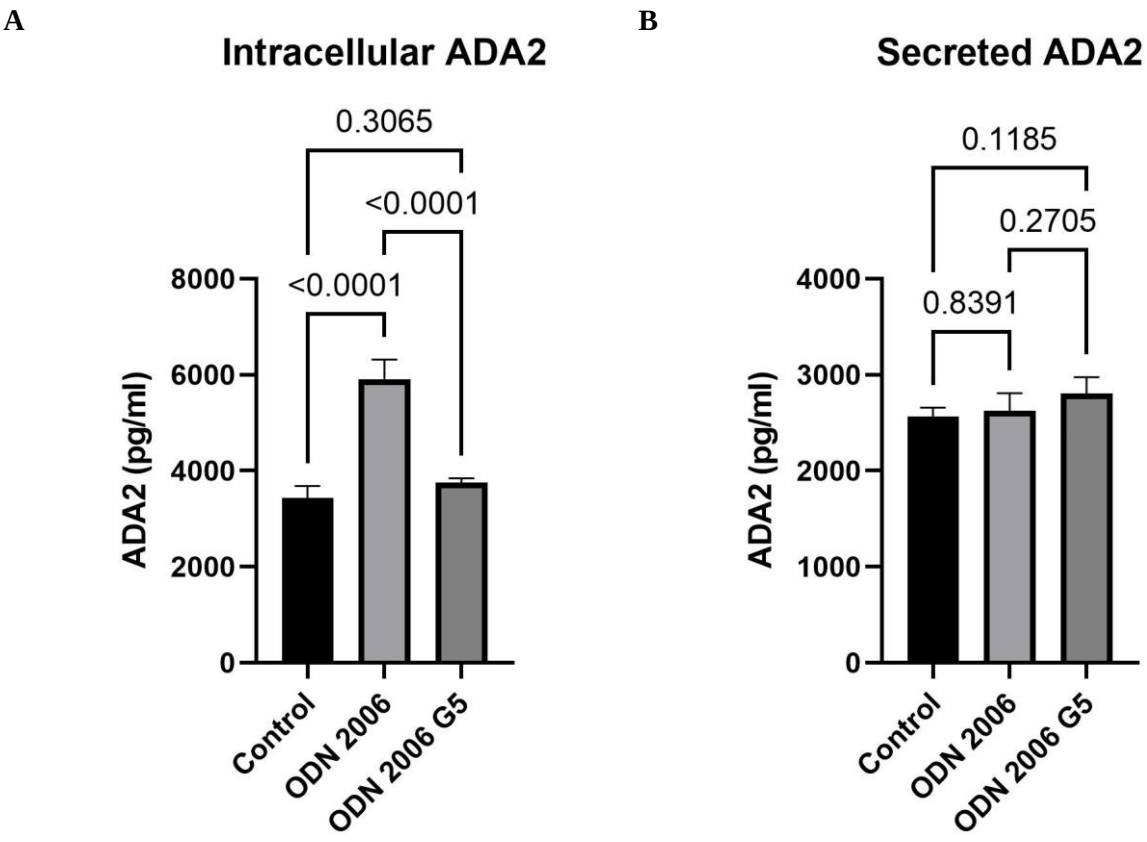
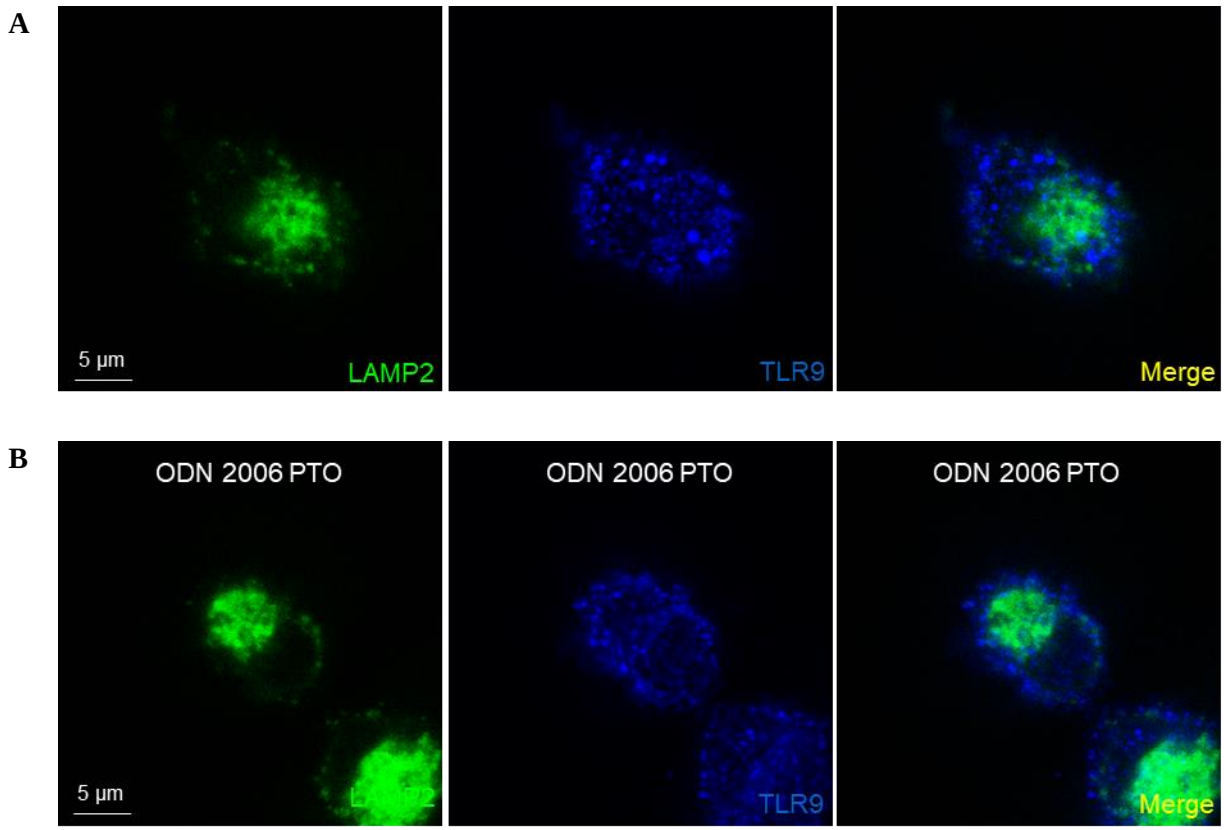


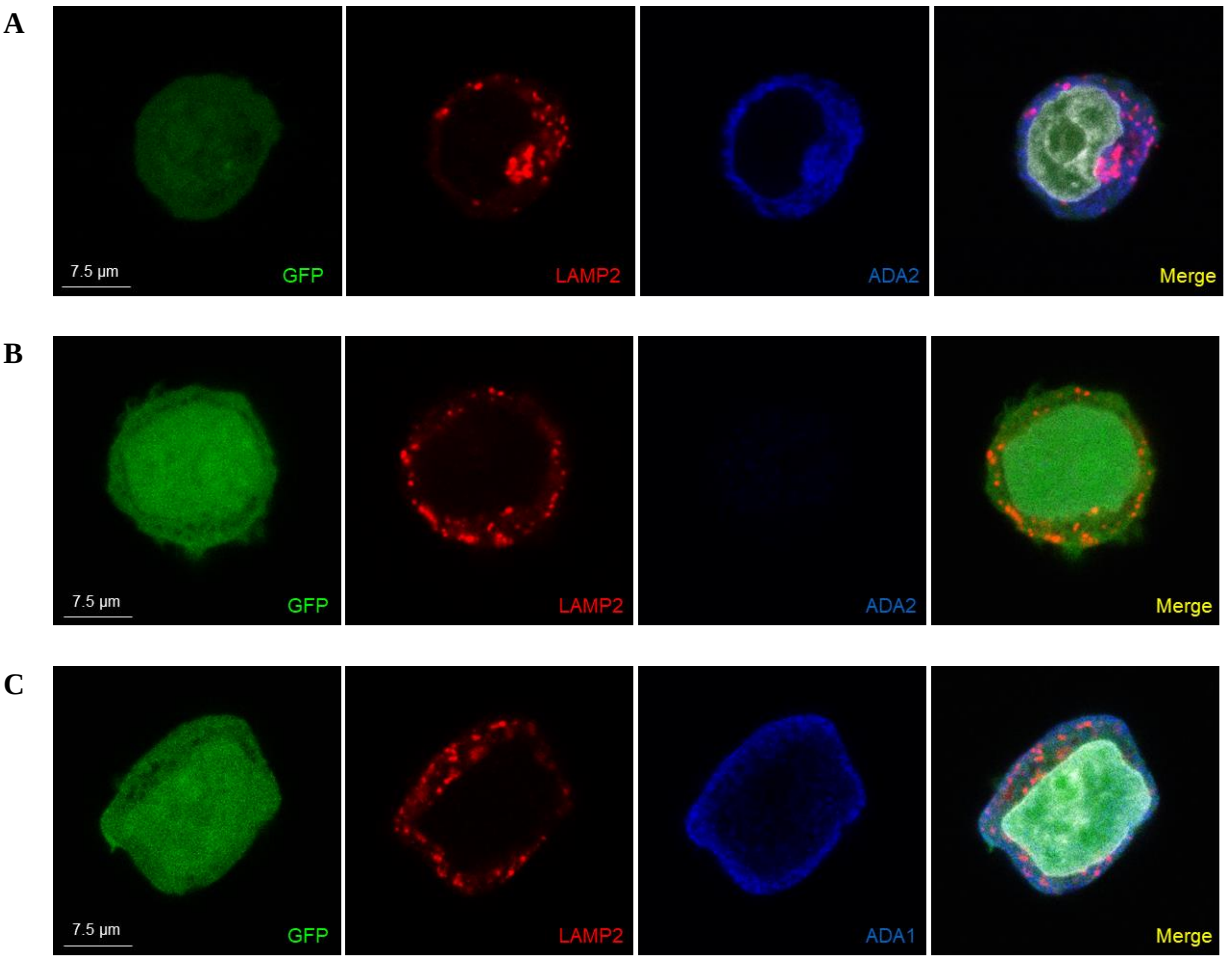
Supplementary Fig. 6. The effect of ODN 2006 PTO incubation on ADA2 density inside lysosomes of macrophages. The fluorescence images from Fig. 4C and D were analyzed using the IntDen tool of Image J Software (A). The analysis resulted in a more than two-fold increase ($p<0.001$) in ADA2 density inside macrophages treated with ODN 2006 PTO compared to the control group.



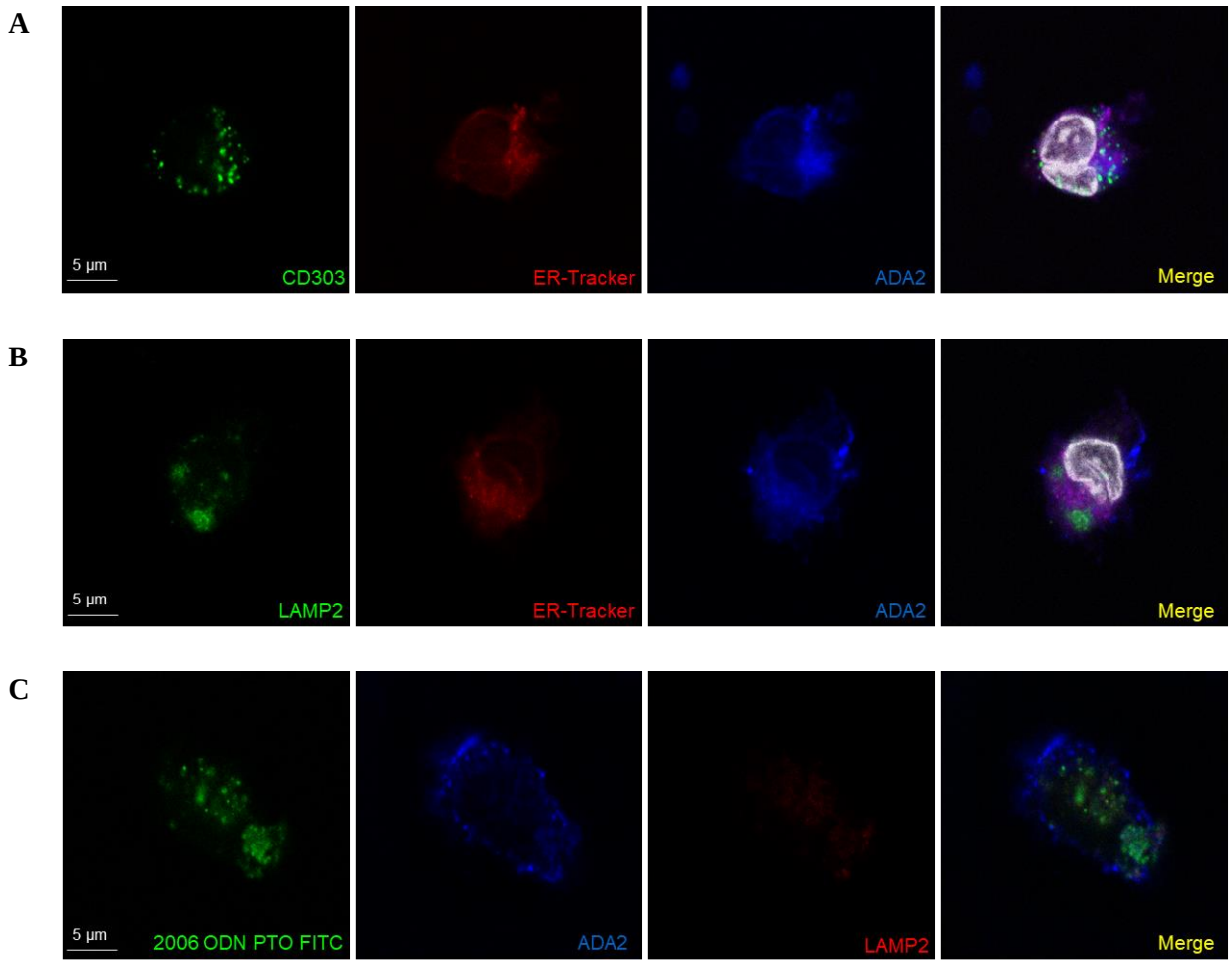
Supplementary Fig. 7. Concentration of ADA2 inside the cells and in the cell culture medium of GM-CSF differentiated monocytes after 6 days of differentiation. Monocytes (0.4 x 10^6 cells/ml) were isolated from PBMCs and differentiated into macrophages for 6 days with 40 ng/ml GM-CSF. The cells were washed with the fresh medium and either left untreated or treated with 0.5 μM CpG ODN 2006 PTO or CpG ODN 2006 G5 PD for 24 hours. The concentration of ADA2 in the cell lysates (A) or cell culture medium (B) was analyzed with ELISA (11).



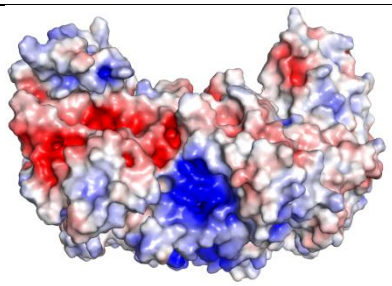
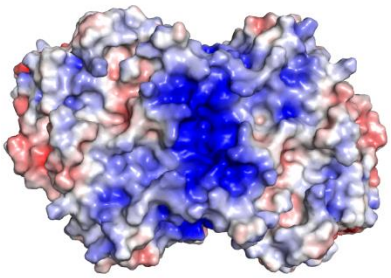
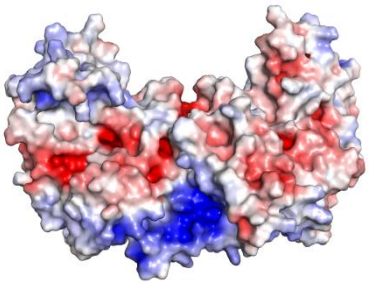
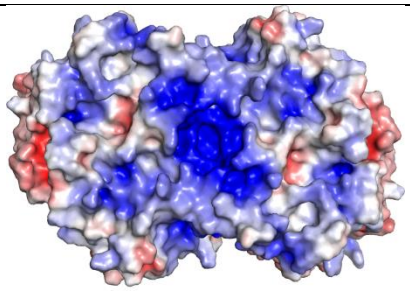
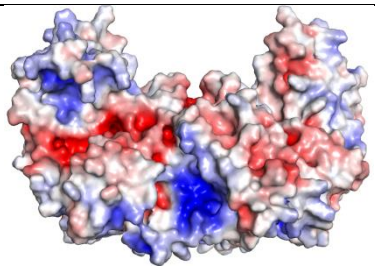
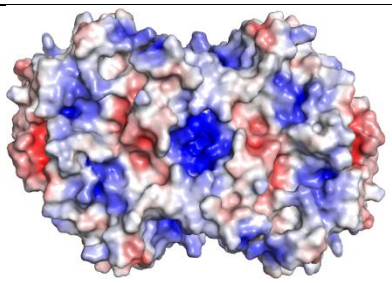
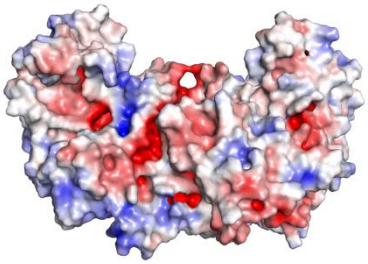
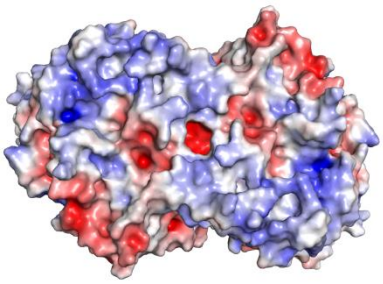
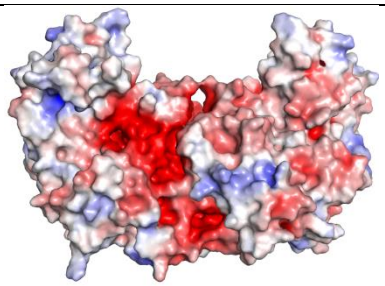
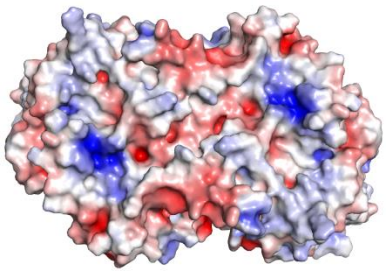
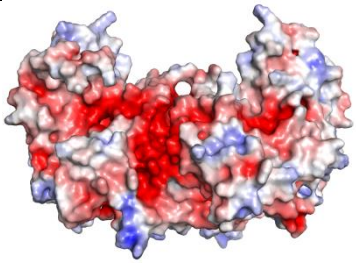
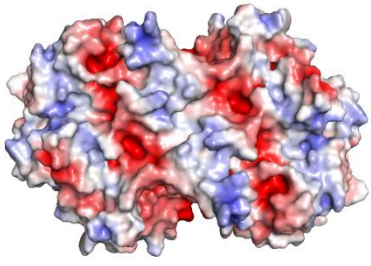
Supplementary Fig. 8. Localization of TLR9 in monocytes-derived macrophages. Monocytes were obtained from PBMCs and differentiated into macrophages for 6 days using 40 ng/ml GM-CSF. The macrophages were then exposed to 0.5 µM ODN 2006 PTO for 24 hours. The cells were stained with anti-LAMP2 (in green) and anti-TLR9 antibodies (in blue) after fixation. Both (A) and (B) indicate that TLR9 does not colocalize with the lysosomal marker LAMP2.

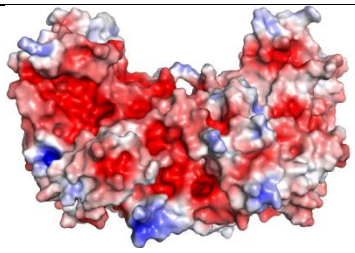
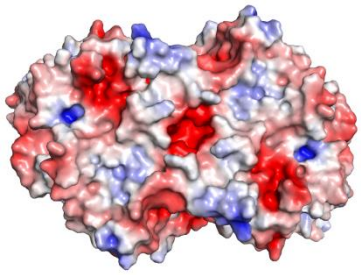


Supplementary Fig. 9. Confocal microscopy of 293T cells overexpressing ADA1 and ADA2. (**A**) 293T cells expressing ADA2 and GFP were fixed and stained with anti-LAMP2 (red) and anti-ADA2 antibodies (blue). (**B**) 293T cells expressing ADA1 and GFP were fixed and stained with anti-LAMP2 (red) and anti-ADA2 antibodies (blue). (C) 293T cells expressing ADA1 and GFP were fixed and stained with anti-LAMP2 (red) and anti-ADA1 antibodies (blue).

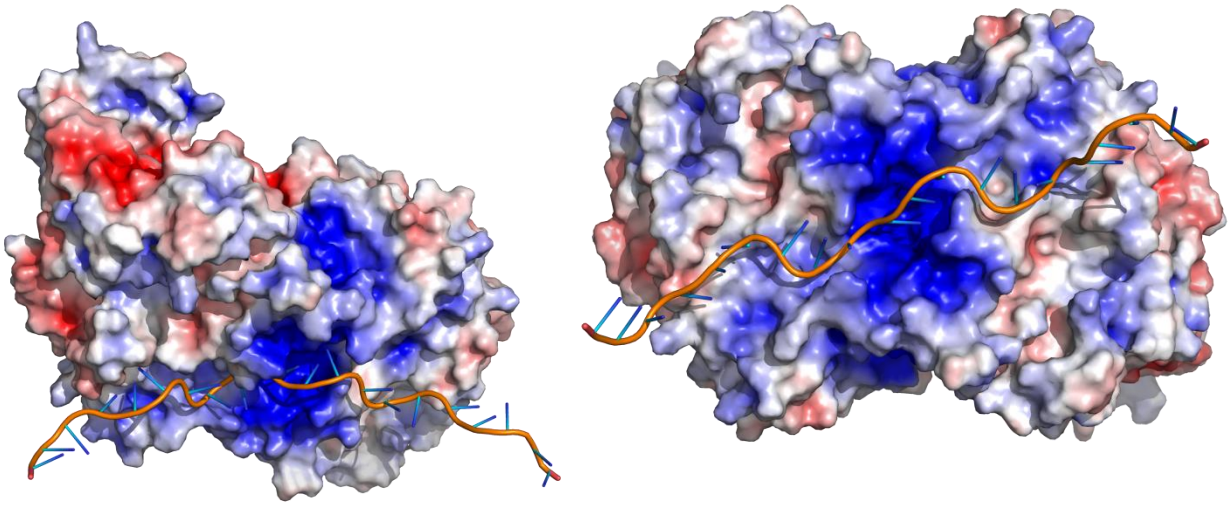


Supplementary Fig. 10. In this study, the effects of IL-3 on plasmacytoid dendritic cells (pDCs) in humans were analyzed after 48 hours of cell culture. For the first 24 hours, the pDCs were cultured in an 8-well chamber with 10 ng/ml IL-3. After that, the cells were either untreated (A-B) or treated with 0.5 µM ODN 2006 PTO FITC for 24 hours (C). Next, the pDCs that were attached to the plastic were washed, fixed, and stained with anti-ADA2 antibodies (in blue), ER-tracker (in red), DAPI (in white), anti-LAMP2 antibodies (in green and red), and anti-CD303, which is a cell surface marker of pDC.

Homo sapiens		
Pongo abelii		
Macaca mulatta		
Sus domesticus		
Gallus gallus		
Danio rerio		

Drosophila melanogaster		

Supplementary Fig. 11. Electrostatic surfaces of ADA2 molecules from different species. A significant evolutionary change is observed when comparing the electrostatic surface distribution of ADA2 between flies and humans. Positive and negative charges are respectively represented by blue and red on the surface of the molecule. Models were created using the Phyre2 Protein Fold Recognition Server [1]. Confidence scores in all models were 100%. Visualization and electrostatic surface calculations were done in the PyMOL Molecular Graphics System, Version 2.5.4 Schrödinger, LLC.



Supplementary Fig. 12. The model predicts a potential binding site for a poly T (20) sequence on hADA2. The model was generated using the HDOCK web server, which utilizes a template-based modeling and free docking hybrid algorithm [2]. The confidence score for the model is 92.43%. The possible binding pattern is shown from various angles, and the molecule's surface is colored to represent positive and negative charges (blue and red, respectively). The poly T oligonucleotide chain is depicted as a cartoon.

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References

1. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 2015; 10(6): 845–858 doi:10.1038/nprot.2015.053 PMID:25950237

2. Yan Y, Zhang D, Zhou P, Li B, Huang SY. HDOCK: a web server for protein-protein and protein-DNA/RNA docking based on a hybrid strategy. Nucleic Acids Res 2017; 45(W1): W365–W373 doi:10.1093/nar/gkx407 PMID:28521030