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LETTER TO THE EDITOR

Polyvalent immunoglobulins, platelet lysate and lenalidomide: cocktail for polyfunctional NK cells expansion for multiple myeloma

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Despite the rapid development of new agents, myeloma (MM) continues to be an incurable disease. Growing bodies of literature suggest a key role of natural killer (NK) cells, a unique subset of CD3⁻CD56⁺ innate lymphoid cells in the control of newly diagnosed MM.¹ MM cells promote a state of progressive immune dysregulation and suppression of NK cell functions, which support a growing interest for NK cell-based immunotherapy.² A major challenge to the broader application of adoptive NK cell therapy is to improve methods for *in vitro* expansion of low-frequency NK cells. Large-scale expansions have been obtained with various feeder cell-based systems but whereas these methods have merit, they also have major drawbacks including complicated procedures, safety issues for human administration and/or the expansion of exhausted NK cells.³ Developing innovative strategies to generate clinically efficient NK cells with minimal *in vitro* manipulation, in large numbers, would provide an important breakthrough in NK cell-based immunotherapy. In the present studies, we report a strategy designed specifically for MM through expansion with polyvalent immunoglobulins (polyIgs) and platelet lysate (PL) in the presence of lenalidomide.

NK cells were isolated from the non-mobilized apheresis product of 15 healthy donors who had given informed consent. The apheresis product started with $8.1 \pm 4.4\%$ NK cells. After depletion of CD3⁺ T cells and enrichment of CD56⁺ NK cells using the MACS selection system with CD3 and CD56 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), the mean frequency of CD3⁻CD56⁺ NK cells was $94.6 \pm 1.9\%$, yielding an NK cell recovery of 35% with a mean T-cell depletion of 2.99 log.

Freshly isolated CD3⁻CD56⁺ cells were cultured in 24-well plates (Thermo Scientific Nunc A/S, Roskilde, Denmark) at a concentration of 0.5×10^6 cells/mL, in CellGro SCGM Medium good manufacturing practice (Cellgenix, Freiburg, Germany) containing 10 µg/mL ciprofloxacin (Bayer Santé, Puteaux, France) and 5 IU/mL Fragmine (Pfizer, Paris, France). It was previously suggested that IL-2 predisposed NK cells to the activation of induced cell death, whereas IL-15 promoted NK cell development and survival.⁴ In the present study, a median of 7.1 ± 0.7 -fold NK cell expansion was obtained with IL-15 (Miltenyi Biotec, Bergisch Gladbach, Germany; 10 ng/mL) alone. In combination with IL-2 (Proleukin, Novartis Pharma, Rueil-Malmaison, France), NK cell growth increased significantly in a dose-dependent manner from 24.6 ± 2.5 (at 100 IU/mL) to 55.5 ± 6.0 -fold (at 600 IU/mL) ($P = 0.017$) (Figure 1a, upper panel). Subsequently, cultures were performed in the presence of IL-15 (10 ng/mL) and IL-2 (600 IU/mL).

Concomitantly, we tested NK cell expansion through coating. It has been suggested that anti-CD3 can potentiate the proliferation of NK cells through an indirect effect on T cells² or alternatively, we postulate the association of the Fc fragment with FcγRIII CD16, mainly expressed on mature NK cells. To test this hypothesis, we used human polyclonal immunoglobulins (PolyIgs; Tégéline, LFB Biomédicaments, Les Ulis, France) in place

of good manufacturing practice anti-CD3 mAb (Miltenyi).⁵ We also evaluated the replacement of human ABs by human platelet-derived growth factor that is allowed for the good manufacturing practice-compliant implementation of various clinical trials using mesenchymal stromal cells, but without evidence of its influence on immunomodulatory cells.⁶ The cells were stimulated by surface immobilized anti-CD3 mAb (5 µg/mL) or surface immobilized human polyIgs (50 mg/mL) in the presence of 5% heat-inactivated ABs (EFS) or 8% PL (Macopharma, Tourcoing, France) for 7 days; cultures were extended for a second week using similar conditions except coating. Coating with anti-CD3 mAb or PolyIgs increased expansion of NK cells to, respectively, 72.1 ± 38.5 and 80.2 ± 20.5 -fold compared with expansion without coating (7.5 ± 0.5 -fold increase). Overall, in these cultures of NK cells, PL and polyvalent Ig could replace 'Gold standard' ABs and anti-CD3 with similar NK cells expansion (Figure 1a, middle and bottom panels).

In these different culture conditions, NK cells were viable, highly activated (CD69, NKp46 and NKp44) (Figure 1c) and expressed activating (NKp30 and NKG2D) and chemokine receptors (data not shown), previously described to participate in the regulation of NK cell trafficking *in vivo*.⁷ Of note, the percentage of contaminating CD3⁺ T cells remained stable (data not shown). We and others previously reported no significant change of inhibitory KIR receptors in expanded NK cells from healthy individuals.⁸

We next assessed the functional capacities of expanded NK cells in six independent experiments using a standard 4-h ⁵¹Cr-release assay and a polyfunctionality assay with simultaneous detection of degranulation (anti-CD107a mAb, H4A3; BD Biosciences, Le Pont de Claix, France) and cytokine production (intracellular expression IFN-γ (B27; BD Biosciences) and TNF-α (E-Biosciences, Paris, France). In four different conditions: anti-CD3 mAb+5% ABs, anti-CD3 mAb+8% PL, polyIgs+5% ABs or PolyIgs+8% PL, there was a trend to increased-specific lysis after expansion with polyIgs compared with anti-CD3 mAb (Figure 2a). This lytic activity with polyIgs is in agreement with previous results reported in Kawasaki disease, CMV and EBV infection in which IVIG contribute to both expansion and activity of circulating CD16⁺ mature NK cells.⁹ This could open the way to the administration of polyIgs in combination with NK cells to promote their *in vivo* functional activities. More importantly, addition of PL significantly improved cytotoxicity both in the presence of anti-CD3 Ab or polyIgs (Figure 2a, right panel). This effect was reproducibly obtained and statistically significant (Friedman test; $P = 0.0024$). Of note, those functional capacities of cultured NK cells were similar to those obtained by us and others with fresh CD3⁻CD56⁺ NK cells activated by IL-2.¹⁰ A significant elevation of the degranulation capacities was also observed in the presence of PL ($P = 0.03$) (Figure 1d, lower panel). These data are in accordance with the interaction between NK cells and platelets. Although the knowledge about their crosstalk is still fragmentary, storage granules in platelets contain proteins involved in cell proliferation as well as several immune modulators and chemokines (that is, neutrophil-activating peptide 2, PF4, CCL-3, CCL-5 or CCL-7) that control activation of NK cells and regulate their motility and chemotaxis.¹¹

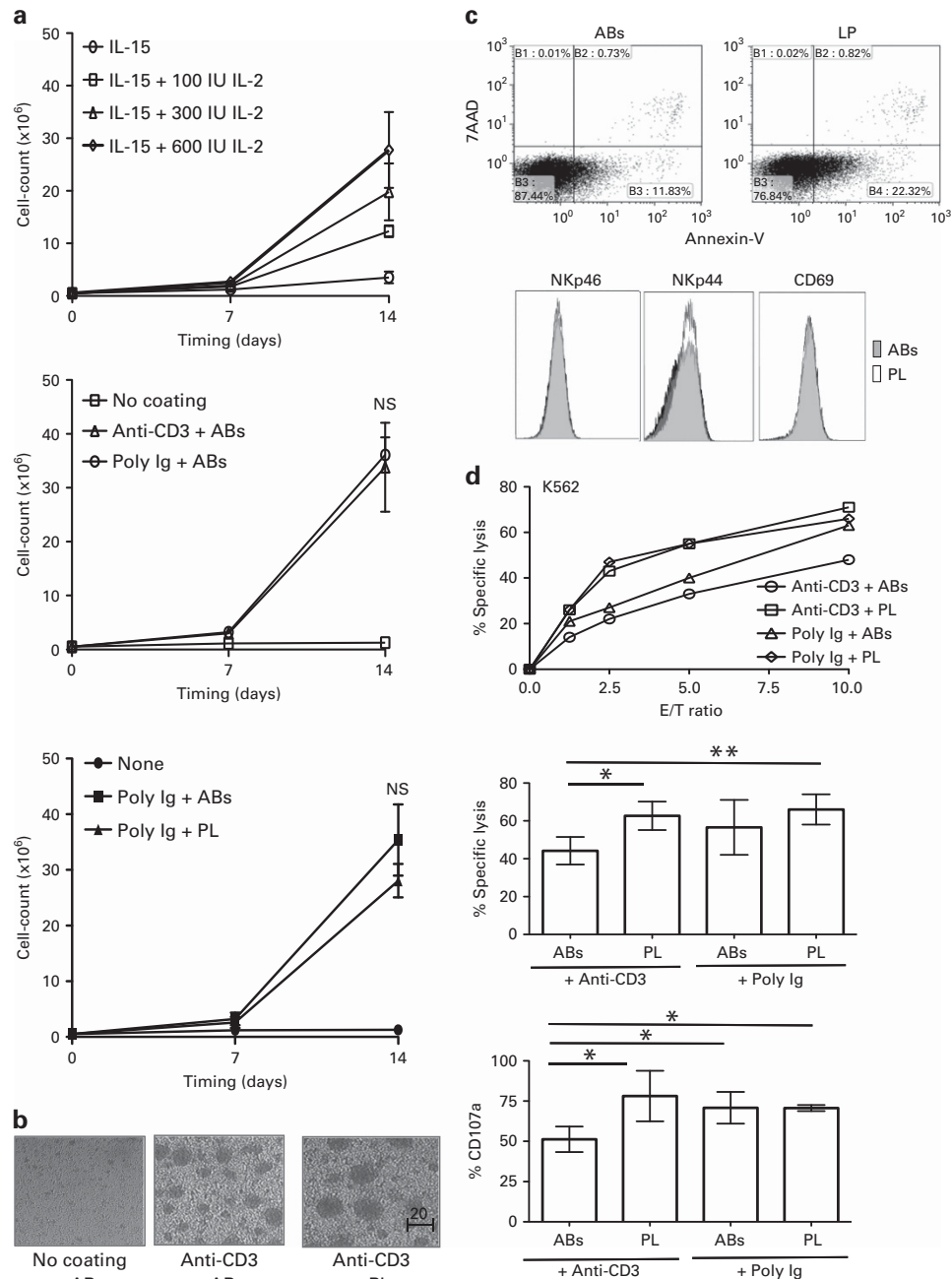


Figure 1. Effect of coating by anti-CD3 or polyvalent Ig and medium additives, AB serum (ABs) or PL (**a**) *Ex vivo* expansion of purified NK cells. Upper panel: effect of cytokines. Purified CD3⁺CD56⁺ cells from healthy donor were expanded 14 days in the presence of 10 ng/mL IL-15 alone (circle) or supplemented with 100 (square), 300 (triangle) or 600 IU/mL (diamond) of IL-2. Note the significant difference between IL-15 and IL-15+ 600 IU IL-2 ($P=0.017$ with Dun post-Friedman test). Middle panel: effect of PolyIg. Purified CD3⁺CD56⁺ cultured in the presence of 10 ng/mL IL-15+600 IU/mL IL-2 and 5% human ABs were tested without coating (square) or after coating with anti-CD3 mAb (triangle) or polyIg (circle). Bottom panel: effect of PL. Purified CD3⁺CD56⁺ cultured in the presence of 10 ng/mL IL-15+600 IU/mL IL-2 and coated with polyIg were tested in the presence of 5% ABs (square) or 8% PL (triangle), and compared with untreated cells (circle). NS: non significant. (**b**) Microscopic observation of the expansion of purified CD3⁺CD56⁺ after 14 days of culture with or without coating by anti-CD3 and addition of 5% ABs or 8% PL. Note that coating supported larger clones. Original magnification $\times 20$. (**c**) Cytometric analysis. A combination of anti-human CD45, CD3, CD16 and CD56 were used to characterize NK cell population. In this population, the Annexin-V Apoptosis Detection Kit with 7-AAD showed at day 14 the same proportions of living cells in presence or 5% ABs or 8% PL and were highly activated (CD69). (**d**) NK cell cytotoxicity and degranulation against K562 target cells. Functional studies of NK cells against K562 target cells were performed at day 14 after expansion in different cell-cultured conditions. Upper panel: specific lysis of NK cells determined by a standard 4-h ⁵¹Cr-release assay at different effector/target (E:T) ratio. Middle panel. Compilation of six independent experiments of specific lysis of NK cells determined by a standard 4-h ⁵¹Cr-release assay at E:T ratio = 10/1; $P=0.0024$ between human ABs and PL. Bottom panel: compilation of six independent experiments for the degranulation of NK cells determined by flow cytometry analysis of CD107a cell-surface expression at E:T ratio = 1/1; $*P < 0.05$. All values in mean \pm s.d.; statistical comparison by a Friedman test for repeated data to compare serum additive with ABs to PL after coating or anti-CD3 to polyvalent Ig.

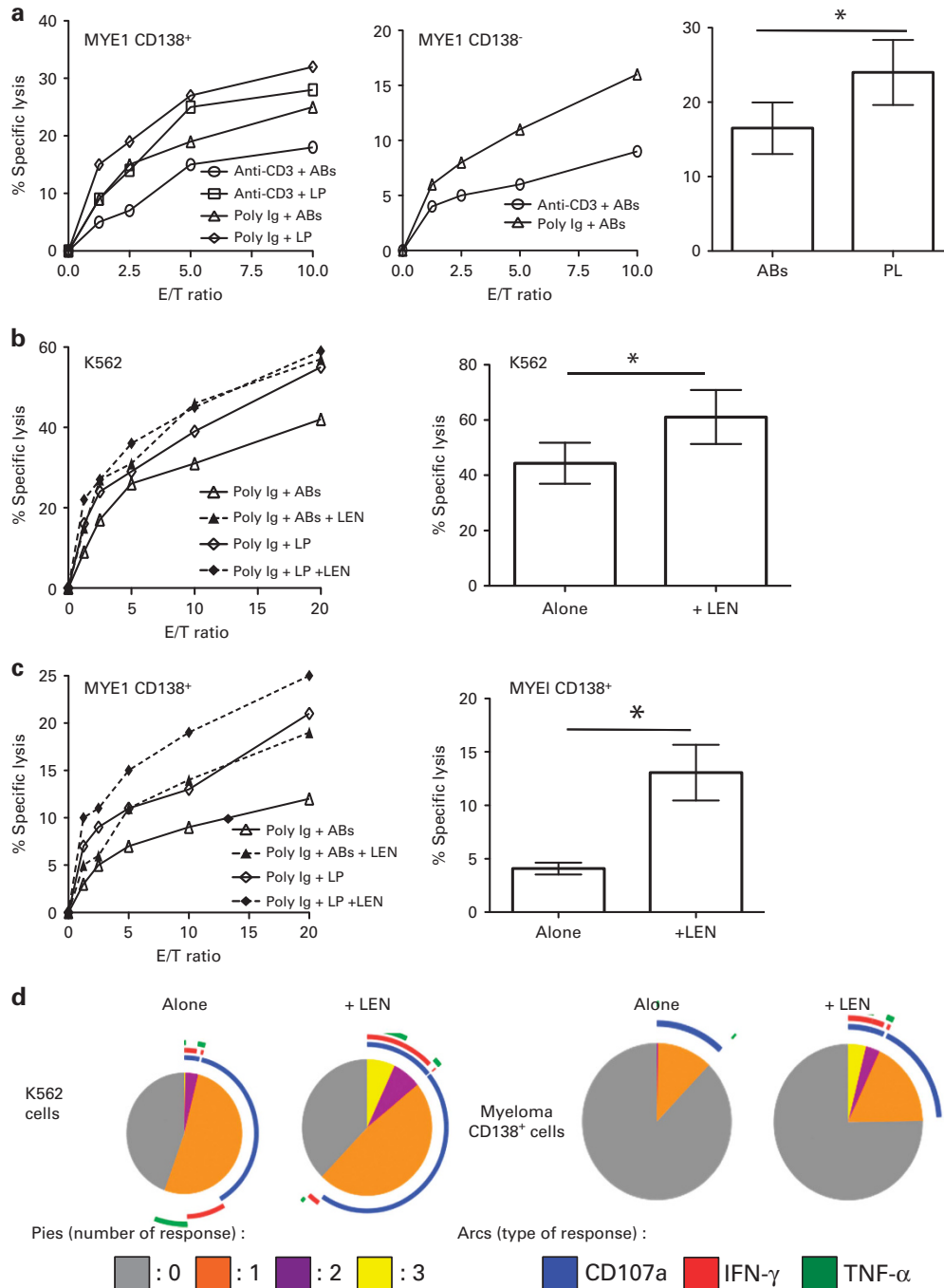


Figure 2. NK cell functional activities against primary purified CD138⁺ myeloma target cells. **(a)** Effect of coating by anti-CD3 or polyvalent Ig and medium additives, ABs or PL. Specific lysis of NK cells against purified myeloma cell from MM patients in different culture conditions was tested with a standard 4-h ⁵¹Cr-release assay. Left panel: specific lysis of NK cells against purified CD38⁺ myeloma cells and the negative fraction CD38⁻ from MYE1 MM patient, at different E:T ratio. Right panel: compilation of four independent experiments of specific lysis of NK cells against purified CD38⁺ myeloma cells from different MM patients, at E:T ratio = 10:1. Statistical analysis was performed using a Wilcoxon matched pair test. Note that PL significantly amplified cytotoxicity against different target cells, including CD138⁺ myeloma cells. All values in mean \pm s.d.; statistical analysis by a Wilcoxon signed rank test; **P* < 0.05. **(b)** Influence of lenalidomide on K562 lysis. Left panel: cytotoxicity against K562 target cells in absence (alone) or presence of lenalidomide (+LEN) for the last 48 h. Test was performed at day 14 by a standard 4-h ⁵¹Cr-release assay at different E:T ratio. Right panel: compilation of four experiments of specific lysis of K562 target cells at E:T ratio = 10:1. **(c)** Influence of Lenalidomide on myeloma lysis. Left panel: cytotoxicity against myeloma CD138⁺ target cells in absence (alone) or presence of lenalidomide (+LEN) for the last 48 h. Test was performed at day 14 by a standard 4-h ⁵¹Cr-release assay at different E:T ratio. Right panel: compilation of four experiments of specific lysis of K562 target cells at E:T ratio = 10:1. Note that lenalidomide significantly upregulated polyfunctional activity of expanded NK cells. **(d)** Polyfunctional activity of NK cell cells (able to performed degranulation+production of IFN- γ and/or TNF- α). The values were analyzed with a Boolean gate algorithm (FlowJo; Tree Star, Ashland, OR, USA). Pie and arc charts were generated using SPICE software (National Institute of Allergy and Infectious Diseases freeware). Pies represent the frequency of NK cells positive for 0, 1, 2 or 3 responses (to CD107a, IFN- γ and TNF- α). Arcs depict for which functions the cells are functional or polyfunctional. All values in mean \pm s.d.; statistical analysis by a Wilcoxon test; **P* < 0.05.

The role of NK cells in MM provided a rationale for testing the functional activity of expanded NK cells against myeloma cells.¹² In the presence of purified primary CD138⁺ myeloma cell subset, from patients at diagnosis or in relapse, we observed a robust increase of the specific cytotoxicity in the presence of PL compared with ABs, whatever the E:T ratio in accordance with data observed with K562 target cells. This effect was reproducibly observed and statistically significant in four experiments (Wilcoxon test; $P=0.035$) (Figure 2a). Of note, ⁵¹Cr-release assay is complex against CD138⁺ primary myeloma cells with low level of cytotoxicity.¹³ In addition, in the presence of the purified CD138⁻ cells from MM patients, we observed that NK cells were also more efficient in the presence of PL (Figure 2a). Together, this suggested that PL amplified cytotoxicity against different target cells, including CD138⁺ myeloma tumor cells.

There is growing evidence that lenalidomide, an immunomodulatory drug active against myeloma, also modulates the cellular immune system, particularly NK cells¹⁴ and this prompted us to test its influence on functional activity of expanded NK cells against myeloma cells. To access the effect of lenalidomide on cytotoxicity, NK cells were maintained in culture with lenalidomide at 5 or 50 μM during the last 48-h period of culture, to avoid toxic effect generated by a more long-term culture. In the presence of polyIgs and PL, lenalidomide increased both cytotoxicity and polyfunctional activity against primary CD138⁺ myeloma cells, and this effect was enhanced in the presence of PL, compared with ABs ($P=0.0218$). (Figure 2c) in dose-response manner (5 vs 50 μM ; data non shown). Interestingly, using Wilcoxon test, lenalidomide significantly increased lysis of K562 target cells ($P=0.008$) and the frequency of polyfunctional NK cells ($P=0.0136$) and decreased the frequency of non-functional NK cells ($P=0.03$) in seven experiments performed with K562 target cells (Figure 2b). Taken together, the expansion of polyfunctional NK cells against target cells, including CD138⁺ myeloma cells from MM patients, could be enhanced by lenalidomide via a putative early apoptotic NK cells rescuing effect. In contrast with previous studies suggesting an effect of NK cells towards T cells,¹⁵ our findings show a direct effect of lenalidomide on expanded NK cells.

In conclusion, we developed a novel and safe strategy requiring only minimal *in vitro* manipulation, which includes polyIgs, PL and lenalidomide to generate large number of functional NK cells. This approach is now being upgraded to complete good manufacturing practice conditions for a clinical trial of adoptive transfer of alloreactive polyfunctional NK cells specifically designed to fight myeloma tumor cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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