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Hierarchical recognition of CpG motifs expressed by immunostimulatory oligodeoxynucleotides

D. M. KLINMAN* & D. CURRIE* **Section of Retroviral Immunology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, USA*

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SUMMARY

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs trigger human PBMC to proliferate and secrete Ig, cytokines and chemokines. CpG ODN have entered clinical trials, and show promise as vaccine adjuvants, antiallergens, and for the treatment of infectious diseases and cancer. ODNs under consideration for human use vary in the sequence, number and location of the CpG motifs they contain. Yet little is known of the magnitude of the immune response elicited by these diverse ODNs, or the rules governing their interaction with immune cells. This work compares the proliferative, IgM, IL-6 and IP-10 response of PBMC from normal donors to a diverse panel of CpG ODNs. Results indicate that ODNs expressing 3–4 different CpG motifs are strongly stimulatory. The location of these motifs is important, with those at the 5′ end exerting the greatest influence on ODN activity. These findings provide a basis for the rational design of ODNs optimized for clinical use.

Keywords CpG oligonucleotides human PBMC immune response innate immunity cytokine Ig

INTRODUCTION

Bacterial DNA contains immunostimulatory CpG motifs that directly or indirectly trigger B cells, NK cells, monocytes, macrophages and dendritic cells to proliferate, mature, and/or secrete a variety of cytokines, chemokines and Ig [1–4]. Synthetic oligodeoxynucleotides (ODN) containing CpG motifs mimic the activity of bacterial DNA [3–6]. In murine models, these ODN show promise as vaccine adjuvants, antiallergens, and in the treatment of infectious diseases and cancer [7–10].

Clinical trials for many of these indications are under way [11]. The success of such trials will depend upon the ability of CpG ODN to broadly activate the human immune system. Unfortunately, data derived from murine studies are of limited value in the selection of such ODN, since the CpG motifs that are most active in mice are poorly immunostimulatory in primates, due to evolutionary divergence in CpG recognition between species [12–14]. Similarly, studies of cloned cell lines may not reflect the complex response of the multiple cell types triggered by CpG ODN *in vivo*, or the heterogeneity in response to CpG ODN by a diverse pool of human donors.

It is widely accepted that CpG ODN capable of stimulating a strong and diverse immune response *in vivo* can be identified by studying human PBMC. To date, ODN that vary with respect to the sequence, type, and number of the CpG motifs they contain have been described by different groups, but without any consensus regarding optimal CpG content or location [12–15]. The present work explores the importance of CpG diversity and location on immune responsiveness. Results indicate that incorporating 3–4 different CpG motifs in a single ODN, and locating the most stimulatory motif at the 5′ end, yields a molecule with the highest activity.

Correspondence: Dennis Klinman, Bldg 29 A Rm 3D10, CBER/FDA, Bethesda, MD 20892, USA.

E-mail: Klinman@cber.fda.gov

MATERIALS AND METHODS

Cells

Normal PBMC were obtained from the NIH Department of Transfusion Medicine. Mononuclear cells were isolated by density gradient centrifugation over Ficoll-Hypaque as described [14]. The human myeloma cell line RPMI 8226 (CCL-155; American Type Culture Collection, Manassa, VA, USA) and mononuclear cells were cultured for 72 h in RPMI supplemented with 10% heat inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Reagents

Phosphorothioate ODNs were synthesized at the CBER Core Facility. All ODN contained less than 0·1 EU/mg of endotoxin as measured by the Limulus amoebocyte lysate assay (QCL-1000, BioWhittaker, East Rutherford, NJ, USA).

ELISAs

5×10^5 PBMC or RPMI 8226 cells were stimulated *in vitro* for 24–72 h with 1 μM ODN. IgM and IL-6 in culture supernatants were detected by ELISA, as previously described [14]. IP-10 was detected using Immulon-2 microtitre plates (Thermo Labsystems, Franklin, MA, USA) coated with anti-IP-10 (2 μg/ml, R & D Systems, Minneapolis, MN, USA) and blocked with PBS-5% BSA. IP-10 levels in the culture supernatants were detected colourimetrically using biotin-labelled secondary anti-IP-10 Ab (R & D Systems) followed by phosphatase-conjugated avidin followed by a phosphatase-specific substrate. ELISA results were quantified using standard curves generated using recombinant IL-6, IP-10 and purified IgM. The limit of detection of the assays was 5–20 pg/ml. To compensate for variability in the magnitude of the response between individual PBMC samples, results were standardized by calculating the relative response of each ODN in comparison to the most stimulatory ODN in each experiment.

Proliferation assays

10^5 PBMC were stimulated for 72 h with 1 μM ODN. For the last 4 h, 1 μCi of ^{3}H-thymidine was added to the cultures. Incorporated label was quantified using liquid scintillation (Perkin Elmer/Wallac, Gaithersburg, MD, USA). All assays were performed in triplicate.

Statistical analysis

Non-parametric ANOVA was used to compare differences in the magnitude of the response induced by incorporating specific motifs at defined locations in each ODN.

RESULTS

*Immunostimulatory activity is influenced by the **total number** of CpG motifs present in an ODN*

Many ODNs capable of stimulating human PBMC have been identified [12–14]. Yet efforts to establish the contribution of motif number and location to ODN activity have been complicated by the large number of ODNs required for such an analysis, the need to monitor multiple types of immune activation, and heterogeneity in the nature and magnitude of the response by different donors [16].

To identify ODNs capable of stimulating physiologically relevant immune responses *in vivo*, a large number of phosphorothioate ODNs were synthesized containing 1–5 different CpG motifs (Table 1). Preliminary experiments were conducted to identify the most relevant ODNs for analysis, select the dose of ODN for study, and determine how data from individual donors could be compared. As previously reported, PBMC from different donors varied in the magnitude of both their baseline and CpG-induced responses [14,16,17]. The range of responses observed in various assays is shown in the legend to Table 2. To standardize results between donors, the response of each PBMC sample to individual ODNs was compared to the strongest response elicited by any ODN in the same experiment. For example, if the most stimulatory ODN triggered a response 20-fold above background, an ODN that induced a 6-fold increase was considered to have generated a 30% maximal response. All ODNs were studied at 1 μM, a concentration previously shown to stimulate strong immune responses by human PBMC *in vitro* (significantly exceeding background) [14,16,18]. As previously reported, control ODN (lacking CpG motifs) typically elicited a response <10% of the maximal response of individual PBMC samples [14,16,18].

The effect of increasing the number of CpG motifs expressed by an ODN was examined. As seen in Table 2, the average response elicited by ODNs that contained only one CpG motif was $25 \pm 4\%$ of the maximum response elicited by the most stimulatory ODN. When two identical CpG motifs were present on a single ODN, the average level of immune activation rose to $47 \pm 9\%$ ($P < 0{\cdot}01$). Increasing the number of motifs to 3 or 4 resulted in 66–70% maximal stimulation ($P < 0{\cdot}01$, Table 2). ODNs in which CpG motifs were incorporated at 5 sites were somewhat less active ($49 \pm 7\%$), suggesting that 3–4 was the optimal number of CpG motifs that could be effectively recognized in ODNs up to 35 bases in length. These results were not significantly affected by the spacing between motifs. We found that separating individual CpG motifs by 1, 2 or 3 nucleotides, or changing the length of an ODN by up to 8 bases, did not alter the relative magnitude of the immune response induced by different ODNs (data not shown).

Immunostimulatory activity is influenced by the number of different motifs expressed by an ODN

The effect of incorporating different CpG motifs within an individual ODN was then examined. As seen in Table 3, ODNs containing multiple different motifs were significantly more immunostimulatory than those expressing a single motif multiple times. Molecules of the same size containing 3–4 different motifs were nearly three times as active as ODNs in which the same motif was present at 3–5 sites ($P < 0{\cdot}01$, Table 3).

Immunostimulatory activity is influenced by the location of CpG motifs within an ODN

The above findings are consistent with evidence showing that human PBMC can recognize and respond to an array of CpG motifs [13,14,19]. The next experiment evaluated whether the $5' \rightarrow 3'$ order of these motifs affected the stimulation induced by an ODN. ODNs of similar length were synthesized in which either a relatively strong CpG motif (such as GGCGTT) or a relatively weak motif (such as CTCGAC) was inserted at any of 5 sites along the length of the ODN (site 1 being nearest the 5′ end and site 5 being nearest the 3′ end). ODNs containing the stronger motif at site 1 induced significantly greater immune activation than those containing a less active motif at the same position (63% *versus* 19%, $P < 0{\cdot}01$, Table 4). This positional effect was observed in all assays of immune activation (proliferation, IgM, IL-6 and IP-10 production), indicating that ODNs with a strong CpG motif at the 5′ end were broadly immunostimulatory. A similar but smaller positional effect was observed at site 3, where inclusion of the more stimulatory motif generated significantly greater immune responses (59% *versus* 33%, $P < 0{\cdot}02$, Table 4). At site 5 (nearest the 3′ end), incorporation of a weaker motif generated ODNs of significantly greater activity (56% *versus* 42%, $P < 0{\cdot}02$). In contrast, the use of strong *versus* weakly stimulatory motifs at sites 2 and 4 had no significant effect on the activity of an ODN.

This pattern of reactivity was confirmed in studies of the RPMI 8226 human B cell line. IP-10 production was significantly higher when RPMI cells were stimulated with ODNs containing a highly stimulatory CpG motif at sites 1 and 3, whereas the same motif at sites 2 and 4 did not improve ODN activity (Table 5).

Table 1. List of ODNs studied

	Study inclusion	
	Table 2	Table 3
Series 1 5′ → 3′		
AT**CG**ACTCT**CG**AG**CG**TTCTC	*	
T**CG**AG**CG**TTCTC	*	*
T**CG**AGGCTTCTC	*	*
TGCAGGCTTCTC	*	*
T**CG**ACTCT**CG**AG**CG**TTCTC	*	*
TGCACTCT**CG**AGGCTTCTC	*	*
TGCACTCT**CG**AG**CG**TTCTC	*	*
ACTCT**CG**AG**CG**TTCTC	*	
TCT**CG**AG**CG**TTCTC	*	*
T**CG**AG**CG**TTCTC	*	*
AT**CG**ACTCT**CG**AG**CG**TTCTC	*	
TAGG**CG**TTT**CG**TTT**CG**ACT**CG**TACT	*	*
T**CG**AGTG**CG**TTGT**CG**TTGT**CG**ACAT**CG**TACT	*	*
T**CG**AGGTG**CG**TTATT**CG**TTGAT**CG**ACTGT**CG**TACT	*	*
T**CG**TTTT**CG**TAAG**CG**TTTT**CG**AGAT**CG**ACCT	*	*
T**CG**TATT**CG**ACAT**CG**TTTG**CG**TTAT**CG**AGCT	*	*
T**CG**AGTT**CG**AGAT**CG**AGTG**CG**TTAG**CG**TTCT	*	*
G**CG**TTTG**CG**TTAG**CG**TTTT**CG**AGAT**CG**AGCT	*	*
T**CG**AGTG**CG**TTGT**CG**TTGT**CG**ACAT**CG**TATT**CG**ATCT	*	*
T**CG**ACTG**CG**TTGT**CG**TTGTGCACAT**CG**TACT	*	*
T**CG**AGTG**CG**TTGT**CG**TTGTGCACATGCTACT	*	*
T**CG**AGTG**CG**TTGTGCTTGTGCACATGCTACT	*	*
T**CG**AGT**CG**AGT**CG**AGT**CG**AGT**CG**AGCT	*	*
G**CG**TTG**CG**TTG**CG**TTG**CG**TTG**CG**TTCT	*	*
T**CG**TTT**CG**TTT**CG**TTTCGTTT**CG**TTCT	*	*
Series 2 5′ → 3′		
[site 1] [site 5]		
T**CG**ACT**CG**ACT**CG**ACT**CG**ACT**CG**ACCT		
T**CG**TAT**CG**TAT**CG**TAT**CG**TAT**CG**TACT		
T**CG**AGTCGAGT**CG**AGT**CG**AGG**CG**TTCT		
T**CG**AGT**CG**AGG**CG**TTG**CG**TTG**CG**TTCT		
T**CG**AGG**CG**TTG**CG**TTG**CG**TTG**CG**TTCT		
G**CG**TTG**CG**TTG**CG**TTG**CG**TTT**CG**AGCT		
G**CG**TTG**CG**TTT**CG**AGT**CG**AGT**CG**AGCT		
G**CG**TTT**CG**AGT**CG**AGT**CG**AGT**CG**AGCT		
T**CG**AGG**CG**TTT**CG**AGG**CG**TTT**CG**AGCT		
G**CG**TTT**CG**AGG**CG**TTT**CG**AGG**CG**TTCT		
T**CG**AGT**CG**AGT**CG**AGG**CG**TTG**CG**TTCT		
G**CG**TTG**CG**TTG**CG**TTT**CG**AGTCGAGCT		
G**CG**TTG**CG**TTG**CG**TTT**CG**AGG**CG**TTCT		
T**CG**AGG**CG**TTT**CG**AGT**CG**AGT**CG**AGCT		
T**CG**AGT**CG**AGG**CG**TTT**CG**AGT**CG**AGCT		
G**CG**TTT**CG**AGG**CG**TTG**CG**TTG**CG**TTCT		
T**CG**AGT**CG**AGT**CG**AGG**CG**TTT**CG**AGCT		
G**CG**TTG**CG**TTT**CG**AGG**CG**TTG**CG**TTCT		
T**CG**AGTGCAGT**CG**AGTGCAGT**CG**AGCT		
G**CG**TTTGCAGG**CG**TTTGCAGG**CG**TTCT		
T**CG**AGT**CG**AGTGCAGG**CG**TTG**CG**TTCT		
T**CG**AGTGCAGT**CG**AGG**CG**TTG**CG**TTCT		
T**CG**AGTGCAGT**CG**AGG**CG**TTTGCAGCT		
T**CG**AGG**CG**TTTGCAGTGCAGTGCAGCT		
T**CG**AGTGCAGTGCAGTGCAGG**CG**TTCT		
G**CG**TTTGCAGTGCAGTGCAGT**CG**AGCT		
TGCAGTGCAGTGCAGT**CG**AGG**CG**TTCT		
TGCAGTGCAGTGCAGG**CG**TTT**CG**AGCT		

The response of PBMC to the phosphorothioate ODN shown above were examined. ODNs in series 1 were used in experiments presented in Table 2 and/or 3, as shown. All of the ODNs in series 2 were used to evaluate the role of motif position on immune stimulation, shown in Tables 4 and 5. CpG dinucleotides are shown in bold.

Table 2. Effect of increasing the total number of CpG motifs expressed on the stimulatory activity of an ODN

Total no. of CpG Motifs	% maximal response				
	Prolif	IL-6	IgM	IP-10	Mean
1	30 ± 3	27 ± 2	25 ± 2	21 ± 1	25 ± 4
2	54 ± 16	38 ± 5	59 ± 14	39 ± 7	47 ± 9*
3	73 ± 13	57 ± 7	84 ± 12	50 ± 11	66 ± 13*†
4	62 ± 7	68 ± 2	88 ± 5	63 ± 1	70 ± 11*†
5	53 ± 4	56 ± 5	46 ± 2	41 ± 4	49 ± 7*

The level of immune activation induced by 1 μM of 32 different ODNs (see Table 1, series 1) was monitored in PBMC from ≥6 donors. IL-6, IgM and IP-10 levels in culture supernatants were measured by ELISA, while proliferation was measured by ^{3}H-thymidine incorporation. The maximum background *versus* immune stimulation observed in the samples studied was: IL-6; 0·2 *versus* 6 ng/ml, IgM; 0·3 *versus* 15 μg/ml, IP-10; 0·1 *versus* 2·2 ng/ml and proliferation 1100 *versus* 27 100 cpm. To facilitate comparison between donors, the maximum response in each assay was set to 100, and the relative strength of each ODN then calculated by the formula: (response to ODN – background)/(maximum response – background) × 100%. The mean and std deviation for each group is shown. *Significantly greater than one motif, $P < 0{\cdot}01$. †Significantly greater than two motifs, $P < 0{\cdot}01$.

Table 3. Effect of increased CpG motif heterogeneity on an ODN's stimulatory activity

No. of different CpG Motifs	% maximal response				
	Prolif	IL-6	IgM	IP-10	Mean
1	27 ± 2	26 ± 3	19 ± 4	21 ± 3	23 ± 4
2	40 ± 16	44 ± 5	50 ± 14	42 ± 7	44 ± 4*
3	63 ± 4	51 ± 5	78 ± 2	56 ± 4	62 ± 10*†
4	69 ± 7	55 ± 2	74 ± 5	63 ± 1	65 ± 7*†
5	48 ± 6	50 ± 3	42 ± 4	48 ±	54 ± 3*

22 ODNs (see Table 1, series 1) were synthesized that contained 1–5 different CpG motifs. The level of immune activation induced by 1 μM of each ODN was measured using PBMC from 6 to 10 donors, as described in the legend to Table 2. The average level of immune stimulation induced by each group of ODNs is shown. *Signficantly greater than one motif, $P < 0{\cdot}01$. †Significantly greater than two motifs, $P < 0{\cdot}01$.

DISCUSSION

Ongoing clinical studies, as well as preclinical research in animal models, suggests that CpG ODN may be therapeutically useful as vaccine adjuvants, anti-allergens, and for the treatment of infectious disease and cancer [7–10,20–25]. Since the precise motifs that are most active in mice are poorly immunostimulatory in humans [12–14], considerable effort has been invested in identifying CpG ODN that strongly activate human cells. The current work evaluates the role of CpG location and number on the immunostimulatory activity of ODNs. Multiple parameters of immune activation were measured to detect the effect of CpG ODN, including the production of IgM, IL-6, IP-10 and proliferation.

Table 4. Effect of CpG motif position on an ODN's stimulatory activity

Site	Motif	Prolif	IL-6	IgM	IP-10	Mean
1	Strong	70 ± 9	67 ± 5	63 ± 7	64 ± 7	63 ± 7*
1	Weak	21 ± 1	16 ± 3	18 ± 2	21 ± 1	19 ± 2
1	Control	26 ± 5	23 ± 5	21 ± 3	13 ± 1	21 ± 6
2	Strong	49 ± 8	45 ± 6	42 ± 6	44 ± 10	45 ± 2
2	Weak	42 ± 7	65 ± 9	36 ± 4	40 ± 5	46 ± 11
3	Strong	61 ± 10	66 ± 7	50 ± 7	60 ± 10	59 ± 6*
3	Weak	35 ± 3	38 ± 3	38 ± 3	22 ± 2	33 ± 7
4	Strong	42 ± 4	60 ± 7	47 ± 3	47 ± 8	49 ± 8
4	Weak	51 ± 7	50 ± 5	61 ± 6	37 ± 3	50 ± 8
5	Strong	45 ± 7	49 ± 6	40 ± 3	47 ± 7	42 ± 7*
5	Weak	53 ± 5	52 ± 4	60 ± 6	45 ± 4	56 ± 9

ODNs containing a strong, weak or control (non-CpG) motif at sites 1–5 (Table 1, series 2) were synthesized. The level of immune activation induced by 1 μM of each ODN was measured in PBMC from 6 donors, as described in the legend to Table 2. The mean level of immune stimulation induced by all ODNs with a specific motif at each site is shown. *Significant difference between strong *versus* weak motifs, $P < 0{\cdot}02$.

Table 5. Effect of CpG motif position on stimulatory activity: Analysis of the RPMI 8226 B cell line

Site	Motif	IP-10 levels (ng/ml)
1	Strong	81 ± 22*
1	Weak	18 ± 9
1	Control	4 ± 2
2	Strong	38 ± 8
2	Weak	43 ± 15
3	Strong	74 ± 18*
3	Weak	22 ± 10
4	Strong	31 ± 11
4	Weak	42 ± 13
5	Strong	18 ± 7*
5	Weak	55 ± 18

RPMI 8226 human B cells were stimulated as described in Table 4. IP-10 levels in 24 h culture supernatants were measured by ELISA. Results represent the mean ± std error from 3 independent experiments. *Significant difference between the strong and weak motifs, $P < 0{\cdot}01$.

Consistent with previous studies, we found that the response of PBMC from different donors to CpG stimulation was heterogeneous [16,17,19,26]. No single ODN was maximally stimulatory in all assays or on immune cells from all donors. This heterogeneity was not due to interassay variability, since the response of individual donors to the same ODNs was reproducible over time [17]. To minimize sources of variability, all ODNs were synthesized and studied under identical conditions at a concentration of 1 μM (at which CpG-induced responses significantly exceeded those induced by non-CpG ODN) [14,18]. Since phosphorothioate ODNs can induce low-level sequence nonspecific immune stimulation, most ODNs used in these studies were 27–35 bases in length. Due to the large number of samples analysed, we were unable to examine the activity of all of these ODNs at multiple concentrations. However, a subset of ODNs (ranging in size from 20 to 35 bases) was studied at multiple concentrations, confirming

the basic results of this work: that increasing the total number of CpG motifs/ODN, the diversity of CpG motifs/ODN, and locating strongly stimulatory motifs at the 5′ end, contributed to increased activity.

Due to differences in the baseline response of PBMC from different donors, the relative response induced by each ODN was calculated as a percentage of the strongest response generated by each sample in each experiment. This method of analysis minimized the need to study non-CpG ODN (which typically induced <10% of the immune stimulation elicited by the most active CpG ODN) [14,18]. As ODNs with greater stimulatory activity were synthesized, the comparative activity of other ODNs in the same experiment fell. Thus, hierarchies of activity, but not relative levels of immune activation, should be compared between experiments.

We and others previously observed that mixtures of ODNs expressing several different CpG motifs induced stronger immune responses in a greater fraction of PBMC donors than ODNs expressing a single motif [14,16,17,19]. In general, CpG dinucleotides flanked by a 5′ T and a 3′ TT, TA or AT generally stimulated human immune cells most effectively, although no single motif or single ODN has been optimally active on PBMC from all donors in all assays ([14,16], and data not shown). The current work explored additional parameters that influence ODN activity. Results indicate that ODNs expressing several different motifs are more stimulatory than those expressing only a single motif. While this outcome might have been influenced by differences in the size of the ODNs tested, the same result was observed when the total number of motifs and size of the ODN was held constant. This observation is consistent with evidence that different individuals may respond optimally to different CpG motifs, and that a mixture of CpG motifs will therefore induce the broadest immune response in a diverse pool of donors [16,17].

Current findings strongly suggest that the location of a motif within an ODN influences its immunostimulatory activity. Placing a more stimulatory motif at the 5′ terminus significantly increases cytokine/Ig production and proliferation. The motif at sites 3 and 5 also had a significant impact on ODN activity. Surprisingly, the use of strong *versus* weak CpG motifs at sites 2 and 4 had little influence on ODN activity. These studies evaluated the activity of suboptimal CpG motifs, since the immune stimulation induced when the strongest motif identified in preliminary studies was inserted at the most 5′ site reduced our ability to detect the more modest contribution of motifs at 3′ sites. These findings suggest that CpG recognition proceeds in a 5′ → 3′ direction, and that recognition of the first motif hinders recognition of a CpG that is immediately 3′ (i.e. binding to site 1 hinders recognition of site 2, while binding to site 3 hinders recognition of site 4). The observation that a 'weaker' CpG motif can be more stimulatory at site 5 is consistent with the evidence that ODNs expressing multiple different motifs are more active than those expressing only a single motif. Thus, the overall ability of an ODN with a 'strong' motif at sites 1 or 3 will be improved by incorporating a different motif at site 5.

This work examines the response of whole, unfractionated PBMC to a large set of phosphorothioate CpG ODN. Our goal was to gain insight into the likely *in vivo* behaviour of the type of ODNs being used in clinical trials. Given that multiple cell types interact in complex ways following *in vivo* CpG administration, such insight could not be gained by analysing the response of purified cell populations or cloned cell lines. However, to verify the effect of motif location on ODN activity, experiments were repeated using the human RPMI 8226 B cell line. Those studies confirmed the conclusion that motif location had a significant impact on ODN activity.

Current results have important implications for the rational design of ODNs for clinical use. The most stimulatory CpG motif should be placed at the 5′ end of an ODN, with the next most stimulatory motif located approximately 10 bases downstream. To maximize the response of an outbred population, ODNs containing a total of 3–4 different motifs should be utilized. Ongoing studies will determine whether such 'complex' ODN are more efficient than a mixture of simple ODNs (each expressing multiple copies of a single CpG motif) at activating donor PBMC.

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