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Regulatory CD8⁺ T lymphocytes
Regulační CD8⁺ T lymfocyty

Bachelor's thesis

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Poděkování

Na tomto místě bych chtěla především poděkovat svému školiteli Mgr. Ondřeji Štěpánkovi, Ph.D. za trpělivost a pomoc při psaní této bakalářské práce. Dále bych ráda poděkovala všem členům laboratoře adaptivní imunity a svým blízkým.

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstrakt

Regulační T-buňky jsou nezbytné pro udržování periferní imunitní tolerance, zabraňování aloimunity a prevenci imunopatologie způsobené patogeny. Zatímco regulační CD4⁺ T lymfocyty jsou studovány po více než dvě desetiletí, existence regulačních T lymfocytů mezi CD8⁺ buňkami zůstává nadále kontroverzní. V průběhu let byla možná regulační aktivita zpozorována u několika subpopulací CD8⁺ T lymfocytů, nicméně žádná z nich nebyla dosud kanonicky uznána. Cílem této práce je přezkoumat současné znalosti týkajících se těchto předpokládaných CD8⁺ regulačních lymfocytů a zhodnotit, zda tyto podskupiny mohou být zavedeny jako regulační T buňky. Důraz bude kladen na fenotypovou charakteristiku, regulační aktivitu a inhibiční mechanismy těchto buněk.

Klíčová slova: CD8⁺ regulační T lymfocyty, imunologická autotolerance, imunosuprese

Abstract

Regulatory T cells are essential for the maintenance of peripheral immune tolerance, preventing alloimmunity and pathogen-induced immunopathology. While regulatory CD4⁺ T lymphocytes have been studied for over two decades, the existence of regulatory T lymphocytes in the CD8⁺ compartment is still controversial. Through the years, multiple CD8⁺ T lymphocytes subpopulations have been reported to exhibit regulatory activity, yet none has been canonically recognized. The aim of this thesis is to review current knowledge of these putative CD8⁺ regulatory lymphocytes and evaluate whether or not these subsets can be established as regulatory T cells. The emphasis of this thesis will be put on the phenotypic characteristics, regulatory activity and inhibitory mechanisms of these cells.

Keywords: CD8⁺ Regulatory T lymphocytes, immunological self-tolerance, immunosuppression

Table of contents

1 Introduction	6
2 Regulatory cells.....	7
2.1. CD4 ⁺ regulatory T cells.....	7
2.2. Definition of a regulatory T cell.....	7
3 CD8 ⁺ CD122 ⁺ T cells	8
3.1. Characterization.....	8
3.2. Regulatory activity	10
3.3. Possible mechanisms of suppression.....	10
3.4. Virtual memory T cells.....	12
3.5. Summary	13
4 CD8 $\alpha\alpha$ ⁺ T cells	14
4.1. Characterization.....	14
4.2. Regulatory activity	15
4.3. Qa-1 restricted CD8 ⁺ T cells	15
4.4. Summary	17
5 CD8 ⁺ CD28 ⁻ and CD8 ⁺ CD28 ^{low} T cells.....	18
5.1. Characterization of CD8 ⁺ CD28 ⁻ T cells.....	18
5.2. Regulatory activity of CD8 ⁺ CD28 ⁻ T cells	19
5.3. Murine CD8 ⁺ CD28 ^{-/low} T cells.....	20
5.4. Summary	22
6 CD8 ⁺ CD25 ⁺ FOXP3 ⁺	23
7 CD8 ⁺ CD103 ⁺ T cells	25
8 CD8 ⁺ CD45RC ^{low} T cells.....	26
9 Summary	28
10 Conclusion.....	31
Abbreviations	32
References	33

1 Introduction

The immune system is an incredibly complex network of cells, molecules, and tissues working together in order to protect the body from diseases and other possibly harmful elements. One of the key players of the immune system is the T-lymphocytes population. T lymphocytes (also known as T cells) are essential in cell-mediated immunity, defending our body by fighting pathogens and removing infected and cancer cells. However, their activity can become a double-edged sword, harming the own body if not regulated.

One level of such regulation is mediated by regulatory T cells. These cells prevent possible damage done by an immune response by suppressing conventional lymphocytes. The regulatory activity can be performed either by preventing differentiation of self-reactive naive T cells into effector T cells or by producing inhibitory cytokines that limit the effector responses of other T-cell subsets.

Regulatory T lymphocytes are crucial for maintaining self-tolerance, preventing pathogen-induced immunopathology and alloimmunity including fetomaternal tolerance. On the other hand, they can contribute to the growth of tumors, by protecting malignant cancer cells from being removed by conventional T cells.

Suppressor T cells were extensively studied in the 1970s. However, the lack of success in finding specific markers and gene expression regulators to distinguish them from other subsets led to an abandonment of these studies. Interest in regulatory T cells was revived by the discovery of CD4⁺CD25⁺ regulatory T cells in 1995 (Sakaguchi et al., 1995). Their universal recognition led to a search for regulatory cells in the CD8⁺ subset. So far, multiple CD8⁺ T-cell subsets have been considered to have a suppressive function. These putative regulatory T-cell subsets are: CD8⁺CD122⁺, CD8⁺CD28⁻, CD8⁺CD25⁺, CD8 $\alpha\alpha^+$ TCR $\alpha\beta^+$, CD8⁺CD103⁺, and CD8⁺CD45RC^{low} T cells. However, the existence of the regulatory CD8⁺ regulatory T cells is still controversial, despite being studied for almost 2 decades. In this thesis, I will review published data about these putative CD8⁺ regulatory cells and assess whether or not there is sufficient evidence to establish them as specific regulatory T-cell subpopulations.

2 Regulatory cells

2.1. CD4⁺ regulatory T cells

The first canonical T-cell population with immunoregulatory function was the CD4⁺CD25⁺ subset which is defined by its expression of a transcription factor FOXP3. FOXP3 is a master regulator of CD4⁺ Treg development and function and thus is recognized as their signature marker. CD4⁺ Tregs develop both extrathymically from CD4⁺Foxp3⁻ T cells (induced CD4⁺ Tregs) and intrathymically (natural CD4⁺ Tregs). CD4⁺ Tregs circulate in the periphery where they help to maintain immune tolerance. This can be done by either preventing differentiation of self-reactive naive T cells into effector T cells, inhibiting proliferation of various types of immune cells or by their direct killing.

Mechanisms of immunosuppression used by CD4⁺ Tregs have been intensively studied. It has been found that CD4⁺ Tregs restrain immune response in various ways, either by cytokine production and/or by a physical cell-cell contact. Contact-independent suppression mechanisms are mediated by secretion of immunosuppressive cytokines IL-10, TGFβ, and IL-35 (*reviewed in* Yong et al., 2007, Shalev et al., 2011, Liberal et al., 2015). Another mechanism is called 'IL-2 stealing'. CD4⁺ Tregs absorb local IL-2 which is required for differentiation of effector T cells. The deprivation of this essential cytokine causes apoptosis of naive and effector T cells (Chinen et al., 2016). Direct cell-cell interaction is needed for suppression of proliferation mediated by CTLA-4 and LAG-3. These two proteins target antigen presenting cells preventing activation of effector T cells (*reviewed in* Shalev et al., 2011, Liberal et al., 2015). CD4⁺ Tregs can also suppress immune response by killing natural killer cells in a granzyme-B and perforin-dependent manner (*reviewed in* Chen et al., 2016).

2.2. Definition of a regulatory T cell

To consider whether currently published data are sufficient to generally accept the concept of CD8⁺ regulatory T cells, I need a definition of regulatory T-cell population. Based on the parallel with the established CD4⁺Foxp3⁺ Tregs, I defined 4 criteria that a population of T lymphocytes should meet in order to be considered regulatory:

- They can be recognized and defined as a specific T-cell subset in vivo
- They are capable of suppressing immune response of conventional T lymphocytes
- Their specific mechanism of suppression is known (e.g., killing of effector cells, blocking their activation and/or suppressing their proliferation)
- They are self-tolerant and do not promote the immune response (i.e, they are not proinflammatory)

Based on these criteria I will review the putative CD8⁺ regulatory T lymphocyte subsets and evaluate them whether or not they should be established as regulatory T cells.

3 CD8⁺CD122⁺ T cells

It was observed that IL-2R β (alias CD122)-deficient mice show signs of abnormal phenotype such as increased expression of the early activation marker CD69 on T cells, autoimmune hemolytic anemia, and decreased number of CD4⁺CD8⁺ cells in the thymus (Suzuki et al., 1995). These mice died prematurely at the age of ca. 12 weeks. These results suggested that IL-2R β is essential for T-cell regulation, homeostasis, and self-tolerance.

One possible explanation of the phenotype of the CD122-deficient mice is potential involvement of IL-2 in the FAS-mediated T-cell death (Refaeli et al., 1998). Another factor contributing to the dysregulation of conventional T cells in the CD122^{-/-} mice might be a defective function of CD4⁺CD25⁺ Tregs. Indeed, the autoimmunity and associated abnormalities in CD122-deficient mice were rescued by adoptive transfer of 3 x 10⁴ wildtype CD4⁺CD25⁺ T cells (Malek et al., 2002). However, Suzuki's group could not rescue the autoimmune phenotype in CD122^{-/-} mice even when they transferred 5 x 10⁴ CD4⁺CD25⁺ cells (Rifa'i et al., 2004). Interestingly, adoptive transfer of the same number of CD8⁺CD122⁺ T cells prevented expansion of activated memory-type T cells and rescued all signs of the CD122^{-/-} phenotype.

Therefore, the authors suggested that CD8⁺CD122⁺ T cells could represent a novel regulatory T-cell population that has a capacity to suppress conventional T cells. Currently, the CD8⁺CD122⁺ T-cell population is one of the best characterized putative CD8⁺ Treg populations.

3.1. Characterization

CD122 molecule is the β chain of the IL-2 receptor. This makes CD8⁺CD122⁺ population a potential counterpart to CD4⁺CD25⁺ Tregs since CD25 is the α chain of the same receptor. However, CD122 is not a very specific marker since it is expressed on resting T cells at low density and it is up-regulated by stimulation of the T-cell receptor (TCR). Moreover, it is expressed on memory CD8⁺ T cells.

Subsequent studies revealed that CD8⁺CD122⁺ T cells lack expression of FOXP3 and exhibit CD44^{high}CD62L^{high} phenotype (Dai et al., 2010). The CD8⁺CD44^{high}CD62L^{high}CD122^{high} profile strikingly resembles central memory CD8⁺ T cells. Thus, additional markers are required to distinguish CD8⁺CD122⁺ regulatory cells and central memory T cells.

One way of discriminating CD8⁺CD122⁺ Treg cells from T_{CM} could be based on different expression levels of CD49, creating two subsets: CD49d^{low} and CD49d^{high}. Akane et al. showed that CD8⁺CD122⁺CD49d^{low} T cells, but not CD8⁺CD122⁺CD49d^{high} T cells, suppress conventional T cells (Akane et al., 2016). However, the very same phenotype has been recently associated with a T-cell population called virtual memory cells (Haluszczak et al., 2009). Virtual memory T cells are CD8⁺ cells that have a memory phenotype despite no previous antigen exposure.

	CD122	CD44	CD49d	CD62L	PD-1
CD8⁺CD122⁺ Tregs	++	++	+	++	+!
CD8⁺ T_{CM}	++	++	++	++	-
CD8⁺ VM	++	++	+	+	-

Figure 1: Comparison of regulatory and memory T cell phenotypes (LI ET AL., 2014, DECMAN ET AL., 2012 and references in the main text)

(++: expressed, +: lowly expressed, -: not expressed, !: controversial data)

Another marker for distinguishing putative regulatory CD8⁺CD122⁺ T cells from memory cells could be PD-1 (programmed death-1 protein). CD8⁺CD122⁺PD-1⁺ T cells suppressed proliferation of CD3⁺CD25⁻CD44^{low} cells in vitro, whereas CD8⁺CD122⁺PD-1⁻ T cells failed to do so (Dai et al., 2010). Accordingly, CD8⁺CD122⁺PD-1⁺ T cells, but not CD8⁺CD122⁺PD-1⁻ T cells, significantly suppressed T cell-mediated skin allograft rejection mediated by CD3⁺CD25⁻CD44^{low} T cells. However, this finding is rather controversial, since this single study has not been reproduced by others. Moreover, Akane et al. expressed skepticism about these results (Akane et al., 2016).

It was suggested that regulatory CD8⁺ T cells can be defined by their expression of CXCR3 (CD183). In vitro experiments showed that CD8⁺CXCR3⁺ T cells are capable of suppressing IFN- γ production of CD8⁺CXCR3⁻ T cells (Shi et al., 2009). Accordingly, lymphocyte-deficient SCID mice which were injected with CD8⁺CXCR3⁺ T cell with CD8⁺CXCR3⁻ T cells had lower percentage of activated memory T cells than mice that obtained CD8⁺CXCR3⁻ T cells alone. However, murine CD8⁺CD122⁺CXCR3⁺, but not CD8⁺CD122⁻CXCR3⁺, T cells exerted suppressive activity, implying that CXCR3 alone cannot be used as a specific marker of CD8⁺ Tregs (Shi et al., 2009).

CD122 cannot be used for the identification of regulatory T cells in humans, because no CD8⁺CD122⁺ cells are preset in human blood (Shi et al., 2009). For this reason, the putative human regulatory T cells were defined as CD8⁺CXCR3⁺ (Shi et al., 2009). Indeed, human CD8⁺CXCR3⁺ population suppressed proliferation of naive CD8⁺CXCR3⁻ T cells when co-cultured in vitro (Shi et al., 2009). However, the CXCR3 marker alone is insufficient to define a pure regulatory population, since effector CD8⁺ T cells express CXCR3 as well (Liu et al., 2015). Therefore, no human counterparts to murine CD8⁺CD122⁺ T cells have been unambiguously identified so far.

3.2. Regulatory activity

Suppressive activity of CD8⁺CD122⁺ cells was first observed in experiments with CD122-deficient mice that exhibit severe autoimmune disorder. Transfer of CD8⁺CD122⁺ T cells prevented expansion of activated memory–phenotype T cells and rescued all autoimmune symptoms in CD122^{-/-} neonates (Rifa'i et al., 2004).

In order to find out whether CD8⁺CD122⁺ T cells are capable of suppressing conventional CD8⁺CD122⁻ T cells, CD8⁺CD122⁺ T cells were co-cultured with naive CD8⁺CD122⁻ T cells. After 72 hours of the co-culture, low numbers of CD8⁺CD122⁻ T cells survived in the presence of CD8⁺CD122⁺ T cells (Akane et al., 2016). In the *in vivo* assay, T cells were transferred into RAG2^{-/-} mice. While adoptive transfer of CD8⁺CD122⁻ T cells alone caused premature death of mice, mice that received CD8⁺CD122⁻ T cells with CD8⁺CD122⁺ T cells stayed alive and healthy with a survival rate similar to mice with no T-cell transfer (Rifa'i et al., 2004). In another experiment, allogeneic donor pancreatic islets were transplanted into a kidney of a RAG^{-/-} mouse, followed by an adoptive transfer of unfractionated T cells. A co-transfer of CD8⁺CD122⁺ T cells delayed islet allograft rejection mediated by conventional T cells (Dai et al., 2014). The effect of CD8⁺CD122⁺ T cells was even more pronounced than the protection mediated by the same number of CD4⁺CD25⁺ Tregs.

Further research of suppressive activity of CD8⁺CD122⁺ T cells was done using animal models of autoimmune diseases. Adoptive transfer of CD8⁺CD122⁺ T cells prevented the development of colitis mediated by CD4⁺ T cells showing that CD8⁺CD122⁺ T cells are capable of regulating CD8⁺ as well as CD4⁺ cells (Endharti et al., 2011). The regulatory function was also observed in a model of experimental autoimmune encephalomyelitis (EAE), an experimental animal model for human multiple sclerosis. EAE was induced by immunization with MOG peptide (Lee et al., 2008). Subsequently, CD8⁺CD122⁺ T cells were or were not depleted by an anti-CD122 antibody. While the onset and peak values did not differ between the experimental groups, the disease score in the later phase was much higher in CD122-depleted mice. Accordingly, transfer of CD8⁺CD122⁺ T cells into CD122-depleted hosts did not influence the initial phase of the illness, but the symptoms in the later phase were less severe and went away faster. This finding indicated that CD8⁺CD122⁺ T cells do not inhibit the onset of the disease, but they contribute to the recovery.

3.3. Possible mechanisms of suppression

Suppressed proliferation and low expression of IFN- γ in CD8⁺CD122⁻ and CD4⁺CD25⁻ T cells was observed when they were co-cultured with CD8⁺CD122⁺ (Rifa'i et al., 2004). However, the mechanism of suppression is unclear. Multiple studies suggested that the key mediator could be IL-10 produced by CD8⁺CD122⁺ T cells. The expression level of this molecule is about 8 fold higher in CD8⁺CD122⁺ than in CD8⁺CD122⁻ T cells and IL-10 was detected in the medium obtained from the culture of CD8⁺CD122⁺ T cells (Endharti et al., 2005). This medium showed an ability to suppress proliferation

and production of IFN- γ naive CD8⁺CD122⁻ cells in vitro. Interestingly, this ability was lost when anti-IL-10 antibody was added to the medium. Accordingly, when anti-IL-10 antibody was added to the co-culture of CD8⁺CD122⁺ T cells with naive target cells the regulatory activity was blocked. Following these results, CD8⁺CD122⁺ T cells from IL-10 deficient mice were tested for their regulatory activity. CD8⁺CD122⁺ from IL-10 deficient mice did not suppress cell proliferation and production of IFN- γ in vitro (Endharti et al., 2005).

However, when co-transferred with CD8⁺ naive T cells into RAG^{-/-} mouse, IL-10 deficient CD8⁺CD122⁺ T cells are still capable of a considerable level of suppressive activity. The IL-10^{-/-} CD8⁺CD122⁺ cells, despite not being able to suppress colitis, still improved the mice condition compared to mice that were given CD4⁺CD45RB^{high} alone (Endharti et al., 2011). The IL-10^{-/-} CD8⁺CD122⁺ cells partially reduced weight loss and improved tissue pathology in the colon. The IL-10^{-/-} CD8⁺CD122⁺ cells were also able to delay rejection of allograft although not as much as those derived from WT mice, again showing that IL-10 production is probably one of the mechanisms of CD8⁺CD122⁺ T cell-mediated suppression (Dai et al., 2014). Overall, these in vivo results imply that IL-10 plays a role in the suppression activity of CD8⁺CD122⁺ T cells, but it is not the only mechanism used.

Other proposed effector molecule of suppressive activity by CD8⁺CD122⁺ was TGF- β . It was reported that CD8⁺CD122⁺ T cells produce increased levels of TGF- β (Mangalam et al., 2012). Despite that, anti-TGF- β antibody added to co-culture of CD8⁺CD122⁺ T cells with naive CD8⁺ cells did not influence the level of suppression (Endharti et al., 2005). Medium obtained from a culture of CD8⁺CD122⁺ T cells with absorbed TGF- β still maintained the same suppression level as a medium without TGF- β -blocking antibody. Accordingly, no TGF- β production by CD8⁺CD122⁺ T cells has been detected in a mouse colitis model (Endharti et al., 2011). Overall, CD8⁺CD122⁺ T cells do not suppress conventional T cells via producing TGF- β .

Another suppression mechanism used by the CD8⁺CD122⁺ could be the Fas/FasL system. It was observed that CD8⁺CD122⁺ T cells cannot efficiently suppress Fas-deficient naïve CD8⁺ T cells ex vivo (Akane et al., 2016). The same result was obtained when FasL-deficient CD8⁺CD122⁺ regulatory cells with WT target cells were used. These results were supported in vivo. When naive cells from Fas-deficient mutant mice were co-transferred with WT CD8⁺CD122⁻ naive cells into RAG-2- deficient mice, the survival of these mice was similar to mice that received only CD8⁺CD122⁻ naive cells. Same results were obtained when CD8⁺CD122⁺ T lymphocytes from FasL-deficient mice were co-transferred together with WT target cells. These findings indicate that the suppression by CD8⁺CD122⁺ could be mediated by Fas-mediated killing of target cells.

3.4. Virtual memory T cells

Virtual memory cells are a recently described population of T cells that obtains memory phenotype despite no previous antigen exposure (Haluszczak et al., 2009). These cells arise via physiological homeostatic processes. They are defined as $CD8^+CD44^{high}CD49d^{low}CD62L^+$ and were also described to express a high level of CD122 (Sosinowski et al., 2013). This characteristic is identical to the one of the putative $CD8^+CD122^+CD49d^{low}$ regulatory cells.

Experiments done with the virtual memory population showed that this population has some interesting properties. Some of them are, however, in disagreement with the hypothesis of the $CD8^+CD122^+$ regulatory cells. One of these properties is the rapid production of IFN- γ . Splenocytes from B6 mice were stimulated for 18 hours with a mixture of proinflammatory cytokines IL-12, IL-18, and IL-2. This exposure led to a production of IFN- γ by $CD8^+CD44^{high}$ T cells (Haluszczak et al., 2009). Accordingly, when virtual memory cells were stimulated with their cognate antigen, they could rapidly produce IFN- γ (Lee et al., 2013). However, the production of IFN- γ by virtual memory T cells was weaker, when compared to true memory population.

Another interesting property of virtual memory cells is their fast expansion in the early phase of immune response to infection and robust differentiation into short-lived effector $KLRG1^+CD127^lo$ T cells (Lee et al., 2013).

Virtual memory subset also showed an ability to control pathogen infection by *Listeria monocytogenes* (Lee et al., 2013). Ovalbumin-specific virtual memory population was transferred into a host mouse that was subsequently infected with LM-OVA (*Listeria monocytogenes* expressing ovalbumin). These mice were analyzed 5 days later. Numbers of colony forming units of *L. monocytogenes* in mice that obtained virtual memory cells was lower than in mice that received ovalbumin-specific naive cells. The level of protection against the bacteria by virtual memory T cells was comparable with antigen-experienced true memory T cells (Lee et al., 2013). These findings are in conflict with the theory of $CD8^+CD122^+$ T cells being regulatory cells since one of the characteristics of regulatory cells is that they should not actively participate in the immune protection and inflammation.

If $CD8^+CD122^+$ regulatory and virtual memory cells are the identical T-cell subset, $CD8^+CD122^+$ cells do not fulfill all criteria of a regulatory T-cell population as defined in chapter 2.2. However, it does not exclude the possibility that these cells can promote protective immunity as well as immunological tolerance, possibly depending on the circumstances. Alternatively, $CD8^+CD122^+$ regulatory T cells and virtual memory $CD8^+$ T cells could represent two distinct populations that we are currently unable to distinguish.

3.5. Summary

Suppressive capacity of CD8⁺CD122⁺ putative regulatory T cells has been observed in in vitro and in vivo experiments. This subset suppressed proliferation of target CD8⁺ and CD4⁺ cells and their IFN- γ production. This regulatory activity is likely mediated by IL-10 cytokine and not by TGF β . Another possible mechanism is the Fas/FasL system, which was proposed in a recent experiment with CD8⁺CD122⁺CD49^{low} T-cell subset. Also, CD8⁺CD122⁺ T cells were reported to produce IFN- γ themselves (Mangalam et al., 2012). Since IFN- γ is considered to be a proinflammatory cytokine, this finding rather contradicts hypothesis of them being regulatory cells and more corresponds with the theory of virtual memory cells. The relationship of putative regulatory CD8⁺CD122⁺ T cells, virtual memory T cells and central memory T cells needs to be clarified.

Despite evidence of regulatory activity by CD8⁺CD122⁺ T cells, and known mechanisms of suppression, the inability to distinguish them as specific population combined with conflicting findings regarding their proinflammatory capacity forbids me from declaring them as a regulatory subset.

4 CD8 $\alpha\alpha^+$ T cells

Mouse T cells expressing CD8 $\alpha\alpha$ homodimer and not CD8 $\alpha\beta$ heterodimer form an unconventional T-cell subset that was proposed to possess immunoregulatory activity. These cells were originally described in intestinal intraepithelium. Whether these cells are present outside of intestinal intraepithelium is not certain. It was originally thought that CD8 $\alpha\alpha^+$ T cells were generated extrathymically, but they are most likely derived from thymic double negative cells (Nambu et al., 2012). Progenitors of CD8 $\alpha\alpha^+$ T cells arise in the thymus as CD4/CD8 double positive cells. They undergo a so called agonist selection, which rescues highly self-reactive T cells from being negatively selected and triggers their differentiation into a specific unconventional T-cell subset. These positively selected self-reactive cells then become double negative cells and migrate into the intestinal epithelium where they complete their differentiation program and become CD8 $\alpha\alpha^+$ T cells (Rocha et al., 1992, Gangadharan et al., 2006). Furthermore, it has been proposed that CD8 $\alpha\alpha^+$ T cells can develop from naïve CD4 $^+$ T cells in the peripheral lymphoid tissues (Nambu et al., 2012).

4.1. Characterization

CD8 $^+$ T cells that express exclusively CD8 $\alpha\alpha$ homodimer can be found among intestine intraepithelial lymphocytes (iIELs). CD8 $\alpha\alpha$ decreases antigen sensitivity of the TCR, therefore, CD8 $\alpha\alpha^+$ TCR $\alpha\beta^+$ T cells are anergic following TCR engagement (Mowat et al., 1986). Despite the high reactivity of their TCR to self-antigens, there are no records of CD8 $\alpha\alpha^+$ T-cell-provoked autoimmunity. However, stimulation of CD8 $\alpha\alpha^+$ T cells with anti-CD3 antibody resulted in rapid secretion of IFN- γ and IL-2 (Yamagata et al., 2004).

Analysis of mRNA expression showed that CD8 $\alpha\alpha^+$ T cells have lower expression of CD2, CD5, CD28 compared to their CD8 $\alpha\beta^+$ counterparts (Denning et al., 2007). Also, their *FoxP3* mRNA expression was low in comparison to CD8 $\alpha\beta^+$ T cells. CD8 $\alpha\alpha^+$ T cells expressed increased mRNA for TGF- β_1 and TGF- β_3 ; but not for IL-10. Interestingly, CD8 $\alpha\alpha^+$ T cells seem to express Fc ϵ RI γ chain, which is usually expressed on antigen presenting cells. Further analyses revealed the presence of molecules associated with natural killer cells at the surface of CD8 $\alpha\alpha^+$ T cells. These molecules are NK1.1, CD94 and the Ly49 family of natural killer cell receptors (Yamagata et al., 2004, Denning et al., 2007, Holler et al., 2007).

CD8 $\alpha\alpha^+$ T cells are known to reside mostly in the intestinal intraepithelium. Although non-IEL CD8 $\alpha\alpha^+$ T cells were isolated from peripheral lymph nodes, their relation to IEL CD8 $\alpha\alpha^+$ T cells is uncertain. According to some studies, these non-IEL CD8 $\alpha\alpha^+$ T cell clones exhibit different phenotype than IEL CD8 $\alpha\alpha^+$ T cells. Unlike IEL CD8 $\alpha\alpha^+$ T cells, non-IEL CD8 $\alpha\alpha^+$ T cells are NK1.1 $^-$, Ly49A $^-$, and CD28 $^+$ (Tang et al., 2006). These cells secrete IFN- γ and TNF- α , but no IL-2 or IL-10. Although it has been

suggested that these non-IEL CD8 $\alpha\alpha^+$ T cells are circulating IEL CD8 $\alpha\alpha^+$ T cells, it is not certain that non-IEL and IEL CD8 $\alpha\alpha^+$ T cells represent the same T cell lineage.

4.2. Regulatory activity

Regulatory activity of IEL CD8 $\alpha\alpha^+$ T cells was demonstrated in vivo. Colitis was induced by transfer of TCR $\alpha\beta^+$ CD4 $^+$ CD45RB hi T cells in SCID mice (Poussier et al., 2002). Mice that obtained 0.5×10^6 of splenic TCR $\alpha\beta^+$ CD4 $^+$ CD45RB hi cell with 5×10^6 TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$ did not develop colitis. However, the ratio of target cells to TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$ undermines this result. Either CD8 $\alpha\alpha^+$ T cells are very inefficient in suppression or the apparent suppression is rather caused by competition between conventional and CD8 $\alpha\alpha^+$ T cells for the niche.

Regulatory activity of non-IEL CD8 $\alpha\alpha^+$ T-cell clones was examined in vivo in a model of EAE which was induced by injection of pertussis toxin (Tang et al., 2006). Subsequent adoptive transfer of 1×10^6 non-IEL CD8 $\alpha\alpha^+$ cells diminished EAE symptoms and speeded up recovery compared to control group. Transfer of 5×10^6 non-IEL CD8 $\alpha\alpha^+$ cells protected mouse from development of EAE completely.

The evidence of regulatory activity was challenged by findings in a mouse model of chronic colitis. WT CD8 $\alpha\alpha^+$ T cells transferred into TCR $\beta\gamma\delta^{-/-}$ recipients were unable to prevent or delay the onset of the disease induced by WT CD4 $^+$ CD45RB high T cells (Ostanin et al., 2010). The transfer even seemed to worsen the illness. CD8 $\alpha\alpha^+$ T cells were also unable to suppress activated CD4 $^+$ Foxp3 $^-$ responder T cells in vitro (Ostanin et al., 2010).

The mechanisms of possible suppression used by CD8 $\alpha\alpha^+$ T cells are unknown.

4.3. Qa-1 restricted CD8 $^+$ T cells

Qa-1 is a non-classical MHC class Ib molecule, homologous to human leukocyte antigen E. Qa-1 forms a heterodimer with $\beta 2$ microglobulin and can present peptides from both self and foreign antigens.

Experiments with Qa-1 $^{-/-}$ mice revealed that Qa-1-dependent inhibition is needed for protection from a pathogenic expansion of autoreactive CD4 $^+$ T-cell populations and consequent autoimmune disease. Obtained data showed that Qa-1 $^{-/-}$ mice displayed enhanced immunopathology caused by CD4 $^+$ T cells after herpes simplex virus injection compared to WT mice (Hu et al., 2004). Furthermore, Qa-1 $^{-/-}$ mice were more susceptible to high-dose *Mycobacterium tuberculosis* infection than their WT counterparts thus resulting in higher mortality (Bian et al., 2017). The increased susceptibility of Qa-1 $^{-/-}$ mice was associated with rise of activated T cells and higher levels of IFN γ .

Activity of Qa-1 restricted T cells was further tested in T-cell vaccination assays. Mice were vaccinated with CD4 $^+$ T cells isolated from mice that had developed herpes stromal keratitis (HSK). These mice acquired protection from further HSK induction by herpes simplex virus injection (Panoutsakopoulou et al., 2004). This protection could be abolished by CD8 depletion or by use of CD4 $^+$ T cells incubated

with anti Qa-1 antibody. Therefore, these findings imply that Qa-1 restricted regulatory CD8⁺ T cells develop during the primary immune response and can control secondary responses. This hypothesis is in accordance with experiments performed with a murine model of ConA-induced hepatitis. CD8⁺ cells were purified from mice with developed ConA-induced hepatitis and transferred into WT mouse followed by induction of hepatitis. WT mouse was protected from the development of hepatitis by CD8⁺ cells, whereas Qa-1^{-/-} mice developed hepatitis (Varthaman et al., 2010).

Regulatory activity of CD8⁺ cells that specifically recognize Qa-1-bound peptides expressed by autoreactive CD4⁺ cells was observed. However, there is no known surface marker characterizing this population. Therefore, the phenotype of Qa-1 restricted regulatory T cells is still unidentified. Since reduced numbers of IEL CD8 $\alpha\alpha$ ⁺ T cells compared to WT mice were observed in β 2 microglobulin^{-/-} mice but not in MHC I^{-/-} mice, Qa-1 seems to be needed in the generation and maintenance of CD8 $\alpha\alpha$ ⁺ T cells (Ruscher et al., 2017). Therefore, some authors use the term Qa-1 restricted T cells as a synonym for CD8 $\alpha\alpha$ ⁺ T cells. However, in another study, Qa-1 restricted T cells were defined as CD8⁺CD44⁺CD122⁺Ly49⁺ T-cell subset expressing CD8 $\alpha\beta$ ⁺ but not CD8 $\alpha\alpha$ ⁺ (Kim et al., 2011). These cells efficiently suppressed WT CD4⁺ T cells, but not Qa-1^{-/-} CD4⁺ T cells. Further analysis showed that this suppression could be mediated by perforin (Yao et al., 2017). Whether CD8⁺CD44⁺CD122⁺Ly49⁺ T-cell subset is the same as CD8⁺CD122⁺ T-cell subset is uncertain. Despite the fact that they share high expression of CD122 and CD44, studies of CD8⁺CD44⁺CD122⁺Ly49⁺ T-cell subsets contradict our knowledge of CD8⁺CD122⁺ T cells (see chapter 3). CD8⁺CD122⁺ T cell-mediated suppression was not inhibited by anti-Qa-1 antibody and is thought to be conventional MHC class I-restricted (Rifa'i et al., 2008). Also, CD8⁺CD122⁺ T cells seem to implement different suppressive mechanism than Qa-1 restricted cells.

A characteristic feature of the Qa-1-dependent CD8⁺ T cells is that they require priming by the activated CD4⁺ T cells during the primary immune response to regulate the secondary immune response. This was shown in vivo in models of HSK and ConA induced colitis. However, the exact identity of Qa-1 restricted T cells waits to be determined.

4.4. Summary

Although CD8 $\alpha\alpha^+$ T cells were proposed to possess immunosuppressive activity, the only available in vivo experiment indicating their regulatory activity was performed with a high ratio of CD8 $\alpha\alpha^+$ T cells to target cells. Although more evidence of suppression can be found within studies of non-IEL CD8 $\alpha\alpha^+$ T cell lines, their relationship with IEL CD8 $\alpha\alpha^+$ T cells has not been addressed. Moreover, production of proinflammatory molecules by both of these cell subsets was reported.

Quite a few interesting in vivo experiments have been done with Qa-1 restricted T cells. These findings imply that there is a T-cell subset exerting immunoregulatory activity within the Qa-1 restricted cells. However, phenotype of these T cells remains to be specified. Although CD8 $\alpha\alpha^+$ T cells have been associated with Qa-1 restricted cells, it is not certain how these two T-cell subsets relate to each other.

So far there is little known about the regulatory activity of CD8 $\alpha\alpha^+$ T-cell subset. There is barely any available evidence of their regulatory activity and subsequently no suppressive mechanism is known. Furthermore, they were reported to secrete proinflammatory cytokines. Therefore, it can be concluded that based on published data these cells do not fit criteria defined in chapter 2.2.

5 CD8⁺CD28⁻ and CD8⁺CD28^{low} T cells

Another putative regulatory CD8⁺ T-cell subset is the CD8⁺CD28⁻ subpopulation. They were first described in 1998 as human CD8⁺ suppressor T lymphocytes (CD8⁺ Ts) (Liu et al., 1998). Since then their role in cancer and allograft rejection has been studied. This subset contains cells of a very heterogeneous activity, including immunosuppressive and cytotoxic T cells.

CD28 is a co-stimulatory signaling receptor that binds to CD80 and CD86 ligands on proinflammatory APCs. This interaction is important in promoting T-cell proliferation, cytokine production, and cell survival. The absence of CD28 expression in CD8⁺CD28⁻ T cells is likely caused by their repeated stimulation and proliferation that causes gradual down-regulation of CD28 expression (Merino et al., 1998). It does not seem that this phenomenon occurs in mice.

Although immunosuppressive CD8⁺CD28⁻ T-cell population in mice has been reported, the term CD28⁻ is inaccurate. These cells actually express CD28 at low, but detectable level. Despite being mistaken for murine homolog of human CD8⁺CD28⁻ T cells (Manavalan et al., 2004, Smith and Kumar, 2008, Pomie et al., 2011), murine CD8⁺CD28^{low} T cells more likely correspond to human CD8⁺CD28^{low} T cells (Vuddamalay et al., 2016).

5.1. Characterization of CD8⁺CD28⁻ T cells

Down-regulation of CD28 in human CD8⁺CD28⁻ T cells is the result of cell ageing. This decrease in CD28 expression is connected with the upregulation of CD57 (Merino et al., 1998), therefore CD8⁺CD28⁻ T cells have been sometimes referred to as CD8⁺CD57⁺ T cells.

Three studies showed that CD8⁺CD28⁻ T cells are FOXP3⁻ and CD25⁻ when unprimed, but they upregulate FOXP3 and CD25 upon priming (Manavalan et al., 2004, Scotto et al., 2004, Simone et al., 2008). This finding is important in regards to their possible overlap with another putative regulatory subset - CD8⁺CD25⁺ T cells.

CD8⁺CD28⁻ T cells form a very heterogeneous population whose reported abilities range from immunosuppressive to cytotoxic. This could be explained by the existence of not only regulatory but also cytotoxic cells in the CD8⁺CD28⁻ T-cell subset. Since the loss of CD27 expression correlates with the expression of perforin, it was suggested that these two populations could be divided into effector CD8⁺CD28⁻ perforin⁺ T cells and regulatory CD8⁺CD28⁻ CD27⁺ T cells (Colovai et al., 2003). However, this is in dispute with study by Filaci et al. that defines putative regulatory CD8⁺CD28⁻ T cells as CD27⁻ (Filaci et al., 2004). Another proposed way of discriminating CD8⁺CD28⁻ T cells into two subsets could be based on their expression of CD62L, since CD8⁺CD28⁻CD62L⁺ T cells showed more potent immunosuppressive capacity than their CD62L⁻ counterparts (Manavalan et al., 2004). However, this finding was mentioned only in one single study with no follow up.

CD8⁺CD28⁻ T cells were reported to produce IL-10 (Filaci et al., 2004, Filaci et al., 2007, Tulunay et al., 2008), but other reports deny any production of IL-10 or TGF- β by these cells (Manavalan et al., 2004, Scotto et al., 2004, Li et al., 2017). Moreover, multiple studies mention their production of cytolytic and proinflammatory molecules IFN- γ and granzyme B (Filaci et al., 2007, Sun et al., 2008, Engela et al., 2013, Pandya et al., 2016, Li et al., 2017).

The major issues concerning CD8⁺CD28⁻ T cells are the lack of consensual definition and the subsequent contradictory findings regarding their cytokine production. Their relationship with cytotoxic cells needs resolving in order to progress in the study of these putative regulatory cells.

5.2. Regulatory activity of CD8⁺CD28⁻ T cells

CD8⁺CD28⁻ T cells were studied in various disease contexts. An increased proportion of CD8⁺CD28⁻ T cells were observed in lung cancer patients in comparison to healthy patients (Meloni et al., 2006, Karagoz et al., 2010). Expansion of CD8⁺CD28⁻ T cells was also observed in heart transplant recipients compared to healthy controls (Colovai et al., 2003). While heart transplant patients with acute rejection had a high frequency of CD8⁺CD28⁻ perforin⁺ T cells, recipients without rejection had more regulatory CD8⁺CD28⁻ T cells. This indicated that CD8⁺CD28⁻ T cells could play a role in the protection of the graft.

The ratio of CD8⁺CD28⁻ T cells was also measured in patients with various autoimmune diseases. Analysis of blood of systemic lupus erythematosus (SLE) patients brought contradictory results. While one study reports that patients with SLE had a significantly lower amount of CD8⁺CD28⁻ T cells than healthy control (Tulunay et al., 2008), other study claims that the number of CD8⁺CD28⁻ T cells was higher in SLE patients (Zabinska et al., 2016). Elevated levels of CD8⁺CD28⁻ T cells compared to healthy groups were found in patients with Graves' disease (Sun et al., 2008), systemic sclerosis (Li et al., 2017), and autoimmune ankylosing spondylitis (Schirmer et al., 2002). However, no correlation was found between the duration of ankylosing spondylitis and percentage of CD8⁺CD28⁻ T cells.

Analysis of human tumors revealed the presence of intratumoral CD8⁺CD28⁻ T cells (Filaci et al., 2007). These tumor-infiltrating cells were incubated with autologous peripheral blood cells that were previously stimulated with anti-CD3 antibody. CD8⁺CD28⁻ T cells suppressed target T-cell proliferation. CD8⁺CD28⁻ T cells were shown to suppress CD4⁺CD25⁻ in the presence of PHA stimulation and APCs at the level of CD4⁺CD25⁺ T cells (Simone et al., 2008). On the other hand, unstimulated CD8⁺CD28⁻ T cells of polymyositis patients in co-culture with calcein-labeled autologous muscle cells induced more muscle cell death than did their CD28⁺ counterparts (Pandya et al., 2016).

Since primed CD8⁺CD28⁻ T cells inhibited proliferation of not only CD4⁺ T cells but also of inhibited antigen presenting activity of dendritic cells (Filaci et al., 2004), it has been proposed that the mechanism of suppression by CD8⁺CD28⁻ T cells could be mediated by antigen presenting cells. Co-culture of

dendritic cells with CD8⁺CD28⁻ T cells inhibited capacity of APCs to stimulate T-cell proliferation (Chang et al., 2002). Further analysis showed that co-incubation of DCs with CD8⁺CD28⁻ T cells leads to decreased expression of costimulatory molecules CD40 and CD58, and induced expression of inhibitory receptors ILT4 and ILT3 (Chang et al., 2002). Accordingly, when the same experiment was performed with IFN- γ activated human endothelial cells instead of dendritic cells, their expression of CD40, CD54, and CD83 was down-regulated, and expression of ILT4 and ILT3 was upregulated (Manavalan et al., 2004). The addition of anti-ILT3 or anti-ILT4 antibody partially abrogated inhibitory effect of CD8⁺CD28⁻ T cells, thus demonstrating their involvement in the suppression (Manavalan et al., 2004).

Suppressive activity of T-cell proliferation was blocked by adding anti-IL-10 antibody, therefore, indicating possible use of IL-10 dependent mechanism of suppression (Filaci et al., 2007). This is in discrepancy with findings that CD8⁺CD28⁻ T cells do not express IL-10 (Manavalan et al., 2004, Scotto et al., 2004, Li et al., 2017).

Analysis of CD8⁺CD28⁻ T cells in disease and in vitro experiments suggested regulatory as well as cytotoxic capacity of these cells. Two possible mechanisms of suppression were proposed. The first one - direct suppression of T cells by producing IL-10 - is rather controversial. Second possible mechanism of suppression involves antigen presenting cells since APCs co-incubated with CD8⁺CD28⁻ T cells are insufficient in activating CD4⁺ T cells.

5.3. Murine CD8⁺CD28^{-/low} T cells

Another putative regulatory CD8⁺ T-cell subset is the population of CD8⁺CD28^{low} T cells. These cells have been inaccurately termed as CD28⁻. However, a thorough phenotypic analysis showed that expression of CD28 in CD8⁺CD28^{low} T cells is low but still detectable (Menager-Marcq et al., 2006).

Since CD8⁺CD28^{low} T cells express low levels of CD122, and are CD25 and FOXP3 negative, it is clear that the putative regulatory subset of CD8⁺CD28^{low} T cells does not overlap with the other two putative regulatory T lymphocyte subsets (i.e., CD8⁺CD122⁺ or CD8⁺CD25⁺ T cells) and forms a distinct population (Pomie et al., 2011, Vuddamalay et al., 2016).

As mentioned above, murine CD8⁺CD28^{low} T cells and human CD8⁺CD28⁻ T cells are not homologous. Murine CD8⁺CD28^{low} T cells arise in the thymus, developing simultaneously with CD8⁺CD28⁺ T cells during ontogeny (Vuddamalay et al., 2016). It was proposed that human counterparts to murine CD8⁺CD28^{low} T cells can be found in the human CD8⁺CD28^{low} population. This population expresses IL-10 and TGF β and is described as CD45RA^{high} (Vuddamalay et al., 2016).

In vitro experiments showed a suppressive activity of murine CD8⁺CD28^{low} T cells. CD4⁺ T cells stimulated with allogeneic APCs in vitro were co-cultured with CD8⁺CD28^{low} T cells. This inhibited

proliferation of CD4⁺ T cells and reduced the fraction of CD4⁺ T cells that produced IFN γ (Vuddamalaya et al., 2016, Menager-Marcq et al., 2006).

Suppressive activity of CD8⁺CD28^{low} T cells was observed in the experimental colitis model. Colitis was induced by injection of CD4⁺CD45RB^{high} T cells in RAG-2^{-/-} mice (Menager-Marcq et al., 2006). When these cells were administrated in combination with CD8⁺CD28^{low} T cells, no signs of colitis were observed. Effector cells and putative regulatory cells were injected in 2:1 ratio. If such high number of CD8⁺CD28^{low} T cells is required to achieve this result, their suppressive activity is not very efficient. Contradicting result was obtained in PLP peptide-induced EAE model. No suppression of proliferation of PLP-specific CD4⁺ cells by CD8⁺CD28^{low} T cells was detected (Mangalam et al., 2012). Immunosuppressive activity was observed only in CD8⁺CD122⁺ T cells.

One of the studies linked to research of CD8⁺CD28^{low} T cells analyzed CD28^{-/-} mice. These mice are resistant to the induction of experimental autoimmune encephalomyelitis by administration of MOG peptide (Najafian et al., 2003). Depletion of CD8⁺ cells by anti-CD8 antibody rendered these mice susceptible to EAE, although the disease was less severe compared to a WT mouse. In vitro, CD8⁺ T cells of CD28^{-/-} mouse showed contact-dependent suppression of IFN- γ production of responder cells from MOG-immunized CD8^{-/-} splenocytes (Najafian et al., 2003). Authors explain these finding by the existence of regulatory CD8⁺CD28^{-/low} T cells enriched in CD28^{-/-} mice. However, the interpretation of these results is challenging as this model does not allow us to distinguish cells by their CD28 expression (all are CD28 negative). Therefore, the results could be potentially explained by the effect of CD28⁻ deficiency on conventional CD8⁺ subsets.

Since ex vivo activated CD8⁺CD28^{low} T cells produce IL-10 and TGF β , it has been proposed that these molecules could participate in the suppression of target cells. CD8⁺CD28^{low} T cells obtained from IL-10^{-/-} mice were capable of a considerable level of suppression, although the suppression was less efficient than when WT cells were used (Menager-Marcq et al., 2006). Accordingly, IL-10^{-/-} CD8⁺CD28^{low} T cells in WT mouse were not able to protect mice from colitis. Less efficient suppression was also achieved when target cells of dnT β RII transgenic mice (these cells that do not respond to TGF β) were used in co-culture with WT CD8⁺CD28^{low} T cells. WT CD8⁺CD28^{low} T cells were not able to prevent colitis in a dnT β RII transgenic mouse (Menager-Marcq et al., 2006). These findings indicated that regulatory activity of CD8⁺CD28^{low} T cells uses multiple mechanisms. Two of these mechanisms could use IL-10 and TGF β molecules.

CD8⁺CD28^{low} T cells were detected in mice and human. Their immunosuppressive activity was demonstrated in vitro and in vivo. However, the evidence from experiments done in vivo is not convincing since it contains only one in vivo experiment which uses more regulatory cells than effector T cells. Overall, data concerning regulatory activity of CD8⁺CD28^{low} T cells is sparse and not convincing enough to establish these cells a stand-alone regulatory T-cell population.

5.4. Summary

CD8⁺CD28⁻ T cells population has been detected only in human so far, so the research possibilities are quite limited. All available data comes from analysis of peripheral blood and in vitro suppression assays. While a few of these experiments imply suppressive activity of these cells, more recently published works indicate that these cells possess cytotoxic character that is able to cause autoimmunity. Accordingly, production of proinflammatory molecules perforin and granzyme B was recorded. This means that either these putative regulatory T cells actually possess cytotoxic properties or cytotoxic and regulatory T cells overlap within the CD8⁺CD28⁻ T-cell subset. If the CD8⁺CD28⁻ T-cell population truly involves two functionally different subsets, markers are needed for their possible distinction. Although there were efforts in the past, no such marker was found.

Another problematic part is the use of the same term (CD8⁺CD28⁻ T cells) for murine thymic CD8⁺CD28^{low} T cells and human peripheral CD8⁺CD28⁻ T cells. Although these two populations are not homologous, some reviews and articles do not distinguish these two T cell lineages and mix findings of these two different subsets together (Manavalan et al., 2004, Smith and Kumar, 2008, Pomie et al., 2011), therefore making an impression of more evidence than there actually is.

In conclusion, based on the current state of knowledge of CD8⁺CD28⁻ and CD8⁺CD28^{low} T cells, neither of these T-cell subsets meets the criteria of a regulatory T-cell population defined in chapter 2.2 and therefore cannot be established as a regulatory T-cell subset.

6 CD8⁺CD25⁺FOXP3⁺

CD8⁺CD25⁺FoxP3⁺ T cells share phenotypic and developmental characteristics with induced CD4⁺ Tregs. They are both induced in the periphery and can also be induced ex vivo in the presence of TGF- β (Mayer et al., 2011). Common phenotypic features of CD8⁺CD25⁺FoxP3⁺ T cells and CD4⁺ Tregs include expression of CD25, GITR, CTLA4 and CD103 (Mayer et al., 2011, Churlaud et al., 2015). It is possible that CD8⁺CD25⁺FoxP3⁺ and CD8⁺CD28⁻ T-cell populations overlap since they are both characterized by expression of markers: Foxp3, GITR, CTLA4. Unfortunately, the reports concerning CD28 expression of CD8⁺CD25⁺FoxP3⁺ T cells are not consistent. While some consider CD8⁺CD25⁺FoxP3⁺ T cells to be CD28⁺ (Mahic et al., 2008, Mayer et al., 2011), others state that CD8⁺CD25⁺FoxP3⁺ T cells express lower level of CD28 than their CD25⁻ counterparts (Correale and Villa, 2010, Lerret et al., 2012).

Analysis of peripheral blood showed that CD8⁺CD25⁺FoxP3⁺ T cells are present in very low numbers. In mice CD8⁺CD25⁺FoxP3⁺ T cells make up only 0.1% of all CD8⁺ T cells, in human their number varies from 0.1 to 1% (Churlaud et al., 2015). Some researchers were not able to detect this population in peripheral blood of healthy donors at all (Peng et al., 2012). Induction of CD8⁺CD25⁺FoxP3⁺ T-cell population was observed during the early stages of Graft versus Host disease (Beres et al., 2012). Also percentage of intratumoral CD8⁺CD25⁺FoxP3⁺ T cells in patients with gastric cancer was reported to grow with tumor progression (Peng et al., 2012). Generation of CD8⁺CD25⁺FoxP3⁺ T cells can be also induced in vitro by TGF β (Mayer et al., 2011, Lerret et al., 2012, Peng et al., 2012) or by continuous stimulation for 4 days with staphylococcal enterotoxin B (Mahic et al., 2008).

The reported suppressive activity of CD8⁺ responder cells exerted by CD8⁺CD25⁺FoxP3⁺ T cells was comparable to level of suppression by their FoxP3⁻ counterparts (Mayer et al., 2011). One of the possible explanations of this result could be dependence on CD28 co-stimulation of CD8⁺CD25⁺FoxP3⁺ T cells. In a similar assay that used stimulating anti-CD28 antibody, TGF- β -induced CD8⁺Foxp3⁺ T cells showed suppression of proliferation of activated CD4⁺ T cells equivalent to CD4⁺Foxp3⁺ T cells (Churlaud et al., 2015). Intratumoral CD8⁺CD25⁺FoxP3⁺ T cells isolated from tumors were able to suppress proliferation and IFN- γ production of autologous peripheral CD4⁺ T cells as well (Peng et al., 2012). In vitro induced CD8⁺CD25⁺FoxP3⁺ T cells were shown to suppress both CD4⁺ and CD8⁺ target cells (Lerret et al., 2012)

In the in vivo experiment, T cells were adoptively transferred into RAG^{-/-} mouse following skin transplantation. While transfer of unfractionated T cells led to rejection of graft, co-injection of 2×10^5 T cells and 1×10^6 TGF β induced CD8⁺CD25⁺FoxP3⁺ T cells prolonged allograft survival. The amount of CD8⁺CD25⁺FoxP3⁺ T cells required to achieve this effect contrasts with their very low occurrence, since the number of adoptively transferred cells exceeds the expected size of their population present in a single mouse.

The regulatory mechanisms of CD8⁺CD25⁺FoxP3⁺ T cells are still rather unexplored. Since blocking anti-IL-10 antibody did not abrogate the suppressive effect of CD8⁺CD25⁺FoxP3⁺ T cells, IL-10 does not seem to be involved in suppressive activity (Mahic et al., 2008). However, their supernatant preserved some suppressive activity, thus indicating involvement of a soluble factor in the mechanism (Mahic et al., 2008). This finding was challenged by results of transwell experiments, which showed that CD8⁺CD25⁺FoxP3⁺ T cells require cell to cell contact for their regulatory activity (Correale and Villa, 2010, Lerret et al., 2012). Furthermore, it was observed that CD8⁺CD25⁺FoxP3⁺ T cells are not capable of suppression in the absence of DCs. It was proposed that the possible mechanism could be downregulation of co-stimulatory molecules (Correale and Villa, 2010).

Contact-dependent suppression by CD8⁺CD25⁺FoxP3⁺ T cells was showed in vitro. However, overall evidence of regulatory activity of CD8⁺CD25⁺FoxP3⁺ T cells is very sparse and contains almost no relevant in vivo experiments. Their very low occurrence in combination with conflicting reports on their characteristics and mechanism of suppression provoke doubts about the relevance of this putative regulatory subset.

7 CD8⁺CD103⁺ T cells

Another proposed CD8⁺ T cell regulatory subset is a population of CD8⁺CD103⁺ T cells. CD103 is an important integrin for lymphocyte homing into the epithelial layer, especially the intestinal sites. The CD8⁺CD103⁺ T cells population represents around 4% of CD8⁺ T cells from freshly isolated human blood (Uss et al., 2006). This T-cell subset has been identified also in mice and rats (Lu et al., 2009, Liu et al., 2014). CD8⁺CD103⁺ T-cell population can be induced by TGF- β ex vivo (Uss et al., 2006, Lu et al., 2009, Liu et al., 2014).

The phenotypic characterization of ex vivo stimulated CD8⁺CD103⁺ T-cell population is rather controversial. While one research group reported that CD8⁺CD103⁺ T cells are CD25⁻, CTLA-4⁻, GITR⁻ (Koch et al., 2008), other claims the opposite (i.e., CD25⁺, CTLA4⁺, GITR⁺) (Liu et al., 2014). Furthermore, it seems that the phenotype of ex vivo stimulated CD8⁺CD103⁺ T cells differs from in vivo stimulated CD8⁺CD103⁺ T-cell population (Koch et al., 2008). Another issue is that CD8⁺CD103⁺ T-cell population was reported to exert cytotoxic activity (Djenidi et al., 2015). Therefore, it is unclear whether CD8⁺CD103⁺ T cells form a cytotoxic and a regulatory subset.

Ex vivo stimulated CD8⁺CD103⁺ T cells suppressed proliferation of CD4⁺ and CD8⁺ T cells in vitro (Uss et al., 2006, Lu et al., 2009, Liu et al., 2014). These results were supported by an in vivo experiment using colitis model (Liu et al., 2014). RAG^{-/-} mouse was injected with 0.5 x 10⁶ CD4⁺CD45RB^{hi} T cells. Co-transfer of 1 x 10⁶ of CD8⁺CD103⁺ T cells inhibited colitis progression.

Despite the expression of TGF- β and IL-10 by CD8⁺CD103⁺ T cells, the addition of anti-IL-10 or anti-TGF antibodies did not affect the inhibition of proliferation of alloactivated T cells (Uss et al., 2006). Transwell experiments revealed that CD8⁺CD103⁺ T cell mediated inhibition requires cell to cell contact (Uss et al., 2006, Liu et al., 2014). The mechanism of suppression remains unknown.

Suppressive activity of ex vivo CD8⁺CD103⁺ T cells was hinted. Their mechanism and capacity of this suppression are largely unknown. However, since all studies used only ex vivo stimulated CD8⁺CD103⁺ T cells, it is not certain that CD8⁺CD103⁺ T cells present in vivo are also capable of this activity. Moreover, CD8⁺CD103⁺ T cells have been reported to possess cytotoxic abilities. Whether or not the cytotoxic and the putative regulatory T cells are the same remains to be addressed.

8 CD8⁺CD45RC^{low} T cells

Last putative CD8⁺ regulatory T-cell subset is a CD8⁺CD45RC^{low} T-cell population isolated from rats. These cells represent around 20% of rat CD8⁺ T cells (Xystrakis et al., 2004a). Existence of this T-cell population in mice has not been addressed, and its existence in human is not clear. Although it is possible that there is a CD8⁺CD45RC^{low} T-cell population present in human, its immunoregulatory properties are unknown (Xystrakis et al., 2004a).

CD8⁺CD45RC^{low} T cells produce IL-10 and are not cytotoxic (Xystrakis et al., 2004b). In vitro experiments revealed that CD8⁺CD45RC^{low} inhibited proliferation and IFN- γ production of CD8⁺CD45RC^{high} and CD4⁺ T cells (Xystrakis et al., 2004a, Xystrakis et al., 2004b). Suppressive activity of CD8⁺CD45RC^{low} was tested also in vivo. CD8⁺CD45RC^{low} T cells were studied in model of experimental autoimmune uveitis (EAU) which is induced by immunization with R-16 peptide (Han et al., 2007). R-16 specific T cells from rats that recovered from monophasic EAU were transferred into recipient rats to induce EAU. When R-16 specific T cells were co-transferred with CD8⁺CD45RC^{low} T cells, their pathogenic activity was significantly inhibited. In order to find out if CD8⁺CD45RC^{low} T cells are able to suppress CD4⁺ T cells in vivo, irradiated rats were injected with purified CD4⁺ T cells from semiallogeneic rats (Xystrakis et al., 2004b). Transplant recipients developed severe signs of graft versus host disease and most of them died within the first month. When CD4⁺ T cells were co-injected with semiallogeneic CD8⁺CD45RC^{low} T cells, the mice survived for more than 2 months. Further transplantation experiments were done with mice that underwent anti-45RC treatment that should enrich CD8⁺CD45RC^{low} T cells. This treatment caused a permanent survival of a heart transplant (Picarda et al., 2017). However, this achieved result could be the result of an inability of the immune system to reject graft after the depletion of CD8⁺CD45RC^{high} T cells.

The mechanism of suppression by CD8⁺CD45RC^{low} is not known. Despite reported IL-10 production, it does not seem that it is mediated by IL-10, since suppressive activity of CD8⁺CD45RC^{low} T cells was not abrogated by addition of anti-IL-10 antibody (Xystrakis et al., 2004b). Use of anti-TGF- β antibody had no effect as well. Transwell experiments indicated that CD8⁺CD45RC^{low} T cells require cell to cell contact for their suppressive activity (Xystrakis et al., 2004b). Accordingly, culture supernatants did not show any suppressive activity (Han et al., 2007). Thus, it can be estimated that regulatory activity by CD8⁺CD45RC^{low} is not mediated by secreting soluble suppressor cytokines but by cell to cell contact. However, the precise mechanism remains unclear.

Suppressive activity of CD8⁺CD45RC^{low} T cells was shown both in vitro and in vivo. However, there is not a lot of information about mechanism of suppression or the phenotypic characterization of CD8⁺CD45RC^{low} T cells. Most studies concerning CD8⁺CD45RC^{low} T cells were published more than ten years ago. Since then, researchers have been focused on study of CD40Ig induced CD8⁺CD45RC^{low} T cells rather than naïve CD8⁺CD45RC^{low} T cells. CD40Ig treatments blocks CD40-CD40L interaction

which leads to induction of CD8⁺CD45RC^{low} T cells and a subsequent permanent allograft survival. Transfer of CD8⁺CD45RC^{low} T cells from CD40Ig treated mice induces tolerance in naive transplant recipients (Picarda et al., 2017).

9 Summary

In this chapter, I will overview gathered evidence and evaluate each putative regulatory subset it based on criteria defined in chapter 2.2.

CD8⁺CD122⁺ T cells are likely the most promising putative regulatory CD8⁺ T-cell subset. Its immunoregulatory capacity was shown in vitro and in vivo even when low numbers were used. This activity seems to implement more than one mechanism. Proposed mechanisms involve IL-10 and Fas/FasL system. The major problem is their unknown relationship with virtual memory T cells, cells that were reported to promote immune response. Until this is resolved, this T-cell population does not fulfill criteria of regulatory T-cell subset.

The following subset (CD8 $\alpha\alpha$ ⁺ T cells) is a lot less explored. CD8 $\alpha\alpha$ ⁺ T cells form an enigmatic T-cell subset that was suggested to retain immunoregulatory ability. However, poor evidence of suppression and reported IFN γ production are reasons why these T cells do not fit set criteria of a regulatory T-cell subset. Interestingly, it seems that there is an unidentified population of Qa-1 restricted T cells in mice that possesses an immunoregulatory activity. Although, CD8 $\alpha\alpha$ ⁺ T-cell subset has been linked to Qa-1 restricted T cells, how these two subsets are related is uncertain.

	Organism			Phenotype characteristics						Mechanism		
	Mouse	Human	Rat	FOXP3	CD25	CD28	CD103	CD122	CTLA4	IL-10	TGF β	Cell to cell
CD8 ⁺ CD122 ⁺	✓	✗	✗	-	-	++	++	+++	++	+	-	+
CD8 $\alpha\alpha$ ⁺	✓	✗	✗	-	++	++	++	++	N/A	-	N/A	N/A
CD8 ⁺ CD28 ^{low}	✓	✓	✗	-	-	-	++	++	-	+	+	!
CD8 ⁺ CD28 ⁻	✗	✓	✗	!	!	-	++	++	++	!	-	+
CD8 ⁺ CD25 ⁺	✓	✓	✗	++	++	!	+++	!	++	-	N/A	+
CD8 ⁺ CD103 ⁺	✓	✓	✓	-	!	!	++	N/A	!	-	-	+
CD8 ⁺ CD45RC ^{low}	✗	✗	✓	++	-	-	-	N/A	++	-	-	+

FIGURE 2: Comparison of putative regulatory CD8⁺ T cell subsets

(✓: present in the organism, ✗: not present in the organism; -: not expressed; ++: expressed; +++: highly expressed; N/A: data not available; !: controversial data; +: possible mechanism of suppression)

Data of murine CD8⁺CD28^{low} T cells and human CD8⁺CD28⁻ T cells have been mixed up. However, when separated the evidence of their suppressive activity is rather disappointing. There is not enough convincing data from in vivo experiments as well as lack of recently published work. While there is no recorded cytotoxicity by murine CD8⁺CD28^{low} T cells, human CD8⁺CD28⁻ T cells were reported to show not only suppressive but also cytotoxic abilities. This discrepancy between studies could be explained by the coexistence of various subpopulations within the CD8⁺CD28⁻ T cells population. There was no success in distinguishing these two possible subpopulations so far.

Interestingly, there is a possibility that CD8⁺CD28⁻ T cells overlap with the next putative regulatory T-cell subset - CD8⁺CD25⁺FoxP3⁺ T cells. Both of these T-cell subsets were reported to be induced in the periphery following disturbances in the immune system and they share similar phenotype characteristics. Unfortunately, expression of crucial markers is still uncertain.

CD8⁺CD25⁺FoxP3⁺ T cells were described in mice and also in human, however in both species their occurrence is very low (below 1% of all CD8⁺ T cells). Although their cell to cell dependent suppressive activity was observed in vitro, relevant in vivo evidence is lacking.

The penultimate putative regulatory T-cell subset mentioned in this thesis is the subset of CD8⁺CD103⁺ T cells. The ex vivo activated CD8⁺CD103⁺ T cells were reported to exert contact dependent suppression in vivo. Whether there are CD8⁺CD103⁺ T cells present in vivo that are capable of such activity remains to be addressed. If so their involvement with cytotoxic CD8⁺CD103⁺ T cells needs resolving.

Although regulatory activity of CD8⁺CD45RC^{low} T cells was shown in multiple in vivo experiments, the mechanism remains unexplored. It should be noted that only a very small number of studies concerning these cells have been published.

	CD8 ⁺ CD122 ⁺	CD8 ^{αα} ⁺	CD8 ⁺ CD28 ^{low}	CD8 ⁺ CD28 ⁻	CD8 ⁺ CD25 ⁺	CD8 ⁺ CD103 ⁺	CD8 ⁺ CD45RC ^{low}
A specific T-cell subset in vivo	N	Y	Y	N	Y	N	Y
Capable of suppression	Y	N	N	N	N	N	Y
Known mechanism	Y	N	N	N	N	N	N
Not proinflammatory	N	N	Y	N	Y	N	Y

FIGURE 3: Criteria of a regulatory T-cell subset
(Y: fits criteria, N: does not fit criteria)

In conclusion, none of the reviewed CD8⁺ T-cell subset fulfills the criteria defined in chapter 2.2 and therefore none can be currently established as a regulatory subset. Yet, some seem to be closer to possible establishment than others. The most prospective candidates are the subpopulations of CD8⁺CD122⁺ and Qa-1-restricted T cells. Studies of CD8⁺CD122⁺ T cells brought quite convincing data of their regulatory activity, however, their unresolved relationship with virtual memory T cells represent a major obstacle in their establishment as regulatory T-cell population. Promising data have been obtained in research of Qa-1 restricted cells, implying an existence of immunoregulatory T lymphocytes within Qa-1 restricted T-cell group. However, phenotype of these cells has not been precisely identified.

10 Conclusion

Many immunologists are skeptical about the existence of CD8⁺ regulatory T lymphocytes. One of the contributing factors is the fact that, at the current state, the field of CD8⁺ regulatory T lymphocytes is rather confusing. Some putative regulatory T-cell subsets are insufficiently and/or inconsistently characterized thus leading to further confusion. In the past, data of two different T-cell subsets (CD8⁺CD28⁻ and CD8⁺CD28^{low} T cells) were mixed up and it is not excluded that some now separately studied T-cell populations actually represent the same cells.

The major obstacle in study of regulatory T cells is the ambiguity of suppressive assays. Although decrease in numbers of target cells might be observed, it does not necessarily demonstrate targeted regulative activity. This development of T cell count can be caused by some kind of mutual competition between cells and/or conditions which one of the cell subsets benefits from. Therefore, more carefully designed in vivo experiments are needed for recognition of possible regulatory activity.

In this thesis, I reviewed published data coming putative regulatory CD8⁺ T cell subpopulations to come to the conclusion that although some of CD8⁺ subsets seem to have immunosuppressive potential (i.e., CD8⁺CD122⁺ T cells, Qa-1 restricted cells), at the moment there is not sufficient evidence to declare any of the reviewed subpopulations as CD8⁺ regulatory cells based on the criteria I predefined.

Abbreviations

APCs	antigen presenting cells
ConA	Concanavalin A
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cells
EAE	experimental autoimmune encephalomyelitis
FcεRIγ	high-affinity IgE receptor
FOXP3	forkhead box P3
GITR	glucocorticoid-induced TNFR-related protein
HSK	herpes stromal keratitis
ICAM-1	Intercellular Adhesion Molecule 1 (CD54)
IEL	intraepithelial lymphocytes
IL	Interleukin
IL-2Rβ	beta chain of interleukin 2 receptor
ILT3	immunoglobulin-like transcript
IFN	interferon
KLRG1	killer-cell lectin-like receptor G1
LAG-3	lymphocyte-activation gene 3
MHC	major histocompatibility complex
PD-1	programmed death-1 protein
PLP	proteolipid protein
PRF	perforin
SCID	severe combined immunodeficiency mouse
SLE	systemic lupus erythematosus
TCR	T cell receptor
T_{CM}	central memory T lymphocytes
Tregs	regulatory T lymphocytes
TGFβ	transforming growth factor beta
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
WT	wildtype

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