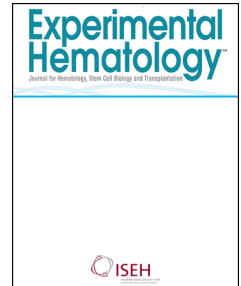# Accepted Manuscript

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NOVEL STRATEGIES OF ADOPTIVE IMMUNOTHERAPY: HOW NATURAL KILLER CELLS MAY CHANGE THE TREATMENT OF ELDERLY PATIENTS WITH ACUTE MYELOBLASTIC LEUKEMIA

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ABSTRACT

Although many attempts have been made for the identification of novel molecular-targeted therapies for acute myeloid leukemia (AML) patients , their translation into clinics have proven of limited impact. In particular, the question of effective and curative treatment of elderly patients, who are not eligible for stem cell transplantation, still represents an unmet medical need. To answer this question, a wide range of immunological therapeutic strategies, mostly T-cell based, have been proposed and investigated. However, at present the clinical results have been largely unsatisfactory. Natural killer (NK) cells have been recently used as a means of adoptive immunotherapy with promising clinical results. Based on recent clinical reports and moving from basic immunobiology of NK cells, here we discuss some open issues about the clinical translation of NK-based adoptive immunotherapy for the management of elderly AML patients.

In the last years, major advances have been made in the understanding of the complex molecular and cellular network regulating the growth and the development of acute myeloid leukemia (AML). In particular, the pivotal role of immunological bone marrow microenvironment is emerging and a wide variety of novel immunological therapies, both pharmacological and cellular-based, are currently under active investigation in the attempt of harnessing the immune system against leukemia (1).

Natural killer (NK) cells represent a unique subset of circulating lymphocytes, which possess potent effector function (2,3). However, differently from T cells, NK cells recognize their targets, including tumor cells, in a MHC- and, more importantly, antigen-independent manner (4-6) (Fig.1). Although for decades tumor antigen-recognition by T cells has been the hallmark for a full characterization of effective anti-tumor cytotoxic T-lymphocytes (CTLs), the identification of tumor-associated antigens, which may critically serve as targets for T-cell immunotherapy, has been largely unsatisfactory. This finding has limited the curative impact of such therapies when translated in the clinical setting. In addition, several pathways and mechanisms have been described which potently tolerize T cells within the tumor microenvironment (1). With this in mind, the use of NK cells as effector cells against leukemia may offer some interesting advantages over T cells in the development of immunological cellular-based therapies. It is known that NK cells play a pivotal role in eradicating residual leukemia cells after haploidentical stem cell transplantation (7-10). These results have prompted several groups to address the capacity of adoptive immunotherapy with NK cells in controlling leukemia. In particular, Passweg and colleagues demonstrated the feasibility of selecting and infusing highly purified, T-cell depleted, NK cells to consolidate engraftment in high risk patients with myeloid malignancies treated by haploidentical transplantation (11). These data were confirmed by Koehl *et al*, who reported about infusion of haploidentical, highly purified, T-cell depleted, NK cells after haploidentical SCT in three pediatric

ALL and AML relapsed/refractory patients. All patients obtained complete response after NK cell infusion, without any GVHD signs or symptoms (12). Lee and colleagues performed a phase I study with the goal of augmenting GvL effects without exacerbating GVHD in a cohort of patients with high risk myeloid malignancies infused with haploidentical NK cells before stem cell infusion (13). More recently, moving from the pioneering study by Miller *et al.* (14), we and others consistently and reproducibly described the safety and feasibility of infusing allogeneic NK cells into AML patients outside the transplantation setting (15,16). We demonstrated that NK cell infusion has the potential to prevent disease relapse in a subset of elderly patients with AML (17). These results have clearly paved the way for designing a new generation of clinical studies aiming to prove the clinical efficacy of NK cell-based immunotherapy in the clinical management of AML patients (18).

Who, when and where?

Although many attempts have been made for the identification of novel molecular-targeted therapies (19), their translation into clinics have proven of limited impact and, consequently, the management of AML still relies on aggressive chemotherapy, followed by allogeneic stem cell transplantation (SCT) (20). If such approach has the potential to cure young patients, the question of effective treatment of elderly patients is still open and unsolved (20). Indeed, although the post-induction remission rate in fit-to-chemo elderly AML patients ranges from 60 to 85%, the disease relapse is still very high, thus reducing overall survival (OS) to 10%. Poor clinical outcome in this patient population is due to an increase in unfavorable biological features and in the presence of co-morbidities, which limit, if not exclude, the possibility of undergoing SCT as a consolidation strategy, whenever CR is achieved. The persistence of minimal residual disease (MRD) is the major problem and its eradication represents an unmet medical need in these patients. In this scenario, the use of an immunological approach to target MRD may significantly impact on the eradication of

4

disease. The proof-of-principle of the capacity of immune cells to eradicate MRD derives from the results of allogeneic SCT, which clearly represents an option for relapse prevention (20). NK cells play a role in the immune control of tumors. They express activating and inhibitory receptors which recognize MHC class I alleles, termed "Killer cell Immunoglobulin-like Receptors" (KIRs) (2). In addition to KIR, other receptors recognizing HLA class I exist, as the inhibitory CD94/NKG2A and activating CD94/NKG2C recognizing HLA-E molecules. The NK cell receptor repertoire is primarily determined by KIR genotype, which is extremely variable in terms of number and identity of KIR gene content, it is clonally distributed and selected in a way that each NK cell expresses at least one inhibitory receptor for self HLA otherwise is hypo-responsive (a phenomenon termed "education"). NK cells are also equipped with activating receptors, including NCR (NKp46, NKp30 and NKp44), NKG2D and DNAM-1, whose ligands are mainly stress-inducible molecules. This great array of activating and inhibitory receptors finely regulates NK cell function. While inhibitory mechanisms predominate when NK cells encounter normal autologous cells, tumor cells can be susceptible to lysis through a mechanism of "missing self recognition", because they down-regulate HLA-class I molecules, and/or "induced self recognition", because they up-regulate ligands for activating receptors. Data from haploidentical T-cell depleted SCT show that alloreactive KIR-L mismatched NK cells are major anti-leukemia effectors, resulting in the protection of AML patients from relapse (7-10). However, the SCT approach has important limitations and it is applicable to a minority of elderly AML patients. For these reasons, it is conceivable to exploit the anti-leukemic potential of NK cells outside transplantation as adoptive immunotherapy. In this view, Miller et al. (14) first reported the safety and feasibility of adoptive immunotherapy with haploidentical NK cells in cancer patients, including AML. Furthermore, Rubnitz et al (16) reported their experience with haploidentical NK cell infusion in a cohort of 10 childhood AML patients with a 2-year event-free survival of 100%. Our group has published the feasibility and safety of adoptive immunotherapy with highly purified NK cells from KIR-L mismatched, haploidentical donors in

5

adult high-risk AML patients, mostly with relapsed or resistant disease (15). Importantly, donor-versus-recipient alloreactive NK cells were demonstrated in vivo by the detection of donor-derived NK clones and adoptively transferred NK cells were alloreactive against recipient's leukemic cells. More recently, we have extended our previous investigation by reporting the biological and clinical results of NK cell infusion from haploidentical KIR-L mismatched donors in elderly AML patients, who had achieved CR after induction/consolidation chemotherapy. Notably, a significantly higher number of donor alloreactive NK cell clones was observed in responders over non-responders. The infusion of higher number of alloreactive NK cells was associated with prolonged disease-free survival. Therefore, we demonstrated, for the first time, that the size of alloreactive donor NK cell repertoire is correlated with reduced relapse rate after NK cell immunotherapy.

Several papers have reported the capacity of non-engrafting alloreactive cells to kill leukemia without inducing graft-versus-host disease (GVHD) (7,8). Therefore, by coupling limited toxicity, with a remarkable anti-tumor activity, alloreactive cells may change the landscape of AML treatment and may represent a viable alternative to allogeneic SCT for elderly patients.

Indeed, Kottaridis *et al*. performed a phase I clinical study demonstrating the feasibility and safety of haploidentical NK cell infusion in a cohort of high risk AML patients not eligible for SCT, while Shaffer conducted a phase II trial among myeloid malignancies patients relapsed after SCT and treated with haploidentical NK cells (21, 22).

We then consider that future trials of adoptive immunotherapy should test the efficacy of alloreactive NK cells in the subset of AML patients not eligible for SCT (especially if over 60 years of age), who have achieved CR, as a means for eradicating MRD and preventing disease recurrence. Indeed, many clinical trials have demonstrated the power of alloreactivity to treat MRD whereas overt disease is often resistant to cellular immunotherapy (23). Moreover, at this stage, well

designed multicenter, randomized clinical studies (see below) should be planned to provide definitive proof of the efficacy of NK-based cellular therapy.

Donor or recipient? Both, please.

Several biological factors, both of recipient and donor origin, may be implicated in the therapeutic effect of NK cells after infusion into AML patients. When recipient-derived factors were addressed, Miller et al (24) demonstrated that NK cell expansion, *in vivo*, after lymphodepleting chemotherapy was positively correlated with the endogenous production of some cytokines, such as IL-15 and IL-35. Both these cytokines are known to critically regulate NK cell effector function and the increase of IL-15 may represent a biomarker for NK cell expansion (15). Moreover, the number of circulating T regulatory cells (T_{regs}) critically influenced the capacity of infused NK cells to expand and to kill AML cells. Thus, a better DFS was observed in patients infused with NK cells and depleted of T_{regs}. It is well-known that T_{regs} play a role in reducing NK cell number and effector function (25). In the transplantation setting, mice undergoing allogeneic SCT and previously depleted of T_{regs}, but not $CD8^+$ T cells, showed an increase of BM graft rejection due to enhanced NK cell activity. Such effect was successfully abrogated when $CD4^+CD25^+$ T_{regs} were transferred to the microenvironment. At the same time, cyclophosphamide-based lymphodepletion, which is commonly used before NK cell infusion, is known to subvert endogenous T-cell repertoire, thus skewing the composition of T-cell subsets by increasing T_{regs} /$T_{effectors}$ ratio (26).

In our previous clinical studies (15,17) , we focused our attention on the donor. In particular, we extensively characterized the alloreactive NK cell repertoire, which was correlated with the clinical response to NK cell immunotherapy (17). Of note, all donors were selected on the basis of KIR-L mismatch with the recipient in the GVH direction. Outside the haploidentical transplantation setting, where KIR-L mismatch between donor and recipient consistently correlates with better

clinical response due to enhanced NK-cell mediated disease control (7,8), few reports clearly demonstrate a significant impact of KIR-L incompatibility (27-33). Miller et al, by performing a retrospective analysis of responders versus non-responders showed that a better response was associated with KIR-L mismatch between donor and recipient (14). These results suggested that donor alloreactivity may impact on the efficacy of adoptively transferred NK cells. Indeed, our results, obtained from a study population of previously selected KIR-L-mismatched donor-recipient pairs, indicate that the frequency of alloreactive NK cell donors, and not only KIR-L mismatch, significantly predicted NK cell response. Thus, we provide the proof-of-concept that also in the setting of adoptive immunotherapy, donor NK alloreactivity may have an important role. Taken together, these data reinforce the concept that both donor selection as well as recipient-derived factors should be taken into consideration to optimize NK cell immunotherapy.

The more the better? The point of a functional NK cell dose

Several investigations have addressed the issue of the "therapeutic dose" of selected cell populations infused in cancer patients. Specifically, early papers have clearly defined the minimum number of hematopoietic CD34$^+$ stem/progenitor cells to be reinfused in patients undergoing autologous stem cell transplantation to predict a fast and durable engraftment (34). Conversely, in the setting of allogeneic stem cell transplantation, much efforts have been devoted to select the optimal dose (and schedule) of CD3$^+$ T cells to achieve a Graft-Versus-Leukemia (GVL) effect, without Graft-versus Host Disease (GVHD), when donor lymphocytes infusions (DLI) are planned for relapsing leukemia (35, 36). However, although the infusion of DLI involves important parameters specific for allogeneic stem cell transplantation (e.g. GVL/GVHD balance, male/female mismatch, effect on donor cell engraftment, timing of cellular therapy after transplantation), adoptive immunotherapy with T cells has taught additional lessons which can be applied to NK cell

therapy. In particular, donor selection, disease stage (i.e. DLI treatment is highly efficient with low tumor burden), the choice of the optimal cell dose and the role of multiple infusions. In this view, our previous experience has shown that the frequency of alloreactive NK cells is highly variable among individuals and, therefore, highly variable doses of alloreactive NK cells were infused (17). However, our data demonstrated that a threshold of >8/100 alloreactive NK clones in the donors effectively discriminated patients at lower versus higher risk of relapse after NK cell infusion. Indeed, the infusion of >8/100 alloreactive NK clones was associated with better RFS versus patients with < 8/100 alloreactive NK clones (mean 43 versus 21.6 months, respectively, p=0.006). Moreover, the absolute number of NK cell clones correlated with clinical outcome. In particular, we found that better outcome was observed when at least 2×10^5 donor alloreactive NK cell clones/kg were infused. Therefore, for future studies we propose the preliminary assessment of donor NK cell repertoires in the attempt to collect and infuse doses of functional donor-versus-recipient alloreactive NK cells that are in the range of those our previous studies suggested to be effective at eradicating residual leukemia (concept of "functional cell dose") (Fig. 2). To determine the "functional cell dose", KIR-L mismatched donor alloreactive NK cell repertoire will be evaluated through the generation of large numbers of donor alloreactive NK clones and cytotoxicity assays against recipient target cells, as well as flow cytometry analysis (see below) . Such analysis will be performed at baseline, since NK repertoire is known to be stable along time. Thus, the number of donor versus recipient alloreactive NK cells may become a predictive biomarker for clinical response to NK immunotherapy. Moreover, few patients in our series were infused a second time upon relapse and molecular analysis demonstrated the achievement of second molecular remission. Therefore, one may hypothesize the storage of multiple vials of highly purified alloreactive NK cells to be used "pre-emptive" during the course of the disease.

9

Much ado about methods!

The methods we used to investigate the alloreactive NK cell repertoire pose some concerns. Indeed, they are based on NK cell limiting dilution cloning and ^{51}Cr-release assay, which may be considered complex, time-consuming and expensive. For these reasons, one major challenge is to develop alternative methods, more widely applicable, while maintaining the same diagnostic potential. When monoclonal antibodies are not able to discriminate between activating and inhibitory KIRs, some studies showed the alloreactive NK cell repertoire could be analyzed only in individuals who are homozygous for the group A *KIR* haplotype (37, 38). Subsequently, new anti-KIR antibodies were developed that could distinguish inhibitory versus activating KIRs when used in appropriate combinations, thus allowing the identification of alloreactive NK subpopulations in all individuals. One persistent limitation is that no monoclonal antibody can currently distinguish KIR2DS2 from KIR2DL2 cells when both are expressed in the KIR genotype (10). Because of these limitations, in previous studies, we decided to use cloning assays. However, one of the endpoints of our future studies will be the comparison of functional assays with the phenotypic identification of alloreactive NK cell repertoire/CD107 assay, as also proposed by other authors (37, 38, 10). Specifically, to define the size of alloreactive NK cell subset, multi-colour cytofluorimetric analysis and appropriate anti-KIR and anti-NKG2A mAb combinations will be used to detect NK cells expressing, as inhibitory receptor, only the KIR specific for the KIR-L mismatch. Thus, the phenotypically defined size of alloreactive cell subset (i.e. NK cells expressing, as inhibitory receptor, only the KIR specific for the KIR-L mismatch) will be compared with the frequency of NK cell clones with ability to kill patient derived cells (10).

Moreover, the molecular interactions between NK and AML cells should be fully evaluated by phenotypic and functional assays to further optimize cell therapy. In particular, the relevance of NK triggering receptors as NCRs (i.e. NKp46, NKp30 and NKp44), NKG2C and activating KIR, which

10

can be variably expressed among different individuals should be addressed for optimal donor selection when alternative donors are available, both in alloreactive and non-alloreactive cases. In addition, the evaluation of ligands for triggering receptors (e.g. PVR, Nectin-2 and ULBPs) as well as possible down-regulation of HLA-class I molecules should be performed on AML cells.

Future directions

Alloreactive NK cells have been emerging as a potent effector cell population against AML. The demonstration of the significant clinical activity of alloreactive purified NK cells without stimulating GVHD outside the transplantation setting represents a proof-of-principle for such an anti-leukemia effect, which had been previously demonstrated in the context of haploidentical SCT. Altogether, published data highlight the pivotal role of NK cells for the development of novel immunological approaches in the clinical management of AML. Nevertheless, several biological and immunological issues still require full elucidation. Additional correlative biological and clinical studies are necessary to fully understand the mechanism(s) NK cells exert to kill leukemia cells in the non-transplantation setting. In particular, the careful evaluation of the impact that recipient- and donor-derived factors may have in influencing in vivo NK cell activity is an important point as well as the utility of multiple infusions. The design of future NK-cell based clinical trials, both in the SCT and adoptive immunotherapy settings, should include a correlation between clinical results and biological outputs. Indeed, correlative biological studies may make possible to identify patients who may really benefit from NK cell immunotherapy, in the attempt to tailor cell-based therapies to the patients' characteristics.

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FIGURE LEGENDS

Figure 1. Leukemia recognition and cytotoxicity mechanisms of CTLs and NK cells. CTLs (A) and NK cells (B) share most of the cytotoxic effector mechanisms, including perforin /granzyme production cytokine release and expression of death signals, such as FAS and TRAIL. However, differently from T cells (A), which may also be tolerized within leukemia microenvironment through leukemia-derived soluble factors, such as tolerogenic cytokines (IL-10 and TGF-β), or inhibitory pathways (PD-1-PD-L1 axis, TIM3), NK cells (B) recognize target cells, including leukemic blast, in a MHC- and antigen- independent manner. Other mechanisms (NKG2A/D, MICA/B) regulate the activation (and inhibition) of NK cells. Among them, KIR-KIR-L interaction represents a critical activatory/inhibitory pathway, which is fundamental for NK alloreactivity.

Figure 2. Flow-chart for patient management, NK cell donor screening/selection and definition of NK alloreactive functional cell dose. The frequency of alloreactive NK cells is highly variable among individuals and, therefore, highly variable doses of alloreactive NK cells are infused. Since the threshold of $>2 \times 10^5$/Kg alloreactive NK clones in the donors effectively discriminated patients at lower versus higher risk of relapse after NK cell infusion. For future studies, along with patient induction/consolidation program, we propose the preliminary assessment of donor NK cell repertoires in the attempt to collect and infuse doses of functional donor versus recipient alloreactive NK cells that are in the range of those our previous studies suggested to be effective at eradicating residual leukemia (concept of "functional cell dose"). Donors are screened with physical examination and laboratory tests, including viral sierology, in order to determine their fitness to the apheretic procedure. Patients are treated with standard induction and consolidation chemotherapy, including cytarabine and anthracycline.
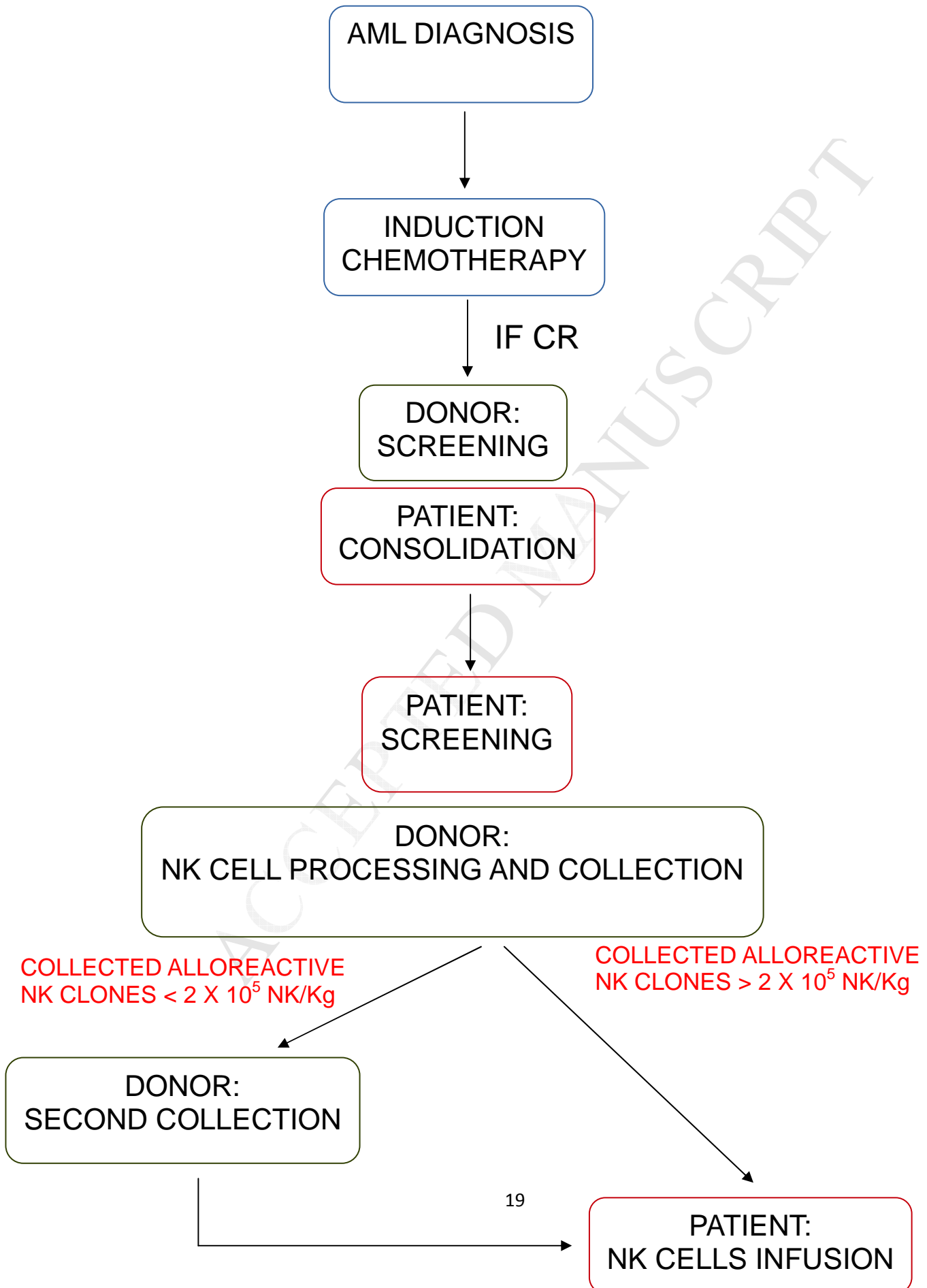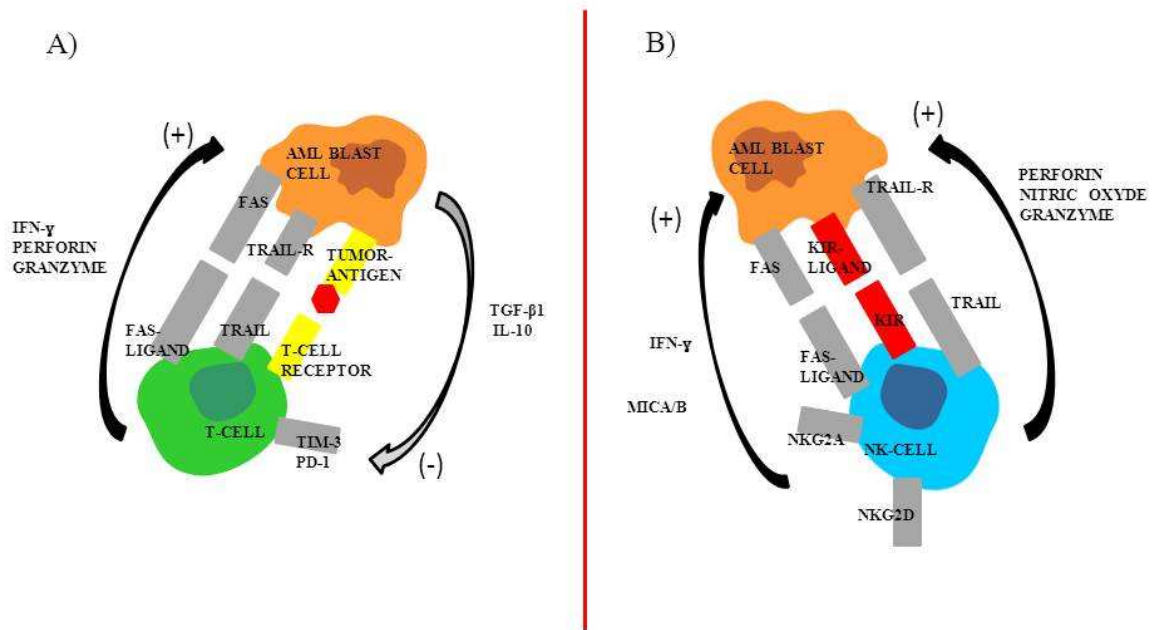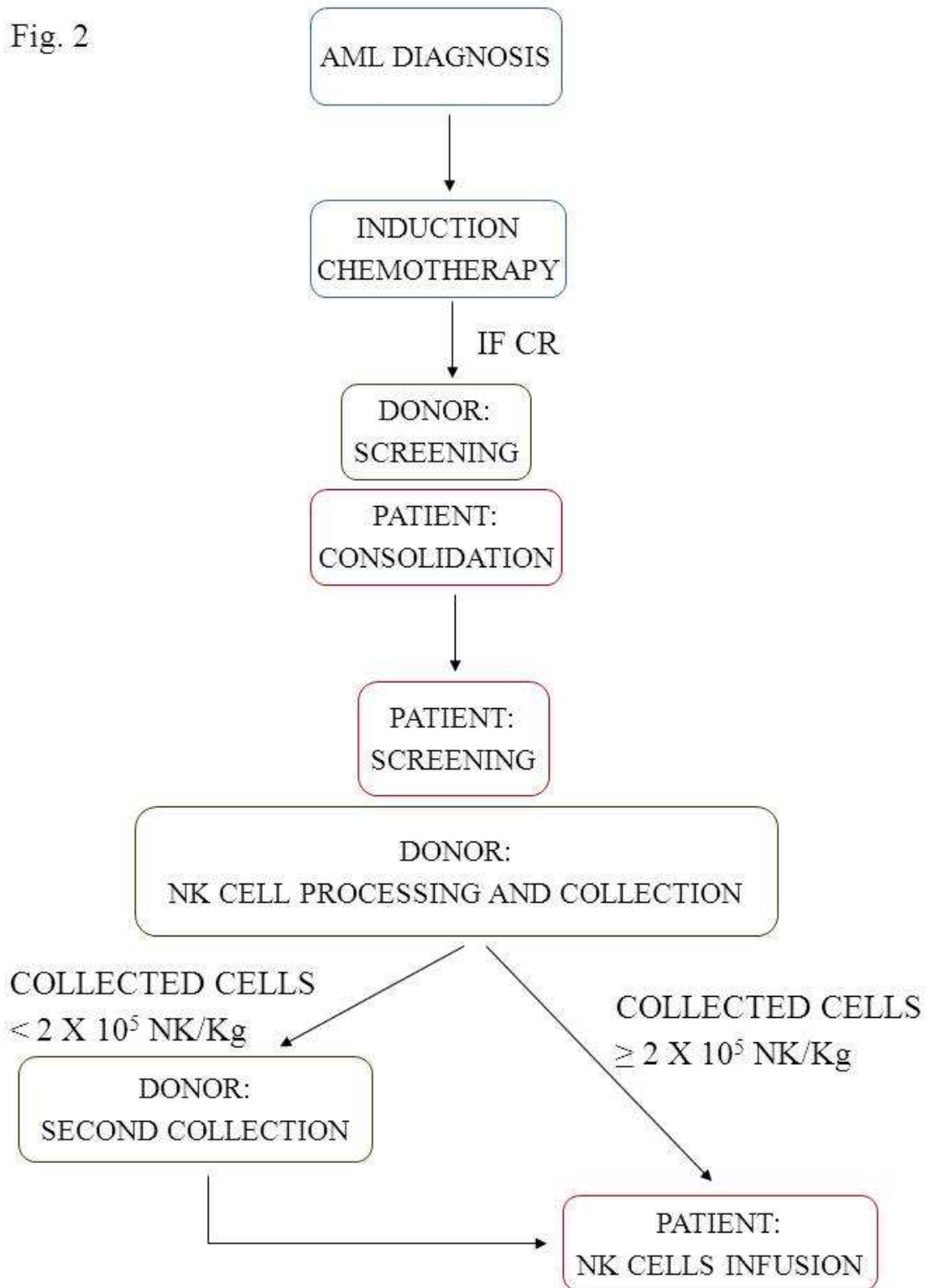
18

Fig. 2

Fig. 1

A)

B)

Fig. 2

1. Alloreactive NK cells have been emerging as potent effectors cell against AML.
2. Several biological and immunological issues still require full elucidation.
3. The impact of donor NK alloreactivity is an important point
4. Future NK trials should correlate clinical results with donor alloreactive NK cell repertoire