RESEARCH ARTICLE

Effect of age on homeostasis of lymphocytes in an interleukin-7-deficient mouse model

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ABSTRACT. Interleukin (IL)-7 is thought to be a non-redundant cytokine for lymphopoiesis as there is a reduction of T and B cells in peripheral blood (PB) and a loss of TCRγδ$^+$ cells in PB and bone marrow (BM) in IL-7$^{-/-}$ mice. To investigate whether the absence of IL-7 influences the organ-dependent distribution of the lymphocytes, we analyzed single cell suspensions of several organs (BM, lung, liver, small intestine, and spleen) at different ages (three and 12 months) of IL-7$^{+/+}$ and IL-7$^{-/-}$ mice using flow cytometry; immunohistochemical staining was performed on frozen sections of various organs. We observed lymphocytopenia in almost all organs of IL-7$^{-/-}$ mice, but normal counts in the liver and the lung of three-month-old IL-7$^{-/-}$ mice. CD4$^+$ and CD8$^+$ cell numbers were decreased in the spleen and the BM. With aging, we found a greater increase in CD4$^+$ and CD8$^+$ cells in the BM of IL-7$^{-/-}$ than in IL-7$^{+/+}$ mice, particularly of memory cells. The spleen of IL-7$^{-/-}$ mice was characterized by lymphocytopenia. We challenge the view that IL-7 is a non-redundant cytokine for lymphocyte development. Some of the changes observed, e.g. partial absence of TCRγδ$^+$ T cells in the PB, BM and small intestine and complete loss in liver, lung and spleen, may be due to the altered organ distribution instead of a defect in γδ$^+$ T cell lymphopoiesis. In this model, aging leads to a significantly altered composition of lymphocyte subsets, and the lack of IL-7 seems to accelerate this process.

Key words: IL-7, homeostasis, memory T-cells, immunodeficiency

IL-7 was first identified and isolated in 1988 [1, 2]. There is an 81% homology between human and murine IL-7-coding sequences [3]. IL-7 is produced by MHC-II-positive thymic epithelial cells [4], BM stromal cells [5], intestinal epithelium cells [6], fetal [7] and liver epithelial cells [8], keratinocytes [9], dendritic cells [10], fibroblasts, smooth muscle cells and follicular dendritic cells [11], but not by lymphocytes [12]. It binds to the IL-7 receptor (IL-7R), which consists of two subunits: the common γ-chain (IL-7Rγ, CD132), which also serves as a receptor subunit for IL-2, IL-4, IL-9, IL-15 and IL-21 [13-16], and the IL-7 receptor α-chain (IL-7Rα, CD127), which is also part of the receptor that binds TSLP (thymic stromal-derived lymphopoietin) [17-19]. IL-7 production underlies an IL-7R-mediated feedback-loop. The IL-7R is expressed on most mature T cells [19], dendritic cells, monocytes [20], subsets of developing B cells and T cells, but not on effector T cells or mature B-cells [3]. A defect in the IL-7Rγ-chain in humans results in a X-linked, severe combined immunodeficiency (B+T-NK-, X-SCID) [21-23], whereas a mutation of the IL-7Rα results in a decrease in T-lymphocytes only (B+T-NK+). Clinical trials showed down-regulation of CD127 (IL-7Rα) expression on mature T cells after administration of IL-7 [24]. This may serve to limit uncontrolled T cell proliferation in the presence of non-physiologically high levels of IL-7 [19]. For murine T and B cell lymphopoiesis, it has been postulated that IL-7 is non-redundant [23]. IL-7-deficient mice have significant lymphopenia in the PB and lymphatic organs, especially the spleen. The number of peripheral B cells is significantly reduced compared to wild-type mice, and B cell production in the BM is abolished [25]. A cessation of B-cell lymphopoiesis in IL-7$^{-/-}$ mice is described at the pro-B to pre-B cell stage [23]. In spite of this termination of B cells development, mature B cells have been detected in IL-7$^{-/-}$ mice. These cells are assumed to be B cells from the marginal zone in the spleen that can survive due to expansion in the periphery [26]. U. von Freeden-Jeffry *et al*. described a reduction in T-lymphocytes in the PB and the thymus of IL-7$^{-/-}$ mice (four-week- to four-month-old mice) [23]. Additionally, in the intestinal epithelium there is a remarkable reduction of TCRγδ$^+$ T cells [27], while the maturation of NK and NK-like T cells is not affected [23]. To investigate whether the absence of IL-7 plus aging, influence the organ-dependent distribution of lymphocytes, we analyzed single cell suspensions of several organs (BM, lung, liver, small intestine, and spleen) from IL-7$^{+/+}$ and IL-7$^{-/-}$ mice of different ages (three and 12 months) using flow cytometry; immunohistochemical staining was performed on frozen sections of various organs.

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METHODS

C57BL/6 wild-type (IL-$7^{+/+}$) and C57BL/6 IL-$7^{-/-}$ mice were bred under conventional housing, non-specific, pathogen-free conditions at the local animal facility. IL-7 genotypes were confirmed by PCR and Southern blot analysis. All animals showed a normal intestinal bacterial flora as documented by microbiological analysis of stool samples. At the time of analysis, IL-$7^{+/+}$ and IL-$7^{-/-}$ mice were three or 12 months old, respectively. Mice were euthanized and organs from five mice from each group were harvested directly. Small intestine, liver, lung, spleen and femur were removed immediately and kept in ice-cold PBS. Organs for immunohistochemical staining were frozen in liquid nitrogen and stored at -80°C.

The production of single cell suspensions was carried out as described in [26, 28]. The numbers of leukocytes in the single cell suspensions were determined by automatic counting. Lymphocyte subpopulations were analyzed by flow cytometry using fluorescence labelled monoclonal antibodies (Becton Dickinson/Pharmingen, Heidelberg, Germany). The following monoclonal antibodies were used: phycoerythrin (PE)-conjugated antibodies: NK1.1 (PK136), TCRγδ (GL3) and CD4 (RM4-5). Fluorescein isothiocyanate (FITC)-conjugated antibodies: CD19 (1D3), NK1.1 (PK136), TCRβ H57(-597), CD45RB (16A). Peridinin chlorophyll-a (PerCP)-conjugated antibodies: CD4 (RM4-5), CD8 (53-6.7) and CD45/RB220 (RA3-6B2). Additionally, we produced cryostat slices that were fixed using established techniques [29, 31]. For the staining, the following monoclonal antibodies were used: TCRβ (H57-597), TCRγδ (GL3), CD4 (H129.19), CD8 (53-6.7), CD19 (1D3) and NK1.1 (PK136) (Santa Cruz, CA, USA).

We determined the absolute number of leukocytes and the percentage of lymphocytes. The absolute numbers of the lymphocyte subpopulations were calculated from these values, and the percentage of each subpopulation determined by flow cytometry (gated on lymphocytes). Statistical analysis was performed using Student's t-test. P-values determined by Student's t-test relate to the mean values of the absolute numbers. Results with $p < 0.05$ are considered to be significant.

RESULTS

Regarding size and weight of the mice, there was no difference between IL-$7^{+/+}$ wild-type and IL-$7^{-/-}$ mice at the age of three months ($^{*}p = 0.174$). However, the older IL-$7^{-/-}$ mice were smaller in size and their body weight was reduced compared to wild-type mice ($^{**}p = 0.093$) *(figure 1)*. While the body weight of IL-$7^{+/+}$ mice increased significantly from three to 12 months of age ($\hat{}p = 0.033$), there was no significant increase for IL-$7^{-/-}$ mice. Macroscopic inspection of the organs showed smaller spleen and liver sizes for the IL-$7^{-/-}$ mice. Peyer's patches were missing in the small intestine in both three- and 12-month-old IL-$7^{-/-}$ mice. Fertility was reduced as observed by smaller litters. The lifespan of the IL-$7^{-/-}$ mice was reduced to one and a half years in contrast to three years for IL-$7^{+/+}$ mice. Compared to wild-type mice, the percentages of total lymphocytes of each organ were reduced in general, and showed significant changes at the age of three months in the spleen (IL-$7^{+/+}$ $64.6 \pm 5.5\%$, IL-$7^{-/-}$ $41.3 \pm 3.3\%$, $p = 0.00004$), and at the age of 12 months in the BM (IL-$7^{+/+}$ $28.9 \pm 2.8\%$, IL-$7^{-/-}$ $22.2 \pm 5.9\%$, $p = 0.0483$), lung (IL-$7^{+/+}$ $49.8 \pm 2.7\%$, IL-$7^{-/-}$ $15.6 \pm 16\%$, $p = 0.0015$) and spleen (IL-$7^{+/+}$ $63.5 \pm 10.4\%$, IL-$7^{-/-}$ $33 \pm 5.2\%$, $p = 0.0004$). In general, the total number of lymphocytes for each organ increased with aging, but differences did not reach significance, either in the wild-type or in the IL-$7^{-/-}$ mice, with the exception of the spleen of the older,

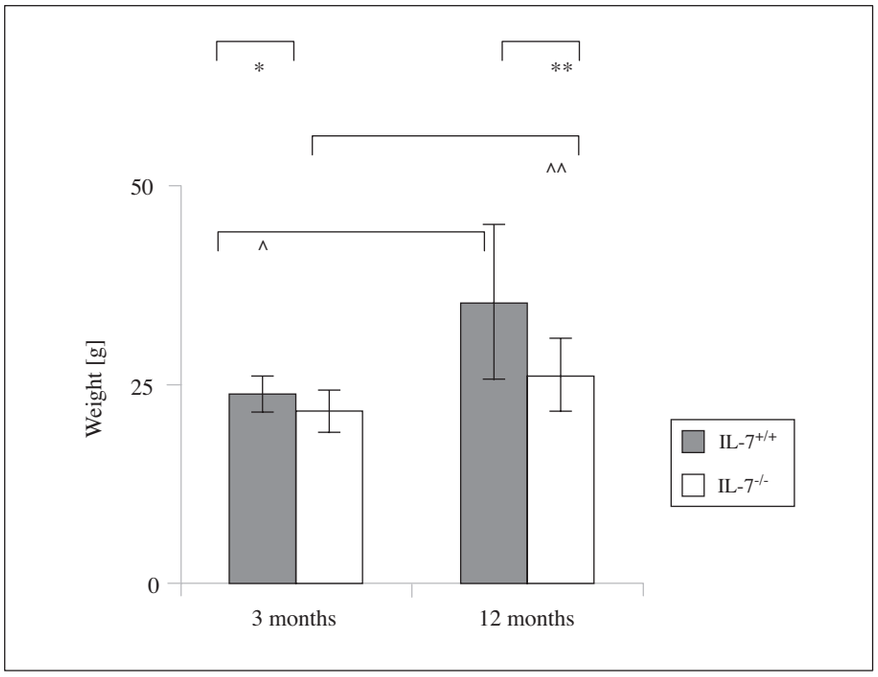

Figure 1
Body weight of IL-$7^{+/+}$ and IL-$7^{-/-}$ mice at three and 12 months of age. While there was no difference between the body weights of three-month-old mice ($^{*}p = 0.174$), the weight of 12-month-old IL-$7^{-/-}$ mice was significantly decreased ($^{**}p = 0.093$). The body weight of IL-$7^{+/+}$ mice increased significantly from three to 12 months of age ($\hat{}p = 0.033$); there was no significant increase for IL-$7^{-/-}$ mice ($\hat{}\hat{}p = 0.08$).

wild-type mice, where the number of total lymphocytes was reduced. Immunohistochemical studies showed no difference in the distribution of lymphocytes in the organs, the typical structure of the organs being maintained *(figures 3F, G and 4F, G)*.

Organ-dependent distribution of lymphocyte subsets

Cell counts of total lymphocytes in the BM of IL-$7^{-/-}$ mice were decreased at three months, but not at 12 months of age. In the BM of three-month-old IL-$7^{-/-}$ mice, the absolute cell count of total lymphocytes was decreased compared to wild-type mice, but there was no difference at the age of 12 months. Absolute numbers of mature $CD4^+$ cells ($TCR\alpha\beta^+/CD4^+$), as well as absolute numbers of naïve ($CD4^+/CD45RB^{high}$) and memory $CD4^+$ cells ($CD4^+/CD45RB^{low}$), were significantly decreased in three-month, but not 12-month-old IL-$7^{-/-}$ mice *(figure 2A)*. The significant decrease in percentages and absolute cell counts of mature $CD8^+$ cells in IL-$7^{-/-}$ mice ($TCR\alpha\beta^+/CD8^+$) seem to be caused by the decrease in naïve $CD8^+$ cells ($CD8^+/CD45RB^{high}$), while memory $CD8^+$ cells ($CD8^+/CD45RB^{low}$) showed no difference. $TCR\gamma\delta^+$ T cell counts were only reduced in three-month-old mice, whereas no difference was seen in 12-month-old mice, compared to IL-$7^{+/+}$ mice *(figure 2A-C)*. The absolute number of DN T cells was significantly reduced in the absence of IL-7 in three-month-old mice only *(figure 2A)*. NK and NK-like T cell counts were significantly decreased in the younger mice but did not differ between IL-$7^{-/-}$ mice and wild-type mice at the age of 12 months. As regards mature B-cells ($CD19^+$), there were significantly reduced percentages (three months IL-$7^{+/+}$ $43 \pm 4.2\%$, IL-$7^{-/-}$ $0.8 \pm 0.4\%$, $p = 0.00000002$; 12 months IL-$7^{+/+}$ $27.6 \pm 5\%$, IL-$7^{-/-}$ $2.1 \pm 1.7\%$, $p = 0.000005$) and absolute numbers in the absence of IL-7 at both ages *(figure 2A, D, E)*.

Lymphocyte subpopulations in the liver

Surprisingly, cell counts of DN-T cells and memory $CD8^+$ T cells in the liver of three-month-old IL-$7^{-/-}$ mice were significantly increased. We also detected a significant increase in NK-like T cell numbers in three- but not 12-month-old IL-$7^{-/-}$ mice. There were no $TCR\gamma\delta^+$ T cells in the liver at either age *(figure 3A-C)*. The absolute cell count of mature B cells was significantly decreased at both ages in the absence of IL-7 *(figure 3D, E)*.

Lymphocyte subpopulations in the lung

Compared to 12-month-old wild-type mice, percentages of memory $CD8^+$ cells increased in the lung of IL-$7^{-/-}$ mice ($p = 0.0335$). The fraction of naïve $CD8^+$ cells was reduced in 3-month-old IL-$7^{-/-}$ mice ($p = 0.0143$), and the numbers of mature $CD4^+$ cells were decreased at 12 months of age, compared to IL-$7^{+/+}$ mice ($p = 0.0095$). We did not detect $TCR\gamma\delta^+$ T cells in the lung of IL-$7^{-/-}$ mice at either age. DN T cell counts increased in 12-month-old mice, but neither reached significance. As regards mature B cells, we observed significantly reduced percentages in the absence of IL-7 at both ages (three months IL-$7^{+/+}$ $12.2 \pm 7.5\%$, IL-$7^{-/-}$ $1.8 \pm 1.1\%$, $p = 0.0149$; 12 months IL-$7^{+/+}$ $6 \pm 1.8\%$, IL-$7^{-/-}$ $2.5 \pm 1.8\%$, $p = 0.0146$).

Lymphocyte subpopulations in the small intestine

The number of DN T cells in the small intestine of IL-$7^{-/-}$ mice surprisingly increased in three-month-old mice compared to wild-type mice, and was significantly increased in 12-month-old mice. Percentages and absolute cell counts of $TCR\gamma\delta^+$ T cells in the small intestine of IL-$7^{-/-}$ mice were significantly decreased at the age of three (IL-$7^{+/+}$ $10.9 \pm 9.6\%$, IL-$7^{-/-}$ $0.1 \pm 0.2\%$, $p = 0.0363$; IL-$7^{+/+}$ $138 \pm 122 \times 10^3$ cells/μL, IL-$7^{-/-}$ $3 \pm 4 \times 10^3$ cells/μL, $p = 0.0371$) and 12 months (IL-$7^{+/+}$ $18.6 \pm 4.8\%$, IL-$7^{-/-}$ $0.3 \pm 0.2\%$, $p = 0.00003$; IL-$7^{+/+}$ $623 \pm 480 \times 10^3$ cells/μL, IL-$7^{-/-}$ $16 \pm 19 \times 10^3$ cells/μL, $p = 0.0222$). The absolute numbers of mature B cells were significantly reduced in the absence of IL-7 at 12 months of age (IL-$7^{+/+}$ $810 \pm 528 \times 10^3$ cells/μL, IL-$7^{-/-}$ $31 \pm 29 \times 10^3$ cells/μL, $p = 0.0109$).

Lymphocyte subpopulations in the spleen are diminished in three- and 12-month-old IL-$7^{-/-}$ mice

The percentages and the absolute cell counts of total lymphocytes in the spleen of IL-$7^{-/-}$ mice were significantly reduced in three- ($p = 0.00004$) and 12-month-old mice ($p = 0.0004$) *(figure 4A)*. Single cell suspensions demonstrated significantly reduced, absolute numbers of $TCR\alpha\beta^+$ T cells in the spleen of both three- and 12-month-old IL-$7^{-/-}$ mice. The numbers of mature $CD4^+$ cells, naïve and memory $CD4^+$ cells were significantly reduced in the spleen of the three- and 12-month-old mice. We did not detect any $TCR\gamma\delta^+$ T cells in the spleen at either age *(figure 4D, E)*. The absolute cell count of DN T cells was significantly decreased in the absence of IL-7 in three-month-old mice. Absolute cell counts of NK and NK-like T cells were decreased in the younger mice, but did not differ between IL-$7^{-/-}$ mice and wild-type at the age of 12 months. Absolute cell counts of mature B cells were significantly decreased in the absence of IL-7 at three and 12 months *(figure 4A-C)*.

General effect of aging on lymphocyte subpopulations in IL-$7^{+/+}$ and IL-$7^{-/-}$ mice

With aging, the total number of lymphocytes in each organ increased in wild-type and IL-$7^{-/-}$ mice, but in the spleen of 12-month-old IL-$7^{+/+}$ mice, the absolute cell count of total lymphocytes was reduced compared to three-month-old IL-$7^{+/+}$ mice.

In the BM, we detected a notable expansion of naïve $CD4^+$ lymphocytes in the older IL-$7^{-/-}$ mice compared to the younger mice *(figure 5A)*, and we also noticed an expansion of memory $CD4^+$ *(figure 5B)*, naïve and memory $CD8^+$ cells in the older mice. In the BM of three-month-old IL-$7^{-/-}$ mice however, we found a significant decrease in the number of almost all lymphocyte subpopulations examined compared to the wild-type mice, even of $TCR\gamma\delta^+$ T cells, the latter increasing with age, such that there was no difference between IL-$7^{-/-}$ and IL-$7^{+/+}$ in older mice *(figure 2A)*.

In the liver of IL-$7^{-/-}$ mice $CD4^+$ *(figure 5C, D)* and $CD8^+$ lymphocytes expanded. There were greater numbers of DN T cells in IL-$7^{-/-}$ mice than in the wild-type at the age of three months, but interestingly, this proportion was reversed at 12 months of age. We saw an increase in all subpopulations in both wild-type and IL-$7^{-/-}$ mice with aging,

A

	BM [10^3 cells/µl]			
Age	3 months		12 months	
IL-7	+/+	-/-	+/+	-/-
TCRαβ$^+$	800±516	259±145	922±460	1063±525
	n.s.		n.s.	
TCRγδ$^+$	239±155	61±40	283±69	266±229
	p = 0.0382‡		n.s.‡‡	
Mature CD4$^+$	321±92	104±71	400±84	1154±948
	p = 0.0031		n.s.	
Naïve CD4$^+$	130±46	29±28	129±62	909±887
	p = 0.0029		n.s.	
Memory CD4$^+$	190±88	75±48	275±101	294±217
	p = 0.0331		n.s.	
Mature CD8$^+$	198±191	53±43	203±54	265±175
	p = 0.0119		n.s.	
Naïve CD8$^+$	143±31	21±13	142±26	145±120
	p = 0.00004		n.s.	
DN T cells	344±192	127±76	426±355	381±123
	p = 0.046		n.s.	
NK cells	314±74	171±109	415±216	339±167
	p = 0.042		n.s.	
NK-T cells	126±45	51±45	164±74	166±95
	p = 0,0278		n.s.	
Mature B cells	1992±1791	20±17	1505±449	122±133
	p = 0.0005		p = 0.0002	
Total lympo-cytes	4602±1701	2287±1498	5460±1346	5320±2992
	n.s.		n.s.	

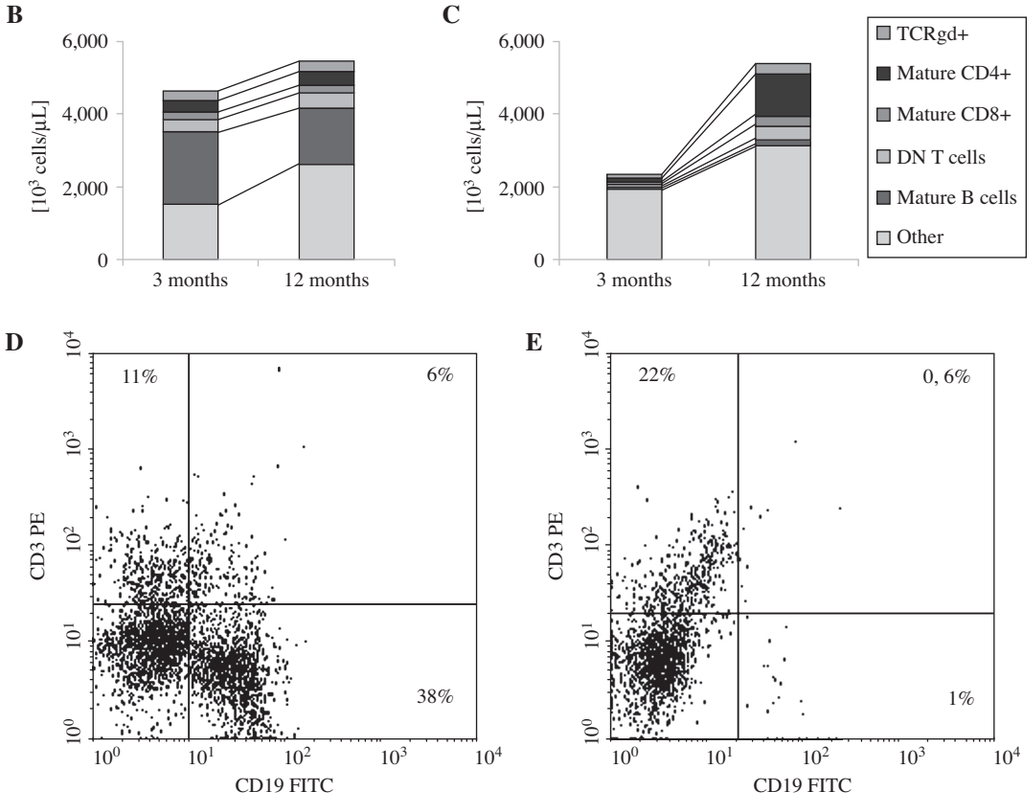

Figure 2
Lymphocyte subpopulations of the BM of IL-7$^{+/+}$ and IL-7$^{-/-}$ mice. **A-C)** Absolute cell counts (MV +/- STDV) calculated by multiplying the absolute lymphocyte number by the percentage of each subpopulation as determined by flow cytometry, gated on lymphocytes. **D, E)** Representative flow cytometry staining for CD3/CD19 of the BM of three-month-old IL-7$^{+/+}$ **(D)** and IL-7$^{-/-}$ **(E)** mice.

A

	Liver [10^3 cells/μL]			
Age	3 months		12 months	
IL-7	+/+	-/-	+/+	-/-
TCRαβ$^+$	3670±3730	4285±3370	12924±6923	7136±4301
	n.s.		n.s.	
TCRγδ$^+$	7±6	0±0	44±32	0±0
	p = 0.0416$^¥$		p = 0.0148$^{¥¥}$	
Mature CD4$^+$	129±80	56±41	378±234	203±76
	n.s.		n.s.	
Mature CD8$^+$	190±88	87±62	300±152	817±871
	n.s.		n.s.	
Memory CD8$^+$	10±7	278±14	23±23	599±837
	p = 0.0424		n.s.	
DN T cells	1635±1796	4013±3248	11944±6412	6249±4037
	n.s.		n.s.	
NK-T cells	58±21	88±42	212±179	218±1380
	p = 0.0081		n.s.	
Mature B cells	167±126	14±16	766±465	58±35
	p = 0.0273		p = 0.0094	
Total lympo-cytes	5340±6906	5478±3702	16370±8098	7513±4286
	n.s.		n.s.	

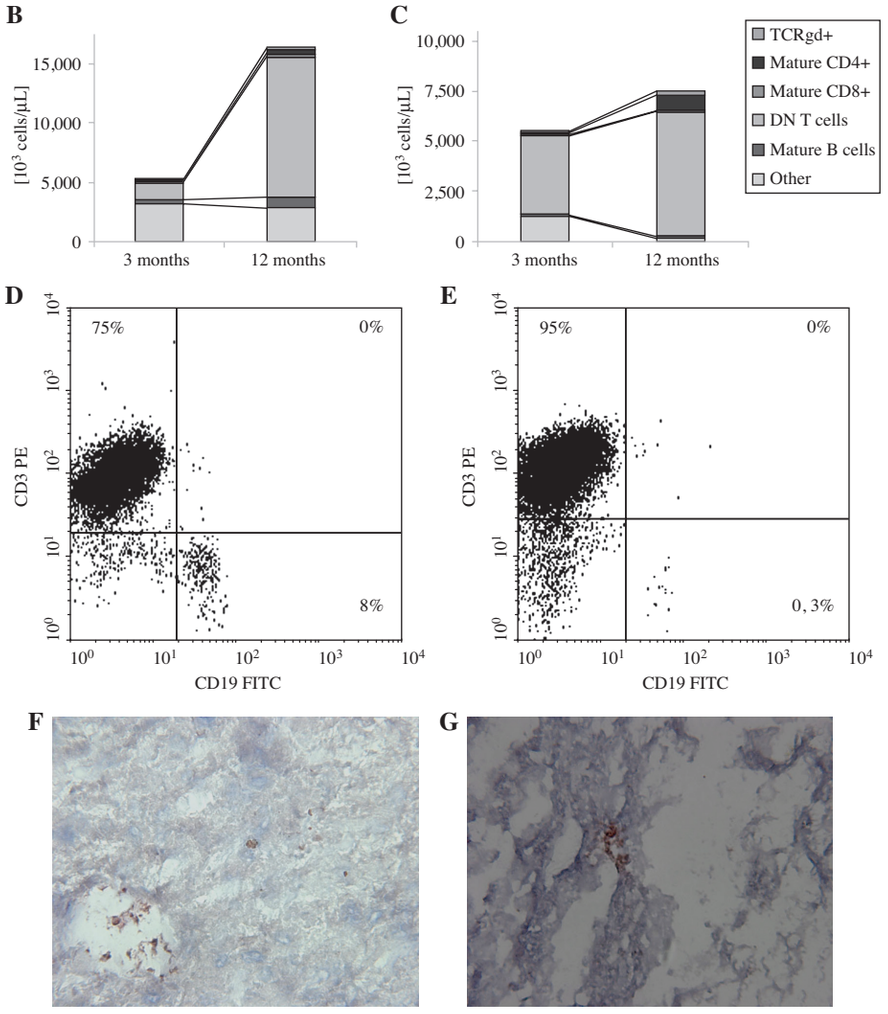

Figure 3

Lymphocyte subpopulations of the liver of IL-7$^{+/+}$ and IL-7$^{-/-}$ mice. **A-C)** Absolute cell counts (MV ± STDV) calculated by multiplying the absolute lymphocyte number by the percentage of each subpopulation as determined by flow cytometry, gated on lymphocytes. TCRγδ$^+$ T cells were absent in IL-7-deficient mice. **D, E)** Representative flow cytometry staining for CD3/CD19 of the liver of three-month-old IL-7$^{+/+}$ **(D)** and IL-7$^{-/-}$ **(E)** mice. **F, G)** Representative immunohistochemistry staining for CD19$^+$ cells of three-month-old IL-7$^{+/+}$ **(F)** and IL-7$^{-/-}$ **(G)** mice (250x).

A

	Spleen [10^3 cells/µL]			
Age	3 months		12 months	
IL-7	+/+	-/-	+/+	-/-
TCRαβ$^+$	33723±19910	2049±1103	20781±4472	3441±1089
		p = 0.0075		p = 0.00003
TCRγδ$^=$	1841±677	18±16	1636±804	3±6
		p = 0.0004		p = 0.0085
Mature CD4$^+$	19411±10598	999±577	11599±2485	1260±376
		p = 0.0047		p = 0.00002
Naive CD4$^+$	4563±3175	209±140	2229±1057	564±242
		p = 0.0155		p = 0.0089
Memory CD4$^+$	14847±7546	789±440	9363±2018	696±327
		p = 0.0032		p = 0.00001
Mature CD8$^+$	11398±6862	696±400	7221±2108	1705±865
		p = 0.0083		p = 0.0006
Naïve CD8$^+$	10284±7015	353±202	6494±2472	549±247
		p = 0.0133		p = 0.0007
DN T cells	1614±783	612±389	1982±661	1143±739
		p = 0.0336		n.s.
NK cells	4340±2347	1494±567	2221±429	1283±900
		p = 0.0299		n.s.
NK-T cells	668±290	46±41	712±151	373±419
		p = 0.0036		n.sl
Mature B cells	45970±23066	1617±1351	39185±16115	2260±1440
		p = 0.0026		p = 0.0009
Total lympo-cytes	94252±48120	8030±4862	66978±20430	10438±1682
		p = 0.004		p = 0.0003

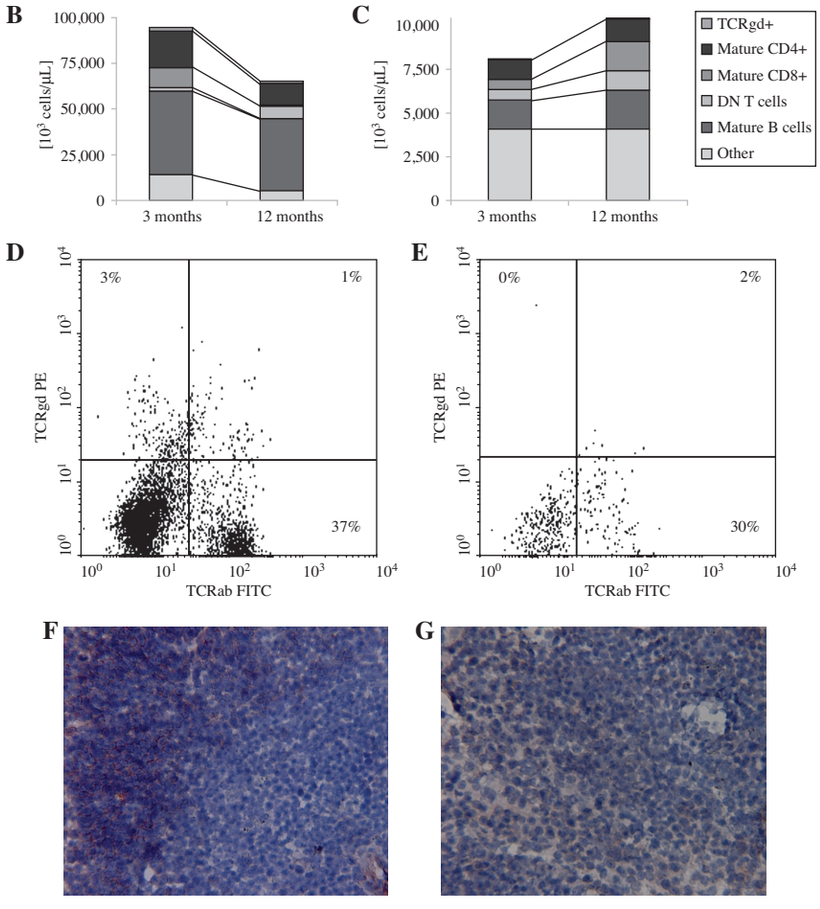

Figure 4

Lymphocyte populations of the spleen of IL-7$^{+/+}$ and IL-7$^{-/-}$ mice. **A-C)** Absolute cell counts (MV +/- STDV) calculated by multiplying the absolute lymphocyte number by the percentage of each subpopulation as determined by flow cytometry, gated on lymphocytes. Significant reduction of cell counts for all lymphocyte subtypes except for DN-, NK-, and NKT-cells. **D, E)** Representative flow cytometry staining for TCRβ/TCRγδ of the spleen of three-month-old IL-7$^{+/+}$ **(D)** and IL-7$^{-/-}$ **(E)** mice. **F, G)** Representative immunohistochemistry staining for CD19$^+$ cells of three-month-old IL-7$^{+/+}$ **(F)** and IL-7$^{-/-}$ **(G)** mice (250x).

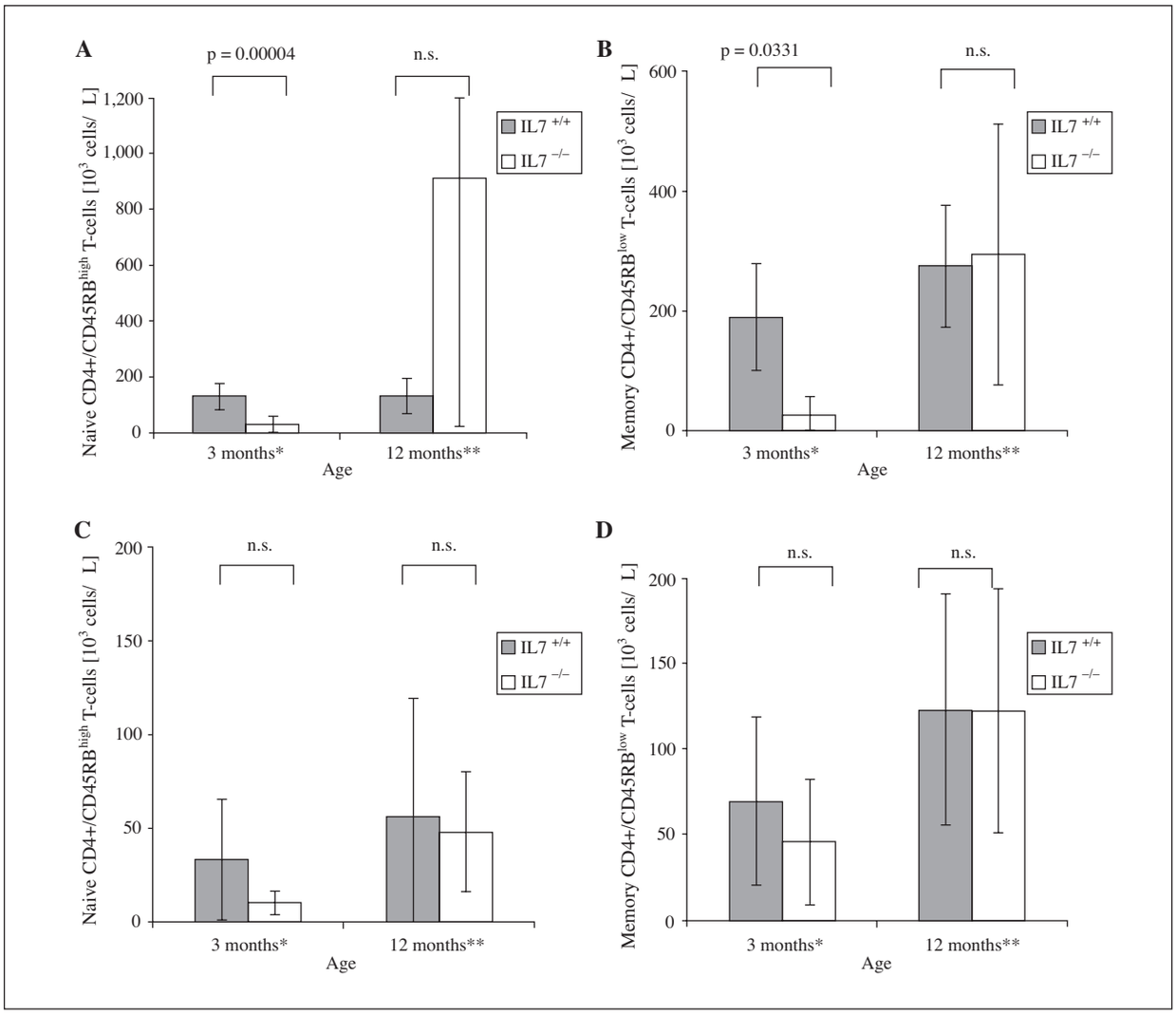

Figure 5

Development of $CD4^+$ T-cells in BM **(A, B)** and liver **(C, D)**. **A)** Whereas in the three-month-old IL-$7^{-/-}$ mice there was a significant reduction of naïve $CD4^+$ T cells ($CD4^+/CD45RB^{high}$) compared to the wild-type mice (*$p = 0.00004$), in the older IL-$7^{-/-}$ mice there was no difference (**$p = 0\,0854$); naïve $CD4^+$ T cells in the BM of IL-$7^{-/-}$ mice expand at the age of 12 months. **B)** BM, memory T cells ($CD4^+/CD45RB^{low}$). While in the three-month-old IL-$7^{-/-}$ mice was a significant reduction of the number of memory $CD4^+$ T cells compared to the wild-type (*p = 0.0331), in the older mice there was no difference (**p = 0.8653); recovery of memory $CD4^+$ T cells takes place in the BM of IL-$7^{-/-}$ mice at the age of 12 months. **C)** There were no significant differences in naïve $CD4^+$ T cells ($CD4^+/CD45RB^{high}$) between IL-$7^{+/+}$ and IL-$7^{-/-}$ mice in the liver. Both, the naive $CD4^+$ T cell population of IL-$7^{+/+}$ mice, and IL-$7^{-/-}$ mice expanded with aging. **D)** Liver, memory T-cells ($CD4^+/CD45RB^{low}$). There were no significant differences for memory $CD4^+$ T cells in the liver between IL-$7^{+/+}$ and IL-$7^{-/-}$ mice.

apart from TCR$\gamma\delta^+$ T cells, which were not detected in three- or 12-month-old IL-$7^{-/-}$ mice *(figure 3A)*.

Investigations of lymphocyte subsets of the lung showed an increase in DN T cells at the age of 12 months compared to the younger mice and compared to wild-type mice.

The analysis of the small intestine showed an increased number of TCR$\alpha\beta^+$, TCR$\gamma\delta^+$ and mature B cells in the 12-month-old wild-type mice. In the absence of IL-7, only TCR$\alpha\beta^+$ T cell populations expanded with aging.

In the spleen of wild-type mice, the numbers of all subpopulations decreased with aging, except for DN T cells and NK-like T cells, which showed an increase in numbers in 12-month-old mice. However, in IL-$7^{-/-}$ mice we observed an increase in the total number of lymphocytes of all subpopulations in the older mice, apart from memory $CD4^+$ cells and NK-cells *(figure 4A)*.

DISCUSSION

Because of its influence on T cell lymphopoiesis in humans, and T and B cell lymphopoiesis in mice, IL-7 is a cytokine of great interest. In their study of IL-$7^{-/-}$ mice, von Freeden-Jeffry *et al*. found decreased cell counts in the PB, spleen and thymus, but normal lymphocyte counts in the BM [23]. Others have also studied lymphocyte populations in IL-$7^{-/-}$ mice in different organs, but the age of the mice used in these studies varied (5-11 months) [26, 32], so that age-related effects might not have been recognized. Aging of the immune system is still not completely understood. We investigated lymphocyte subpopulations in several organs, BM, liver, lung, small intestine and spleen of C57BL/6 IL-$7^{-/-}$ mice and C57BL/6 mice as a control-group, at two time-points (three and 12 months). Many lymphocytes reside in the so-called

mucosa-associated lymphoid tissue (MALT), which is a diffuse system of concentrations of lymphoid tissue found in various sites of the body such as the gastrointestinal tract, lung, thyroid, breasts, salivary glands and skin. A detailed analysis of the PB and BM in a murine syngeneic bone marrow transplantation model was performed in an earlier study and this showed that a substantial number of mice can survive lethal irradiation and can be rescued by bone-marrow transplantation for up to 1.5 years, even in the complete absence of IL-7-producing cells, although there is a trend towards a poorer survival-rate in mice without endogenous IL-7. However, the deaths of these mice were mainly related to infection, and occurred in the early post-transplantation period ($\leq$ 16 days) [28]. Foremost, we noticed the reduced body weight of IL-7$^{-/-}$ mice, not at three-month-old mice but at 12 months of age, and smaller organs (lung, liver and spleen). Reduced spleen size and loss of Peyer's patches has already been described in [23]. One reason for this phenomenon could be diminished lymphopoiesis and a reduced recruitment of lymphocytes into the organs in the absence of IL-7. It could also be related to the smaller body size and therefore reduced blood-volume. However, it remains unknown as to why IL-7$^{-/-}$ mice are smaller. We first hypothesized that this might be due to the partial immunodeficiency in the absence of endogenous IL-7; however, under conventional housing, we did not observe more infections within the IL-7$^{-/-}$ mice than in the wild-type mice. It might also be a direct effect of IL-7 on the body-weight metabolism. However, what we see here seems to conflict with the recent findings of Macia *et al*. [33]. After injecting mice with IL-7 they saw these mice being protected from diet-induced obesity by decreased food intake. They showed that IL-7 targets the hypothalamic arcuate nucleus directly. However, they only saw decreased food intake in an induced obesity model and after starvation, and they did not study the effect of IL-7-deficiency. Non-treated mice did not show any differences in body weight or food uptake. However, IL-7$^{-/-}$ mice have a reduced life span of about one and a half years in contrast to the three years of wild-type mice. This leads to the hypothesis that in absence of IL-7, there is a more rapid aging of the immune system. To address this question, further analysis has to be done, e.g. comparing lymphocytes from one-year-old IL-7$^{-/-}$ mice with three-year-old wild-type mice. A big issue here also is that we still do not know much about senescence of the immune system in general.

In the study of von Freeden Jeffry *et al*. the PB showed leukopenia that was caused by lymphopenia. Further analysis also showed lymphopenia in the thymus and the spleen, but a normal cell count in the BM in absence of IL-7 [23]. Our analysis also demonstrated a normal, absolute lymphocyte count in the BM at the age of 12 months, but a reduced number at the age of three months. Interestingly, a normal cellularity of lymphocytes of the small intestine and the lung was recorded, so we conjecture that the absence of IL-7 is mostly important for lymphatic organs. Not only the organ, but also the age of the mice seems to have an influence on whether the IL-7 deficiency can be counterbalanced, because the composition of the lymphocyte pool differs less between IL-7$^{-/-}$ and IL-7$^{+/+}$ mice in the older groups. Looking at TCR$\alpha\beta^+$ T cells in our study, it seems that IL-7 is mostly important for lymphocyte-development in the BM and the spleen, which is witnessed in the BM by a significant decrease in the TCR$\alpha\beta^+$ T cell count in the three-month-old mice, and in the spleen where TCR$\alpha\beta^+$ T cell counts are decreased at both ages. In the BM of three-month-old mice, we found decreased cell counts for mature CD4$^+$ and CD8$^+$ cells, mainly caused by a decrease in the naïve T-helper and cytotoxic populations; CD4$^+$ memory cells were also decreased. Compared to wild-type mice, the number of memory CD8$^+$ cells in IL-7$^{-/-}$ mice was unchanged in three- and 12-month-old mice in the BM. We assume an effect of IL-15 on memory CD8$^+$ cell development, which is believed to influence the homeostasis of CD8$^+$ lymphocytes [34, 35]. The expansion of mature and naïve CD4$^+$ and CD8$^+$ cells in the absence of IL-7, and the increased number of memory CD4$^+$ cells with aging in IL-7$^{-/-}$ mice in the BM and the liver, seem to be specific for IL-7 due to the missing feedback-loop in its absence, such that other cytokine receptors might get up-regulated suggesting that the lack of IL-7 might be compensated for by other cytokines. Indeed, earlier studies showed that in addition to IL-7, IL-15 is also important for CD8$^+$ development and homeostasis [34, 35]. The function of IL-7 in the BM concerning lymphocyte development is probably compensated for by other cytokines, such as TSLP, which is already known to influence CD4$^+$ cells, and IL-15, which has influence on CD8$^+$ cells. In addition, lymphocytes from the periphery could migrate back into the BM [36].

Partial absence of TCR$\gamma\delta^+$ T-cells

According to our data, we could first show that the number of TCR$\gamma\delta^+$ T cells in IL-7$^{-/-}$ mice was significantly reduced in the BM of younger mice, whereas there was no difference when comparing 12-month-old IL-7$^{-/-}$ and IL-7$^{+/+}$ mice. This expansion of the TCR$\gamma\delta^+$ T cells in the BM also motivates further analysis. A more detailed characterization of the receptor-profile of these cells may give information as to whether there exists another TCR$\gamma\delta^+$-subpopulation. The peculiar position of TCR$\gamma\delta^+$ T cells is further demonstrated because we detected TCR$\gamma\delta^+$ T cells neither in the liver nor in the lung. Earlier studies have already shown reduced TCR$\gamma\delta^+$ T cell counts for the PB, BM, and small intestine [27]. We showed that there is a significant reduction in the small intestine in three- and 12-month-old mice and we could not detect TCR$\gamma\delta^+$ T cells in the spleen. Hence, we suppose that IL-7 has a direct influence on the generation and development of TCR$\gamma\delta^+$ T cells. However, other cytokines are probably also of importance here, *e.g.* IL-15, which is already known to influence the homeostasis of TCR$\gamma\delta^+$ T cells [37]. Furthermore, DN T cells play a peculiar role. Their number increased significantly with aging in absence of IL-7 in the BM, liver, lung and spleen. Therefore, lymphocyte generation in gastrointestinal-associated tissue seems to be influenced by different factors. In the older mice, there were no significant differences between the sizes of the T cell subpopulations of IL-7$^{+/+}$ and IL-7$^{-/-}$ mice. The increased number of DN T cells in the lung and the liver is one indication that further analysis is needed in order to clarify the importance of these organs for lymphopoiesis in IL-7-deficient mice. One could ask whether this subpopulation undergoes extra-thymic lymphopoiesis in the absence of IL-7 or whether it is important in regulating autoimmune reactions.

Carvalho *et al*. showed a reduction in B-cells in the BM of IL-7$^{-/-}$ mice, but not in the periphery, and also found a complete absence of CD19^{+} cells at one year of age. In spite of reduced B-cell counts, Carvalho *et al*. and von Freeden-Jeffry *et al*. found higher levels of immunoglobulin in IL-7$^{-/-}$ mice [23, 26]. We also found significantly decreased absolute cell counts for mature B cells in the BM, the liver and the spleen of three- and 12-month-old mice, as well as in the small intestine of 12-month-old mice, but despite the described cessation of B-lymphopoiesis, mature B cells could be detected albeit at low numbers. Therefore, we can confirm that IL-7 is an essential cytokine for B-lymphopoiesis. The B cells found in IL-7$^{-/-}$ mice are supposed to be B1-cells, which are produced in the fetus and then immigrate from the marginal zones. To prove this hypothesis, further analysis is needed, such as the analysis of B1-specific surface marker. To verify how lymphocyte homeostasis is influenced in the absence of IL-7, which cytokines play a greater role in compensating for some of the functions of IL-7, detailed determination of the cytokine and cytokine-receptor-profile in the absence of IL-7, is needed.

We can confirm the hypothesis that IL-7 is essential for B cell lymphopoiesis in mice, but for T cell lymphopoiesis we make the case that this is not true in general. In three-month-old mice we detected significantly decreased cell counts for several T cell subpopulations. With the exception of the spleen, where this is also the case for 12-month-old mice, there were fewer differences in the older mice. All lymphocyte subsets are also present in IL-7$^{-/-}$ mice, in a similar proportion except for TCR$\gamma\delta^{+}$ T cells. So, IL-7 is non-redundant for the development of TCR$\gamma\delta^{+}$ T cell and is partly redundant for TCR$\alpha\beta^{+}$ T cell lymphopoiesis. As we know from other studies, IL-7 is essential for early thymocyte survival [23], but what we and others [38] found leads to the suggestion that developing T cells receive essential signals from cytokines other than IL-7. One possibility of counterbalancing the missing IL-7 may be that other cytokines, such as TSLP, which can bind to a subunit of the IL-7R, gain importance. Based on our result in older mice, we suggest an effect of IL-7 in limiting senescence of the immune system.

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