Manufacture of Natural Killer Cells as Treatment for Multiple Myeloma

Cliona Rooney

PACT Project 00018 Van Rhee

PI: Frits VanRhee, MD, PhD
University of Arkansas for Medical Sciences
Little Rock, AR

Multiple Myeloma (MM)

- Plasma cell malignancy
- Myeloma cells accumulate in bone and bone marrow
- Diagnosed in 1-4 per 100,000
- Prognosis is 5-7 years with advanced treatment
Natural Killer (NK) cells

- Cytotoxic lymphocytes of innate immune system
  CD56⁺CD3⁻ cells

- Immediate responders to viruses and intracellular pathogens

- Alerted by
  - Ligands of stressed/malignant/infected cells
  - Including lack of MHC class
  - Cytokines produced by activated DCs and MΦs

- Recognize and kill a range of tumor types including MM

NK Cells

- Homeostatic cells
  - Constitutively express TGF-β

- When alerted secrete
  1. MIP1α, MIP1β and Rantes
  2. IFN-γ, TNF-α, GM-CSF
  3. IL-5, IL-10 and IL-13

- Direct effector function

- Activated and guide adaptive immunity
Inhibitory and Activation Signaling Determine NK Function

Protocols for Manufacture NKs

- NK clinical trials require high doses
- For fresh or overnight activated NK cells
  - Use apheresis cells,
  - Deplete of CD3+/CD19+ cells
  - Purity >50%
- Expansion using cytokines only
  - Requires prolonged cultures
- Expansion with feeder cells/ cytokines
  - Rapid expansion
  - High potency cells
Campana Method for NK Cell Expansion

- **K562vr**
  - HLA I and II negative
  - Activated NK cells
  - IL-15 growth factor
  - 4-1BB ligand costimulatory
  - Soluble IL-2

- Master/working cell bank generated
  - PACT (part I)
  - MCB906K562

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**Campana Method for NK Cell Expansion**

Irrad K562vr + PBMC
K562vr: NK cell 10:1

10 U/mL IL-2
SCGM medium

Day 0
2 x 10^6 NK
2 x 10^7 K562vr

~8 days

Day 10

harvest
18 x 10^9 cells
60 – 90%
CD3- CD56+
NK cells

BAGs
Questions for Manufacturing Transfer

- Can we replace bags with G-Rex?

- Shipment of cryopreserved or fresh NK cells?

- Can we generate more potent NKs?

Growth in Bags

- Low ratio of medium to surface area for optimal gas exchange

- Requires large numbers of bags

- Frequent feeding and culture manipulations

- Large harvest volume
Wilson Wolf Manufacturing
Gas Permeable Rapid Expansion Device (G-Rex)

Gas permeable membrane allows optimal exchange of CO2 and O2
Supports cell growth with large volumes of media
No rocking or stirring

Gas Permeable Rapid Expansion Device
(Wilson Wolf - G-Rex)

- Dilution of waste
  - Does not become acidic
- Minimizes manipulation
- Low harvest volume
Optimization of NK Expansion in G-Rex

>200-fold expansion in GRex100 up to 2.0 x10⁹ cells/G-Rex100

Expression of NK Activation Markers

Days 7-10

Count

IgG  NKp44  NKp30  NKG2D

CD56  CD3

Day 0  Day 4  Day 6  Day 10

CD56  CD3
Cytotoxicity of Expanded NK cells

NK Expansion in Bags and G-Rexes

Day 0  Day 2  Day 4  Day 6  Day 8  Day 10
2x10^6 NK Feeding and splitting
Bags

Day 0  Day 8-10
2x10^6 NK
G-Rex

18x10^9 cells in 40 197-mL bags

18x10^9 cells in 20 G-Rex100s

no processing for up to 10 days
NK Expansion in Bags and G-Rexes

In-Process Assay Validation
Click-It Assay

5-7 days post-irradiation

Irradiated K562vr feeder cells do not proliferate
Based on uptake of EdU by 0.05% of cells
In-Process Procedure Validation

Allogeneic Products: Depletion of CD3⁺ T cells using CliniMACS (depletion 2.1 program)

Before CliniMACS

CD3⁻ fraction

Release criterion: <5x10⁵ (<0.2-1%) CD3⁺CD56⁻ T cells per kg for 2-10x10⁷ TNC per kg

Clinical Protocol Logistics

Texas

Arkansas

CAGT: Expansion of NK cells, QC, CoA and shipment of fresh TC-NK product to UAMS
Treatment Schema for Allo-NK
In Myeloma Patients

<table>
<thead>
<tr>
<th>Days</th>
<th>Treatment</th>
<th>Dose</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>-9</td>
<td>Bortezomib</td>
<td>1.0 mg/m²</td>
<td>Reduce HLA, increase TRAIL on MM</td>
</tr>
<tr>
<td>-7</td>
<td>Mesna</td>
<td>30 mg/kg i.v.</td>
<td>Prevention of hemorrhagic cystitis</td>
</tr>
<tr>
<td>-6</td>
<td>Cyclophos</td>
<td>60 mg/kg i.v.</td>
<td>Tumor debulking, immunosuppression</td>
</tr>
<tr>
<td>-4</td>
<td>Dex</td>
<td>40 mg PO</td>
<td>Tumor debulking, immunosuppression</td>
</tr>
<tr>
<td>-2</td>
<td>Fludarabine</td>
<td>25 mg/m² i.v.</td>
<td>Immunosuppression</td>
</tr>
<tr>
<td>0</td>
<td>Exp-NK cells</td>
<td>2-10x10⁷/kg</td>
<td>Lysis of MM</td>
</tr>
<tr>
<td>2</td>
<td>Interleukin-2</td>
<td>3x10⁶ U s.c.</td>
<td>Support NK activity, persistence</td>
</tr>
</tbody>
</table>

NK Cell Production Outline

1. Expansion K562-MCB ~12 days
2. Receive apheresis product
3. Irradiation K562-MCB
4. Ficoll apheresis product
5. Co-culture apheresis product K562-MCB For ~8 days
6. CliniMACS depletion of CD3⁺ T cells
7. Formulation and Shipment
## NK Cell Expansion is Variable

<table>
<thead>
<tr>
<th>Subject</th>
<th>Donor Type</th>
<th>Start NK #</th>
<th>End NK #</th>
<th>NK Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Validation 1</td>
<td>Healthy Donor</td>
<td>9x10^7</td>
<td>1x10^10</td>
<td>114</td>
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<tr>
<td>Validation 2</td>
<td>Healthy Donor</td>
<td>9x10^7</td>
<td>9x10^9</td>
<td>100</td>
</tr>
<tr>
<td>Subject 1</td>
<td>Myeloma Pt</td>
<td>9x10^7</td>
<td>6x10^9</td>
<td>67</td>
</tr>
<tr>
<td>Subject 2A</td>
<td>Healthy Donor</td>
<td>1.25x10^6</td>
<td>3.9x10^9</td>
<td>31</td>
</tr>
<tr>
<td>Subject 2B</td>
<td>Healthy Donor</td>
<td>1.5x10^8</td>
<td>4.5x10^9</td>
<td>30</td>
</tr>
<tr>
<td>Subject 3</td>
<td>Healthy Donor</td>
<td>1.5x10^8</td>
<td>2.4x10^10</td>
<td>160</td>
</tr>
<tr>
<td>Subject 4</td>
<td>Myeloma Pt</td>
<td>1.5x10^8</td>
<td>2.6x10^9</td>
<td>26</td>
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<tr>
<td>Subject 5</td>
<td>Myeloma Pt</td>
<td>1.5x10^8</td>
<td>3.8x10^9</td>
<td>25</td>
</tr>
<tr>
<td>Subject 6</td>
<td>Healthy Donor</td>
<td>1.5x10^8</td>
<td>1.33x10^10</td>
<td>89</td>
</tr>
<tr>
<td>Subject 7</td>
<td>Myeloma Pt</td>
<td>1.5x10^8</td>
<td>1.1x10^10</td>
<td>73</td>
</tr>
</tbody>
</table>

## NK Purity, Potency and Viability

<table>
<thead>
<tr>
<th>Non CD3 Depleted products</th>
<th>Subject</th>
<th>NK %</th>
<th>T %</th>
<th>Viability %</th>
<th>Potency</th>
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</thead>
<tbody>
<tr>
<td>Val1</td>
<td>52</td>
<td>34</td>
<td>72</td>
<td>63.4</td>
<td></td>
</tr>
<tr>
<td>Val2</td>
<td>69</td>
<td>19</td>
<td>86</td>
<td>61.9</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CD3 Depleted products</th>
<th>Subject</th>
<th>NK %</th>
<th>T %</th>
<th>Viability %</th>
<th>Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val 1</td>
<td>93</td>
<td>0.1</td>
<td>91</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Val 2</td>
<td>93</td>
<td>0.04</td>
<td>97</td>
<td>79</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>autologous</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>1</td>
<td>88</td>
<td>4</td>
<td>83</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>6</td>
<td>92</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>allogeneic donors</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>86</td>
<td>0.21</td>
<td>96</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2B</td>
<td>89</td>
<td>1.02</td>
<td>89</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>85</td>
<td>0.09</td>
<td>91</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

Potency = % lysis of K562 at 20:1 E:T ratio
No Increase in Frequency of NK Cells After Infusion

![Graph showing NK cell frequency over time](image)

Recovery After NK Cryopreservation Was Suboptimal

<table>
<thead>
<tr>
<th>Subject</th>
<th>Cryopreserved NK cell dose for infusion</th>
<th>Recovery (%)</th>
<th>Actual infused NK cell Dose</th>
<th>Viability By flow</th>
<th>Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val 1</td>
<td>4.7x10⁷/kg</td>
<td>61</td>
<td>2.9x10⁷/kg</td>
<td>78%</td>
<td>77%</td>
</tr>
<tr>
<td>Val 2</td>
<td>4.9x10⁷/kg</td>
<td>100</td>
<td>4.9x10⁷/kg</td>
<td>99%</td>
<td>90%</td>
</tr>
<tr>
<td>1</td>
<td>4.9x10⁷/kg</td>
<td>65</td>
<td>3.2x10⁷/kg</td>
<td>94%</td>
<td>94%</td>
</tr>
<tr>
<td>2A</td>
<td>2.1x10⁷/kg</td>
<td>100</td>
<td>3.4x10⁷/kg</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>2B</td>
<td>1.3x10⁷/kg</td>
<td>100</td>
<td></td>
<td>83%</td>
<td>79%</td>
</tr>
<tr>
<td>3</td>
<td>5.0x10⁷/kg</td>
<td>100</td>
<td>5.0x10⁷/kg</td>
<td>77%</td>
<td>70%</td>
</tr>
<tr>
<td>4</td>
<td>3.4x10⁷/kg</td>
<td>65</td>
<td>2.2x10⁷/kg</td>
<td>94%</td>
<td>ND</td>
</tr>
</tbody>
</table>
Frozen NK Cells Are Suboptimal

Can we ship fresh NK products?
CERTIFICATE OF ANALYSIS
Center for Cell & Gene Therapy, GMP Cell Processing Facility
Baylor College of Medicine, Houston, Texas 77030
TC-NK CELLS

Overnight release criteria for shipped fresh NK cells

<table>
<thead>
<tr>
<th>Test</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability</td>
<td>&gt;70%</td>
</tr>
<tr>
<td>%CD56+CD3-</td>
<td>&gt;50% for auto-</td>
</tr>
<tr>
<td># CD3+CD56-</td>
<td>&lt;5x10^5 cell/kg for allo-</td>
</tr>
<tr>
<td>%GFP+ K562</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>Gram stain</td>
<td>negative</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>&lt;5.0 EU/mL</td>
</tr>
<tr>
<td>Potency</td>
<td>&gt;20% K562 lysis at 20:1 E:T</td>
</tr>
<tr>
<td>HLA-A,B</td>
<td>Matching donor</td>
</tr>
</tbody>
</table>

Frozen or Fresh NK Products

**Frozen**

- One product for multiple infusions

- Post-thaw
  - Immediately
    - Good viability
    - Poor cytotoxicity
  - 24 hours post thaw
  - Poor viability
  - Good cytotoxicity
  - Expand poorly *in vivo*

**Fresh**

- One product for one infusion

**How do Fresh NK cells ship?**

- Viability?
- Cytotoxicity?
- Subsequent expansion?
Fresh NK Potency is Retained After Shipping

![Graph showing lysis percentage over time for different donors](image)

Fresh NK Continue to Expand During Shipping

<table>
<thead>
<tr>
<th>Time post formulation (h)</th>
<th>0</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Manual Count (M/ml)</td>
<td>10</td>
<td>12.1/121%</td>
<td>13.7/137%</td>
</tr>
<tr>
<td>Donor 1</td>
<td>10</td>
<td>15.0/150%</td>
<td>9.7/97%</td>
</tr>
<tr>
<td>Donor 2</td>
<td>10</td>
<td>18.8/188%</td>
<td>17.1/171%</td>
</tr>
<tr>
<td>Donor 3</td>
<td>10</td>
<td>17.1/171%</td>
<td>13.7/137%</td>
</tr>
</tbody>
</table>

Fresh NK Purity and T cell Content

<table>
<thead>
<tr>
<th>Time post formulation (h)</th>
<th>0</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample CD3-CD56+ (%)</td>
<td>71.1</td>
<td>68.9</td>
<td>70.7</td>
</tr>
<tr>
<td>Donor 1</td>
<td>71.1</td>
<td>68.9</td>
<td>70.7</td>
</tr>
<tr>
<td>Donor 2</td>
<td>62.8</td>
<td>65</td>
<td>67.1</td>
</tr>
<tr>
<td>Donor 3</td>
<td>86.2</td>
<td>85.5</td>
<td>87.3</td>
</tr>
</tbody>
</table>

| Sample CD3+CD56- (%) | 19.7 | 21.8 | 15.9 |
| Donor 1 | 19.7 | 21.8 | 15.9 |
| Donor 2 | 24.7 | 21.8 | 15.9 |
| Donor 3 | 8 | 7.8 | 6.1 |
Fresh Auto-NKs Expand *in vivo*

**Frozen NK**

- NK/µL vs. Day of Protocol

**Fresh NK**

- NK/µL vs. Day of Protocol

---

Fresh Allo-NKs Expand *in vivo*

**Frozen NK**

- NK/µL vs. Day of Protocol

**Fresh NK**

- NK/µL vs. Day of Protocol

---
• Good In Vivo Expansion
• No Tumor Responses

Can we grow more potent NK cells?

Increasing IL-2 In Vitro Improved NK Potency
Similar Rate of Expansion
10 U/mL Vs 500 U/mL IL-2

Expansion in vivo Auto-NKs

Fresh NK low IL-2
Fresh NK high IL-2
Conclusions

- NK cells efficiently expand in G-Rexes within 10 days w/o manipulations
  - Fold expansion is donor dependent (25 to 160-fold)

- Shipment fresh NK cells:
  - Retain viability and potency after 48h in 5% HSA at RT and frozen ice-packs
  - Allow for higher infused NK dose
  - Expand further after infusion in vivo

- Potency is improved with higher IL-2 during manufacturing

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